



UNIWERSYTET
IM. ADAMA MICKIEWICZA
W POZNANIU

Rozprawa doktorska
mgr Dominika Czerwonka

**Synteza i badania aktywności biologicznej pochodnych związków
naturalnych - salinomycyny i kolchicyny**

Synthesis and biological activity of derivatives of natural compounds - salinomycin
and colchicine

Promotor: prof. dr hab. Adam Huczyński

Promotor pomocniczy: dr Michał Antoszczak

Poznań, 2022

MÓJ STAN JEST STABILNY

no dobra
to nie jest depresja
to jest obniżenie nastroju
albo
tak jak pan mówi
przygnębienie spowodowane niespełnieniem
ale to jest
potwornie uporczywe i długotrwałe
właściwie odkąd tylko pamiętam
odkąd tylko skończyłem studia
i poszedłem do pierwszej pracy
to tak się czuję

czyli
możemy uznać
że pana stan jest stabilny

Andrzej Kotański, „Wiersze o moim psychiatrze”

Wszystko co dobre (i złe) kiedyś się kończy. Jestem pewna, że za kilkanaście lat moje wspomnienia o doktoracie się zetrą. Wtedy znajdę tę rozprawę, zakurzoną na półce, przeczytam ten wiersz i przypomnę sobie o emocjach, które towarzyszyły mi podczas tych 5 lat. Emocjach i uczuciach, o których wciąż mało mówi się w Polsce, a które towarzyszą wielu doktorantom (i nie tylko). Temat zdrowia psychicznego nie powinien być powodem do wstydu. Więc jeśli to czytasz, i się z tym utożsamiasz, to hej, nie jesteś sam(a)! Sięgnij po pomoc.

Dlatego moje podziękowania zacznę nieskromnie od podziękowania sobie samej. Dziękuję, że wytrwałaś. Wyszło lepiej niż przypuszczałaś. Brawo ty! Z perspektywy czasu mogę powiedzieć, że to była świetna przygoda.

Jestem pewna, że nie byłabym w miejscu, w którym jestem teraz bez Pani Agaty, mojej psychoterapeutki. Mogłabym napisać elaborat ale napiszę krótko. Dziękuję za pomoc w poznaniu samej siebie, za zmianę perspektywy, za narzędzia, dzięki którym poradziłam sobie ze wszystkimi przeszkodami. Nie było łatwo, ale było warto: zaczynam nowy rozdział z otwartą głową i bez strachu.

Szymon, jestem pewna, że gdyby nie Ty, nie skończyłabym tego doktoratu. Jesteś dla mnie niedoścignionym wzorem naukowca. Wsparcie, miłość i dobroć, jakie od Ciebie otrzymałam w ciągu tych 5 lat, zasługują za znacznie więcej niż na podziękowania.

Ogromne podziękowania należą się mojej wspaniałej rodzinie: Mojej Najlepszej Młodszej Siostrze Irminie, Mamie, Tacie i Babusi. Dziękuję, że byliście moimi największymi kibicami i zawsze mogłam na Was liczyć. Cały czas czułam wasze wsparcie i miłość. Ten doktorat to także Wasza zasługa.

Ola, dziękuję za nasze *umilanie poniedziałków*, które ratowało I rok naszego doktoratu. Tobie nic nie trzeba było tłumaczyć, ty wszystko rozumiałaś „z autopsji”.

Paulina, dziękuję za to, że jesteś. Po prostu. Merci.

Greta, Marta, Michał(y), dziękuję za świetną atmosferę w pracy każdego dnia. Za wzajemną życzliwość i pomoc, nie tylko chemiczną, w każdej sytuacji.

Mojemu Promotorowi, prof. dr. hab. Adamowi Huczyńskiemu, dziękuję za przygarnięcie mnie pod swoje skrzydła i możliwość rozwoju. Dziękuję, że dbałeś o to, żebym miała pracy pod dostatkiem i żeby mi się nie nudziło- jak widać, przyniosło to oczekiwane efekty. Bez Ciebie ten doktorat naprawdę by nie istniał. Ze świecą szukać takiego opiekuna naukowego i drugiej takiej grupy badawczej.

Dziękuję również mojemu Promotorowi Pomocniczemu, dr. Michałowi Antoszczakowi. Praca z Tobą to była sama przyjemność. Dziękuję, że mogłam się od Ciebie uczyć. Jesteś najlepszym możliwym „lab partnerem”, zarówno w labie chemicznym, jak i biologicznym.

Badania przedstawione w niniejszej rozprawie były finansowane przez Narodowe Centrum Nauki w ramach projektów: **ETIUDA 8** (2020/36/T/ST4/00041), **OPUS 11** (2016/21/B/ST5/00111) oraz **SONATA 12** (2016/23/D/ST5/00242).



N A R O D O W E C E N T R U M N A U K I

Badania te były także objęte wsparciem projektu **ChemInter** (POWR.03.02.00-00-I026/16) współfinansowanym przez Unię Europejską ze środków Europejskiego Funduszu Społecznego w ramach Programu Operacyjnego Wiedza Edukacja Rozwój.



Unia Europejska
Europejski Fundusz Społeczny



Spis treści

Streszczenie w języku polskim	1
Streszczenie w języku angielskim	3
Ankieta dorobku naukowego	5
Wstęp	12
Cel pracy	17
Krótkie wprowadzenie do wykorzystanych technik analitycznych	21
Krótkie wprowadzenie do przeprowadzonych badań aktywności biologicznej	23
Modyfikacje struktury salinomycyny i ich wpływ na aktywność przeciwproliferacyjną	27
Modyfikacje struktury kolchicyny i ich wpływ na aktywność przeciwproliferacyjną	36
Wnioski i podsumowania	47
Bibliografia	50
Artykuły naukowe stanowiące rozprawę doktorską	59
PUBLIKACJA I WRAZ Z SUPLEMENTEM	60
PUBLIKACJA II	94
PUBLIKACJA III WRAZ Z SUPLEMENTEM	106
PUBLIKACJA IV WRAZ Z SUPLEMENTEM	148
PUBLIKACJA V WRAZ Z SUPLEMENTEM	188
PUBLIKACJA VI WRAZ Z SUPLEMENTEM	248
Oświadczenia współautorów	292

Streszczenie w języku polskim

W związku ze stale rosnącą liczbą zachorowań na choroby nowotworowe oraz niewystarczającą skutecznością obecnie stosowanych terapii przeciwnowotworowych konieczne jest poszukiwanie nowych związków o aktywności przeciwnowotworowej. Ponieważ chemiczna modyfikacja związków pochodzenia naturalnego wciąż pozostaje jedną z najbardziej efektywnych metod poszukiwania nowych leków, jako obiekt moich badań wybrałam dwa związki pochodzenia naturalnego - salinomycynę i kolchicynę.

Salinomycyna jest naturalnym antybiotykiem jonoforowym o szerokim spektrum aktywności biologicznej izolowanym ze szczepu *Streptomyces albus*. Kolchicyna natomiast jest pseudoalkaloidem o działaniu przeciwzapalnym i przeciwmiotycznym, który występuje w zimowicie jesiennym (*Colchicum autumnale*).

Wyniki przeprowadzonych przeze mnie badań zostały zebrane w cykl 6 artykułów naukowych, które stanowią podstawę mojej rozprawy doktorskiej zatytułowanej „*Synteza i badania aktywności biologicznej pochodnych związków naturalnych - salinomycyny i kolchicyny*”. Modyfikacje struktury cząsteczki salinomycyny obejmowały aminowanie redukcyjne, selektywne utlenienie grupy hydroksylowej w pozycji C20, odwrócenie konfiguracji absolutnej atomu węgla C20, a także reakcje estryfikacji, amidowania oraz acylowania. Modyfikacje struktury kolchicyny obejmowały natomiast selektywne demetylowanie grup metoksyłowych w pozycjach C1 oraz C10, acylowanie, hydrolizę, aminowanie redukcyjne oraz przegrupowanie pierścienia tropolonowego pod wpływem promieniowania UV. W wyniku przeprowadzonych syntez otrzymałam 49 nowych pochodnych salinomycyny oraz 48 nowych pochodnych kolchicyny.

Struktura i czystość otrzymanych pochodnych została wyznaczona z wykorzystaniem spektroskopii magnetycznego rezonansu jądrowego (^1H NMR, ^{13}C NMR, 2D NMR), analizy krystalograficznej, spektrometrii mas (ESI-MS), spektroskopii w podczerwieni (FT-IR), oraz analizy elementarnej (EA).

Wszystkie otrzymane związki zostały poddane badaniom aktywności przeciwnowotworowej we współpracy z naukowcami z polskich i zagranicznych ośrodków naukowych. Wyznaczono cytotoksyczność pochodnych salinomycyny wobec linii komórkowej pierwotnej ostrej białaczki limfoblastycznej (ALL-5), ludzkiego gruczolaka

piersi (MDA-MB-231), pierwotnego raka okrężnicy (SW480), przerzutowego raka okrężnicy (SW620), przerzutowego raka prostaty (PC3), ludzkich mezenchymalnych komórek sutka o właściwościach komórek macierzystych (HMLER CD24^{low}/CD44^{high}), izogenicznej linii epitelialnych komórek sutka niewykazujących właściwości komórek macierzystych (HMLER CD24^{high}/CD44^{low}), a także wobec panelu 60 ludzkich linii komórek nowotworowych (NCI-60). Natomiast pochodne kolchicyny zostały przebadane wobec następujących linii komórek nowotworowych: ludzkiego gruczolaka płuc (A549), ludzkiego gruczolaka piersi (MCF-7), ludzkiego gruczolaka jelita grubego (LoVo) oraz lekoopornej linii ludzkiego gruczolaka jelita grubego (LoVo/DX). Dodatkowo, dla wszystkich badanych związków wyznaczono również współczynniki selektywności i oporności. Pozwoliło to na wyznaczenie korelacji pomiędzy strukturą nowych pochodnych a ich aktywnością biologiczną (SAR).

Przeprowadzone przeze mnie badania dowiodły słuszności wyboru obiektów badawczych – związków naturalnych o dużym potencjale przeciwnowotworowym. Ich modyfikacje pozwoliły mi wykazać, że dobrze zaplanowana synteza analogów salinomycyny i kolchicyny może doprowadzić do otrzymania nowych i bardziej aktywnych pochodnych udowadniając, że potęgę tkwiącą w naturze można udoskonalić.

Streszczenie w języku angielskim

Facing continuously growing number of cancer cases and insufficient effectiveness of the currently used anti-cancer therapies, it is necessary to search for new compounds showing anti-cancer activity. Since chemical modification of natural compounds is still one of the most effective methods of searching for new drugs, I chose two compounds of natural origin - salinomycin and colchicine, as the objects of my research.

Salinomycin is a natural ionophore antibiotic with a broad spectrum of biological activity, isolated from *Streptomyces albus*. Colchicine, on the other hand, is a pseudoalkaloid with an anti-inflammatory and antimitotic effect, which can be found in autumn crocus (*Colchicum autumnale*).

The results of my research have been presented in a series of 6 scientific articles, which constitute the basis of my doctoral dissertation entitled "*Synthesis and biological activity of derivatives of natural compounds- salinomycin and colchicine*". Modifications of the salinomycin molecule included reductive amination, selective oxidation of the C20 hydroxyl group, inversion of the absolute configuration of the C20 carbon atom, as well as esterification, amidation and acylation reactions. Modifications of colchicine, on the other hand, included selective demethylation of methoxy groups at C1 and C10 positions, acylation, hydrolysis, reductive amination and rearrangement of the tropolone ring under the influence of UV radiation. As a result of the performed syntheses, I obtained 49 new derivatives of salinomycin and 48 new derivatives of colchicine.

The structure and purity of the derivatives obtained were determined using nuclear magnetic resonance spectroscopy (^1H NMR, ^{13}C NMR, 2D NMR), crystallographic analysis, mass spectrometry (ESI-MS), infrared spectroscopy (FT-IR), and elemental analysis (EA).

All obtained compounds were tested for anti-cancer activity in cooperation with scientists from Polish and foreign research centers. The cytotoxicity of the salinomycin derivatives was determined against the primary acute lymphoblastic leukemia (ALL-5), human breast adenocarcinoma (MDA-MB-231), primary colon cancer (SW480), metastatic colon cancer (SW620), metastatic prostate cancer (PC3), human cell lines breast mesenchymal cells with stem cell properties (HMLER CD24^{low} / CD44^{high}), an isogenic non-stem cell epithelial line of breast cells (HMLER CD24^{high} / CD44^{low}), and against a panel of 60 human tumor cell lines (NCI-60). On the other hand, the obtained colchicine derivatives have been tested against

the following cancer cell lines: human lung adenoma (A549), human breast adenoma (MCF-7), human colorectal adenoma (LoVo) and drug-resistant human colorectal adenoma (LoVo / DX) line. In addition, selectivity and resistance indexes were also determined for all tested compounds. This allowed us to determine the correlation between the structure of the new derivatives and their biological activity (*SAR*).

The obtained results have proven the right choice of the research objects - natural compounds with high anti-cancer potential. Their modifications allowed me to show that a well-planned synthesis of salinomycin and colchicine analogs can lead to new and more active derivatives, proving that the power of nature can be improved.

Ankieta dorobku naukowego

A. Wykaz opublikowanych i powiązanych tematycznie artykułów naukowych stanowiących rozprawę doktorską pt. „Synteza i badania aktywności biologicznej pochodnych związków naturalnych - salinomycyny i kolchicyny”:

- I. **Dominika Czerwonka**, Alicja Urbaniak, Szymon Sobczak, Sergio Piña-Oviedo, Timothy C. Chambers, Michał Antoszczak, Adam Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, **ChemMedChem** 2020, 15, 236–246. IF₂₀₂₀ = 3,466.¹

Mój udział w publikacji obejmował syntezę oraz oczyszczenie 21 pochodnych salinomycyny (w tym 13 nowo otrzymanych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam także odpowiedzialna za ich krystalizację. Przygotowałam również wstęp oraz opis syntezy do manuskryptu, a także suplement. Mój udział procentowy stanowi 30%.

Udział procentowy współautorów wynosi: Alicja Urbaniak 30%, Szymon Sobczak 5%, Sergio Piña-Oviedo 5%, Timothy C. Chambers 10%, Michał Antoszczak 10%, Adam Huczyński 10%.

- II. **Dominika Czerwonka**, Magdalena Mielczarek-Putra, Michał Antoszczak, Aleksandra Cioch, Marta Struga, Adam Huczyński, „*Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin*”, **European Journal of Pharmacology** 2021, 908, 174347. IF₂₀₂₀ = 4,432.²

Mój udział w publikacji obejmował syntezę oraz oczyszczenie 20 pochodnych salinomycyny (w tym 18 nowo otrzymanych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Przygotowałam również roboczą wersję manuskryptu oraz suplement.

Mój udział procentowy stanowi 60%.

Udział procentowy współautorów wynosi: Magdalena Mielczarek-Putra 10%, Michał Antoszczak 10%, Aleksandra Cioch 5%, Marta Struga 5%, Adam Huczyński 10%.

- III. **Dominika Czerwinka**, Sebastian Müller, Tatiana Cañeque, Ludovic Colombeau, Adam Huczyński, Michał Antoszczak, Raphaël Rodriguez, „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*”, **ACS Organic & Inorganic Au** 2022, DOI: 10.1021/acsorginorgau.1c00046.³

Nowo utworzone czasopismo bez IF.

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 18 nowych pochodnych salinomycyny. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za przeprowadzenie części badań biologicznych *in vitro* na liniach komórek nowotworowych w trakcie 3-miesięcznego stażu w Insytucie Curie w Paryżu, a także za analizę i opracowanie wyników przeprowadzonych badań. Przygotowałam manuskrypt, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement.

Mój udział procentowy stanowi 50%.

Udział procentowy współautorów wynosi: Sebastian Müller 10%, Tatiana Cañeque 5%, Ludovic Colombeau 5%, Adam Huczyński 10%, Michał Antoszczak 10%, Raphaël Rodriguez 10%.

- IV. **Dominika Czerwinka**, Szymon Sobczak, Ewa Maj, Joanna Wietrzyk, Andrzej Katrusiak, Adam Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, **Molecules** 2020, 25, 1180. IF₂₀₂₀ = 4,412.⁴

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 12 pochodnych kolchicyny (w tym 9 nowych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za ich krystalizację. Przygotowałam kolejne wersje manuskryptu, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement, a także brałam aktywny udział w dyskusji z recenzentami.

Mój udział procentowy stanowi 65%.

Udział procentowy współautorów wynosi: Szymon Sobczak 5%, Ewa Maj 10%, Joanna Wietrzyk 5%, Andrzej Katrusiak 5%, Adam Huczyński 10%.

- V. **Dominika Czerwonka**, Szymon Sobczak, Tomasz Pędziński, Ewa Maj, Joanna Wietrzyk, Lech Celewicz, Andrzej Katrusiak, Adam Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, **Journal of Organic Chemistry** 2021, 86, 16, 11029–11039. IF₂₀₂₀ = 4,354.⁵

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 17 nowych pochodnych kolchicyny, w tym syntezę 6 nowych lumipochodnych. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za ich krystalizację. Przygotowałam kolejne wersje manuskryptu, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement, a także brałam aktywny udział w dyskusji z recenzentami.

Mój udział procentowy stanowi 55%.

Udział procentowy współautorów wynosi: Szymon Sobczak 5%, Tomasz Pędziński 5%, Ewa Maj 10%, Joanna Wietrzyk 5%, Lech Celewicz 5%, Andrzej Katrusiak 5%, Adam Huczyński 10%.

- VI. **Dominika Czerwonka**, Ewa Maj, Joanna Wietrzyk, Adam Huczyński, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, **Bioorganic & Medicinal Chemistry Letters** 2021, 52, 128382. IF₂₀₂₀ = 2,823.⁶

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 22 nowych pochodnych kolchicyny. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Przygotowałam manuskrypt, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement, a także brałam aktywny udział w dyskusji z recenzentami.

Mój udział procentowy stanowi 70%.

Udział procentowy współautorów wynosi: Ewa Maj 15%, Joanna Wietrzyk 5%, Adam Huczyński 10%.

B. Publikacje naukowe niebędące częścią rozprawy doktorskiej:

1. **Dominika Czerwonka**, Yzobelle Barcelos, Dietmar Steverding, Aleksandra Cioch, Michał Antoszczak, Adam Huczyński, „*Singly and doubly modified analogues of C20-epi-salinomycin: A new group of antiparasitic agents against Trypanosoma brucei*”, **European Journal of Medicinal Chemistry** 2021, 209, 112900, DOI: 10.1016/j.ejmech.2020.112900.
2. **Dominika Czerwonka**, Joanna Domagalska, Krystian Pyta, Marcelina M. Kubicka, Paulina Pecyna, Marzena Gajeczka, Piotr Przybylski, „*Structure–activity relationship studies of new rifamycins containing L-amino acid esters as inhibitors of bacterial RNA polymerases*”, **European Journal of Medicinal Chemistry** 2016, 116, 216-221. DOI: 10.1016/j.ejmech.2016.03.061.
3. Piotr Przybylski, Krystian Pyta, **Dominika Czerwonka**, Marcelina M. Kubicka, Marzena Gajeczka, „*The effect of complexation of 3-formylrifamycin SV macrocyclic ether derivatives with metal cations and small nitrogen-containing organic molecules on antibacterial activity against S. aureus and S. epidermidis*”, **Bioorganic & Medicinal Chemistry Letters** 2015, 25, 3903, DOI: 10.1016/j.bmcl.2015.07.043.

C. Staże naukowe

1. **3,5-miesięczny** staż w grupie badawczej prof. Thomasa B. Poulsena na Wydziale Chemii w **Aarhus Universitet (Aarhus, Dania)** w ramach projektu CHEMINTER finansowanego przez program POWR.03.02.00e00-I026/16. Realizacja projektu badawczego „*Towards the total synthesis of natural product Hatomamicin and its isomer*” (1.09.2021 - 17.12.2021);
2. **3-miesięczny** staż w grupie badawczej prof. Raphaëla Rodrigueza w **Instytucie Curie (Paryż, Francja)** sfinansowany w ramach stypendium Narodowego Centrum Nauki w programie **ETIUDA 8** (2020/36/T/ST4/00041): „*Synteza i badania aktywności biologicznej pochodnych związków naturalnych - salinomycyny i kolchicyny*” (15.03.2021 – 18.06.2021).

D. Realizowane projekty badawcze:

1. Kierownik projektu w grantie Narodowego Centrum Nauki **ETIUDA 8** (2020/36/T/ST4/00041): „*Synteza i badania aktywności biologicznej pochodnych związków naturalnych - salinomycyny i kolchicyny*”;
2. Wykonawca w grantie Narodowego Centrum Nauki **OPUS 11** (2016/21/B/ST5/00111): „*Aktywność przeciwnowotworowa nowych inhibitorów polimeryzacji tubuliny otrzymanych poprzez chemiczną modyfikację kolchicyny*”; kierownik projektu: prof. Adam Huczyński;
3. Wykonawca w grantie Narodowego Centrum Nauki **SONATA 12** (2016/23/D/ST5/00242): „*Drugie życie salinomycyny: Chemo- i regioselektywne modyfikacje salinomycyny oraz ich wpływ na aktywność przeciwnowotworową i przeciwdrobnoustrojową*”; kierownik projektu: dr Michał Antoszczak;
4. Wykonawca w grantie Narodowego Centrum Nauki **OPUS 2** (2011/03/B/ST5/01014): „*Projektowanie i synteza nowych analogów rifampicyny jako źródło efektywnych inhibitorów bakteryjnej polimerazy RNA*”; kierownik projektu: prof. Piotr Przybylski;
5. Wykonawca w projekcie firmy FiLeClo Sp. z o.o. finansowanym w ramach Programu Operacyjnego Inteligentny Rozwój (POIR.01.01.01-00-0906/19-00): „*Opracowanie nowego terapeutycznego o działaniu przeciwnowotworowym o wysokiej aktywności i selektywności w porównaniu do obecnie stosowanych leków cytostatycznych*”;
6. Wykonawca w projekcie firmy FiLeClo Sp. z o.o. finansowanym w ramach Programu Operacyjnego Inteligentny Rozwój (POIR.02.03.02-10-0022/19): „*Pochodne salinomycyny ze zredukowanym wiązaniem podwójnym, jako prekursorzy leków w terapii nowotworów*”.

E. Udział w konferencjach:

- Konferencje międzynarodowe:

1. 20th Tetrahedron Symposium, 18 – 21.06.2019, Bangkok, Tajlandia;
D. Czerwonka, M. Antoszczak, A. Urbaniak, T.C. Chambers, A. Huczyński,
„Synthesis and biological activity of novel double-modified salinomycin derivatives”, poster;
2. 2nd Molecules Medicinal Chemistry Symposium, 15 – 17.05.2019, Barcelona, Hiszpania; M. Antoszczak, **D. Czerwonka**, A. Urbaniak, S. Sobczak, T.C. Chambers, A. Huczyński, *„Double modification of polyether ionophores: Synthesis and biological activity of novel salinomycin derivatives”*, poster;
3. The 2nd PSL Chemical Biology symposium, 17 – 18.01.2019, Paryż, Francja;
D. Czerwonka, M. Antoszczak, A. Urbaniak, M. Delgado, T.C. Chambers, A. Huczyński, *„Synthesis and anticancer activity of double modified salinomycin derivatives”*, poster;

- Konferencje krajowe:

1. IX Konwersatorium Chemii Medycznej, 13 – 15.09.2018, Lublin; **D. Czerwonka**, G. Klejborowska, U. Majcher, E. Maj, J. Wietrzyk, A. Huczyński, *„Synthesis and biological evaluation of 4-chlorothiocolchicine derivatives as potent tubulin-targeting anticancer agents”*, poster;
2. VI Łódzkie Sympozjum Doktorantów Chemii, 10 – 11.05.2018, Łódź;
D. Czerwonka, U. Majcher, G. Klejborowska, E. Maj, J. Wietrzyk, A. Huczyński
„Synteza i właściwości przeciwproliferacyjne nowych potrójnie modyfikowanych pochodnych kolchicyny”, poster;
3. 59 Zjazd Polskiego Towarzystwa Chemicznego, 19 – 23.09.2016, Poznań;
D. Czerwonka, J. Domagalska, M. Kubicka, M. Gajecka, K. Pyta, P. Przybylski
„Structure and antibacterial activity of new amino derivatives of 3-formylrifamicin SV containing crown ethers and L-aminoacids”, poster;
4. 58 Zjazd Polskiego Towarzystwa Chemicznego, 21 – 25.09.2015, Gdańsk;
D. Czerwonka, J. Domagalska, M. Kubicka, M. Gajecka, K. Pyta, P. Przybylski,
„Structure and antibacterial activity of new amino derivatives of 3-formylrifamicin SV containing crown ethers and L-aminoacids”, poster;

5. XIII Ogólnopolskie Seminarium Doktorantów „Na pograniczu chemii i biologii”, 31.05 – 03.06.2015, Karpacz; **D. Czerwonka**, „*Makrolidy- struktura i właściwości koordynacyjne a aktywność biologiczna*”, wystąpienie ustne.

F. Nagrody i wyróżnienia:

1. Stypendium Rektora UAM za wybitne osiągnięcia w pracy naukowej w roku 2020;
2. Stypendium Rektora UAM dla najlepszych doktorantów (2020 – 2021 & 2021 – 2022);
3. Nagroda za najlepszy poster, VI Łódzkie Sympozjum Doktorantów Chemii, Łódź (10 – 11.05.2018).

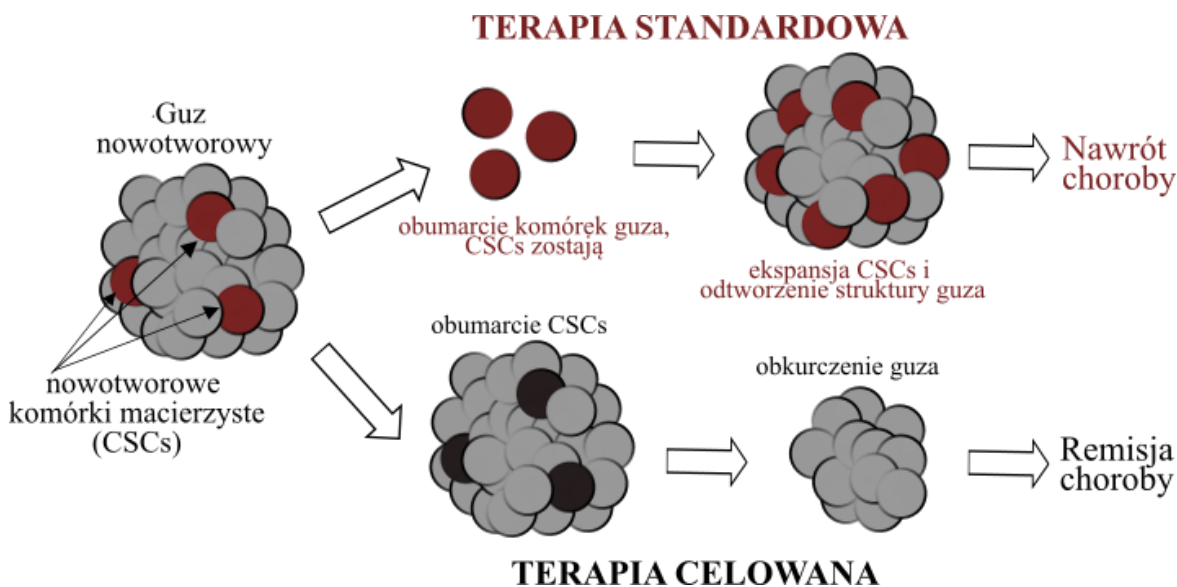
Wstęp

Walka z chorobami nowotworowymi jest jednym z najważniejszych i najtrudniejszych wyzwań jakie zostało postawione przed współczesną nauką. Choroby nowotworowe, choć są problemem współczesnym, znane są ludziom od dawna. Przypadki zachorowań na nowotwory, a także próby ich leczenia przy pomocy chirurgii były opisywane już w starożytności przez Hipokratesa (a nawet wcześniej przez starożytnych Egipcjan około 3 tysiące lat przed naszą erą), czego potwierdzeniem są guzy nowotworowe odnajdywane w z mumifikowanych ciałach pochodzących sprzed tysięcy lat.^{7,8} Terminem „nowotwór” określa się grupę chorób powstających na skutek utraty kontroli organizmu nad wzrostem, podziałem i rozprzestrzenieniem się komórek, co w konsekwencji prowadzi do powstania guza pierwotnego, atakującego i niszczącego sąsiednie tkanki. Nowotwór może dalej rozprzestrzeniać się na inne tkanki i narządy w wyniku procesu przerzutowania, który jest główną przyczyną śmierci z powodu raka.⁹ Według danych Światowej Organizacji Zdrowia (ang. *World Health Organization, WHO*) obecnie niemal co szósty zgon na świecie spowodowany jest przez choroby nowotworowe. W 2018 roku nowotwór zdiagnozowano u 18,1 miliona osób, jednak szacuje się, że do roku 2040 liczba ta wzrośnie do ponad 29 milionów przypadków rocznie.¹⁰

Wśród stosowanych obecnie terapii przeciwnowotworowych wymienić można radioterapię, leczenie chirurgiczne oraz chemioterapię, której działanie oparte jest na wykorzystaniu substancji hamujących podziały komórek nowotworowych (tzw. leków cytostatycznych). Powszechnie stosowanie leki cytostatyczne często charakteryzują się niską selektywnością działania, oddziałując zarówno na komórki nowotworowe, jak i na komórki zdrowe, tym samym powodując dotkliwe skutki uboczne. Szacuje się, że terapie te są nieskuteczne u niemal połowy chorych; nie prowadzą do wyleczenia, a jedynie do nieznacznego wydłużenia życia pacjentów.^{9,11,12}

Ograniczoną skuteczność obecnie stosowanych terapii przeciwnowotworowych można wytłumaczyć w oparciu o teorię nowotworowych komórek macierzystych (ang. *Cancer Stem Cells, CSCs*), według której w obrębie wzrastającego guza nowotworowego znaleźć można nieliczne, dobrze ukryte komórki o właściwościach nowotworowych komórek macierzystych, odpowiedzialne za nawroty choroby i przerzutowanie (**Rysunek 1**). W przeciwieństwie do niemacierzystych komórek nowotworowych, CSCs nie ulegają tak szybkim podziałom i dzięki

temu są odporne na działanie typowych leków cytostatycznych.^{13,14} Ponadto, komórki te wykazują nadekspresję specyficznych białek odpowiedzialnych za usuwanie leków z ich wnętrza. Zwiększone stężenie glutationu komórkowego i białek chroniących przed reaktywnymi formami tlenu (ang. *Reactive Oxygen Species, ROS*) zwiększa dodatkowo ich odporność na radioterapię.^{15–17}



Rysunek 1. Uprozczone przedstawienie różnicy w działaniu terapii standardowej i celowanej w walce z nowotworowymi komórkami macierzystymi (CSCs).

Związki pochodzenia naturalnego od wieków stanowią dla człowieka cenne źródło substancji biologicznie czynnych, wykorzystywanych bezpośrednio jako związki lecznicze lub struktury wiodące w opracowywaniu leków.¹⁸ Związki wiodące wykazują określone kierunki działania biologicznego (niekoniecznie silnego) i mogą wywoływać pewne działania niepożądane, stanowią jednak doskonały punkt wyjścia w projektowaniu nowych substancji leczniczych.^{19,20} Z raportu opublikowanego w 2016 roku przez Davida Newmana i Gordona Cragga wynika, że związki pochodzenia naturalnego wciąż pozostają głównym źródłem nowych leków. Od końca lat 30-tych XX wieku do końca 2014 roku, spośród 175 nowych, małocząsteczkowych leków przeciwnowotworowych wprowadzonych na rynek, aż 49% stanowiły związki pochodzenia naturalnego lub ich pochodne.²¹

Przewaga produktów naturalnych nad związkami syntetycznymi wynika z faktu, że jako metabolity pierwotne lub wtórne, oprócz aktywności biologicznej, mogą również działać jako substraty dla transporterów dostarczających związki do tkanek i ich

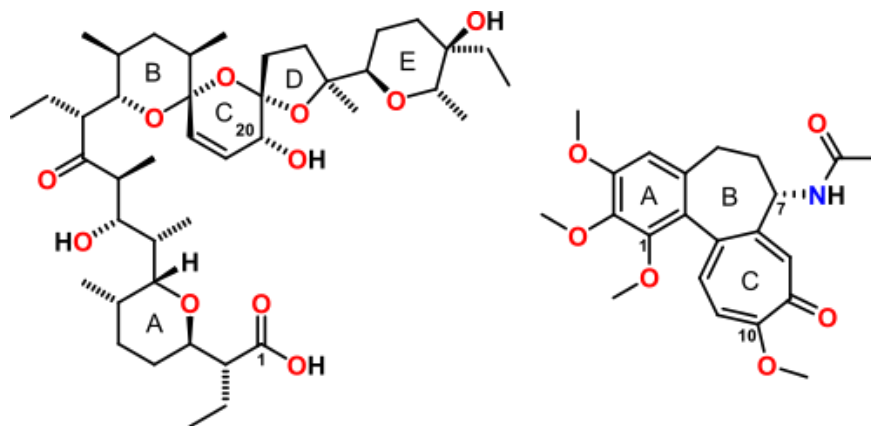
wewnątrzkomórkowego miejsca działania.^{22,23} Z drugiej jednak strony, pozyskiwane w wyniku racjonalnie zaplanowanej syntezy chemicznej pochodne produktów naturalnych często charakteryzują się większą skutecznością i selektywnością działania, niższą toksycznością, jak również zoptymalizowanymi właściwościami farmakokinetycznymi w porównaniu do związków wyjściowych.²⁴⁻²⁷ Produkty naturalne i ich pochodne są zatem kluczowe w projektowaniu nowych leków, ponieważ dostarczają informacji na temat zależności między strukturą a aktywnością biologiczną (ang. *Structure-Activity Relationship, SAR*).^{28,29}

W tym miejscu nie sposób nie wspomnieć o innej, istotnej ścieżce poszukiwania nowych leków jaką jest tzw. repozycjonowanie (ang. *drug repurposing*). Polega ono na odnajdywaniu nowego zastosowania dla znanych już leków czy substancji zatwierdzonych do użytku przez amerykańską Agencję Żywności i Leków (ang. *Food and Drug Administration, FDA*).³⁰⁻³² Podejście to pozwala na szybsze wprowadzenie leku na rynek, ponieważ większość badań przedklinicznych, ocena bezpieczeństwa czy też formuacja zostały już przeprowadzone. Co więcej, ze względu na fakt, że substancja została już uznana za bezpieczną w badaniach przedklinicznych, ryzyko niepowodzenia jest znacznie niższe. Całkowite koszty badań również mogą zostać obniżone, chociażby dzięki oszczędności w kosztach przedklinicznych i badaniach I i II fazy.³⁰⁻³² Repozycjonowanie leków cieszy się obecnie dużym zainteresowaniem.

Rosnąca liczba zachorowań na choroby nowotworowe w połączeniu z ograniczoną skutecznością obecnie dostępnych terapii sprawia, że konieczne jest poszukiwanie nowych leków przeciwnowotworowych. Nie ulega także wątpliwości, że bogatym źródłem substancji aktywnych biologicznie są związki pochodzenia naturalnego. Dlatego na obiekt moich badań wybrałam salinomycynę i kolchicynę (**Rysunek 2**), które nie tylko należą do grupy produktów pochodzenia naturalnego, ale są również wykorzystywane jako leki w medycynie i weterynarii.

Pierwszy ze związków, salinomycyna, jest naturalnym antybiotykiem jonoforowym o szerokim spektrum aktywności biologicznej izolowanym ze szczepu *Streptomyces albus*. Salinomycyna wykazuje właściwości przeciwpasożytnicze, przeciwmalaryczne, przeciwwirusowe oraz przeciwgrzybicze, dzięki czemu jest stosowana powszechnie jako dodatek paszowy i niehormonalny stymulator wzrostu drobiu i bydła (SACOX, Bio-Cox).^{33,34} Wysoka aktywność biologiczna salinomycyny jest związana z jej naturalną zdolnością do kompleksowania i transportowania kationów metali przez błony biologiczne, co prowadzi do

zaburzenia wewnątrzkomórkowej równowagi jonowej oraz wewnątrzkomórkowego pH. W konsekwencji dochodzi do uszkodzenia mitochondriów, obrzęku komórki, a w końcu do jej śmierci na drodze apoptozy.^{35,36} Popularność salinomycyny wzrosła w 2009 roku, kiedy badania przesiewowe przeprowadzone przez Gupta i współpracowników wskazały salinomycynę jako niezwykle obiecujący lek przeciwnowotworowy.³⁷ Spośród około 16 tysięcy substancji biologicznie aktywnych, w tym wszystkich dotychczas stosowanych leków przeciwnowotworowych, jedynie 32 związki niszczyły komórki macierzyste raka piersi, a wśród nich najbardziej efektywna była salinomycyna; jej aktywność była blisko 100-krotnie większa od tej wykazywanej przez powszechnie stosowany lek przeciwnowotworowy - paclitaxel.³⁷ Na przestrzeni lat wykazano więcej niezwykłych właściwości salinomycyny, takich jak chociażby zdolność do indukowania programowanej śmierci zarówno lekowrażliwych komórek nowotworowych, jak i tych wykazujących oporność wielolekową (ang. *Multi-Drug Resistance*, *MDR*).³⁸ Dowiedziono również, że salinomycyna ma zdolność do zaburzania funkcjonowania mitochondriów, obniżania poziomu komórkowego ATP oraz zaburzania procesu autofagocytozy.³⁹⁻⁴¹ Ponadto, poprzez zaburzenie homeostazy kationów Ca^{2+} wywołuje stres w retikulum endoplazmatycznym.⁴² W 2017 roku grupa badawcza profesora Rodrigueza zaproponowała mechanizm przeciwnowotworowego działania salinomycyny i jej pochodnych oparty na zaburzeniu homeostazy żelaza w CSCs, które wykazują dużo większe zapotrzebowanie na kationy Fe^{2+} niż zwykłe komórki. Salinomycyna i jej pochodne, gromadząc się w lizosomach, powodują sekwestrację (wiązaną) kationów Fe^{2+} , co może w konsekwencji prowadzić do powstawania ROS, peroksydacji lipidów i śmierci komórki na drodze ferroptozy.^{43,44}



Rysunek 2. Wzory strukturalne salinomycyny (z lewej) oraz kolchicyny (z prawej).

Drugim z moich obiektów badawczych była kolchicina (**Rysunek 2**). Związek ten należy do grupy pseudoalkaloidów i jest izolowany głównie z zimowitu jesiennego (łac. *Colchicum autumnale*) i gloriozy wspaniałej (łac. *Gloriosa superba*). Korzystne właściwości kolchicyny były wykorzystywane już przez starożytnych Greków i Egipcjan do leczenia obrzęków i stanów zapalnych.^{45–48} Obecnie kolchicina jest stosowana jako lek w leczeniu dny moczanowej, zapalenia osierdzia i rodzinnej gorączki śródziemnomorskiej.^{49–51} Kolchicina wykazuje również wysoką aktywność przeciwnowotworową; należy do grupy tzw. przeciwmitytyków, czyli czynników oddziałujących z mikrotubulami (ang. *microtubule interfering agents*, *MIA*), których działanie wpływa bezpośrednio na proces polimeryzacji tubuliny.⁵² Kolchicina wiąże się z mikrotubulami w metafazie podziału komórki hamując ich polimeryzację, co w rezultacie uniemożliwia utworzenie wrzeciona kariokinetycznego i skutkuje zahamowaniem mitozy (podziału) komórek nowotworowych.⁵³ Niestety, stosunkowo wysoka toksyczność i niska biodostępność wyklucza stosowanie kolchicyny w chemioterapii.^{9,54,55}

Cel pracy

Za cel prowadzonych badań postawiłam sobie: (1) zaplanowanie oraz przeprowadzenie syntezy nowych pochodnych salinomycyny oraz kolchicyny, (2) potwierdzenie struktury i czystości otrzymanych związków przy pomocy szczegółowej analizy spektroskopowej, spektrometrycznej oraz rentgenograficznej, następnie (3) przebadanie ich pod kątem aktywności przeciwnowotworowej, co w konsekwencji pozwoliłoby mi na (4) wyznaczenie korelacji pomiędzy strukturą nowych pochodnych a ich aktywnością biologiczną (SAR).

Stale rosnąca liczba zachorowań na choroby nowotworowe w połączeniu z niewystarczającą skutecznością obecnie stosowanych terapii przeciwnowotworowych powoduje, że konieczne jest poszukiwanie nowych substancji o aktywności przeciwnowotworowej. Chemiczna modyfikacja związków pochodzenia naturalnego wciąż pozostaje jedną z najbardziej efektywnych metod poszukiwania nowych leków. Ponieważ nawet niewielka zmiana w strukturze związku niejednokrotnie niesie ze sobą znaczącą zmianę właściwości biologicznych, postanowiłam zbadać wpływ przeprowadzonych przeze mnie modyfikacji na aktywność przeciwaproliferacyjną nowych pochodnych salinomycyny i kolchicyny. W tym celu podjęłam współpracę z badaczami z krajowych i zagranicznych ośrodków naukowych:

- a) Z prof. Joanną Wietrzyk i dr Ewą Maj z Instytutu Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda Polskiej Akademii Nauk we Wrocławiu;
- b) Z prof. Martą Strugą i dr Magdaleną Mielczarek-Putą z Katedry i Zakładu Biochemii Wydziału Lekarskiego Warszawskiego Uniwersytetu Medycznego;
- c) Z dr. Timothym Chambersem, dr. Sergio Piña-Oviedo i dr Alicją Urbaniak z Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Stany Zjednoczone;
- d) Z prof. Raphaëlem Rodriguezem, dr. Sebastianem Müllerem, dr Tatianą Cañeque i dr. Ludoviciem Colombeau z Chemical Biology of Cancer, Institute Curie, Paryż, Francja.

Natomiast w celu określenia struktury związków otrzymanych w postaci monokrystalicznej przeprowadzono badania rentgenograficzne we współpracy z prof. Andrzejem Katrusiakiem i dr. Szymonem Sobczakiem z Zakładu Chemii Materiałów Uniwersytetu im. Adama Mickiewicza w Poznaniu.

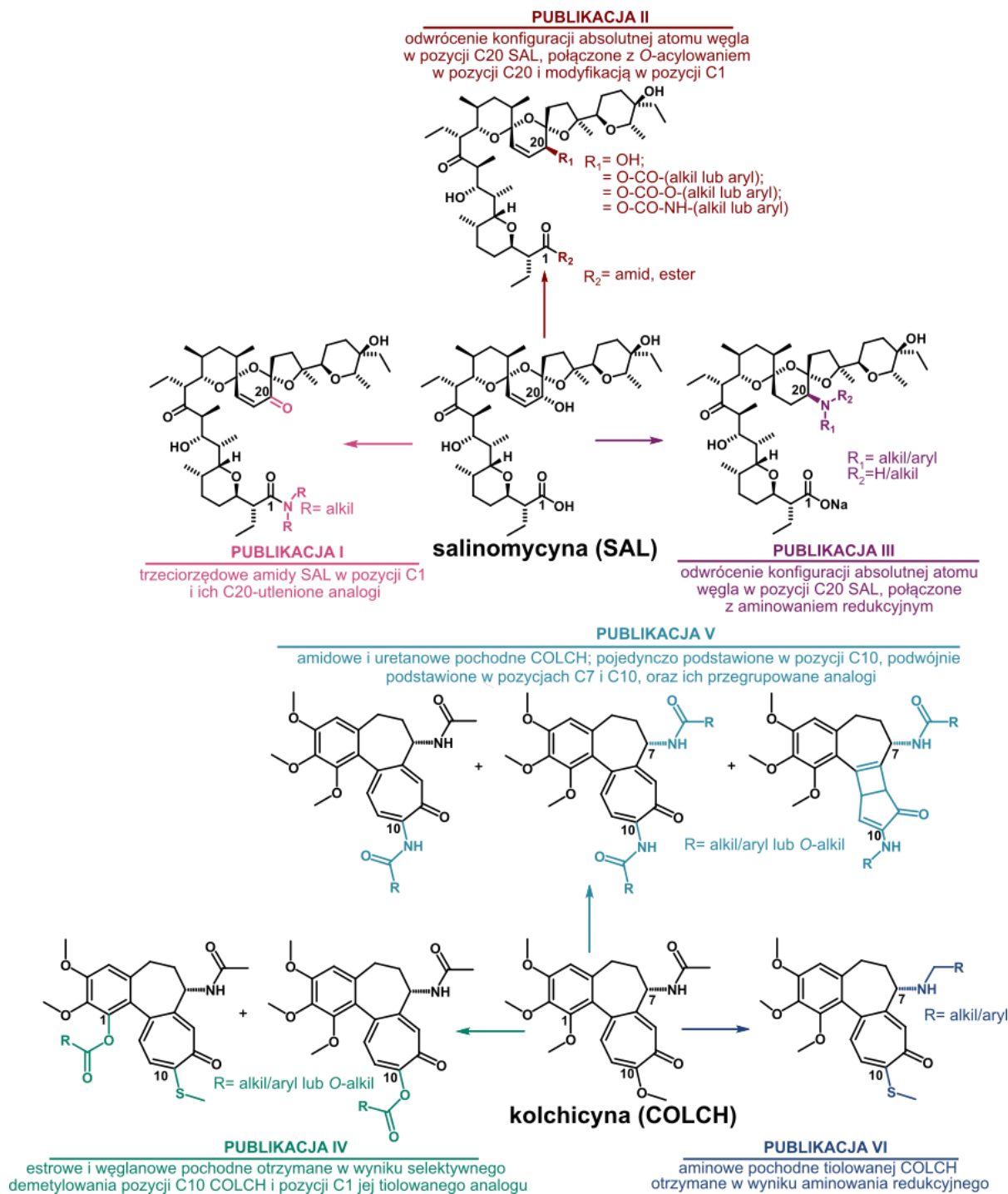
Badania fotochemiczne dotyczące mechanizmu przegrupowania *N*-podstawionych pochodnych kolchicyny przeprowadzono we współpracy z prof. Tomaszem Pędzińskim z Zakładu Fizyki Chemicznej Uniwersytetu im. Adama Mickiewicza w Poznaniu.

Realizacja wszystkich zaplanowanych celów **(1-4)** pozwoliłaby w przyszłości na bardziej racjonalne projektowanie nowych pochodnych salinomycyny i kolchicyny, co mogłoby doprowadzić do otrzymania związków o wysokim potencjale terapeutycznym, które w przyszłości stałyby się kandydatami na leki przeciwnowotworowe.

Wyniki przeprowadzonych przeze mnie badań zostały zebrane w cykl 6 artykułów naukowych, które stanowią podstawę mojej rozprawy doktorskiej. Kopie tych artykułów wraz z suplementami zostały załączone do niniejszej rozprawy:

- I. **Dominika Czerwonka**, Alicja Urbaniak, Szymon Sobczak, Sergio Piña-Oviedo, Timothy C. Chambers, Michał Antoszczak, Adam Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem 2020, 15, 236 – 246.
- II. **Dominika Czerwonka**, Magdalena Mielczarek-Puta, Michał Antoszczak, Aleksandra Cioch, Marta Struga, Adam Huczyński, „*Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin*”, European Journal of Pharmacology 2021, 908, 174347.
- III. **Dominika Czerwonka**, Sebastian Müller, Tatiana Cañeque, Ludovic Colombeau, Adam Huczyński, Michał Antoszczak, Raphaël Rodriguez, „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*”, ACS Organic & Inorganic Au 2022, DOI: 10.1021/acsorginorgau.1c00046.
- IV. **Dominika Czerwonka**, Szymon Sobczak, Ewa Maj, Joanna Wietrzyk, Andrzej Katrusiak, Adam Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, Molecules 2020, 25, 1180.
- V. **Dominika Czerwonka**, Szymon Sobczak, Tomasz Pędziński, Ewa Maj, Joanna Wietrzyk, Lech Celewicz, Andrzej Katrusiak, Adam Huczyński, „*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, Journal of Organic Chemistry 2021, 86, 16, 11029 – 11039.
- VI. **Dominika Czerwonka**, Ewa Maj, Joanna Wietrzyk, Adam Huczyński, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, Bioorganic & Medicinal Chemistry Letters 2021, 52, 128382.

Celem większej przejrzystości, każda publikacja otrzymała swój kolor przewodni. Na **Rysunku 3** w sposób uogólniony przedstawiłam modyfikacje przeprowadzone w każdej z prac składających się na niniejszą rozprawę.



Rysunek 3. Modyfikacje struktur salinomycyny i kolchicyny przedstawione w publikacjach I – VI.

Krótkie wprowadzenie do wykorzystanych technik analitycznych

Wszystkie związki przedstawione w niniejszej rozprawie zostały poddane szczegółowej analizie spektroskopowej, spektrometrycznej, a w przypadku związków krystalicznych również rentgenograficznej, co pozwoliło na dokładne określenie ich czystości i struktury. Szczegółowe dane na temat wykorzystywanych aparatów znajdują się w publikacjach i/lub suplementach do nich, jednakże dla większej jasności poniżej omówione zostały wykorzystane techniki.

Piki molekularne i pseudomolekularne $[M+Na]^+$ oraz $[M+H]^+$ analizowanych związków uwidoczniono wykorzystując spektroskopię mas z jonizacją przez elektrorozpylenie (**ESI-MS**). Metoda ta polega na pomiarze stosunku masy do ładunku danego jonu, a zastosowanie łagodnej metody jonizacji pozwala wyeliminować zjawisko fragmentacji badanych cząstek.⁵⁶ Widma masowe wszystkich otrzymanych pochodnych wykonano w acetonitrylowym roztworze badanego związku o stężeniu $10^{-5} - 10^{-4} \text{ mol} \cdot \text{dm}^{-3}$ z dodatkiem roztworu chloranu(VII) sodu o stężeniu $0,01 \text{ mol} \cdot \text{dm}^{-3}$. Dodatkowo, dla związków przedstawionych w publikacji III, zastosowanie wysokorozdzielczej spektrometrii mas (**HRMS**) pozwoliło na wyznaczenie mas cząsteczkowych z dokładnością do czwartego miejsca po przecinku. W publikacji V wykorzystano dodatkowo **analizę elementarną**, co pozwoliło na potwierdzenie procentowej zawartości azotu, węgla oraz wodoru w przedstawionych pochodnych kolchicyny.

Określenie czystości oraz struktury analizowanych związków było możliwe dzięki wykorzystaniu spektroskopii magnetycznego rezonansu jądrowego (**NMR**). Technika ta wykorzystuje właściwości magnetyczne jąder atomowych i polega na wzbudzaniu spinów jądrowych znajdujących się w zewnętrznym polu magnetycznym, a następnie rejestrację promieniowania elektromagnetycznego powstającego na skutek powrotu układu spinów jądrowych do stanu równowagi (relaksacja).⁵⁷ Widma ^1H NMR i ^{13}C NMR, zarejestrowane w roztworach deuterowanego chloroformu, chlorku metylenu lub dimetylosulfotlenku, umieszczono w suplementach do publikacji I – VI. W celu dokładnego przypisania sygnałów w przegrupowanych pochodnych zaprezentowanych w publikacji V wykorzystano także techniki dwuwymiarowe (HMBC, HSQC, COSY), które pozwalają na obserwację korelacji między jądrami atomów.

Obecność określonych grup funkcyjnych w strukturze analizowanych pochodnych została określona dzięki wykorzystaniu spektroskopii w podczerwieni z transformacją Fouriera (**FT-IR**), w której obserwuje się pasma pochodzące od drgań rozciągających (ν) i drgań deformacyjnych (δ) wiązań, przy czym drgania pochodzące od różnych grup funkcyjnych występują w określonych zakresach spektralnych. Pozwala to na śledzenie przebiegu reakcji poprzez obserwację zaniku pasm od drgań grup funkcyjnych substratów i pojawiania się pasm od grup funkcyjnych produktu.⁵⁷ Wszystkie widma przedstawionych związków zarejestrowano wykorzystując pastylkę zawierającą 200 mg KBr i 2 mg badanego związku.

W publikacjach I, IV oraz V wykorzystano również **analizę rentgenograficzną** polegającą na rejestracji obrazów dyfrakcyjnych promieni rentgenowskich, które powstają w skutek interakcji promieniowania z chmurami elektronowymi atomów w kryształach. Pozwoliło to na dokładne ustalenie struktury związków otrzymanych w postaci monokrystalicznej, w tym ustalenia oddziaływań międzycząsteczkowych, dokładnych kątów oraz długości wiązań między atomami.

Krótkie wprowadzenie do przeprowadzonych badań aktywności biologicznej

Wszystkie otrzymane związki zostały poddane badaniom mającym na celu określenie ich aktywności cytotoksycznej we współpracy z badaczami z krajowych i zagranicznych ośrodków. W badaniach aktywności przeciwnowotworowej *in vitro*, terminem **cytotoksyczność** określamy zdolność danej substancji do wpływania na żywotność komórek, co może się odbywać poprzez hamowanie wzrostu/podziału lub śmierci komórkowej. Miarą cytotoksyczności jest wartość stężenia powodującego zahamowanie wzrostu i namnażania (prolifracji) połowy komórek w hodowli (**IC₅₀**).⁵⁸

W celu oceny przeżywalności komórek stosuje się różnego rodzaju testy pozwalające w sposób pośredni lub bezpośredni na śledzenie zmian aktywności metabolicznej komórek w hodowli *in vitro* po 72 godzinnej inkubacji z potencjalną substancją cytotoksyczną o określonym stężeniu. W prezentowanych badaniach wykorzystano trzy różne metody pomiaru cytotoksyczności: test **SRB**, test **MTT** oraz test **CTB**. W metodzie **SRB** wykorzystuje się barwnik, sulforodaminę B, który wykazuje zdolność do wiązania białek w odpowiednich warunkach pH. Ponieważ ilość białka jest proporcjonalna do absorpcji sulforodaminy B, pozwala to na bezpośrednie wyznaczenie ilości żywych komórek w hodowli. W metodzie **MTT** wykorzystuje się pomarańczową, rozpuszczalną w wodzie sól tetrazolową (bromek 3-(4,5-dimetylo-tiazol-2-ilo)-2,5-difenylo-tetrazolu), która pod wpływem enzymu, dehydrogenazy mitochondrialnej, jest przekształcana w nierozpuszczalne w wodzie, fioletowe kryształy formazanu. W wyniku ich rozpuszczenia w DMSO powstaje barwny roztwór, którego intensywność zabarwienia jest proporcjonalna do liczby żywych komórek w hodowli. Natomiast metoda **CTB** polegająca na dodaniu pojedynczego odczynnika bezpośrednio do medium hodowlanego oparta jest na zdolności żywych komórek do przekształcania barwnika redoks (resazuryny) we fluorescencyjny produkt końcowy (rezorufinę). Martwe komórki szybko tracą aktywność metaboliczną i tym samym nie wpływają na intensywność sygnału fluorescencyjnego.

W kontekście określania zahamowania proliferacji komórek nowotworowych niezwykle istotny jest indeks selektywności (ang. *selectivity index*, *SI*), który pozwala na porównanie cytotoksyczności wobec komórek nowotworowych z cytotoksycznością wobec komórek zdrowych według wzoru: $SI = IC_{50} \text{ dla normalnej linii komórkowej} / IC_{50} \text{ dla linii}$

komórek nowotworowych. Za korzystne uznaje się wartości $SI > 1$, co oznacza, że substancja działa silniej na komórki nowotworowe niż na komórki zdrowe.

Natomiast indeks oporności (ang. *resistance index*, *RI*) wskazuje, ile razy lekooporna linia komórkowa jest mniej podatna na działanie leku od linii komórek rodzicielskich. Wartość $RI < 1$ oznacza, że komórki są wrażliwe na testowany związek, wartości RI w zakresie 1-10 oznaczają średnią wrażliwość na lek, natomiast $RI > 10$ oznacza silną lekooporność. Nabywanie oporności przez komórki nowotworowe powoduje zmniejszenie skuteczności stosowanych leków. Problem lekooporności jest dodatkowym czynnikiem uzasadniającym konieczność poszukiwania nowych związków o działaniu terapeutycznym.

W publikacji I, dzięki współpracy z dr Alicją Urbaniak i dr. Timothy Chambersem z University of Arkansas w Stanach Zjednoczonych, wybrane pochodne zostały poddane badaniom na **panelu 60 ludzkich linii komórek nowotworowych (NCI-60)** przez National Cancer Institute (NCI). W panelu NCI-60, utworzonym pod koniec lat osiemdziesiątych ubiegłego wieku, znajdują się linie komórkowe dziewięciu ludzkich nowotworów: piersi, ośrodkowego układu nerwowego, okrężnicy, nerek, białaczki, płuc, czerniaka, jajnika oraz prostaty. Panel ten pozwolił dotychczas na przebadanie tysięcy związków pod kątem aktywności przeciwnowotworowych oraz utworzenie obszernej bazy danych farmakologii onkologicznej. NCI-60 dostarcza również wiedzy na temat biologii nowotworów, mutacji genomu i interakcji lek-guz.^{59,60}

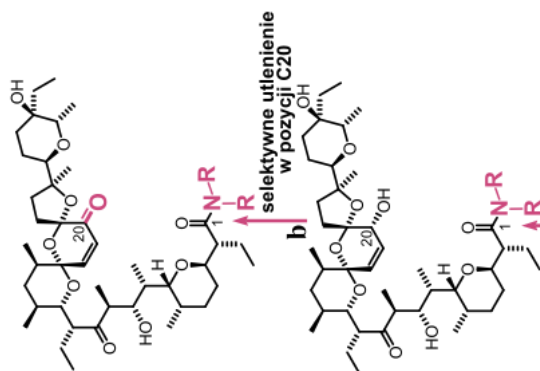
Dodatkowo, we współpracy z prof. Martą Strugą i dr Magdaleną Mielczarek-Putą z Warszawskiego Uniwersytetu Medycznego, dla najbardziej aktywnych pochodnych przedstawionych w publikacji II przeprowadzono badania indukowania wczesnej i późnej apoptozy oraz martwicy w celu rozszerzenia badań nad wyznaczaniem przeciwnowotworowego mechanizmu działania pochodnych salinomycyny. **Apoptoza** i **martwica** to dwa podstawowe typy śmierci komórkowej, przy czym martwica polega zwykle na pasywnym pęknięciu komórki spowodowanym nadmiernym uszkodzeniem egzogennym, natomiast apoptoza jest procesem aktywnym, na który składa się wiele skoordynowanych działań, którym towarzyszy modyfikacja powierzchni komórki. Komórki znajdujące się w fazie wczesnej apoptozy zachowują integralność błony komórkowej zatrzymując w swoim wnętrzu potencjalnie szkodliwą zawartość komórkową. Jeżeli w tej fazie komórka nie zostanie prawidłowo wchłonięta przez fagocyty, przechodzi w fazę późnej apoptozy lub wtórnej

martwicy, a jej błona komórkowa staje się przepuszczalna i powstający wyciek cząsteczek wewnątrzkomórkowych wywołuje reakcję zapalną.⁶¹ Badania nad indukcją apoptozy i martwicy zostały przeprowadzone metodą cytometrii przepływowej. Dodatkowo, w celu lepszego zrozumienia działania cytotoksycznego, po raz pierwszy sprawdziliśmy wpływ pochodnych salinomycyny na poziom **interleukiny IL-6**, która jest wskaźnikiem stanu zapalnego. IL-6 wykazuje działanie onkogenne poprzez aktywację wielu ścieżek sygnałowych.⁶² Badania wykazały, że pacjenci w zaawansowanym i przerzutowym stadium choroby nowotworowej mają wyższy poziom IL-6 we krwi, co często koreluje ze słabymi wskaźnikami przeżywalności.^{63,64} Powoduje to, że terapie celujące w obniżenie poziomu IL-6 jawią się jako obiecujące.

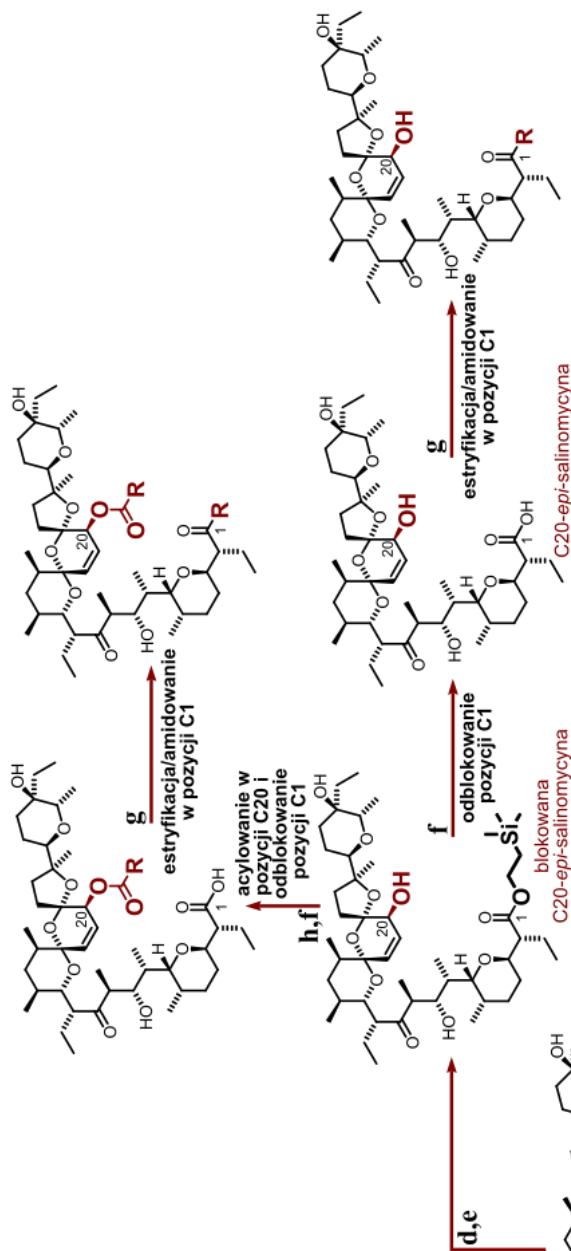
W celu oceny cytotoksyczności zsyntezowanych związków wykorzystano następujące linie komórkowe:

- 1) ALL-5 – pierwotna ostra białaczka limfoblastyczna;
- 2) MDA-MB-231 – ludzki gruczolakorak piersi;
- 3) MCF 10A – prawidłowa linia komórkowa nabłonka sutka;
- 4) SW480 – pierwotny rak okrężnicy;
- 5) SW620 – przerzutowy rak okrężnicy;
- 6) PC3 – przerzutowy rak prostaty;
- 7) HaCaT – linia ludzkich keratynocytów;
- 8) HMLER CD24^{low}/CD44^{high} – ludzkie mezenchymalne komórki sutka wykazujące właściwości komórek macierzystych;
- 9) HMLER CD24^{high}/CD44^{low} – izogeniczna linia epitelialnych komórek sutka niewykazująca właściwości komórek macierzystych;
- 10) A549 – ludzki gruczolak płuc;
- 11) MCF-7 – ludzki gruczolak piersi;
- 12) LoVo – ludzki gruczolak jelita grubego;
- 13) LoVo/DX – lekooporna linia ludzkiego gruczolaka jelita grubego;
- 14) BALB/3T3 – prawidłowa linia mysich fibroblastów;
- 15) NCI-60 – panel 60 ludzkich linii komórek nowotworowych.

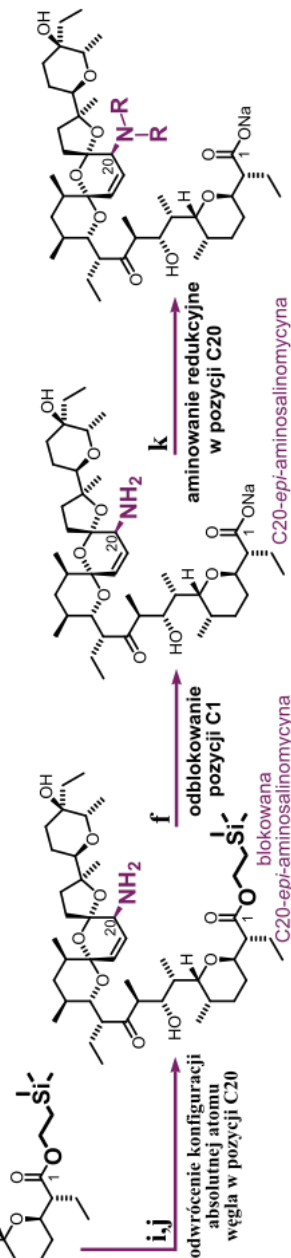
Publikacja I



Publikacja II



SALINOMYCYN A



Publikacja III

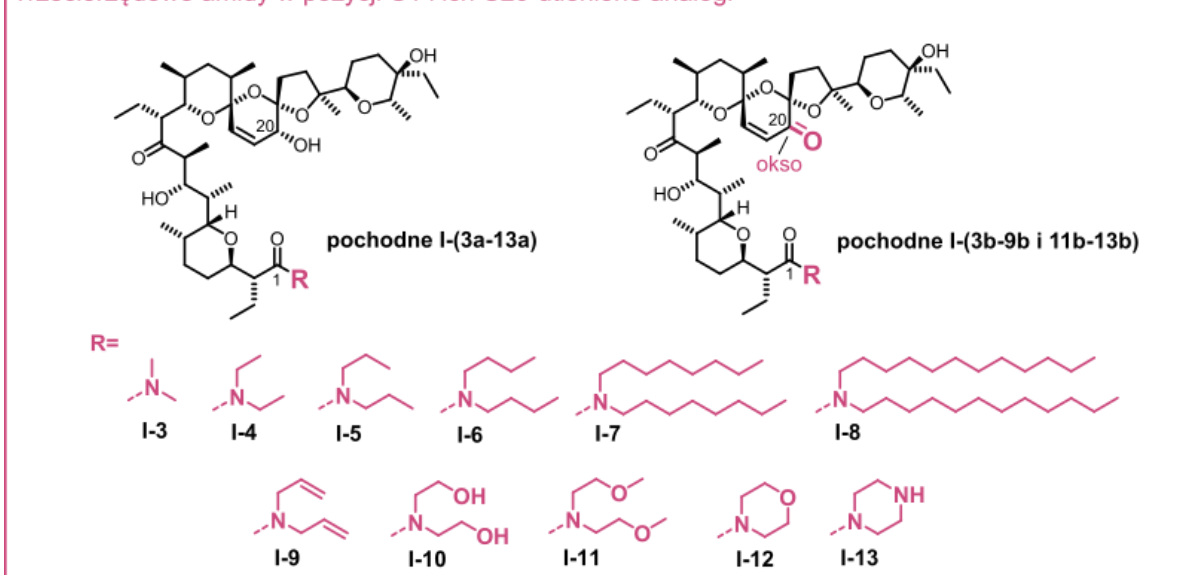
Schemat 1. Ścieżki syntetyczne podjęte w celu modyfikacji struktury salinomycyny. Warunki reakcji: (a) DCC, HOBt, 2° amina, CH_2Cl_2 , od 0°C do temp. pokojowej (tp); (b) MnO_2 , CH_2Cl_2 , tp; (c) TMSEtOH , DMAP, TCFH, CH_2Cl_2 , tp; (d) kwas 4-nitrobenzoesowy, PPh_3 , DIAD, bezwodny THF, od 0°C do tp; (e) K_2CO_3 ; (f) TBAF, bezwodny THF, tp; (g) DCC, HOBt, amina, CH_2Cl_2 , tp lub bromek propargilu, DBU, toluen, 90°C lub DCC, DMAP, kwas hydroksamowy, CH_2Cl_2 , tp; (h) chlorek kwasowy/chloromrówczan, DMAP, Et_3N , CH_2Cl_2 , od 0°C do tp lub izocyanian, CuCl , DMF, tp; (i) PPh_3 , DIAD, DPPA, THF, tp; (j) PPh_3 , H_2O , THF; (k) aldehyd, NaBH_3CN , $\text{CH}_2\text{Cl}_2/\text{MeOH}$, tp.

Modyfikacje struktury salinomycyny i ich wpływ na aktywność przeciwproliferacyjną

W publikacjach I - III opisane zostały badania związane z chemiczną modyfikacją struktury salinomycyny. Przeprowadzone przeze mnie modyfikacje wraz z warunkami podjętych reakcji przedstawiono na **Schemacie 1**. W wyniku przeprowadzonych syntez otrzymałam łącznie 60 pochodnych salinomycyny, spośród których aż 49 otrzymano po raz pierwszy. Dane spektroskopowe znajdują się na końcu niniejszej rozprawy, w publikacjach i/lub suplementach dołączonych do tych publikacji. Tabele zawierające wyniki przeprowadzonych badań aktywności przeciwproliferacyjnej *in vitro* także znajdują się w załączonych publikacjach.

Publikacja I

Trzeciorzędowe amidy w pozycji C1 i ich C20-utlenione analogi



Rysunek 4. Struktury pochodnych salinomycyny opisanych w publikacji I.

W publikacji I przedstawiono syntezę 21 pochodnych salinomycyny, w tym 11 pojedynczo modyfikowanych, trzeciorzędowych amidów (**I-(3a-13a)**) w pozycji C1 oraz 10 podwójnie modyfikowanych analogów (**I-3b-9b; 11b-13b**) w pozycjach C1 i C20 (**Rysunek 4**). Syntezy powyższych związków oznaczono **ścieżką** na **Schemacie 1**. Związki **I-(3a-13a)** otrzymano w reakcji salinomycyny z drugorzędową aminą, w obecności *N,N'*-dicykloheksylokarbodiimidu (DCC) oraz 1-hydroksybenzotriazolu (HOBt). Procedura ta została wcześniej opisana w literaturze i pozwoliła na otrzymanie trzeciorzędowych amidów

salinomycyny z zadowalającą wydajnością (14 – 95%).⁶⁵ Pochodne **I-(3a-13a)** zostały następnie poddane reakcji z aktywowanym tlenkiem manganu(IV) w celu chemoselektywnego utlenienia allilowej grupy hydroksylowej w pozycji C20. W wyniku tej reakcji otrzymano 10 podwójnie modyfikowanych analogów **I-(3b-9b;11b-13b)** z wydajnością rzędu 26 – 97%. Ze względu na obecność pierwszorzędowych grup hydroksylowych przy ugrupowaniu amidowym w pozycji C1 związku **I-10a**, nie otrzymano analogu **I-10b**. Próby odwrócenia kolejności prowadzonych reakcji, polegające na otrzymaniu w pierwszym etapie analogu salinomycyny zawierającego keton w pozycji C20, a następnie wprowadzeniu trzeciorzędowego amidu **I-10** w pozycję C1 również okazały się nieskuteczne.

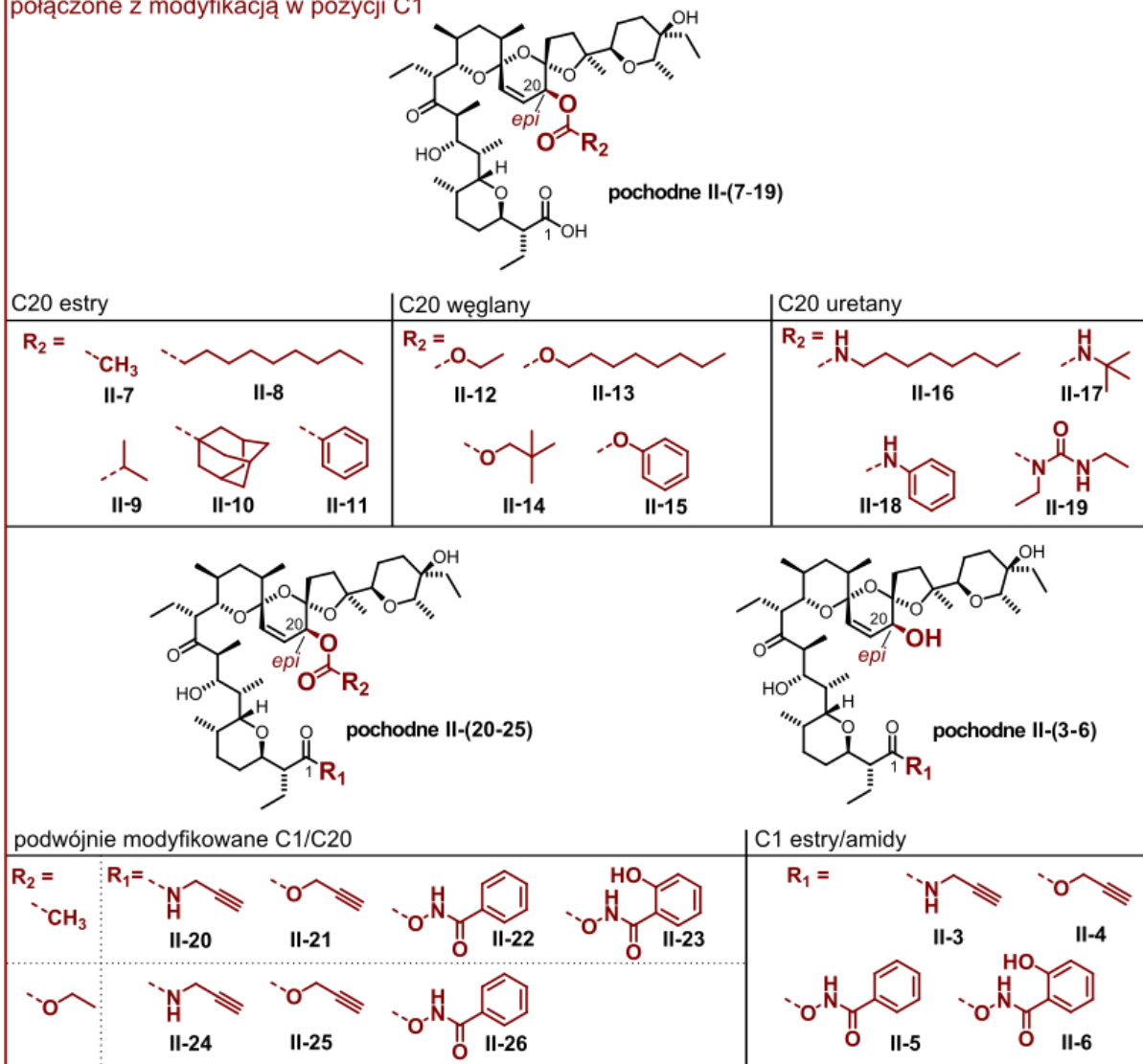
Powstający w wyniku reakcji nowy układ sprzężonych wiązań podwójnych umożliwił kontrolę reakcji przy zastosowaniu chromatografii cienkowarstwowej (TLC), wykorzystując lampę emitującą promieniowanie UV o długości fali 254 nm. Generowało to również bardzo widoczną zmianę w widmach węglowego magnetycznego rezonansu jądrowego ¹³C NMR, w których pojawiał się sygnał pomiędzy 190,3 a 190,6 ppm charakterystyczny dla α,β-nienasyconych ketonów. Pojawienie się dodatkowej grupy karbonylowej potwierdzało także wzrost intensywności pasma drgań rozciągających ν(C=O) w widmach FT-IR przy liczbie falowej ~ 1700 cm⁻¹. Co więcej, analiza rentgenograficzna wykonana dla monokryształów związków **I-3a**, **I-6b** oraz **I-9b** pozwoliła wykazać, że utlenienie grupy hydroksylowej w pozycji C20 nie wpływa znacząco na zmianę konformacji cząsteczki. Zarówno trzeciorzędowe amidy, jak i ich utlenione analogi wykazują duże podobieństwo konformacyjne, natomiast charakter podstawnika wprowadzonego w pozycję C1 może istotnie tę konformację zmienić.

Związki przedstawione w publikacji I zostały przebadane metodą MTT we współpracy z naukowcami z Uniwersytetu w Arkansas w Stanach Zjednoczonych. Do badań wybrano dwie nowotworowe linie komórkowe: linię pierwotnej ostrej białaczki limfoblastycznej (ALL-5) oraz linię ludzkiego gruczolakoraka piersi (MDA-MB-231). W celu wyznaczenia selektywności działania wykorzystano także prawidłową linię komórkową nabłonka sutka (MCF 10A). Wśród wszystkich przebadanych związków warto zwrócić uwagę na parę pochodnych **I-5a** (ALL-5 IC₅₀ = 1.02 ± 0.17 μM, SI = 10.12; MDA-MB-231 IC₅₀ = 4.11 ± 2.19 μM, SI = 2.51) oraz **I-5b** (ALL-5 IC₅₀ = 4.05 ± 0.71 μM, SI = 0.07; MDA-MB-231 IC₅₀ = 1.94 ± 0.14 μM, SI = 0.14), które posiadają to samo ugrupowanie *N,N*-dipropyloamidowe w pozycji

C1. Pomimo zbliżonych wartości aktywności cytotoksycznej, wprowadzenie ketonu w pozycję C20 prowadzi do znacznego spadku wartości współczynnika selektywności, sprawiając, że utleniony analog wykazują ten sam efekt wobec komórek zmienionych chorobowo oraz zdrowych. Zauważalne jest, że związki zawierające krótkie alkilowe łańcuchy (od 1 do 4 atomów węgla) wprowadzone w pozycję C1 wykazują większą aktywność wobec wybranych linii komórkowych niż pochodne zawierające długie łańcuchy alkilowe (8 i 12 atomów węgla), które nie wykazały żadnego działania cytotoksycznego w wybranym zakresie stężeń.

Publikacja II

Odwrośnienie konfiguracji absolutnej atomu węgla C20 oraz O-acylowanie w pozycji C20, połączone z modyfikacją w pozycji C1



Rysunek 5. Struktury pochodnych salinomycyny opisanych w publikacji II.

W 2016 roku opracowana została regio- i stereoselektywna ścieżka syntezy pozwalająca na otrzymanie C20-*epi*-salinomycyny charakteryzującej się odwróconą konfiguracją absolutną atomu węgla C20. Otrzymane C20-*epi*-estry salinomycyny wykazywały zarówno większą aktywność, jak i selektywność działania wobec komórek nowotworowych.⁶⁶ Doniesienia te zainspirowały mnie do przeprowadzenia syntezy nowej grupy 24 pochodnych salinomycyny przedstawionych w publikacji II (**Rysunek 5**). Wśród tych pochodnych wyróżnić można 4 pojedynczo modyfikowane związki w pozycji C1 (**II-(3-6)**), 13 pojedynczo modyfikowanych w pozycji C20 *epi*-estrów, *epi*-węglanów oraz *epi*-uretanów (**II-(7-19)**) oraz 7 analogów podwójnie modyfikowanych jednocześnie w pozycjach C1 i C20 (**II-(20-26)**). Syntezy powyższych związków oznaczono **ścieżką** na **Schemacie 1**.

Konformacja niemodyfikowanej cząsteczki salinomycyny nie umożliwia bezpośredniego odwrócenia konfiguracji grupy hydroksylowej w pozycji C20 w warunkach reakcji Mitsunobu; jest to spowodowane pseudocykliczną strukturą cząsteczki, która niejako chroni tę grupę hydroksylową. Aby to zmienić, konieczne było wprowadzenie dużego objętościowego podstawnika w pozycję C1. W celu zablokowania grupy karboksylowej w pozycji C1 wykorzystuje się 2-(trimetylsilyl)etanol (TMSEtOH) w obecności soli chloroformamidyniowej (TCFH) oraz 4-dimetyloaminopirydyny (DMAP). Otrzymany w wyniku tej reakcji ester poddawany jest, w warunkach reakcji Mitsunobu, działaniu kwasu 4-nitrobenzoesowego, azydokarboksylanu diizopropylu (DIAD) oraz trifenylofosfiny (PPh₃). Powstający C20-*epi*-ester 4-nitrobenzoesowy poddawany jest następnie hydrolizie przy użyciu węglanu potasu. Otrzymana w ten sposób C20-*epi*-salinomycyna z zablokowaną grupą karboksylową w pozycji C1 stanowiła dla mnie punkt wyjścia do dalszych modyfikacji. Warto podkreślić, że w opisanych warunkach obserwuje się pełną regioselektywność; pomimo że salinomycyna w swojej strukturze zawiera dwie dodatkowe grupy hydroksylowe, opisane przemiany wpływają wyłącznie na grupę hydroksylową w pozycji C20.

Dalsze modyfikacje rozpocząłam od wykorzystania roztworu fluorku tetra-*n*-butyloamoniowego (TBAF) w tetrahydrofuranie, w celu odblokowania grupy karboksylowej w pozycji C1. Następnie otrzymałam cztery pochodne (**II-(3-6)**) modyfikowane przy atomie węgla C1: amid **II-3** w reakcji z propargiloaminą, DCC oraz HOBt; ester **II-4** w reakcji z bromkiem propargilu w obecności 1,8-diazabicyklo[5.4.0]undek-7-enu (DBU); oraz dwa koniugaty z kwasami hydroksamowymi **II-5** oraz **II-6**, wykorzystując odpowiedni

kwaski hydroksamowy, DCC i DMAP. Sygnał od atomu węgla niemodyfikowanej grupy karboksylanowej C20-*epi*-salinomycyny obserwuje się w widmie ^{13}C NMR przy wartości 184,1 ppm, natomiast jej modyfikacja powoduje przesunięcie tego sygnału do wartości 174,6 ppm dla amidu propargilowego **II-3**, a w przypadku estrów **II-(4-6)** sygnał ten obserwowano w przedziale 173,4–174,8 ppm.

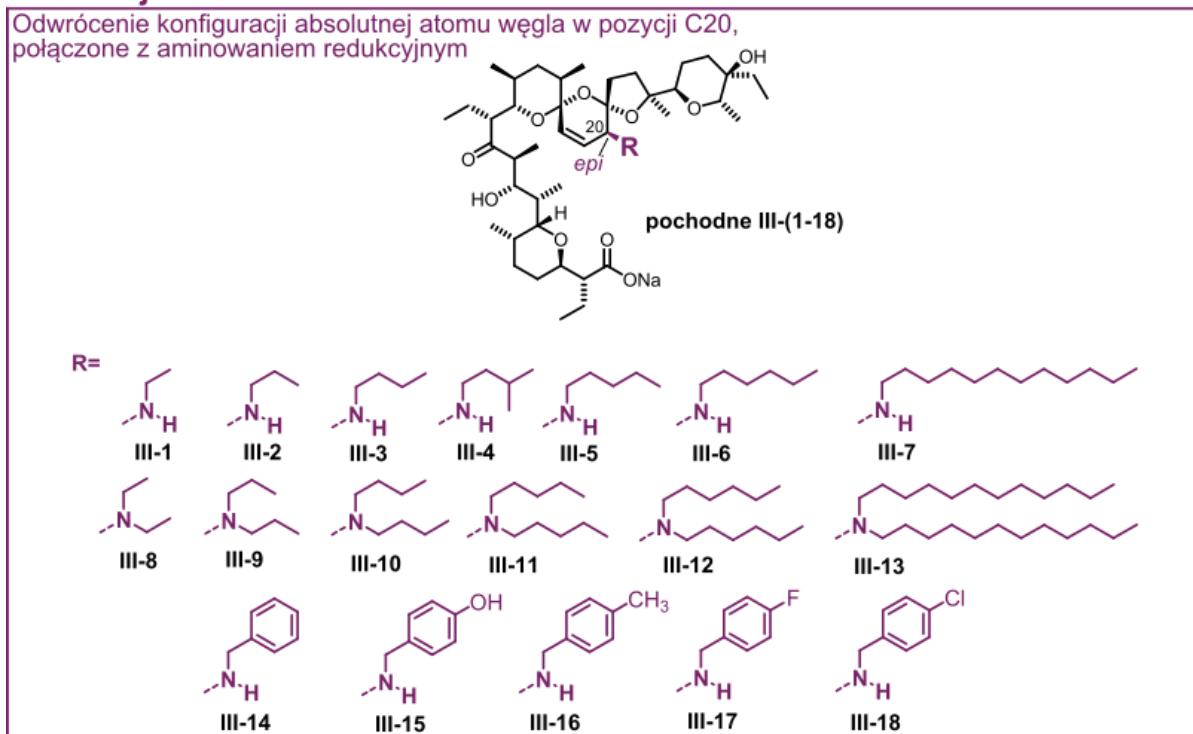
Mając do dyspozycji blokową C20-*epi*-salinomycynę, przeprowadziłam reakcje *O*-acylowania pozycji C20, a następnie odblokowania pozycji C1 przy użyciu roztworu TBAF, co pozwoliło na otrzymanie 5 C20-*epi*-estrów **II-(7-11)**, 4 C20-*epi*-węglanów **II-(12-15)** oraz 4 C20-*epi*-uretanów **II-(16-19)**. W celu jak największej dywersyfikacji pochodnych otrzymanych w pozycji C20, w każdej grupie znalazł się związek zawierający podstawnik o krótkim, długim (w tym z rozgałęzionym) łańcuchu alkilowym oraz podstawnik z pierścieniem aromatycznym. Potwierdzeniem wprowadzenia grupy *O*-acylowej do struktury w pozycji C20 była obecność dodatkowego sygnału od karbonylowego atomu węgla w widmach ^{13}C NMR odpowiednio w regionie 172,8–176,5 ppm dla C20 *epi*-estrów, 153,1–154,9 ppm dla C20-*epi*-węglanów oraz 152,7–156,0 ppm dla C20-*epi*-uretanów. W przypadkach podwójnego uretanu **II-19** odpowiednie sygnały zaobserwowano przy wartości 155,8 ppm.

Otrzymałam także podwójnie modyfikowane pochodne wykorzystując C20-*epi*-*O*-acylowane pochodne **II-7** oraz **II-12** zawierające odpowiednio ester metylowy oraz węglan etylowy w pozycji C20 i poddając je działaniu warunków reakcji użytych wcześniej dla pojedynczo modyfikowanych pochodnych (**II-(3-6)**).

Związki przedstawione w publikacji II również zostały przebadane z użyciem metody MTT we współpracy z naukowczyniami z Warszawskiego Uniwersytetu Medycznego. Do badań wybrano następujące nowotworowe linie komórkowe: linię pierwotnego raka okrężnicy (SW480), linię przerzutowego raka okrężnicy (SW620) oraz linię przerzutowego raka prostaty (PC3). Wykorzystano także prawidłową linię komórkową ludzkich keratynocytów (HaCaT). Wszystkie związki, oprócz tych zawierających w strukturze ester propargilowy (**II-4**, **II-21** oraz **II-25**), charakteryzowały się niskimi wartościami IC_{50} nieprzekraczającymi wartości 20 μM . Korzystnym właściwościom cytotoksycznym nie towarzyszyła wysoka selektywność, co uniemożliwiło preferencyjne działanie wyłącznie przeciwko komórkom nowotworowym. Jednakże, dokładniejsze badania wykazały,

że pochodne tej serii mają zdolność do indukowania późnej apoptozy w komórkach raka okrężnicy oraz nekrozy w komórkach raka prostaty, a także do hamowania wydzielania prozapalnej, onkogennej interleukiny-6 (IL-6). Analiza zależności między strukturą a aktywnością biologiczną uwiarygodniła, że przemiana grupy karboksylowej w amid propargilowy jest korzystniejsza dla aktywności przeciwproliferacyjnej w porównaniu z przemianą w ester propargilowy. Natomiast w przypadku modyfikacji pozycji C20, aktywność uzyskanych pochodnych przedstawia się następująco: C20-*epi*-węglany > C20-*epi*-estry > C20-*epi*-uretany, przy czym wprowadzenie łańcucha alkilowego w tę pozycję generuje dużo większą cytotoksyczność niż wprowadzenie pierścienia aromatycznego.

Publikacja III



Rysunek 6. Struktury pochodnych salinomycyny opisanych w publikacji III.

Pochodne **III-(1-18)** przedstawione w publikacji III (**Rysunek 6**) są dla mnie szczególnie istotne. Jest to seria 18 zupełnie nowych analogów salinomycyny, których ścieżka syntezy została przedstawiona **ścieżką** na **Schemacie 1**. Ich zaprojektowanie oraz synteza były podyktowane wynikami otrzymanymi w 2017 roku w grupie badawczej prof. Raphaëla Rodrigueza. Zaprezentowali oni na łamach czasopisma *Nature Chemistry* aminowe pochodne salinomycyny, otrzymane w wyniku stereoselektywnej reakcji aminowania redukcyjnego

C20-okso-salinomycyny zawierającej keton w pozycji C20.⁴³ Co istotne, pochodne te charakteryzowały się niezmienioną konfiguracją atomu węgla w pozycji C20 w porównaniu do samej salinomycyny. W szczególności jedna z tych pochodnych, nazwana ironomycyną, zawierająca w swojej strukturze ugrupowanie aminopropargilowe wykazała blisko 10-krotnie wyższą aktywność *in vitro* oraz *in vivo* wobec komórek macierzystych raka piersi od niemodyfikowanej salinomycyny. Pochodne te pozwoliły na zaproponowanie przeciwnowotworowego mechanizmu działania salinomycyny opartego na zaburzaniu homeostazy żelaza.⁴³

Bazując na pozytywnym wpływie zmiany konfiguracji atomu węgla w pozycji C20 na aktywność przeciwo proliferacyjną pochodnych salinomycyny, uzasadnione wydawało się sprawdzenie wpływu zmiany stereochemii atomu węgla C20 na potencjał przeciwnowotworowy aminowych pochodnych. W ramach projektu ETIUDA 8 (2020/36/T/ST4/00041) pt. „*Synteza i badania aktywności biologicznej pochodnych związków naturalnych - salinomycyny i kolchicyny*” otrzymałam serię nowych, aminowych pochodnych salinomycyny o odwróconej konfiguracji w pozycji C20, których aktywność przeciwnowotworową określiłam podczas 3-miesięcznego stażu w Instytucie Curie w Paryżu w grupie badawczej prof. Raphaëla Rodrigueza.

Czteroetapowa ścieżka syntezy C20-*epi*-aminosalinomycyny, została uprzednio opisana w literaturze⁶⁷ i jest ona zbliżona do ścieżki otrzymywania C20-*epi*-salinomycyny wykorzystanej w publikacji II. W pierwszej kolejności zablokowana została grupa karboksylowa w pozycji C1 (TMSEtOH, DMAP, TCFH). Następnie, wykorzystując reakcję Mitsunobu, wprowadzono w pozycję C20 grupę azydkową, wykorzystując azydek difenylofosforylowy (DPPA), DIAD oraz PPh₃. W kolejnym etapie przeprowadzono reakcję Staudingera, pozwalającą na przekształcenie grupy azydkowej w grupę aminową w obecności PPh₃ i niewielkiej ilości wody. Na koniec grupę karboksylową w pozycji C1 odblokowuje się z wykorzystaniem roztworu TBAF.

W moich badaniach wprowadziłam jednak pewną modyfikację do opisanej w literaturze metody syntezy C20-*epi*-aminosalinomycyny. Mianowicie, możliwe stało się pominięcie etapu wyodrębniania produktu reakcji Mitsunobu, tj. C20-*epi*-azydosalinomycyny, przeprowadzając reakcje Staudingera bez wyodrębniania produktu reakcji Mitsunobu. Pominięcie tego etapu pozwoliło znacząco skrócić czas syntezy, a także ograniczyć ilość

rozpuszczalników, które byłyby niezbędne do chromatograficznego oczyszczenia produktu tej reakcji. Poprawiło to także całkowitą wydajność procesu, gdyż każdy dodatkowy etap syntezy generował mniejsze lub większe straty. Wprowadzona przeze mnie modyfikacja pozwoliła mi na szybsze i efektywniejsze otrzymanie C20-*epi*-aminosalinomycyny w skali kilkugramowej.

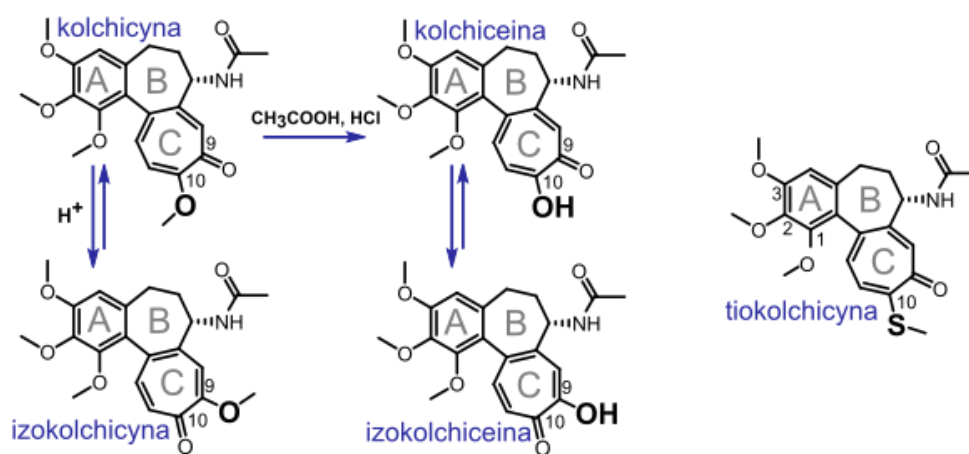
Mając do dyspozycji C20-*epi*-aminosalinomycynę przeprowadziłam reakcję aminowania (alkilowania) redukcyjnego, wykorzystując zróżnicowane strukturalnie aldehydy i cyjanoborowoderek sodu (NaBH_3CN) jako łagodny czynnik redukujący. Zmiana ilości wprowadzonego do reakcji aldehydu pozwoliła na sterowanie stechiometrią reakcji alkilowania redukcyjnego, generując odpowiednio drugorzędowe (**III-(1-7);(14-18)**) oraz trzeciorzędowe aminy (**III-(8-13)**) w pozycji C20.

W czasie mojego pobytu w Instytucie Curie przeszłam szkolenie w laboratorium biologicznym i miałam możliwość samodzielnego przeprowadzenia badań oceny cytotoksyczności pochodnych **III-(1-18)**. Co więcej, badania te wykonałam na liniach komórek nowotworowych o cechach komórek macierzystych (HMLER CD24^{low}/CD44^{high}) oraz ich odpowiedników niewykazujących cech komórek macierzystych (HMLER CD24^{high}/CD44^{low}), które są trudno dostępne w Polsce. Wszystkie przebadane pochodne wykazały większą aktywność cytotoksyczną wobec tych linii komórkowych niż niemodyfikowana salinomycyna. Dodatkowo, najaktywniejsze pochodne **III-8** oraz **III-9** charakteryzowały się wyjątkowo niskimi wartościami stężeń IC_{50} (odpowiednio 3 oraz 9 nM) w porównaniu do ironomycyny (170 nM) dla linii HMLER CD24^{low}/CD44^{high}. Co więcej, wskazane związki działały dużo silniej na linię komórkową o cechach komórek macierzystych niż na ich niemacierzyste odpowiedniki, z wartościami indeksu selektywności odpowiednio $\text{SI} = 90$ dla **III-8** oraz $\text{SI} = 20$ dla **III-9**. Związki przedstawione w publikacji III posiadają niezwykle potencjał terapeutyczny, który mógłby zostać wykorzystany w poszukiwaniu nowych substancji celujących w macierzyste komórki nowotworowe (CSCs). W związku z tym, Instytut Curie w Paryżu w porozumieniu z Uniwersytetem im. Adama Mickiewicza w Poznaniu dokonał międzynarodowego zgłoszenia patentowego dla pochodnych przedstawionych w publikacji III.

Modyfikacje struktury kolchicyny i ich wpływ na aktywność przeciwproliferacyjną

Nie sposób przejść do omawiania modyfikacji struktury kolchicyny bez krótkiego wyjaśnienia niekorzystnych przemian jakim ulega ten związek. Ich przedstawienie wydaje mi się niezbędne w kontekście przedstawienia wyników prowadzonych przeze mnie badań.

Niemodyfikowana kolchicyna wykazuje tendencję do izomeryzacji w środowisku kwasowym i podwyższonej temperaturze (**Rysunek 7**). Obecny w jej strukturze pierścień 10-metoksytropolonowy (pierścień C), w którym przy sąsiadujących atomach węgla C9 oraz C10 znajduje się grupa metoksylova oraz ketonowa, ulega zjawisku tautomerii prowadząc do powstania izokolchicyny. Jest to zjawisko o tyle niekorzystne, że forma izo tego związku wiąże się z tubuliną 500 razy słabiej niż związek pierwotny.⁶⁸ Ten niekorzystny proces można zatrzymać podstawiając grupę -OCH₃ w pozycji C10 kolchicyny grupą -SCH₃, stosując roztwór metanotiolanu sodu. Otrzymana tym sposobem tiokolchicyna nie ulega wspomnianej wyżej tautomerizacji, a ponadto charakteryzuje się niższą toksycznością i silniej wiąże się z tubuliną od niemodyfikowanej kolchicyny (**Rysunek 7**).^{69–71}

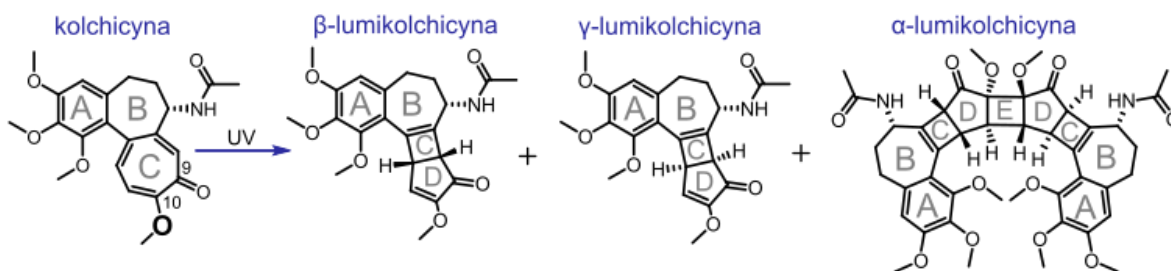


Rysunek 7. Struktura kolchicyny, izokolchicyny, kolchiceiny, izokolchiceiny i tiokolchicyny.

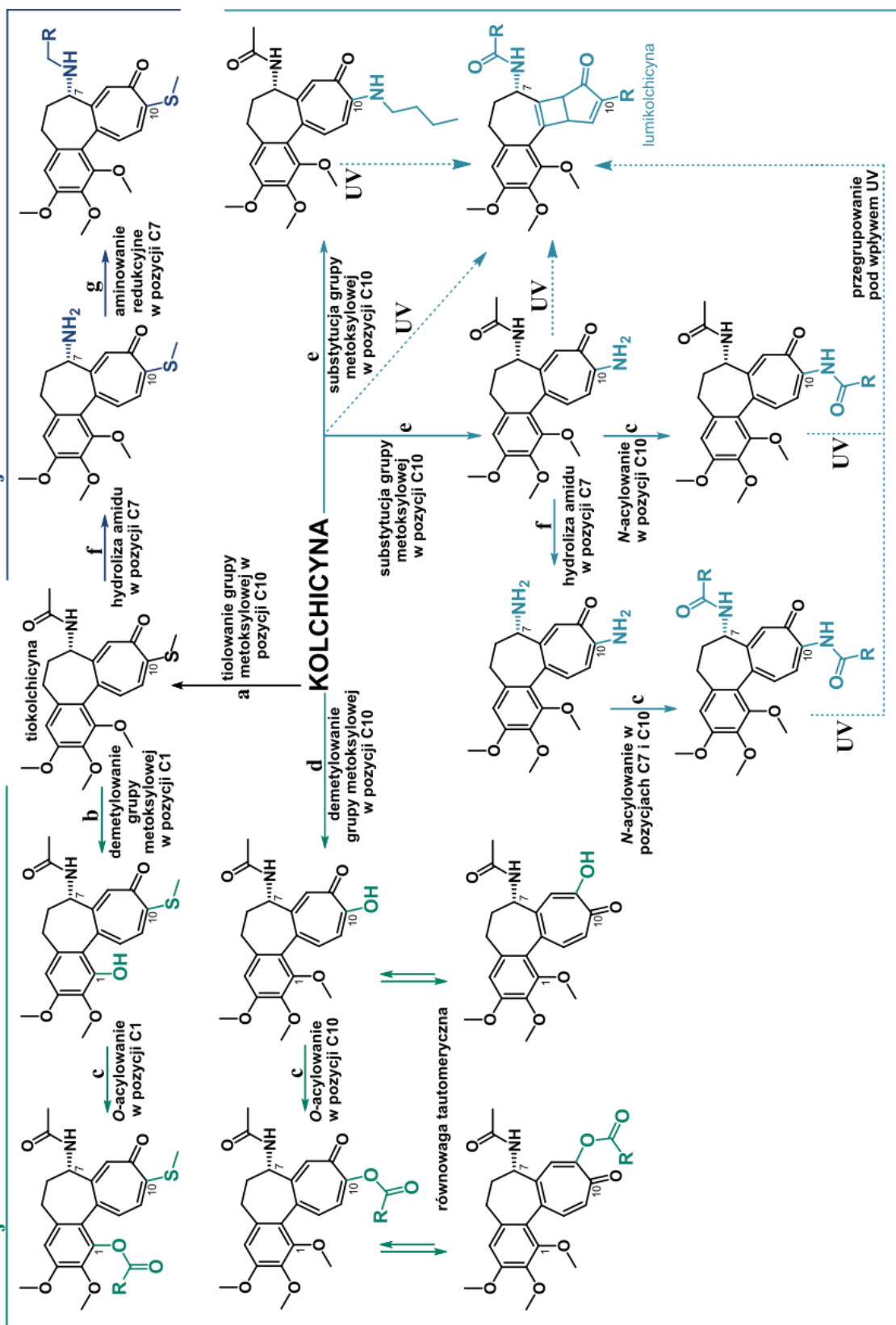
Celem modyfikacji chemicznej kolchicyny jest przede wszystkim zmniejszenie jej ogólnej toksyczności, przy jednoczesnym zachowaniu jej właściwości przeciwmiotycznych.^{45,68–77} Wykazano, że grupy metoksylova w pozycjach C1, C2 i C10, a także grupa karbonylova w pozycji C9 są kluczowe dla oddziaływania z tubuliną i zachowania przeciwmiotycznego działania kolchicyny.⁸² Co interesujące, demetylacja

jednej lub kilku grup metoksyłowych, oprócz obniżenia siły wiązania z tubuliną, prowadzi zarazem do zmniejszenia toksyczności analogów kolchicyny.^{71,83–85} Na przykład, działając mieszaniną lodowatego kwasu octowego i 0,1-molowego roztworu kwasu solnego na kolchicynę można przeprowadzić regioselektywną demetylację grupy metoksyłowej przy atomie węgla C10 otrzymując kolchiceinę (**Rysunek 7**). Jest ona głównym produktem degradacji kolchicyny i występuje naturalnie w ekstraktach roślinnych razem z kolchicyną.⁸⁶ Podobnie jak związek wyjściowy, również kolchiceina wykazuje skłonność do izomeryzacji ze względu na sąsiedztwo wolnej grupy hydroksylowej i ketonowej w pierścieniu tropolonowym. Badania rentgenograficzne wykazały, że równowaga tautomeryczna kolchiceiny i izokolchiceiny występuje również w ciele stałym (**Rysunek 7**). Najprawdopodobniej to właśnie tendencja kolchiceiny do tautomeryzacji w połączeniu z demetylacją w pozycji C10 odpowiada za jej słabą aktywność przeciwmityotyczną.⁶⁸

Struktura kolchicyny może ulegać jeszcze jednej istotnej przemianie. Pod wpływem promieniowania UV zawartego w świetle słonecznym metoksytropolonowy pierścień C kolchicyny ulega przegrupowaniu w sprzężony układ cztero- i pięcioczłonowych pierścieni C i D.^{87,88} Zaobserwowano, że w wyniku działania promieniowania UV powstawać mogą aż 3 produkty: α -, β - i γ -lumikolchicyna (**Rysunek 8**). Co ciekawe, sam pierścień tropolonowy i metoksytropolonowy nie przegrupowują się w opisany wyżej sposób, co może sugerować wpływ pozostałych pierścieni kolchicyny na to przegrupowanie, w szczególności trimetoksybenzenowego pierścienia A.^{89,90} Przegrupowanie metoksytropolonowego pierścienia C w kolchicynie skutkuje całkowitą utratą właściwości przeciwnowotworowych, ponieważ struktura tego związku nie umożliwia mu wiązania się z kolchicynowym miejscem wiążącym w tubulinie.⁹¹ Tiokolchicyna nie ulega temu przegrupowaniu, co dodatkowo potwierdza jej zalety i większą stabilność od niemodyfikowanej kolchicyny.⁹²



Rysunek 8. Struktury kolchicyny i jej fotoizomerów: α -, β i γ -lumikolchicyny.

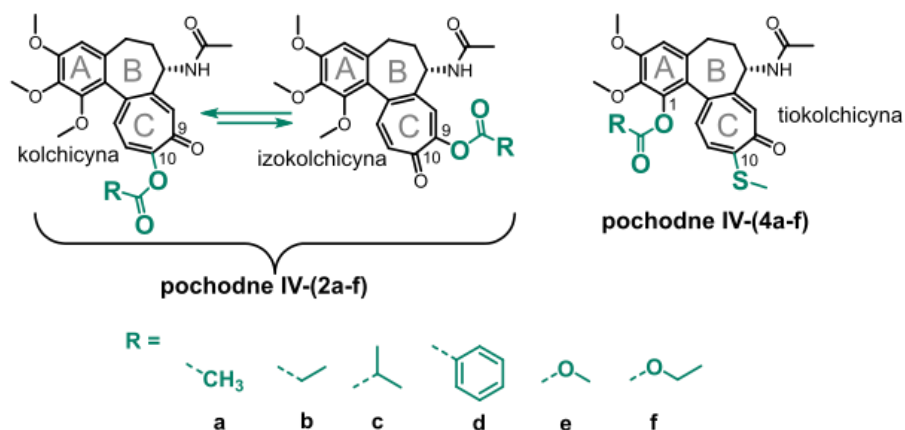


Schemat 2. Ścieżki syntetyczne podjęte w celu modyfikacji struktury kolchicyny. Warunki reakcji: (a) CH_3SNa , $\text{MeOH}/\text{H}_2\text{O}$, temp. pokojowa (tp); (b) chlorek acetylu, SnCl_4 , CH_2Cl_2 , 0°C do tp, następnie $\text{MeOH}/\text{H}_2\text{O}$, LiOH , tp; (c) chlorek kwasowy lub chloromówczan, Et_3N , CH_2Cl_2 , 0°C do tp; (d) $\text{CH}_3\text{COOH}/\text{HCl}$, 100°C ; (e) *n*-butoamina/amoniak, CH_3CN , temp. wrzenia; (f) HCl , MeOH , temp. wrzenia; (g) aldehyd, NaBH_3CN , $\text{CH}_2\text{Cl}_2/\text{MeOH}$, tp.

Moje badania związane z chemiczną modyfikacją struktury kolchicyny zostały opisane w publikacjach IV - VI. Przeprowadzone przeze mnie modyfikacje wraz z warunkami podjętych reakcji przedstawiono na **Schemacie 2**. W wyniku przeprowadzonych syntez otrzymałam łącznie 52 analogi kolchicyny, spośród których aż 48 otrzymano po raz pierwszy, a pozostałe związki były niezbędnymi produktami pośrednimi do dalszych modyfikacji. Dane spektroskopowe znajdują się na końcu niniejszej rozprawy, w publikacjach i/lub suplementach dołączonych do tych publikacji. Tabele zawierające wyniki przeprowadzonych badań aktywności przeciwproliferacyjnej *in vitro* także można znaleźć w załączonych publikacjach.

Publikacja IV

Estrowe i węglanowe pochodne otrzymane w wyniku selektywnego demetylowania grupy metoksyłowej w pozycji C1 tiokolchicyny oraz w pozycji C10 kolchicyny



Rysunek 9. Struktury pochodnych kolchicyny opisanych w publikacji IV.

Doniesienia o wpływie demetylowania grup metoksyłowych na aktywność przeciwnowotworową kolchicyny były dla mnie inspiracją do sprawdzenia, jaki efekt wywoła wprowadzenie w miejsce jednej z grup metoksyłowych większego objętościowo podstawnika węglanowego lub estrowego. W tym celu otrzymałam serię pochodnych kolchicyny przedstawionych w publikacji IV (**Rysunek 9**), których ścieżka syntezy została przedstawiona **ścieżką** na **Schemacie 2**.

W pierwszej kolejności przeprowadziłam selektywne demetylowanie grupy metoksyłowej przy atomie węgla C10 kolchicyny, otrzymując kolchiceinę, korzystając z uprzednio opisanej w literaturze metody.⁹³ Potwierdzeniem przeprowadzenia demetylowania był zanik sygnału przy wartości 56,5 ppm w widmie ^{13}C NMR pochodzącego od grupy

metoksylowej w pozycji C10. Potwierdzenie było również możliwe dzięki analizie rentgenograficznej otrzymanych kryształów kolchiceiny. Następnie, przeprowadziłam O-acylowanie wolnej grupy hydroksylowej w obecności trietyloaminy (TEA), stosując zróżnicowane strukturalnie chlorki kwasowe lub chloromrówczany. W ten sposób otrzymałam cztery estry **IV-(2a-d)** oraz dwa węglany **IV-(2e-f)**. Ze względu na dużą podatność samej kolchiceiny na tautomeryzację, jej O-acylowane pochodne również pozostają ze sobą w równowadze tautomerycznej (**Rysunek 9**), co odzwierciedlają widma NMR, w których wartości przesunięć chemicznych sygnałów pochodzących od atomów węgla miały wartości pośrednie między jedną a drugą formą. Co ciekawe, pochodne te nie wykazywały tendencji do krystalizacji, w przeciwieństwie do niemodyfikowanej kolchiceiny, której monokryształy były tożsame z tymi otrzymanymi w 1985 roku.⁹⁴

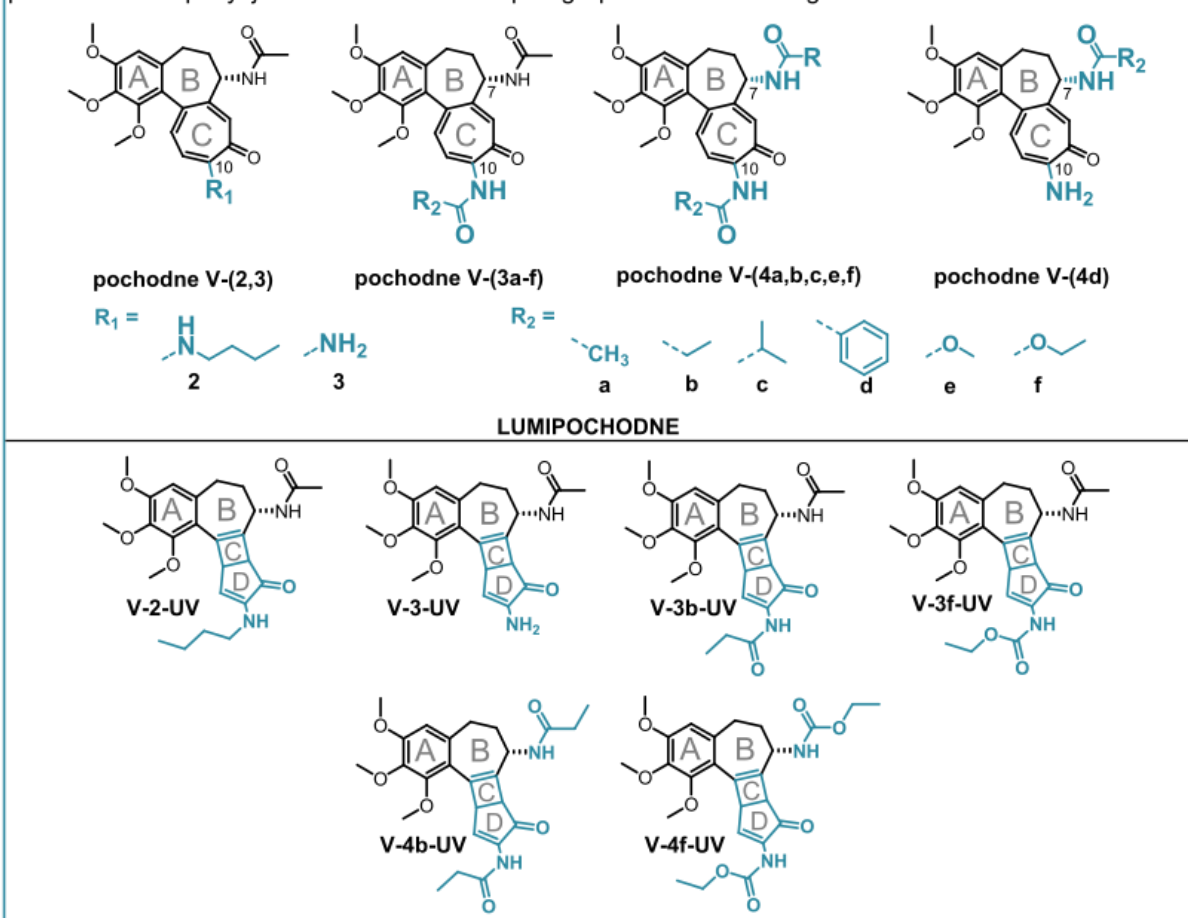
Ponieważ tiokolchicina posiadająca grupę tiometylową w pozycji C10 nie jest podatna na tautomeryzację, to właśnie na niej postanowiłam przeprowadzić kolejne selektywne demetylowanie. Wprowadzenie ugrupowania tiometylowego do struktury kolchicyny bardzo łatwo zaobserwować w widmie ¹³C NMR, ponieważ sygnał pochodzący od grupy metoksylowej w pozycji C10 ulega przesunięciu z 56,5 ppm do 15,1 ppm. Poddając tiokolchicinę działaniu chlorku acetylu w obecności chlorku cyny(IV), a następnie przeprowadzając hydrolizę powstałego estru wykorzystując wodorotlenek litu, otrzymałam 1-demetylotiokolchicinę z wolną grupą hydroksylową w pozycji C1. Potwierdzeniem struktury produktu był zanik sygnału w widmie ¹³C NMR przy wartości około 61 ppm, odpowiadającego grupie metoksylowej przy atomie węgla C1, a także przeprowadzona analiza rentgenograficzna otrzymanych kryształów.⁹⁵ Następnie przeprowadziłam O-acylowanie otrzymanej 1-demetylotiokolchiciny w sposób opisany powyżej, co doprowadziło do otrzymania pochodnych **IV-(4a-f)**.

Związki przedstawione w publikacji IV zostały przebadane pod kątem aktywności przeciwproliferacyjnej z użyciem metody SRB, we współpracy z grupą badawczą prof. Wietrzyk. Do badań wybrano następujące nowotworowe linie komórkowe: linię ludzkiego gruczolaka płuc (A549), linię ludzkiego gruczolaka piersi (MCF-7) oraz linię ludzkiego gruczolaka jelita grubego (LoVo). Wykorzystano także prawidłową linię mysich fibroblastów (BALB/3T3). Jako odnośnik wykorzystano powszechnie stosowane leki przeciwnowotworowe - cisplatynę oraz doksorubicynę. Wszystkie przebadane związki

wykazały potencjał terapeutyczny przewyższający aktywność cisplatyny względem wskazanych linii komórkowych. Co istotne, zaobserwowano, że *O*-acylowanie może prowadzić do zwiększenia cytotoksyczności analogów kolchicyny; niemal wszystkie związki okazały się bardziej aktywne niż demetylowane związki wyjściowe – kolchiceina i 1-demetylotiokolchicyna. Nie przewyższyły one jednak aktywnością niemodyfikowanej kolchicyny.

Publikacja V

Amidowe i uretanowe pochodne kolchicyny; pojedynczo podstawione w pozycji C10, podwójnie podstawione w pozycjach C7 i C10 oraz ich przegrupowane lumianalogi



Rysunek 10. Struktury pochodnych kolchicyny opisanych w publikacji V.

Kontynuując pracę nad syntezą bardziej stabilnych analogów kolchicyny, postanowiłam otrzymać jej *N*-acylowane pochodne, które zostały przedstawione w publikacji V (**Rysunek 10**). Ścieżkę syntezy przedstawiono **kolorem** na **Schemacie 2**. W tym celu, w pierwszej kolejności poddałam kolchicynę działaniu *n*-butyloaminy (**V-2**) oraz wodnego roztworu amoniaku (**V-3**), w celu wprowadzenia ugrupowania aminowego w pozycję C10,

czego potwierdzeniem ponownie był zanik sygnału przy wartości 56,5 ppm w widmie ^{13}C NMR pochodzącego od grupy metoksylovej przy atomie węgla C10. Mając dostęp do 10-aminokolchicyny z wolną grupą aminową w pozycji C10, przeprowadziłam jej *N*-acylowanie, korzystając z metody przedstawionej w publikacji IV, otrzymując pojedynczo modyfikowane amidowe i uretanowe pochodne **V-(3a-f)**. W celu poszerzenia biblioteki związków, postanowiłam także otrzymać podwójnie modyfikowane pochodne kolchicyny, przeprowadzając hydrolizę amidu w pozycji C7 10-aminokolchicyny. Skutkowało to zanikiem kolejnych dwóch sygnałów w widmie ^{13}C NMR, odpowiednio przy wartościach 168,4 ppm i 22,6 ppm, przypisanych do grupy acetylovej. Powstała 7,10-diaminokolchicinę posiadającą dwie wolne grupy aminowe również poddałam reakcji acylowania, otrzymując podwójnie modyfikowane pochodne **V-(4b,c,e,f)** oraz pojedynczo modyfikowaną pochodną **V-4d**; w tym przypadku nie doszło do podwójnego podstawienia ugrupowania benzoilowego, pozostawiając jedną wolną grupę aminową w pozycji C10. Dla pochodnych **V-(3a-f)** oraz **V-4d** zaobserwowano dodatkowy sygnał w widmie ^{13}C NMR pochodzący od jednego karbonylovego atomu węgla, natomiast w widmach związków **V-(4b,c,e,f)** obecne były dwa takie sygnały.

W celu potwierdzenia struktury otrzymanych *N*-acylowanych pochodnych podjęłam próby ich krystalizacji. Jednak struktury uzyskane dla monokryształów związków **V-4b** oraz **V-4f** odpowiadały ich fotoizomerom **V-4b-UV** i **V-4f-UV** (**Rysunek 10**); już pośrednia obecność światła słonecznego docierająca do roztworu przez ścianki przezroczystych probówek była wystarczająca do przegrupowania pierścienia tropolonowego C. Odkrycie tak niskiej stabilności azotowych analogów kolchicyny było dla nas zaskoczeniem i skłoniło mnie do podjęcia próby dokładnego przebadania tego zjawiska na innych pochodnych tej serii. Do naświetlenia metanolowych roztworów pochodnych kolchicyny wykorzystałam fotoreaktor wyposażony w średnociśnieniową lampę rtęciową TQ 150W z filtrem pyreksowym przepuszczającym promieniowanie o $\lambda > 290$ nm, co pozwoliło na otrzymanie sześciu lumipochodnych z wydajnością izolacyjną 31 – 60% (**Rysunek 10**). Otrzymałam również β -lumikolchicinę w formie monokrystalicznej, co pozwoliło po raz pierwszy na określenie jej struktury krystalicznej z wykorzystaniem metod dyfrakcji rentgenowskiej. Badania strukturalne wykonane we współpracy z prof. Katrusiakiem wykazały, że podczas prowadzenia reakcji powstaje tylko jeden fotoprodukt, odpowiadający formie β ; jedynie w tym

izomerze możliwa jest stabilizacja poprzez wytworzenie wewnątrzcząsteczkowego wiązania wodorowego pomiędzy grupą karbonylową nowo powstałego pierścienia D a ugrupowaniem amidowym w pozycji C7 kolchicyny.

Przegrupowanie w pierścieniu tropolonowym C kolchicyny nie może zostać zaobserwowane przy użyciu spektrometrii mas, ponieważ przegrupowane pochodne charakteryzują się dokładnie tą samą masą, co nieprzegrupowane związki. Lumipochodne są jednak mniej polarne, co łatwo zaobserwować przy zastosowaniu chromatografii cienkowarstwowej (TLC). Roztwory przegrupowanych pochodnych kolchicyny są częściej brązowe lub zielone niż żółte i pomarańczowe, co byłoby charakterystyczne dla związków nieprzegrupowanych. Przegrupowanie można było również zaobserwować w widmach absorpcyjnych UV-VIS; zanikowi pasma o maksimum absorpcji przy długości fali 350 nm pochodzącego od tropolonowego pierścienia C towarzyszyło pojawienie się nowego pasma przy długości fali 280 nm. Widma UV-VIS znajdują się w suplemencie do publikacji V. Istotne różnice są również widoczne w widmach NMR i związane są z utratą aromatyczności tropolonowego pierścienia C; m.in. widoczne jest przesunięcie sygnału pochodzącego od atomu węgla C9 z 175,5 ppm do 202,5 ppm.

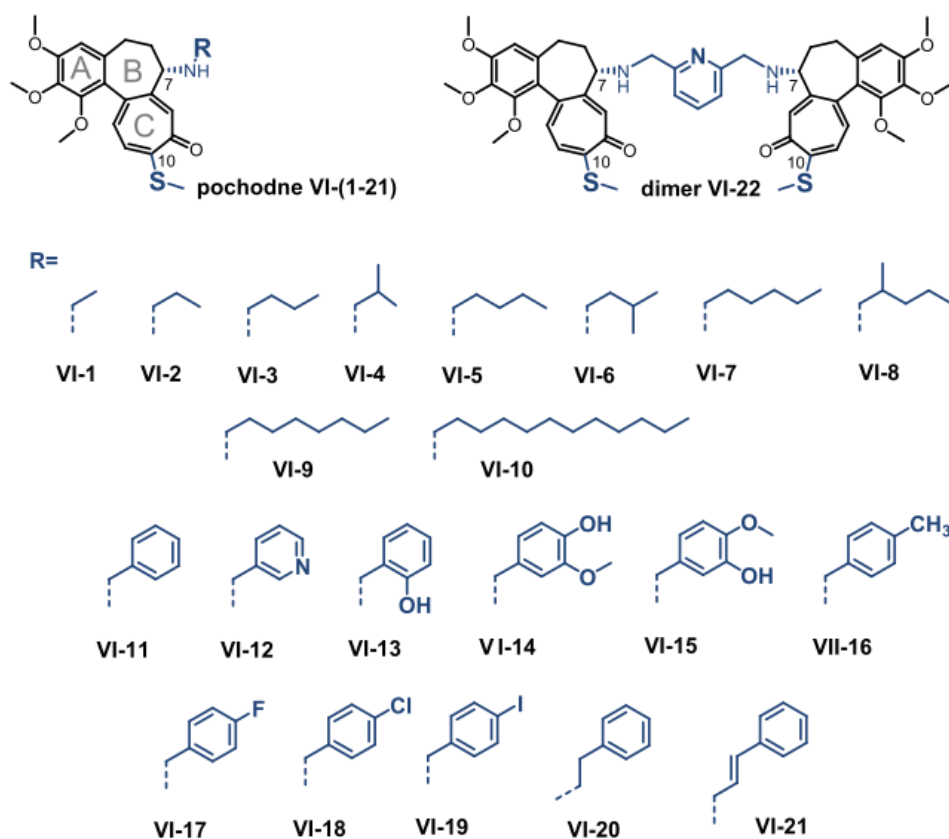
We współpracy z prof. Pędzińskim ustaliliśmy również fotochemiczny mechanizm przegrupowania *N*-podstawionych pochodnych kolchicyny. Wykorzystując technikę femtosekundowej laserowej fotolizy błyskowej, nie potwierdziliśmy wcześniejszych doniesień literaturowych, według których przegrupowanie miałoby zachodzić z udziałem tripletowego stanu wzbudzonego. Zaobserwowano natomiast szybki zanik najniższego wzbudzonego stanu singletowego S₁, który został przypisany do częściowo odwracalnego procesu wewnątrzcząsteczkowego przeniesienia protonu w stanie wzbudzonym (ang. *Excited State Intramolecular Proton Transfer, ESIPT*). W badanym układzie *N*-podstawionych kolchicyn, 5-członowy układ wiązań wodorowych tworzący się pomiędzy karbonylowym atomem tlenu w pozycji C9 a protonem przy atomie azotu w pozycji C9 mógłby umożliwiać przeniesienie protonu i w konsekwencji fotoizomeryzację pierścienia C.

Zastanawiającym jest fakt, iż w licznych doniesieniach literaturowych na temat nowych pochodnych kolchicyny brakuje badań ich fotostabilności. Zaobserwowane przeze mnie zjawisko przegrupowania tropolonowego pierścienia C jest natomiast niezwykle istotne w kontekście poszukiwania nowych inhibitorów polimeryzacji tubuliny. Badania biologiczne

przeprowadzone we współpracy z grupą badawczą prof. Wietrzyk potwierdziły, że fotoizomeryzacja prowadzi do utraty właściwości przeciwmiotycznych kolchicyny. Jak wskazałam wyżej, zjawisko to może być spowodowane nawet światłem słonecznym. Niedostateczne zabezpieczenie pochodnych kolchicyny przed działaniem światła może prowadzić do ich przegrupowania, np. w czasie między syntezą a badaniami biologicznymi, co może prowadzić do otrzymania dużo gorszych niż zakładane wyników tych badań; w przypadku związków z publikacji V, lumipochodne charakteryzowały się nawet 1000-krotnie niższą aktywnością cytotoksyczną niż ich nieprzegrupowane analogi.

Publikacja VI

Aminowe pochodne tiokolchicyny w pozycji C7 otrzymane w wyniku aminowania redukcyjnego



Rysunek 11. Struktury pochodnych kolchicyny opisanych w publikacji VI.

Publikacja VI (**Rysunek 11**) opisuje syntezę 22 analogów tiokolchicyny **VI-(1-22)**, których ścieżka syntezy została przedstawiona **kolorem** na **Schemacie 2**. Praca ta zawiera

element wspólny z publikacją III; w obydwu przypadkach w syntezie pochodnych wykorzystano tę samą reakcję alkilowania redukcyjnego (aminowania redukcyjnego).

Syntezę tej serii pochodnych rozpoczęłam, wykorzystując reakcje opisane wcześniej w publikacjach IV i V; w pierwszym kroku przeprowadziłam tiolowanie pozycji C10 kolchicyny, a następnie hydrolizę amidu w pozycji C7. Otrzymaną 7-deacetylotiokolchicynę z wolną grupą aminową przy atomie węgla C7 poddałam następnie alkilowaniu redukcyjnemu, stosując odpowiednie aldehydy alifatyczne oraz aromatyczne w obecności cyjanoborowodoru sodu (NaBH_3CN) jako czynnika redukującego. W ten sposób otrzymałam 10 nowych pochodnych tiokolchicyny z łańcuchami alifatycznymi **VI-(1–10)** oraz 11 nowych pochodnych z podstawnikami aromatycznymi **VI-(11–21)**. Dodatkowo, wykorzystanie pirydyno-2,6-dikarbaldehydu pozwoliło mi na zsyntetyzowanie dimeru **VI-22**. Czystość otrzymanych związków została potwierdzona spektroskopowo (^1H i ^{13}C NMR) oraz spektrometrycznie (ESI-MS).

Pochodne przedstawione w publikacji VI również zostały przebadane pod kątem aktywności przeciwproliferacyjnej we współpracy z grupą badawczą prof. Wietrzyk, wykorzystując wspomniane wcześniej linie komórkowe (A549, MCF-7, LoVo, BALB/3T3), ale także jedną dodatkową linię ludzkiego gruczolaka jelita grubego oporną na doksorubicynę (LoVo/DX). Wiele spośród pochodnych **VI-(1-22)** wykazało silniejsze działanie przeciwproliferacyjne od niemodyfikowanej kolchicyny, a także cisplatyny i doksorubicyny. Wszystkie związki (z wyjątkiem dimeru **VI-22**) przełamywały lekooporność linii LoVo/DX ($\text{RI} < 10$), a aż 10 pochodnych charakteryzowało się wartościami $\text{RI} < 2$. Co ważne, zaobserwowano wyraźną zależność między długością wprowadzonego łańcucha alkilowego a aktywnością cytostatyczną; dla długich łańcuchów alifatycznych tj. *n*-oktyłowego i *n*-dodecyłowego, obserwowano nawet 100-krotny spadek wartości współczynnika IC_{50} w stosunku do krótkich łańcuchów. Natomiast wśród podstawników benzyłowych podstawionych w pozycji *para* odpowiednio atomem fluoru, chloru i jodu (**VI-(17-19)**) zauważono spadek aktywności wraz ze wzrostem masy atomowej halogenu. Warto również zwrócić uwagę na związki **VI-14** oraz **VI-15** zawierające tożsame ugrupowania izowanilionowe i wanilinowe; pochodne te wykazywały zbliżone wartości IC_{50} w stosunku do linii A549 i MCF-7, podczas gdy **VI-14** wykazywał 6-krotnie wyższą aktywność wobec linii komórkowej LoVo niż **VI-15** (odpowiednio $\text{IC}_{50} = 8,8 \pm 4,6$ i $\text{IC}_{50} = 53,3 \pm 16,5$ nM).

Za najbardziej aktywny spośród tej serii związków należy uznać **VI-15**, który charakteryzował się najbardziej obiecującą aktywnością ($IC_{50} = 7,1 - 85,6$ nM) wobec wszystkich testowanych linii komórek nowotworowych (A549, MCF-7, LoVo i LoVo/DX), ponadprzeciętną selektywnością ($SI = 4,5 - 54,2$) oraz bardzo dobrym wskaźnikiem lekooporności ($RI = 1,6$). Związek **VI-15** niewątpliwie jest obiecującym kandydatem do dalszych badań.

Wnioski i podsumowania

- W wyniku prowadzonych badań zsyntezowałam 60 pochodnych salinomycyny, spośród których 49 otrzymano po raz pierwszy;
- Sprawdzono cytotoksyczność związków (**I-(3a-13a)**) oraz (**I-3b-9b; 11b-13b**) wobec linii komórkowej pierwotnej ostrej białaczki limfoblastycznej (ALL-5) oraz ludzkiego gruczolaka piersi (MDA-MB-231). Zauważono, że związki zawierające w pozycji C1 trzeciorzędowe amidy o krótkich podstawnikach alkilowych wykazują większą aktywność wobec wybranych linii komórkowych niż związki zawierające w tej pozycji długie podstawniki alkilowe;
- Dla pochodnych salinomycyny **I-5a** i **I-5b** posiadających w pozycji C1 to samo ugrupowanie *N,N*-dipropyloamidowe i charakteryzujących się porównywalną cytotoksycznością zauważono, że utlenienie grupy hydroksylowej w pozycji C20 salinomycyny prowadzi do znaczącego spadku selektywności;
- Aktywność pochodnych salinomycyny **II-(3-26)** przebadano wobec linii komórkowej pierwotnego raka okrężnicy (SW480), przerzutowego raka okrężnicy (SW620) oraz przerzutowego raka prostaty (PC3). Wykazano, że przemiana grupy karboksylowej w pozycji C1 w amid propargilowy jest korzystniejsza dla aktywności przeciwproliferacyjnej w porównaniu z przemianą w ester propargilowy;
- Aktywność pochodnych salinomycyny **II-(3-26)** modyfikowanych w pozycji C20 przedstawić można w następujący szereg: C20-*epi*-węglany > C20-*epi*-estry > C20-*epi*-uretany. Co więcej, wprowadzenie w tę pozycję łańcucha alkilowego generuje dużo większą cytotoksyczność niż wprowadzenie pierścienia aromatycznego;
- Pochodne salinomycyny **II-(3-26)** były także zdolne do indukowania późnej apoptozy w komórkach raka okrężnicy oraz nekrozy w komórkach raka prostaty, a także do hamowania wydzielania prozapalnej, onkogennej interleukiny-6 (IL-6);
- Wprowadziłam modyfikację do znanej uprzednio ścieżki syntezy C20-*epi*-aminosalinomycyny, przeprowadzając reakcje Staudingera bez wyodrębniania produktu reakcji Mitsunobu (reakcja one-pot). Pozwoliło to na poprawę całkowitej wydajności tej syntezy, ograniczenie ilości potrzebnych do oczyszczania

rozpuszczalników i skrócenie czasu całego procesu otrzymywania C20-*epi*-aminosalinomycyny w skali gramowej;

- Podczas 3-miesięcznego stażu w Instytucie Curie w Paryżu samodzielnie przeprowadziłam badania oceny cytotoksyczności pochodnych salinomycyny **III-(1-18)**. Badania te przeprowadziłam na liniach komórek nowotworowych o cechach komórek macierzystych (HMLER CD24^{low}/CD44^{high}) oraz ich odpowiedników niewykazujących cech komórek macierzystych (HMLER CD24^{high}/CD44^{low}), które są trudno dostępne w Polsce;
- Najaktywniejsze pochodne **III-8** oraz **III-9** charakteryzowały się wyjątkowo niskimi wartościami stężeń IC₅₀ (odpowiednio 3 nM oraz 9 nM) oraz wysokimi wartościami indeksu selektywności (SI = 90 dla **III-8** oraz SI = 20 dla **III-9**);
- Ze względu na niezwykle potężny potencjał terapeutyczny, pochodne salinomycyny **III-(1-18)** stały się przedmiotem międzynarodowego zgłoszenia patentowego dokonanego pomiędzy Instytutem Curie w Paryżu a Uniwersytetem im. Adama Mickiewicza w Poznaniu. Postępowanie patentowe jest obecnie w toku;
- Zsyntezowałam również 52 analogi kolchicyny, spośród których aż 48 otrzymano po raz pierwszy;
- Aktywność przeciwproliferacyjną pochodnych kolchicyny wyznaczono wobec linii ludzkiego gruczolaka płuc (A549), ludzkiego gruczolaka piersi (MCF-7), ludzkiego gruczolaka jelita grubego (LoVo), a także ludzkiego gruczolaka jelita grubego opornego na doksorubicynę (LoVo/DX);
- *O*-acylowane pochodne kolchicyny **IV-(2a-f)** oraz **IV-(4a-f)** były bardziej aktywne niż demetylowane związki wyjściowe – kolchiceina i 1-demetylotiokolchicyna, nie przewyższyły one jednak aktywnością niemodyfikowanej kolchicyny;
- Wykazałam, że *N*-podstawione pochodne kolchicyny w pozycjach C7 i C10 charakteryzują się bardzo niską fotostabilnością; do przegrupowania pierścienia tropolonowego C wystarczy ekspozycja na światło słoneczne.
- Odtworzyłam efekt jaki promieniowanie UV zawarte w świetle słonecznym wywiera na *N*-podstawione pochodne kolchicyny, wykorzystując średniociśnieniową lampę rtęciową. W wyniku naświetlania ich metanolowych roztworów otrzymałam sześć przegrupowanych lumikolchicyn;

- Po raz pierwszy otrzymałam także β -lumikolchicynę w formie monokrystalicznej, co pozwoliło na rentgenograficzne potwierdzenie jej struktury. Struktury krystalograficzne zostały wyznaczone także dla dwóch *N*-podstawionych lumipochodnych **V-4b-UV** i **V-4f-UV**;
- Potwierdziliśmy także doniesienia literaturowe dotyczące przewagi izomeru β nad izomrem γ -lumikolchicyny; jedynie w izomerze β możliwa jest stabilizacja poprzez wytworzenie wewnątrzcząsteczkowego wiązania wodorowego pomiędzy grupą karbonylową nowopowstałego pierścienia D a ugrupowaniem amidowym w pozycji C7 kolchicyny;
- Wykazałam także, że fotostabilność jest ważnym parametrem, który powinien być brany pod uwagę podczas otrzymywania nowych inhibitorów polimeryzacji tubuliny wywodzących się z kolchicyny. Przeprowadzone badania biologiczne jednoznacznie wykazały, że lumipochodne kolchicyny charakteryzowały się nawet 1000-krotnie niższą aktywnością cytotoksyczną niż ich nieprzegrupowane analogi;
- Dla aminowych analogów kolchicyny **VI-(1-22)** zauważono, że aktywność cytotoksyczna spada wraz z wydłużaniem łańcucha alkilowego oraz ze wzrostem masy halogenu przy pierścieniu benzylovym w pozycji C7;
- Wszystkie pochodne kolchicyny **VI-(1-22)** przełamywały lekooporność linii LoVo/DX, wykazując wartości RI w zakresie 8,5 – 0,8 (z wyjątkiem **VI-22**, RI = 14,3);
- Najbardziej obiecującym kandydatem do dalszych badań jest związek **VI-15**, wykazujący cytotoksyczność w zakresie $IC_{50} = 7,1 - 85,6$ nM wobec linii A549, MCF-7, LoVo i LoVo/DX, ponadprzeciętną selektywność (SI = 4,5 - 54,2) oraz bardzo dobrą wartością współczynnika przełamywania lekooporności RI = 1,6.
- Przeprowadzone przeze mnie badania dowiodły także słuszności wyboru obiektów badawczych – związków naturalnych o dużym potencjale przeciwnowotworowym. Ich modyfikacje pozwoliły mi wykazać, że dobrze zaplanowana synteza analogów salinomycyny i kolchicyny może doprowadzić do otrzymania nowych i niejednokrotnie bardziej aktywnych pochodnych, zaś potęgę tkwiącą w naturze można udoskonalić.

Bibliografia

- (1) Czerwonka, D.; Urbaniak, A.; Sobczak, S.; Piña-Oviedo, S.; Chambers, T. C.; Antoszczak, M.; Huczyński, A. Synthesis and Anticancer Activity of Tertiary Amides of Salinomycin and Their C20-Oxo Analogues. *ChemMedChem* **2020**, *15* (2), 236–246. <https://doi.org/10.1002/cmdc.201900593>.
- (2) Czerwonka, D.; Mielczarek-Putra, M.; Antoszczak, M.; Cioch, A.; Struga, M.; Huczyński, A. Evaluation of the Anticancer Activity of Singly and Doubly Modified Analogues of C20-Epi-Salinomycin. *Eur. J. Pharmacol.* **2021**, *908*, 174347. <https://doi.org/10.1016/j.ejphar.2021.174347>.
- (3) Czerwonka, D.; Müller, S.; Cañeque, T.; Colombeau, L.; Huczyński, A.; Antoszczak, M.; Rodriguez, R. Expeditive Synthesis of Potent C20- Epi -Amino Derivatives of Salinomycin against Cancer Stem-Like Cells . *ACS Org. Inorg. Au* **2022**. <https://doi.org/10.1021/acsorginorgau.1c00046>.
- (4) Czerwonka, D.; Sobczak, S.; Maj, E.; Wietrzyk, J.; Katrusiak, A.; Huczyński, A. Synthesis and Antiproliferative Screening of Novel Analogs of Regioselectively Demethylated Colchicine and Thiocolchicine. *Molecules* **2020**, *25* (5). <https://doi.org/10.3390/molecules25051180>.
- (5) Czerwonka, D.; Sobczak, S.; Pędziński, T.; Maj, E.; Wietrzyk, J.; Celewicz, L.; Katrusiak, A.; Huczyński, A. Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives. *J. Org. Chem.* **2021**, *86* (16), 11029–11039. <https://doi.org/10.1021/acs.joc.0c02507>.
- (6) Czerwonka, D.; Maj, E.; Wietrzyk, J.; Huczyński, A. Synthesis of Thiocolchicine Amine Derivatives and Evaluation of Their Antiproliferative Activity. *Bioorg. Med. Chem. Lett.* **2021**, *52*, 128382. <https://doi.org/10.1016/j.bmcl.2021.128382>.
- (7) Hajdu, S. I. A Note from History: Landmarks in History of Cancer, Part 1. *Cancer* **2011**, *117* (5), 1097–1102. <https://doi.org/10.1002/cncr.25553>.
- (8) Halperin, E. C. PALEO-ONCOLOGY: The Role of Ancient Remains in the Study of Cancer. *Perspect. Biol. Med.* **2004**, *47* (1), 1–14. <https://doi.org/10.1353/pbm.2004.0010>.
- (9) Avendaño, C.; Menéndez, J. C. *Medicinal Chemistry of Anticancer Drugs*; Elsevier, 2008. <https://doi.org/10.1016/B978-0-444-52824-7.X0001-7>.
- (10) WHO report on cancer. Setting priorities, investing wisely and providing care for all <https://apps.who.int/iris/handle/10665/330745> (accessed Feb 23, 2021).
- (11) Chabner, B. A.; Roberts, T. G. Chemotherapy and the War on Cancer. *Nature Reviews Cancer*. Nature Publishing Group January 2005, pp 65–72. <https://doi.org/10.1038/nrc1529>.
- (12) DeVita, V. T.; Chu, E. A History of Cancer Chemotherapy. *Cancer Res.* **2008**, *68* (21),

8643–8653. <https://doi.org/10.1158/0008-5472.CAN-07-6611>.

- (13) Szaryńska, M. Rola Nowotworowych Komórek Macierzystych w Patogenezie i Terapii Chorób Nowotworowych. *Forum Med. Rodz.* **2011**, 5 (1), 47–56.
- (14) Kwasniewski, W.; Stupak, A.; Kotarski, J.; Gozdzicak-Jozefiak, A. Chaos and Cancers. Theories Concerning Carcinogenesis. *Ginekol. Pol.* **2021**, 92 (4), 318–321. <https://doi.org/10.5603/GP.a2021.0003>.
- (15) Kreso, A.; Dick, J. E. Evolution of the Cancer Stem Cell Model. *Cell Stem Cell* **2014**, 14 (3), 275–291. <https://doi.org/10.1016/j.stem.2014.02.006>.
- (16) Han, L.; Shi, S.; Gong, T.; Zhang, Z.; Sun, X. Cancer Stem Cells: Therapeutic Implications and Perspectives in Cancer Therapy. *Acta Pharm. Sin. B* **2013**, 3 (2), 65–75. <https://doi.org/10.1016/j.apsb.2013.02.006>.
- (17) Nguyen, L. V.; Vanner, R.; Dirks, P.; Eaves, C. J. Cancer Stem Cells: An Evolving Concept. *Nat. Rev. Cancer* **2012**, 12 (2), 133–143. <https://doi.org/10.1038/nrc3184>.
- (18) Dias, D. A.; Urban, S.; Roessner, U. A Historical Overview of Natural Products in Drug Discovery. *Metabolites* **2012**, 2 (2), 303–336. <https://doi.org/10.3390/metabo2020303>.
- (19) Strategie w opracowywaniu nowych substancji leczniczych - Artykuły - Laboratoria.net <http://laboratoria.net/artikul/22233.html> (accessed Feb 7, 2022).
- (20) Opracowywanie nowych substancji leczniczych - projektowanie leków - Artykuły - Biotechnologia.pl - łączymy wszystkie strony biobiznesu <https://biotechnologia.pl/farmacja/opracowywanie-nowych-substancji-leczniczych-projektowanie-lekow,11442> (accessed Feb 7, 2022).
- (21) Newman, D. J.; Cragg, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products*. American Chemical Society March 25, 2016, pp 629–661. <https://doi.org/10.1021/acs.jnatprod.5b01055>.
- (22) Hert, J.; Irwin, J. J.; Laggner, C.; Keiser, M. J.; Shoichet, B. K. Quantifying Biogenic Bias in Screening Libraries. *Nat. Chem. Biol.* 2009 57 **2009**, 5 (7), 479–483. <https://doi.org/10.1038/nchembio.180>.
- (23) Harvey, A. L.; Edrada-Ebel, R.; Quinn, R. J. The Re-Emergence of Natural Products for Drug Discovery in the Genomics Era. *Nat. Rev. Drug Discov.* **2015**, 14 (2), 111–129. <https://doi.org/10.1038/nrd4510>.
- (24) Rodrigues, T.; Reker, D.; Schneider, P.; Schneider, G. Counting on Natural Products for Drug Design. *Nat. Chem.* 2016 86 **2016**, 8 (6), 531–541. <https://doi.org/10.1038/nchem.2479>.
- (25) Xiao, Z.; ... S. M.-M. research; 2016, undefined. Strategies for the Optimization of Natural Leads to Anticancer Drugs or Drug Candidates. *Wiley Online Libr.* **2015**, 36 (1), 32–91. <https://doi.org/10.1002/med.21377>.

- (26) Zhang, Y.; Zhang, Z.; Wang, B.; Liu, L.; Che, Y. Design and Synthesis of Natural Product Derivatives with Selective and Improved Cytotoxicity Based on a Sesquiterpene Scaffold. *Bioorg. Med. Chem. Lett.* **2016**, *26* (8), 1885–1888. <https://doi.org/10.1016/J.BMCL.2016.03.022>.
- (27) Li, G.; Lou, H. X. Strategies to Diversify Natural Products for Drug Discovery. *Med. Res. Rev.* **2018**, *38* (4), 1255–1294. <https://doi.org/10.1002/med.21474>.
- (28) Kim, E.; Moore, B. S.; Yoon, Y. J. Reinvigorating Natural Product Combinatorial Biosynthesis with Synthetic Biology. *Nat. Chem. Biol.* **2015**, *11* (9), 649–659. <https://doi.org/10.1038/nchembio.1893>.
- (29) Sears, J.; research, D. B.-A. of chemical; 2015, undefined. Total Synthesis of Vinblastine, Related Natural Products, and Key Analogues and Development of Inspired Methodology Suitable for the Systematic Study of Their Structure. *ACS Publ.* **2015**, *48* (3), 653–662. <https://doi.org/10.1021/ar500400w>.
- (30) Oprea, T. I.; Mestres, J. Drug Repurposing: Far beyond New Targets for Old Drugs. *AAPS J.* **2012**, *14* (4), 759–763. <https://doi.org/10.1208/s12248-012-9390-1>.
- (31) Pushpakom, S.; Iorio, F.; Eyers, P. A.; Escott, K. J.; Hopper, S.; Wells, A.; Doig, A.; Guilliams, T.; Latimer, J.; McNamee, C.; Norris, A.; Sanseau, P.; Cavalla, D.; Pirmohamed, M. Drug Repurposing: Progress, Challenges and Recommendations. *Nat. Rev. Drug Discov.* **2018**, *18* (1), 41–58. <https://doi.org/10.1038/nrd.2018.168>.
- (32) Sleire, L.; Førde-Tislevoll, H. E.; Netland, I. A.; Leiss, L.; Skeie, B. S.; Enger, P. Ø. Drug Repurposing in Cancer. *Pharmacol. Res.* **2017**, *124*, 74–91. <https://doi.org/10.1016/j.phrs.2017.07.013>.
- (33) Antoszczak, M.; Rutkowski, J.; Huczyński, A. Structure and Biological Activity of Polyether Ionophores and Their Semisynthetic Derivatives. *Bioact. Nat. Prod. Chem. Biol.* **2015**, 107–170. <https://doi.org/10.1002/9783527684403.ch6>.
- (34) Butaye, P.; Devriese, L. A.; Haesebrouck, F. Antimicrobial Growth Promoters Used in Animal Feed: Effects of Less Well Known Antibiotics on Gram-Positive Bacteria. *Clin. Microbiol. Rev.* **2003**, *16* (2), 175–188. <https://doi.org/10.1128/CMR.16.2.175-188.2003>.
- (35) Dutton, C. J.; Banks, B. J.; Cooper, C. B. Polyether Ionophores. *Nat. Prod. Rep.* **1995**, *12* (2), 165–181. <https://doi.org/10.1039/NP9951200165>.
- (36) Huczynski, A. Salinomycin - A New Cancer Drug Candidate. *Chem. Biol. Drug Des.* **2012**, *79* (3), 235–238. <https://doi.org/10.1111/j.1747-0285.2011.01287.x>.
- (37) Gupta, P. B.; Onder, T. T.; Jiang, G.; Tao, K.; Kuperwasser, C.; Weinberg, R. A.; Lander, E. S. Identification of Selective Inhibitors of Cancer Stem Cells by High-Throughput Screening. *Cell* **2009**, *138* (4), 645–659. <https://doi.org/10.1016/j.cell.2009.06.034>.
- (38) Fuchs, D.; Heinold, A.; Opelz, G.; Daniel, V.; Naujokat, C. Salinomycin Induces

Apoptosis and Overcomes Apoptosis Resistance in Human Cancer Cells. *Biochem. Biophys. Res. Commun.* **2009**, 390 (3), 743–749. <https://doi.org/10.1016/j.bbrc.2009.10.042>.

- (39) Jangamreddy, J. R.; Ghavami, S.; Grabarek, J.; Kratz, G.; Wiechec, E.; Fredriksson, B. A.; Pariti, R. K. R.; Cieřlar-Pobuda, A.; Panigrahi, S.; Łos, M. J. Salinomycin Induces Activation of Autophagy, Mitophagy and Affects Mitochondrial Polarity: Differences between Primary and Cancer Cells. *Biochim. Biophys. Acta - Mol. Cell Res.* **2013**, 1833 (9), 2057–2069. <https://doi.org/10.1016/j.bbamcr.2013.04.011>.
- (40) Boehmerle, W.; Endres, M. Salinomycin Induces Calpain and Cytochrome C-Mediated Neuronal Cell Death. *Cell Death Dis.* **2011**, 2 (6). <https://doi.org/10.1038/cddis.2011.46>.
- (41) Kim, S. H.; Choi, Y. J.; Kim, K. Y.; Yu, S. N.; Seo, Y. K.; Chun, S. S.; Noh, K. T.; Suh, J. T.; Ahn, S. C. Salinomycin Simultaneously Induces Apoptosis and Autophagy through Generation of Reactive Oxygen Species in Osteosarcoma U2OS Cells. *Biochem. Biophys. Res. Commun.* **2016**, 473 (2), 607–613. <https://doi.org/10.1016/j.bbrc.2016.03.132>.
- (42) Huang, X.; Borgström, B.; Stegmayr, J.; Abassi, Y.; Kruszyk, M.; Leffler, H.; Persson, L.; Albinsson, S.; Massoumi, R.; Scheblykin, I. G.; Hegardt, C.; Oredsson, S.; Strand, D. The Molecular Basis for Inhibition of Stemlike Cancer Cells by Salinomycin. *ACS Cent. Sci.* **2018**, 4 (6), 760–767. <https://doi.org/10.1021/acscentsci.8b00257>.
- (43) Mai, T. T.; Hamäi, A.; Hienzschi, A.; Cañeque, T.; Müller, S.; Wicinski, J.; Cabaud, O.; Leroy, C.; David, A.; Acevedo, V.; Ryo, A.; Ginestier, C.; Birnbaum, D.; Charafe-Jauffret, E.; Codogno, P.; Mehrpour, M.; Rodriguez, R. Salinomycin Kills Cancer Stem Cells by Sequestering Iron in Lysosomes. *Nat. Chem.* **2017**, 9 (10), 1025–1033. <https://doi.org/10.1038/nchem.2778>.
- (44) Müller, S.; Sindikubwabo, F.; Cañeque, T.; Lafon, A.; Versini, A.; Lombard, B.; Loew, D.; Wu, T. Di; Ginestier, C.; Charafe-Jauffret, E.; Durand, A.; Vallot, C.; Baulande, S.; Servant, N.; Rodriguez, R. CD44 Regulates Epigenetic Plasticity by Mediating Iron Endocytosis. *Nat. Chem.* **2020**, 12 (10), 929–938. <https://doi.org/10.1038/s41557-020-0513-5>.
- (45) Slobodnick, A.; Shah, B.; Pillinger, M. H.; Krasnokutsky, S. Colchicine: Old and New. *American Journal of Medicine*. Elsevier Inc. May 1, 2015, pp 461–470. <https://doi.org/10.1016/j.amjmed.2014.12.010>.
- (46) GRAHAM, W.; ROBERTS, J. B. Intravenous Colchicine in the Management of Gouty Arthritis. *Ann. Rheum. Dis.* **1953**, 12 (1), 16–19. <https://doi.org/10.1136/ard.12.1.16>.
- (47) Dasgeb, B.; Kornreich, D.; McGuinn, K.; Okon, L.; Brownell, I.; Sackett, D. L. Colchicine: An Ancient Drug with Novel Applications. *Br. J. Dermatol.* **2018**, 178 (2), 350–356. <https://doi.org/10.1111/bjd.15896>.
- (48) Nerlekar, N.; Beale, A.; Harper, R. W. Colchicine — A Short History of an Ancient

Drug. Med. J. Aust. **2014**, 201 (11), 687–688. <https://doi.org/10.5694/mja14.00846>.

- (49) Khanna, D.; Fitzgerald, J. D.; Khanna, P. P.; Bae, S.; Singh, M. K.; Neogi, T.; Pillinger, M. H.; Merrill, J.; Lee, S.; Prakash, S.; Kaldas, M.; Gogia, M.; Perez-Ruiz, F.; Taylor, W.; Lioté, F.; Choi, H.; Singh, J. A.; Dalbeth, N.; Kaplan, S.; Niyar, V.; Jones, D.; Yarows, S. A.; Roessler, B.; Kerr, G.; King, C.; Levy, G.; Furst, D. E.; Edwards, N. L.; Mandell, B.; Schumacher, H. R.; Robbins, M.; Wenger, N.; Terkeltaub, R. 2012 American College of Rheumatology Guidelines for Management of Gout. Part 1: Systematic Nonpharmacologic and Pharmacologic Therapeutic Approaches to Hyperuricemia. *Arthritis Care Res.* **2012**, 64 (10), 1431–1446. <https://doi.org/10.1002/acr.21772>.
- (50) Grattagliano, I.; Bonfrate, L.; Ruggiero, V.; Scaccianoce, G.; Palasciano, G.; Portincasa, P. Novel Therapeutics for the Treatment of Familial Mediterranean Fever: From Colchicine to Biologics. *Clin. Pharmacol. Ther.* **2014**, 95 (1), 89–97. <https://doi.org/10.1038/clpt.2013.148>.
- (51) Imazio, M.; Gaita, F.; LeWinter, M. Evaluation and Treatment of Pericarditis: A Systematic Review. *JAMA - Journal of the American Medical Association*. American Medical Association October 13, 2015, pp 1498–1506. <https://doi.org/10.1001/jama.2015.12763>.
- (52) Subbaramaiah, K.; Hart, J. C.; Norton, L.; Dannenberg, A. J. Microtubule-Interfering Agents Stimulate the Transcription of Cyclooxygenase-2. Evidence for Involvement of ERK1/2 and P38 Mitogen- Activated Protein Kinase Pathways. *J. Biol. Chem.* **2000**, 275 (20), 14838–14845. <https://doi.org/10.1074/jbc.275.20.14838>.
- (53) Seligmann, J.; Twelves, C. Tubulin: An Example of Targeted Chemotherapy. *Future Medicinal Chemistry*. March 2013, pp 339–352. <https://doi.org/10.4155/fmc.12.217>.
- (54) Cocco, G.; Chu, D. C. C.; Pandolfi, S. Colchicine in Clinical Medicine. A Guide for Internists. *European Journal of Internal Medicine*. Elsevier B.V. 2010, pp 503–508. <https://doi.org/10.1016/j.ejim.2010.09.010>.
- (55) Yang, L. P. H. Oral Colchicine (Colcris®: In the Treatment and Prophylaxis of Gout. *Drugs*. 2010, pp 1603–1613. <https://doi.org/10.2165/11205470-000000000-00000>.
- (56) Gross, J. H. Mass Spectrometry: A Textbook: Second Edition. *Mass Spectrom. A Textb. Second Ed.* **2011**, 1–753. <https://doi.org/10.1007/978-3-642-10711-5>.
- (57) Skibiński, A.; Rajca, A.; Bieg, T.; Salwińska, E.; Mazurkiewicz, R.; Zieliński, W.; Suwiński, J. Metody Spektroskopowe i Spektrometria Mas w Zastosowaniu Do Identyfikacji Związków Organicznych : Praca Zbiorowa. T. 2, Tabele Korelacyjne / Pod Redakcją Wojciecha Zielińskiego i Andrzeja Rajcy ; Autorzy.
- (58) Istifli, E. S.; Hüsunet, M. T.; İla, H. B. Cell Division, Cytotoxicity, and the Assays Used in the Detection of Cytotoxicity. *Cytotox. - Defin. Identification, Cytotoxic Compd.* **2019**. <https://doi.org/10.5772/INTECHOPEN.88368>.

- (59) NCI-60 Human Tumor Cell Lines Screen | Discovery & Development Services | Developmental Therapeutics Program (DTP) https://dtp.cancer.gov/discovery_development/nci-60/ (accessed Feb 23, 2022).
- (60) Abaan, O. D.; Polley, E. C.; Davis, S. R.; Zhu, Y. J.; Bilke, S.; Walker, R. L.; Pineda, M.; Gindin, Y.; Jiang, Y.; Reinhold, W. C.; Holbeck, S. L.; Simon, R. M.; Doroshow, J. H.; Pommier, Y.; Meltzer, P. S. The Exomes of the NCI-60 Panel: A Genomic Resource for Cancer Biology and Systems Pharmacology. *Cancer Res.* **2013**, 73 (14), 4372–4382. <https://doi.org/10.1158/0008-5472.CAN-12-3342/659272/P/THE-EXOMES-OF-THE-NCI-60-PANEL-A-GENOMIC-RESOURCE>.
- (61) Stepanek, O.; Brdicka, T.; Angelisova, P.; Horvath, O.; Spicka, J.; Stockbauer, P.; Man, P.; Horejsi, V. Interaction of Late Apoptotic and Necrotic Cells with Vitronectin. *PLoS One* **2011**, 6 (5). <https://doi.org/10.1371/JOURNAL.PONE.0019243>.
- (62) Heikkilä, K.; Ebrahim, S.; Lawlor, D. A. Systematic Review of the Association between Circulating Interleukin-6 (IL-6) and Cancer. *Eur. J. Cancer* **2008**, 44 (7), 937–945. <https://doi.org/10.1016/J.EJCA.2008.02.047>.
- (63) Cancer Patients Typically Have Increased Interleukin-6 Levels <https://www.medscape.com/viewarticle/537309> (accessed Feb 24, 2022).
- (64) Bellone, G.; Smirne, C.; Mauri, F. A.; Tonel, E.; Carbone, A.; Buffolino, A.; Dughera, L.; Robecchi, A.; Pirisi, M.; Emanuelli, G. Cytokine Expression Profile in Human Pancreatic Carcinoma Cells and in Surgical Specimens: Implications for Survival. *Cancer Immunol. Immunother.* 2005 556 **2005**, 55 (6), 684–698. <https://doi.org/10.1007/S00262-005-0047-0>.
- (65) Stefańska, J.; Antoszczak, M.; Stępień, K.; Bartoszcze, M.; Mirski, T.; Huczyński, A. Tertiary Amides of Salinomycin: A New Group of Antibacterial Agents against *Bacillus Anthracis* and Methicillin-Resistant *Staphylococcus Epidermidis*. *Bioorganic Med. Chem. Lett.* **2015**, 25 (10), 2082–2088. <https://doi.org/10.1016/j.bmcl.2015.03.085>.
- (66) Zhang, W.; Wu, J.; Li, B.; Xia, J.; Wu, H.; Wang, L.; Hao, J.; Zhou, Q.; Wu, S. Synthesis and Biological Activity Evaluation of 20-: Epi -Salinomycin and Its 20- O -Acyl Derivatives. *RSC Adv.* **2016**, 6 (48), 41885–41890. <https://doi.org/10.1039/c6ra08967d>.
- (67) Li, Y.; Shi, Q.; Shao, J.; Yuan, Y.; Yang, Z.; Chen, S.; Zhou, X.; Wen, S.; Jiang, Z. X. Synthesis and Biological Evaluation of 20-Epi-Amino-20-Deoxysalinomycin Derivatives. *Eur. J. Med. Chem.* **2018**, 148, 279–290. <https://doi.org/10.1016/j.ejmech.2018.02.004>.
- (68) Hastie, S. B.; Williams, R. C.; Puett, D.; Macdonald, T. L. The Binding of Isocolchicine to Tubulin. Mechanisms of Ligand Association with Tubulin. *J. Biol. Chem.* **1989**, 264 (12), 6682–6688.
- (69) Banerjee, A.; Kasmala, L. T.; Hamel, E.; Sun, L.; Lee, K. H. Interaction of Novel Thiocolchicine Analogs with the Tubulin Isoforms from Bovine Brain. *Biochem. Biophys. Res. Commun.* **1999**, 254 (2), 334–337.

<https://doi.org/10.1006/bbrc.1998.9943>.

- (70) Shi, Q.; Chen, K.; Morris-Natschke, S. L.; Lee, K. H. Recent Progress in the Development of Tubulin Inhibitors as Antimitotic Antitumor Agents. *Curr. Pharm. Des.* **1998**, *4* (3), 219–248.
- (71) Kerekes, P.; Sharma, P. N.; Brossi, A.; Chignell, C. F.; Quinn, F. R. Synthesis and Biological Effects of Novel Thiocolchicines. 3. Evaluation of N-Acyldeacetylthiocolchicines, N-(Alkoxy carbonyl)Deacetylthiocolchicines, and O-Ethyl demethylthiocolchicines. New Synthesis of Thiodemecolcine and Antileukemic Effects of 2-Demeth. *J. Med. Chem.* **1985**, *28* (9), 1204–1208. <https://doi.org/10.1021/jm00147a014>.
- (72) Kurek, J.; Kwaśniewska-Sip, P.; Myszkowski, K.; Cofta, G.; Barczyński, P.; Murias, M.; Kurczab, R.; Śliwa, P.; Przybylski, P. Antifungal, Anticancer, and Docking Studies of Colchicine Complexes with Monovalent Metal Cation Salts. *Chem. Biol. Drug Des.* **2019**, *94* (5), 1930–1943. <https://doi.org/10.1111/cbdd.13583>.
- (73) Huczyński, A.; Majcher, U.; Maj, E.; Wietrzyk, J.; Janczak, J.; Moshari, M.; Tuszynski, J. A.; Bartl, F. Synthesis, Antiproliferative Activity and Molecular Docking of Colchicine Derivatives. *Bioorg. Chem.* **2016**, *64*, 103–112. <https://doi.org/10.1016/j.bioorg.2016.01.002>.
- (74) Shchegravina, E. S.; Maleev, A. A.; Ignatov, S. K.; Gracheva, I. A.; Stein, A.; Schmalz, H. G.; Gavryushin, A. E.; Zubareva, A. A.; Svirshchevskaya, E. V.; Fedorov, A. Y. Synthesis and Biological Evaluation of Novel Non-Racemic Indole-Containing Alcolchicinoids. *Eur. J. Med. Chem.* **2017**, *141*, 51–60. <https://doi.org/10.1016/j.ejmech.2017.09.055>.
- (75) Krzywik, J.; Aminpour, M.; Janczak, J.; Maj, E.; Moshari, M.; Mozga, W.; Wietrzyk, J.; Tuszynski, J. A.; Huczyński, A. An Insight into the Anticancer Potential of Carbamates and Thiocarbamates of 10-Demethoxy-10-Methylaminocolchicine. *Eur. J. Med. Chem.* **2021**, *215*, 113282. <https://doi.org/10.1016/j.ejmech.2021.113282>.
- (76) Majcher, U.; Urbaniak, A.; Maj, E.; Moshari, M.; Delgado, M.; Wietrzyk, J.; Bartl, F.; Chambers, T. C.; Tuszynski, J. A.; Huczyński, A. Synthesis, Antiproliferative Activity and Molecular Docking of Thiocolchicine Urethanes. *Bioorg. Chem.* **2018**, *81*, 553–566. <https://doi.org/10.1016/j.bioorg.2018.09.004>.
- (77) Yasobu, N.; Kitajima, M.; Kogure, N.; Shishido, Y.; Matsuzaki, T.; Nagaoka, M.; Takayama, H. Design, Synthesis, and Antitumor Activity of 4-Halocolchicines and Their pro-Drugs Activated by Cathepsin B. *ACS Med. Chem. Lett.* **2011**, *2* (5), 348–352. <https://doi.org/10.1021/ml100287y>.
- (78) Zhang, X.; Kong, Y.; Zhang, J.; Su, M.; Zhou, Y.; Zang, Y.; Li, J.; Chen, Y.; Fang, Y.; Zhang, X.; Lu, W. Design, Synthesis and Biological Evaluation of Colchicine Derivatives as Novel Tubulin and Histone Deacetylase Dual Inhibitors. *Eur. J. Med. Chem.* **2015**, *95*, 127–135. <https://doi.org/10.1016/j.ejmech.2015.03.035>.

- (79) Johnson, L.; Goping, I. S.; Rieger, A.; Mane, J. Y.; Huzil, T.; Banerjee, A.; Luduena, R.; Hassani, B.; Winter, P.; Tuszynski, J. A. Novel Colchicine Derivatives and Their Anti-Cancer Activity. *Curr. Top. Med. Chem.* **2017**, *17* (22). <https://doi.org/10.2174/1568026617666170104143618>.
- (80) Ghawanmeh, A. A.; Al-Bajalan, H. M.; Mackeen, M. M.; Alali, F. Q.; Chong, K. F. Recent Developments on (–)-Colchicine Derivatives: Synthesis and Structure-Activity Relationship. *Eur. J. Med. Chem.* **2020**, *185*, 111788. <https://doi.org/10.1016/j.ejmech.2019.111788>.
- (81) Kumar, A.; Sharma, P. R.; Mondhe, D. M. Potential Anticancer Role of Colchicine-Based Derivatives: An Overview. *Anti-Cancer Drugs*. Lippincott Williams and Wilkins December 27, 2016, pp 250–262. <https://doi.org/10.1097/CAD.0000000000000464>.
- (82) Andreu, J. M.; Timasheff, S. N. Tubulin Bound to Colchicine Forms Polymers Different from Microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79* (22 I), 6753–6756. <https://doi.org/10.1073/pnas.79.22.6753>.
- (83) Guan, J.; Zhu, X. K.; Tachibana, Y.; Bastow, K. F.; Brossi, A.; Hamel, E.; Lee, K. H. Antitumor Agents. 185. Synthesis and Biological Evaluation of Tridemethylthiocolchicine Analogues as Novel Topoisomerase II Inhibitors. *J. Med. Chem.* **1998**, *41* (11), 1956–1961. <https://doi.org/10.1021/jm980007f>.
- (84) Rósner, M.; Capraro, H. G.; Jacobson, A. E.; Atwell, L.; Brossi, A.; Iorio, M. A.; Williams, T. H.; Sik, R. H.; Chignell, C. F. Biological Effects of Modified Colchicines. Improved Preparation of 2-Demethylcolchicine, 3-Demethylcolchicine, and (+)-Colchicine and Reassignment of the Position of the Double Bond in Dehydro-7-Deacetamidocolchicines. *J. Med. Chem.* **1981**, *24* (3), 257–261. <https://doi.org/10.1021/jm00135a005>.
- (85) Hastie, S. B.; Macdonald, T. L. Binding of Colchicine to Tubulin. Mechanisms of Ligand Association with Tubulin. *Biochem. Pharmacol.* **1990**, *39* (8), 1271–1276. [https://doi.org/10.1016/0006-2952\(90\)90002-3](https://doi.org/10.1016/0006-2952(90)90002-3).
- (86) Klein, A. E.; Davis, P. J. Determination of Colchicine and Colchicine in Microbial Cultures by High-Performance Liquid Chromatography. *Anal. Chem.* **1980**, *52* (14), 2432–2435. <https://doi.org/10.1021/ac50064a048>.
- (87) Bussotti, L.; Cacelli, I.; D’Auria, M.; Foggi, P.; Lesma, G.; Silvani, A.; Villani, V. Photochemical Isomerization of Colchicine and Thiocolchicine. *J. Phys. Chem. A* **2003**, *107* (43), 9079–9085. <https://doi.org/10.1021/jp035507l>.
- (88) Ertel, N. H.; Wallace, S. L. Purification of Colchicine, Its Photoisomer(s), and Some Congeners by Paper and Thin-Layer Chromatography. *Biochem. Med.* **1970**, *4* (3–4), 181–192. [https://doi.org/10.1016/0006-2944\(70\)90048-7](https://doi.org/10.1016/0006-2944(70)90048-7).
- (89) Day, A. C.; Ledlie, M. A. The Photoisomerisation of α -Tropolone. *J. Chem. Soc. D Chem. Commun.* **1970**, No. 19, 1265–1266. <https://doi.org/10.1039/C2970001265b>.

- (90) Pauson, P. L. Tropones and Tropolones. *Chem. Rev.* **1955**, 55 (1), 9–136. <https://doi.org/10.1021/cr50001a002>.
- (91) Sagorin, C.; Ertel, N. H.; Wallace, S. L. Photoisomerization of Colchicine. Loss of Significant Antimitotic Activity in Human Lymphocytes. *Arthritis Rheum.* **1972**, 15 (2), 213–217. <https://doi.org/10.1002/art.1780150213>.
- (92) Cacelli, I.; D'Auria, M.; Villani, V. Why Thiocolchicine Does Not Undergo Photochemical Isomerization: A Theoretical Study. *Chem. Phys. Lett.* **2008**, 459 (1–6), 167–171. <https://doi.org/10.1016/j.cplett.2008.05.048>.
- (93) Boyland, E.; Mawson, E. H. The Conversion of Colchicine into Colchicine. *Biochem. J.* **1938**, 32 (7), 1204–1206. <https://doi.org/10.1042/bj0321204>.
- (94) F Mackay, M.; D Morrison, J.; M Gulbis, J. Crystal Structure of Triclinic Colchicine Hemihydrate. *Aust. J. Phys.* **1985**, 38 (3), 413. <https://doi.org/10.1071/ph850413>.
- (95) Elguero, J.; Muller, R. N.; Blade-Font, A.; Faure, R.; Vincent, E. J. Carbon-13 Magnetic Resonance Spectroscopy. A Study Of Colchicine And Related Compounds. *Bull. des Sociétés Chim. Belges* **1980**, 89 (3), 193–204. <https://doi.org/10.1002/bscb.19800890307>.

Artykuły naukowe stanowiące rozprawę doktorską

PUBLIKACJA I wraz z suplementem

Dominika Czerwotka, Alicja Urbaniak Szymon Sobczak, Sergio Piña-Oviedo,
Timothy C. Chambers, Michał Antoszczak, Adam Huczyński

„Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs”

ChemMedChem 2020, 15, 236–246

Synthesis and Anticancer Activity of Tertiary Amides of Salinomycin and Their C20-oxo Analogues

Dominika Czerwona^{+, [a]} Alicja Urbaniak^{+, [b]} Szymon Sobczak^[c] Sergio Piña-Oviedo^[d]
Timothy C. Chambers^[b] Michał Antoszczak^{*, [a]} and Adam Huczyński^[a]

The polyether ionophore salinomycin (SAL) has captured much interest because of its potent activity against cancer cells and cancer stem cells. Our previous studies have indicated that C1/C20 double-modification of SAL is a useful strategy to generate diverse agents with promising biological activity profiles. Thus, herein we describe the synthesis of a new class of SAL analogues that combine key modifications at the C1 and C20 positions. The activity of the obtained SAL derivatives was evaluated using primary acute lymphoblastic leukemia, human breast adenocarcinoma and normal mammary epithelial cells. One single- [N,N-dipropyl amide of salinomycin (5a)] and two novel double-modified analogues [N,N-dipropyl amide of C20-

oxosalinomycin (5b) and piperazine amide of C20-oxosalinomycin (13b)] were found to be more potent toward the MDA-MB-231 cell line than SAL or its C20-oxo analogue 2. When select analogues were tested against the NCI-60 human tumor cell line panel, 4a [N,N-diethyl amide of salinomycin] showed particular activity toward the ovarian cancer cell line SK-OV-3. Additionally, both SAL and 2 were found to be potent ex vivo against human ER/PR⁺, Her2⁻ invasive mammary carcinoma, with 2 showing minimal toxicity toward normal epithelial cells. The present findings highlight the therapeutic potential of SAL derivatives for select targeting of different cancer types.

Introduction

Cancer is a leading cause of death worldwide,^[1] and it is estimated that about 10 million people died from cancer in 2018.^[2] Among currently used cancer therapies, surgical removal and radiation treatment, followed by systemic chemotherapy, are the most commonly used strategies.^[3–5] However, the major disadvantages of chemotherapy are the recurrence of cancer, associated with drug resistance, and also severe side effects that significantly limit dosing^[6,7] and impair a patient's quality of life.^[3] Despite these limitations, chemotherapy is still widely employed at all stages of cancer progression.^[8,9] Identification of new chemotherapeutic agents that effectively and selectively attack cancer cells thus remains a priority. Many well-known

anticancer drugs are of natural origin, and these include bleomycin, dactinomycin, daunorubicin, doxorubicin, paclitaxel, pentostatin, phleomycin, streptozotecin, valrubicin, and vincristine.^[10]

Emerging more recently as novel anticancer agents of natural origin are select polyether ionophores, such as salinomycin.^[11–14] Salinomycin (SAL, 1, Scheme 1), isolated from *Streptomyces albus*,^[15] exhibits a broad spectrum of bioactivity.^[16,17] SAL is commercially used as a food additive and a growth promoter due to its potent coccidiostatic and antibacterial properties.^[18] More recently, SAL has been also found to exhibit potent antifungal, antiparasitic and antiviral activity,^[16,17] and extensive screening studies by Gupta et al. identified SAL as a very promising anticancer agent.^[19] Among about 16,000 compounds tested, SAL has shown the most potent activity against breast cancer stem cells (CSCs),^[19] a small sub-population of cells within tumors with capabilities of self-renewal, differentiation, and tumorigenicity.^[20] Of note is that the anticancer activity of SAL was nearly 100-fold higher than that of the commonly used chemotherapeutic drug paclitaxel.^[19] Further, potent in vitro activity of SAL against other cancer cell types and CSCs has been demonstrated.^[11] SAL was also successfully used to induce partial clinical regression during pre-clinical studies in the treatment of a small group of patients with invasive carcinoma of the head, neck, breast and ovary.^[21]

Because SAL exhibits so many diverse biological activities, there is significant interest in developing bioactive derivatives, especially since several SAL analogues examined to date exhibit superior biological activity than the parent structure.^[22] For example, our previous studies have indicated that derivatization of the C1 carboxylate moiety of SAL with amide, ester, or bioconjugate modifications generates compounds with higher

[a] D. Czerwona,⁺ Dr. M. Antoszczak, Prof. A. Huczyński
Department of Bioorganic Chemistry
Faculty of Chemistry
Adam Mickiewicz University
Uniwersytetu Poznańskiego 8, 61-614 Poznań (Poland)
E-mail: michant@amu.edu.pl

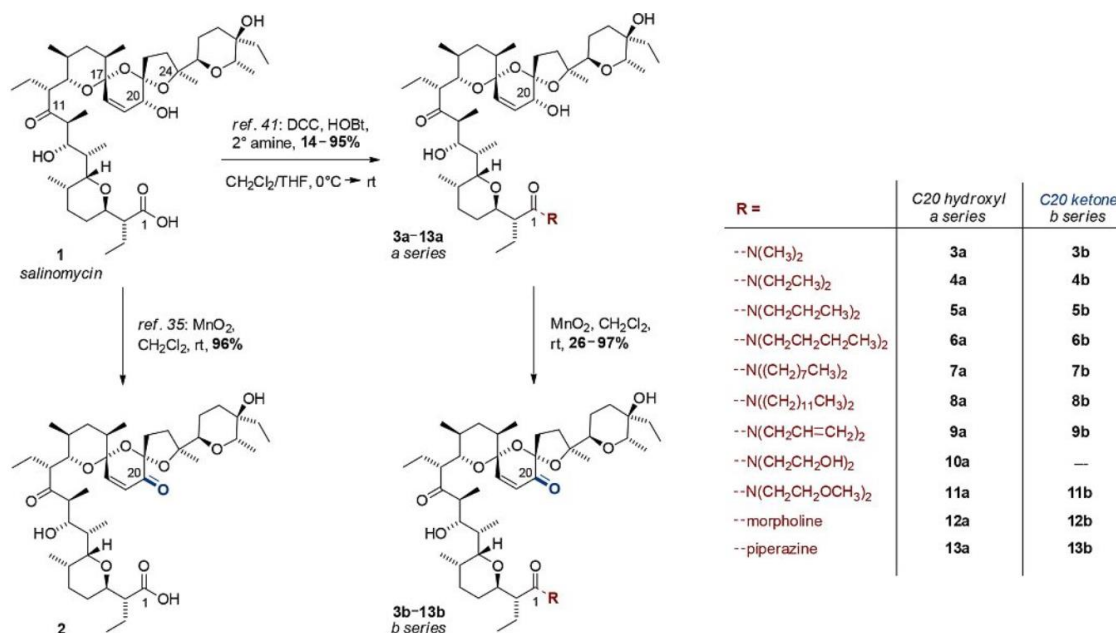
[b] Dr. A. Urbaniak,⁺ Dr. T. C. Chambers
Department of Biochemistry and Molecular Biology
University of Arkansas for Medical Sciences
Little Rock, AR 72205 (USA)

[c] S. Sobczak
Department of Materials Chemistry
Faculty of Chemistry
Adam Mickiewicz University
Uniwersytetu Poznańskiego 8
61-614 Poznań (Poland)

[d] Dr. S. Piña-Oviedo
Department of Pathology
University of Arkansas for Medical Sciences
Little Rock, AR 72205 (USA)

[*] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cmdc.201900593>



Scheme 1. Synthesis of C20-oxo analogues of salinomycin and its C1 tertiary amides.

potency and selectivity compared to commonly used chemotherapeutics, such as cisplatin or doxorubicin.^[23–29] Similarly, chemical modification of **SAL** at the C20 hydroxy group generates analogues superior to the starting compound.^[30–39]

We have recently exploited these findings to synthesize doubly modified **SAL** derivatives at the C1 and C20 positions that are highly promising anticancer agents both in vitro and ex vivo.^[40] Here, we designed and synthesized a new series of double-modified **SAL** analogues, with oxo modification at the C20 position and a cohort of tertiary amides at the C1 position. Compounds were tested in cell viability assays against human primary acute lymphoblastic leukemia cells, the breast cancer cell line MDA-MB-231, and MCF 10A normal breast epithelial cells. Select analogues were also screened against the NCI panel of 60 human tumor cell lines. Moreover, we studied the activity of **SAL** and its C20-oxo form in an ex vivo model of human ER/PR⁺, Her2⁺ invasive mammary carcinoma.

Results and Discussion

Design and Synthesis

Based on previous observations that **SAL** and select derivatives exhibit broad bioactivity ranging from antibacterial to anticancer,^[16] we postulated that C1 tertiary amides of **SAL** could also exhibit potent activity against cancer cells. Thus, a library of **SAL** analogues was devised based on the most active **SAL** C1 tertiary amides obtained in previous studies (com-

pounds **4a–7a** and **10a–13a**, Scheme 1).^[41] To expand the structural diversity at the C1 position, three novel tertiary amides of **SAL** (*N,N*-dimethyl, *N,N*-didodecyl and *N,N*-diallyl) were also included in this series (compound **3a**, **8a** and **9a**, respectively, Scheme 1). With respect to modification of the C20 position, we elected to transform the hydroxy group to the ketone group because it was anticipated that such modification would lead to enhanced anticancer activity. As a result, C20-oxosalinomycin (compound **2**, Scheme 1), and a completely novel series of C1/C20 doubly modified **SAL** analogues was obtained (compounds **3b–13b**, Scheme 1).

Synthetically, the general procedure for the synthesis of C1 tertiary amides of **SAL** was as described previously.^[41] With access to the series of **SAL** amides (compounds **3a–13a**, Scheme 1), the allylic C20 hydroxy group was chemoselectively oxidized to the corresponding C20 ketone with moderate to good yields (26–97%) using activated manganese(IV) oxide (compounds **3b–13b**, Scheme 1). For better structure-activity relationship (SAR) studies, unmodified **SAL** was also quantitatively oxidized to C20-oxosalinomycin **2**, using the same reaction conditions (Scheme 1).^[35] Of note, full selectivity toward the C20 hydroxy was observed in all cases. Despite extensive experimentation, this method unfortunately failed for compound **10a** (Scheme 1) having the additional primary hydroxy groups at the C1 amide moiety. In this case, chemoselective oxidation of C20 hydroxy followed by amidation reaction of the C1 carboxyl was also unsuccessful. The purity and structure of all **SAL** derivatives, including its novel double-modified analogues, were determined by NMR, FT-IR and ESI MS studies

(Supporting Information, Figures S1–S16), but also by single-crystal X-ray diffraction (scXRD) analysis.

X-ray Analysis

To gain insight into SAR for **SAL** derivatives, we characterized C20-oxosalinomycin **2**, *N,N*-dimethyl amide of salinomycin **3a** as well as *N,N*-dibutyl and *N,N*-diallyl amide of C20-oxosalinomycin (compounds **6b** and **9b**, Scheme 1, respectively) using scXRD method (Figure 1 and Figure 2).

Crystal structure of C20-oxosalinomycin **2** in the sodium salt form, known as SY-9, was solved by Paulus and Vértessy.^[42] However, the structure of this analogue in the acid form has been unknown until now. Compound **2** in the acid form (Scheme 1) crystallized in orthorhombic space group $P2_12_12_1$, with one molecule in the asymmetric part of the unit cell. With the exception of the unsaturated six-membered ring which adopted an envelope conformation with the C21 atom shifted out of the plane by 0.422 Å, the other six-membered rings were in the chair conformation. The unsaturated ring had a central position in the tricyclic spiroketal system and is connected by

the two tetrahedral spiro-heads, i.e. by C17 and C21 atoms with six- and five-membered rings, respectively. The oxidation of the hydroxy group reduced the distance between O7 and C20 atoms to 1.235 Å. The resulting conjugation of C20 ketone with C18=C19 olefinic system elongated the double bond to 1.322 Å. Further, the pseudo-cyclic conformation of **2** molecule is stabilized by three OH...O contacts arranged in the form of the twelve-member ring, involving O2H...O11, O11H...O5 and O4H...O1 bonds (Figure 1A).

Of the **SAL** analogues with amide substituent at the C1 position and a hydroxy group at C20 (Scheme 1, *a* series), we have characterized the crystal structure of **3a** (Figure 1B). Amide **3a** co-crystallized in monoclinic group C2 with one disordered acetone molecule in the asymmetric part of the unit-cell. Despite the spiroketal ring system, the conformation of **3a** significantly differed from that observed in **2**. Within this group only slight changes in the shift of C21 (to 0.571 Å) and the C18=C19 distance (to 1.317 Å) were noticeable. The most significant conformational changes are observed for the torsion angle τ_1 (C11–C10–C9–O8) which rotated to -177.6° ($\tau_1 = 169^\circ$ in **2**), and τ_2 (C23–C24–C25–O26) that changed to 71.8° ($\tau_2 = -68^\circ$ in **2**). The intramolecular H-bonds, O3–H...O4, O3–H...O8 and O7–H...O9, stabilized the pseudo-cyclic conformation of **3a** (Figure 1B). The **3a** molecule can also create intermolecular hydrogen bonds; this is shown by the green lines in Figure 1B. These unique O10H...O1¹ (1¹ symmetry code $x,1+y,z$) bonds have the potential to connect many **3a** molecules into one supramolecular aggregate (not shown).

After oxidation of the C20 hydroxy group, single crystals of doubly modified analogues **6b** and **9b** were also obtained (Figure 2A and Figure 2B). Their conformations were remarkably similar, despite the character and size of the amide group at the C1 position. Together with increasing number of carbon atoms, the crystal symmetry of studied compounds lowered from the orthorhombic space group $P2_12_12_1$ in **9b** to monoclinic $P2_1$ in **6b**. Additionally, the long alkyl groups with rotational freedom gave a rise to the disorder presented in both structures. Nonetheless, in molecule **9b**, the double bonds C44=C45 (1.349 Å or 1.356 Å) and C47=C48 (1.359 Å or 1.391 Å) in the C1 amide moiety are clearly observed. The asymmetric part of the unit-cell in both crystal structures is occupied by one molecule in cyclic conformation. What is worth noting, the molecular conformation of compounds **6b** and **9b** did not remain the conformation of C20-oxosalinomycin **2**. However, without the obvious changes connected with the formation of amide at the C1 position, conformation of molecules **6b** and **9b** can be compared to those of **3a**. The major difference is in τ_2 angle, which is changed from 54.9° in **6b** to 53.5° in **9b**.

The size of the substituent seemed to have only a slight influence on the spiroketal ring system, shifting the C21 atom from 0.466 Å in **9b** to 0.441 Å in **6b**. The C18=C19 distance in central spiro-ring system varied between 1.320 Å and 1.323 Å for **9b** and **6b**, respectively. Also, the H-bonds scheme was similar in both structures. The hydroxy group at C3 position is a proton-donor connecting with O4, O8 and O9 atoms, but in compound **6b** all lengths were significantly longer (Table 1).

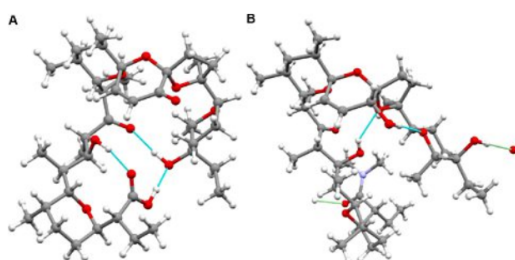


Figure 1. Molecule of (A) C20-oxosalinomycin **2** and (B) *N,N*-dimethyl amide of salinomycin **3a** in the pseudo-cyclic conformation with intra- and intermolecular H-bonds indicated by blue and green lines, respectively. The solvent molecule in compound **3a** was omitted for clarity.

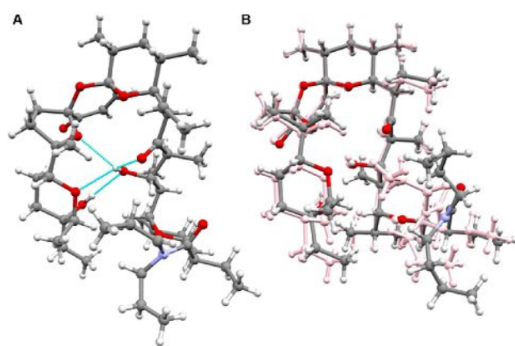


Figure 2. (A) The system of H-bonds present in molecule of *N,N*-diallyl amide of C20-oxosalinomycin **9b**, and (B) the superimposed molecule **9b** (marked with pink color) and *N,N*-dibutyl amide of C20-oxosalinomycin **6b** (colors by atom) with respect to the spiroketal ring system. Both molecules are shown in one of their disordered positions.

Table 1. H-bonds present in molecules **2**, **3a**, **6b** and **9b**. The asterisk marks the intermolecular H-bond.

Compound	H-bond	Length [Å]	Angle [°]
2	O4–H...O1	2.804	162.28
	O2–H...O11	2.705	173.65
	O11–H...O5	2.890	164.73
3a	O3–H...O8	2.925	142.80
	O3–H...O4	2.841	81.35
	O7–H...O9	2.858	174.11
6b	O10–H...O1*	3.069	167.37
	O3–H...O8	2.956	104.51
	O3–H...O9	2.922	149.13
9b	O3–H...O4	2.892	111.04
	O10–H...O3	2.956	145.49
	O3–H...O8	2.976	126.29
	O3–H...O9	2.949	128.14
	O3–H...O4	2.916	122.27
	O10–H...O3	2.968	168.48

Activity Against Primary Acute Lymphoblastic Leukemia Cells, MDA-MB-231 Breast Cancer Cell Line and Normal MCF 10A Breast Cell Line

Salinomycin (**1**), its analogues (**3a–13a**), as well as C20-oxosalinomycin (**2**) and its analogues (**3b–9b**; **11b–13b**) were evaluated for their in vitro antiproliferative effect against primary acute lymphoblastic leukemia cells (ALL-5), human breast adenocarcinoma (MDA-MB-231) and normal mammary epithelial (MCF 10A) cell lines. Since SAL was previously proved to be highly potent toward CSCs,^[43–45] it was of particular interest to investigate effects on the MDA-MB-231 cell line, which harbors a subpopulation of cells with stem-like properties.^[46] Standard viability assays were conducted over a range of concentrations and the data expressed as IC₅₀ (mean ± SD) of the tested compounds (Table 2). For the full set of cell viability curves, see Figure S17 (Supporting Information).

Most of the compounds were more potent toward primary ALL-5 cells than MDA-MB-231 cells (Table 2). However, none of the novel analogues of **1** or its C20-oxo form **2** expressed higher activity toward primary ALL-5 cells than their parent molecules **1** and **2** (Table 2). However, one single- (**5a**) and two novel double-modified analogues (**5b** and **13b**) showed higher cytotoxic activity toward MDA-MB-231 cells than **1** (Table 2). Interestingly, both compounds **5a** and **5b** have the same *N,N*-dipropyl amide substituent at the C1 position (Scheme 1), suggesting that this moiety would positively enhance anti-cancer activity of SAL derivatives against the breast cancer cell line. The recorded IC₅₀ value for unmodified **1** stays in a good agreement with previously reported one.^[47]

Selectivity index (SI) measures the ratio between cytotoxicity and anticancer activity by dividing the given IC₅₀ values for the normal MCF 10A cell line and respective IC₅₀ values for cancerous cells. Higher SI values are associated with preferable toxicity toward cancerous *versus* normal cells. Calculated SI numbers have been presented in Table 2. Beneficial SI of > 1.00 on primary ALL-5 were observed for all active analogues of **1** and six analogues of **2**, namely **3b**, **4b**, **6b**, **9b**, **11b** and **12b** (Table 2). Importantly, those compounds were characterized by higher SI than unmodified **1** (Table 2). In general, SI values

Table 2. * symmetry code x,1 + y,zTable 2
Antiproliferative activity (IC₅₀) and selectivity index (SI) values of salinomycin (**1**), its analogues (**3a–13a**), C20-oxosalinomycin (**2**) and its analogues (**3b–9b**; **11b–13b**).

Compound	ALL-5 IC ₅₀ [μM]	SI	MDA-MB-231 IC ₅₀ [μM]	SI	MCF 10A IC ₅₀ [μM]
1	0.13 ± 0.00	1.00	4.69 ± 0.31	0.03	0.13 ± 0.02
3a	–*	n/a	–*	n/a	n/a
4a	1.28 ± 0.51	7.37	9.58 ± 0.24	0.98	9.44 ± 2.89
5a	1.02 ± 0.17	10.12	4.11 ± 2.19	2.51	10.32 ± 2.14
6a	0.63 ± 0.13	1.36	9.51 ± 1.87	0.09	0.86 ± 0.11
7a	–*	n/a	–*	n/a	n/a
8a	–*	n/a	–*	n/a	n/a
9a	1.71 ± 0.42	5.42	9.44 ± 0.11	0.98	9.27 ± 6.13
10a	–*	n/a	–*	n/a	n/a
11a	3.08 ± 0.07	3.32	–*	n/a	10.23 ± 2.42
12a	–*	n/a	–*	n/a	n/a
13a	–*	n/a	–*	n/a	n/a
2	0.38 ± 0.31	0.29	9.43 ± 0.31	0.01	0.11 ± 0.05
3b	3.69 ± 0.16	< 2.71	–*	n/a	< 10
4b	1.23 ± 0.02	< 8.13	–*	n/a	< 10
5b	4.05 ± 0.71	0.07	1.94 ± 0.42	0.14	0.28 ± 0.14
6b	1.00 ± 0.18	< 10	–*	n/a	< 10
7b	–*	n/a	–*	n/a	n/a
8b	–*	n/a	–*	n/a	n/a
9b	1.69 ± 0.63	5.71	9.70 ± 3.76	0.99	9.65 ± 3.37
11b	1.17 ± 0.08	< 8.55	–*	n/a	< 10
12b	8.20 ± 0.16	< 1.22	–*	n/a	< 10
13b	2.72 ± 0.44	0.96	3.49 ± 0.28	0.75	2.62 ± 0.52

The IC₅₀ value is defined as the concentration of a compound at which 50% growth inhibition is observed. *Inhibition of proliferation did not exceed 50% at the highest concentration tested of 10 μM. SI was calculated for each compound using the formula: SI = IC₅₀ for normal cell line MCF 10A/IC₅₀ for respective cancerous cell line. A beneficial SI > 1.00 indicates a drug with efficacy against tumor cells greater than the toxicity against normal cells.

observed on MDA-MB-231 cell line were much lower, with one compound **5a** showing a very favorable SI = 2.51 which is much higher than the unfavorable value observed for parent molecule **1** (SI = 0.03, Table 2). Thus, with both higher potency and superior SI value compared to **1**, **5a** is a promising lead molecule for future development. Note that SI values are given as a numerical ratio without an estimate of error, because error values become unreliable when numerator and/or denominator values are low.

In vitro Growth Inhibition Against a Panel of Human Cancer Cell Lines

The most active compounds pre-selected in our in vitro cell viability screening (based on the lowest IC₅₀ values, see Table 2) were submitted to the National Cancer Institute (NCI) for screening against their 60 human tumor cell lines panel representing leukemia, non-small cell lung, colon, central nervous system (CNS), melanoma, ovarian, renal, prostate and breast cancer. Firstly, compounds **1**, **4a**, **5a**, **6a**, **9a**, **2**, **4b**, **5b**, **6b** and **9b** were tested at a single concentration of 10^{–5} M on the various cancer subtypes. These data represent percent of growth of the compound treated cells relative to vehicle, and relative to “time zero” cell number. The subsequent growth

percent is utilized to calculate growth inhibition (numbers between 0 and 100) and lethality (numbers < 0). The data are presented in Supporting Information (Table S2). Compounds which showed 60% or higher growth inhibition in at least eight cell lines during one-dose screening were evaluated against the same panel in 5-dose concentrations (see Experimental Section).

Based on the initial screening, eight compounds (**1**, **4a**, **5a**, **6a**, **9a**, **2**, **4b** and **9b**) were selected for 5-dose testing over the range of 10^{-4} to 10^{-8} M. For each cell line dose-response curves were created by plotting cytotoxic activity against the $\log_{10}$ of the compound concentration. For each compound, cytotoxic effect was determined as the GI_{50} value, which refers to the concentration causing 50% growth inhibition. Additionally, due to their promising activity, compounds **1** and **2** were selected for the repeat of 5-dose assay. Results from the 5-dose study are presented in Supporting Information (Table S3).

One derivative **4a** showed higher activity than the parent molecule toward ovarian cancer cell line SK-OV-3 (Supporting Information, Table S3). However, compound **2**, so the C20-oxo form of **SAL**, was in general more potent than other compounds toward OVCAR-3, and -5 (Supporting Information, Table S3). Additionally, analogue **2** was generally more potent than **1** toward cell lines in the colon and breast sub-panels. On the other hand, **1** was characterized by the highest activity toward the panel of CNS cancer cell lines (except U251, where **2** was again more potent).

Activity in an ex Vivo Model of Breast Cancer

Since tumor microenvironment plays an important role in drug responsiveness, it was of particular interest to study the activity of the investigated compounds in an intact tumor system.^[48,49] For this purpose we employed an ex vivo explant model of breast cancer, as we and others have described previously.^[40,48] In this model, fresh unfrozen human breast tumor slices were either fixed immediately or incubated in the presence of vehicle or compounds **1** or **2**.

Since the amount of slices obtained from each individual specimen is limited by the tumor volume and there is non-uniform distribution of cancer cells within each slice requiring multiple replicates for each treatment, only parent molecule **1** and its C20-oxo form **2**, which were the most active toward panel of breast cancer cell lines (Supporting Information, Table S3), were selected for testing. A representative image of an H&E stained section of specimen sample fixed immediately upon receipt is presented in Figure 3A at 10x and 20x magnifications. Slices of the same tumor were treated with vehicle (0.1% DMSO) or **1** or **2** at 8 μ M. This concentration was selected based on previous findings of van der Kuip et al.,^[48] where 8 μ M was found to be optimal for this ex vivo model. Representative images of H&E stained slices at 10x and 20x magnifications are shown in Figure 3B.

Viable tumor cells are distinguished by intact cytology of the nucleus and cytoplasm preservation, whereas dead tumor cells are characterized by apoptotic nuclear debris and coagu-

lative necrosis. No changes in the amount of dead tumor cells were observed when comparing untreated slices (Figure 3A) and vehicle treated (Figure 3B, first column), indicating that culturing conditions had no effect on cell viability. As can be observed in Figure 3B, the number of dead tumor cells significantly increased after treatment with **1** or **2** comparing with vehicle (Figure 3C). Although no statistically significant differences in the amount of dead tumor cells were observed between treatments, compound **2** was less toxic toward normal epithelial cells, as evidenced by the preserved morphology of ductal cells with only a few cells showing vacuolization of the cytoplasm (Figure 3B, third column, indicated by the arrow).

Moreover, immunohistochemical stain for the proliferation marker, Ki-67, was used in the formalin-fixed paraffin-embedded tissue.^[50] Representative images at 10x and 20x magnifications are presented in Figure 3D. The Ki-67 proliferation index is high in tumor cells (~90%) and after treatment with vehicle (similar to untreated tumor samples, not shown), and the Ki-67 proliferation index is intermediate (40–50%) in those samples that were incubated with compounds.

Conclusions

A library of novel doubly modified salinomycin (**SAL**) analogues, merging the previously identified beneficial modifications at C1 and C20 positions, was devised. All 10 targeted compounds were synthesized by chemoselective oxidation of the C20 hydroxy of a cohort of C1 tertiary amides of **SAL** in moderate to good yields (26–97%). Synthesis was facilitated by straightforward protocols that allowed simple reaction workup and convenient isolation of the analogues.

X-ray single crystal analyses of both doubly modified **SAL** derivatives **6b** and **9b** showed their remarkable conformational similarity to the C1 tertiary amide **3a**. The chemical modification of the C1 position and oxidation of the C20 hydroxy did not change the molecular conformation in a significant way. Essential for changing the molecule arrangement seems to be a strong donor-acceptor group at C1 position.

Compound **5a** (single-modified analogue), **5b** and **13b** (double-modified analogues) were found to be more potent in cell viability assay toward the MDA-MB-231 breast cancer cell line than their parent molecule. Moreover, **5a** was simultaneously more active and more selective toward cancerous versus normal cells than **SAL** and its C20-oxo form (compound **2**). Interestingly, a pair **5a/5b** has the same respective *N,N*-dipropyl amide substituent at the C1 position, suggesting that this moiety may play a role in enhancing anticancer activity of **SAL** against breast cancer cell lines. Analogue **4a** showed particular selectivity for ovarian cancer cell line SK-OV-3, when all the compounds were screened against the NCI-60 human tumor cell lines panel. Furthermore, **SAL** and its C20-oxo form **2** were very potent when tested ex vivo against human ER/PR⁺, Her2⁺ invasive mammary carcinoma, with **2** exhibiting minimal toxicity toward normal epithelial cells. Our findings highlight the potential of **SAL** analogues for the treatment of various types of cancer, and provide promising lead compounds for

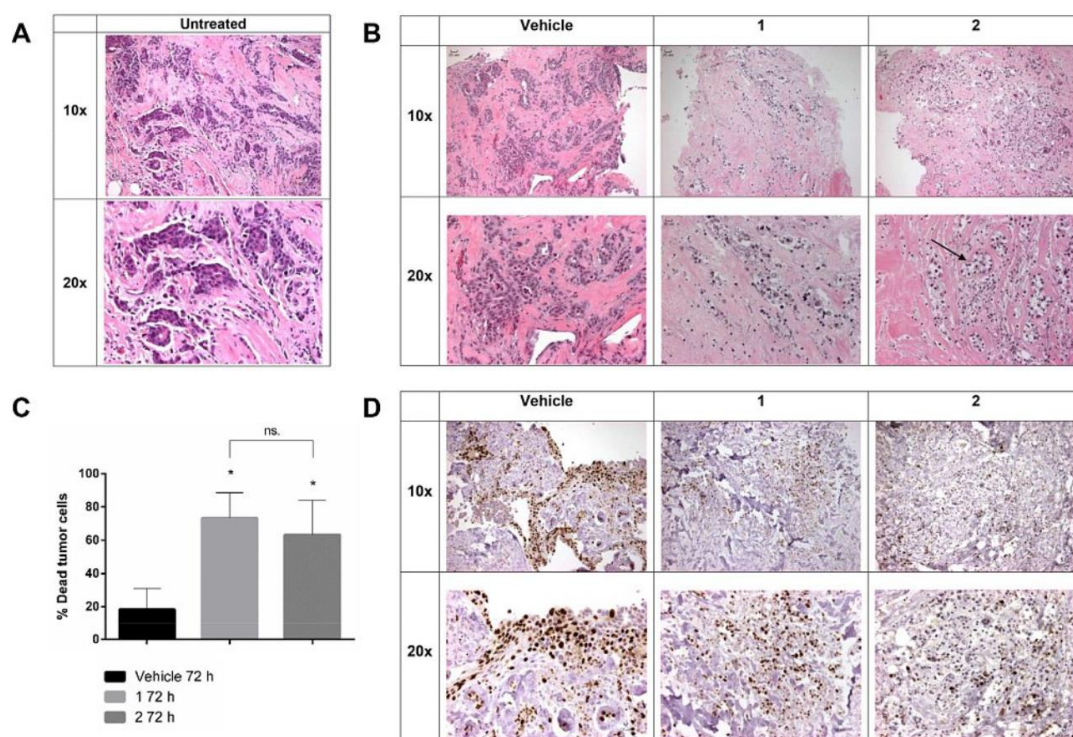


Figure 3. Cytotoxic effects of salinomycin (1) and C20-oxosalinomycin (2) in an ex vivo model of breast cancer. (A) H&E stain of representative untreated sections of infiltrating breast carcinoma. (B) H&E stain of infiltrating breast carcinoma samples treated with vehicle, 1 or 2 after 72 h (Bar = 20 μ m). Right lower panel: the arrow points to normal ductal epithelial cells. (C) Quantification of dead tumor cells was performed as described in Experimental Section. Data represent mean $\pm$ SD (n = 3), *p < 0.05 treatment versus control. (D) Ki-67 stain of representative vehicle, 1 or 2 treated sections for 72 h. Two magnifications are shown, with bars representing 10 μ m.

future development, including in vivo testing of pharmacodynamics and pharmacokinetics.

Experimental Section

General Procedures

All reagents and all solvents were obtained from Merck or Trimex Chemicals S.A. (Poland), and were used as received without further purification. CDCl_3 spectral grade solvent were stored over 3 Å molecular sieves for several days. All manipulations were carried out under nitrogen atmosphere in oven-dried glassware. Reaction mixtures were stirred using teflon-coated magnetic stir bars. Reaction mixtures were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60 F_{254}). TLC plates were visualized by UV-light (254 nm), followed by treatment with phosphomolybdic acid (PMA, 5% in absolute EtOH) and gentle heating. Products of the reactions were purified using CombiFlash® Rf + Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. Solvents were removed using a rotary evaporator.

NMR spectra were recorded on a Varian 400 (^1H NMR at 403 MHz, and ^{13}C NMR at 101 MHz) magnetic resonance spectrometer. ^1H NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl_3 δ 7.26 ppm). ^1H NMR spectra are reported as follows: chemical shift (δ , ppm), multiplicity (s=singlet, d=doublet, q=quartet, dd=doublet of doublets, dt=doublet of triplets, ddd=doublet of doublet of doublets, tdd=triplet of doublet of doublets, m=multiplet), coupling constant(s) in Hz, and integration. Significant peaks are reported within the overlapping ~ 2.00 – 0.50 ppm region of the ^1H NMR spectra. ^{13}C NMR spectra are reported in chemical shifts downfield from TMS using the respective residual solvent peak as internal standard (CDCl_3 δ 77.16 ppm). Line broadening parameters were 0.5 or 1.0 Hz, while the error of chemical shift value was 0.1 ppm.

Infrared spectra in the mid infrared region were recorded in KBr tablets on an IFS 113v FT-IR spectrophotometer (Bruker) equipped with a DTGS detector, and are reported as follows: wavenumbers (cm^{-1}), description (w=weak, m=medium, s=strong, br=broad). The spectra were taken on resolution 2 cm^{-1} , NSS=64. The Happ-Genzel apodization function was used.

Electrospray ionization (ESI) mass spectra were recorded on a Waters/Micromass ZQ mass spectrometer (Waters Alliance) equipped with a Harvard syringe pump. Samples were prepared in

dry acetonitrile, and were infused into the ESI source using a Harvard pump at a flow rate of 20 mL min⁻¹. The ESI source potentials were: capillary 3 kV, lens 0.5 kV, and extractor 4 V. Standard ESI mass spectra were recorded at the cone voltages of 10 and 30 V. The source temperature was 120 °C and the desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 dm³ h⁻¹. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from m/z = 500 to m/z = 1300.

Synthesis

SAL sodium salt was isolated from commercially available veterinary premix SACOX[®] following acidic extraction, using the procedure described by us previously.^[24,25] Briefly, isolated sodium salt of **SAL** was dissolved in CH₂Cl₂ and stirred vigorously with a layer of aqueous sulfuric acid (pH = 1.0). The organic layer containing **SAL** was washed with distilled water, and then CH₂Cl₂ was evaporated under reduced pressure to dryness giving **SAL** as a clear oil. After 3-times repeated evaporation with *n*-pentane this oil was transformed to white amorphous solid. The spectroscopic data of **SAL** were in agreement with previously published data.^[51]

All tertiary amides of **SAL** were prepared using the procedure described by us previously,^[41] while C20-oxosalinomycin **2** (Scheme 1) was prepared following the procedure described by Rodriguez et al.^[35] The NMR data of all the compounds were in good agreement with those found in the reference literature.

General procedure for preparation of novel C1 tertiary amides of salinomycin (analogues 3a, 8a and 9a). To a stirred solution of **SAL** (1.0 equiv.) in CH₂Cl₂ at 0 °C, the following compounds were added: DCC (1.2 equiv.), HOBT (0.5 equiv., dissolved in few ml of THF) and secondary amine (2.5 equiv.). If the amine was in the form of hydrochloride, TEA (3.0 equiv.) was also added. The reaction mixture was further warmed to room temperature and stirred for additional 48 h. After that, the solvent was evaporated under reduced pressure, and the residue was suspended in CH₂Cl₂ and filtered off. Purification of the filtrate on silica gel using the CombiFlash system (30%→100% EtOAc/*n*-hexane) gave the corresponding products of the reactions, i.e. *N,N*-dimethyl **3a**, *N,N*-didodecyl **8a** and *N,N*-diallyl amide **9a**, as the white amorphous solids or as a colorless oil (54%, 72% and 21% yield for **3a**, **8a** and **9a**, respectively).

***N,N*-dimethyl amide of salinomycin 3a.** Yield: 416 mg, 54%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.56 in 50% EtOAc/*n*-hexane. Stains green with PMA. ¹H NMR (403 MHz, CDCl₃) δ: 6.02 (dd, *J* = 10.8, 1.9 Hz, 1H), 5.95 (dd, *J* = 10.7, 1.1 Hz, 1H), 4.17–4.12 (m, 1H), 3.96 (d, *J* = 8.6 Hz, 2H), 3.87 (d, *J* = 8.4 Hz, 1H), 3.83–3.77 (m, 1H), 3.64–3.52 (m, 3H), 3.25–3.18 (m, 1H), 3.15 (s, 3H), 3.04 (s, 3H), 2.67 (dd, *J* = 8.0, 2.1 Hz, 1H), 2.40–2.30 (m, 2H), 2.21–2.13 (m, 1H), 2.00–0.50 (m, 45H), 1.37 (s, 3H), 1.20 (d, *J* = 6.9 Hz, 3H), 0.67 (d, *J* = 6.8 Hz, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) δ: 215.0, 174.4, 133.0, 120.6, 106.2, 98.9, 87.9, 79.6, 77.2, 76.8, 74.6, 74.1, 72.5, 70.8, 70.5, 67.4, 55.9, 47.9, 40.4, 38.7, 37.5, 36.9, 36.4, 36.0, 33.1, 30.6, 30.3, 29.1, 28.6, 26.9, 25.8, 23.0, 22.1, 20.8, 18.9, 17.5, 15.6, 14.5, 13.2, 12.2, 11.8, 8.2, 6.3 ppm. FT-IR (KBr pellet): 3431 (br, m), 2965 (s), 2936 (s), 2875 (s), 1714 (m), 1630 (s), 1498 (w), 1457 (s), 1392 (m), 1381 (s) cm⁻¹. ESI MS (m/z): [M + Na]⁺ Calcd for C₄₄H₇₃NNaO₁₀ 800.5; Found 800.

***N,N*-didodecyl amide of salinomycin 8a.** Yield: 699 mg, 72%. Isolated as a colorless oil, >95% pure by NMR and a single spot by TLC. Rf: 0.72 in 33% EtOAc/*n*-hexane. Stains green with PMA. ¹H NMR (403 MHz, CDCl₃) δ: 6.06 (d, *J* = 10.4 Hz, 1H), 5.95 (d, *J* = 10.9 Hz, 1H), 4.11 (d, *J* = 8.1 Hz, 1H), 3.96 (d, *J* = 12.0 Hz, 2H), 3.84–3.79 (m, 1H),

3.69–3.61 (m, 2H), 3.52–3.44 (m, 1H), 3.37–3.27 (m, 1H), 3.22–3.14 (m, 1H), 3.06–2.99 (m, 1H), 2.77 (d, *J* = 9.6 Hz, 1H), 2.47 (s, 1H), 2.39–2.32 (m, 1H), 2.22–2.16 (m, 1H), 2.00–0.50 (m, 97H), 1.38 (s, 3H), 0.69 (d, *J* = 6.8 Hz, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) δ: 214.8, 173.8, 132.9, 120.8, 106.3, 99.0, 87.7, 79.4, 77.2, 76.9, 73.9, 73.8, 70.8, 70.6, 67.9, 56.2, 48.4, 47.7, 46.8, 40.4, 39.0, 36.6, 35.3, 33.7, 31.9, 30.6, 30.3, 29.71, 29.65, 29.63, 29.61, 29.57, 29.55, 29.44, 29.40, 29.3, 29.2, 27.8, 27.3, 27.0, 25.5, 22.7, 22.6, 22.1, 19.2, 17.4, 15.6, 14.5, 14.09, 14.08, 12.7, 12.2, 8.7, 6.3 ppm. FT-IR (KBr pellet): 3423 (br, m), 2960 (s), 2932 (s), 2875 (s), 2854 (s), 1717 (m), 1631 (s), 1463 (s), 1381 (s) cm⁻¹. ESI MS (m/z): [M + Na]⁺ Calcd for C₆₆H₁₁₉NNaO₁₀ 1108.9; Found 1109.

***N,N*-diallyl amide of salinomycin 9a.** Yield: 160 mg, 21%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.62 in 50% EtOAc/*n*-hexane. Stains green with PMA. ¹H NMR (403 MHz, CDCl₃) δ: 6.04 (dd, *J* = 10.8, 1.6 Hz, 1H), 5.94 (d, *J* = 10.7 Hz, 1H), 5.91–5.78 (m, 2H), 5.29–5.07 (m, 4H), 4.25 (dd, *J* = 16.9, 4.3 Hz, 1H), 4.15 (dd, *J* = 14.4, 4.8 Hz, 1H), 4.09 (d, *J* = 9.0 Hz, 1H), 4.02–3.86 (m, 4H), 3.81 (dd, *J* = 13.8, 6.8 Hz, 1H), 3.67 (dd, *J* = 9.7, 2.7 Hz, 1H), 3.64–3.56 (m, 2H), 3.13 (s, 1H), 3.00 (dt, *J* = 15.1, 7.4 Hz, 1H), 2.89 (s, 1H), 2.71 (dt, *J* = 10.0, 2.3 Hz, 1H), 2.52 (s, 1H), 2.34 (dt, *J* = 12.6, 9.3 Hz, 1H), 2.17 (ddd, *J* = 12.7, 9.8, 4.2 Hz, 1H), 2.10–0.50 (m, 41H), 1.38 (s, 3H), 1.21 (d, *J* = 6.9 Hz, 3H), 1.08 (dd, *J* = 25.7, 12.9 Hz, 1H), 0.68 (d, *J* = 6.7 Hz, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) δ: 214.4, 174.3, 133.9, 133.0, 120.7, 117.4, 106.3, 98.9, 87.9, 79.5, 77.2, 76.9, 73.93, 73.87, 73.3, 70.8, 70.3, 67.6, 55.8, 49.7, 48.2, 47.6, 40.3, 38.7, 36.6, 35.5, 33.3, 30.5, 30.3, 29.7, 29.1, 27.2, 25.6, 23.2, 22.6, 22.0, 18.8, 17.4, 15.6, 14.5, 14.2, 12.9, 12.1, 8.7, 6.3 ppm. FT-IR (KBr pellet): 3433 (br, m), 2965 (s), 2939 (s), 2877 (s), 1709 (m), 1630 (s), 1457 (s), 1417 (s), 1381 (s) cm⁻¹. ESI MS (m/z): [M + Na]⁺ Calcd for C₄₈H₇₇NNaO₁₀ 852.6; Found 853.

General procedure for preparation of novel C20-oxo analogues of salinomycin tertiary amides (analogues 3b–13b, except 10b). To a stirred solution of the corresponding **SAL** tertiary amide **3a–13a** (1.0 equiv.) in CH₂Cl₂, activated MnO₂ (20.0 equiv.) was added. The suspension was stirred for 2 days at room temperature, and then was filtrated over Celite. The filtrate was concentrated under reduced pressure. Purification of the filtrate on silica gel using the CombiFlash system (30%→100% EtOAc/*n*-hexane) gave the corresponding products of the reactions **3b–13b** (except **10b**) as the white amorphous solids or colorless oils (26–97% yield).

***N,N*-dimethyl amide of C20-oxosalinomycin 3b.** Yield: 110 mg, 96%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.76 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ¹H NMR (403 MHz, CDCl₃) δ: 7.20 (d, *J* = 10.8 Hz, 1H), 6.20 (d, *J* = 10.8 Hz, 1H), 4.09 (d, *J* = 9.7 Hz, 1H), 3.93 (d, *J* = 9.4 Hz, 1H), 3.73 (dd, *J* = 13.6, 6.9 Hz, 1H), 3.66 (d, *J* = 7.5 Hz, 1H), 3.58 (d, *J* = 8.7 Hz, 1H), 3.34 (d, *J* = 10.7 Hz, 1H), 3.12 (s, 3H), 2.96 (s, 3H), 2.86 (dd, *J* = 9.4, 7.2 Hz, 1H), 2.68 (dt, *J* = 8.9, 3.1 Hz, 1H), 2.62–2.53 (m, 2H), 2.20–0.50 (m, 49H), 1.39 (s, 3H), 1.17 (d, *J* = 5.7 Hz, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) δ: 214.5, 190.3, 174.5, 143.5, 127.2, 108.2, 105.3, 97.4, 88.7, 77.2, 76.9, 74.4, 72.2, 72.0, 70.9, 70.5, 56.4, 47.4, 39.7, 38.8, 37.4, 36.4, 36.0, 34.1, 33.7, 30.2, 29.6, 29.2, 28.5, 26.7, 22.8, 22.7, 21.0, 20.6, 17.9, 17.6, 15.8, 14.3, 14.2, 12.4, 11.9, 7.8, 6.4 ppm. FT-IR (KBr pellet): 3586 (br, m), 3427 (br, m), 2965 (s), 2932 (s), 2875 (s), 1705 (s), 1631 (s), 1500 (w), 1455 (s), 1398 (m), 1382 (m) cm⁻¹. ESI MS (m/z): [M + Na]⁺ Calcd for C₄₄H₇₃NNaO₁₀ 798.5; Found 798.

***N,N*-diethyl amide of C20-oxosalinomycin 4b.** Yield: 100 mg, 89%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.73 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ¹H NMR (403 MHz, CDCl₃) δ: 7.25 (d, *J* = 10.6 Hz, 1H), 6.19 (d, *J* = 10.7 Hz, 1H), 4.11–4.03 (m, 1H), 4.00–3.92 (m, 1H), 3.85 (d, *J* = 3.7 Hz, 1H), 3.77–3.68 (m, 2H), 3.68–3.64 (m, 1H),

3.53 (dd, $J = 14.7, 7.3$ Hz, 1H), 3.37–3.24 (m, 2H), 2.89 (dd, $J = 16.4, 7.2$ Hz, 1H), 2.81–2.70 (m, 2H), 2.60–2.55 (m, 1H), 2.16–2.05 (m, 2H), 2.00–0.50 (m, 57H), 1.38 (s, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 214.6, 190.5, 173.7, 143.9, 127.2, 105.5, 97.4, 88.6, 77.6, 77.2, 77.0, 74.3, 72.5, 72.0, 70.9, 56.6, 47.1, 42.1, 40.6, 39.7, 39.2, 35.7, 34.5, 34.2, 34.1, 30.2, 29.7, 29.2, 29.0, 27.8, 22.8, 22.4, 20.5, 18.3, 17.6, 15.8, 14.6, 14.3, 13.9, 13.0, 12.2, 11.6, 8.0, 6.4 ppm. FT-IR (KBr pellet): 3542 (br, m), 3406 (br, m), 2968 (s), 2936 (s), 2876 (s), 1702 (s), 1624 (s), 1586 (s), 1460 (s), 1381 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{46}\text{H}_{77}\text{NNaO}_{10}$ 826.5; Found 826.

***N,N*-dipropyl amide of C20-oxosalinomycin 5b.** Yield: 29 mg, 26%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.75 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.26 (d, $J = 10.8$ Hz, 1H), 6.19 (d, $J = 10.8$ Hz, 1H), 4.11 (s, 1H), 3.92 (dd, $J = 12.5, 7.1$ Hz, 1H), 3.77–3.65 (m, 3H), 3.41–3.31 (m, 2H), 3.31–3.22 (m, 1H), 3.18–3.08 (m, 1H), 2.95–2.88 (m, 1H), 2.83 (s, 1H), 2.66–2.56 (m, 2H), 2.20–0.50 (m, 60H), 1.38 (s, 3H), 1.19 (d, $J = 6.9$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 214.8, 190.6, 174.1, 144.0, 127.2, 105.5, 97.4, 88.6, 77.5, 77.2, 77.1, 74.2, 72.7, 71.9, 71.2, 71.0, 56.8, 50.0, 48.3, 47.0, 39.7, 39.2, 35.4, 34.8, 34.3, 34.1, 30.3, 29.7, 29.2, 28.0, 23.9, 22.8, 22.3, 20.8, 20.5, 18.6, 17.6, 15.7, 14.3, 13.7, 12.2, 11.5, 11.4, 8.2, 6.4 ppm. FT-IR (KBr pellet): 3586 (w), 3414 (br, m), 2960 (s), 2924 (s), 2870 (s), 1741 (w), 1696 (s), 1627 (s), 1586 (s), 1459 (s), 1410 (m), 1381 (m) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{48}\text{H}_{81}\text{NNaO}_{10}$ 854.6; Found 855.

***N,N*-dibutyl amide of C20-oxosalinomycin 6b.** Yield: 29 mg, 26%. Isolated as colorless oil, >95% pure by NMR and a single spot by TLC. Rf: 0.58 in 33% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.26 (d, $J = 10.8$ Hz, 1H), 6.19 (d, $J = 10.7$ Hz, 1H), 4.18–4.06 (m, 1H), 3.93 (d, $J = 7.0$ Hz, 2H), 3.76–3.69 (m, 2H), 3.68–3.63 (m, 1H), 3.44–3.24 (m, 4H), 3.20–3.10 (m, 2H), 2.96–2.80 (m, 2H), 2.65–2.54 (m, 2H), 2.10 (tdd, $J = 10.9, 7.3, 3.9$ Hz, 2H), 2.00–0.50 (m, 56H), 1.37 (s, 3H), 1.18 (d, $J = 6.9$ Hz, 3H), 0.71 (d, $J = 7.0$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 215.0, 190.5, 174.0, 144.0, 127.1, 105.4, 97.4, 88.5, 77.4, 77.2, 77.0, 74.4, 72.6, 71.7, 71.2, 70.9, 56.9, 47.1, 46.4, 39.7, 39.2, 34.9, 34.2, 34.0, 31.7, 30.2, 29.8, 29.1, 28.1, 23.8, 22.9, 22.3, 20.5, 20.3, 20.2, 18.7, 17.6, 15.7, 14.3, 13.9, 13.8, 13.7, 12.2, 11.3, 8.2, 6.4 ppm. FT-IR (KBr pellet): 3541 (br, m), 3434 (br, m), 2965 (s), 2936 (s), 2875 (s), 1712 (s), 1694 (s), 1640 (s), 1460 (s), 1404 (w), 1378 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{50}\text{H}_{85}\text{NNaO}_{10}$ 882.6; Found 883.

***N,N*-dioctyl amide of C20-oxosalinomycin 7b.** Yield: 90 mg, 90%. Isolated as a colorless oil, >95% pure by NMR and a single spot by TLC. Rf: 0.65 in 33% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.27 (d, $J = 10.7$ Hz, 1H), 6.20 (d, $J = 10.7$ Hz, 2H), 4.35–4.24 (m, 1H), 4.18–4.10 (m, 1H), 3.92 (s, 1H), 3.73 (dt, $J = 9.5, 6.4$ Hz, 2H), 3.66 (dd, $J = 9.8, 2.6$ Hz, 1H), 3.35 (d, $J = 10.6$ Hz, 2H), 3.30–3.23 (m, 1H), 3.15 (dd, $J = 15.2, 9.1$ Hz, 1H), 2.95–2.83 (m, 2H), 2.61–2.55 (m, 2H), 2.18–2.07 (m, 2H), 2.00–0.50 (m, 76H), 1.38 (s, 3H), 1.19 (d, $J = 6.9$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 215.1, 190.6, 173.9, 144.1, 127.1, 105.4, 97.4, 88.5, 77.2, 77.0, 74.5, 72.5, 71.9, 71.4, 70.9, 56.9, 47.1, 46.7, 39.7, 39.2, 35.1, 34.2, 34.0, 33.1, 31.8, 31.7, 30.2, 29.7, 29.45, 29.35, 29.29, 29.2, 29.1, 27.2, 26.9, 23.0, 22.6, 22.3, 20.5, 18.7, 17.6, 15.7, 15.5, 14.3, 14.10, 14.08, 12.2, 11.1, 8.2, 6.4 ppm. FT-IR (KBr pellet): 3402 (br, m), 2960 (s), 2932 (s), 2875 (s), 2858 (s), 1705 (s), 1627 (s), 1463 (s), 1377 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{58}\text{H}_{101}\text{NNaO}_{10}$ 994.7; Found 995.

***N,N*-didodecyl amide of C20-oxosalinomycin 8b.** Yield: 70 mg, 90%. Isolated as a colorless oil, >95% pure by NMR and a single spot by TLC. Rf: 0.67 in 33% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.27 (d, $J = 10.1$ Hz, 1H), 6.20 (d, $J = 10.7$ Hz, 1H), 4.39–4.26 (m, 1H), 4.20–4.10 (m, 1H), 3.92 (s, 1H), 3.77–3.69 (m, 2H), 3.67 (dd, $J = 10.6, 8.4$ Hz, 1H), 3.35 (d, $J = 10.4$ Hz,

2H), 3.31–3.23 (m, 1H), 3.19–3.11 (m, 1H), 2.95–2.82 (m, 2H), 2.65–2.55 (m, 2H), 2.15–2.07 (m, 2H), 2.00–0.50 (m, 96H), 1.38 (s, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 215.1, 190.6, 174.0, 144.1, 127.1, 105.4, 97.4, 88.5, 77.2, 77.1, 77.0, 74.5, 72.5, 71.9, 71.4, 70.9, 57.0, 47.1, 46.7, 39.7, 39.2, 35.3, 35.1, 34.2, 34.0, 31.89, 31.88, 30.2, 29.69, 29.66, 29.62, 29.59, 29.53, 29.50, 29.3, 29.1, 27.2, 26.9, 23.8, 23.0, 22.7, 22.3, 20.5, 18.7, 17.6, 15.7, 15.5, 14.3, 14.1, 13.7, 12.2, 11.1, 8.2, 6.4 ppm. FT-IR (KBr pellet): 3406 (br, m), 2960 (s), 2928 (s), 2875 (s), 2854 (s), 1705 (s), 1623 (s), 1459 (s), 1381 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{66}\text{H}_{117}\text{NNaO}_{10}$ 1106.9; Found 1107.

***N,N*-diallyl amide of C20-oxosalinomycin 9b.** Yield: 175 mg, 87%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.73 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (400 MHz, CDCl_3) δ : 7.24 (d, $J = 10.8$ Hz, 1H), 6.20 (d, $J = 10.7$ Hz, 1H), 5.80 (tdd, $J = 16.2, 10.7, 5.9$ Hz, 2H), 5.28–5.17 (m, 2H), 5.17–5.05 (m, 2H), 4.16 (dd, $J = 17.0, 4.5$ Hz, 1H), 4.07 (s, 2H), 3.96 (dd, $J = 10.5, 6.2$ Hz, 1H), 3.86 (dd, $J = 16.9, 5.7$ Hz, 2H), 3.80–3.59 (m, 4H), 3.34 (dd, $J = 10.7, 2.0$ Hz, 1H), 2.89 (dt, $J = 15.2, 7.4$ Hz, 1H), 2.80–2.74 (m, 1H), 2.60 (ddd, $J = 12.0, 11.5, 7.1$ Hz, 1H), 2.15–2.00 (m, 2H), 1.98–0.50 (m, 47H) 1.39 (s, 3H), 1.18 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ : 214.4, 190.4, 174.5, 143.7, 133.6, 127.3, 117.5, 105.5, 97.4, 88.7, 78.1, 77.2, 77.1, 74.2, 72.8, 72.0, 70.9, 70.8, 56.1, 49.6, 48.1, 47.0, 39.7, 38.9, 35.5, 34.3, 34.1, 34.0, 30.3, 29.7, 29.4, 29.2, 27.6, 23.6, 22.7, 20.5, 18.2, 17.5, 15.8, 14.3, 13.9, 12.3, 11.8, 8.3, 6.5 ppm. FT-IR (KBr pellet): 3547 (m), 3450 (br, m), 2962 (s), 2933 (s), 2877 (s), 1694 (s), 1644 (s), 1463 (s), 1408 (m), 1379 (m) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{48}\text{H}_{77}\text{NNaO}_{10}$ 850.5; Found 851.

***N,N*-bis(2-methoxyethyl)amide of C20-oxosalinomycin 11b.** Yield: 139 mg, 97%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.68 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.24 (d, $J = 11.0$ Hz, 1H), 6.19 (d, $J = 10.8$ Hz, 1H), 4.18–3.67 (m, 7H), 3.62 (dd, $J = 9.8, 2.5$ Hz, 2H), 3.57–3.36 (m, 6H), 3.33 (s, 3H), 3.30 (s, 3H), 2.89 (ddd, $J = 14.1, 9.5, 7.0$ Hz, 1H), 2.77 (d, $J = 11.2$ Hz, 2H), 2.65–2.56 (m, 1H), 2.16–2.04 (m, 2H), 2.00–0.50 (m, 46H), 1.38 (s, 3H), 1.19 (d, $J = 6.9$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 214.7, 190.5, 175.0, 143.8, 127.2, 105.4, 97.5, 88.6, 77.8, 77.2, 77.1, 74.1, 73.0, 71.9, 71.0, 70.9, 58.8, 58.6, 56.7, 48.9, 47.1, 47.0, 39.7, 39.1, 34.6, 34.2, 34.0, 30.3, 29.7, 29.2, 27.8, 23.8, 22.9, 22.3, 20.5, 18.5, 17.6, 15.8, 15.3, 14.3, 13.8, 11.8, 11.6, 8.3, 6.4 ppm. FT-IR (KBr pellet): 3545 (br, m), 3480 (br, m), 2960 (s), 2924 (s), 2879 (s), 1714 (s), 1696 (s), 1636 (s), 1455 (s), 1409 (s), 1377 (m) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{48}\text{H}_{81}\text{NNaO}_{12}$ 886.6; Found 887.

Morpholine amide of C20-oxosalinomycin 12b. Yield: 47 mg, 45%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.43 in 50% EtOAc/*n*-hexane. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.22 (d, $J = 10.5$ Hz, 1H), 4.09–4.01 (m, 1H), 3.95 (dd, $J = 9.0, 3.9$ Hz, 1H), 3.74 (dd, $J = 13.5, 6.7$ Hz, 2H), 3.70–3.58 (m, 5H), 3.51–3.47 (m, 1H), 3.37–3.32 (m, 1H), 2.93–2.84 (m, 1H), 2.79 (s, 1H), 2.71–2.67 (m, 1H), 2.64–2.56 (m, 1H), 2.20–0.50 (m, 53H), 1.40 (s, 3H), 1.19 (d, $J = 6.9$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 214.4, 190.3, 173.0, 143.4, 127.2, 105.3, 97.4, 88.6, 78.5, 77.2, 76.8, 74.0, 72.7, 72.0, 70.9, 70.6, 67.2, 66.6, 56.4, 47.3, 46.2, 42.5, 39.7, 38.8, 36.1, 34.1, 34.0, 30.4, 29.2, 29.0, 26.6, 22.7, 22.6, 20.5, 18.3, 17.6, 15.8, 14.3, 14.2, 12.3, 12.2, 8.1, 6.4 ppm; one signal is overlapped. FT-IR (KBr pellet): 3582 (br, m), 3430 (br, m), 2965 (s), 2936 (s), 2879 (s), 1705 (s), 1631 (s), 1463 (s), 1377 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{46}\text{H}_{75}\text{NNaO}_{11}$ 840.5; Found 840.

Piperazine amide of C20-oxosalinomycin 13b. Yield: 60 mg, 73%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. Rf: 0.49 in 50% CHCl_3 /acetone. UV active, and stains green with PMA. ^1H NMR (403 MHz, CDCl_3) δ : 7.32 (d, $J =$

10.4 Hz, 1H), 6.49–6.34 (m, 1H), 4.16–4.07 (m, 1H), 3.96 (d, $J=9.9$ Hz, 1H), 3.73 (dd, $J=9.8$, 2.1 Hz, 2H), 3.68 (d, $J=9.5$ Hz, 2H), 3.34 (dd, $J=11.4$, 2.1 Hz, 1H), 3.16–3.04 (m, 2H), 2.78 (dd, $J=9.8$, 7.1 Hz, 1H), 2.61 (d, $J=9.0$ Hz, 1H), 2.51 (dd, $J=21.5$, 9.3 Hz, 1H), 2.10–0.50 (m, 56H), 1.44 (s, 3H), 1.18 (d, $J=6.9$ Hz, 3H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ : 213.5, 190.4, 173.2, 144.8, 127.2, 105.4, 97.5, 89.0, 77.2, 76.6, 73.7, 73.2, 72.5, 71.4, 70.4, 55.7, 47.7, 44.5, 39.9, 38.6, 36.0, 35.2, 34.2, 33.4, 31.0, 29.6, 29.4, 29.2, 27.0, 24.8, 22.6, 21.7, 20.5, 20.4, 17.8, 16.8, 15.9, 14.3, 13.9, 12.6, 11.6, 8.1, 6.5 ppm; one signal is overlapped. FT-IR (KBr pellet): 3414 (br, m), 2965 (s), 2932 (s), 2875 (s), 1705 (s), 1619 (s), 1463 (s), 1377 (s) cm^{-1} . ESI MS (m/z): $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{46}\text{H}_{76}\text{N}_2\text{O}_{10}$ 816.6; Found: 817.

X-ray Measurements

The X-ray diffraction measurements at room temperature were carried out on Eos X-Calibur using MoK α radiation or on SuperNova Agilent 4-circle diffractometer with a CCD detector as well as a powerful microfocus CuK α ($\lambda=1.5418$ Å) radiation source and multilayer collimation optics. For data collections and their preliminary reduction, CrysAlisPro 171.37.31 software was used.^[52] Structure was solved by direct methods (Shelxs) and refined with the Shelxl^[53,54] the Olex2 interface was used.^[55] The hydrogen atoms bound to carbon atoms were located from the geometry, and the hydrogen atoms of hydroxy groups from difference Fourier maps or refined occupancy. The crystal data and experimental details are listed in Table S1 (Supporting Information).

The structures have been deposited with the Cambridge Crystallographic Data Center in the CIF format, no. 1906102 (2); 1872811 (3a); 1872556 (6b) and 1870765 (9b). Copies of this information can be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 1223 336 033); E-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk.

Cell Culture

Human primary acute lymphoblastic leukemia ALL-5 cells, human breast adenocarcinoma cell line MDA-MB-213, and human breast epithelial cell line MCF 10A, were employed to evaluate antiproliferative activity of studied compounds. Primary ALL-5 cells were derived from the bone marrow of a 37-year old patient and as previously described can be cultured for up to 6 months with no change in their properties.^[56,57] Nevertheless, cells at low passages were exclusively used in present study. Primary ALL-5 cells were cultured in IMDM Modified (HyClone) media supplemented with 10 $\mu\text{g mL}^{-1}$ cholesterol, 6 mg mL^{-1} human serum albumin, 2 mM L-glutamine, 2% v/v amphotericin-B/penicillin/streptomycin, 1 $\mu\text{g mL}^{-1}$ insulin, 200 $\mu\text{g mL}^{-1}$ apo-transferrin, and 50 μM β -mercaptoethanol, and were subcultured to maintain a density of $1\text{--}3 \times 10^6$ cells mL^{-1} . MDA-MB-213 cell line was cultured in DMEM/Ham's Nutrient Mixture F12 1:1 (cat. no. 51445 C, Sigma-Aldrich, Deisenhofen, Germany) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (cat. no. FP-0500-A, Atlas Biologicals, Fort Collins, CO, USA), 1% penicillin/streptomycin solution 100x (cat. no. 30-002-Cl, Corning, Manassas, VA, USA) and 1 mM L-glutamine (cat. no. 25005-Cl, Corning, Manassas, VA, USA). MCF 10 A cell line was purchased from ATCC, Manassas, VA, USA (cat. no. CRL-10317) and cultured in Mammary Epithelial Cell Basal Medium (cat. no. C-21215, Promo Cell, Heidelberg, Germany) supplemented with Mammary Epithelial Cell Supplement Pack (cat. no. C-39110, Promo Cell, Heidelberg, Germany), 100 $\mu\text{g mL}^{-1}$ gentamicin (cat. no. G1397, Sigma-Aldrich, Deisenhofen, Germany) and 0.05 $\mu\text{g mL}^{-1}$ amphotericin B (cat. no. A2942, Sigma-Aldrich, Deisenhofen, Germany). All cell types were routinely maintained at 37 °C in a humidified 5% CO_2 incubator. Validation was performed

via short tandem repeat profiling in July 2018 by Genetica DNA Laboratories (Burlington, NC, USA) and verified as authentic, giving a 100% match when compared to the known reference profile.^[58]

Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay was employed to assess cell viability.^[59,60] All compounds were dissolved in cell culture grade DMSO. ALL-5 cells were seeded at a density of 10^5 cells/well in a 96-well plate (TPP, Trasadingen, Switzerland) and treated with different concentrations of compounds or vehicle (0.1% DMSO) for 120 h. Of note, ALL cells have a fairly long doubling time of ~ 60 h^[57] and the assay was designed to encompass two doubling times for untreated control cells. At the end of the incubation, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well, and the plate was incubated at 37 °C for 24 h in a humidified 5% CO_2 incubator. Then, 100 μL of 10% SDS in 0.01 M HCl was added to each well and the plate was incubated at 37 °C for a further 24 h. Absorbance was recorded at 540 nm using a BioTek Plate Reader. MDA-MB-231 and MCF 10A cells were seeded in 96-well plates (TPP, Trasadingen, Switzerland) at a density of 2×10^3 or 10^4 , respectively, in 100 μL of corresponding medium. After 24 h cells were treated with drugs at concentrations up to 10 μM for 72 h (MDA-MB-231) or 96 h (MCF 10A) with control cells receiving vehicle (0.1% DMSO) alone. After treatment, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well, and the plate was incubated at 37 °C for 4 h in a humidified 5% CO_2 incubator. Medium was then aspirated and 150 μL of DMSO was added to each well and the plate agitated on a shaking platform (150 rpm) for 10 min. Absorbance was recorded at 540 nm using a BioTek Plate Reader. Inhibition of formation of colored MTT formazan was taken as an index of cytotoxicity activity. IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 6 for Windows (GraphPad Software).

NCI-60 Human Tumor Cell Lines Screening Methodology

Anticancer screening assay was performed by the National Cancer Institute employing previously published methodology.^[60] Tumor cell lines were cultured in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well plates microtiter plates in 100 μL of medium at plating densities varying from 5,000 to 40,000 cells per well, depending on the doubling time of each individual cell line. In the next step, plates were incubated at 37 °C, 5% CO_2 , 95% air and 100% relative humidity for 24 h prior the addition of tested compounds. After 24 h, two microtiter plates of each cell line were fixed *in situ* with trichloroacetic acid (TCA). Optical density reading for this point represent a measurement of the cell population for each cell line at the time of compound addition (time zero, Tz). All the compounds studied were dissolved in DMSO at 400-fold the final maximum test concentration and stored frozen prior to use. An aliquot of concentrate was thawed and diluted as required in complete medium containing 50 $\mu\text{g mL}^{-1}$ gentamicin and aliquots of 100 μL added to the respective microtiter wells already containing 100 μL of medium. After 48 h incubation, assays performed on adherent cells were terminated by the addition of 50 μL cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and microtiter plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for another 10 minutes at room temperature. After SRB staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was subsequently dissolved employing 10 mM

Trizma base, and the absorbance was read at 515 nm. The protocol for non-adherent cells was similar except that the assay was terminated by fixing settled cells at the bottom of the wells by gentle addition of 50 μ L of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements (time zero (Tz), control growth (C), and test growth in the presence of compound at five concentration levels (Ti)), the percentage growth was calculated for every compound concentration level. Percentage of growth inhibition was calculated according to the following formulas: $[(Ti - Tz)/(C - Tz)] \times 100$ for concentrations for which $Ti > Tz$; or $[(Ti - Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$.

Tissue Slices Preparation and Culture

Fresh breast cancer tissue was provided by the Cooperative Human Tissue Network (CHTN, <https://www.chtn.org/>), a National Cancer Institute supported resource. Other investigators may have received samples from these same tissue specimens. The specimen used was collected from a 34-year old female patient diagnosed with ER positive, PR positive, Her2 negative invasive mammary carcinoma and delivered within 24 h after surgical removal in UW Cold Storage solution. The tumor was sliced according to our previously established methodology^[40] based on the studies of Van der Kuip et al.^[48] Briefly, 200 μ m slices were cut in sterile, cold PBS (cat. no. 21-030-CM, Corning, Manassas, VA, USA) supplemented with 1% antibiotic/antimycotic solution (cat. no. A5955, Sigma-Aldrich, Deisenhofen, Germany) using a microtome with vibrating blade (Leica Biosystems VT1200, Nussloch, Germany). In order to prevent contamination, blades were steam sterilized before use. Several single slices from different parts of the specimen were immediately placed into embedding cassettes (cat. no. 27158-2B and cat. no. 27154-1, Ted Pella, Redding, CA, USA) and fixed in 10% neutral-buffered formalin for 24 h and further stored in 70% ethanol prior to processing. The remaining slices were cultured in separate wells of a 24 well plate in 1 mL of Mammary Epithelial Cell Basal Medium (cat. no. C-21215, Promo Cell, Heidelberg, Germany) supplemented with Mammary Epithelial Cell Supplement Pack (cat. no. C-39110, Promo Cell, Heidelberg, Germany), 100 μ g mL⁻¹ gentamicin (cat. no. G1397, Sigma-Aldrich, Deisenhofen, Germany) and 0.05 μ g mL⁻¹ amphotericin B (cat. no. A2942, Sigma-Aldrich, Deisenhofen, Germany). The plate was incubated at 37°C in a constant atmosphere of 5% CO₂ on a shaking platform at 150 rpm (Orbi-Shaker Jr, Benchmark Scientific, Sayreville NJ, USA). After 24 h to allow tissue equilibration, treatment with 0.1% DMSO or 8 μ M of **1** or **2** was conducted for 72 h. Three biological replicates were performed for each condition (n = 3). The medium was changed every 24 h. Tissue slices were then fixed and stored as described above.

Histologic Assessment and Immunohistochemical Staining

Paraffin embedded tissue sections (4 μ m) were stained with hematoxylin and eosin (H&E) (cat. no. 7231, Richard-Allan Scientific, Thermo Fisher Scientific, Waltham, MA, USA) for histopathological examination. Immunohistochemical staining was performed by the UAMS Experimental Pathology Core. After deparaffinization and hydration of the tissue, epitope retrieval (cat. no. S1699, Target Retrieval Solution, Dako, Carpinteria, CA, USA) was achieved in a decloaking chamber for 20 min (Biocare medical). Immunohistochemical staining for Ki-67 (1:100, rabbit monoclonal anti-Ki67, cat. no. ab16667, Abcam, Cambridge, MA, USA) was performed with biotinylated goat anti-rabbit second antibody (1:400) (cat. no. BA-1000, Vector, Burlingame, CA, USA) followed by detection using the Vectastain ABC Elite detection system for 30 min (cat. no. PK-6100, Vector, Burlingame, CA, USA). Dako DAB+ (cat. No. K3468, Dako

Liquid, Carpinteria, CA, USA) was used for 3 minutes prior to counterstaining with hematoxylin. Slides were then coverslipped using mounting media.

The estimated proportion of necrotic tumor cells was quantified manually based on changes in cell and nuclear morphology using a Nikon Eclipse E600 microscope. Specifically, tumor cells which were distinctly reduced in size and/or showed nuclear fragmentation were scored. Effects of compounds on normal ductal epithelial cells were similarly assessed by evaluating architectural and cytological features. A minimum of 50 cells were examined per condition. Each evaluation was conducted in triplicate.

Statistical Analysis

1-way ANOVA with Tukey's multiple comparisons test was used to determine statistically significant differences between means in experimental groups and p values of <0.05 were considered significant. Data are presented as a mean $\pm$ SD. Statistical analysis and graphical representations were performed using GraphPad Prism 6 for Windows (GraphPad Software).

Acknowledgements

M.A. acknowledges the Polish Science Centre (NCN) for financial support by a SONATA grant (2016/23/D/ST5/00242) and the Foundation for Polish Science (FNP) for a START scholarship. D.C. and S.S. acknowledge a scholarship (No. POWR. 03.02.00-00-1026/16 and POWR. 03.02.00-00-1023/17, respectively) co-financed by the European Union through the European Social Fund under the Operational Program Knowledge Education Development. Testing was performed by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, US National Cancer Institute (NCI; <http://dtp.cancer.gov>). T.C.C. acknowledges support from the Arkansas Breast Cancer Research Program. The authors thank Mrs. Jennifer James from the UAMS Experimental Pathology Core.

Keywords: Cancer • Chemoselectivity • Crystal structures • In vitro screening • Polyether ionophores

- [1] H. Nagai, Y. H. Kim, *J. Thorac. Dis.* **2017**, *9*, 448–451.
- [2] World Health Organization – Cancer, [on-line access: 2019–09–04]. <http://www.who.int/cancer/en/>.
- [3] A. Rayan, J. Raiyn, M. Falah, *PLoS One* **2017**, *12*, e0187925.
- [4] M. M. Xu, Y. Pu, R. R. Weichselbaum, Y. X. Fu, *Oncogene* **2017**, *36*, 585–592.
- [5] M. Arruebo, N. Vilaboa, B. Sáez-Gutierrez, J. Lambea, A. Tres, M. Valladares, Á. González-Fernández, *Cancers (Basel)* **2011**, *3*, 3279–3330.
- [6] E. Chatelut, J. P. Delord, P. Canal, *Invest. New Drugs* **2003**, *21*, 141–148.
- [7] C. Y. Huang, D. T. Ju, C. F. Chang, P. M. Reddy, B. K. Velmurugan, *Biomedicine (Taipei)* **2017**, *7*, 23.
- [8] B. A. Chabner, T. G. Roberts Jr., *Nature Rev. Cancer* **2005**, *5*, 65–72.
- [9] V. T. DeVita Jr., E. Chu, *Cancer Res.* **2008**, *68*, 8643–8653.
- [10] G. M. Cragg, D. J. Newman, *Biochim. Biophys. Acta* **2013**, *1830*, 3670–3695.
- [11] M. Antoszczak, *Eur. J. Med. Chem.* **2019**, *164*, 366–377.
- [12] M. Huang, B. Liu, R. Liu, J. Li, J. Chen, F. Jiang, H. Ding, Z. Deng, T. Liu, *ACS Pharmacol. Transl. Sci.* **2018**, *1*, 84–95.
- [13] S. Müller, T. Cañeque, V. Acevedo, R. Rodriguez, *Isr. J. Chem.* **2017**, *57*, 239.

- [14] M. Vanneste, Q. Huang, M. Li, D. Moose, L. Zhao, M. A. Stamnes, M. Schultz, M. Wu, M. D. Henry, *Sci. Rep.* **2019**, 9, 1200.
- [15] Y. Miyazaki, M. Shibuya, H. Sugawara, O. Kawaguchi, C. Hirsoe, *J. Antibiot. (Tokyo)* **1974**, 27, 814–821.
- [16] M. Antoszczak, A. Huczyński, *Eur. J. Med. Chem.* **2019**, 176, 208–227.
- [17] M. Antoszczak, D. Steverding, A. Huczyński, *Eur. J. Med. Chem.* **2019**, 166, 32–47.
- [18] J. Dewangan, S. Srivastava, S. K. Rath, *Tumor Biol.* **2017**, 39, 1010428317695035.
- [19] P. B. Gupta, T. T. Onder, G. Jiang, K. Tao, C. Kuperwasser, R. A. Weinberg, E. S. Lander, *Cell* **2009**, 138, 645–659.
- [20] E. Battle, H. Clevers, *Nat. Med.* **2017**, 23, 1124–1134.
- [21] C. Naujokat, R. Steinhart, *J. Biomed. Biotechnol.* **2012**, 2012, 950658.
- [22] M. Antoszczak, *Eur. J. Med. Chem.* **2019**, 166, 48–64.
- [23] M. Antoszczak, E. Maj, A. Napiórkowska, J. Stefańska, E. Augustynowicz-Kopeć, J. Wietrzyk, J. Janczak, B. Brzezinski, A. Huczyński, *Molecules* **2014**, 19, 19435–19459.
- [24] M. Antoszczak, K. Popiel, J. Stefańska, J. Wietrzyk, E. Maj, J. Janczak, G. Michalska, B. Brzezinski, A. Huczyński, *Eur. J. Med. Chem.* **2014**, 76, 435–444.
- [25] M. Antoszczak, E. Maj, J. Stefańska, J. Wietrzyk, J. Janczak, B. Brzezinski, A. Huczyński, *Bioorg. Med. Chem. Lett.* **2014**, 24, 1724–1729.
- [26] A. Urbaniak, M. Delgado, M. Antoszczak, A. Huczyński, T. C. Chambers, *Biomed. Pharmacother.* **2018**, 99, 384–390.
- [27] A. Huczyński, M. Antoszczak, N. Kleczewska, M. Lewandowska, E. Maj, J. Stefańska, J. Wietrzyk, J. Janczak, L. Celewicz, *Eur. J. Med. Chem.* **2015**, 93, 33–41.
- [28] B. Li, J. Wu, W. Zhang, Z. Li, G. Chen, Q. Zhou, S. Wu, *Bioorg. Med. Chem. Lett.* **2017**, 27, 1624–1626.
- [29] B. Borgström, X. Huang, E. Chygorin, S. Oredsson, D. Strand, *ACS Med. Chem. Lett.* **2016**, 7, 635–640.
- [30] B. Borgström, X. Huang, M. Pošta, C. Hegardt, S. Oredsson, D. Strand, *Chem. Commun.* **2013**, 49, 9944–9946.
- [31] W. Zhang, J. Wu, B. Li, J. Xia, H. Wu, L. Wang, J. Hao, Q. Zhou, S. Wu, *RSC Adv.* **2016**, 6, 41885–41890.
- [32] Q. Shi, Y. Li, S. Bo, X. Li, P. Zhao, Q. Liu, Z. Yang, H. Cong, H. Deng, M. Chen, S. Chen, X. Zhou, H. Ding, Z. X. Jiang, *Chem. Commun.* **2016**, 52, 5136–5139.
- [33] Y. Li, Q. Shi, J. Shao, Y. Yuan, Z. Yang, S. Chen, X. Zhou, S. Wen, Z. X. Jiang, *Eur. J. Med. Chem.* **2018**, 148, 279–290.
- [34] B. Borgström, X. Huang, C. Hegardt, S. Oredsson, D. Strand, *Chem. Eur. J.* **2017**, 23, 2077.
- [35] T. T. Mai, A. Hamai, A. Hienzsch, T. Cañeque, S. Müller, J. Wicinski, O. Cabaud, C. Leroy, A. David, V. Acevedo, A. Ryo, C. Ginestier, D. Birnbaum, E. Charafe-Jauffret, P. Codogno, M. Mehrpour, R. Rodriguez, *Nat. Chem.* **2017**, 9, 1025–1033.
- [36] W. Zhang, J. Wu, B. Li, X. Lian, J. Xia, Q. Zhou, S. Wu, *Eur. J. Med. Chem.* **2017**, 138, 353–356.
- [37] W. Zhang, J. Wu, B. Li, H. Wu, L. Wang, J. Hao, S. Wu, Q. Zhou, *Org. Biomol. Chem.* **2016**, 14, 2840–2845.
- [38] M. Huang, Z. Deng, J. Tian, T. Liu, *Eur. J. Med. Chem.* **2017**, 127, 900–908.
- [39] M. Antoszczak, E. Maj, B. Borgström, S. Oredsson, A. Huczyński, D. Strand, *Tetrahedron Lett.* **2017**, 58, 2396–2399.
- [40] M. Antoszczak, A. Urbaniak, M. Delgado, E. Maj, B. Borgström, J. Wietrzyk, A. Huczyński, Y. Yuan, T. C. Chambers, D. Strand, *Eur. J. Med. Chem.* **2018**, 156, 510–523.
- [41] J. Stefańska, M. Antoszczak, K. Stępień, M. Bartoszcze, T. Mirski, A. Huczyński, *Bioorg. Med. Chem. Lett.* **2015**, 25, 2082–2088.
- [42] E. F. Paulus, L. Vértessy, *Z. Kristallogr. NCS* **2004**, 219, 184–186.
- [43] Y. Lu, W. Ma, J. Mao, X. Yu, Z. Hou, S. Fan, B. Song, H. Wang, J. Li, L. Kang, P. Liu, Q. Liu, L. Li, *Chem.-Biol. Interact.* **2015**, 228, 100–107.
- [44] H. An, J. Y. Kim, N. Lee, Y. Cho, E. Oh, J. H. Seo, *Biochem. Biophys. Res. Commun.* **2015**, 466, 696–703.
- [45] H. An, J. Y. Kim, E. Oh, N. Lee, Y. Cho, J. H. Seo, *PLoS One* **2015**, 10, e0141919.
- [46] W. Li, H. Ma, J. Zhang, L. Zhu, C. Wang, Y. Yang, *Sci. Rep.* **2017**, 7, 13856.
- [47] B. Li, J. Wu, W. Zhang, Z. Li, G. Chen, Q. Zhou, *Bioorg. Med. Chem. Lett.* **2017**, 27, 1624–1626.
- [48] H. Van der Kuip, T. E. Mürdter, M. Sonnenberg, M. McClellan, S. Gutzeit, A. Gerteis, W. Simon, P. Fritz, W. E. Aulitzky, *BMC Cancer* **2006**, 6, 86.
- [49] P. J. Morin, *Drug Resist. Updat.* **2003**, 6, 169–172.
- [50] R. Yerushalmi, R. Woods, P. M. Ravdin, M. M. Hayes, K. A. Gelmon, *Lancet Oncol.* **2010**, 11, 174–183.
- [51] A. Huczyński, J. Janczak, J. Stefańska, M. Antoszczak, B. Brzezinski, *Bioorg. Med. Chem. Lett.* **2012**, 22, 4697–4702.
- [52] Xcalibur CCD System. CrysAlisPro Software System, version 1.171.33; Oxford Diffraction Ltd.: Wrocław, Poland, **2009**.
- [53] G. M. Sheldrick, *Acta Crystallogr. C Struct. Chem.* **2015**, 71, 3–8.
- [54] G. M. Sheldrick, *Acta Crystallogr. Sect. A* **2008**, 64, 112–122.
- [55] O. V. Dolomanov, L. J. Bourhis, R. J. Gildea, J. A. K. Howard, H. J. Puschmann, *Appl. Crystallogr.* **2009**, 42, 339–341.
- [56] A. Kothari, W. N. Hittelman, T. C. Chambers, *Cancer Res.* **2016**, 76, 3553–3561.
- [57] B. A. Nijmeijer, K. Suzhai, H. M. Goselink, M. L. J. Van Schie, M. Van der Burg, D. De Jong, E. W. Marijt, O. G. Ottmann, R. Willemze, J. H. F. Falkenburg, *Exp. Hematol.* **2009**, 37, 376–385.
- [58] W. G. Dirks, R. A. MacLeod, Y. Nakamura, A. Kohara, Y. Reid, H. Milch, H. G. Drexler, H. Mizusawa, *Int. J. Cancer* **2010**, 126, 303–304.
- [59] D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, M. R. Boyd, *Canc. Res.* **1988**, 48, 4827–4833.
- [60] M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker, M. R. Boyd, *Canc. Res.* **1988**, 48, 589–601.

Manuscript received: October 23, 2019
Accepted manuscript online: November 8, 2019
Version of record online: November 27, 2019

**Synthesis and Anticancer Activity of Tertiary Amides of
Salinomycin and Their C20-oxo Analogues**

Dominika Czerwonka⁺, Alicja Urbaniak⁺, Szymon Sobczak, Sergio Piña-Oviedo,
Timothy C. Chambers, Michał Antoszczak,^{*} and Adam Huczyński

Index

X-ray analysis.....	– S3 –
ESI MS analysis of C1 tertiary amides of salinomycin and their C20-oxo analogs.....	– S4 –
¹ H and ¹³ C NMR spectra of selected salinomycin analogs.....	– S9 –
Biological activity tests.....	– S21 –

X-ray analysis

Table S1. Detailed crystallographic data of C20-oxosalinomycin (**2**), *N,N*-dimethyl amide of salinomycin (**3a**) as well as *N,N*-dibutyl amide (**6b**) and *N,N*-diallyl amide of C20-oxosalinomycin (**9b**).

Formula	2	3a	6b	9b
Wavelength (Å)	1.54184	0.71075	0.71075	1.54184
Crystal system	orthorhombic	monoclinic	monoclinic	orthorhombic
Space group	P2 ₁ 2 ₁ 2 ₁	C2	P2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions				
<i>a</i> (Å)	23.5880(2)	11.939(3)	9.22259(12)	11.6256(2)
<i>b</i> (Å)	11.06405(12)	11.4323(19)	18.9417(2)	17.2694(4)
<i>c</i> (Å)	18.38600(19)	19.560(4)	24.7717(3)	24.3151(5)
<i>α</i> (°)	90	90	90	90
<i>β</i> (°)	99.8085(9)	104.76(2)	90	90
<i>γ</i> (°)	90	90	90	90
Volume (Å ³)	4327.41(9)	4728.21(8)	2581.7(9)	4881.67(17)
<i>Z</i> / <i>Z'</i>	4/1	4/1	2/1	4/1
<i>D_x</i> (g cm ⁻³)	1.150	1.133	1.111	1.123
Absorption (mm ⁻¹)	0.662	0.079	0.076	0.618
<i>F</i> (000)	1632.0	1761.0	949.0	1808.0
Crystal size (mm)	0.354 × 0.167 × 0.095	0.697 × 0.225 × 0.083	0.531 × 0.139 × 0.063	0.717 × 0.225 × 0.136
2 θ -range (°)	5.874 to 130.174	4.078 to 52.744	5.802 to 52.738	6.278 to 153.546
Min/max indices: <i>h</i> , <i>k</i> , <i>l</i>	−10 ≤ <i>h</i> ≤ 10, −14 ≤ <i>k</i> ≤ 22, −29 ≤ <i>l</i> ≤ 22	−19 ≤ <i>h</i> ≤ 29, −13 ≤ <i>k</i> ≤ 11, −22 ≤ <i>l</i> ≤ 21	−14 ≤ <i>h</i> ≤ 14, −14 ≤ <i>k</i> ≤ 14, −24 ≤ <i>l</i> ≤ 24	−14 ≤ <i>h</i> ≤ 9, −21 ≤ <i>k</i> ≤ 20, −30 ≤ <i>l</i> ≤ 30
Ref. collected/unique	10827/6211	19356/8291	16585/10203	25827/9496
<i>R_{int}</i>	0.0268	0.0223	0.1330	0.0352
Refinement method	least-squares	least-squares	least-squares	least-squares
Completeness (%)	0.90	0.9	0.97	0.97
Data/restraints/parameter	6211/0/491	8291/73/551	10203/889/561	9496/637/584
Goodness-of-fit on <i>F</i> ²	1.057	1.071	0.961	1.036
Final <i>R</i> ₁ / <i>wR</i> ₂ (<i>I</i> > 2σ ₁)	0.0407/0.0956	0.0428/0.1227	0.1267/0.2779	0.0545/0.1503
<i>R</i> ₁ / <i>wR</i> ₂ (all data)	0.0451/0.1004	0.0452/0.1253	0.3826/0.4383	0.0692/0.1642
Largest diff. peak/hole (e.Å ⁻³)	0.19/−0.24	0.31/−0.17	0.28/−0.65	0.19/−0.17

ESI MS analysis of C1 tertiary amides of salinomycin and their C20-oxo analogs

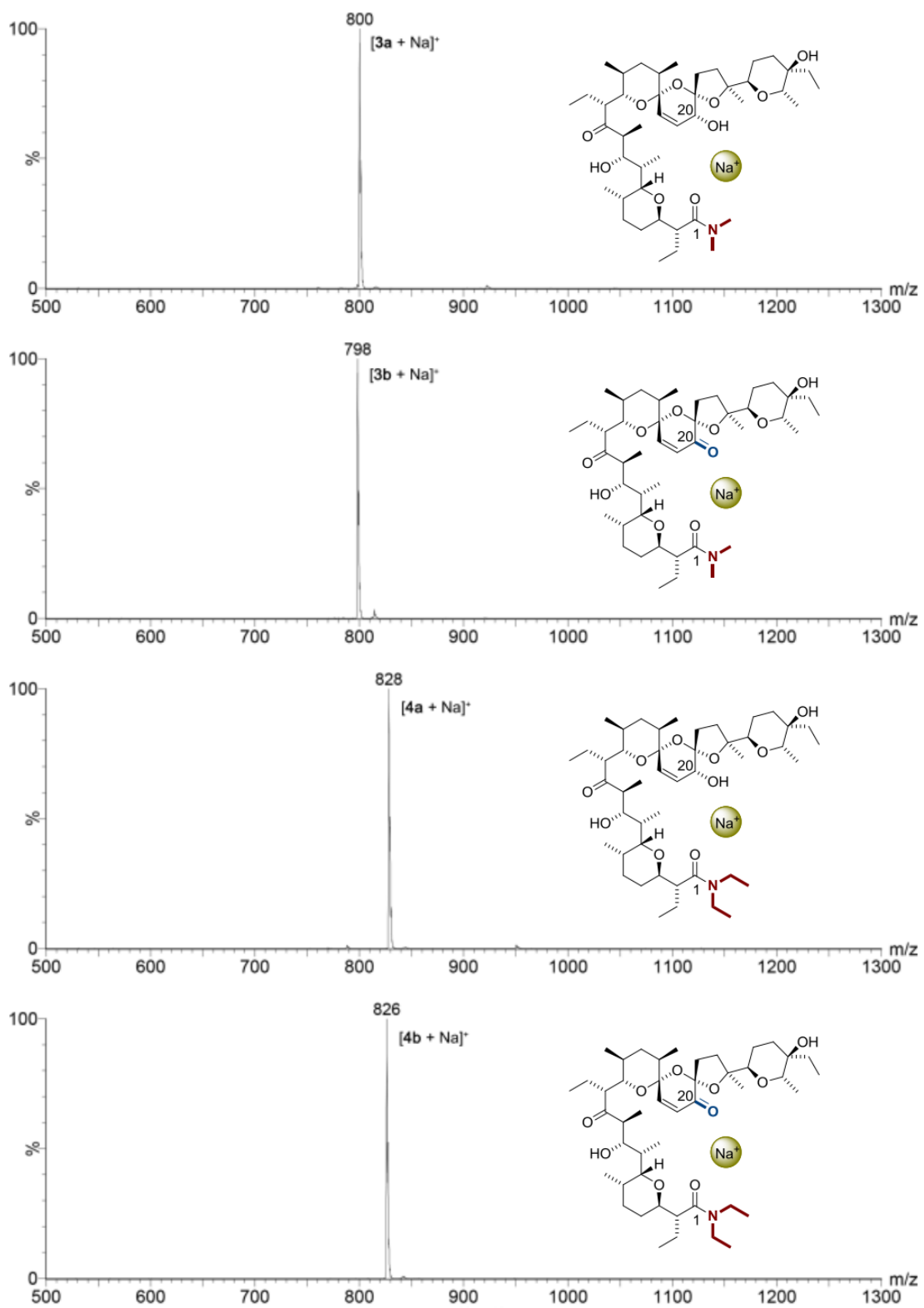


Figure S1. ESI mass spectra of a mixture of **3a**, **3b**, **4a** and **4b** with $NaClO_4$ at $cv = 30$ V.

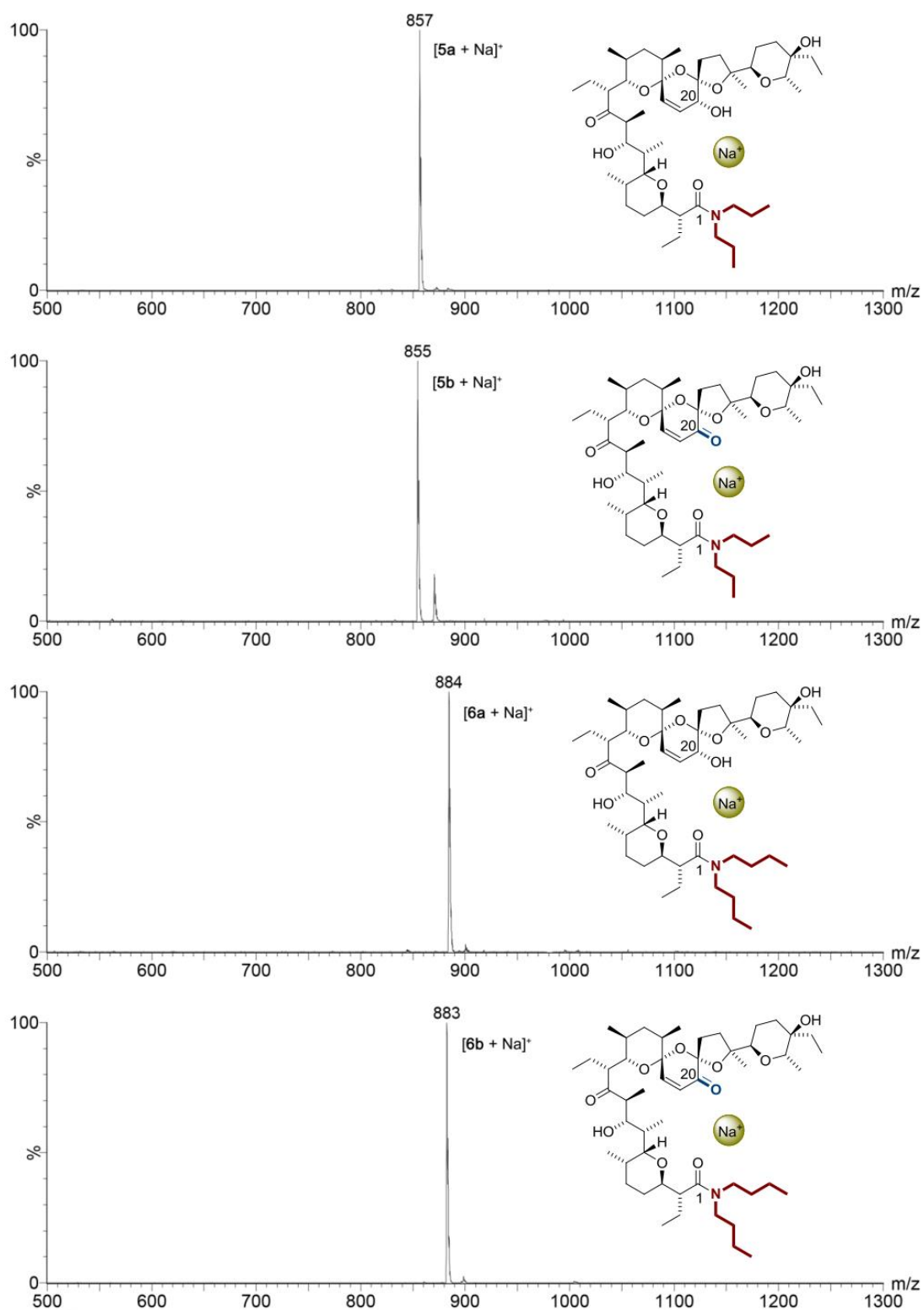


Figure S2. ESI mass spectra of a mixture of **5a**, **5b**, **6a** and **6b** with $NaClO_4$ at $cv = 30$ V.

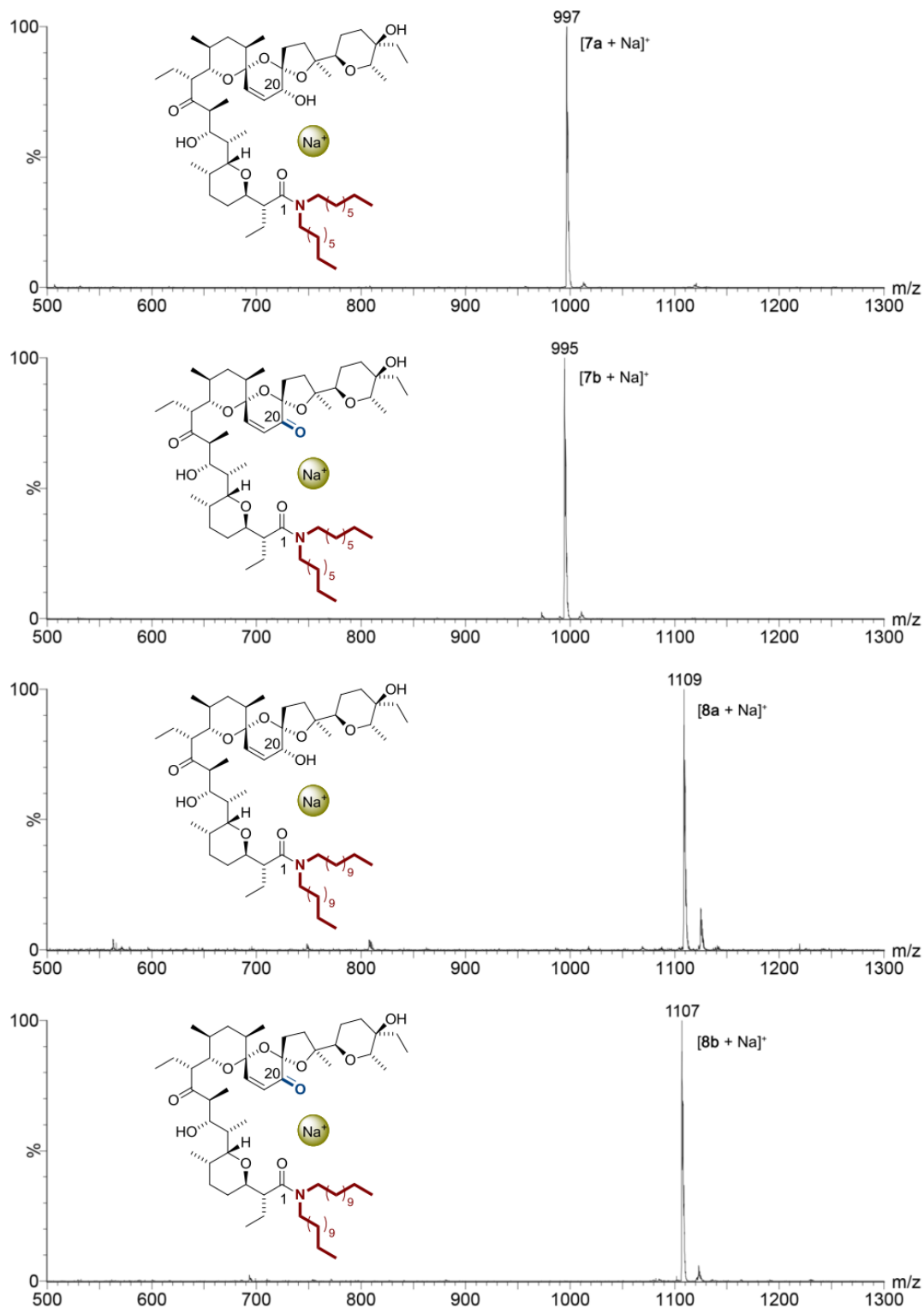


Figure S3. ESI mass spectra of a mixture of **7a**, **7b**, **8a** and **8b** with $NaClO_4$ at $cv = 30$ V.

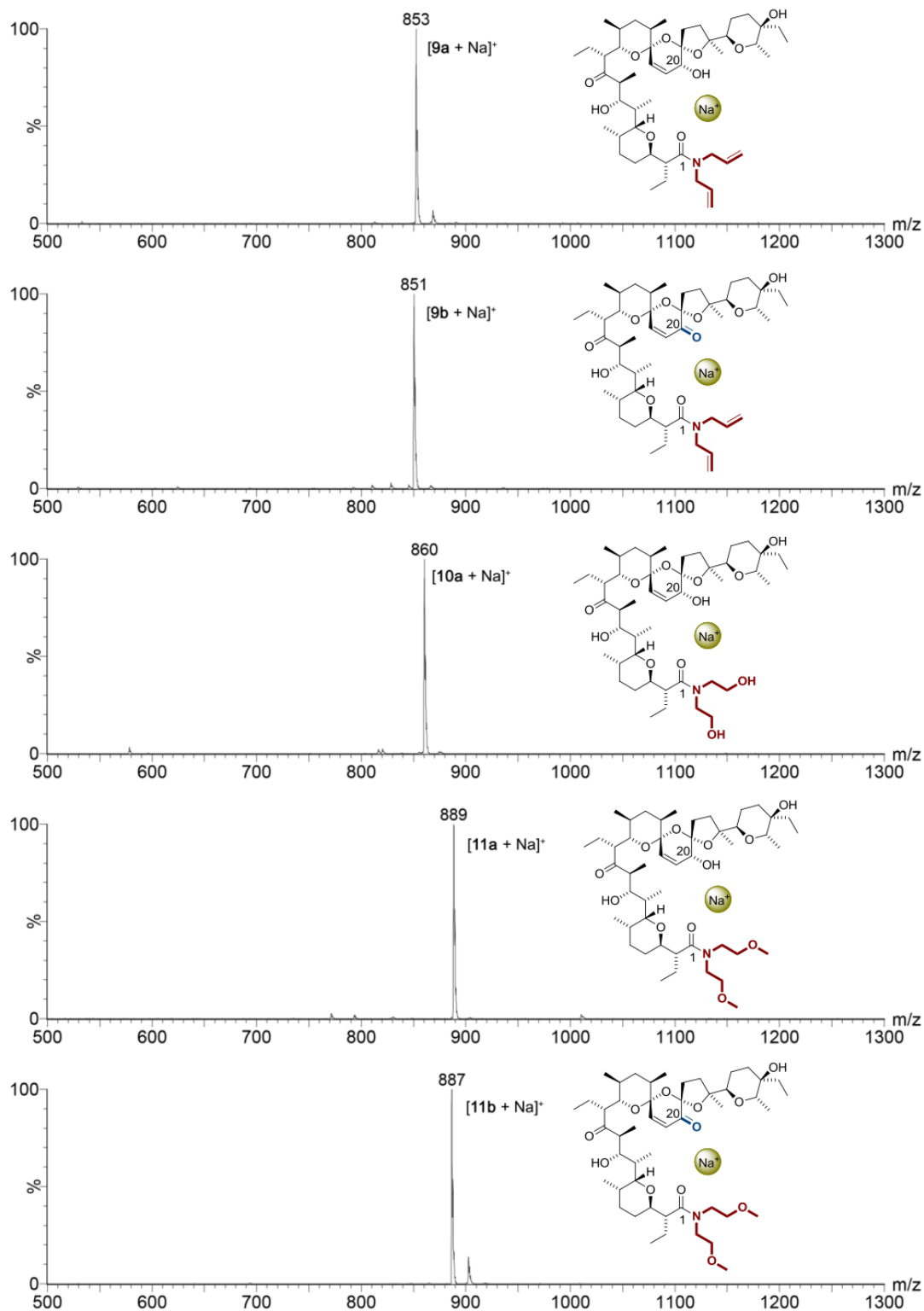


Figure S4. ESI mass spectra of a mixture of **9a**, **9b**, **10a**, **11a** and **11b** with NaClO₄ at cv = 30 V.

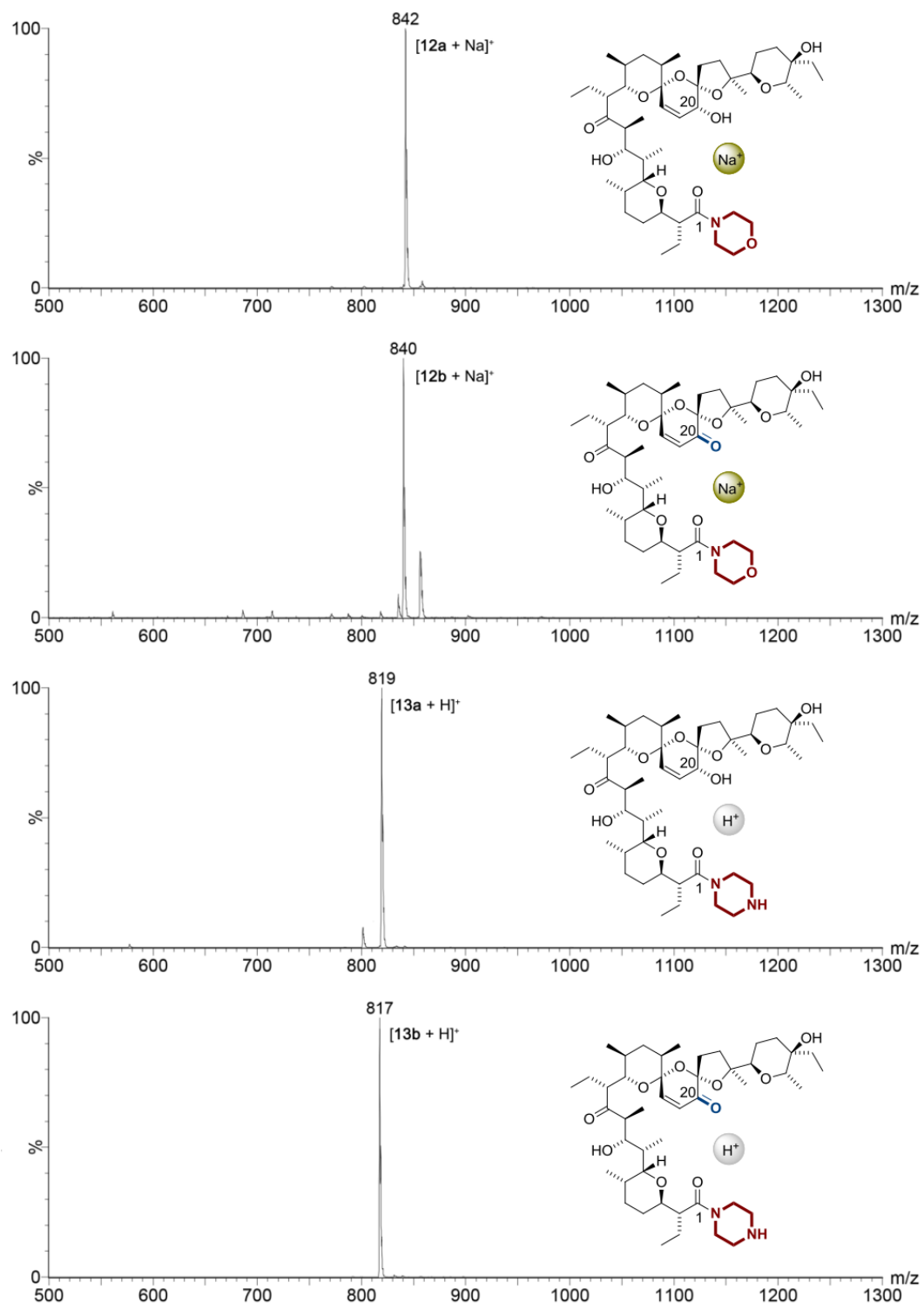


Figure S5. ESI mass spectra of a mixture of 12a, 12b, 13a and 13b with $NaClO_4$ at $cv = 30$ V.

¹H and ¹³C NMR spectra of selected salinomycin analogs

Briefly, the ¹³C NMR signal of the carboxyl group of unmodified **SAL** is observed at 177.7 ppm (**Figure S6**). Thus, the evidence for the formation of C1 tertiary amides of **SAL** is the presence of the characteristic signal of the amide group in their ¹³C NMR spectra in the range of 172.8–175.5 ppm (**Figure S6**). With respect to the C1/C20 doubly modified analogs, the new ¹³C NMR signal assigned to the α,β -unsaturated ketone group at the C20 position is observed in a very narrow range of 190.3–190.6 ppm, while for C20-oxosalinomycin **2** such a signal is observed at 190.7 ppm (**Figure S6**). Moreover, in the ¹³C NMR spectra of **2** and all double-modified **SAL** analogs, the signals assigned to C18 and C19 carbon atoms are observed at higher ppm values ($\sim$ 144.0 ppm and $\sim$ 127.0 ppm, respectively) after chemoselective oxidation of the C20 hydroxyl (**Figure S6**). Similar observations were also found in the ¹H NMR spectra of C20-oxo analogs, where a characteristic signal assigned to C19, and especially to C18 olefinic proton is shifted towards higher ppm values, when compared to its position in the ¹H NMR spectrum of unmodified **SAL** and the corresponding tertiary amides of **SAL** (**Figure S7**).

On the other hand, in the FT-IR spectrum of **SAL**, a broad band with a maximum at 1713 cm⁻¹ is observed, and assigned to the superposition of $\nu(\text{C}=\text{O})$ stretching vibrations of both C11 ketone and C1 carboxyl (**Figure S8**). Of note is that the intensity of this band decreased after chemical modification of the C1 moiety, indicating that carboxyl group had been consumed during amide synthesis (**Figure S8**). In the FT-IR spectra of tertiary amides of **SAL**, the characteristic amide I band is observed in a narrow range of 1621–1632 cm⁻¹ (**Figure S8**). In turn, after modification of C20 hydroxyl, the intensity of the band at about 1713 cm⁻¹ increased again, proving the formation of the corresponding α,β -unsaturated ketone at C20 position (**Figure S8**). Importantly, all **SAL** derivatives, similar to the chemically unmodified ionophore, had the ability to form 1:1 complexes with Na⁺ cations when mixed with the solution of sodium perchlorate (**Figures S1–S5**).

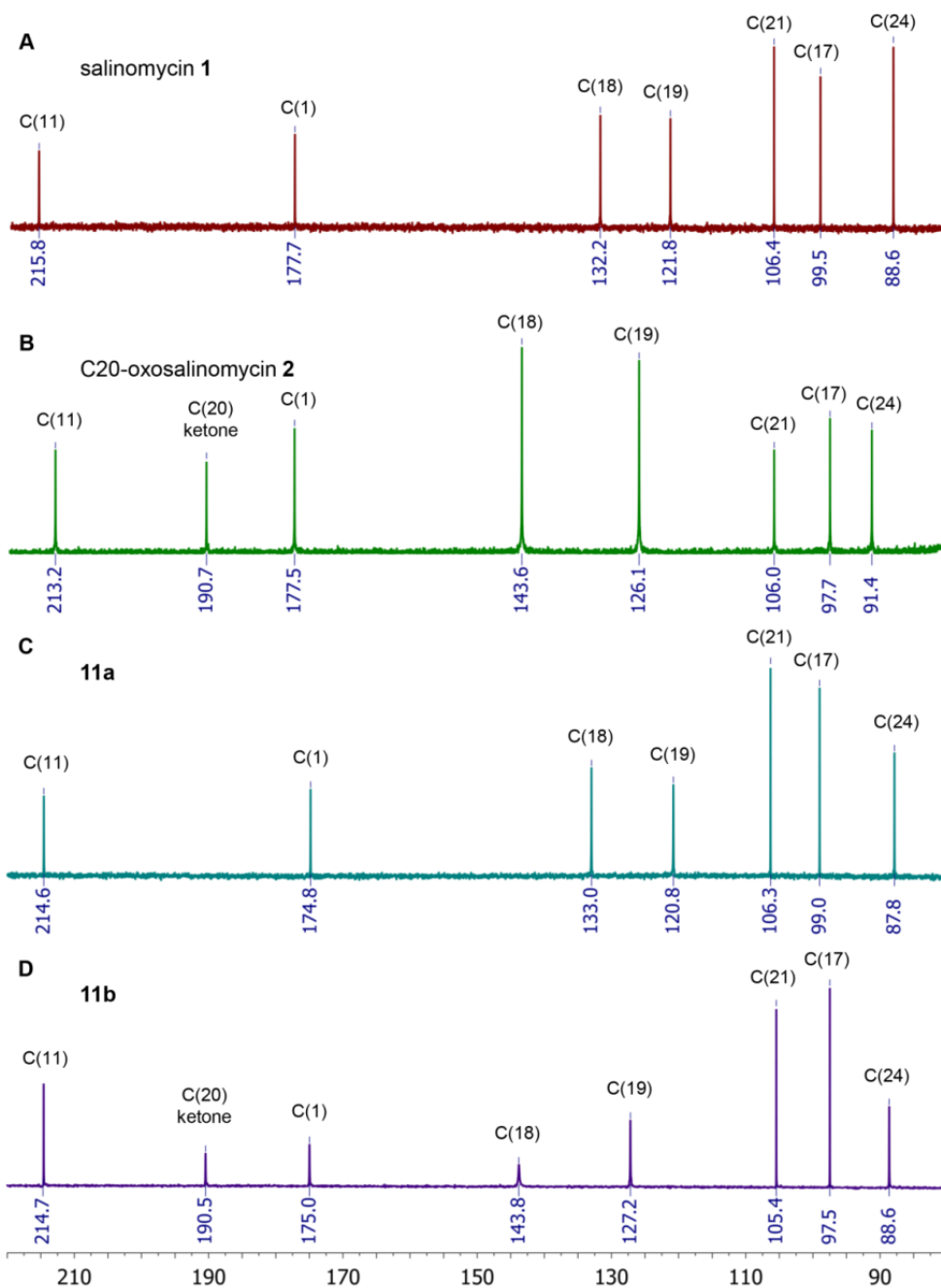


Figure S6. ^{13}C NMR spectra (all in CDCl_3) in the region of 220 and 80 ppm of (A) salinomycin **1**, (B) C20-oxosalinomycin **2**, (C) *N,N*-bis(2-methoxyethyl)amide of salinomycin **11a** and (D) its C20-oxo analog **11b**.

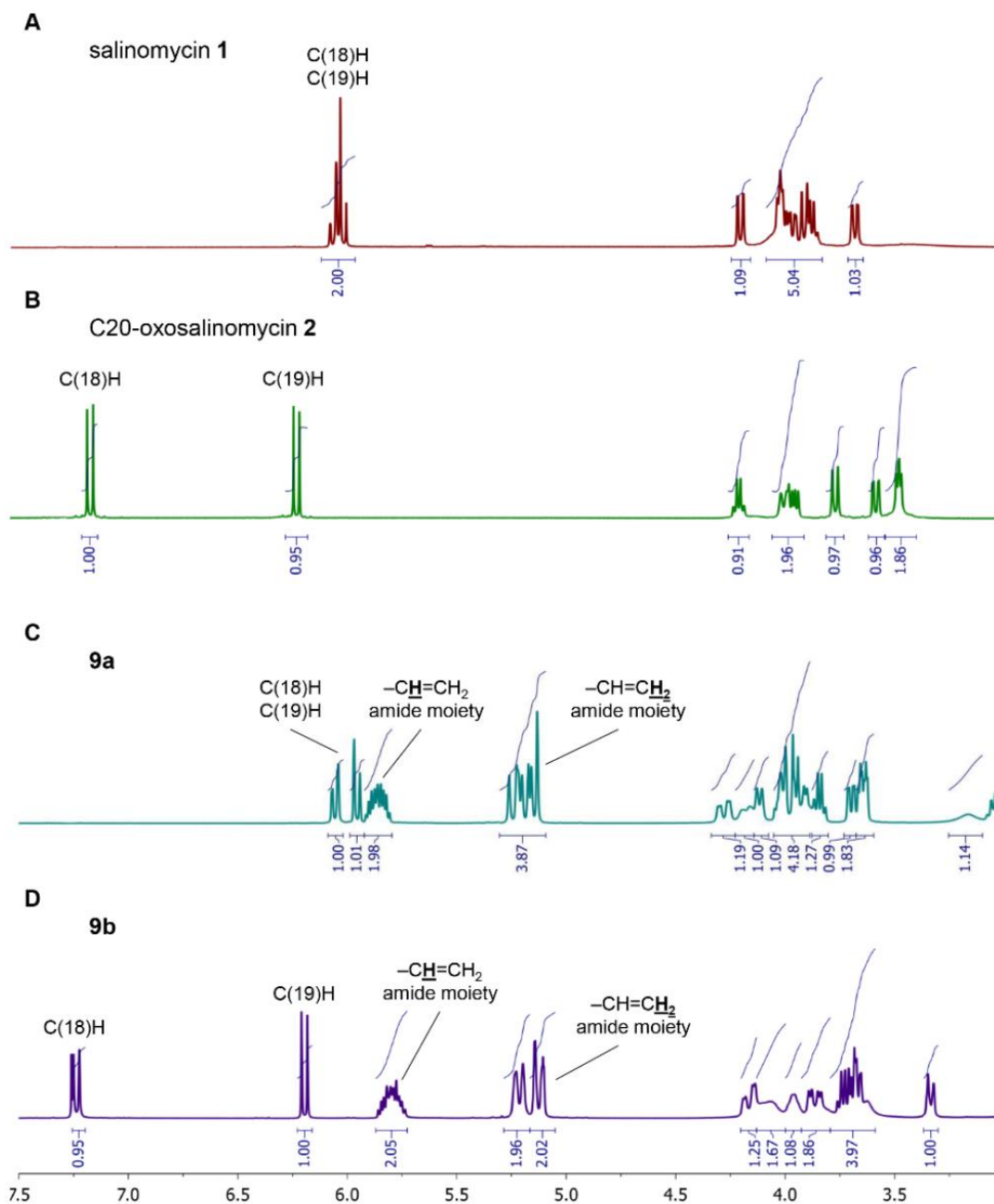


Figure S7. The ^1H NMR spectra (all in CDCl_3) in the region of 7.5 and 3.0 ppm of (A) salinomycin **1**, (B) C20-oxosalinomycin **2**, (C) *N,N*-diallyl amide of salinomycin **9a** and (D) its C20-oxo analog **9b**. For better clarity, the solvent residual signal has been removed.

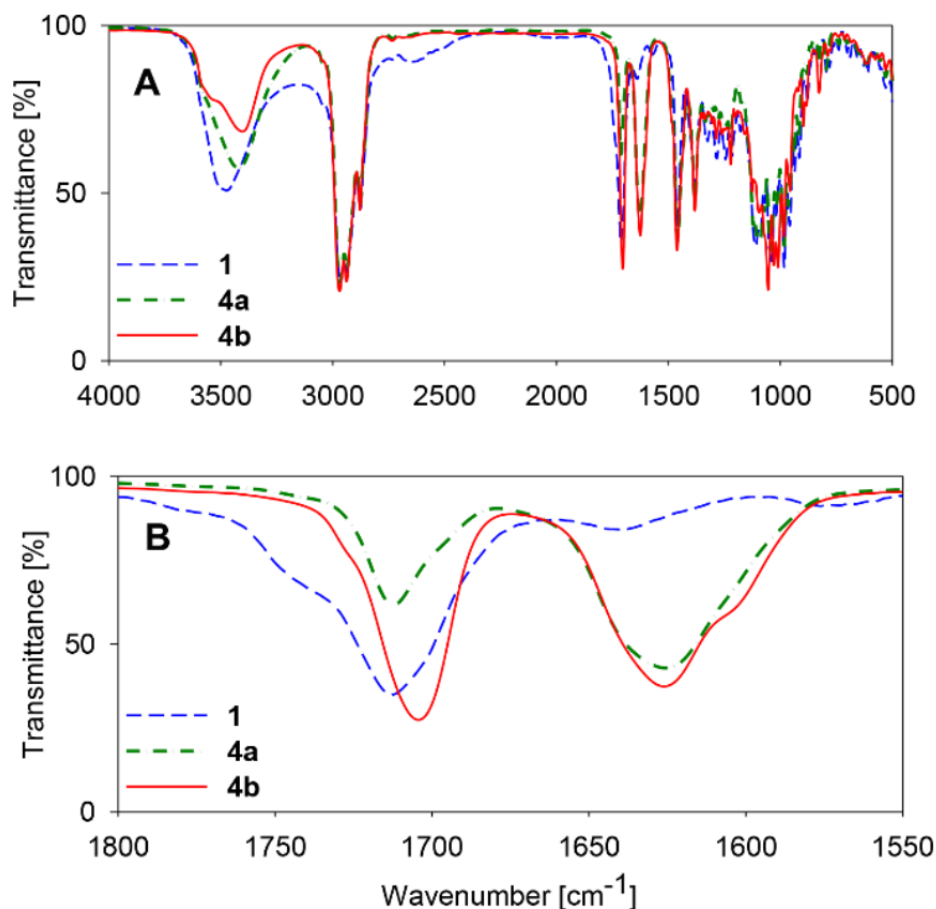
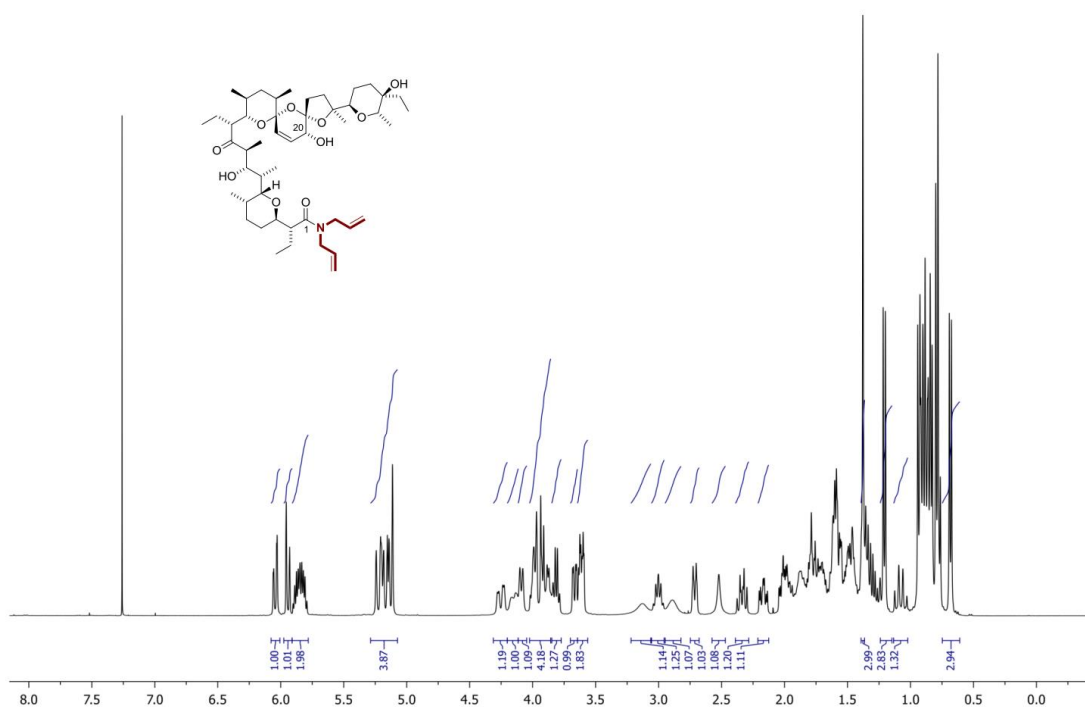


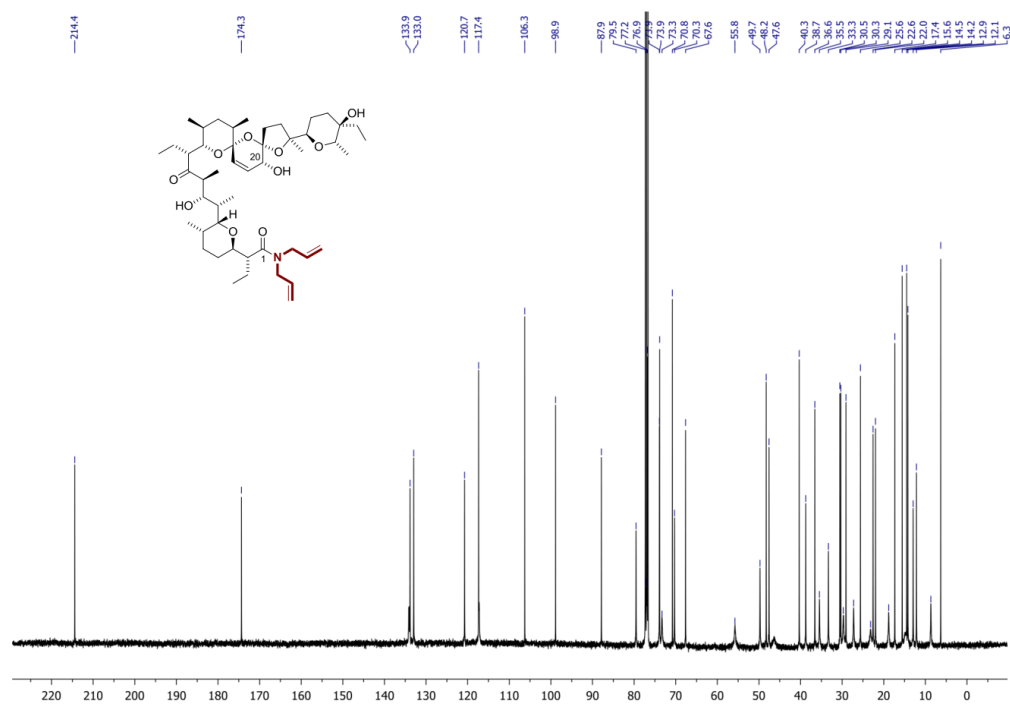
Figure S8. FT-IR spectra of salinomycin **1**, *N,N*-diethyl amide of salinomycin **4a** and its C20-oxo analog **4b** made for KBr tablet; (A) 4000–500 cm^{-1} , and (B) 1800–1550 cm^{-1} .

List of spectra

Figure S9. The ^1H NMR spectrum of 9a in chloroform- <i>d</i>	– S13 –
Figure S10. The ^{13}C NMR spectrum of 9a in chloroform- <i>d</i>	– S14 –
Figure S11. The ^1H NMR spectrum of 9b in chloroform- <i>d</i>	– S15 –
Figure S12. The ^{13}C NMR spectrum of 9b in chloroform- <i>d</i>	– S16 –
Figure S13. The ^1H NMR spectrum of 11a in chloroform- <i>d</i>	– S17 –
Figure S14. The ^{13}C NMR spectrum of 11a in chloroform- <i>d</i>	– S18 –
Figure S15. The ^1H NMR spectrum of 11b in chloroform- <i>d</i>	– S19 –
Figure S16. The ^{13}C NMR spectrum of 11b in chloroform- <i>d</i>	– S20 –

Figure S9. The ^1H NMR spectrum of **9a** in chloroform-*d*.

– S13 –

Czerwonka *et al.*, 2019Figure S10. The ^{13}C NMR spectrum of **9a** in chloroform-*d*.

– S14 –

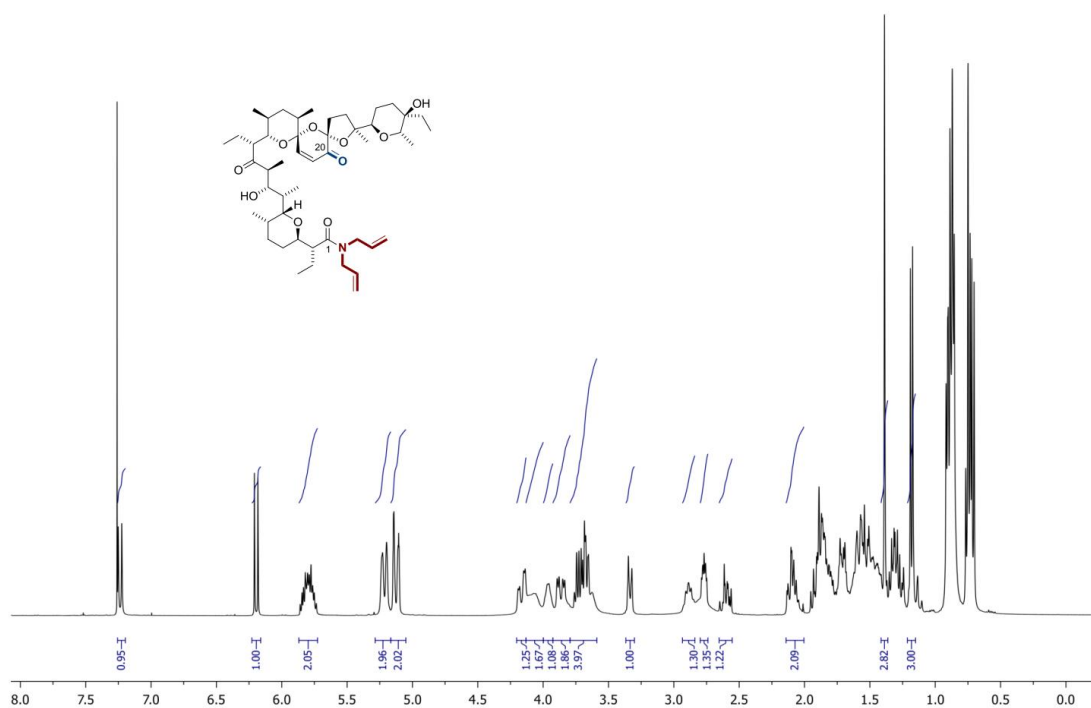


Figure S11. The ^1H NMR spectrum of **9b** in chloroform-*d*.

– S15 –

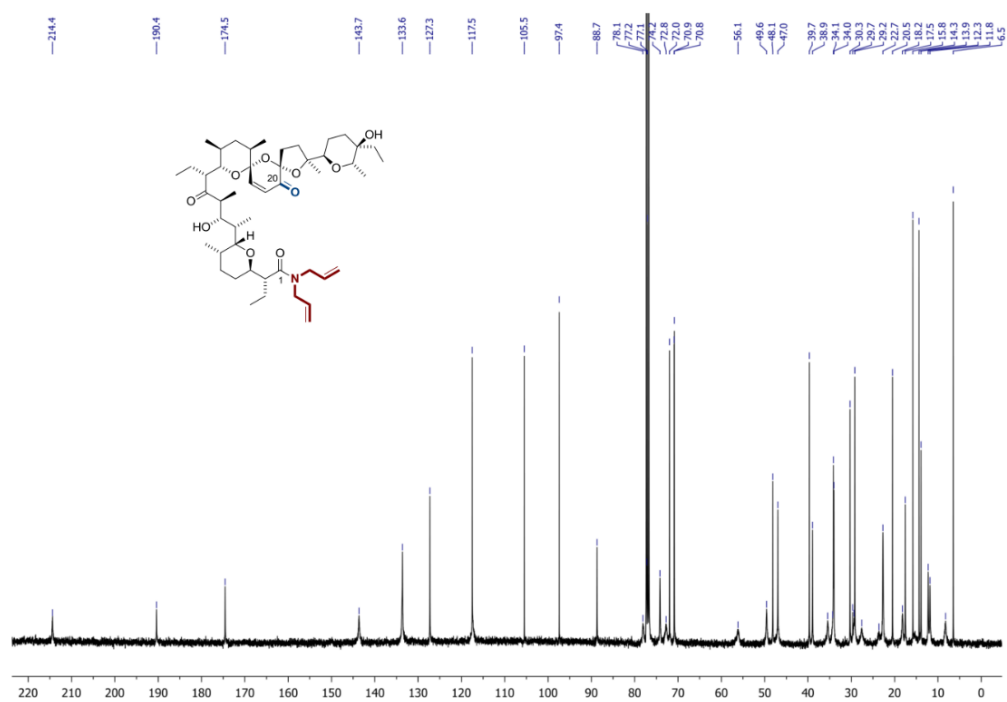


Figure S12. The ^{13}C NMR spectrum of **9b** in chloroform-*d*.

– S16 –

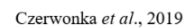


Figure S14. The ^{13}C NMR spectrum of **11a** in chloroform-*d*.

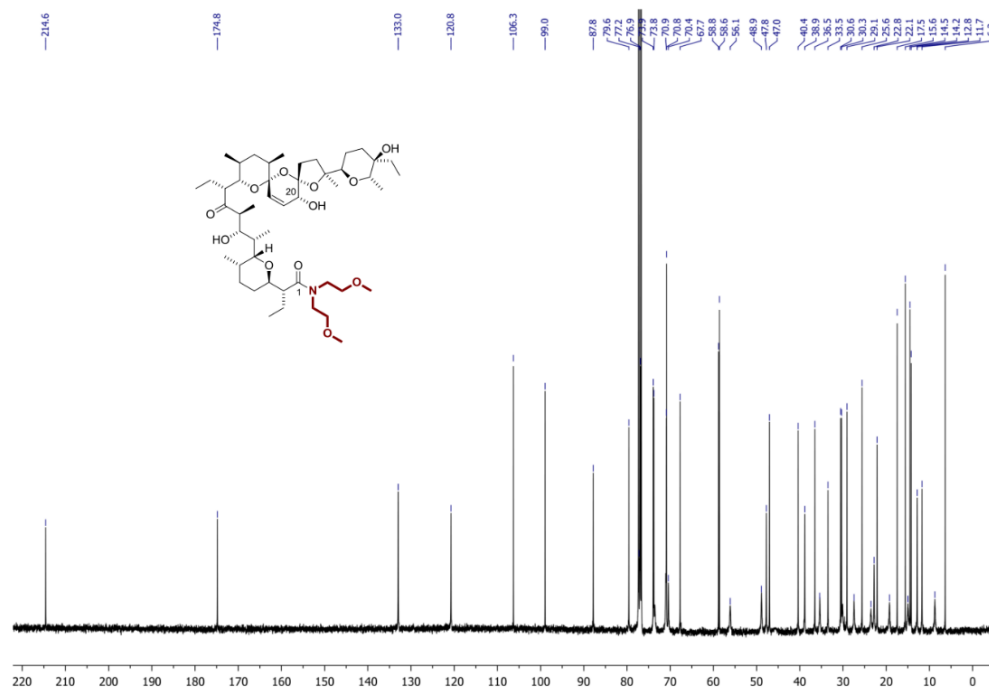
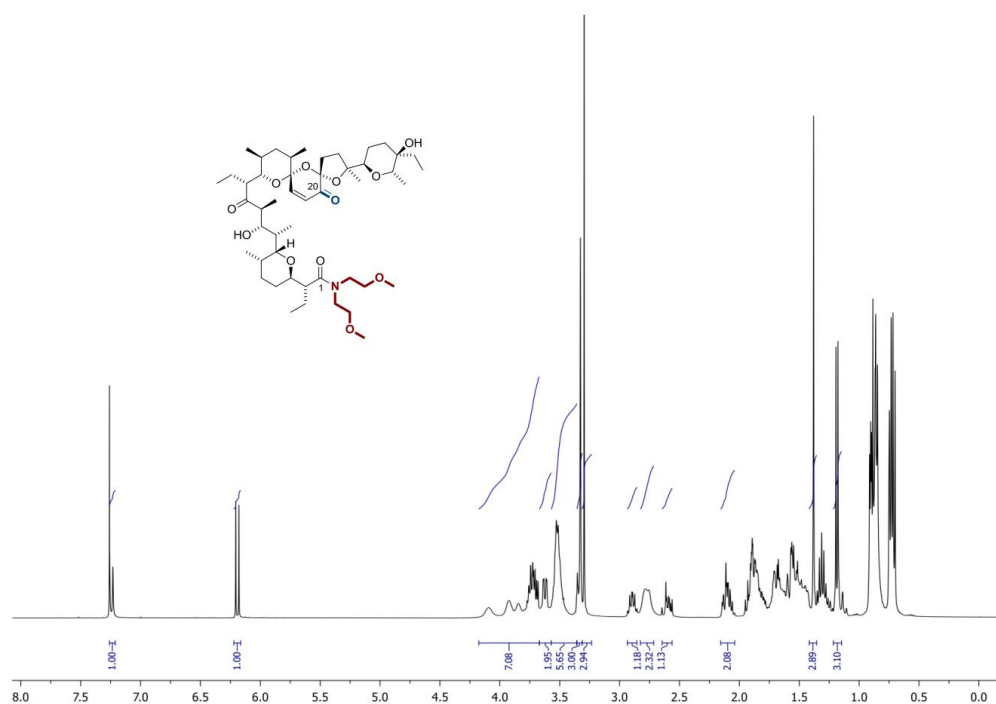
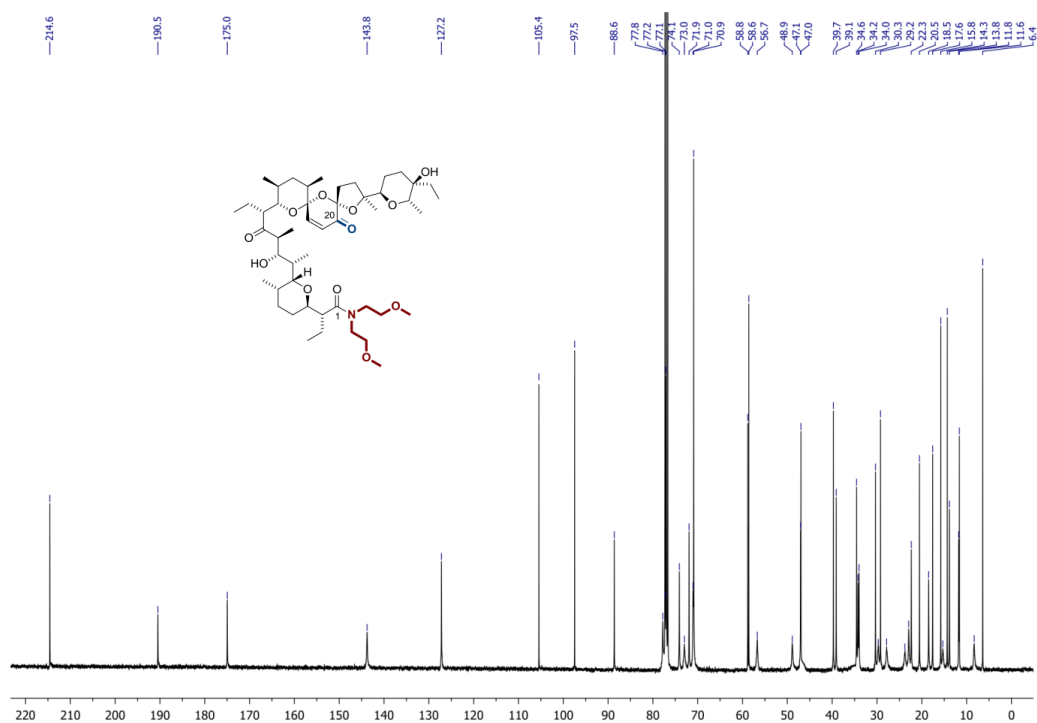


Figure S14. The ^{13}C NMR spectrum of **11a** in chloroform-*d*.

Figure S15. The ¹H NMR spectrum of 11b in chloroform-*d*.

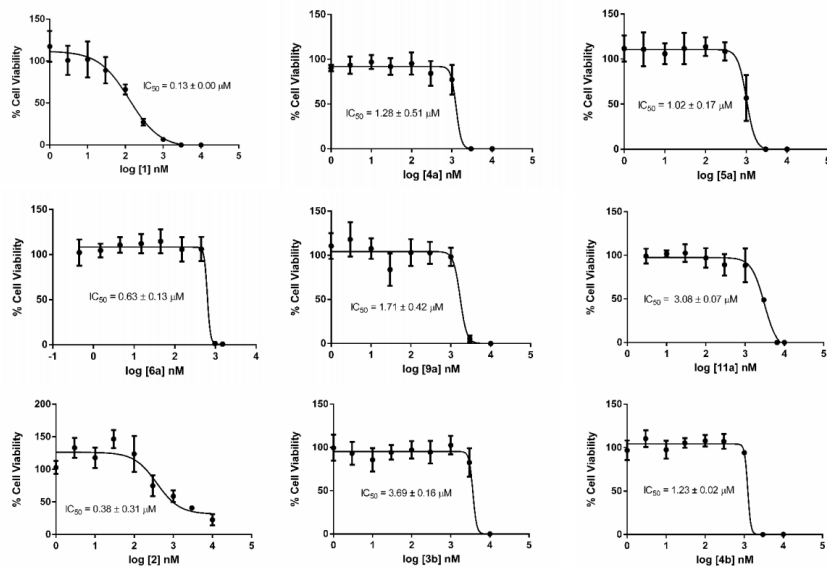
– S19 –

Figure S16. The ¹³C NMR spectrum of 11b in chloroform-*d*.

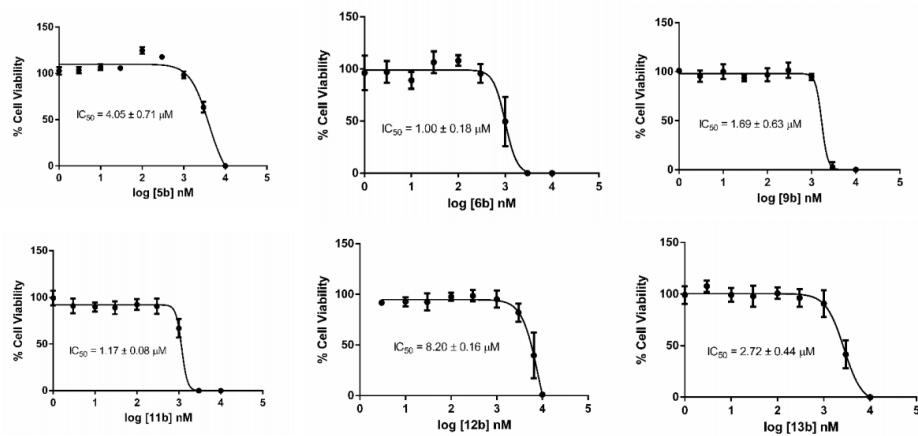
– S20 –

Biological activity tests

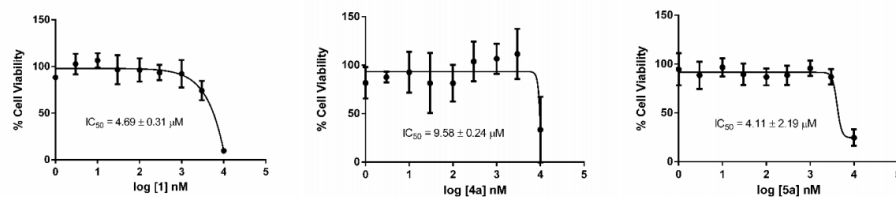
ALL-5



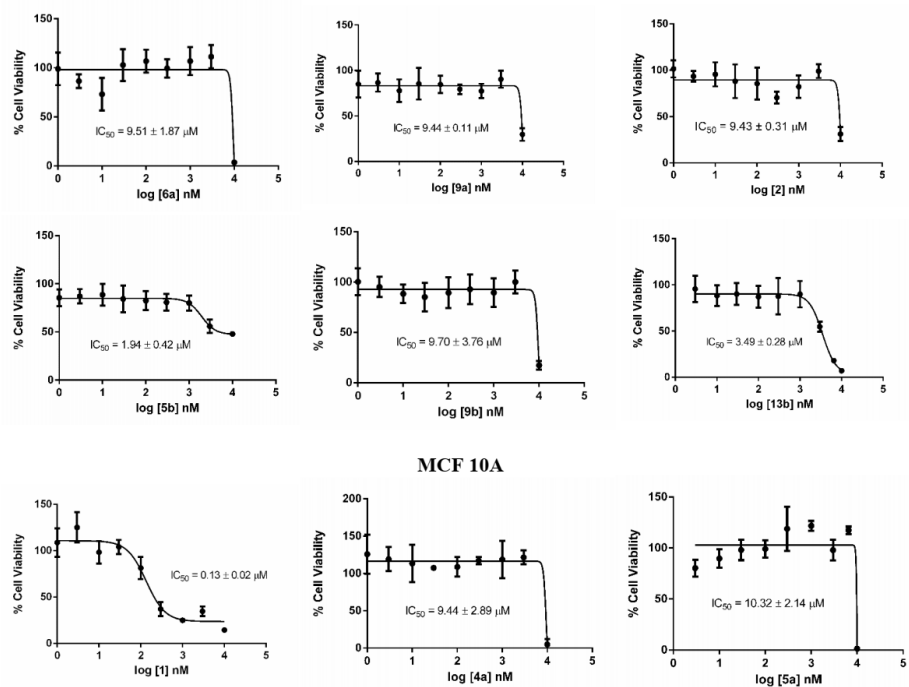
– S21 –

Czerwonka *et al.*, 2019

MDA-MB-231



– S22 –



- S23 -

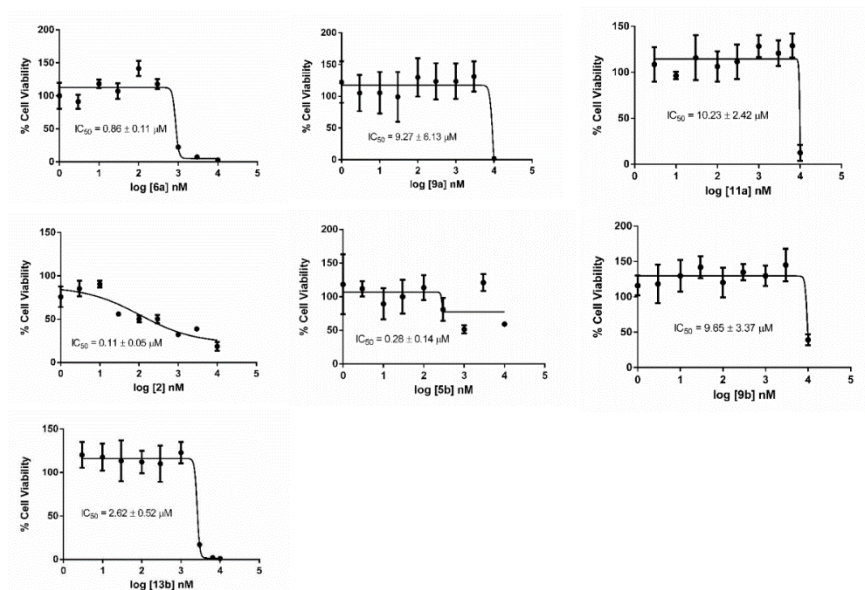


Figure S17. Sensitivity of human primary ALL-5, MDA-MB-231 and MCF 10A cells to SAL (1), its C20-oxo form (2) and their selected analogs. Cell viability was assessed by MTT assay. Cells were treated with vehicle (100% viability) or increasing concentrations of compounds for 120 h (ALL-5), 72 h (MDA-MB-231) or 96 h (MCF 10A). Results are given as mean ± SD (n = 4) and IC₅₀ values are indicated. See Scheme 1 for structures.

- S24 -

Table S2. Growth percent for sixty human cancer cell lines treated with compounds **1**, **4a–6a**, **9a**, **2**, **4b–6b** and **9b** at 10 μ M.

Cell line	Percentage growth relative to untreated control cells									
	1	4a	5a	6a	9a	2	4b	5b	6b	9b
Leukemia										
HL-60 (TB)	–36.85	–28.85	–47.74	–40.80	–36.80	–32.74	–36.43	103.08	–1.52	–25.22
K-562	6.72	16.23	10.37	14.10	14.27	13.83	19.28	107.34	24.37	18.67
MOLT-4	17.32	28.91	15.27	11.79	19.37	40.65	22.73	110.91	39.38	28.78
RPMI-8226	–7.00					4.99		89.71		–23.02
SR	22.49	55.53	15.23	27.04	36.92	31.25	41.14	128.09	75.13	49.08
Non-small cell lung cancer										
A549/ATCC	20.76	20.94	16.64	9.11	11.52	28.28	28.32	92.62	30.27	24.11
EKVX	31.34	1.82	–13.60	–8.73	–5.86	37.96	19.60	85.92	27.74	12.79
HOP-62	36.94	16.73	14.24	7.81	12.02	41.93	31.68	98.25	42.07	31.40
HOP-92	23.99	–26.17	–21.09	–25.59	–23.94	73.53	–17.16	91.54	–6.89	–8.35
NCI-H226	21.44	7.49	–2.40	–2.42	–0.69	28.29	13.41	91.29	15.35	13.75
NCI-H23	13.41	18.58	–9.41	–11.83	6.23	30.49	20.04	94.64	25.80	18.57
NCI-H460	11.45	12.46	1.15	–4.44	3.23	23.80	18.53	99.56	19.69	15.24
NCI-H522	–4.35	5.16	–11.11	–18.41	–2.70	24.61	8.58	91.11	13.25	11.57
Colon cancer										
COLO 205	8.73	–20.19	–64.19	–77.59	–62.39	10.13	12.21	102.23	21.29	1.18
HCC-2998	8.29	22.07	4.75	2.49	12.63	36.08	28.99	94.55	39.03	26.45
Cervical cancer										
HCT-116	22.24	10.85	6.43	2.55	9.64	32.59	5.97	101.08	19.02	13.93
HCT-15	25.73	9.17	–9.51	–5.99	4.64	23.76	11.01	93.22	54.05	11.46
HT29	26.08	13.83	7.02	7.79	9.13	30.48	13.12	104.15	20.91	16.71
KM12	24.47	29.63	19.91	20.57	27.55	36.04	40.91	101.85	55.60	48.80
SW-620	23.99	18.54	9.23	9.41	12.33	35.22	25.33	93.22	25.73	24.84
CNS cancer										
SF-268	38.03	42.07	24.51	19.65	41.73	50.64	46.35	93.30	68.64	58.87
SF-295	–12.56	–6.82	–5.79	–10.44	–15.96	4.02	4.86	93.33	2.72	5.69
SF-539	2.48	38.20	8.44	16.94	29.09	16.24	44.83	91.00	59.61	53.16
SNB-19	–13.27	12.75	17.84	15.30	14.25	11.65	23.41	89.63	27.04	23.83
SNB-75	–12.66	6.47	–12.77	–12.36	–7.45	19.25	30.18	89.63	33.26	38.73
U251	14.16	24.22	21.47	13.88	18.89	23.97	6.72	105.20	32.75	30.47
Melanoma										
LOX IMVI	29.26	30.99	1.21	2.12	16.96	35.44	34.34	95.21	43.52	36.85
MALME-3M	–83.62	–20.13	–13.29	–15.81	–22.44	–64.83	–27.31	85.61	–5.97	–25.08
M14	–38.75	11.72	13.87	14.23	17.88	9.56	13.57	95.21	29.96	17.32
MDA-MB-435	–16.29	26.34	3.17	4.48	6.00	21.04	30.23	99.34	44.60	40.69
SK-MEL-2	13.73	0.11	0.71	–12.39	–7.12	40.69	11.45	101.41	26.16	22.05
SK-MEL-28	–92.97	26.98	24.22	39.82	33.90	–81.22	19.54	98.89	23.79	19.83
SK-MEL-5	–99.19	–74.66	–92.42	–92.02	–85.16	–91.72	–42.90	96.40	17.98	–12.30
UACC-257	35.33	12.44	8.40	0.42	7.88	42.92	19.76	96.58	21.29	20.64
UACC-62	–92.17	14.28	14.95	12.22	14.29	–32.49	13.04	90.97	22.50	10.69

– S25 –

Czerwotka et al., 2019

– S26 –

Ovarian cancer										
IGROV1	41.06	19.77	17.13	20.09	10.72	48.43	34.88	90.56	59.25	43.55
OVCAR-3	25.85	14.28	2.11	4.13	14.59	27.15	21.58	109.28	31.49	29.79
OVCAR-4	43.36	28.22	7.28	9.88	25.42	47.69	34.35	103.60	42.69	42.67
OVCAR-5	41.92	32.01	11.39	23.96	31.88	56.08	29.67	95.50	39.42	32.81
OVCAR-8	49.10	25.37	14.99	12.54	16.03	44.40	28.97	93.28	34.70	32.37
NCI/ADR-RES	44.02	5.44	-11.21	25.36	1.02	39.97	10.49	101.76	78.20	16.78
SK-OV-3	42.45	20.84	3.96	-8.58	4.44	69.08	49.80	98.20	51.93	40.95
Renal cancer										
786-0	27.13	33.45	15.84	17.41	20.02	24.71	34.61	97.85	47.70	44.29
A498	-69.56	13.84	-20.31	-14.57	-2.03	-11.38	36.59	105.09	40.97	22.24
ACHN	31.16	5.95	-2.43	7.05	5.93	31.57	6.10	98.79	13.72	5.47
CAKI-1	22.19	8.82	-8.15	-3.74	1.41	27.03	7.58	77.07	21.59	13.68
RXF 393	-8.61	13.96	-6.32	-3.81	-1.56	25.84	28.16	96.51	46.97	21.38
SN12C	11.84	17.37	3.43	3.56	6.36	27.59	16.78	92.54	28.75	19.75
TK-10	56.52	24.34	20.68	18.57	16.19	57.74	29.08	102.83	42.63	35.55
UO-31	34.53	-5.88	-16.85	-11.68	-12.57	24.33	0.81	72.89	13.39	0.66
Prostate cancer										
PC-3	27.47	3.64	6.69	4.26	2.56	27.43	7.83	85.80	12.47	14.72
DU-145	51.31	55.06	31.50	35.42	53.66	48.03	68.50	104.82	84.86	74.77
Breast cancer										
MCF7	3.70	10.41	-15.29	-14.10	1.92	11.22	5.81	90.37	16.23	8.84
MDA-MB-231/ATCC	17.28	15.90	-40.29	-25.30	-36.32	30.34	25.77	89.36	46.85	23.10

- S27 -

Czerwonka <i>et al.</i> , 2019										
HS 578T	21.89	24.13	11.69	14.46	13.35	30.63	28.35	84.37	43.08	33.92
BT-549	6.06	37.10	6.17	8.46	27.32	34.16	43.81	92.12	62.65	54.84
T-47D	25.48	17.03	11.14	5.04	9.12	26.10	16.20	93.80	20.70	18.19
MDA-MB-468	0.84	-18.26	-24.60	-22.15	-19.06	18.89	-12.93	89.42	-11.53	-17.45

Values ≥ 100 mean no growth inhibition. Values between 0 and <100 represent growth percent of compound-treated cancer cells relative to vehicle control at zero time, and a negative value indicates cell lethality.

- S28 -

Table S3. Anti-growth potency for compounds **1**, **4a–6a**, **9a**, **2**, **4b** and **9b** in the five dose human cancer cell panel assay.

Cell line	GI ₅₀ (μM)							
	1 ± SD*	4a	5a	6a	9a	2 ± SD*	4b	9b
Leukemia								
CCRF-CEM	0.32 ± 0.20	3.39	2.78	3.13	3.19	0.28 ± 0.05	3.01	2.65
HL-60 (TB)	0.06 ± 0.01	2.24	2.26	2.35	2.09	0.04 ± 0.01	2.16	1.86
K-562	0.22 ± 0.03	3.12	2.68	2.66	2.64	0.37 ± 0.06	2.89	2.31
MOLT-4	1.25 ± 0.05	2.55	2.39	2.68	2.33	1.44 ± 0.39	2.66	2.36
RPMI-8226	0.17 ± 0.05	2.05	1.77	1.79	1.62	0.09 ± 0.05	2.12	1.10
SR	0.08 ± 0.01	2.91	2.49	2.89	2.41	0.11 ± 0.08	2.39	2.05
Non-small cell lung cancer								
A549/ATCC	0.27 ± 0.08	2.95	3.18	3.12	3.37	0.35 ± 0.12	3.21	n/a
EKVX	0.36 ± 0.04	2.58	2.59	2.32	2.33	0.56 ± 0.22	2.75	n/a
HOP-62	0.57 ± 0.31	4.33	3.33	3.79	3.21	1.08 ± 1.45	3.75	n/a
HOP-92	0.74 ± 0.38	1.45	1.29	1.20	1.39	1.33 ± 0.12	1.35	1.21
NCI-H226	0.60 ± 0.10	2.69	1.93	2.01	1.86	1.06 ± 0.07	2.21	n/a
NCI-H23	0.33 ± 0.20	2.81	2.52	2.17	2.48	0.10 ± 0.03	2.40	n/a
NCI-H322M	1.87 ± 0.05	2.91	2.38	2.66	2.31	4.12 ± 0.46	3.30	n/a
NCI-H460	0.40 ± 0.04	2.61	2.07	1.99	1.97	0.41 ± 0.02	2.11	2.33
NCI-H522	0.11 ± 0.07	2.34	1.82	2.08	2.08	0.05 ± 0.02	2.02	1.79
Colon cancer								

– S29 –

Czerwonka *et al.*, 2019

COLO 205	0.20	2.50	2.27	2.45	2.56	0.16	2.35	1.96
HCC-2998	0.20 ± 0.12	2.97	2.18	2.01	2.41	0.17 ± 0.10	1.97	2.68
HCT-116	0.74 ± 0.26	2.90	2.16	2.39	2.81	0.69 ± 0.07	3.18	2.76
HCT-15	0.64 ± 0.11	2.64	2.24	2.31	2.05	0.65 ± 0.14	2.02	n/a
HT29	0.36 ± 0.02	3.34	3.10	2.88	3.12	0.28 ± 0.07	2.73	2.31
KM12	0.74 ± 0.22	2.53	1.86	1.83	1.87	0.52 ± 0.10	1.82	2.03
SW-620	0.99 ± 0.27	3.22	3.23	2.75	2.97	0.83 ± 0.01	3.11	n/a
CNS cancer								
SF-268	1.18 ± 0.40	3.60	3.43	3.17	3.27	2.13 ± 0.32	3.25	n/a
SF-295	0.49 ± 0.18	2.49	2.45	2.06	1.87	1.22 ± 0.19	2.26	n/a
SF-539	0.33 ± 0.04	3.01	2.06	1.88	1.97	0.34 ± 0.05	2.26	n/a
SNB-19	0.21 ± 0.05	3.24	3.36	3.46	3.08	0.40 ± 0.06	3.31	n/a
SNB-75	0.89 ± 0.60	1.72	1.73	1.78	1.84	1.23 ± 0.34	1.90	1.57
U251	0.11 ± 0.02	3.33	2.87	2.78	2.78	0.08 ± 0.02	3.10	n/a
Melanoma								
LOX IMVI	0.72 ± 0.20	1.83	1.50	1.43	1.52	0.79 ± 0.48	1.58	n/a
MALME-3M	0.25 ± 0.08	2.21	1.72	1.87	1.76	0.16 ± 0.03	1.93	1.85
M14	1.13	n/a	n/a	n/a	n/a	1.41	n/a	n/a
MDA-MB-435	1.08 ± 0.08	2.81	2.96	2.92	2.71	1.21 ± 0.03	2.91	n/a
SK-MEL-2	1.28 ± 0.23	3.19	2.43	2.67	2.62	1.18 ± 0.79	2.71	n/a
SK-MEL-28	0.84 ± 0.08	4.97	4.50	4.21	4.45	1.26 ± 0.01	4.45	n/a

– S30 –

SK-MEL-5	0.10 ± 0.01	1.76	1.73	1.73	1.72	0.07 ± 0.01	1.69	n/a
UACC-257	0.24 ± 0.09	3.07	2.84	2.90	2.35	0.21 ± 0.17	3.17	n/a
UACC-62	1.06 ± 0.05	2.43	1.71	1.47	1.66	1.29 ± 0.06	1.83	1.65
Ovarian cancer								
IGROV1	2.09 ± 0.41	2.73	2.59	3.00	2.41	2.44 ± 0.06	2.31	n/a
OVCAR-3	0.09 ± 0.05	2.64	1.84	1.87	1.92	0.06 ± 0.03	2.34	1.99
OVCAR-4	0.90 ± 0.52	3.04	2.95	2.85	2.63	1.10 ± 0.13	2.96	2.23
OVCAR-5	1.09 ± 0.33	2.85	2.09	1.90	2.25	0.76 ± 0.26	2.85	n/a
OVCAR-8	1.59 ± 0.02	3.42	3.50	3.42	3.48	2.03 ± 1.50	3.55	n/a
NCI/ADR-RES	0.57 ± 0.16	2.56	2.93	2.50	2.70	0.84 ± 0.49	2.82	n/a
SK-OV-3	4.10	3.06	3.29	3.38	3.51	3.70	3.87	n/a
Renal cancer								
786-0	1.27 ± 0.25	3.55	2.79	2.49	2.19	0.99 ± 0.01	3.22	n/a
A498	0.04	2.44	2.13	1.99	2.07	0.06	2.25	2.41
ACHN	0.78 ± 0.13	2.93	3.18	3.04	2.90	1.33 ± 0.13	2.16	n/a
CAKI-1	0.66 ± 0.26	2.12	1.95	1.91	1.75	0.83 ± 0.46	2.18	1.96
RXF 393	0.08 ± 0.07	2.06	1.71	1.59	1.57	0.04 ± 0.02	1.66	1.90
SN12C	0.18 ± 0.11	2.75	2.13	2.13	2.20	0.23 ± 0.19	2.41	n/a
TK-10	1.69 ± 0.30	3.35	3.30	3.57	3.65	2.72 ± 2.48	3.49	n/a
UO-31	1.42 ± 0.13	2.40	2.12	2.26	2.16	1.19 ± 0.01	2.42	n/a
Prostate cancer								

– S31 –

PC-3	0.07 ± 0.01	2.54	2.05	2.05	2.31	0.07 ± 0.01	2.60	2.02
DU-145	1.21 ± 0.83	3.91	2.05	2.61	2.46	1.46 ± 0.40	3.41	n/a
Breast cancer								
MCF7	0.15 ± 0.02	2.61	2.46	2.40	1.95	0.07 ± 0.01	2.41	n/a
MDA-MB-231/ATCC	0.62 ± 0.30	1.86	1.62	1.57	1.77	0.87 ± 0.26	1.69	n/a
HS 578T	0.27 ± 0.17	2.90	2.75	2.32	2.22	0.22 ± 0.17	2.68	2.55
BT-549	0.24 ± 0.03	3.33	3.05	2.73	3.03	0.26 ± 0.18	3.29	n/a
T-47D	0.14 ± 0.10	2.44	3.08	2.34	2.37	0.06 ± 0.02	2.59	2.07
MDA-MB-468	0.23 ± 0.18	1.81	1.72	1.47	1.53	0.09 ± 0.06	9.43	1.42

* Due to their promising activity compounds **1** and **2** were selected for the repeat of 5-dose assay and n = 2 ± standard deviation is presented.

– S32 –

PUBLIKACJA II

Dominika Czerwotka, Magdalena Mielczarek-Puto, Michał Antoszczak, Aleksandra Cioch, Marta Struga, Adam Huczyński

„Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin”

European Journal of Pharmacology 2021, 908, 174347



Evaluation of the anticancer activity of singly and doubly modified analogues of C20-*epi*-salinomycin

Dominika Czerwonka^a, Magdalena Mielczarek-Puta^b, Michał Antoszczak^a, Aleksandra Cioch^a, Marta Struga^b, Adam Huczyński^{a,*}

^a Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61–614, Poznań, Poland

^b Department of Biochemistry, Medical University of Warsaw, Banacha 1, 02–097, Warszawa, Poland

ARTICLE INFO

Keywords:

Polyether ionophores
O-acylated derivatives
Antiproliferative activity
Apoptosis
Colon cancer
Prostate cancer
Interleukin 6

ABSTRACT

In developed countries, cancer is the second leading cause of death, with colon and prostate cancer belonging to the group of most often diagnosed types of neoplastic diseases. The search for new treatment strategies against these types of cancer is thus of top current interest. In this context, salinomycin (SAL), a naturally occurring polyether ionophore, has been identified recently as a very promising anticancer drug candidate towards several tumour cells. In the present work, a broad library of 24 derivatives of C20-*epi*-salinomycin (2), including C1 singly, C20 singly and C1/C20 doubly modified analogue structures, was screened to identify compounds with improved activity against colon and prostate cancer cells. Our study demonstrated that the growth inhibitory potency of the parent compound on both primary and metastatic colon cancer cells was similar to that of the semisynthetic products derived from SAL, and simultaneously the SAL analogues showed more potent toxic action on metastatic prostate cancer cells than that of the chemically unmodified ionophore. In contrast to the widely used oncological drug doxorubicin, some of the SAL derivatives demonstrated promising anticancer activity with no toxic effects on non-tumour cells, and with more favourable cytotoxicity than that of a reference agent 5-fluorouracil. Mechanistically, the SAL analogues induced late apoptosis in colon cancer cells and necrosis in prostate cancer cells, as well as reduced secretion of interleukin 6 (IL-6) in these cells.

1. Introduction

According to the World Health Organization, in 2018, nearly 18 million people were diagnosed with cancer, and simultaneously, almost 10 million patients died from neoplastic diseases globally (World Health Organization, 2020). It is also predicted that the number of cancer patients will exceed 30 million new cases per year in the next 20 years (World Health Organization, 2020). The most frequently diagnosed cancers in 2018 were lung, female breast, colon and prostate cancer, and at the same time, lung and colon cancers were the cause of the greatest number of cancer-related deaths (Nagai and Kim, 2017; World Health Organization, 2020). Despite many limitations, chemotherapy, together with surgical removal and radiotherapy, belongs to the group of the most commonly employed anticancer therapeutic strategies (Chabner and Roberts, 2005; DeVita and Chu, 2008; Schirmacher, 2019). Unfortunately, systemic therapy is often insufficient against cancer stem cells (CSCs), a small subpopulation of cancer cells within tumour,

exhibiting self-renewal and proliferative properties as well as the ability to differentiate which are often responsible for cancer relapse and poor prognosis (Batlle and Clevers, 2017; Clevers, 2011). As ~60% of all currently used anticancer agents are derived from natural products, it is reasonable to search in the nature for other promising drug candidates effective against cancer cells and CSCs (Sarker et al., 2020).

One of such promising anticancer drug candidates is salinomycin (SAL, Scheme 1), a polyether ionophore antibiotic with potent antiparasitic, antimalarial, antiviral and antifungal activities, commercially used in veterinary medicine as a food additive and growth promoter (Huczyński, 2012; Kevin et al., 2009; Miyazaki et al., 1974; Pan et al., 2010; Yvoré et al., 1980). In 2009, SAL was identified as an effective tumour-targeting agent in the extensive screening study performed by Gupta and co-workers (Gupta et al., 2009). Among the ~16,000 biologically active compounds tested, SAL was found to be the most selective inhibitor of breast CSCs, with nearly 100-fold greater activity than that of the commonly used cytostatic drug paclitaxel (Gupta et al.,

* Corresponding author.

E-mail address: adhucz@amu.edu.pl (A. Huczyński).

<https://doi.org/10.1016/j.ejphar.2021.174347>

Received 22 April 2021; Received in revised form 6 July 2021; Accepted 12 July 2021

Available online 13 July 2021

0014-2999/© 2021 The Authors.

Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

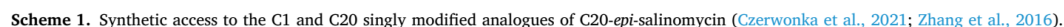
(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

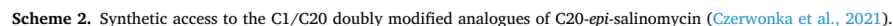
in the effects of multiple derivatization of **SAL** molecule on the biological activity of the resulting products, a series of C1/C20 doubly modified analogues (**Scheme 2**) was also included and tested for their activity. All **SAL** derivatives were assessed for their *in vitro* antiproliferative activity against selected human primary and metastatic cancer cell lines as well as non-tumour cells. The cytotoxic properties of the synthesized compounds were compared to those of two commonly used oncological drugs – 5-fluorouracil and doxorubicin. Additionally, the most promising analogue structures were evaluated for their ability to induce apoptosis and release interleukin-6 (IL-6) in both cancer and normal cells.

2. Materials and methods

2.1. General procedures

All reagents and solvents were obtained from Merck or Trimer Chemicals S.A. (Poland) and were used as received without further purification. CDCl_3 spectral grade solvent was stored over 3 Å molecular sieves for several days. All manipulations were carried out under nitrogen atmosphere in an oven-dried glassware. The reaction mixtures were stirred using Teflon-coated magnetic stir bars, and the progress of the reaction was monitored by thin-layer chromatography (TLC) using aluminium-backed plates (Merck 60 F₂₅₄). The TLC plates were visualized by UV-light (254 nm), as well as treated with phosphomolybdic acid (PMA, 5% in absolute EtOH) and gentle heating. The products of the reactions were purified using CombiFlash® Rf+ Lumen Flash Chromatography System (Teledyne Isco) with integrated ELS and UV detectors. All solvents used in flash chromatography were of HPLC grade (Merck) and were used as received. The solvents were removed using a rotary





C1 benzhydroxamic acid conjugate of C20 ethyl carbonate of C20-epi-salinomycin 26 ((2S,5S,7R,9S,10S,12R,15S)-9-((3R,5S,6S,7S)-7-((2R,3S,6R)-6-((R)-1-(benzamidoxy)-1-methoxybutan-2-yl)-3-methyltetrahydro-2H-pyran-2-yl)-6-hydroxy-5-oxabutyl-4-oxooctano-3-yl)-2-((2R,5R,6S)-5-ethyl-5-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)-2,10,12-trimethyl-1,6,8-trioxaspiro[4.1.5.7.35]pentadec-13-en-15-yl ethyl carbonate): Yield: 74 mg, 54%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC. UV-active and stains green with PMA; ¹H NMR (401 MHz, CDCl₃) δ 11.06 (s, 1H),

8.17–8.13 (m, 2H), 7.56–7.51 (m, 1H), 7.46–7.41 (m, 2H), 6.46 (d, $J = 10.6$ Hz, 1H), 6.20 (dd, $J = 10.5, 5.4$ Hz, 1H), 4.72 (d, $J = 5.4$ Hz, 1H), 4.14 (q, $J = 7.1$ Hz, 2H), 4.03 (ddd, $J = 17.9, 10.5, 6.6$ Hz, 2H), 3.84 (dd, $J = 10.2, 2.5$ Hz, 1H), 3.73 (dt, $J = 6.9, 4.1$ Hz, 2H), 3.49 (dd, $J = 11.9, 2.3$ Hz, 1H), 3.16 (ddd, $J = 10.8, 9.7, 5.1$ Hz, 1H), 2.99–2.89 (m, 1H), 2.68 (s, 1H), 2.57 (dt, $J = 9.4, 2.2$ Hz, 1H), 2.20 (d, $J = 7.2$ Hz, 1H), 2.08–0.50 (m, 57H) ppm; ^{13}C NMR (101 MHz, CDCl_3) δ 216.3, 174.1, 165.6, 155.1, 132.5, 131.7, 128.9 (2C), 128.5 (2C), 127.8, 125.7, 106.5, 99.0, 90.5, 80.3, 77.3, 75.2, 73.7, 72.6, 71.3, 70.6, 69.2, 64.7, 54.1, 46.9, 46.7, 40.2, 38.9, 37.3, 36.9, 33.0, 31.4, 31.3, 30.3, 30.1, 28.8, 27.1, 26.1, 23.8, 22.4, 17.4, 17.1, 16.6, 15.1, 14.9, 14.8, 14.6, 12.2, 11.3, 8.2, 6.9 ppm; ESI MS (m/z): $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{52}\text{H}_{79}\text{NNaO}_4^+$ 964.5; Found 965.

2.3. Cell culture

The anticancer activity studies were conducted on primary (SW480) and metastatic (SW620) colon cancer, metastatic prostate cancer (PC3), and human keratinocyte (HaCaT) cell lines obtained from the American Type Culture Collection (ATCC, Rockville, USA). SW480 cells were established from a primary adenocarcinoma grade 3–4 of Caucasian male, whereas SW620 cells derived from the lymph node of the same patient. PC3 cells were initiated from a bone metastasis of a grade 4 prostatic adenocarcinoma from Caucasian male, and HaCaT cells were aneuploid immortal keratinocytes from adult human skin. The SW480, SW620, PC3, and HaCaT cells were cultured in EMEM, RPMI1640, and DMEM High Glucose medium, respectively (Biowest SAS, France) supplemented with 10% FBS (Biowest SAS, France), penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$) in $37^\circ\text{C}/5\% \text{ CO}_2$ humidified incubator. After reaching 80% confluence, the cells were harvested using trypsin-EDTA and used for the experiments.

2.4. MTT assay

MTT assay, based on the enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) to insoluble formazan crystals by mitochondrial dehydrogenases present in viable cells, was used to determine the cytotoxicity of SAL, **2** and a cohort of its derivatives. The cells were seeded at 96-well plates (1×10^4 cells per well) and after 24 h incubation, treated with various concentrations of the studied compounds (1 – $100 \mu\text{M}$) for 72 h in $37^\circ\text{C}/5\% \text{ CO}_2$. The untreated cells were used as the control. Next, the medium was removed, MTT solution (0.5 mg ml^{-1}) was added to each well, and the cells were incubated for 4 h in $37^\circ\text{C}/5\% \text{ CO}_2$. After that, the obtained formazan crystals were dissolved in DMSO and isopropanol (1:1, v/v). The optical density of the solution was measured using MultiscanGo (Thermo Scientific) at 570 nm wavelength. The experiments were repeated three times.

The cell viability was presented as a percent of MTT reduction in the treated cells versus control cells. The number of viable cells cultured without the studied compounds was assumed as 100%. The relative MTT level (in %) was calculated using the formula $[\text{A}]/[\text{B}] \times 100$, where [A] was the absorbance of the tested sample, and [B] was the absorbance of the control sample containing the untreated cells. The decreased relative MTT level indicated the decreased cell viability. On the basis of the values of the IC_{50} parameter, the most promising compounds were selected and studied in further experiments.

2.5. Annexin V-FITC binding assay

The mechanisms of action of selected SAL derivatives were inferred from the results of Annexin V-FITC binding and IL-6 (see paragraph 2.6) assays. The cells were seeded at 12-well plates (1×10^5 cells per well), incubated 24 h, and treated with selected compounds at IC_{50} concentrations. The untreated cells were used as the control. After 72 h incubation, the apoptotic effect of the compounds was analyzed both in cell

culture medium and harvested cells by dual staining with Annexin V-FITC and propidium iodide (PI) detection kit (BD Biosciences Pharmingen), according to the manufacturer's protocol and using flow cytometry (Becton Dickinson). The Annexin V-FITC positive and PI negative cells were identified as early apoptosis, while Annexin V-FITC positive/negative and PI positive cells as late apoptosis or necrosis. The experiments were repeated three times.

2.6. IL-6 assay

The level of interleukin-6 (IL-6) released by the cells after treatment with selected compounds at IC_{50} concentrations was estimated by enzyme-linked immunosorbent assay (ELISA) (Diaclon SAS Besancon Cedex, France). The untreated cells were used as the control. After 72 h incubation, the cell culture medium was collected and used both for dual staining Annexin V-FITC and IL-6 assay, according to the manufacturer's protocol. The experiments were repeated twice.

2.7. Statistical analysis

The statistical calculation was performed using Statistica 13.0 (StatSoft, Inc, USA) programme. Quantitative comparisons were made using Student's t-test. The IC_{50} values were estimated by CompuSyn version 1.0. All presented experiments were expressed as the mean $\pm$ S.D. and considered as statistically significant at $P < 0.05$.

3. Results

3.1. Analogue design and synthesis

In 2016, Wu and co-workers successfully applied the Mitsunobu reaction to synthesize C20-*epi*-salinomycin (**2**), followed by further C20 hydroxyl esterification; while **2** showed similar antiproliferative activity as starting SAL, its C20-esters were even 2–10 times more potent (Zhang et al., 2016). Inspired by this finding, we decided to expand the structural diversity at the C20 position and synthesized a broad series of analogues of **2**, including not only its C20-esters, but also other regioselectively *O*-acylated derivatives (carbonates, carbamates and allophanate) (Czerwonka et al., 2021). Evaluation of the antiparasitic activity of these products led us to identify several analogue structures that met the activity criteria for hit compounds for *Trypanosoma brucei*, a species of parasitic kinetoplastid which is responsible for African trypanosomiasis in humans and animals (sleeping sickness and nagana disease, respectively) (Czerwonka et al., 2021). However, the ability of this class of compounds to selectively target colon and prostate cancer cells, including their metastatic variants, remains unknown.

The synthetic access to C20-*epi*-salinomycin (**2**) and the analogues studied in this work, both C1 (compounds **3**–**6**) and C20 singly modified (compounds **7**–**19**, except carbonate **13**) (Scheme 1), as well as the C1/C20 doubly modified products (compounds **20**–**25**) (Scheme 2), is described thoroughly elsewhere (Czerwonka et al., 2021; Zhang et al., 2016). Very briefly, a broad library of derivatives of **2** was devised based on the compounds that showed antitrypanosomal activity in our recent study (Czerwonka et al., 2021). It included a series of 22 semisynthetic products, i.e., C1 amides and esters, C20-*O*-acylated analogues and C1/C20 doubly modified derivatives of **2**, merging desirable structural features to produce possible drug candidates with activity on cancer cells. Moreover, for better studies of the relationship between the structure of the synthesized analogues and their anticancer activity, we extended this series and synthesized two completely new derivatives of **2** (compound **13**, Scheme 1, and compound **26**, Scheme 2). The spectroscopic data of all resynthesized products were in line with previously published data (Czerwonka et al., 2021; Zhang et al., 2016).

3.2. Cytotoxic activity studies

The aim of our biological study was the evaluation of the cytotoxicity of **SAL**, **2** and its derivatives **3–26** (Scheme 1 and Scheme 2) as well as assessment of the potential usefulness of these compounds in cancer treatment. The cell viability was determined by the MTT assay for both cancer (SW480, SW620, and PC3) and non-tumour (HaCaT) cells. The cytotoxic activity was established by calculation of the half maximal inhibitory concentration IC_{50} , i.e., the concentration of the compound at which the inhibition is equal to 50%; as shown in Table 1. Additionally, a cytotoxic capacity of the tested compounds against tumour cells was expressed as the values of selectivity index (SI) (Table 1). Doxorubicin (DOX) and 5-fluorouracil (5FU), two commonly used oncological chemotherapeutics, were used as the references.

In the MTT assay, compound **2** showed ~3–4 times lower anti-proliferative activity than **SAL** against both colon cancer cell lines, and simultaneously it was nearly twice more active than the native structure towards the prostate cancer cell line, showing similar selectivity of action (Table 1). This is slightly in contrast to the results obtained by Wu and co-workers who have identified **SAL** and its C20-epimer as agents with similar anticancer activity (Zhang et al., 2016), which suggests that the therapeutic potential of these compounds strongly depends on the type of cancer cell line used.

On the other hand, almost all studied compounds derived from **SAL** revealed high cytotoxic activity against the three cancer cell lines (Table 1). Except for propargyl esters **4**, **21** and **25** that showed rather low antiproliferative activity ($\geq 20 \mu M$) towards selected cancer cell lines, the IC_{50} values of other semisynthetic products for SW480 cells ranged from 0.15 to 17 μM , SW620 cells from 0.13 to 19 μM , and PC3 cells from 0.34 to 10 μM . However, despite relatively high inhibitory effects on cancer cells, **SAL**, **2** and its derivatives showed also low tumour-targeting selectivity. The IC_{50} values for HaCaT cells (0.05–16 μM , except **4**, **21** and **25**) were similar to those achieved for cancer cells, therefore the SI values were below 1.0 in most cases (Table 1); it is in line with the results reported by other authors who have documented the considerable neurotoxicity of compound **2** in their studies (Zhang et al., 2016).

Prostate cancer cells were identified generally as more sensitive to the toxic action of **SAL** derivatives than colon cancer cells. As far as the anti-prostate cancer activity is concerned, the activity of **14** ($IC_{50} = 1.6 \pm 0.81 \mu M$, SI = 5.2), **20** ($IC_{50} = 7.6 \pm 0.43 \mu M$, SI = 2.1) and **21** ($IC_{50} = 9.4 \pm 2.7 \mu M$, SI = 9.4) seemed to be the highest. With respect to colon cancer cells, while propargyl ester **21** showed favourable activity against the metastatic variant of colon cancer (SW620, $IC_{50} = 5.5 \pm 2.1 \mu M$, SI = 16), its amide counterpart **20** was identified as an agent that might be used in first-line therapy of this type of cancer (SW480, $IC_{50} = 8.9 \pm 1.6 \mu M$, SI = 1.8).

Finally, in contrast to the starting **SAL**, **2** and the reference doxorubicin, some of the analogues inhibited the proliferation of cancer cells with no toxic effects on non-tumour cells. On the other hand, the semisynthetic products showed comparable tumour-targeting selectivity to that of 5-fluorouracil, but with more favourable IC_{50} values. Taking into consideration the results from our preliminary MTT assay (Table 1) and the structural diversity of the compounds screened, we identified 11 analogues (**5**, **8**, **9**, **12**, **14**, **16**, **17**, **19**, **20**, **21**, and **26**) for further experiments.

3.3. Apoptotic activity studies

To deepen the *in vitro* anticancer mechanism of action of **SAL** derivatives, the effects of the most promising analogue structures (**5**, **8**, **9**, **12**, **14**, **16**, **17**, **19**, **20**, **21**, and **26**) on early and late apoptosis or necrosis were analyzed by flow cytometry (Fig. 1 and Fig. 2); compounds **13** and **18** were also included in the study, as they exhibited antiproliferative activity towards prostate cancer cells at low micromolar concentrations ($IC_{50} = 0.34 \mu M$ and $IC_{50} = 1.2 \mu M$, respectively,

Table 1

Cytotoxicity (IC_{50} , μM) and tumour-targeting selectivity (SI) of the studied compounds estimated by the MTT assay. **SAL** = salinomycin, 5FU = 5-fluorouracil, DOX = doxorubicin.

Compound	Cancer cells						Non-tumour cells
	SW480		SW620		PC-3		
	IC ₅₀	SI	IC ₅₀	SI	IC ₅₀	SI	IC ₅₀
SAL	6.2 ± 2.1	0.5	6.4 ± 1.5	0.5	30 ± 1.4	0.1	3.3 ± 0.85
2	28 ± 1.4	0.4	18 ± 0.67	0.6	16 ± 1.8	0.7	11 ± 2.8
3	17 ± 5.1	0.3	6.8 ± 2.0	0.8	5.1 ± 0.21	1.0	5.2 ± 0.55
4	23 ± 2.0	0.8	20 ± 2.9	1.0	14 ± 1.5	1.4	19 ± 2.0
5	5.4 ± 1.6	0.8	3.5 ± 0.51	1.2	4.0 ± 1.3	1.1	4.3 ± 1.5
6	7.3 ± 1.2	0.4	13 ± 0.43	0.2	8.9 ± 3.4	0.4	3.3 ± 1.4
7	7.3 ± 2.6	0.7	13 ± 2.9	0.4	7.3 ± 1.3	0.7	5.3 ± 2.0
8	2.6 ± 0.99	1.2	4.4 ± 1.7	0.7	5.4 ± 1.1	0.6	3.1 ± 0.87
9	2.3 ± 1.3	0.4	7.2 ± 1.1	0.1	4.7 ± 0.78	0.2	0.84 ± 0.23
10	5.0 ± 2.5	0.6	4.8 ± 1.0	0.6	6.2 ± 2.2	0.4	2.8 ± 0.91
11	6.4 ± 2.3	0.3	2.8 ± 0.06	0.6	10 ± 3.0	0.2	1.8 ± 0.94
12	3.1 ± 0.97	1.8	1.4 ± 0.27	4.0	1.9 ± 0.53	2.9	5.6 ± 1.7
13	11 ± 3.6	<0.1	5.4 ± 1.6	<0.1	0.34 ± 0.11	0.3	0.11 ± 0.01
14	7.8 ± 2.0	1.1	8.5 ± 0.36	1.0	1.6 ± 0.81	5.2	8.3 ± 2.3
15	10 ± 2.2	0.1	12 ± 1.9	0.1	9.6 ± 1.6	0.1	0.72 ± 0.02
16	0.15 ± 0.07	0.3	0.13 ± 0.01	0.4	0.63 ± 0.37	0.1	0.05 ± 0.01
17	0.74 ± 0.12	0.8	0.80 ± 0.13	0.8	4.4 ± 1.4	0.1	0.62 ± 0.11
18	7.8 ± 0.98	0.1	12 ± 0.41	0.1	1.2 ± 0.26	0.8	0.95 ± 0.28
19	2.4 ± 0.18	0.6	2.4 ± 0.43	0.6	6.6 ± 2.8	0.2	1.5 ± 0.54
20	8.9 ± 1.6	1.8	19 ± 2.2	0.8	7.6 ± 0.43	2.1	16 ± 0.32
21	100 ± 17	0.9	5.5 ± 2.1	16	9.4 ± 2.7	9.4	88 ± 0.21
22	9.3 ± 1.9	0.1	7.9 ± 1.7	0.2	9.9 ± 3.5	0.1	1.3 ± 0.48
23	6.0 ± 1.9	0.3	6.7 ± 0.68	0.2	3.6 ± 2.1	0.5	1.7 ± 0.42
24	10 ± 1.9	0.4	8.4 ± 0.34	0.4	9.1 ± 3.1	0.4	3.7 ± 0.91
25	39 ± 8.2	0.1	13 ± 5.5	0.3	5.9 ± 0.08	0.8	4.6 ± 2.0
26	3.0 ± 0.72	0.2	3.9 ± 1.1	0.1	1.7 ± 0.25	0.3	0.45 ± 0.22
5FU ^a	43 ± 3.1	1.2	39 ± 1.2	1.3	11 ± 0.97	4.7	52 ± 1.6
DOX ^a	0.29 ± 0.08	0.8	0.31 ± 0.07	0.8	0.59 ± 0.02	0.4	0.24 ± 0.03

Data are expressed as mean ± S.D.; IC_{50} (μM), the concentration of the compound that corresponds to a 50% growth inhibition of the cell line (compared to the control) after culturing the cells for 72 h with the individual compound; SI (selectivity index) was calculated using the formula: $SI = IC_{50}$ for normal cell line (HaCaT)/ IC_{50} for respective cancer cell lines (SW480, SW620, or PC-3); SW480, human primary colon cancer cell line; SW620, human metastatic colon cancer cell line; PC-3, human metastatic prostate cancer cell line; HaCaT, human immortalized keratinocyte cell line; ^a selected reference compounds commonly used in cancer treatment.

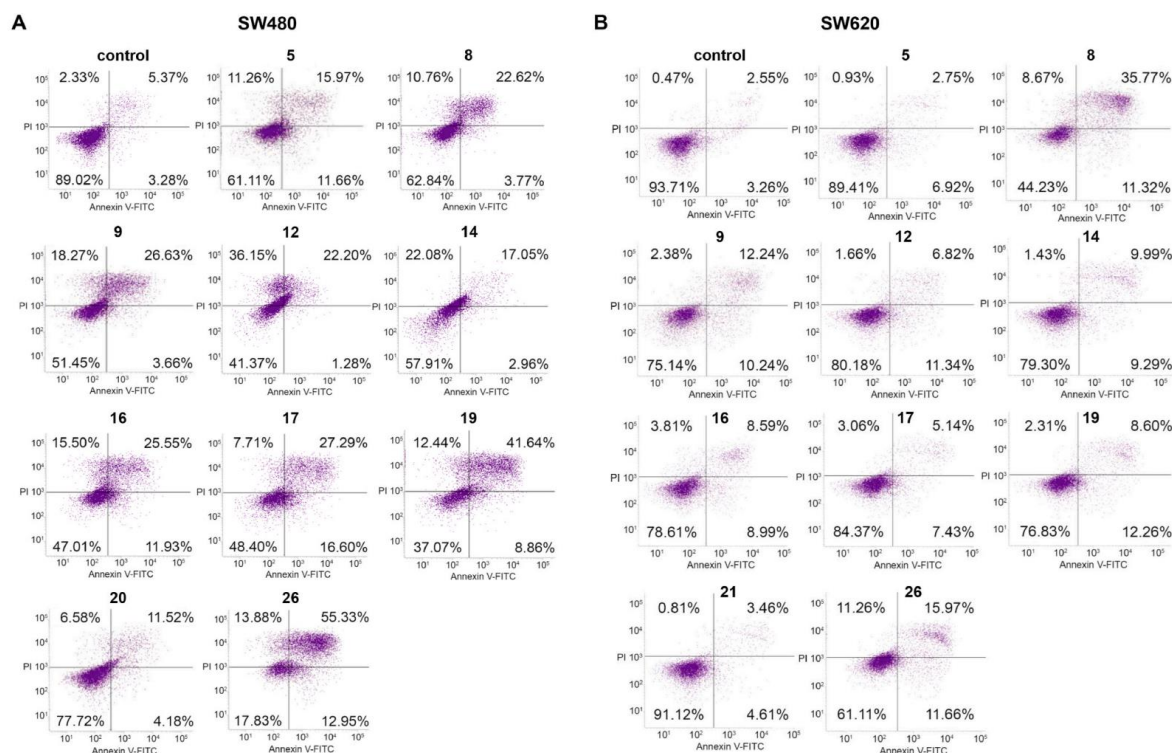


Fig. 1. The effects of salinomycin derivatives on early and late apoptosis or necrosis in (A) SW480, (B) SW620, (C) PC3, and (D) HaCaT cells detected with Annexin V-FITC/PI by flow cytometry. Diagrams show representative experiments. The lower right quadrant shows early apoptotic cells (Annexin V-FITC positive and PI negative staining), while the upper right and upper left quadrants represent the late stage of apoptotic or necrotic cells, respectively (Annexin V-FITC positive and PI positive, or Annexin V-FITC negative and PI positive staining, respectively).

Table 1).

For ester **8**, the strongest late apoptosis-inducing effect was found in SW620 ($35.44\% \pm 0.46$) and PC3 cells ($60.38\% \pm 7.10$), while the compound **5** showed the highest necrotic activity only in PC3 cells ($27.78\% \pm 0.40$). Allophanate **19** induced significant late apoptosis in SW480 cells ($46.96\% \pm 0.46$) and early apoptosis in SW620 cells ($13.20\% \pm 1.33$), whereas **16** was the most potent activator of late apoptosis and necrosis in PC3 cells ($36.29\% \pm 4.46$ and $23.59\% \pm 4.46$, respectively). Doubly modified analogue **26** had strong proapoptotic activity in primary colon cancer cells ($56.21\% \pm 1.24$) and weaker in metastatic variant of this cancer ($18.86\% \pm 4.08$). On the other hand, **20** induced stronger late apoptosis in PC3 ($25.05\% \pm 0.24$) than SW480 cells ($12.22\% \pm 0.99$). Compound **14** gave a similar percentage of late apoptotic and necrotic cells in SW480 ($20.43\% \pm 0.85$ and $28.65\% \pm 1.66$, respectively) and PC3 cancer cell lines ($15.38\% \pm 4.78$ and $22.44\% \pm 0.70$), whereas in SW620 cells this analogue structure induced mainly early and late apoptosis ($9.92\% \pm 0.89$ and $10.06\% \pm 0.11$, respectively). Moreover, ester **9** affected both late apoptosis and necrosis only in SW480 cells ($24.31\% \pm 3.27$ and $18.63\% \pm 0.51$, respectively), as well as early and late apoptosis in SW620 cells ($10.75\% \pm 0.72$ and $12.69\% \pm 0.63$, respectively). The strongest necrotic activity ($43.12\% \pm 3.99$) was found for **21** towards PC3 cancer cells. Of note, carbamate **16** induced early apoptosis in both SW480 ($14.41\% \pm 3.51$) and PC3 ($23.59\% \pm 4.46$) cells; high percent of early apoptotic SW480 cells were found for **17** ($16.60\% \pm 0.77$) and **26** ($14.21\% \pm 1.78$) which induced early apoptosis also in SW620 cells ($12.18\% \pm 0.74$).

The treatment of HaCaT non-tumour cells with SAL derivatives also revealed high levels of late apoptosis or necrosis in some cases. Four

compounds (**5**, **17**, **19** and **26**) were the strongest activators of late apoptosis that varied from 39.23% to 78.10% . In addition, a similar level of proapoptotic and necrotic cells was observed for **8** ($38.91\% \pm 3.47$ and $35.33\% \pm 6.47$, respectively) and **9** ($27.88\% \pm 0.71$ and $26.55\% \pm 0.38$, respectively), while **12** and **19** were stronger inducers of late apoptosis than necrosis ($32.28\% \pm 3.62$ versus $22.35\% \pm 5.21$ and $48.66\% \pm 4.64$ versus $32.0\% \pm 5.48$, respectively).

3.4. IL-6 level studies

Finally, to better understand the cytotoxic mechanism triggered by SAL derivatives, an interleukin-6 (IL-6) assay was also performed (Fig. 3). There is much evidence that a multifunctional pro-inflammatory cytokine IL-6 is involved in the pathogenesis of various cancers, including colon and prostate cancers (Culig and Puhr, 2018; Kumari et al., 2016; Waldner et al., 2012). IL-6 has been found in multiple cancer cell lines and in tissues obtained from cancer patients (Culig, 2014). The increased expression of IL-6 has been related to advanced stage of the disease and decreased survival in colorectal cancer patients, and its serum levels correlated with prostate tumour burden and patient morbidity (Smith et al., 2001; Waldner et al., 2012). It is well known that IL-6 exerts oncogenic effects in inflammation-associated cancers by activation of multiple signalling pathways, such as Janus kinases/signal transducers and activators of transcription (STAT), mitogen-activated protein kinases and phosphatidylinositol 3-kinase (Culig, 2014; Hodge et al., 2005). The role of STAT transcription factors in the development and survival of tumour cells varies with each type of cancer (Konjevic et al., 2013). The aberrant

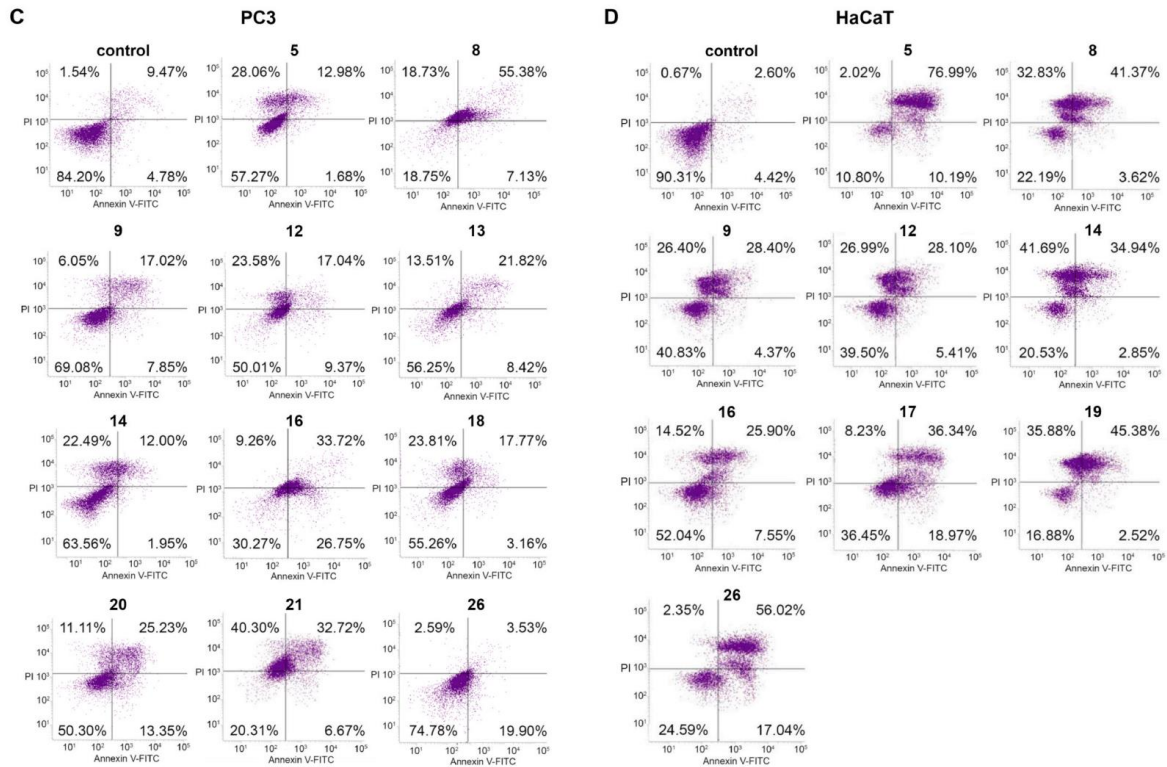


Fig. 1. (continued).

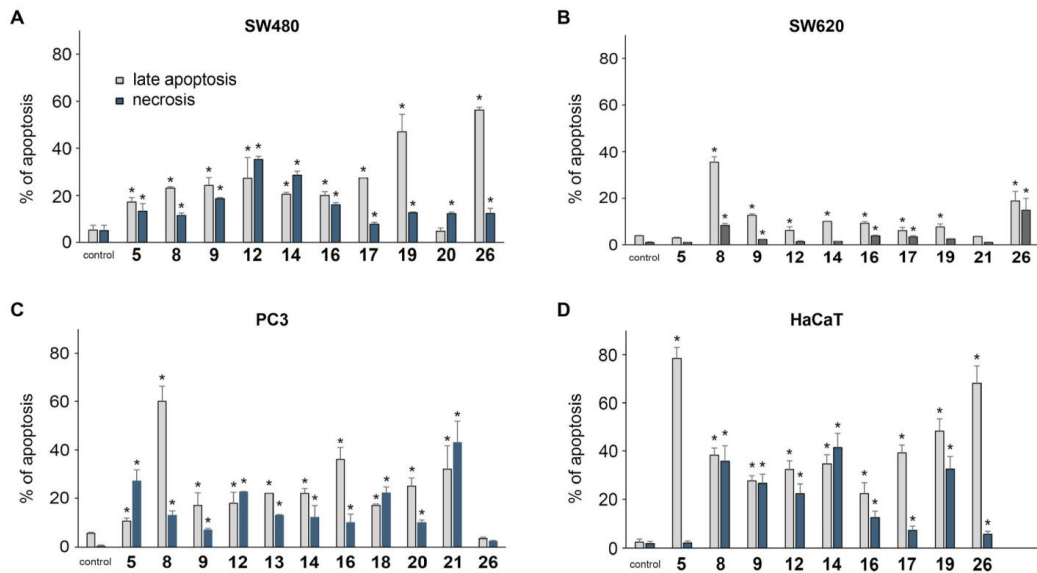


Fig. 2. The effect of salinomycin derivatives on late apoptosis and necrosis in (A) SW480, (B) SW620, (C) PC3, and (D) HaCaT cells detected with Annexin V-FITC/PI by flow cytometry. Cells were incubated for 72 h with selected salinomycin analogues at their IC_{50} concentrations. Data are expressed as % of cells in late apoptosis or necrosis, as means $\pm$ S.D. * $P < 0.001$ compared to the control.

activation of STAT3 has been described, e.g., in breast, lung, renal, prostate, colon, ovarian cancer as well as hepatocellular carcinoma and chronic myelogenous leukaemia (Konjevic et al., 2013; Lee et al., 2019). STAT3 enhances proliferation and angiogenesis, contributes to the promotion of metastasis and invasion, but also maintains CSCs (Hodge et al., 2005; Lee et al., 2019). Therefore, several components of the IL-6 signalling pathway, like IL-6, IL-6 receptor, JAK and/or STAT, have been proposed as very promising targets for effective cancer therapy (Waldner et al., 2012). However, to the best of our knowledge, there are no published data on the changes in IL-6 levels induced by SAL derivatives in colon and prostate cancers.

In our experiment, cancer cell lines were treated with IC₅₀ doses of selected SAL derivatives (5, 8, 9, 12, 13, 14, 16, 17, 18, 19, 20, 21, and 26). A noticeable inhibition of IL-6 was observed in cancer cell lines for almost all compounds used. A deeper analysis of the results shows that both SW480 and PC3 cells were more susceptible to the inhibition of IL-6 release, compared to the respective SW620 cells (Fig. 3). In SW480 cells, the highest inhibition of IL-6 secretion was observed for 8, 12, 16, 17 and 19. Allophanate 19 was also the most effective in SW620 cells, whereas 13 and 16 were the strongest inhibitors in PC3 cells. The mentioned analogues reduced IL-6 levels about twice more effectively when compared to the control.

4. Discussion

As demonstrated, also by us, the rationally designed chemical modification of the C1 carboxyl or C20 hydroxyl of salinomycin (SAL) alone, together with the multiple derivatization of both these functional groups may result in the improvement of the bioactivity of the resulting semisynthetic products (Antoszczak et al., 2014a, 2014b, 2014c, 2018, 2019, 2018; Borgström et al., 2013, 2016, 2017; Czerwonka et al., 2020, 2021; Li et al., 2017, 2018; Mai et al., 2017; Shi et al., 2016; Sulik et al., 2020; Versini et al., 2020; Zhang et al., 2016). From among the studied compounds, the most promising therapeutic potential against selected cancer cell lines was shown by four analogues (12, 14, 20 and 21) (Scheme 1 and Scheme 2), with IC₅₀ values (1.4–9.4 µM) (Table 1) that could be potentially achievable in serum or cancer tissue after their intravenous administration, and favourable toxicity towards non-tumour HaCaT cells (SI values between 1.8 and 16) (Table 1). Looking closer at the results, the most universal cancer growth inhibitor seems to be carbonate 12 that was found to exhibit significant cytotoxic activity against all studied cancer cell lines, together with high tumour-targeting selectivity (SW480, IC₅₀ = 3.1 ± 0.97 µM, SI = 1.8; SW620, IC₅₀ = 1.4 ± 0.27 µM, SI = 4.0; PC3, IC₅₀ = 1.9 ± 0.53 µM, SI = 2.9). The analysis of the structure-activity relationship (SAR) of the C20-O-acylated derivatives 7–19 clearly confirmed that the introduction of the aliphatic moiety improved the antiproliferative potential of the compounds when compared to that of the compounds with the aromatic (phenyl) substituent. Our finding seems to be in contrast to that of Wu and co-workers who have identified C20 phenyl and 4-fluorophenyl esters of C20-*epi*-salinomycin (2) as the most promising

anticancer drug candidates (Zhang et al., 2016), which may be most likely due to the different cancer cell lines used in the tests.

The incubation of SW480, SW620 and PC3 cells with the products derived from SAL showed a significantly higher percentage of late apoptotic or necrotic cells compared to the control (untreated cells) (Fig. 2). The results obtained for SW620 cells were not so pronounced as for SW480 and PC3 cells, however, some of the tested compounds still exhibited late apoptotic properties. It should be regarded that most of the studied compounds (9, 16, 17, 19 and 26) were generally characterized by low tumour-targeting selectivity (SI ≤ 1.0), therefore the results obtained in the apoptosis assay for HaCaT cells were similar or higher to those observed in the tests on cancer cells. As 5, 12 and 14 exhibited SI > 1.0, higher concentrations of these compounds (equal to IC₅₀ values) were used for HaCaT than for the corresponding cancer cells, which could explain the stronger apoptotic or necrotic effects. Nevertheless, the concentrations cytotoxic for cancer cells that would actually be used in clinical practice are much lower than those for HaCaT cells, e.g., 14, IC₅₀ = 1.6 µM ± 0.81 for PC3 cancer cells versus IC₅₀ = 8.3 ± 2.3 µM for HaCaT non-tumour cells (Table 1), thus the genuine proapoptotic effects for normal cells should be considerably weaker than those achieved in the present study.

It is known that interleukin 6 (IL-6) exerts both local and systemic pro-oncogenic effects; IL-6 release from cancer cells leads to autocrine and paracrine stimulation of tumour cell growth. Moreover, IL-6 has a pro-inflammatory systemic effect resulting in increased synthesis of acute phase proteins in the liver (Heinrich et al., 1990), and more important, it stimulates skeletal muscle and adipose tissue catabolism which has a negative impact on the quality of life and survival of cancer patients (Carson and Baltgalvis, 2010; Han et al., 2018). Therefore, the observed inhibition of IL-6 release by studied compounds seems to be beneficial both in patient therapy and care.

5. Conclusions

Cancer remains a major burden of disease around the world. Each year, tens of millions of individuals are diagnosed with cancer worldwide, and unfortunately, more than half of the cancer patients eventually die. In developed countries, cancer ranks the second most common cause of death following cardiovascular diseases. In light of the recent findings showing that the natural polyether ionophore salinomycin (SAL) induces apoptosis and overcomes multidrug resistance in various human cancer cells, the design and synthesis of more active and selective derivatives of SAL is of top interest.

In the current study, we have investigated the anticancer potential of a series of 24 derivatives of C20-*epi*-salinomycin (2), including the regioselectively O-acylated analogue structures, using several independent bioassays. Evaluation of the biological activity of the SAL derivatives has shown that some of the semisynthetic products inhibit the proliferation of colon and/or prostate cancer cells with non-toxic effects on non-tumour cells at low micromolar concentration range, which may be potentially achieved in serum of cancer patients. Importantly, the

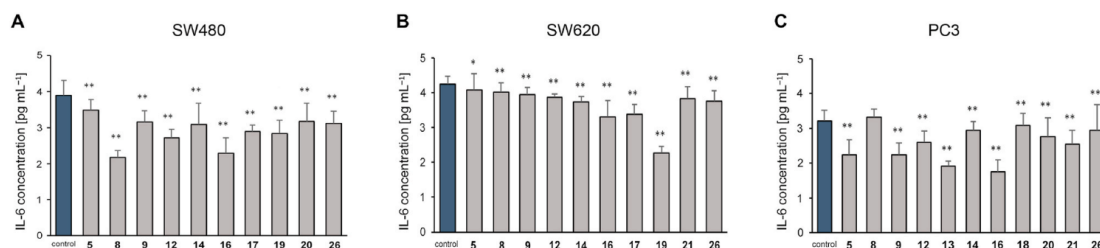


Fig. 3. The effect of salinomycin derivatives on IL-6 levels in (A) SW480, (B) SW620, and (C) PC3 cancer cells. Cells were incubated for 72 h with selected salinomycin analogues at their IC₅₀ concentrations. IL-6 levels in culture medium were measured by ELISA test. Data are expressed as the mean ± S.D. **P < 0.001, *P < 0.01 compared to the control.

most promising compounds have shown lower cytotoxic activity and higher tumour-targeting selectivity than those of doxorubicin, and simultaneously more favourable antiproliferative activity when compared to 5-fluorouracil. The selected SAL derivatives have induced mainly late apoptosis in colon cancer cells and necrosis in the prostate cancer cells, as well as have inhibited the secretion of interleukin 6 (IL-6) with stronger/higher effects in primary colon and metastatic prostate cancer cells.

With respect to the structure-activity relationships (SARs), we have clearly shown that (i) the C1 amides of **2** are more effective antiproliferative agents than the respective C1 esters, (ii) the C20 *O*-acylated derivatives containing aliphatic substituents are more potent anticancer compounds than those with aromatic (phenyl) moieties, as well as (iii) C20 carbonates are more selective analogues than C20 esters, which in turn are more promising than C20 carbamates or allophanate. The present findings highlight the therapeutic potential of SAL derivatives against colon and prostate cancer cells and identify compounds that merit further study and clinical development.

CCRediT authorship contribution statement

Dominika Czerwonka: Synthesis, purification and characterization of salinomycin derivatives, Formal analysis, Writing – original draft. **Magdalena Mielczarek-Put:** Bioassays performing, Formal analysis, Writing – original draft. **Michał Antoszczak:** Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Aleksandra Cioch:** Synthesis and purification of salinomycin derivatives. **Marta Struga:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, Supervision. **Adam Huczynski:** Conceptualization, Writing – review & editing, Resources, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

D.C. wishes to acknowledge the scholarship no. POWR. 03.02.00-00-1026/16, co-financed by the European Union through the European Social Fund under the Operational Program Knowledge Education Development. D.C. also wishes to acknowledge the Polish Science Center (NCN) for the doctoral scholarship ETIUDA (2020/36/T/ST4/00041).

M.A. wishes to acknowledge the NCN for financial support by a grant SONATA (2016/23/D/ST5/00242). M.A. also wishes to acknowledge the NCN and the Polish National Agency for Academic Exchange (NAWA) for the scholarships under the UWERTURA (2019/32/U/ST4/00092) and the BEKKER program (PPN/BEK/2019/1/00034), respectively, and the Polish Ministry of Science and Higher Education (MNiSW) for the scholarship for outstanding young scientists in the years 2020–2023 (STYP/15/1665/E-336/2020).

References

- Antoszczak, M., 2019. A comprehensive review of salinomycin derivatives as potent anticancer and anti-CSCs agents. *Eur. J. Med. Chem.* 166, 48–64. <https://doi.org/10.1016/j.ejmech.2019.01.034>.
- Antoszczak, M., Huczynski, A., 2019. Salinomycin and its derivatives – a new class of multiple-targeted “magic bullets”. *Eur. J. Med. Chem.* 176, 208–227. <https://doi.org/10.1016/j.ejmech.2019.05.031>.
- Antoszczak, M., Maj, E., Napiórkowska, A., Stefańska, J., Augustynowicz-Kopeć, E., Wietrzyk, J., Janczak, J., Brzezinski, B., Huczynski, A., 2014a. Synthesis, anticancer and antibacterial activity of salinomycin *N*-benzyl amides. *Molecules* 19, 19435–19459. <https://doi.org/10.3390/molecules191219435>.
- Antoszczak, M., Maj, E., Stefańska, J., Wietrzyk, J., Janczak, J., Brzezinski, B., Huczynski, A., 2014b. Synthesis, antiproliferative and antibacterial activity of new amides of salinomycin. *Bioorg. Med. Chem. Lett* 24, 1724–1729. <https://doi.org/10.1016/j.bmcl.2014.02.042>.
- Antoszczak, M., Popiel, K., Stefańska, J., Wietrzyk, J., Maj, E., Janczak, J., Michalska, G., Brzezinski, B., Huczynski, A., 2014c. Synthesis, cytotoxicity and antibacterial activity of new esters of polyether antibiotic – Salinomycin. *Eur. J. Med. Chem.* 76, 435–444. <https://doi.org/10.1016/j.ejmech.2014.02.031>.
- Antoszczak, M., Steverding, D., Sulik, M., Janczak, J., Huczynski, A., 2019. Antitrypanosomal activity of doubly modified salinomycin derivatives. *Eur. J. Med. Chem.* 173, 90–98. <https://doi.org/10.1016/j.ejmech.2019.03.061>.
- Antoszczak, M., Urbaniak, A., Delgado, M., Maj, E., Borgström, B., Wietrzyk, J., Huczynski, A., Yuan, Y., Chambers, T.C., Strand, D., 2018. Biological activity of doubly modified salinomycin analogs – evaluation *in vitro* and *ex vivo*. *Eur. J. Med. Chem.* 156, 510–523. <https://doi.org/10.1016/j.ejmech.2018.07.021>.
- Barrett, S.V., Barrett, M.P., 2000. Anti-sleeping sickness drugs and cancer chemotherapy. *Parasitol. Today*. [https://doi.org/10.1016/S0169-4758\(99\)01560-4](https://doi.org/10.1016/S0169-4758(99)01560-4).
- Battle, E., Clevers, H., 2017. Cancer stem cells revisited. *Nat. Med.* <https://doi.org/10.1038/nm.4409>.
- Borgström, B., Huang, X., Chygorin, E., Oredsson, S., Strand, D., 2016. Salinomycin hydroxamic acids: synthesis, structure, and biological activity of polyether ionophore hybrids. *ACS Med. Chem. Lett.* 7, 635–640. <https://doi.org/10.1021/acsmchemlett.6b00079>.
- Borgström, B., Huang, X., Hegardt, C., Oredsson, S., Strand, D., 2017. Structure-activity relationships in salinomycin: cytotoxicity and phenotype selectivity of semi-synthetic derivatives. *Chem. Eur. J.* 23, 2077–2083. <https://doi.org/10.1002/chem.201603621>.
- Borgström, B., Huang, X., Pošta, M., Hegardt, C., Oredsson, S., Strand, D., 2013. Synthetic modification of salinomycin: selective *O*-acylation and biological evaluation. *Chem. Commun.* 49, 9944. <https://doi.org/10.1039/c3cc45983g>.
- Carson, J., Baltgalvis, K., 2010. Interleukin-6 as a key regulator of muscle mass during cachexia. *Exerc. Sport Sci. Rev.* 38, 168–176. <https://doi.org/10.1097/JES.0b013e3181f44f11>.
- Chabner, B.A., Roberts, T.G., 2005. Chemotherapy and the war on cancer. *Nat. Rev. Canc.* 5, 65–72. <https://doi.org/10.1038/nrc1529>.
- Clevers, H., 2011. The cancer stem cell: premises, promises and challenges. *Nat. Med.* 17, 313–319. <https://doi.org/10.1038/nm.2304>.
- Culig, Z., 2014. Proinflammatory cytokine interleukin-6 in prostate carcinogenesis. *Am. J. Clin. Exp. Urol.* 2, 231–238.
- Culig, Z., Puh, M., 2018. Interleukin-6 and prostate cancer: current developments and unsolved questions. *Mol. Cell. Endocrinol.* 462, 25–30. <https://doi.org/10.1016/j.mce.2017.03.012>.
- Czerwonka, D., Barcelos, Y., Steverding, D., Cioch, A., Huczynski, A., Antoszczak, M., 2021. Singly and doubly modified analogues of C20-*epi*-salinomycin: a new group of antiparasitic agents against *Trypanosoma brucei*. *Eur. J. Med. Chem.* 209, 112900. <https://doi.org/10.1016/j.ejmech.2020.112900>.
- Czerwonka, D., Urbaniak, A., Sobczak, S., Piña-Oviedo, S., Chambers, T.C., Antoszczak, M., Huczynski, A., 2020. Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogues. *ChemMedChem* 15, 236–246. <https://doi.org/10.1002/cmdc.201900593>.
- DeVita, V.T., Chu, E., 2008. A history of cancer chemotherapy. *Canc. Res.* 68, 8643–8653. <https://doi.org/10.1158/0008-5472.CAN-07-6611>.
- Fuchs, D., Heinold, A., Opelz, G., Daniel, V., Naujokat, C., 2009. Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cells. *Biochem. Biophys. Res. Commun.* 390, 743–749. <https://doi.org/10.1016/j.bbrc.2009.10.042>.
- Guilford, F.T., Yu, S., 2019. Antiparasitic and antifungal medications for targeting cancer cells literature review and case studies [WWW Document]. *Altern. Ther. Health Med.* URL: <https://pubmed.ncbi.nlm.nih.gov/31202208/>, accessed 4.20.21.
- Gupta, P.B., Onder, T.T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R.A., Lander, E. S., 2009. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 138, 645–659. <https://doi.org/10.1016/j.cell.2009.06.034>.
- Han, J., Meng, Q., Shen, L., Wu, G., 2018. Interleukin-6 induces fat loss in cancer cachexia by promoting white adipose tissue lipolysis and browning. *Lipids Health Dis.* 17, 14. <https://doi.org/10.1186/s12944-018-0657-0>.
- Heinrich, P., Castell, J., Andust, T., 1990. Interleukin-6 and the acute phase response. *Biochem. J.* 265, 621–636. <https://doi.org/10.1042/bj2650621>.
- Hodge, D.R., Hurt, E.M., Farrar, W.L., 2005. The role of IL-6 and STAT3 in inflammation and cancer. *Eur. J. Canc.* 41, 2502–2512. <https://doi.org/10.1016/j.ejca.2005.08.016>.
- Huczynski, A., 2012. Polyether ionophores - promising bioactive molecules for cancer therapy. *Bioorg. Med. Chem. Lett* 22, 7002–7010. <https://doi.org/10.1016/j.bmcl.2012.09.046>.
- Kevin II, D.A., Meujo, D.A., Hamann, M.T., 2009. Polyether ionophores: broad-spectrum and promising biologically active molecules for the control of drug-resistant bacteria and parasites. *Expet Opin. Drug Discov.* 4, 109–146. <https://doi.org/10.1517/17460440802661443>.
- Klinkert, M., Heussler, V., 2006. The use of anticancer drugs in antiparasitic chemotherapy. *Mini Rev. Med. Chem.* 6, 131–143. <https://doi.org/10.2174/138955706775475939>.
- Konjevic, G., Radenkovic, S., Vuletic, A., Mirjagic, K., Jurisic, V., Srdic, T., 2013. STAT transcription factors in tumor development and targeted therapy of malignancies. In: *Oncogene and Cancer - from Bench to Clinic. InTech*. <https://doi.org/10.5772/54893>.
- Kumari, N., Dwarakanath, B.S., Das, A., Bhatt, A.N., 2016. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumor Biol.* <https://doi.org/10.1007/s13277-016-5098-7>.

- Lee, H., Jeong, A.J., Ye, S.K., 2019. Highlighted STAT3 as a potential drug target for cancer therapy. *BMB Rep.* <https://doi.org/10.5483/BMBRep.2019.52.7.152>.
- Li, B., Wu, J., Zhang, W., Li, Z., Chen, G., Zhou, Q., Wu, S., 2017. Synthesis and biological activity of salinomycin-hydroxamic acid conjugates. *Bioorg. Med. Chem. Lett.* 27, 1624–1626. <https://doi.org/10.1016/j.bmcl.2017.01.080>.
- Li, Y., Shi, Q., Shao, J., Yuan, Y., Yang, Z., Chen, S., Zhou, X., Wen, S., Jiang, Z.-X., 2018. Synthesis and biological evaluation of 20-*epi*-amino-20-deoxysalinomycin derivatives. *Eur. J. Med. Chem.* 148, 279–290. <https://doi.org/10.1016/j.ejmech.2018.02.004>.
- Mai, T.T., Hamai, A., Hienzschi, A., Cañeque, T., Müller, S., Wicinski, J., Cabaud, O., Leroy, C., David, A., Acevedo, V., Ryo, A., Ginestier, C., Birnbaum, D., Charafe-Jauffret, E., Codogno, P., Mehrpour, M., Rodriguez, R., 2017. Salinomycin kills cancer stem cells by sequestering iron in lysosomes. *Nat. Chem.* 9, 1025–1033. <https://doi.org/10.1038/nchem.2778>.
- Miyazaki, Y., Shibuya, M., Sugawara, H., Kawaguchi, O., Hirsoe, C., Hirose, C., Nagatsu, J., Esumi, S., 1974. Salinomycin, a new polyether antibiotic. *J. Antibiot. (Tokyo)* 27, 814–821. <https://doi.org/10.7164/antibiotics.27.814>.
- Nagai, H., Kim, Y.H., 2017. Cancer prevention from the perspective of global cancer burden patterns. *J. Thorac. Dis.* <https://doi.org/10.21037/jtd.2017.02.75>.
- Naujokat, C., Steinhart, R., 2012. Salinomycin as a drug for targeting human cancer stem cells. *J. Biomed. Biotechnol.* <https://doi.org/10.1155/2012/950658>.
- Pan, Z.M., Geng, S.Z., Zhou, Y.Q., Liu, Z.Y., Fang, Q., Liu, B.B., Jiao, X.A., 2010. Journal of animal and veterinary advances. *J. Anim. Vet. Adv. (Medwell Online)*.
- Sarker, S.D., Nahar, L., Miron, A., Guo, M., 2020. Anticancer natural products. In: *Annual Reports in Medicinal Chemistry*. Academic Press Inc., pp. 45–75. <https://doi.org/10.1016/bs.armac.2020.02.001>.
- Schirmacher, V., 2019. From chemotherapy to biological therapy: a review of novel concepts to reduce the side effects of systemic cancer treatment (Review). *Int. J. Oncol.* 54, 407–419. <https://doi.org/10.3892/ijo.2018.4661>.
- Shi, Q., Li, Y., Bo, S., Li, X., Zhao, P., Liu, Q., Yang, Z., Cong, H., Deng, H., Chen, M., Chen, S., Zhou, X., Ding, H., Jiang, Z.-X., 2016. Discovery of a (19)F MRI sensitive salinomycin derivative with high cytotoxicity towards cancer cells. *Chem. Commun.* 52, 5136–5139. <https://doi.org/10.1039/c6cc01508e>.
- Smith, P.C., Hobisch, A., Lin, D.L., Culig, Z., Keller, E.T., 2001. Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev.* [https://doi.org/10.1016/S1359-6101\(00\)00021-6](https://doi.org/10.1016/S1359-6101(00)00021-6).
- Sulik, M., Stepień, K., Stefańska, J., Huczyński, A., Antoszczak, M., 2020. Antibacterial activity of singly and doubly modified salinomycin derivatives. *Bioorg. Med. Chem. Lett.* 30, 127062. <https://doi.org/10.1016/j.bmcl.2020.127062>.
- Versini, A., Colombeau, L., Hienzschi, A., Gaillet, C., Retailliau, P., Debieu, S., Müller, S., Cañeque, T., Rodriguez, R., 2020. Salinomycin derivatives kill breast cancer stem cells by lysosomal iron targeting. *Chem. Eur. J.* 26, 7416–7424. <https://doi.org/10.1002/chem.202000335>.
- Versini, A., Saier, L., Sindikubwabo, F., Müller, S., Cañeque, T., Rodriguez, R., 2018. Chemical biology of salinomycin. *Tetrahedron* 74, 5585–5614. <https://doi.org/10.1016/j.tet.2018.07.028>.
- Waldner, M.J., Foersch, S., Neurath, M.F., 2012. Interleukin-6 - a key regulator of colorectal cancer development. *Int. J. Biol. Sci.* <https://doi.org/10.7150/ijbs.4614>.
- World Health Organization, 2020. WHO report on cancer: setting priorities, investing wisely and providing care for all [WWW Document]. *Die Gynäkologie*. URL: <http://www.who.int/publications/i/item/who-report-on-cancer-setting-priorities-investing-wisely-and-providing-care-for-all>. accessed 2.23.21.
- Yvoré, P., Raynaud, J.P., Conan, L., Naciri, M., 1980. Evaluation of the efficacy of salinomycin in the control of coccidiosis in chicks. *Poultry Sci.* 59, 2412–2416. <https://doi.org/10.3382/ps.0592412>.
- Zhang, W., Wu, J., Li, B., Xia, J., Wu, H., Wang, L., Hao, J., Zhou, Q., Wu, S., 2016. Synthesis and biological activity evaluation of 20-*epi*-salinomycin and its 20-O-acyl derivatives. *RSC Adv.* 6, 41885–41890. <https://doi.org/10.1039/C6RA08967D>.

PUBLIKACJA III wraz z suplementem

Dominika Czerwonka, Sebastian Müller, Tatiana Cañeque, Ludovic Colombeau,
Adam Huczyński, Michał Antoszczak, Raphaël Rodriguez

„Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against
Cancer Stem-Like Cells”

ACS Organic & Inorganic Au 2022, DOI: 10.1021/acsorginorgau.1c00046.

Reprinted with permission from *ACS Org. Inorg. Au* 2022, XXXX, XXX, XXX-XXX, Publication Date: January 5, 2022, <https://doi.org/10.1021/acsorginorgau.1c00046>. **Copyright © 2022 The Authors. Published by American Chemical Society**

Expeditive Synthesis of Potent C20-*epi*-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells

Dominika Czerwonka, Sebastian Müller, Tatiana Cañeque, Ludovic Colombeau, Adam Huczyński, Michał Antoszczak,* and Raphaël Rodriguez*



Cite This: <https://doi.org/10.1021/acsorginorgau.1c00046>



Read Online

ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: As a continuation of our studies toward the development of small molecules to selectively target cancer stem cells (CSCs), a library of 18 novel derivatives of salinomycin (**Sal**), a naturally occurring polyether ionophore, was synthesized with a good overall yield using a one-pot Mitsunobu–Staudinger procedure. Compared to the parent structure, the newly synthesized products contained the mono- or disubstituted C20-*epi*-amine groups. The biological activity of these compounds was evaluated against human mammary mesenchymal HMLER CD24^{low}/CD44^{high} cells, a well-established model of breast CSCs, and its isogenic epithelial cell line (HMLER CD24^{high}/CD44^{low}) lacking CSC properties. Importantly, the vast majority of **Sal** derivatives were characterized by low nanomolar activities, comparing favorably with previous data in the literature. Furthermore, some of these derivatives exhibited a higher selectivity for the mesenchymal state compared to the reference **Sal** and ironomycin, representing a promising new series of compounds with anti-CSC activity.

KEYWORDS: salinomycin, Mitsunobu reaction, Staudinger reaction, cancer stem cells, anticancer activity

1. INTRODUCTION

There is an urgent need to develop new cancer therapeutics.¹ Particularly interesting in this context are molecules that could preferentially target a fraction of cancer cells, known as cancer stem cells (CSCs).^{2,3} These cells can be refractory to conventional chemotherapy and radiation therapy, leading to disease recurrence and metastasis.^{4,5}

Using a high-throughput screening method, Gupta et al. identified the natural product salinomycin (**Sal**) as a selective inhibitor of breast CSCs among ~16 000 compounds tested.⁶ Since **Sal** was reported to be active against CSCs of various tissue types,^{7,8} intensive studies have been performed to elucidate the mechanism of action (MoA) of **Sal**. In addition to other effects, **Sal** has been shown to alter mitochondrial functions, decrease ATP levels, and alter autophagy.^{9–11} Moreover, **Sal** has also been evidenced to induce stress in the endoplasmic reticulum (ER) by altering Ca²⁺ homeostasis.¹²

Regarding its promising anticancer potential, many research groups have attempted to develop more effective chemical modifications of **Sal**,¹³ particularly through the derivatization of the C1-carboxyl^{14–19} or C20-hydroxyl.^{20–24} Using a series of chemo- and stereocontrolled reactions, we successfully modified the C20-hydroxyl of **Sal** to obtain a series of potent C20-amino analogs.^{20,21} The highly promising molecule in this group, ironomycin (Figure 1), had been found to be approximately 10-fold more active against breast CSCs than the unmodified **Sal** both *in vitro* and *in vivo*.²¹

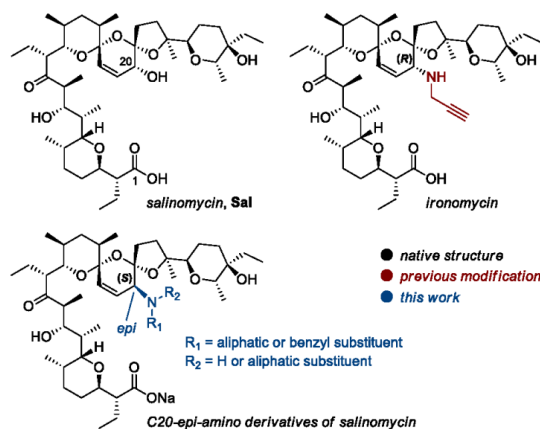


Figure 1. Molecular structures of the parent natural product salinomycin, a potent C20-amino derivative previously reported by us, and a novel series with C20-*epi*-amino substituents.

Received: November 3, 2021

Revised: December 18, 2021

Accepted: December 21, 2021

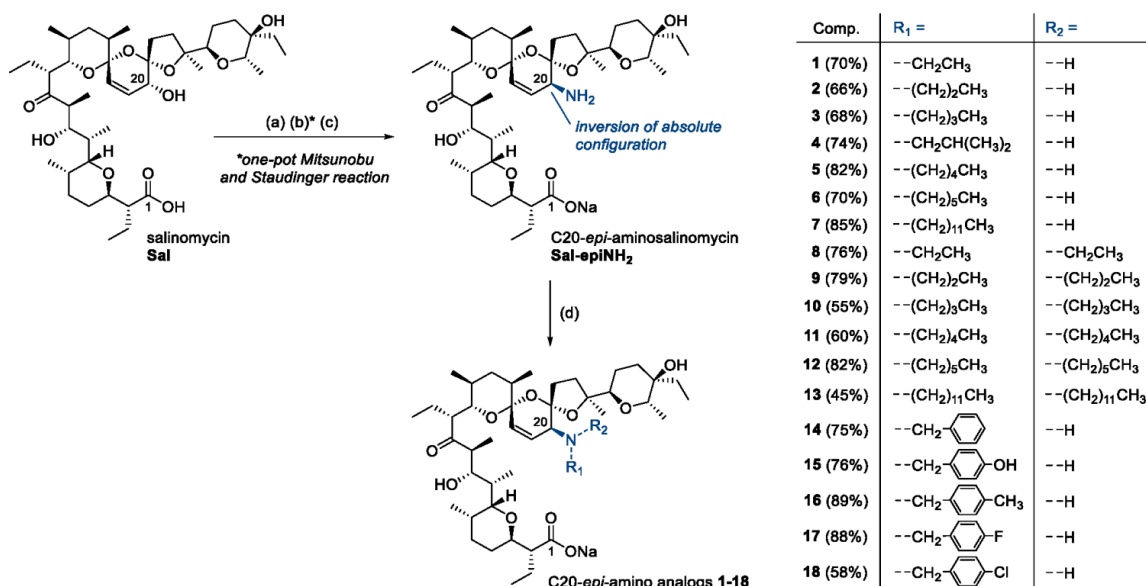


ACS Publications

© XXXX The Authors. Published by
American Chemical Society

A

<https://doi.org/10.1021/acsorginorgau.1c00046>
ACS Org. Inorg. Au XXXX, XXX, XXX–XXX

Scheme 1. Synthesis of C20-*epi*-Amino Derivatives of Salinomycin^a

^aReagents and conditions are as follows: (a) DMAP, TMSEtOH, TCFH, CH₂Cl₂, 0 °C to RT; (b) TPP, DIAD, DPPA, THF, RT, then TPP, H₂O, THF, RT; (c) TBAF, THF, RT, then aq. Na₂CO₃; (d) RCHO, CH₂Cl₂, RT, then NaBH₃CN, MeOH, RT.

Table 1. Antiproliferative Activity (IC₅₀, μM) with Standard Deviation and Selectivity Index (SI) Values of the C20-*epi*-Amino Derivatives of Salinomycin Measured at 72 h in HMLER CD24^{low}/CD44^{high}, HMLER CD24^{high}/CD44^{low}, and MCF10A Cells^a

	HMLER CD24 ^{low} /CD44 ^{high}	HMLER CD24 ^{high} /CD44 ^{low}	SI (HMLER)	MCF10A
Sal	1.39 ± 0.10	4.36 ± 0.50	3.1	1.13 ± 0.14
Sal-epiNH ₂	20.48 ± 3.30	>50	n.d.	>12.5
ironomycin	0.17 ± 0.02	2.28 ± 0.16	13.4	0.12 ± 0.02
1	0.53 ± 0.08	3.25 ± 3.30	6.1	2.61 ± 0.78
2	0.17 ± 0.02	1.71 ± 0.40	10.1	1.56 ± 0.68
3	0.12 ± 0.02	1.83 ± 0.70	15.2	1.46 ± 0.08
4	0.07 ± 0.05	0.77 ± 0.46	11.0	1.30 ± 0.46
5	0.11 ± 0.03	1.19 ± 0.60	10.8	0.46 ± 0.27
6	0.03 ± 0.01	0.74 ± 0.29	24.7	1.50 ± 0.50
7	0.17 ± 0.04	0.69 ± 0.05	4.1	n.d.
8	0.003 ± 0.0005	0.27 ± 0.04	90.0	1.39 ± 0.18
9	0.009 ± 0.0002	0.18 ± 0.02	20.0	0.47 ± 0.19
10	0.85 ± 0.22	6.48 ± 0.50	7.6	n.d.
11	0.33 ± 0.12	2.53 ± 1.00	7.7	3.93 ± 0.29
12	0.30 ± 0.16	3.98 ± 2.20	13.3	1.70 ± 0.44
13	1.92 ± 0.50	10.02 ± 3.60	5.2	n.d.
14	0.12 ± 0.003	0.75 ± 0.12	6.2	4.67 ± 0.73
15	0.10 ± 0.01	1.24 ± 0.02	12.4	4.16 ± 0.57
16	0.07 ± 0.04	0.89 ± 0.28	12.7	1.26 ± 0.18
17	0.06 ± 0.01	0.61 ± 0.01	10.2	1.88 ± 0.11
18	0.08 ± 0.02	0.88 ± 0.54	11.0	1.86 ± 0.31

^aThe selectivity index (SI) was defined as IC₅₀(HMLER CD24^{high}/CD44^{low})/IC₅₀(HMLER CD24^{low}/CD44^{high}). Each IC₅₀ value was determined in biological triplicate (three independent biological experiments), and each triplicate was determined in at least technical duplicate; n.d., not determined.

Mechanistically, we have provided robust evidence that Sal, ironomycin, and other analogs exert their activity by accumulating in lysosomes and sequestering iron in this organelle, which can lead to the production of reactive oxygen species (ROS), lipid peroxidation, and cell death reminiscent of ferroptosis.²¹ As cells in the mesenchymal state and CSCs are

addicted to iron showing a higher load,²⁵ this cell state has a pronounced vulnerability to cell death induced by ironomycin and Sal.

Given that Sal and ironomycin have been found to target lysosomal iron, we hypothesized that a distinct orientation of the C20-amino substituents might affect its stability and target

engagement as well as efficacy and selectivity toward the CSC populations. In this context, the synthetic strategy for the diastereoselective inversion of the absolute configuration at the C20-position of **Sal** has been reported previously.^{26–29} The esters of C20-*epi*-salinomycin showed a potent activity toward colorectal, gastric, and triple-negative breast cancer cells,²⁸ while the corresponding C20-*epi*-carbonates and carbamates were identified as inducers of late apoptosis in colon cancer and necrosis in prostate cancer cells.²⁹ Jiang and co-workers synthesized a series of C20-*epi*-*N*-acyl²⁶ and C20-*epi*-triazole²⁷ analogs of **Sal**, showing improved properties compared to **Sal**.

Thus, we were interested in evaluating the effect of combining the presence of an amine at C20 together with the opposite stereochemistry. Here, we describe rapid access to the C20-*epi*-amino derivatives of **Sal** (Figure 1 and Scheme 1). We evaluated a library of 18 novel **Sal** derivatives against a well-established model of breast CSCs together with the corresponding cells deprived of stem-like properties (Table 1). We identified three compounds that have the ability to preferentially kill the cancer stem-like cells with remarkably low IC₅₀ values that compete favorably with our reference compound ironomycin. All products were also less toxic against the normal breast cell line MCF10A compared to ironomycin, illustrating some degree of improvement (Table 1).

2. RESULTS AND DISCUSSION

2.1. Synthesis

Although the synthetic access to our key precursor, C20-*epi*-aminosalinomycin **Sal-epiNH₂**, has been previously reported,²⁶ we were able to improve on this procedure. It was conveniently afforded using a one-pot Mitsunobu and Staudinger methodology (Scheme 1), which is important when it comes to scaling up biologically active compounds for therapeutic use. Briefly, in the first step we masked the C1-functionality of **Sal** by converting the C1-carboxyl to a TMS ethyl ester. Next, using a Mitsunobu–Staudinger reaction sequence, followed by quantitative deprotection of the C1-position with TBAF, we obtained **Sal-epiNH₂** on a gram scale.

Having facile access to the starting material, a library of 18 C20-*epi*-amino derivatives of **Sal** was synthesized with a good overall yield by means of a chemoselective reductive amination, reacting **Sal-epiNH₂** with structurally diverse aldehydes (Scheme 1). Specifically, we used aldehydes that differed in polarity and flexibility, selecting both aliphatic and aromatic substrates. We selected aldehydes with shorter or longer aliphatic chains, from 2 to up to 12 carbon atoms, and various benzyl aldehydes substituted at the *para*-position with a methyl group, a hydroxyl group, or halogens. Importantly, we were able to obtain the corresponding secondary and tertiary amines at the C20-position by varying the amount of aldehyde employed in the reaction mixture. The NMR data of analogs **1–18** obtained as sodium salts are presented in the Supporting Information. The HRMS analysis (Supporting Information) also supported the formation of the products.

2.2. Biological Evaluation

The activity of our benchmark compounds **Sal** and ironomycin, together with those of **Sal-epiNH₂** and the new derivatives **1–18**, was measured *in vitro* against transformed human mammary mesenchymal HMLER CD24^{low}/CD44^{high} cells, an established model of human breast CSCs.^{30,31} We also evaluated these products for their selectivity toward the corresponding epithelial

counterparts (HMLER CD24^{high}/CD44^{low}), which lacked features of CSCs (Table 1).

Notably, C20-*epi*-amino derivatives exhibited higher anti-CSC potentials compared to **Sal**. Of note, most of these products also showed an improved potency compared to that of the reference ironomycin, among which compounds **4**, **6**, **8**, **9**, and **16–18** were the most potent of this series. A deeper analysis of the results revealed that tertiary amine-containing **Sal** derivatives were essentially less potent than their corresponding secondary amine counterparts, with the exceptions of **8** and **9** bearing diethyl and dipropyl substituents, respectively. These derivatives were the most potent compounds, with remarkably low IC₅₀ values of 3 and 9 nM against mesenchymal HMLER CD24^{low}/CD44^{high} cells, respectively. Remarkably, the anti-proliferative activity of **8** and **9** was accompanied by their higher selectivity for the mesenchymal state, with SIs of 90.0 and 20.0, respectively. This is consistent with iron-targeting and the higher iron demand of that cell state. These data indicate the promising therapeutic potential for **8** and **9** in light of preclinical results already obtained for the less potent lead structure ironomycin. With respect to the other tertiary amines, further elongation of the aliphatic chains resulted in reduced antiproliferative activity, with the didodecyl derivative **13** identified as the least potent of the series. This argues in favor of a model whereby an optimal apolar alkane is required for this biological activity. Analogs with alkanes that are too long might be retained in lipid membranes, up to a point where their capacity to accumulate in the lumen of lysosomes and thus engage with iron is reduced. On the other hand, a critically short side chain may not allow the derivative to effectively cross lipid membranes.

Interestingly, this class of compounds showed generally lower toxicities against the normal breast cell line MCF10A (Table 1), highlighting the potential for the development of these compounds to target CSCs selectively. All analogs were less active against the MCF10A cell line than reference ironomycin, indicating that an inversion of the absolute configuration at the C20-position may improve not only the antiproliferative activity against cancer cells but also the selectivity.

Although monosubstituted C20-*epi*-amino analogs **1** and **2** were found to be less effective than their corresponding disubstituted counterparts **8** and **9**, compound **6** bearing an *n*-hexyl aliphatic chain showed potent antiproliferative activity and selectivity for the mesenchymal state of cells. With an IC₅₀ value of 30 nM toward HMLER CD24^{low}/CD44^{high} cells and a good selectivity (SI = 24.7), this compound was the most potent among all secondary amine products. It exhibited a potency comparable to those of **8** and **9**, supporting the importance of an optimal overall lipophilicity, which remains comparable for these three derivatives. It is consistent with the capacity of these derivatives to effectively cross lipid membranes to reach their functional target. Finally, C20-*epi*-amino derivatives **14–18** with a benzyl moiety exhibited comparable activities against HMLER CD24^{low}/CD44^{high} cells, with IC₅₀ values in the range of 60–120 nM. Interestingly, in all cases the introduction of the substituents (methyl, hydroxyl, or halogen) at the *para*-position resulted in increased selectivity. Thus, the evaluation of the activity of other monosubstituted products (*para*-position versus *ortho*- and *meta*-positions) or multiple-substituted analogs might be worth considering in the future.

3. CONCLUSIONS

In summary, prompted by the improved therapeutic potential of C20-amino analogs of **Sal** (e.g., ironomycin) and the fact that

C20-functionalized epimers retain their efficacy *in vitro*, we synthesized a series of 18 C20-*epi*-amino derivatives of **Sal** that combined both structural modifications using a straightforward and scalable protocol.

All derivatives were assessed for their antiproliferative activity and selectivity toward a well-established model of mesenchymal CSCs (HMLER CD24^{low}/CD44^{high}) together with their epithelial counterparts (HMLER CD24^{high}/CD44^{low}) lacking CSC properties. Most of these derivatives were found to be more potent and more selective against the mesenchymal state compared to the references **Sal** and ironomycin. Specifically, compounds **6**, **8**, and **9** were identified to be particularly interesting in this context, with IC₅₀ values of 30, 3, and 9 nM, respectively, together with their outstanding selectivities (SIs between 20.0 and 90.0). Concerning a structure–activity relationship (SAR), we found the following: (i) monosubstituted C20-*epi*-amino derivatives of **Sal** are essentially more active than their corresponding disubstituted counterparts; (ii) with respect to secondary amine products, *n*-pentyl and *n*-hexyl substituents are more potent against the mesenchymal state, (iii) regarding tertiary amine derivatives, elongation of the aliphatic chains results in a decrease of the antiproliferative activity; and (iv) the introduction of the nonpolar or polar substituent at the *para*-position of the benzyl motif increases the selectivity.

Here, we have reported a convenient synthetic scheme that can readily afford potent derivatives in a short number of steps. Importantly, we describe the most potent and selective derivatives of **Sal** reported so far. Future work will involve the preclinical evaluation of the most promising compounds in various cancer settings.

4. EXPERIMENTAL SECTION

4.1. General Information

Detailed descriptions of the general procedures, equipment, and measurement parameters can be found in the Supporting Information. C20-*epi*-aminosalinomycin (**Sal-epiNH₂**) was resynthesized following the previously reported procedure,²⁶ with slight modifications.

Briefly, in the first step, to a stirred solution of salinomycin (**Sal**) (3.00 g, 1.0 equiv) in 50 mL of CH₂Cl₂ in an ice bath were added DMAP (2.38 g, 5.0 equiv), TMSEtOH (2.77 g, 6.0 equiv), and TCFH (1.31 g, 1.2 equiv). The resulting mixture was stirred at RT overnight. The reaction mixture was then concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 40% EtOAc/*n*-hexane) gave the C1-EtTMS ester of **Sal** as a yellow oil (1.70 g, 50% yield).

Next, to a solution of the C1-EtTMS ester of **Sal** (1.70 g, 1.0 equiv) in 20 mL of anhydrous THF in an ice bath was added TPP (784 mg, 1.5 equiv). After 20 min, DIAD (481 mg, 1.2 equiv) was slowly added to the mixture, followed by DPPA (605 mg, 1.1 equiv). The solution was stirred at RT for 24 h. After this time, TPP (1.57 g, 3.0 equiv) was added to the mixture in one portion, followed by the addition of 0.5 mL of water. The mixture was stirred at RT for next 24 h, and the reaction progress was monitored by TLC. The reaction mixture was then concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 50% acetone/CHCl₃) gave the C1-EtTMS ester of C20-*epi*-aminosalinomycin as a yellow oil (716 mg, 42% yield).

Finally, the C1-masked C20-*epi*-aminosalinomycin (716 mg, 1.0 equiv) was dissolved in 15 mL of anhydrous THF at RT. A 1.0 M solution of TBAF in THF (2.53 mL, 3.0 equiv) was then added dropwise. The solution was left to be stirred at RT for next 24 h. The reaction mixture was then concentrated under reduced pressure. Purification on silica gel using the CombiFlash system (0 → 50% acetone/CHCl₃) gave the reaction product as a yellow oil. The product was then dissolved in CH₂Cl₂ and washed with a 0.1 M solution of Na₂CO₃. The separated organic layers were dried over MgSO₄ and

concentrated under reduced pressure. The residue was then evaporated several times with *n*-pentane to quantitatively give the sodium salt of **Sal-epiNH₂** as a white amorphous solid (648 mg). The data for the obtained material were in accordance with the literature.

4.2. General Procedure for the Preparation of Analogs 1–18

To a stirred solution of **Sal-epiNH₂** (50 mg, 0.07 mmol, 1.0 equiv) in 5 mL of CH₂Cl₂ was added the corresponding aldehyde (1.0 equiv for singly substituted analogs **1**–**7** and **14**–**18** or 5.0 equiv for the doubly substituted counterparts **8**–**13**). The solution was stirred at RT for 24 h. After that time, a solution of NaBH₃CN (5 mg, 0.08 mmol, 1.2 equiv; in 2 mL of MeOH) was added to the mixture drop by drop. The reaction mixture was stirred further at RT for an additional 30 min. Next, the solvent was evaporated under reduced pressure. Purification of the crude material on silica gel using the CombiFlash system gave the C20-*epi*-amino analogs **1**–**18** as white amorphous solids after evaporation with *n*-pentane. The NMR and HRMS spectra of compounds **1**–**18** are included in the Supporting Information (Figures S1–S54).

4.2.1. Compound 1. Yield: 35 mg, 70%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.57 in 60% acetone/CHCl₃. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 6.31 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.12 (d, *J* = 10.8 Hz, 1H), 4.29 (q, *J* = 6.8 Hz, 1H), 4.12 (d, *J* = 10.4 Hz, 1H), 3.77 (dt, *J* = 13.8, 6.9 Hz, 1H), 3.64 (dd, *J* = 10.1, 2.1 Hz, 1H), 3.60 (d, *J* = 10.2 Hz, 1H), 3.37 (dd, *J* = 11.9, 2.1 Hz, 1H), 2.86 (d, *J* = 5.6 Hz, 1H), 2.81–2.73 (m, 2H), 2.72–2.68 (m, 1H), 2.67–2.57 (m, 2H), 2.00–0.50 (m, 60H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.4, 184.1, 129.9, 122.7, 110.1, 99.7, 89.2, 76.4, 76.3, 75.5, 75.1, 71.8, 70.2, 67.7, 56.1, 55.5, 51.6, 50.9, 42.8, 40.7, 39.5, 37.5, 36.5, 33.4, 33.1, 33.0, 29.8, 28.6, 28.3, 27.4, 24.2, 21.4, 20.6, 17.9, 17.3, 16.5, 16.4, 15.1, 13.5, 12.8, 12.4, 11.0, 7.0, 6.8 ppm; FT-IR (KBr) 3319, 2963, 2935, 2875, 1714, 1568, 1460, 1407 cm^{−1}. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₄H₇₅NO₁₀Na⁺ 800.5289, found 800.5283; [M − Na + 2H]⁺ Calcd for C₄₄H₇₆NO₁₀⁺ 778.5464, found 778.5460.

4.2.2. Compound 2. Yield: 35 mg, 66%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.23 in 66% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 6.27 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.08 (d, *J* = 10.8 Hz, 1H), 4.28 (q, *J* = 6.7 Hz, 1H), 4.11 (d, *J* = 10.2 Hz, 1H), 3.78 (dd, *J* = 11.1, 4.8 Hz, 1H), 3.64 (dd, *J* = 16.6, 6.3 Hz, 2H), 3.37 (dd, *J* = 11.9, 2.1 Hz, 1H), 2.97–2.87 (m, 2H), 2.79–2.68 (m, 2H), 2.68–2.61 (m, 1H), 2.11–2.04 (m, 2H), 2.00–0.50 (m, 61H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.6, 184.1, 130.3, 122.0, 110.4, 99.6, 89.22, 76.5, 76.4, 75.4, 75.1, 71.8, 70.2, 67.8, 56.1, 53.3, 51.6, 50.9, 47.6, 40.7, 39.5, 37.7, 36.5, 33.3, 33.2, 33.0, 30.0, 28.6, 28.2, 27.5, 24.8, 24.1, 23.2, 21.3, 20.6, 17.9, 17.4, 16.6, 15.1, 13.5, 12.8, 12.4, 11.0, 7.1, 6.8 ppm; FT-IR (KBr) 3289, 2962, 2933, 2874, 1713, 1660, 1568, 1459, 1407 cm^{−1}. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₅H₇₇NO₁₀Na⁺ 814.5440, found 814.5426; [M − Na + 2H]⁺ Calcd for C₄₅H₇₈NO₁₀⁺ 792.5620, found 792.5619.

4.2.3. Compound 3. Yield: 36 mg, 68%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.29 in 66% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 6.31 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.11 (d, *J* = 10.8 Hz, 1H), 4.29 (q, *J* = 6.7 Hz, 1H), 4.12 (d, *J* = 10.4 Hz, 1H), 3.78 (dd, *J* = 11.1, 4.8 Hz, 1H), 3.64 (dd, *J* = 9.9, 1.8 Hz, 1H), 3.60 (d, *J* = 10.2 Hz, 1H), 3.37 (dd, *J* = 11.9, 2.1 Hz, 1H), 2.84 (d, *J* = 5.6 Hz, 1H), 2.80–2.69 (m, 3H), 2.68–2.62 (m, 1H), 2.60–2.52 (m, 1H), 2.00–0.50 (m, 64H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.4, 184.1, 130.0, 122.6, 110.1, 99.7, 89.2, 76.4, 76.3, 75.5, 75.0, 71.8, 70.2, 67.7, 56.1, 55.8, 51.6, 50.9, 48.3, 40.7, 39.4, 37.5, 36.5, 33.5, 33.4, 33.1, 33.0, 29.8, 28.6, 28.3, 27.4, 24.2, 21.4, 20.8, 20.6, 17.8, 17.3, 16.5, 15.1, 14.3, 13.5, 12.8, 12.4, 11.0, 7.0, 6.8 ppm; FT-IR (KBr) 3288, 2960, 2931, 2873, 1713, 1661, 1567, 1459, 1406 cm^{−1}. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₆H₇₉NO₁₀Na⁺ 828.5596, found 828.5577; [M − Na + 2H]⁺ Calcd for C₄₆H₈₀NO₁₀⁺ 806.5777, found 806.5772.

4.2.4. Compound 4. Yield: 40 mg, 74%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.50 in 66% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400

MHz, CD_2Cl_2) δ 6.35 (dd, J = 10.7, 5.6 Hz, 1H), 6.15 (d, J = 10.8 Hz, 1H), 4.33 (q, J = 6.8 Hz, 1H), 4.16 (d, J = 9.4 Hz, 1H), 3.81 (dd, J = 11.1, 4.9 Hz, 1H), 3.72–3.60 (m, 2H), 3.41 (dd, J = 12.0, 2.1 Hz, 1H), 2.85 (d, J = 5.6 Hz, 1H), 2.82–2.73 (m, 2H), 2.72–2.65 (m, 1H), 2.62 (dd, J = 11.3, 6.7 Hz, 1H), 2.41 (dd, J = 11.3, 6.6 Hz, 1H), 2.15–2.09 (m, 2H), 2.05–0.50 (m, 62H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.3, 184.1, 130.0, 122.5, 110.2, 99.7, 89.2, 76.4, 76.3, 75.5, 75.0, 71.8, 70.2, 67.7, 56.7, 56.0 (2C), 51.6, 50.8, 40.7, 39.4, 37.6, 36.5, 33.4, 33.2, 33.0, 29.8, 29.7, 28.6, 28.3, 27.4, 24.2, 21.3, 20.8, 20.7, 20.5, 17.8, 17.4, 16.5, 15.1, 13.5, 12.8, 12.4, 11.0, 7.0, 6.8 ppm; FT-IR (KBr) 3289, 2960, 2931, 2874, 1713, 1660, 1568, 1459, 1407 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{46}\text{H}_{79}\text{NO}_{10}\text{Na}^+$ 828.5596, found 828.5578; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{46}\text{H}_{80}\text{NO}_{10}^+$ 806.5777, found 806.5771.

4.2.5. Compound 5. Yield: 45 mg, 82%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.30 in 66% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.23 (dd, J = 10.7, 5.6 Hz, 1H), 6.03 (d, J = 10.8 Hz, 1H), 4.21 (q, J = 6.7 Hz, 1H), 4.04 (d, J = 10.4 Hz, 1H), 3.70 (dd, J = 11.1, 4.8 Hz, 1H), 3.62–3.49 (m, 2H), 3.29 (dd, J = 11.9, 2.1 Hz, 1H), 2.76 (d, J = 5.6 Hz, 1H), 2.71–2.60 (m, 3H), 2.60–2.53 (m, 1H), 2.48 (ddd, J = 11.3, 7.5, 6.4 Hz, 1H), 2.02–1.96 (m, 2H), 1.90–0.50 (m, 64H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.4, 184.1, 130.0, 122.6, 110.1, 99.7, 89.2, 76.5, 76.3, 75.5, 75.1, 71.8, 70.2, 67.7, 56.1, 55.8, 51.6, 50.9, 48.6, 40.7, 39.5, 37.5, 36.5, 33.4, 33.2, 33.0, 31.1, 29.9, 29.8, 28.6, 28.3, 27.5, 24.2, 23.1, 21.4, 20.6, 17.9, 17.4, 16.6, 15.1, 14.4, 13.5, 12.8, 12.4, 11.0, 7.0, 6.8 ppm; FT-IR (KBr) 3292, 2961, 2932, 2873, 1713, 1660, 1567, 1459, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{47}\text{H}_{81}\text{NO}_{10}\text{Na}^+$ 842.5753, found 842.5728; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{47}\text{H}_{82}\text{NO}_{10}^+$ 820.5933, found 820.5926.

4.2.6. Compound 6. Yield: 37 mg, 70%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.31 in 66% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.31 (dd, J = 10.7, 5.6 Hz, 1H), 6.11 (d, J = 10.7 Hz, 1H), 4.29 (q, J = 6.7 Hz, 1H), 4.12 (d, J = 10.3 Hz, 1H), 3.78 (dd, J = 11.0, 4.9 Hz, 1H), 3.64 (dd, J = 10.0, 1.7 Hz, 1H), 3.60 (d, J = 10.1 Hz, 1H), 3.37 (dd, J = 11.9, 1.7 Hz, 1H), 2.84 (d, J = 5.6 Hz, 1H), 2.79–2.69 (m, 3H), 2.68–2.62 (m, 1H), 2.56 (dt, J = 11.3, 6.8 Hz, 1H), 2.08–2.02 (m, 2H), 2.00–0.50 (m, 66H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 217.8, 183.5, 129.4, 122.0, 109.5, 99.1, 88.6, 75.9, 75.7, 74.9, 74.5, 71.2, 69.6, 67.1, 55.5, 55.2, 51.0, 50.3, 48.0, 40.1, 38.9, 36.9, 35.9, 32.8, 32.6, 32.4, 31.7, 30.7, 29.2, 28.0, 27.7, 26.9, 26.8, 23.6, 22.6, 20.8, 20.0, 17.3, 16.8, 16.0, 14.5, 13.8, 12.9, 12.3, 11.8, 10.4, 6.5, 6.2 ppm; FT-IR (KBr) 3297, 2958, 2930, 2872, 2858, 1714, 1668, 1565, 1459, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{48}\text{H}_{83}\text{NO}_{10}\text{Na}^+$ 856.5909, found 856.5889; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{48}\text{H}_{84}\text{NO}_{10}^+$ 834.6090, found 834.6084.

4.2.7. Compound 7. Yield: 60 mg, 85%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.40 in 66% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.31 (dd, J = 10.7, 5.6 Hz, 1H), 6.11 (d, J = 10.8 Hz, 1H), 4.28 (dd, J = 13.5, 6.7 Hz, 1H), 4.12 (d, J = 10.4 Hz, 1H), 3.77 (dd, J = 11.1, 4.7 Hz, 1H), 3.64 (dd, J = 10.1, 2.0 Hz, 1H), 3.59 (d, J = 10.2 Hz, 1H), 3.37 (dd, J = 11.9, 2.1 Hz, 1H), 2.83 (d, J = 5.6 Hz, 1H), 2.79–2.73 (m, 1H), 2.74–2.68 (m, 2H), 2.68–2.61 (m, 1H), 2.55 (ddd, J = 11.3, 7.4, 6.3 Hz, 1H), 2.10–2.03 (m, 2H), 2.00–0.50 (m, 78H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.1, 183.9, 129.7, 122.4, 109.9, 99.5, 89.0, 76.2, 76.0, 75.3, 74.8, 71.6, 70.0, 67.4, 55.8, 55.5, 51.3, 50.6, 48.3, 40.5, 39.2, 37.3, 36.3, 33.2, 32.9, 32.8, 32.3, 31.2, 30.0 (3C), 29.9, 29.7, 29.6, 28.4, 28.1, 27.5, 27.2, 24.0, 23.1, 21.1, 20.3, 17.6, 17.2, 16.3, 14.9, 14.3, 13.3, 12.6, 12.2, 10.8, 6.8, 6.6 ppm, one signal overlapped; FT-IR (KBr) 3288, 2960, 2927, 2854, 1713, 1568, 1459, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{54}\text{H}_{95}\text{NO}_{10}\text{Na}^+$ 940.6848, found 940.6829; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{54}\text{H}_{96}\text{NO}_{10}^+$ 918.7029, found 918.7024.

4.2.8. Compound 8. Yield: 41 mg, 76%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.42 in 33% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.28 (dd, J = 11.0, 1.6 Hz, 1H), 5.98 (dd, J = 11.0, 4.2 Hz, 1H), 4.22 (q, J = 6.7 Hz, 1H), 4.11 (d, J = 10.2 Hz, 1H), 3.79 (dd, J = 11.1, 4.8 Hz, 1H), 3.63 (d, J = 10.1 Hz, 2H), 3.38 (dd, J = 12.0, 2.1 Hz,

2H), 2.75 (td, J = 11.2, 3.3 Hz, 1H), 2.69–2.64 (m, 2H), 2.56–2.43 (m, 3H), 2.31 (dq, J = 13.4, 6.7 Hz, 2H), 2.20–0.50 (m, 61H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.4, 183.7, 125.6, 123.9, 110.9, 98.0, 87.3, 76.1, 75.8, 75.1, 74.9, 71.2, 69.5, 67.1, 56.4, 55.7, 51.0, 50.1, 44.4, 40.4, 38.6, 36.7, 35.9, 33.0, 32.6, 32.4, 29.8, 28.0, 27.6, 26.9 (2C), 23.6, 21.1, 19.9, 17.3, 16.8, 15.7, 14.6, 13.7 (2C), 12.8, 12.2, 12.0, 10.4, 6.5, 6.2 ppm; FT-IR (KBr) 3300, 2962, 2933, 2874, 1714, 1566, 1458, 1405 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{46}\text{H}_{79}\text{NO}_{10}\text{Na}^+$ 828.5596, found 828.5582; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{46}\text{H}_{80}\text{NO}_{10}^+$ 806.5777, found 806.5774.

4.2.9. Compound 9. Yield: 45 mg, 79%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.57 in 33% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.28 (dd, J = 11.0, 1.7 Hz, 1H), 6.00 (dd, J = 11.0, 4.2 Hz, 1H), 4.22 (q, J = 6.8 Hz, 1H), 4.11 (d, J = 10.3 Hz, 1H), 3.79 (dd, J = 11.1, 4.8 Hz, 1H), 3.68–3.59 (m, 2H), 3.37 (dd, J = 12.0, 2.1 Hz, 1H), 3.30 (dd, J = 4.2, 1.8 Hz, 1H), 2.77–2.70 (m, 1H), 2.69–2.63 (m, 2H), 2.52–2.41 (m, 1H), 2.40–2.25 (m, 4H), 2.00–0.50 (m, 65H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 219.0, 184.2, 126.3, 124.4, 111.6, 98.7, 87.9, 76.7, 76.4, 75.6, 75.4, 71.8, 70.0, 67.7, 57.9, 56.3, 53.7 (2C), 51.6, 50.8, 41.1, 39.2, 37.3, 36.5, 33.6, 33.3, 33.0, 30.4, 28.6, 28.2, 27.5, 24.1, 22.1 (2C), 21.7, 20.5, 17.9, 17.5, 16.3, 15.2, 13.4, 12.8, 12.6, 12.1 (2C), 11.0, 7.1, 6.7 ppm; FT-IR (KBr) 3297, 2960, 2933, 2873, 1714, 1566, 1459, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{48}\text{H}_{83}\text{NO}_{10}\text{Na}^+$ 856.5909, found 856.5892; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{48}\text{H}_{84}\text{NO}_{10}^+$ 834.6090, found 834.6089.

4.2.10. Compound 10. Yield: 30 mg, 55%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.59 in 33% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.29 (dd, J = 11.0, 1.7 Hz, 1H), 6.01 (dd, J = 11.0, 4.2 Hz, 1H), 4.20 (q, J = 6.7 Hz, 1H), 4.11 (d, J = 10.2 Hz, 1H), 3.79 (dd, J = 11.1, 4.8 Hz, 1H), 3.66–3.60 (m, 2H), 3.37 (dd, J = 12.0, 2.1 Hz, 1H), 3.30 (dd, J = 4.2, 1.7 Hz, 1H), 2.77–2.64 (m, 3H), 2.51–2.35 (m, 3H), 2.34–2.25 (m, 2H), 2.00–0.50 (m, 69H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.9, 184.2, 126.1, 124.4, 111.6, 98.7, 87.9, 76.9, 76.4, 75.6, 75.3, 71.8, 69.9, 67.6, 57.7, 56.2, 51.6, 51.4 (2C), 50.8, 41.1, 39.2, 37.4, 36.5, 33.6, 33.3, 32.9, 31.5 (2C), 30.2, 28.6, 28.2, 27.5, 24.2, 21.7, 21.2 (2C), 20.5, 17.9, 17.5, 16.3, 15.2, 14.5 (2C), 13.4, 12.9, 12.6, 11.0, 7.1, 6.7 ppm; FT-IR (KBr) 3300, 2960, 2933, 2873, 2860, 1712, 1566, 1457, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{50}\text{H}_{87}\text{NO}_{10}\text{Na}^+$ 884.6222, found 884.6212; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{50}\text{H}_{88}\text{NO}_{10}^+$ 862.6403, found 862.6410.

4.2.11. Compound 11. Yield: 35 mg, 60%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.62 in 33% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.29 (dd, J = 11.0, 1.6 Hz, 1H), 6.01 (dd, J = 11.0, 4.2 Hz, 1H), 4.19 (q, J = 6.7 Hz, 1H), 4.11 (d, J = 10.3 Hz, 1H), 3.79 (dd, J = 11.0, 4.7 Hz, 1H), 3.69–3.60 (m, 2H), 3.37 (dd, J = 12.0, 1.9 Hz, 1H), 3.29 (dd, J = 4.1, 1.6 Hz, 1H), 2.70 (tdd, J = 10.3, 9.3, 3.1 Hz, 3H), 2.45 (ddd, J = 22.3, 13.0, 7.1 Hz, 2H), 2.39–2.25 (m, 3H), 2.00–0.50 (m, 73H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.9, 184.2, 126.1, 124.4, 111.6, 98.7, 87.9, 77.0, 76.4, 75.6, 75.3, 71.8, 69.9, 67.7, 57.8, 56.3, 51.7 (2C), 51.6, 50.8, 41.1, 39.2, 37.4, 36.5, 33.6, 33.3, 32.9, 30.3 (2C), 30.1, 29.0 (2C), 28.6, 28.2, 27.5, 24.2, 23.2 (2C), 21.7, 20.5, 17.9, 17.5, 16.3, 15.2, 14.5 (2C), 13.4, 12.9, 12.6, 11.0, 7.1, 6.7 ppm; FT-IR (KBr) 3297, 2959, 2931, 2872, 2860, 1714, 1567, 1459, 1406 cm^{-1} . HRMS (ESI^+) m/z [$\text{M} + \text{H}$] $^+$ Calcd for $\text{C}_{52}\text{H}_{91}\text{NO}_{10}\text{Na}^+$ 912.6535, found 912.6524; [$\text{M} - \text{Na} + 2\text{H}$] $^+$ Calcd for $\text{C}_{52}\text{H}_{92}\text{NO}_{10}^+$ 890.6716, found 890.6724.

4.2.12. Compound 12. Yield: 50 mg, 82%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; R_f = 0.64 in 33% EtOAc/*n*-hexane. Strain green with PMA; ^1H NMR (400 MHz, CD_2Cl_2) δ 6.29 (dd, J = 11.0, 1.6 Hz, 1H), 6.01 (dd, J = 11.0, 4.2 Hz, 1H), 4.20 (q, J = 6.7 Hz, 1H), 4.12 (d, J = 10.3 Hz, 1H), 3.78 (dt, J = 13.2, 6.5 Hz, 1H), 3.68–3.59 (m, 2H), 3.38 (dd, J = 12.0, 1.9 Hz, 1H), 3.29 (dd, J = 4.1, 1.6 Hz, 1H), 2.72 (ddd, J = 11.4, 8.5, 3.4 Hz, 2H), 2.68–2.63 (m, 1H), 2.52–2.43 (m, 1H), 2.43–2.36 (m, 2H), 2.36–2.27 (m, 2H), 2.00–0.50 (m, 77H) ppm; ^{13}C NMR (101 MHz, CD_2Cl_2) δ 218.9, 184.2, 126.1, 124.4, 111.6, 98.7, 87.9, 77.0, 76.4, 75.6, 75.3, 71.8, 69.9, 67.7, 57.8, 56.3, 51.7 (2C), 51.6, 50.8, 41.1, 39.2, 37.4, 36.5, 33.6, 33.3, 33.0, 32.5 (2C), 30.0, 29.3 (2C), 28.6, 28.2, 27.8 (2C),

27.5, 24.2, 23.4 (2C), 21.7, 20.5, 17.9, 17.6, 16.3, 15.2, 14.5 (2C), 13.5, 12.9, 12.6, 11.0, 7.1, 6.8 ppm; FT-IR (KBr) 3286, 2960, 2931, 2873, 2859, 1713, 1568, 1459, 1407 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₅₄H₉₅NO₁₀Na⁺ 940.6848, found 940.6839; [M - Na + 2H]⁺ Calcd for C₅₄H₉₆NO₁₀⁺ 918.7029, found 918.7038.

4.2.13. Compound 13. Yield: 21 mg, 45%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.79 in 33% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 6.28 (dd, *J* = 11.0, 1.6 Hz, 1H), 6.00 (dd, *J* = 11.0, 4.2 Hz, 1H), 4.20 (q, *J* = 6.6 Hz, 1H), 4.11 (d, *J* = 10.3 Hz, 1H), 3.79 (dd, *J* = 11.0, 4.6 Hz, 1H), 3.71–3.59 (m, 2H), 3.38 (dd, *J* = 12.0, 1.9 Hz, 1H), 3.29 (dd, *J* = 4.1, 1.5 Hz, 1H), 2.75 (dd, *J* = 11.1, 3.2 Hz, 1H), 2.71–2.64 (m, 2H), 2.47 (dd, *J* = 12.5, 3.6 Hz, 1H), 2.43–2.38 (m, 1H), 2.36 (d, *J* = 8.1 Hz, 1H), 2.34–2.25 (m, 2H), 2.00–0.50 (m, 10H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.9, 184.2, 126.1, 124.4, 111.6, 98.7, 87.9, 76.9, 76.4, 75.6, 75.3, 71.7, 69.9, 67.6, 57.7, 56.3, 51.7 (2C), 51.6, 50.8, 41.1 (2C), 39.2, 37.4, 36.5, 33.6, 33.3, 32.9, 32.5 (2C), 30.6, 30.4 (3C), 30.3, 30.2 (4C), 30.0 (3C), 29.9, 29.3, 28.6, 28.2, 28.1 (2C), 27.5, 24.2, 23.3 (2C), 21.7, 20.5, 17.9, 17.6, 16.3, 15.2, 14.5 (2C), 13.4, 12.9, 12.6, 11.0, 7.1, 6.8 ppm; FT-IR (KBr) 3295, 2958, 2926, 2872, 2854, 1714, 1566, 1459, 1405 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₆₆H₁₁₉NO₁₀Na⁺ 1108.8726, found 1108.8715; [M - Na + 2H]⁺ Calcd for C₆₆H₁₂₀NO₁₀⁺ 1086.8907, found 1086.8920.

4.2.14. Compound 14. Yield: 48 mg, 75%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.65 in 50% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 7.19 (d, *J* = 4.4 Hz, 4H), 7.12 (dt, *J* = 5.9, 4.2 Hz, 1H), 6.28 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.09 (d, *J* = 10.7 Hz, 1H), 4.18 (q, *J* = 6.7 Hz, 1H), 4.04 (d, *J* = 10.4 Hz, 1H), 3.83 (d, *J* = 12.7 Hz, 1H), 3.74–3.63 (m, 2H), 3.61–3.50 (m, 2H), 3.26 (dd, *J* = 11.8, 1.8 Hz, 1H), 2.90 (d, *J* = 5.5 Hz, 1H), 2.52–2.65 (m, 1H), 2.64–2.54 (m, 2H), 2.00–0.50 (m, 57H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.4, 184.1, 141.5, 129.3, 128.8 (4C), 127.3, 123.1, 110.0, 99.7, 89.3, 76.5, 76.3, 75.5, 75.0, 71.8, 70.2, 67.7, 56.1, 55.1, 52.5, 51.6, 50.9, 40.7, 39.4, 37.6, 36.5, 33.4, 33.1, 33.0, 29.7, 28.6, 28.2, 27.4, 24.2, 21.3, 20.5, 17.8, 17.4, 16.5, 15.1, 13.5, 12.9, 12.4, 11.0, 7.0, 6.8 ppm; FT-IR (KBr) 3317, 2961, 2933, 2874, 1713, 1567, 1495, 1458, 1406 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₉H₇₇NO₁₀Na⁺ 862.5440, found 862.5432; [M - Na + 2H]⁺ Calcd for C₄₉H₇₈NO₁₀⁺ 840.5620, found 840.5628.

4.2.15. Compound 15. Yield: 43 mg, 76%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.60 in 50% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CD₂Cl₂) δ 7.12 (td, *J* = 7.9, 1.6 Hz, 1H), 6.98 (d, *J* = 6.3 Hz, 1H), 6.77–6.72 (m, 2H), 6.43 (dd, *J* = 10.7, 5.5 Hz, 1H), 6.29 (d, *J* = 10.8 Hz, 1H), 4.29 (q, *J* = 6.7 Hz, 1H), 4.13 (d, *J* = 10.5 Hz, 1H), 4.05 (d, *J* = 13.5 Hz, 1H), 3.97 (d, *J* = 13.4 Hz, 1H), 3.77 (dd, *J* = 11.1, 4.8 Hz, 1H), 3.63 (dd, *J* = 16.2, 6.0 Hz, 2H), 3.38 (dd, *J* = 11.9, 1.9 Hz, 1H), 3.10 (d, *J* = 5.4 Hz, 1H), 2.78 (dd, *J* = 11.0, 2.9 Hz, 1H), 2.73 (dd, *J* = 10.8, 2.4 Hz, 1H), 2.66 (dd, *J* = 10.2, 7.4 Hz, 1H), 2.00–0.50 (m, 58H) ppm; ¹³C NMR (101 MHz, CD₂Cl₂) δ 218.4, 184.3, 158.4, 129.4, 129.1, 127.7, 125.1, 123.5, 119.7, 116.7, 108.8, 99.9, 89.8, 76.5, 76.2, 75.8, 75.0, 71.8, 70.2, 67.8, 56.0, 55.1, 51.6, 51.2, 50.8, 40.5, 39.3, 37.8, 36.5, 33.4, 33.1, 32.9, 29.7, 28.6, 28.3, 27.4, 24.3, 21.4, 20.5, 17.8, 17.4, 16.5, 15.1, 13.5, 12.9, 12.4, 10.9, 7.0, 6.7 ppm; FT-IR (KBr) 3433, 3320, 2953, 2932, 2872, 1713, 1563, 1456, 1428, 1405 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₉H₇₇NO₁₀Na⁺ 878.5389, found 878.5381; [M - Na + 2H]⁺ Calcd for C₄₉H₇₉NO₁₀⁺ 856.5569, found 856.5575.

4.2.16. Compound 16. Yield: 51 mg, 89%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.63 in 50% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.15 (d, *J* = 7.9 Hz, 2H), 7.08 (d, *J* = 8.2 Hz, 2H), 6.49 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.19 (d, *J* = 10.8 Hz, 1H), 4.39 (dd, *J* = 13.8, 6.9 Hz, 1H), 4.24 (d, *J* = 10.3 Hz, 1H), 3.90 (dd, *J* = 11.0, 4.9 Hz, 1H), 3.85 (d, *J* = 12.5 Hz, 1H), 3.72 (dd, *J* = 15.5, 7.2 Hz, 2H), 3.56 (d, *J* = 10.0 Hz, 1H), 3.34–3.29 (m, 1H), 3.03 (d, *J* = 5.7 Hz, 1H), 2.87 (td, *J* = 11.0, 3.3 Hz, 1H), 2.68 (dd, *J* = 11.0, 2.6 Hz, 1H), 2.64–2.59 (m, 1H), 2.31 (s, 3H), 2.20–0.50 (m, 57H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 216.8, 184.1, 137.7, 136.3, 129.0 (2C), 128.2 (2C), 122.6, 109.3, 99.1, 88.7, 75.7, 75.6, 74.8, 74.5, 71.5, 69.8, 67.1, 55.2, 54.6, 51.6, 51.2, 50.4, 40.1, 38.9, 37.2, 36.0, 32.9, 32.5, 32.4, 29.7, 29.0, 27.9 (2C), 26.9, 23.9,

21.1, 20.8, 19.9, 17.5, 17.0, 16.0, 14.6, 13.2, 12.5, 11.8, 10.6, 6.7, 6.5 ppm; FT-IR (KBr) 3295, 2961, 2931, 2874, 1713, 1567, 1458, 1406 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₅₀H₇₉NO₁₁Na⁺ 876.5596, found 876.5593; [M - Na + 2H]⁺ Calcd for C₅₀H₈₀NO₁₁⁺ 854.5777, found 854.5777.

4.2.17. Compound 17. Yield: 70 mg, 88%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.67 in 50% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.22 (dd, *J* = 8.5, 5.6 Hz, 2H), 6.95 (dd, *J* = 12.0, 5.3 Hz, 2H), 6.48 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.20 (d, *J* = 10.8 Hz, 1H), 4.37 (q, *J* = 6.6 Hz, 1H), 4.23 (d, *J* = 10.3 Hz, 1H), 3.89 (dd, *J* = 11.1, 4.9 Hz, 1H), 3.85 (d, *J* = 11.9 Hz, 1H), 3.74–3.68 (m, 2H), 3.56 (d, *J* = 10.1 Hz, 1H), 3.30 (dd, *J* = 11.9, 1.9 Hz, 1H), 3.00 (d, *J* = 5.6 Hz, 1H), 2.87 (td, *J* = 10.9, 3.1 Hz, 1H), 2.67 (dt, *J* = 7.1, 3.6 Hz, 1H), 2.62 (dd, *J* = 10.2, 7.4 Hz, 1H), 2.20–0.50 (m, 57H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 216.8, 184.1, 161.9 (d, *J*_{C-F} = 191.9 Hz, 1C), 136.4, 129.9 (d, *J*_{C-F} = 6.2 Hz, 1C), 128.8, 122.8, 115.1 (d, *J*_{C-F} = 20.2 Hz, 1C), 109.3, 99.1, 88.8, 75.7, 75.7, 74.9, 74.5, 71.6, 69.8, 67.2, 55.3, 54.3, 51.3, 51.0, 50.4, 40.1, 38.9, 37.3, 36.0, 34.1, 32.9, 32.5, 32.4, 29.0, 27.9, 26.9, 24.0, 22.3, 20.9, 20.0, 17.5, 17.0, 16.0, 14.6, 14.1, 13.2, 12.5, 11.8, 10.6, 6.7, 6.5 ppm; FT-IR (KBr) 3321, 2962, 2932, 2874, 1713, 1567, 1510, 1459, 1406 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₉H₇₆FNO₁₀Na⁺ 880.5345, found 880.5337; [M - Na + 2H]⁺ Calcd for C₄₉H₇₇FNO₁₀⁺ 858.5526, found 858.5532.

4.2.18. Compound 18. Yield: 34 mg, 58%. Isolated as a white amorphous solid, >95% pure by NMR and a single spot by TLC; *R_f* = 0.58 in 50% EtOAc/*n*-hexane. Strain green with PMA; ¹H NMR (400 MHz, CDCl₃) δ 7.18 (d, *J* = 8.8 Hz, 2H), 7.13 (d, *J* = 8.5 Hz, 2H), 6.42 (dd, *J* = 10.7, 5.6 Hz, 1H), 6.14 (d, *J* = 10.8 Hz, 1H), 4.30 (q, *J* = 6.7 Hz, 1H), 4.17 (d, *J* = 10.2 Hz, 1H), 3.83 (dd, *J* = 10.6, 4.2 Hz, 1H), 3.78 (d, *J* = 12.9 Hz, 1H), 3.68–3.61 (m, 2H), 3.49 (d, *J* = 10.1 Hz, 1H), 3.23 (d, *J* = 11.6 Hz, 1H), 2.93 (d, *J* = 5.6 Hz, 1H), 2.80 (td, *J* = 10.9, 3.1 Hz, 1H), 2.61 (dd, *J* = 11.0, 2.4 Hz, 1H), 2.55 (dd, *J* = 10.2, 7.4 Hz, 1H), 2.20–0.50 (m, 57H) ppm; ¹³C NMR (101 MHz, CDCl₃) δ 216.7, 184.2, 139.1, 132.5, 129.7 (2C), 128.7, 128.4 (2C), 122.8, 109.2, 99.0, 88.7, 75.7, 75.6, 74.9, 74.5, 71.5, 69.8, 67.1, 55.2, 54.3, 51.2, 51.0, 50.4, 40.1, 38.8, 37.3, 35.9, 32.9, 32.5, 32.4, 29.7, 28.9, 27.9 (2C), 23.9, 20.8, 19.9, 17.4, 17.0, 16.0, 14.6, 13.2, 12.5, 11.8, 10.6, 6.7, 6.5 ppm; FT-IR (KBr) 3322, 2962, 2932, 2874, 1713, 1663, 1567, 1492, 1459, 1407 cm⁻¹. HRMS (ESI⁺) *m/z* [M + H]⁺ Calcd for C₄₉H₇₆ClNO₁₀Na⁺ 896.5050, found 896.5043; [M - Na + 2H]⁺ Calcd for C₄₉H₇₇ClNO₁₀⁺ 874.5231, found 874.5238.

4.3. Cell Culture

HMLER cells naturally repressing E-cadherin, obtained from human mammary epithelial cells infected with a retrovirus carrying hTERT, SV40, and the oncogenic allele *H-ras*V12, were cultured in DMEM/F12 (Gibco, 31331–028) supplemented with 10% FBS, 10 μg/mL insulin (Sigma-Aldrich, I0516), 0.5 μg/mL hydrocortisone (Sigma-Aldrich, H0888), and 0.5 μg/mL puromycin (Life Technologies, A11138-02); cells were a generous gift from Alain Puisieux (INSERM). All cells were incubated at 37 °C with 5% CO₂. HMLER CD24^{low}/CD44^{high} cells stained with CD24-APC and CD44-PE antibodies were sorted by FACS using an Aria IIu (BD Biosciences) to obtain isolated CD24^{low}/CD44^{high} and CD24^{high}/CD44^{low} cell populations. HMLER CD24^{low}/CD44^{high} cells were supplemented with 10 ng/mL human epidermal growth factor (EGF, Miltenyi Biotec, 130-093-750, 100 ng/mL), while HMLER CD24^{high}/CD44^{low} cells were grown without EGF. MCF10A cells (ATCC, CRL-10317) were cultured in DMEM/F12 supplemented with 10% horse serum (Invitrogen, 16050-122), 10 μg/mL insulin, 10 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma-Aldrich, C8052), and 1 × PenStrep (Invitrogen, 15070-063).

4.4. Cell Viability Assay (IC₅₀)

The cell viability assay was carried out by plating 1000 cells per well in 96-well plates. The cells were treated for 72 h in a range between 12 nM and 50 μM or 0.3 nM and 4 μM using serial dilutions following the manufacturer's protocol. Very briefly, the CellTiter-Blue reagent (G8081, Promega) was added to the wells after 72 h of treatment, and cells were incubated for 3 h before fluorescence intensities (*λ*_{ex} =

560/20 nm; λ_{em} = 590/10 nm) were recorded using a PerkinElmer Wallac 1420 Victor2 microplate reader.

The IC₅₀ cell viability curves were plotted using the Prism 8 software for the synthesized compounds against HMLER CD24^{low}/CD44^{high} and the isogenic cell line HMLER CD24^{high}/CD44^{low}; MCF10A cells are included in the Supporting Information (Figures S55–S56).

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsorginorgau.1c00046>.

Detailed descriptions of general procedures, equipment, and measurement parameters; ¹H NMR, ¹³C NMR, and HRMS spectra of new C20-*epi*-amino analogs of salinomycin (**1–18**); and IC₅₀ cell viability curves (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Michał Antoszczak – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France; Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, 61–614 Poznań, Poland; orcid.org/0000-0003-0877-1726; Phone: +48 61 829 1754; Email: michant@amu.edu.pl

Raphaël Rodriguez – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France; orcid.org/0000-0001-7668-446X; Phone: +33 648 482 191; Email: raphael.rodriguez@curie.fr

Authors

Dominika Czerwonka – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France; Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, 61–614 Poznań, Poland

Sebastian Müller – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France

Tatiana Cañeque – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France; orcid.org/0000-0002-1110-0643

Ludovic Colombeau – Department of Chemical Biology Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université, 75005 Paris, France

Adam Huczyński – Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, 61–614 Poznań, Poland

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acsorginorgau.1c00046>

Author Contributions

R.R. and A.H. directed the research. D.C. designed and synthesized the derivatives. T.C. did the stability tests. D.C., M.A., and S.M. evaluated the compounds *in vitro*. M.A., D.C., and R.R. wrote the article with contributions from S.M., T.C., L.C., and A.H.

Funding

This research was funded by the European Research Council under the European Union's Horizon 2020 research and

innovation program Grant 647973 (R.R.), the Foundation Charles Defforey-Institut de France (R.R.), Ligue Contre le Cancer (R.R., Equipe Labelisée), and Region IdF for NMR infrastructure (R.R.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

D.C. wishes to acknowledge the Polish Science Center (NCN) for the doctoral scholarship ETIUDA (2020/36/T/ST4/00041) and scholarship no. POWR.03.02.00e00-I026/16, cofinanced by the European Union through the European Social Fund under the Operational Program Knowledge Education Development. M.A. thanks the NCN and the Polish National Agency for Academic Exchange (NAWA) for scholarships under the UWERTURA (2019/32/U/ST4/00092) and the BEKKER programs (PPN/BEK/2019/1/00034), respectively, and the Polish Ministry of Science and Higher Education (MNiSW) for the scholarship for outstanding young scientists in the years 2020–2023 (STYP/15/1665/E-336/2020).

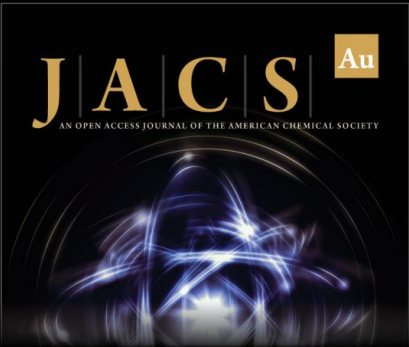
■ ABBREVIATIONS

aq., aqueous
ATP, adenosine triphosphate
CSCs, cancer stem cells
DIAD, diisopropyl azodicarboxylate
DMAP, 4-dimethylaminopyridine
DPPA, diphenylphosphoryl azide
equiv, equivalent(s)
ER, endoplasmic reticulum
ESI, electrospray ionization
EtOAc, ethyl acetate
FT-IR, Fourier-transform infrared spectroscopy
HRMS, high-resolution mass spectroscopy
MeOH, methanol
MoA, mechanism of action
NMR, nuclear magnetic resonance
PMA, phosphomolybdic acid hydrate
ROS, reactive oxygen species
RT, room temperature
Sal, salinomycin
Sal-*epi*NH₂, C20-*epi*-aminosalinomycin
SAR, structure–activity relationship
SI, selectivity index
TBAF, tetrabutylammonium fluoride
TCFH, chloro-*N,N,N',N'*-tetramethylformamidine hexafluorophosphate
THF, tetrahydrofuran
TLC, thin-layer chromatography
TMSEtOH, 2-(trimethylsilyl)ethanol
TPP, triphenylphosphine.

■ REFERENCES

- (1) World Health Organization. WHO Report on Cancer, 2020. <https://www.who.int/publications/i/item/who-report-on-cancer-setting-priorities-investing-wisely-and-providing-care-for-all> (accessed 2021-10-07).
- (2) Clevers, H. The cancer stem cell: Premises, promises and challenges. *Nat. Med.* **2011**, *17*, 313–319.
- (3) Battle, E.; Clevers, H. Cancer stem cells revisited. *Nat. Med.* **2017**, *23*, 1124–1134.
- (4) Reya, T.; Morrison, S. J.; Clarke, M. F.; Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **2001**, *414*, 105–111.

- (5) Kaiser, J. The cancer stem cell gamble. *Science* **2015**, *347*, 226–229.
- (6) Gupta, P. B.; Onder, T. T.; Jiang, G.; Tao, K.; Kuperwasser, C.; Weinberg, R. A.; Lander, E. S. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **2009**, *138*, 645–659.
- (7) Antoszczak, M. A medicinal chemistry perspective on salinomycin as a potent anticancer and anti-CSCs agent. *Eur. J. Med. Chem.* **2019**, *164*, 366–377.
- (8) Versini, A.; Saier, L.; Sindikubwabo, F.; Müller, S.; Cañeque, T.; Rodriguez, R. Chemical biology of salinomycin. *Tetrahedron* **2018**, *74*, 5585–5614.
- (9) Jangamreddy, J. R.; Ghavami, S.; Grabarek, J.; Kratz, G.; Wiechec, E.; Fredriksson, B. A.; Rao Pariti, R. K.; Ciešlar-Pobuda, A.; Panigrahi, S.; Los, M. J. Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: Differences between primary and cancer cells. *Biochim. Biophys. Acta Mol. Cell Res.* **2013**, *1833*, 2057–2069.
- (10) Boehmerle, W.; Endres, M. Salinomycin induces calpain and cytochrome c-mediated neuronal cell death. *Cell Death Dis.* **2011**, *2*, e168.
- (11) Kim, S. H.; Choi, Y. J.; Kim, K. Y.; Yu, S. N.; Seo, Y. K.; Chun, S. S.; Noh, K. T.; Suh, J. T.; Ahn, S. C. Salinomycin simultaneously induces apoptosis and autophagy through generation of reactive oxygen species in osteosarcoma U2OS cells. *Biochem. Biophys. Res. Commun.* **2016**, *473*, 607–613.
- (12) Huang, X.; Borgström, B.; Stegmayr, J.; Abassi, Y.; Kruszyk, M.; Leffler, H.; Persson, L.; Albinsson, S.; Massoumi, R.; Scheblykin, I. G.; Hegardt, C.; Oredsson, S.; Strand, D. The molecular basis for inhibition of stemlike cancer cells by salinomycin. *ACS Cent. Sci.* **2018**, *4*, 760–767.
- (13) Antoszczak, M. A comprehensive review of salinomycin derivatives as potent anticancer and anti-CSCs agents. *Eur. J. Med. Chem.* **2019**, *166*, 48–64.
- (14) Li, B.; Wu, J.; Zhang, W.; Li, Z.; Chen, G.; Zhou, Q.; Wu, S. Synthesis and biological activity of salinomycin-hydroxamic acid conjugates. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 1624–1626.
- (15) Borgström, B.; Huang, X.; Chygorin, E.; Oredsson, S.; Strand, D. Salinomycin hydroxamic acids: Synthesis, structure, and biological activity of polyether ionophore hybrids. *ACS Med. Chem. Lett.* **2016**, *7*, 635–640.
- (16) Kuran, D.; Flis, S.; Antoszczak, M.; Piskorek, M.; Huczyński, A. Ester derivatives of salinomycin efficiently eliminate breast cancer cells via ER-stress-induced apoptosis. *Eur. J. Pharmacol.* **2021**, *893*, 173824.
- (17) Czerwonka, D.; Urbaniak, A.; Sobczak, S.; Piña-Oviedo, S.; Chambers, T. C.; Antoszczak, M.; Huczyński, A. Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogues. *ChemMedChem* **2020**, *15*, 236–246.
- (18) Antoszczak, M.; Popiel, K.; Stefańska, J.; Wietrzyk, J.; Maj, E.; Janczak, J.; Michalska, G.; Brzezinski, B.; Huczyński, A. Synthesis, cytotoxicity and antibacterial activity of new esters of polyether antibiotic – salinomycin. *Eur. J. Med. Chem.* **2014**, *76*, 435–444.
- (19) Huczyński, A.; Antoszczak, M.; Kleczewska, N.; Lewandowska, M.; Maj, E.; Stefańska, J.; Wietrzyk, J.; Janczak, J.; Celewicz, L. Synthesis and biological activity of salinomycin conjugates with floxuridine. *Eur. J. Med. Chem.* **2015**, *93*, 33–41.
- (20) Versini, A.; Colombeau, L.; Hienzsche, A.; Gaillet, C.; Retailleau, P.; Debieu, S.; Müller, S.; Cañeque, T.; Rodriguez, R. Salinomycin derivatives kill breast cancer stem cells by lysosomal iron targeting. *Chem. Eur. J.* **2020**, *26*, 7416–7424.
- (21) Mai, T. T.; Hamaï, A.; Hienzsche, A.; Cañeque, T.; Müller, S.; Wicinski, J.; Cabaud, O.; Leroy, C.; David, A.; Acevedo, V.; Ryo, A.; Ginestier, C.; Birnbaum, D.; Charafe-Jauffret, E.; Codogno, P.; Mehrpour, M.; Rodriguez, R. Salinomycin kills cancer stem cells by sequestering iron in lysosomes. *Nat. Chem.* **2017**, *9*, 1025–1033.
- (22) Borgström, B.; Huang, X.; Hegardt, C.; Oredsson, S.; Strand, D. Structure-activity relationships in salinomycin: Cytotoxicity and phenotype selectivity of semi-synthetic derivatives. *Chem. Eur. J.* **2017**, *23*, 2077–2083.
- (23) Borgström, B.; Huang, X.; Pošta, M.; Hegardt, C.; Oredsson, S.; Strand, D. Synthetic modification of salinomycin: Selective O-acylation and biological evaluation. *Chem. Commun.* **2013**, *49*, 9944–9946.
- (24) Huang, M.; Deng, Z.; Tian, J.; Liu, T. Synthesis and biological evaluation of salinomycin triazole analogues as anticancer agents. *Eur. J. Med. Chem.* **2017**, *127*, 900–908.
- (25) Müller, S.; Sindikubwabo, F.; Cañeque, T.; Lafon, A.; Versini, A.; Lombard, B.; Loew, D.; Wu, T. D.; Ginestier, C.; Charafe-Jauffret, E.; Durand, A.; Vallot, C.; Baulande, S.; Servant, N.; Rodriguez, R. CD44 regulates epigenetic plasticity by mediating iron endocytosis. *Nat. Chem.* **2020**, *12*, 929–938.
- (26) Li, Y.; Shi, Q.; Shao, J.; Yuan, Y.; Yang, Z.; Chen, S.; Zhou, X.; Wen, S.; Jiang, Z. X. Synthesis and biological evaluation of 20-epi-amino-20-deoxysalinomycin derivatives. *Eur. J. Med. Chem.* **2018**, *148*, 279–290.
- (27) Shi, Q.; Li, Y.; Bo, S.; Li, X.; Zhao, P.; Liu, Q.; Yang, Z.; Cong, H.; Deng, H.; Chen, M.; Chen, S.; Zhou, X.; Ding, H.; Jiang, Z. X. Discovery of a ^{19}F MRI sensitive salinomycin derivative with high cytotoxicity towards cancer cells. *Chem. Commun.* **2016**, *52*, 5136–5139.
- (28) Zhang, W.; Wu, J.; Li, B.; Xia, J.; Wu, H.; Wang, L.; Hao, J.; Zhou, Q.; Wu, S. Synthesis and biological activity evaluation of 20-epi-salinomycin and its 20-O-acyl derivatives. *RSC Adv.* **2016**, *6*, 41885–41890.
- (29) Czerwonka, D.; Mielczarek-Puta, M.; Antoszczak, M.; Cioch, A.; Struga, M.; Huczyński, A. Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin. *Eur. J. Pharmacol.* **2021**, *908*, 174347.
- (30) Morel, A. P.; Lièvre, M.; Thomas, C.; Hinkal, G.; Ansieau, S.; Puisieux, A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* **2008**, *3*, e2888.
- (31) Elenbaas, B.; Spirio, L.; Koerner, F.; Fleming, M. D.; Zimonjic, D. B.; Donaher, J. L.; Popescu, N. C.; Hahn, W. C.; Weinberg, R. A. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.* **2001**, *15*, 50–65.



JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Editor-in-Chief
Prof. Christopher W. Jones
Georgia Institute of Technology, USA

Open for Submissions

pubs.acs.org/jacsau

ACS Publications
Most Trusted. Most Cited. Most Read.

Supporting Information

Expeditive synthesis of potent C20-*epi*-amino derivatives of salinomycin against cancer stem-like cells

Dominika Czerwonka,^{a,b} Sebastian Müller,^a Tatiana Cañeque,^a Ludovic Colombeau,^a

Adam Huczyński,^b Michał Antoszczak,^{,a,b} and Raphaël Rodriguez^{*,a}*

^a Department of Chemical Biology

Institut Curie, CNRS UMR 3666, INSERM U1143, PSL Université

26 rue d'Ulm, 75005 Paris, France

^b Department of Medical Chemistry

Faculty of Chemistry, Adam Mickiewicz University

Uniwersytetu Poznańskiego 8, 61–614 Poznań, Poland

Index

General procedures	– S2 –
Spectroscopic and spectrometric analysis of newly synthesized compounds	– S3 –
Biological activity studies.....	– S59 –

General procedures

All solvents and chemicals were purchased from commercially available sources and used without further purification, or purified according to Purification of Laboratory Chemicals (Armarego, W.L.F.; Chai, C.L.L. 5th Ed.). Solvents were dried under standard conditions. Salinomycin (**Sal**) technical grade (~10%, Sacox 120) was extracted with CH₂Cl₂, washed with an aqueous solution of H₂SO₄ (15 mM), and purified on silica gel flash chromatography (cyclohexane/EtOAc 100:0 to 50:50). Reactions were monitored by thin layer chromatography (TLC) using precoated silica on aluminum plates from Merck (60F₂₅₄). TLC plates were visualized with UV-light and/or by treatment with phosphomolybdic acid (PMA, 5% in absolute EtOH) and heating. Products were purified using the CombiFlash Rf+ Teledyne Isco system fitted with pre-packed silica gel columns (Interchim).

NMR spectroscopy was performed on Bruker spectrometer. Spectra were run in CD₂Cl₂ or CDCl₃, at 298 K. ¹H NMR were recorded at 400 MHz, and chemical shifts δ are expressed in ppm using the residual non-deuterated solvent signal as internal standard and the coupling constants *J* are specified in Hz. The following abbreviations are used: s, singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; dq, doublet of quartets; ddd, doublet of doublet of doublets; td, triplet of doublets; tdd, triplet of doublet of doublets; q, quartet; m, multiplet. We only reported labile protons that could be clearly identified in the spectra. ¹³C NMR were recorded at 101 MHz, and chemical shifts δ are expressed in ppm using deuterated solvent signal as internal standard.

Infrared spectra in the mid infrared region were recorded for KBr tablets on an IFS 113v FT-IR spectrophotometer (Bruker) equipped with a DTGS detector, and are reported by wavenumbers (cm⁻¹). The spectra were taken at a resolution 2 cm⁻¹, NSS = 64. The Happ-Genzel apodization function was used.

The UPLC mass spectra were recorded on a Waters Acquity H-class equipped with a Photodiode array detector and SQ Detector 2 with a reverse phase column (Aquity UPLC BEH C18 1.7 μ m, 2.1×50 mm). High-resolution mass spectra (HRMS) were recorded on a Thermo Fisher Scientific Q-Exactive Plus equipped with a Robotic TriVersa NanoMate Advion.

Spectroscopic and spectrometric analysis of newly synthesized compounds

List of spectra

Figure S1. The ^1H NMR spectrum of 1 in dichloromethane- d_2	- S5 -
Figure S2. The ^{13}C NMR spectrum of 1 in dichloromethane- d_2	- S6 -
Figure S3. The HRMS spectrum of 1	- S7 -
Figure S4. The ^1H NMR spectrum of 2 in dichloromethane- d_2	- S8 -
Figure S5. The ^{13}C NMR spectrum of 2 in dichloromethane- d_2	- S9 -
Figure S6. The HRMS spectrum of 2	- S10 -
Figure S7. The ^1H NMR spectrum of 3 in dichloromethane- d_2	- S11 -
Figure S8. The ^{13}C NMR spectrum of 3 in dichloromethane- d_2	- S12 -
Figure S9. The HRMS spectrum of 3	- S13 -
Figure S10. The ^1H NMR spectrum of 4 in dichloromethane- d_2	- S14 -
Figure S11. The ^{13}C NMR spectrum of 4 in dichloromethane- d_2	- S15 -
Figure S12. The HRMS spectrum of 4	- S16 -
Figure S13. The ^1H NMR spectrum of 5 in dichloromethane- d_2	- S17 -
Figure S14. The ^{13}C NMR spectrum of 5 in dichloromethane- d_2	- S18 -
Figure S15. The HRMS spectrum of 5	- S19 -
Figure S16. The ^1H NMR spectrum of 6 in dichloromethane- d_2	- S20 -
Figure S17. The ^{13}C NMR spectrum of 6 in dichloromethane- d_2	- S21 -
Figure S18. The HRMS spectrum of 6	- S22 -
Figure S19. The ^1H NMR spectrum of 7 in dichloromethane- d_2	- S23 -
Figure S20. The ^{13}C NMR spectrum of 7 in dichloromethane- d_2	- S24 -
Figure S21. The HRMS spectrum of 7	- S25 -
Figure S22. The ^1H NMR spectrum of 8 in dichloromethane- d_2	- S26 -
Figure S23. The ^{13}C NMR spectrum of 8 in dichloromethane- d_2	- S27 -
Figure S24. The HRMS spectrum of 8	- S28 -
Figure S25. The ^1H NMR spectrum of 9 in dichloromethane- d_2	- S29 -
Figure S26. The ^{13}C NMR spectrum of 9 in dichloromethane- d_2	- S30 -
Figure S27. The HRMS spectrum of 9	- S31 -
Figure S28. The ^1H NMR spectrum of 10 in dichloromethane- d_2	- S32 -
Figure S29. The ^{13}C NMR spectrum of 10 in dichloromethane- d_2	- S33 -
Figure S30. The HRMS spectrum of 10	- S34 -
Figure S31. The ^1H NMR spectrum of 11 in dichloromethane- d_2	- S35 -
Figure S32. The ^{13}C NMR spectrum of 11 in dichloromethane- d_2	- S36 -
Figure S33. The HRMS spectrum of 11	- S37 -
Figure S34. The ^1H NMR spectrum of 12 in dichloromethane- d_2	- S38 -
Figure S35. The ^{13}C NMR spectrum of 12 in dichloromethane- d_2	- S39 -
Figure S36. The HRMS spectrum of 12	- S40 -
Figure S37. The ^1H NMR spectrum of 13 in dichloromethane- d_2	- S41 -
Figure S38. The ^{13}C NMR spectrum of 13 in dichloromethane- d_2	- S42 -
Figure S39. The HRMS spectrum of 13	- S43 -
	- S3 -

Figure S40. The ^1H NMR spectrum of 14 in dichloromethane- d_2	- S44 -
Figure S41. The ^{13}C NMR spectrum of 14 in dichloromethane- d_2	- S45 -
Figure S42. The HRMS spectrum of 14	- S46 -
Figure S43. The ^1H NMR spectrum of 15 in dichloromethane- d_2	- S47 -
Figure S44. The ^{13}C NMR spectrum of 15 in dichloromethane- d_2	- S48 -
Figure S45. The HRMS spectrum of 15	- S49 -
Figure S46. The ^1H NMR spectrum of 16 in chloroform- d	- S50 -
Figure S47. The ^{13}C NMR spectrum of 16 in chloroform- d	- S51 -
Figure S48. The HRMS spectrum of 16	- S52 -
Figure S49. The ^1H NMR spectrum of 17 in chloroform- d	- S53 -
Figure S50. The ^{13}C NMR spectrum of 17 in chloroform- d	- S54 -
Figure S51. The HRMS spectrum of 17	- S55 -
Figure S52. The ^1H NMR spectrum of 18 in chloroform- d	- S56 -
Figure S53. The ^{13}C NMR spectrum of 18 in chloroform- d	- S57 -
Figure S54. The HRMS spectrum of 18	- S58 -

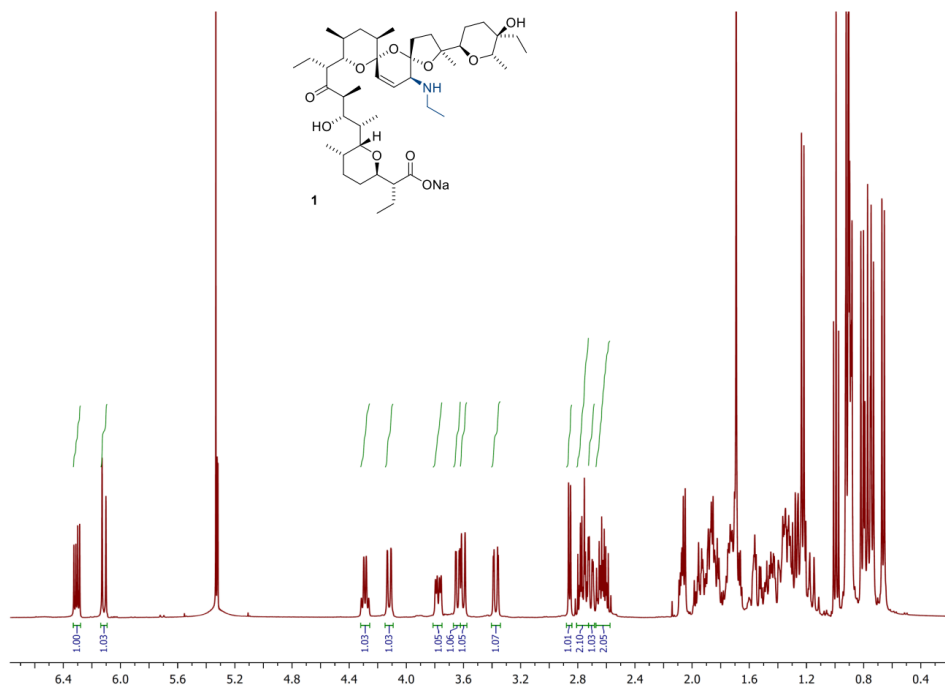


Figure S1. The ^1H NMR spectrum of **1** in dichloromethane- d_2 .

– S5 –

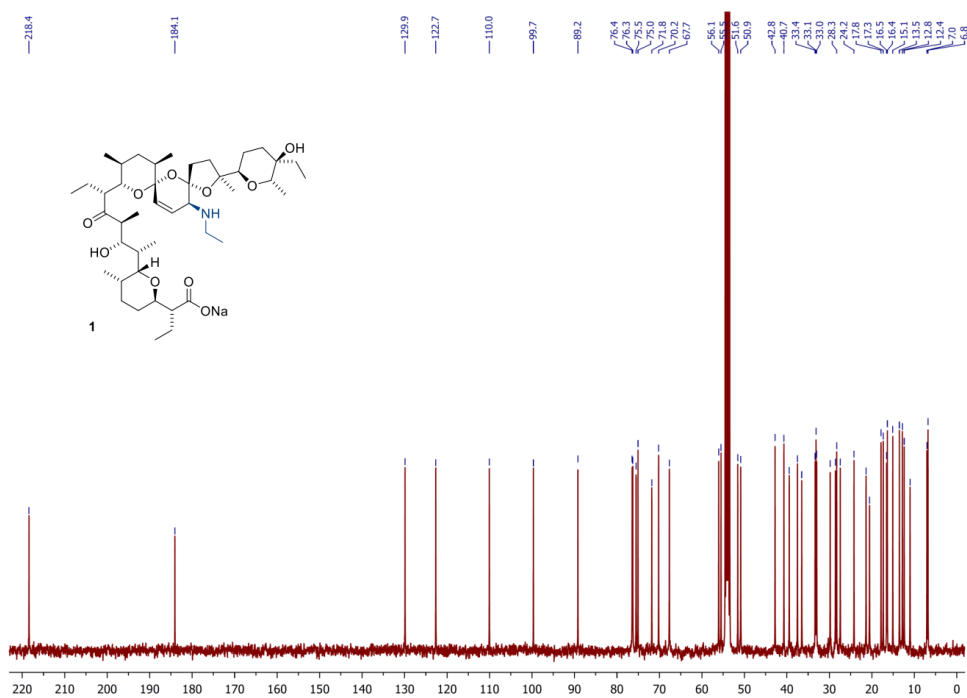


Figure S2. The ^{13}C NMR spectrum of **1** in dichloromethane- d_2 .

– S6 –

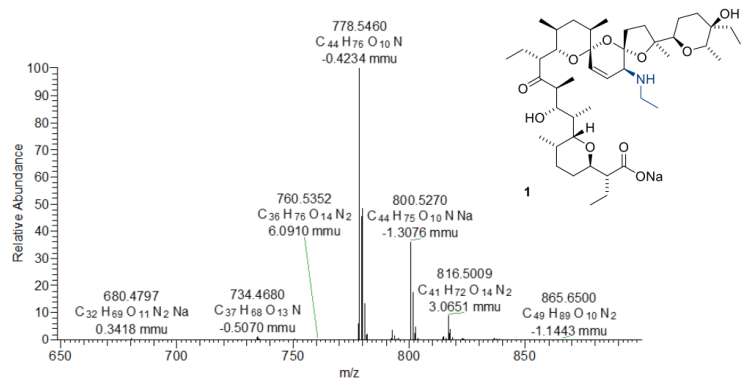


Figure S3. The HRMS spectrum of **1**.

– S7 –

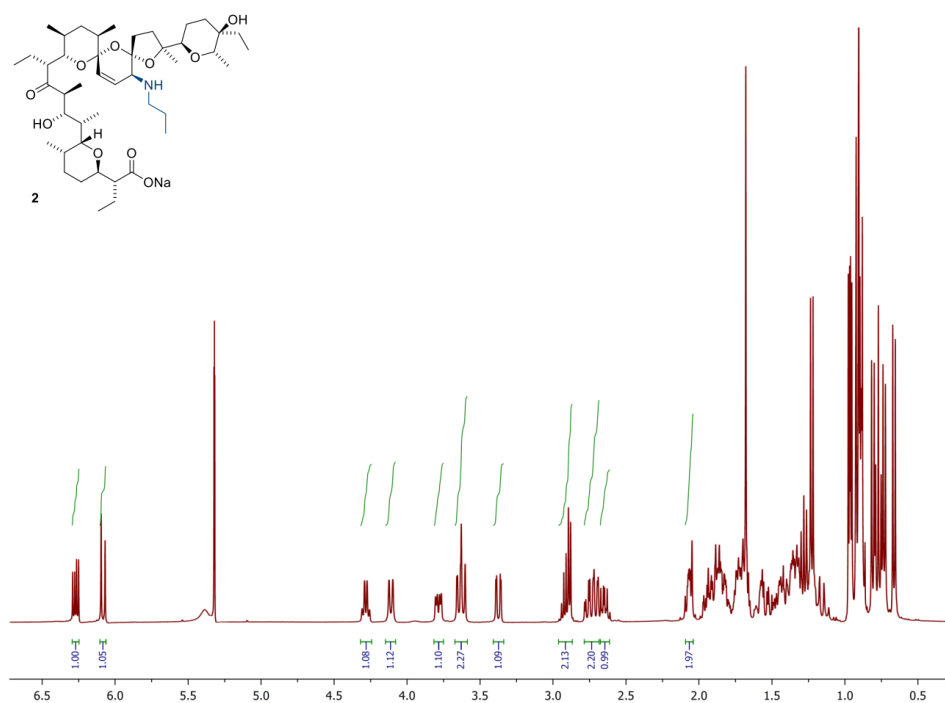


Figure S4. The 1H NMR spectrum of **2** in dichloromethane- d_2 .

– S8 –

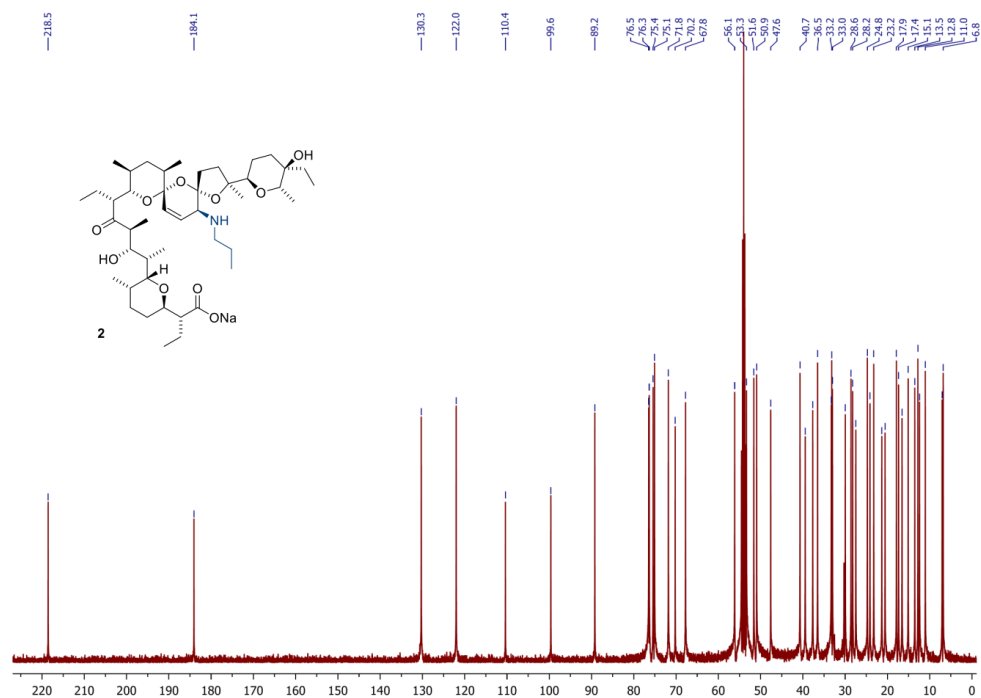


Figure S5. The ^{13}C NMR spectrum of **2** in dichloromethane- d_2 .

– S9 –

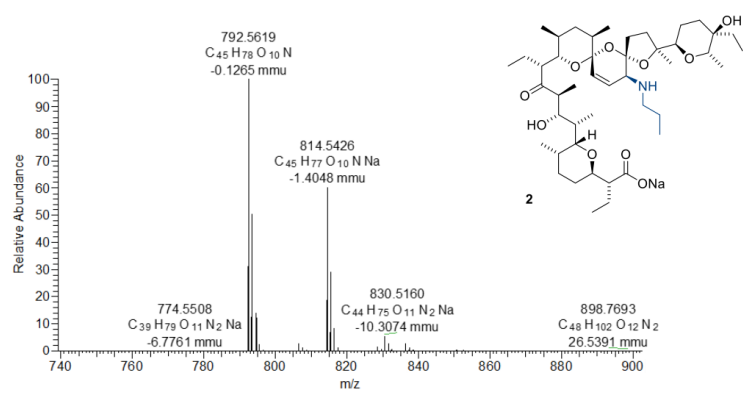


Figure S6. The HRMS spectrum of **2**.

– S10 –

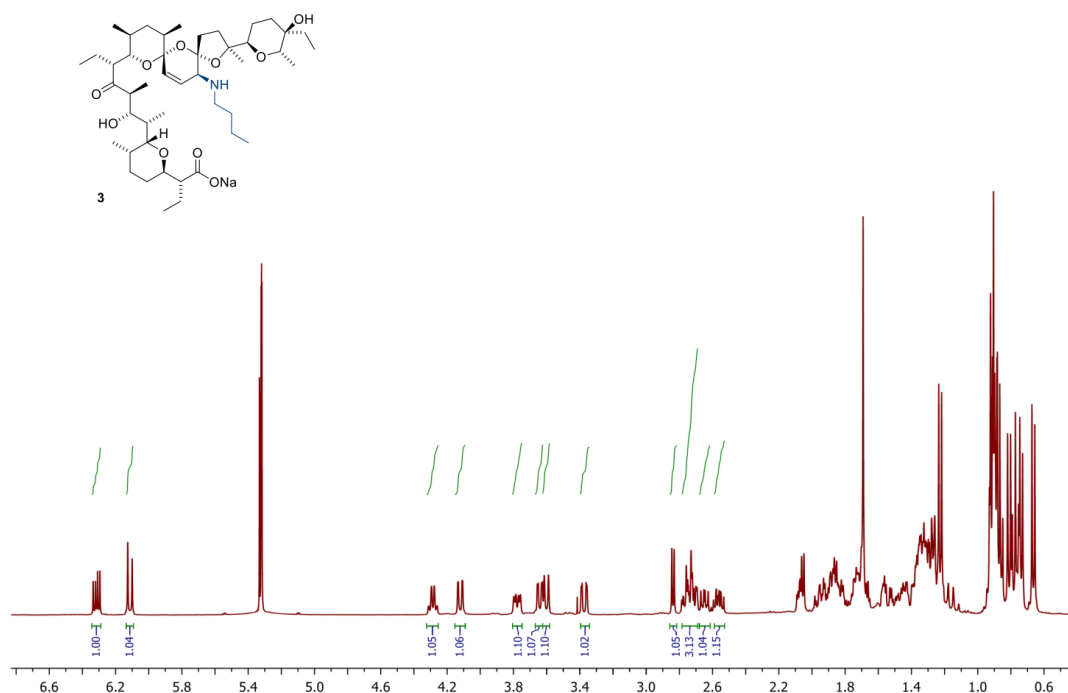


Figure S7. The ^1H NMR spectrum of **3** in dichloromethane- d_2 .

– S11 –

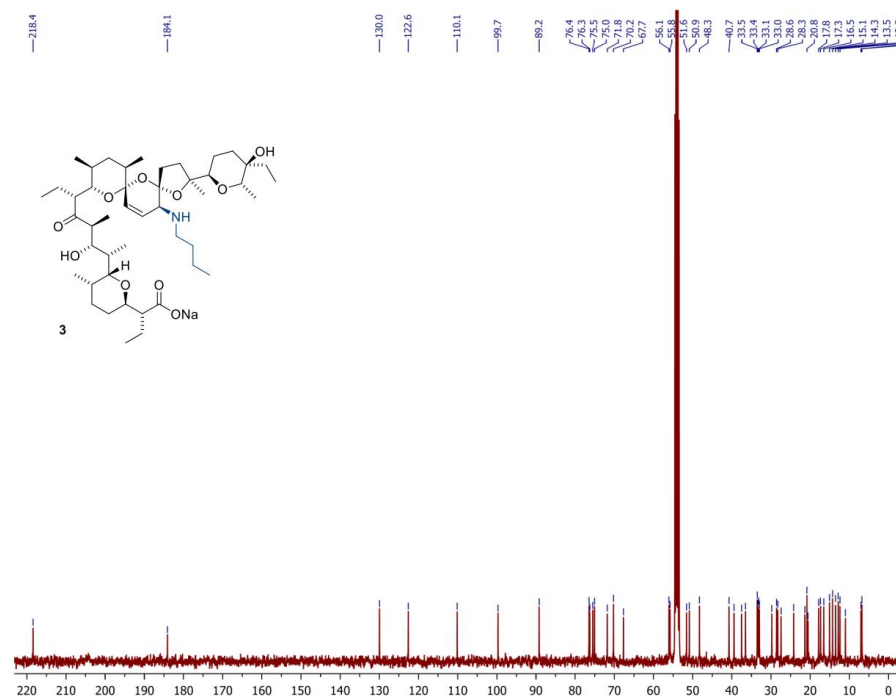


Figure S8. The ^{13}C NMR spectrum of **3** in dichloromethane- d_2 .

– S12 –

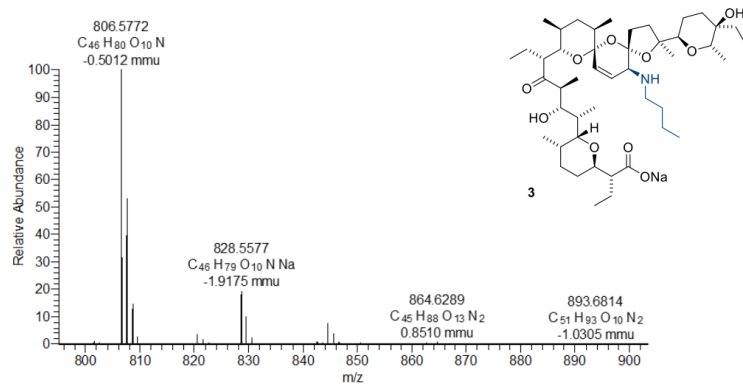


Figure S9. The HRMS spectrum of **3**.

– S13 –

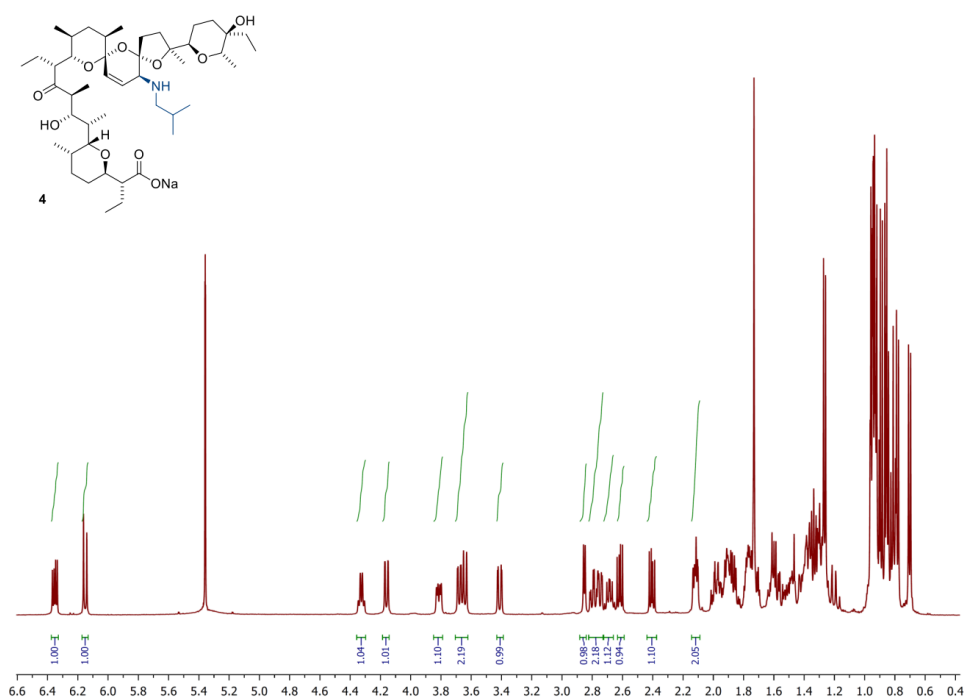


Figure S10. The 1H NMR spectrum of **4** in dichloromethane- d_2 .

– S14 –

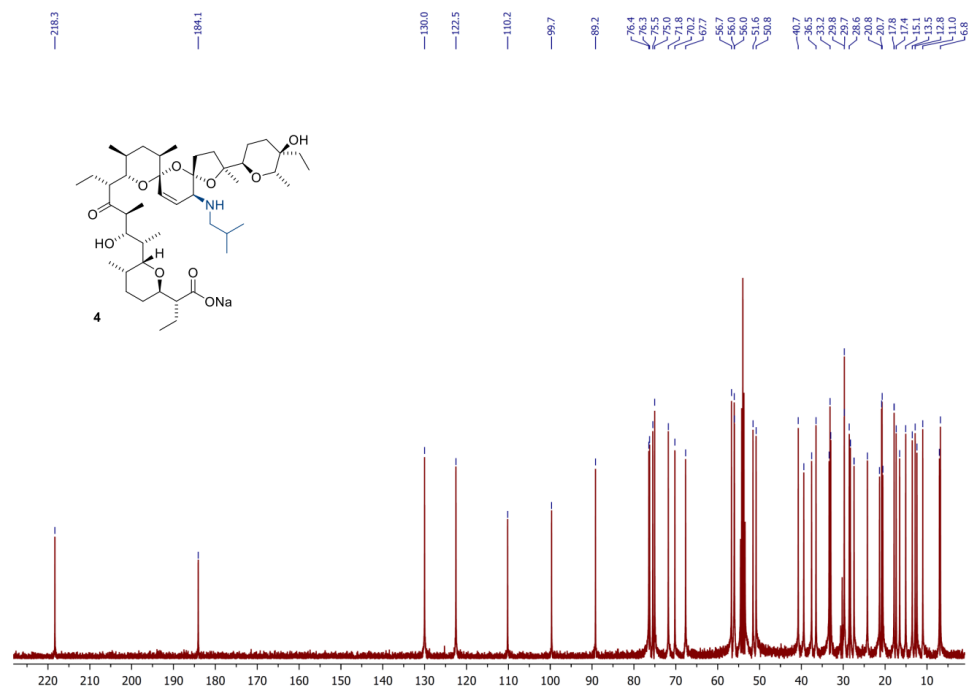


Figure S11. The ^{13}C NMR spectrum of **4** in dichloromethane- d_2 .

– S15 –

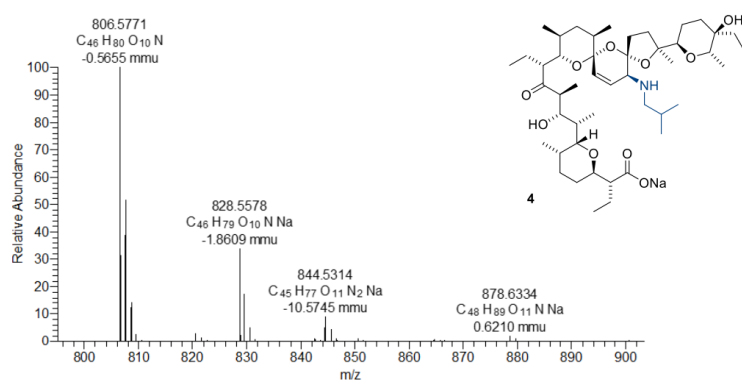


Figure S12. The HRMS spectrum of **4**.

– S16 –

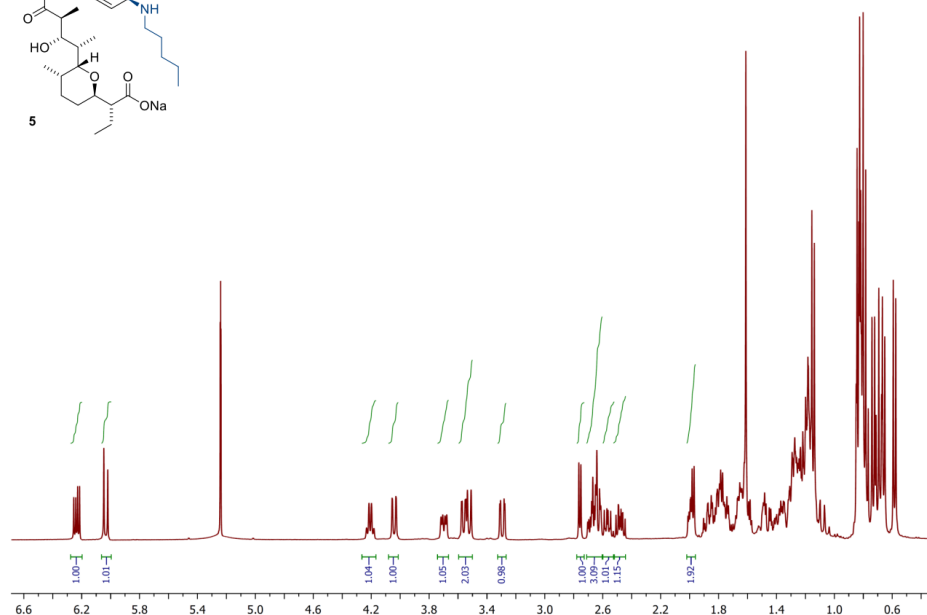
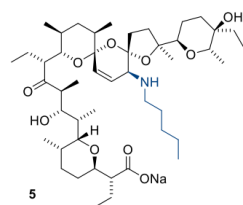


Figure S13. The ^1H NMR spectrum of **5** in dichloromethane- d_2 .

– S17 –

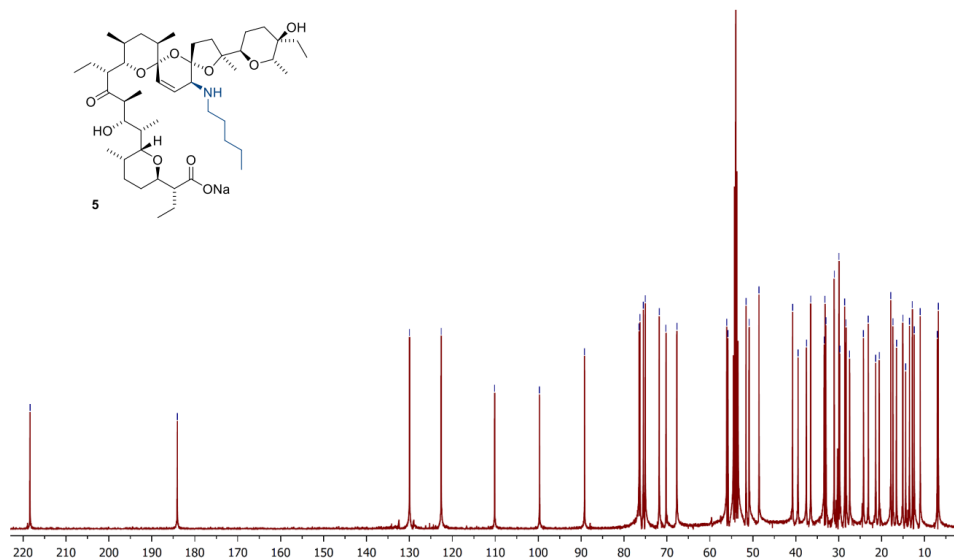
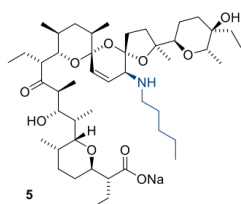


Figure S14. The ^{13}C NMR spectrum of **5** in dichloromethane- d_2 .

– S18 –

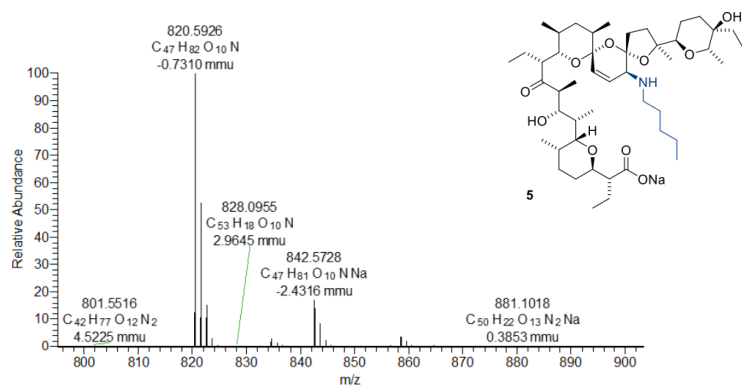


Figure S15. The HRMS spectrum of **5**.

– S19 –

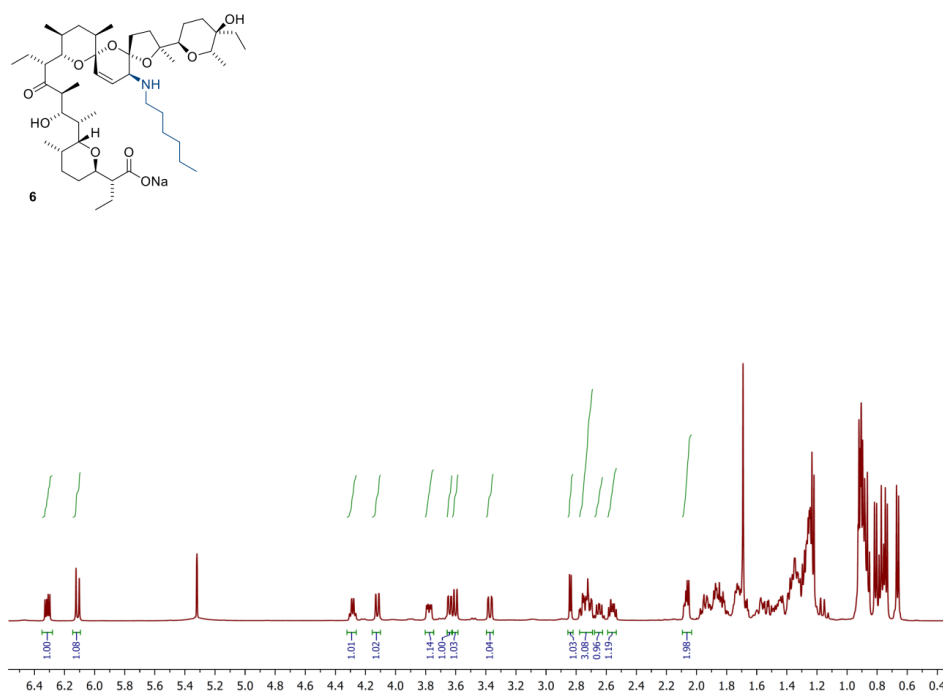


Figure S16. The 1H NMR spectrum of **6** in dichloromethane- d_2 .

– S20 –

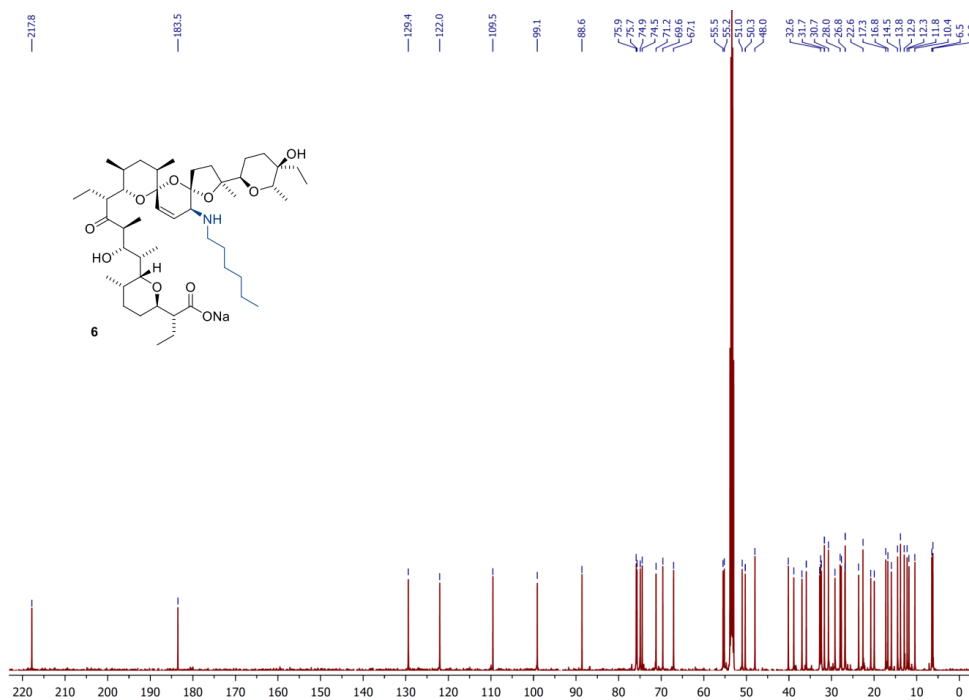


Figure S17. The ^{13}C NMR spectrum of **6** in dichloromethane- d_2 .

– S21 –

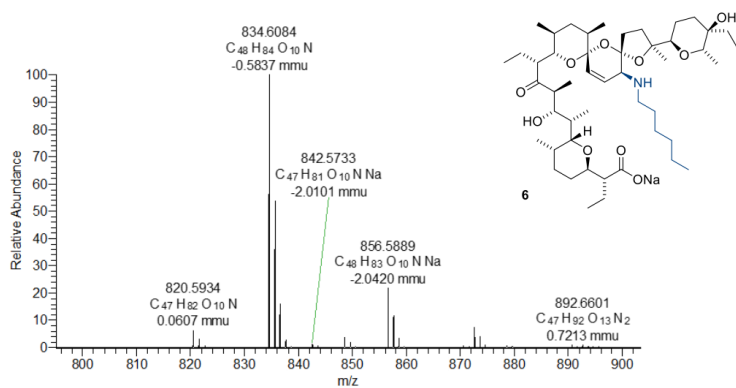


Figure S18. The HRMS spectrum of **6**.

– S22 –

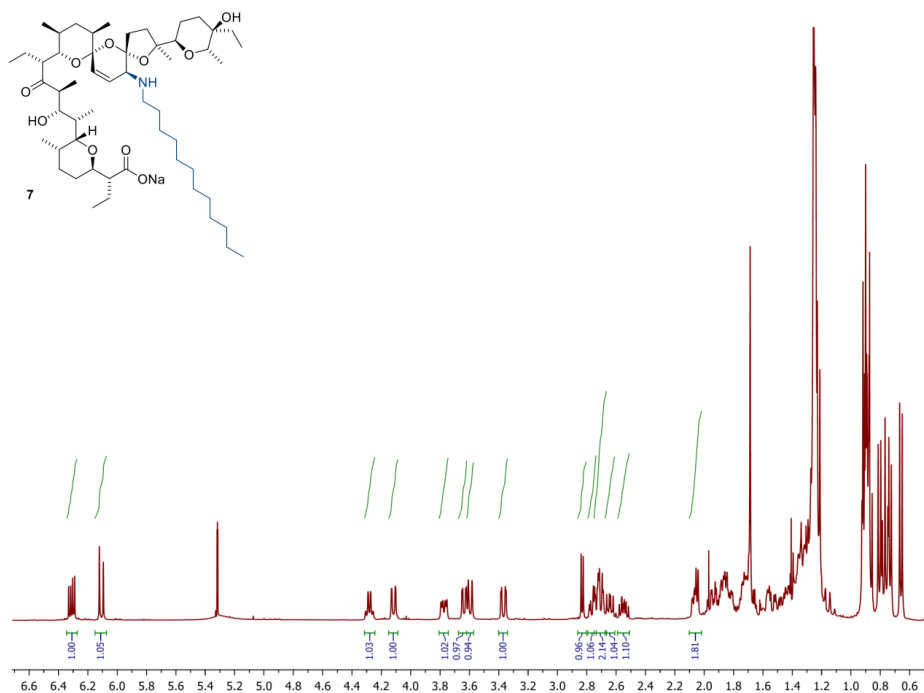


Figure S19. The ^1H NMR spectrum of **7** in dichloromethane- d_2 .

– S23 –

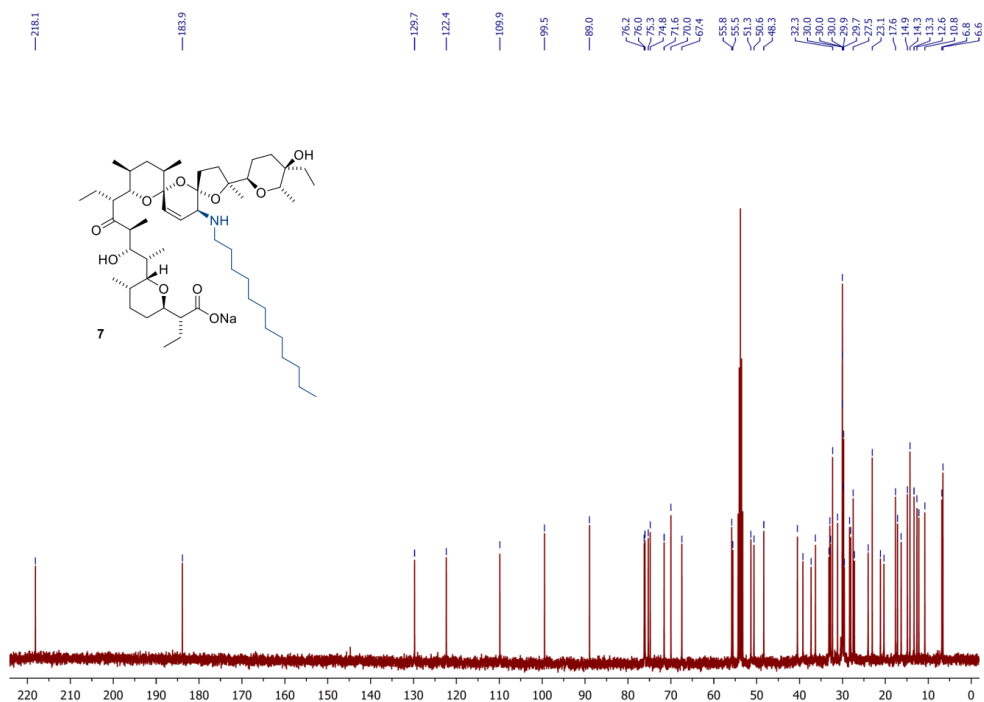


Figure S20. The ^{13}C NMR spectrum of **7** in dichloromethane- d_2 .

– S24 –

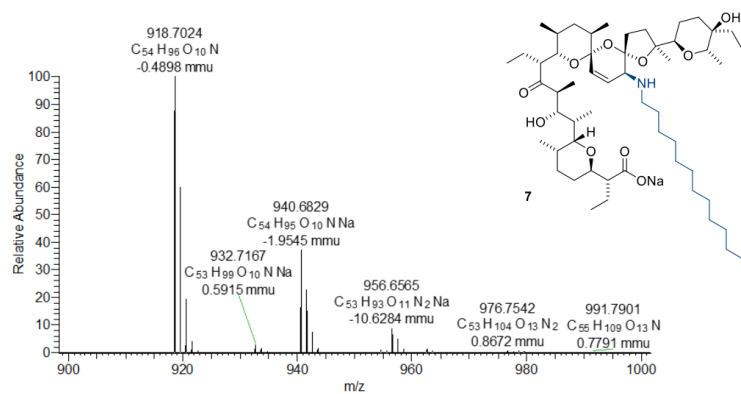


Figure S21. The HRMS spectrum of **7**.

– S25 –

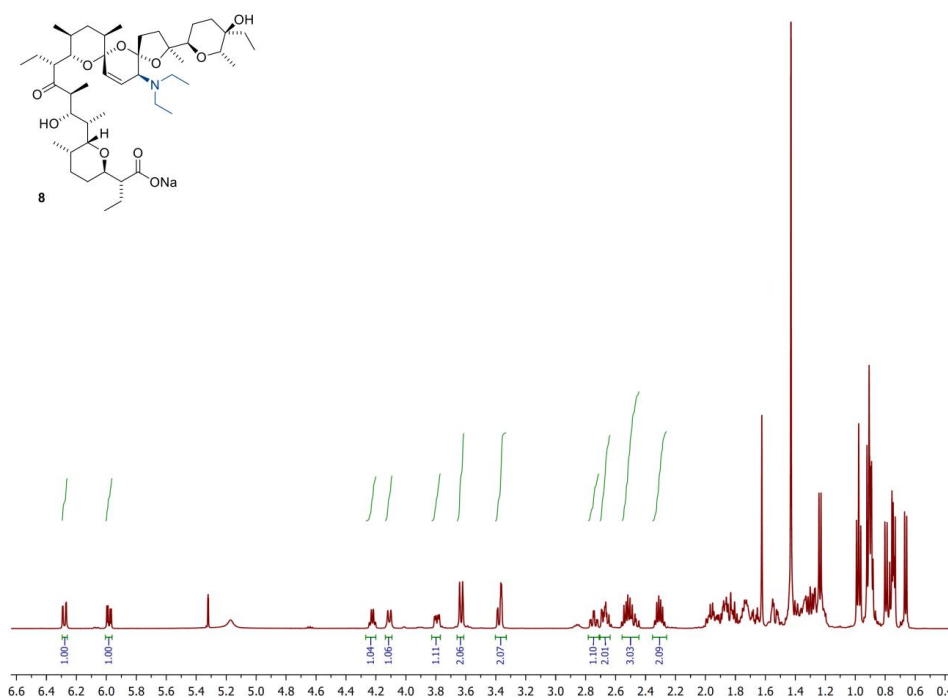
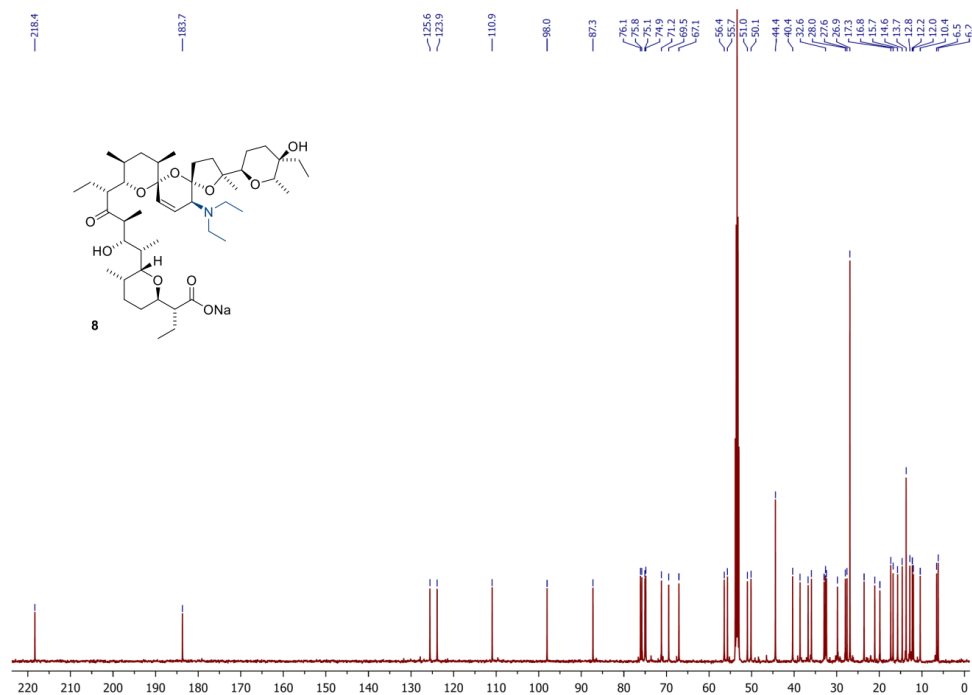
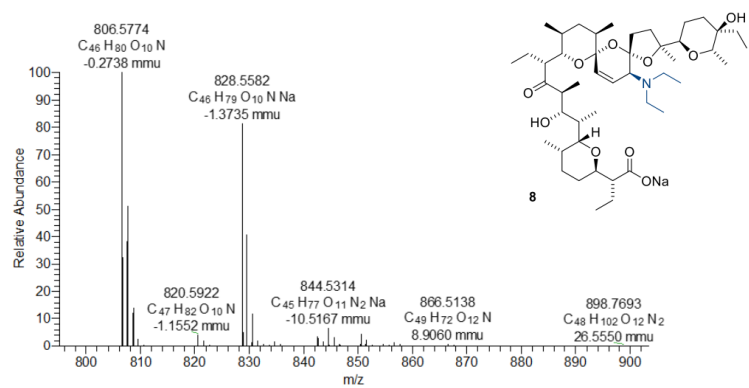


Figure S22. The 1H NMR spectrum of **8** in dichloromethane- d_2 .

– S26 –



- S27 -



– S28 –

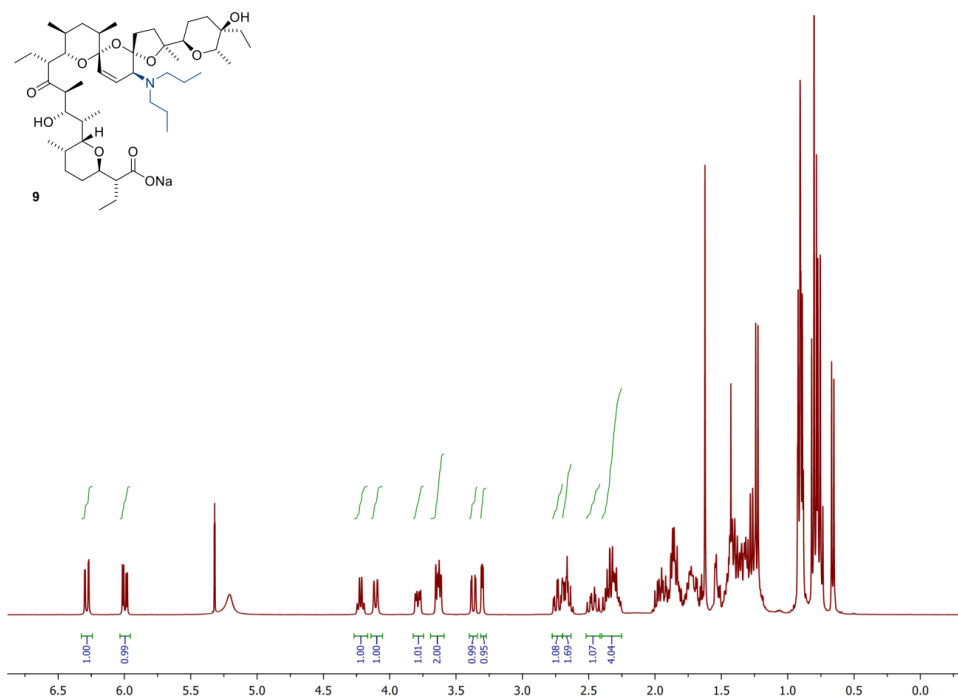


Figure S25. The ^1H NMR spectrum of **9** in dichloromethane- d_2 .

– S29 –

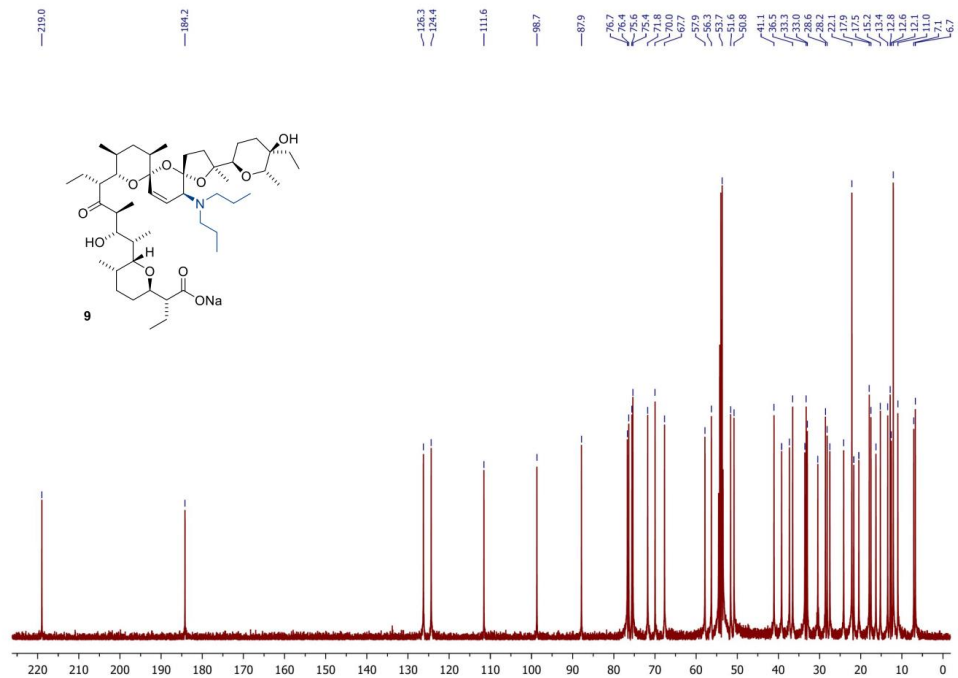


Figure S26. The ^{13}C NMR spectrum of **9** in dichloromethane- d_2 .

– S30 –

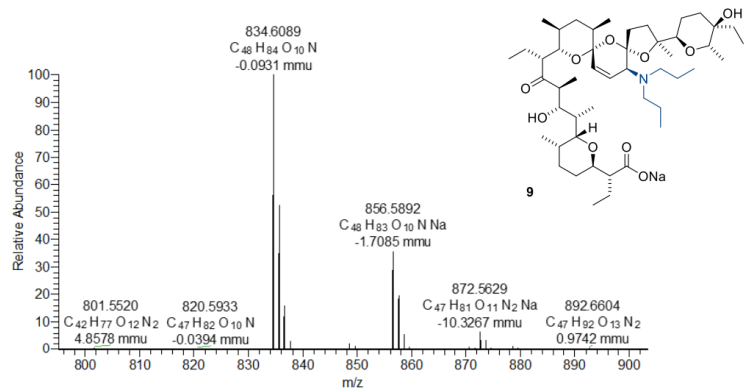


Figure S27. The HRMS spectrum of **9**.

– S31 –

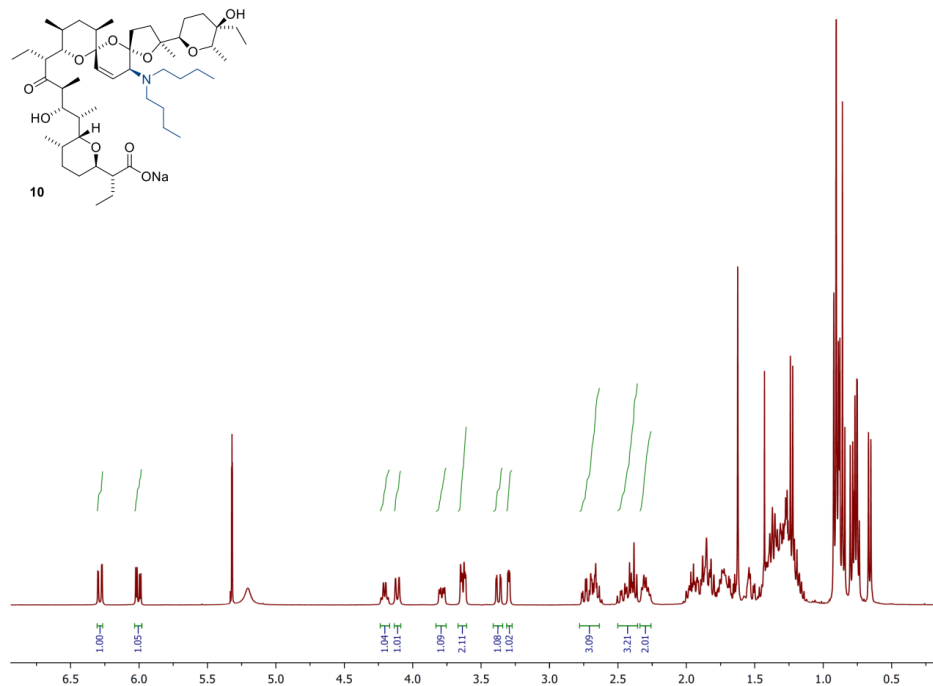


Figure S28. The 1H NMR spectrum of **10** in dichloromethane- d_2 .

– S32 –

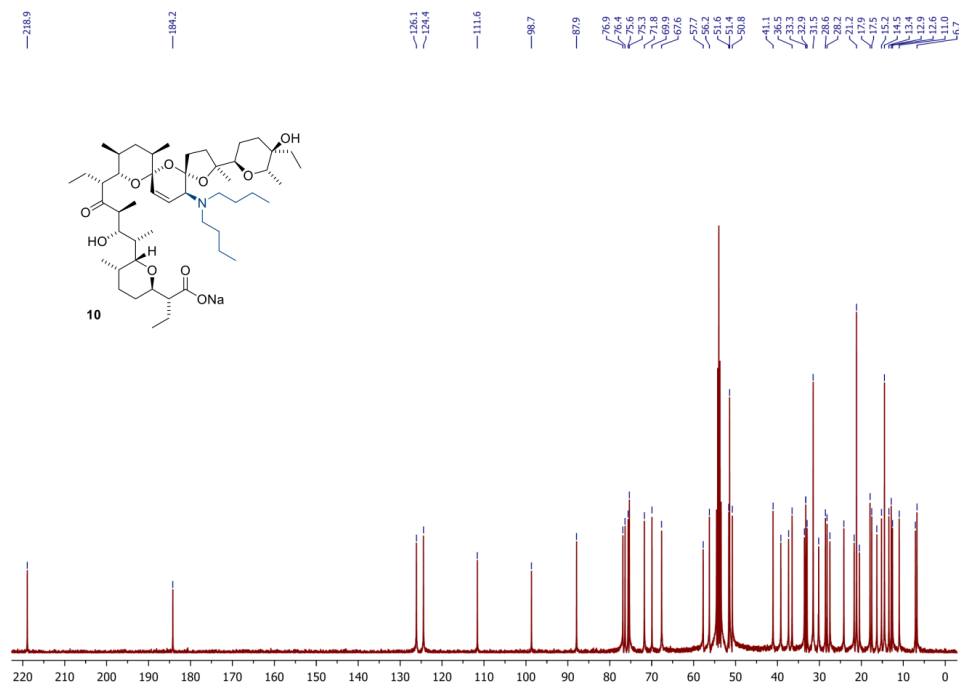


Figure S29. The ¹³C NMR spectrum of **10** in dichloromethane-d₂.

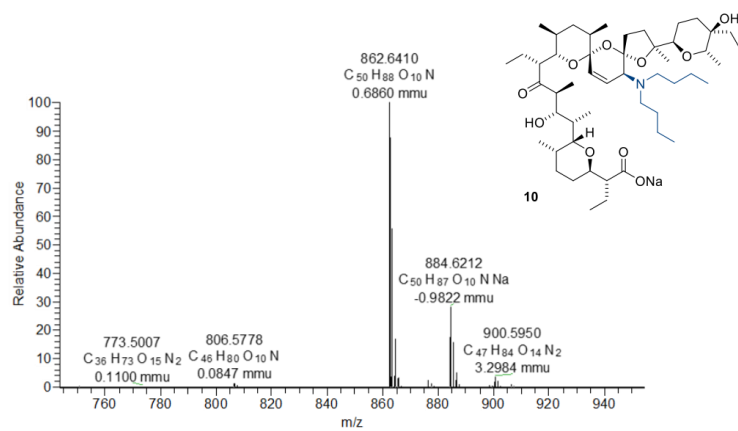


Figure S30. The HRMS spectrum of **10**.

– S33 –

– S34 –

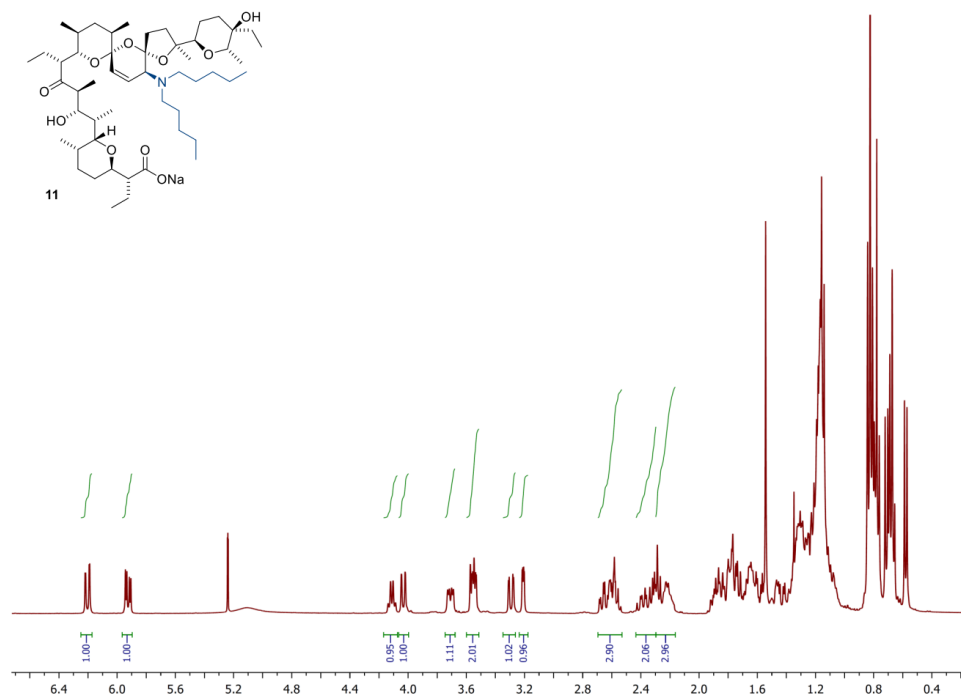


Figure S31. The ^1H NMR spectrum of **11** in dichloromethane- d_2 .

– S35 –

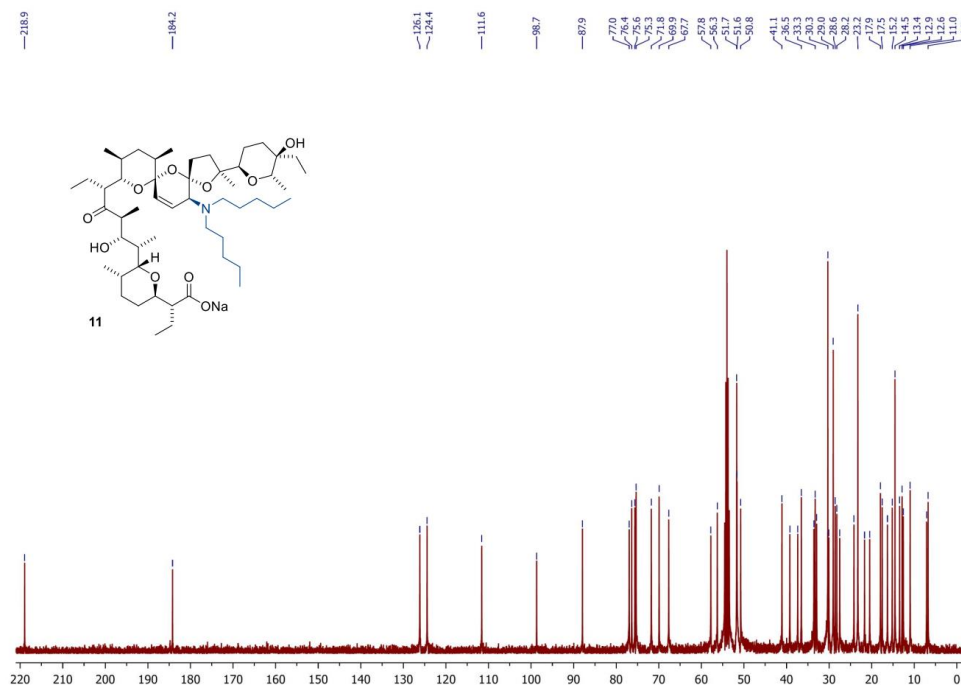


Figure S32. The ^{13}C NMR spectrum of **11** in dichloromethane- d_2 .

– S36 –

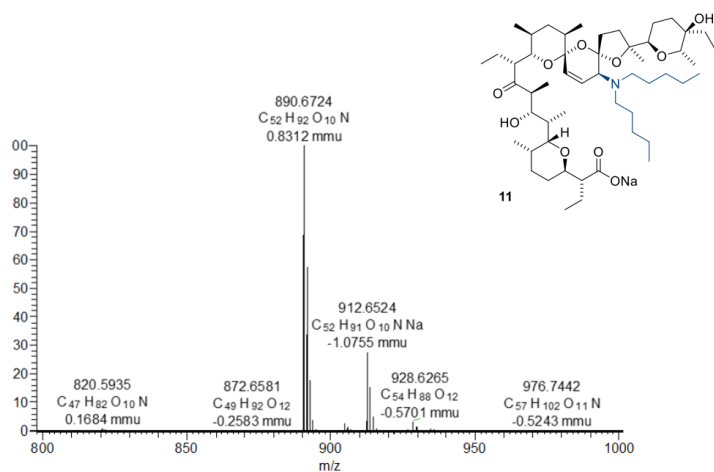


Figure S33. The HRMS spectrum of **11**.

– S37 –

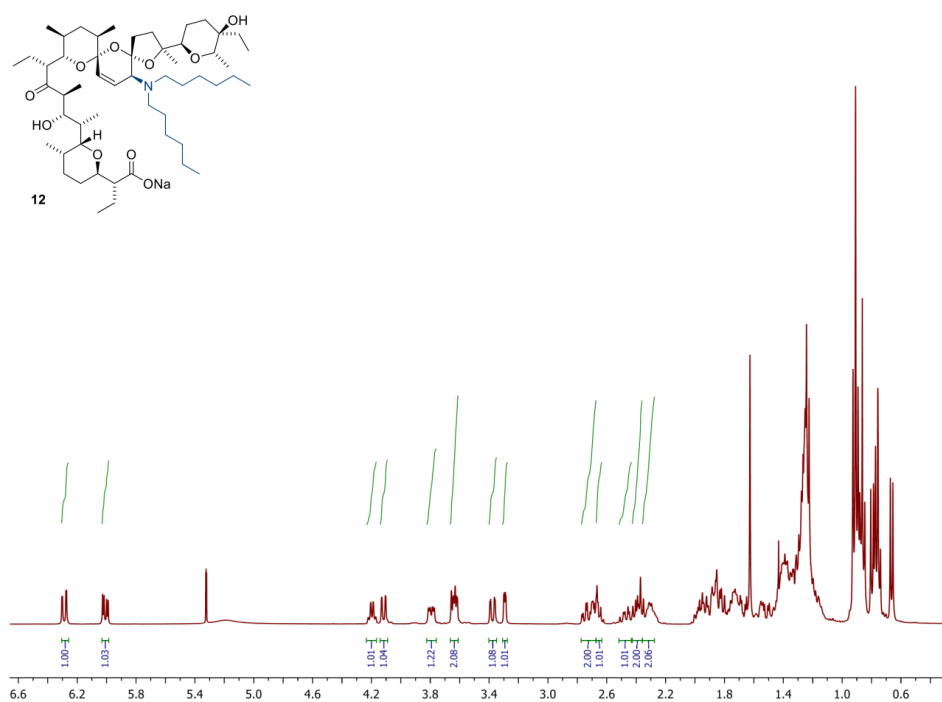


Figure S34. The ¹H NMR spectrum of **12** in dichloromethane-d₂.

– S38 –

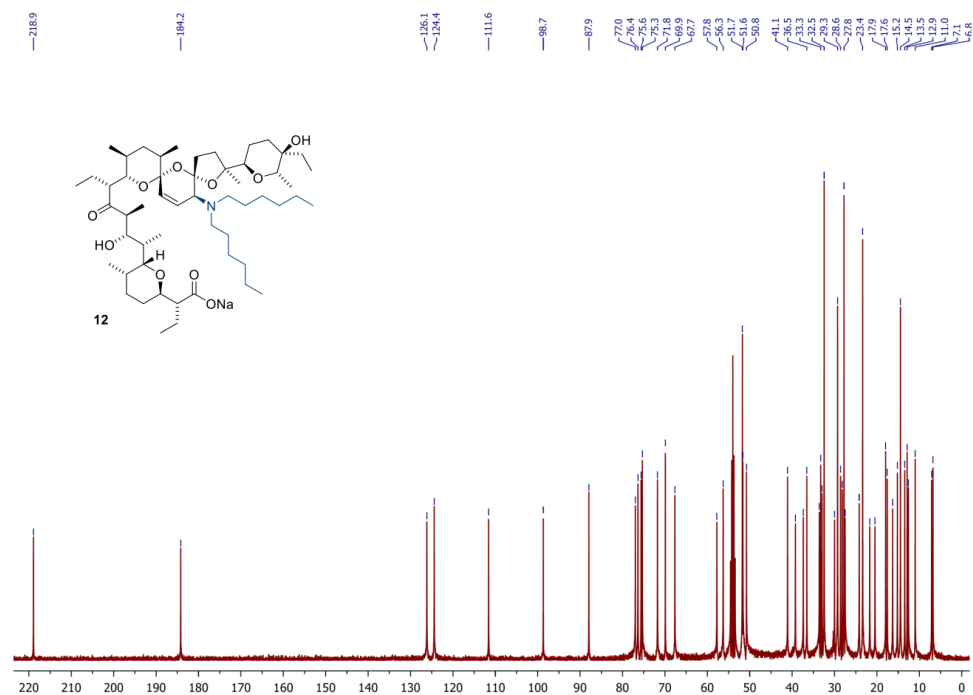


Figure S35. The ^{13}C NMR spectrum of **12** in dichloromethane- d_2 .

– S39 –

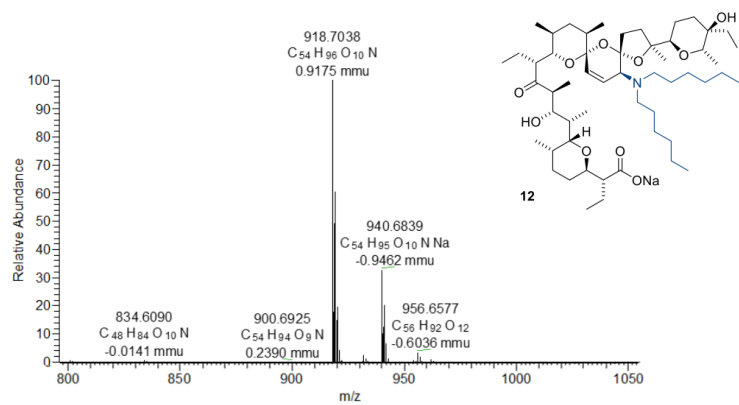


Figure S36. The HRMS spectrum of **12**.

– S40 –

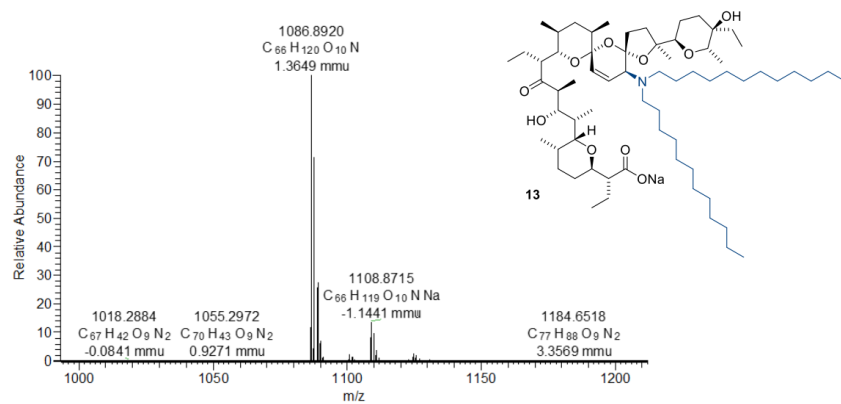


Figure S39. The HRMS spectrum of **13**.

– S43 –

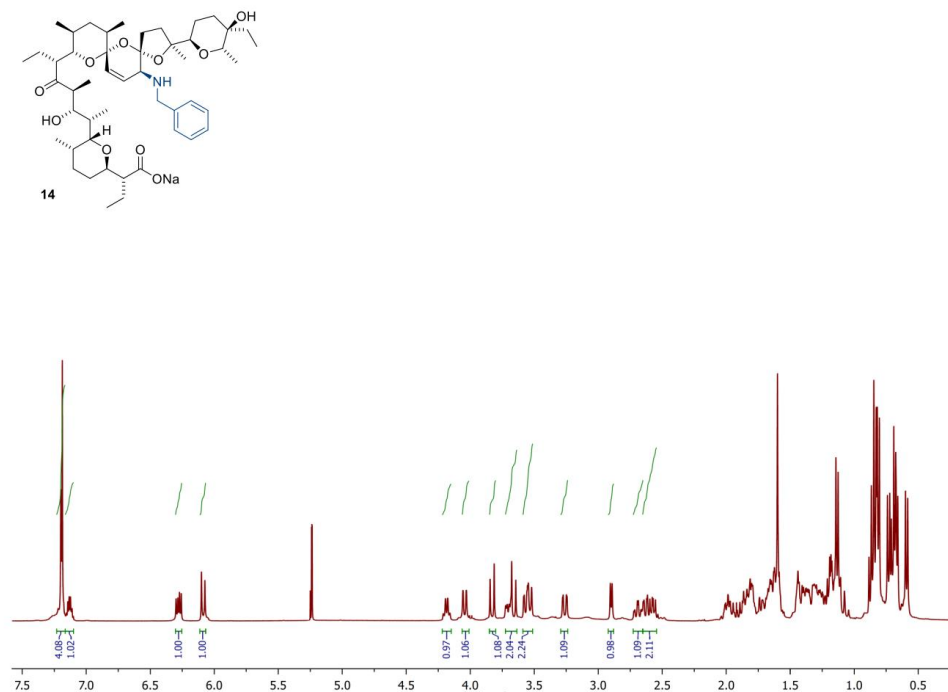


Figure S40. The ¹H NMR spectrum of **14** in dichloromethane-d₂.

– S44 –

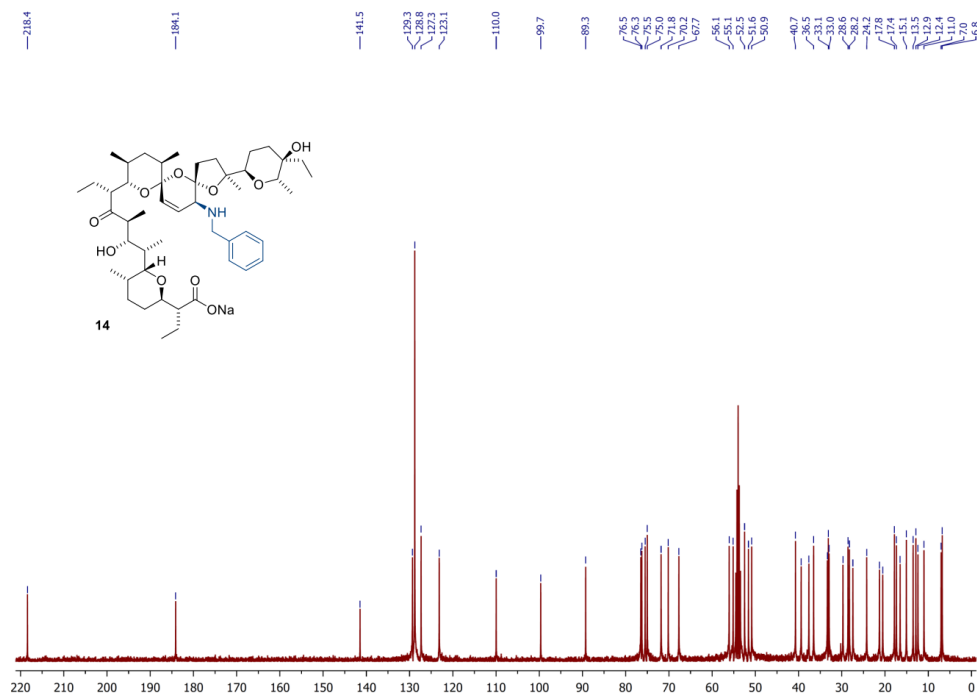


Figure S41. The ^{13}C NMR spectrum of **14** in dichloromethane- d_2 .

– S45 –

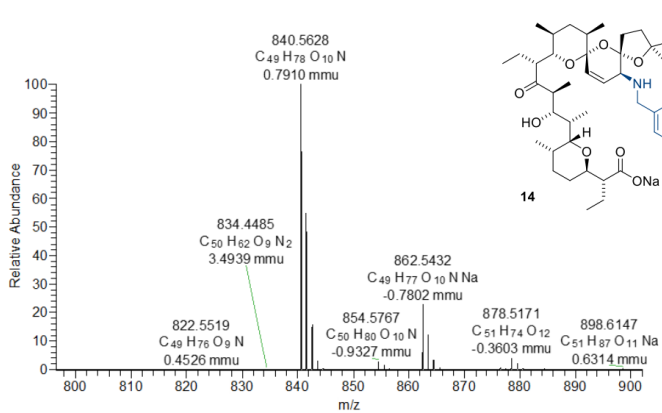


Figure S42. The HRMS spectrum of **14**.

– S46 –

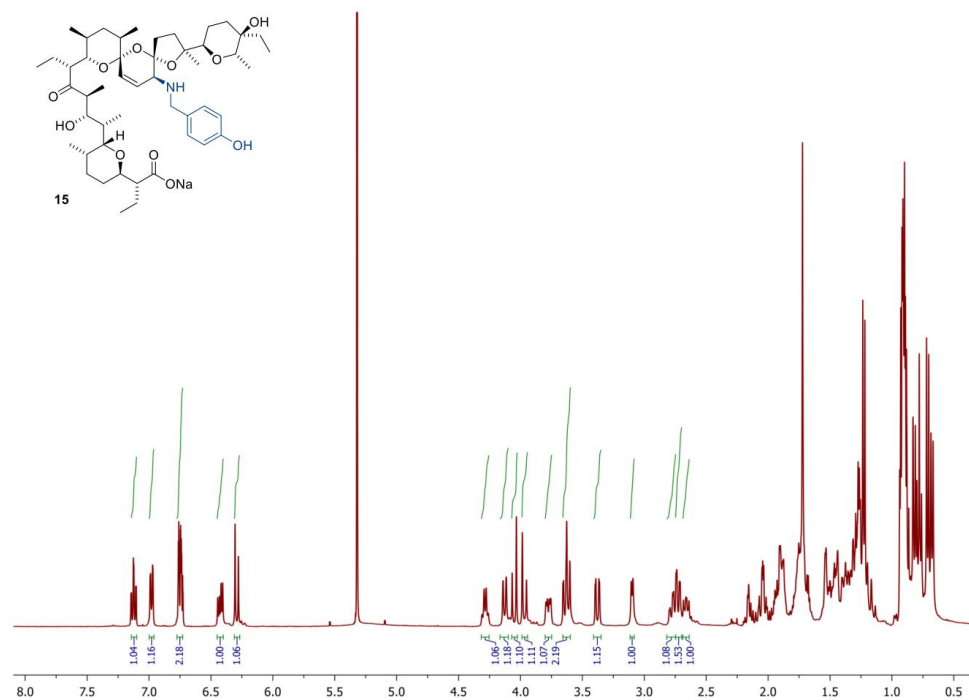


Figure S43. The ^1H NMR spectrum of **15** in dichloromethane- d_2 .

– S47 –

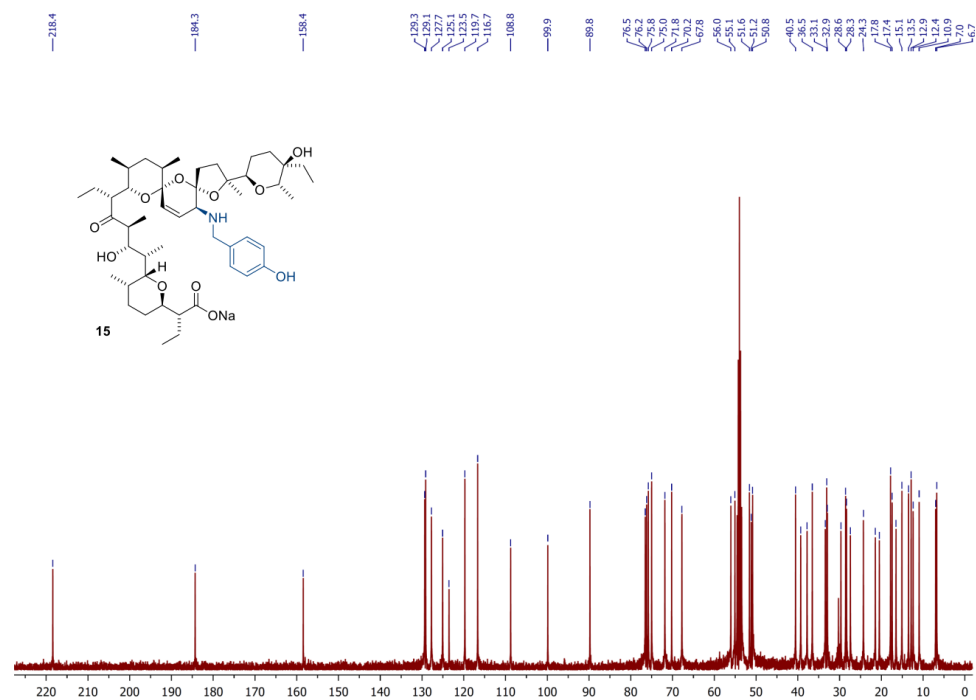


Figure S44. The ^{13}C NMR spectrum of **15** in dichloromethane- d_2 .

– S48 –

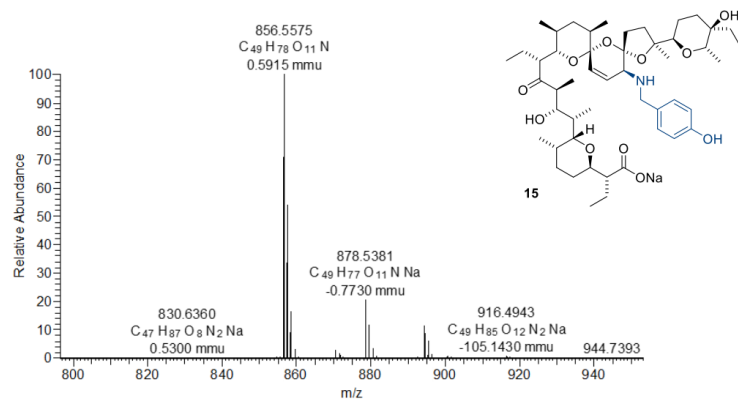


Figure S45. The HRMS spectrum of **15**.

– S49 –

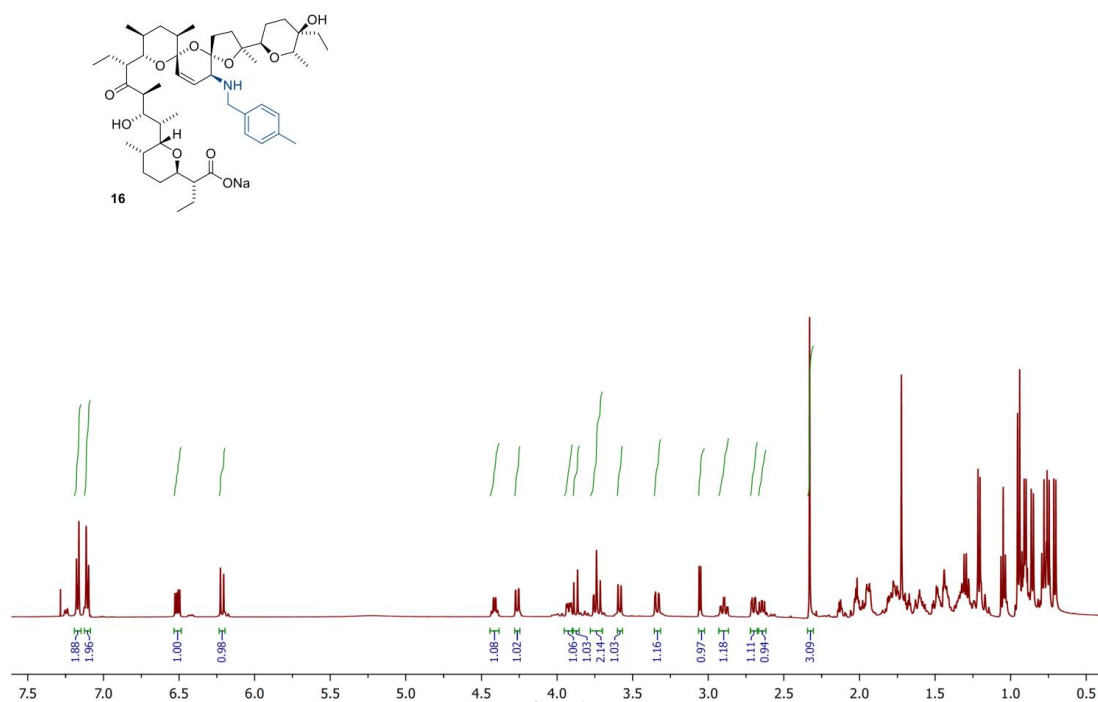


Figure S46. The 1H NMR spectrum of **16** in chloroform- d .

– S50 –

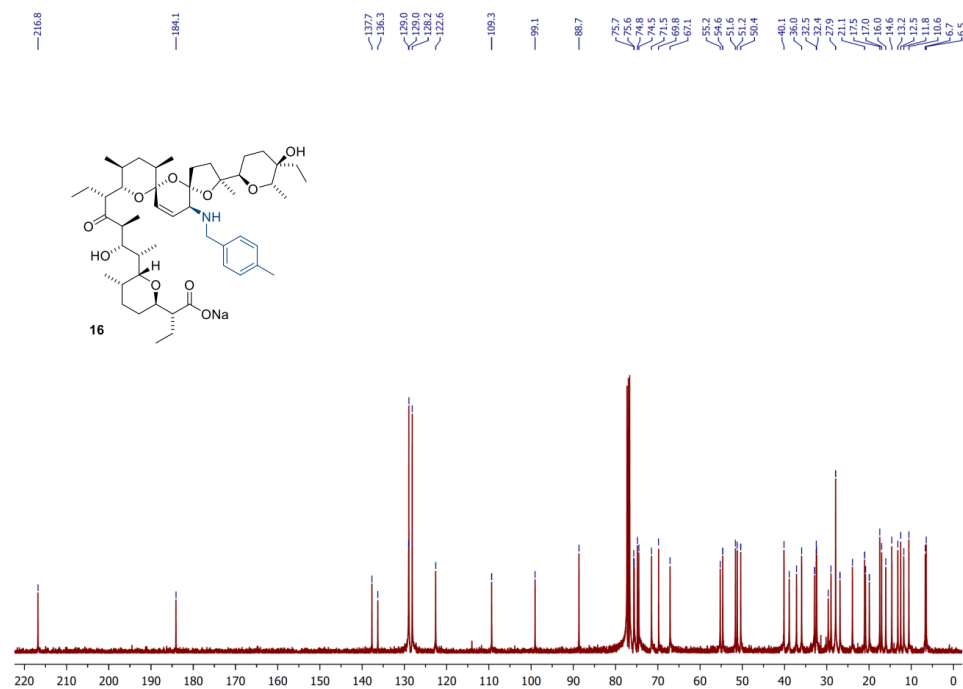


Figure S47. The ^{13}C NMR spectrum of **16** in chloroform- d .

– S51 –

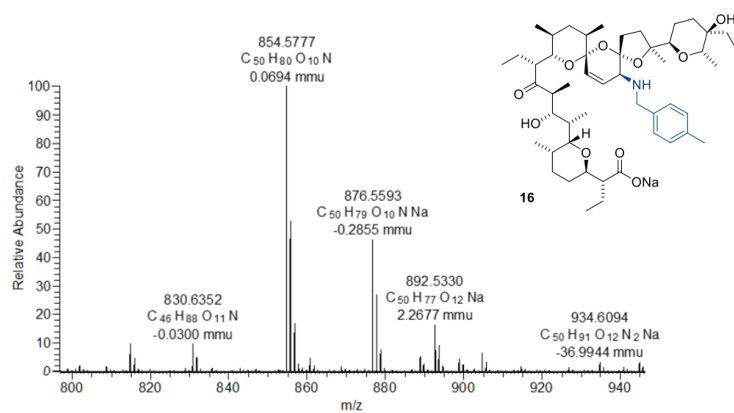


Figure S48. The HRMS spectrum of **16**.

– S52 –

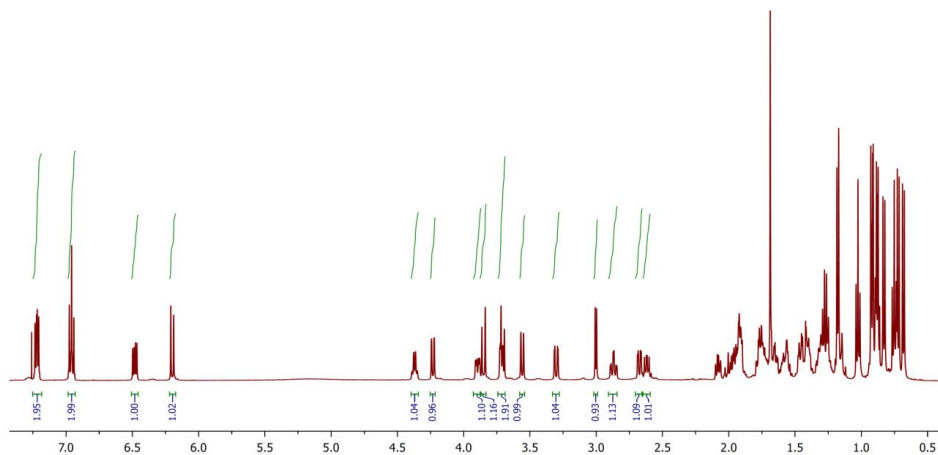
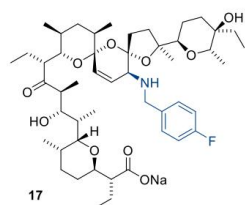


Figure S49. The ^1H NMR spectrum of **17** in chloroform- d .

– S53 –

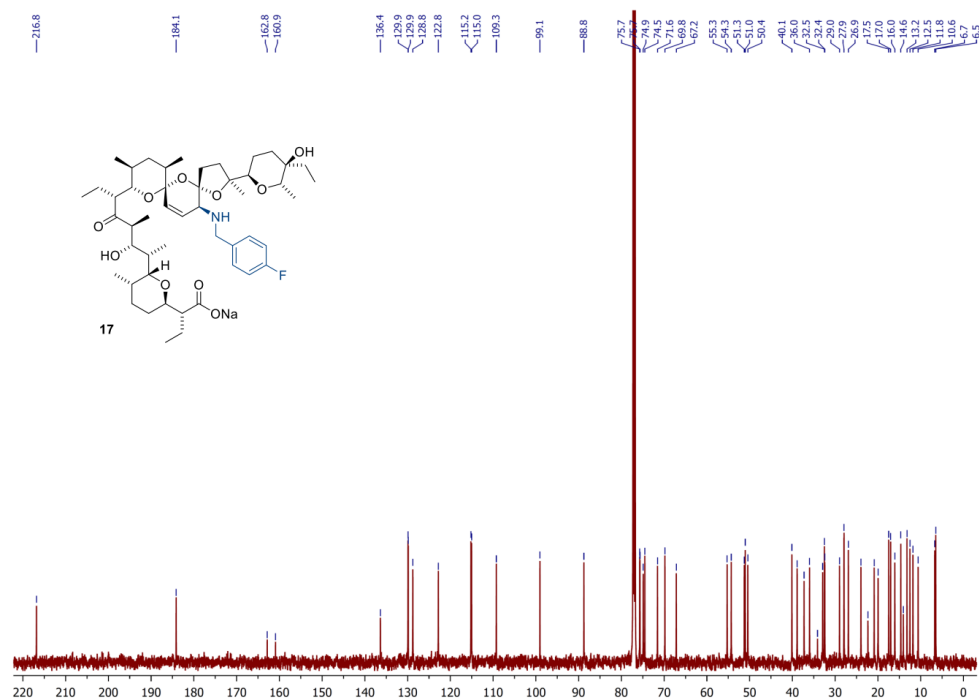


Figure S50. The ^{13}C NMR spectrum of **17** in chloroform- d .

– S54 –

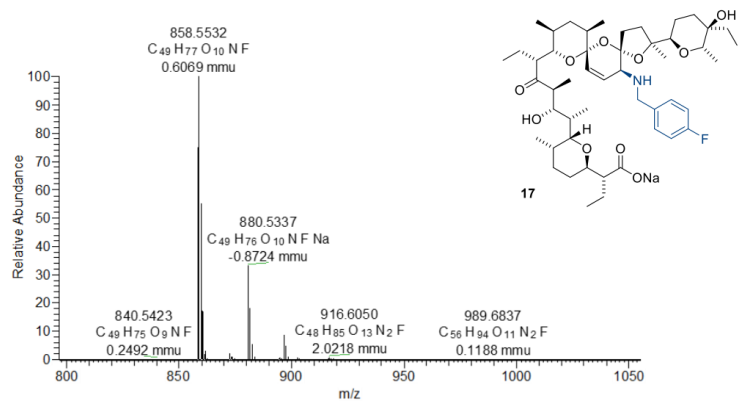


Figure S51. The HRMS spectrum of **17**.

– S55 –

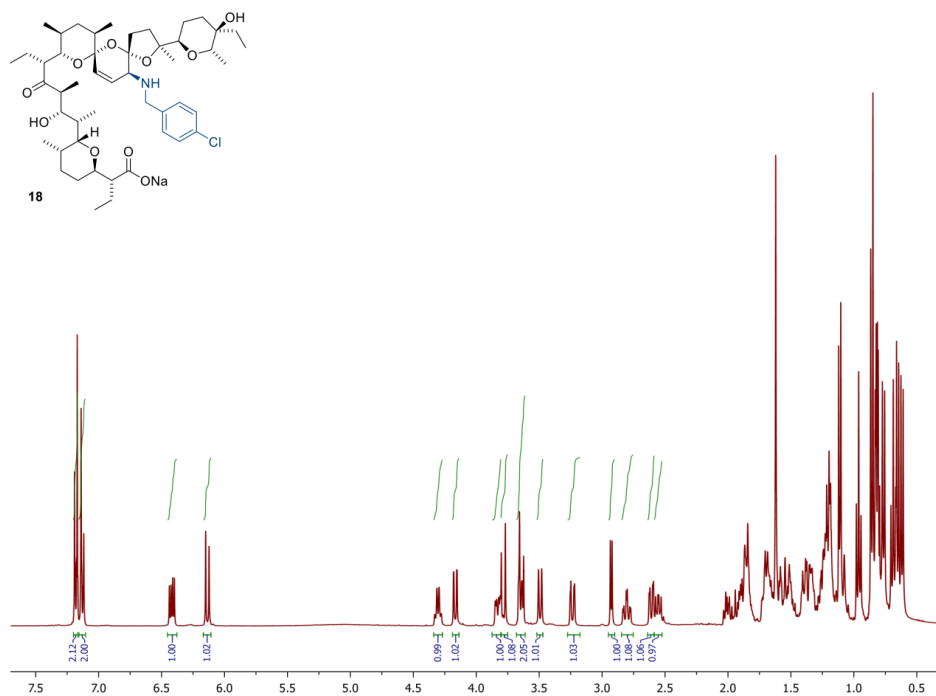


Figure S52. The 1H NMR spectrum of **18** in chloroform- d .

– S56 –

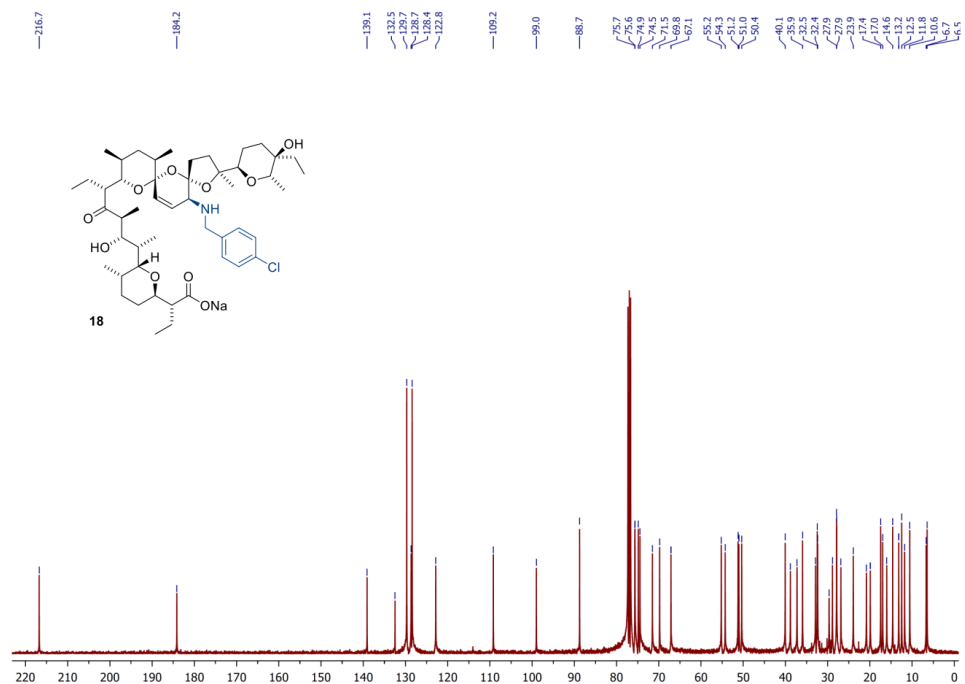


Figure S53. The ^{13}C NMR spectrum of **18** in chloroform-d.

– S57 –

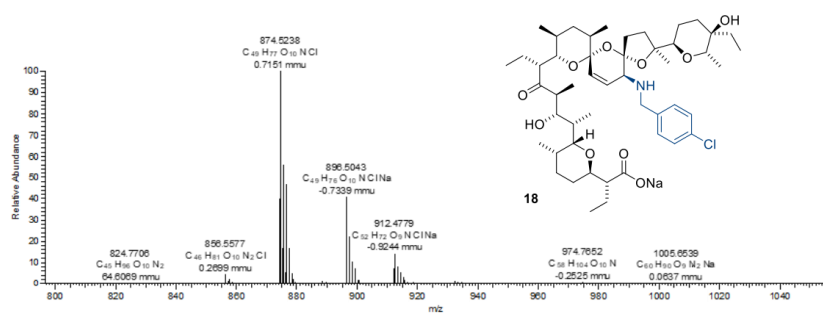


Figure S54. The HRMS spectrum of **18**.

– S58 –

Biological activity studies

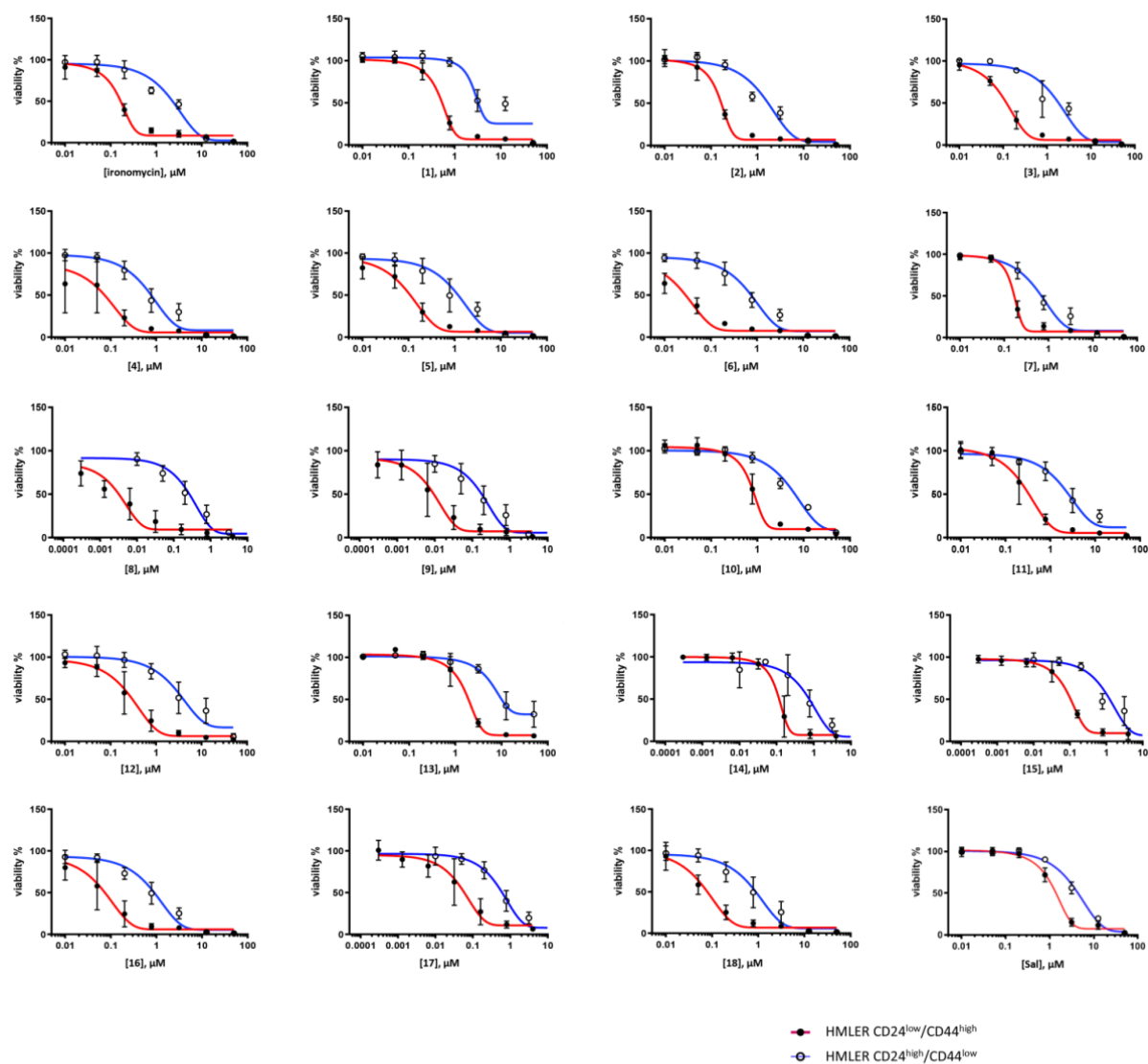


Figure S55. The IC₅₀ cell viability curves for synthesized compounds against HMLER CD24^{low}/CD44^{high} and its isogenic cell line HMLER CD24^{high}/CD44^{low}.

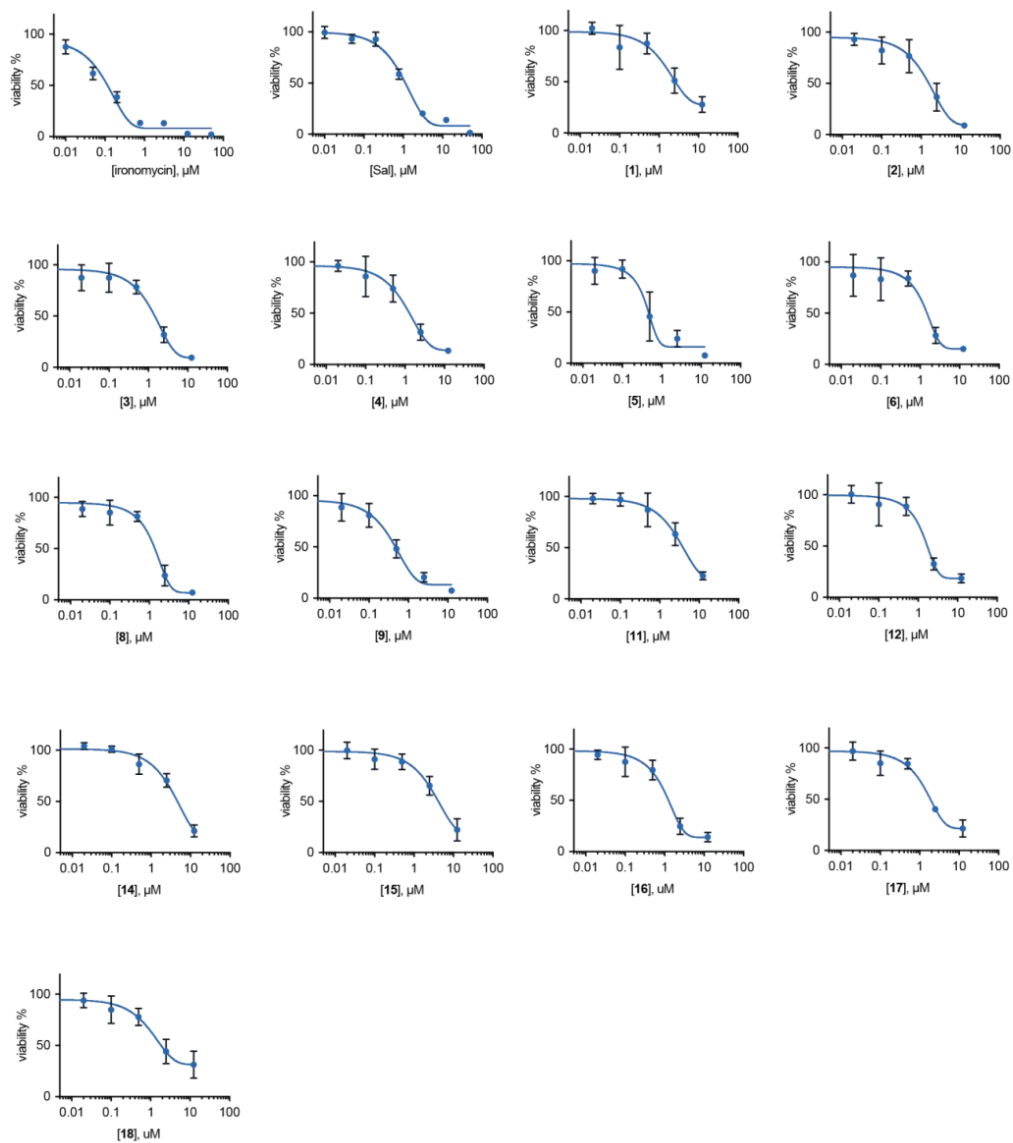


Figure S56. The IC₅₀ cell viability curves for synthesized compounds against MCF10A cells.

PUBLIKACJA IV wraz z suplementem

Dominika Czerwonka, Szymon Sobczak, Ewa Maj, Joanna Wietrzyk, Andrzej
Katrusiak, Adam Huczyński

„Synthesis and antiproliferative screening of novel analogs of regioselectively
demethylated colchicine and thiocolchicine”

Molecules 2020, 25, 1180

Article

Synthesis and Antiproliferative Screening Of Novel Analogs of Regioselectively Demethylated Colchicine and Thiocolchicine

Dominika Czerwonka ¹, Szymon Sobczak ², Ewa Maj ³, Joanna Wietrzyk ³, Andrzej Katrusiak ² and Adam Huczynski ^{1,*}

¹ Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland; dominika.czerwonka@amu.edu.pl

² Department of Materials Chemistry, Faculty of Chemistry Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland; szymon.sobczak@amu.edu.pl (S.S.); katran@amu.edu.pl (A.K.)

³ Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland; ewa.maj@hirsfeld.pl (E.M.); joanna.wietrzyk@hirsfeld.pl (J.W.)

* Correspondence: adhucz@amu.edu.pl; Tel.: +48-618291673

Academic Editor: Qiao-Hong Chen

Received: 29 January 2020; Accepted: 3 March 2020; Published: 5 March 2020



Abstract: Colchicine, a pseudoalkaloid isolated from *Colchicum autumnale*, has been identified as a potent anticancer agent because of its strong antimitotic activity. It was shown that colchicine modifications by regioselective demethylation affected its biological properties. For demethylated colchicine analogs, 10-demethylcolchicine (colchiceine, **1**) and 1-demethylthiocolchicine (**3**), a series of 12 colchicine derivatives including 5 novel esters (**2b–c** and **4b–d**) and 4 carbonates (**2e–f** and **4e–f**) were synthesized. The antiproliferative activity assay, together with in silico evaluation of physicochemical properties, confirmed attractive biological profiles for all obtained compounds. The substitutions of H-donor and H-acceptor sites at C1 in thiocolchicine position provide an efficient control of the hydration affinity and solubility, as demonstrated for anhydrate **3**, hemihydrate **4e** and monohydrate **4a**.

Keywords: colchicine analogs; thiocolchicine; colchiceine; antimitotic agents; antiproliferative activity; hydrates

1. Introduction

Colchicine (Figure 1) is a pseudoalkaloid produced by *Colchicum autumnale* [1]. Its beneficial applications have been known for centuries, as colchicine was used by ancient Greeks and Egyptians to treat swelling and inflammation [2]. Presently, colchicine is considered as the first-line therapy for gout, pericarditis and familial Mediterranean fever [1,3–5]. The most interesting property of colchicine is its high affinity for binding to the tubulin in cancer cells, leading to the inhibition of microtubule polymerization and mitosis arrest [6–8]. These unique properties have attracted great interest to colchicine as an anticancer-drug candidate, however its use is limited by its toxicity towards normal cells [9–11]. For this reason, new derivatives obtained through chemical modifications guided by structure–activity relations are intensively investigated. This research is aimed at reducing the colchicine toxicity and preserving the antimitotic and anticancer properties [12–24]. As it turns out, tubulin interacts with trimethoxyphenyl ring A and tropolone ring C, making the methoxy groups at C1, C2 and C10, as well as the C9-keto group, crucial for colchicine’s antimitotic activity [25–27].

Unfortunately, colchicine is also prone to isomerization, resulting in isocolchicine (Figure 1) with 500 times lower affinity for binding to tubulin than colchicine itself [28].

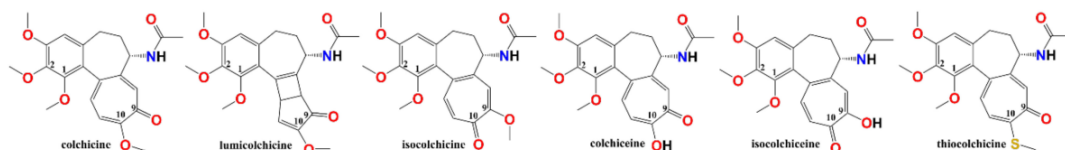


Figure 1. Structures of colchicine, lumicolchicine, isocolchicine, colchiceine, isocolchiceine and thiocolchicine.

Colchiceine (Figure 1) is a C10 demethylated colchicine analog characterized by much lower toxicity [29,30]. It is, next to lumicolchicine (Figure 1), the main product of colchicine degradation and can naturally occur in plant extracts together with colchicine [31]. Elguero et al. [32] have established that colchiceine (as well as its C10-acylated analog) can occur in two tautomeric forms, from which the isoform is slightly more dominant. This equilibrium between colchiceine and isocolchiceine (Figure 1) exists also in the solid state, which has been confirmed by X-ray diffraction studies [33]. The tendency of colchiceine towards tautomerization, in combination with the loss of the C10-methoxy group, may be responsible for its poor antimitotic activity. In contrast, colchiceine shows better bacteriostatic and antifungal activity than colchicine, because its free hydroxyl group may increase its binding affinity towards the cell walls of pathogens [29,33]. Interestingly, the replacement of the C10-methoxy group with thiomethyl in a colchicine analog thiocolchicine (Figure 1) decreases its isomerization. Thiocolchicine is more stable, exhibits lower toxicity and has a higher binding affinity to tubulin [34–36]. As could be expected, demethylation of thiocolchicine at C1/C2/C3 positions leads to a lower binding ability to tubulin and lower toxicity [37–39]. As the removal of the methoxy groups from colchicine's structure reduces its toxicity but also lowers its binding affinity to tubulin, we decided to replace these groups with a bigger substituent, such as an ester or carbonate moiety, to investigate an effect of this modification on biological activity [40].

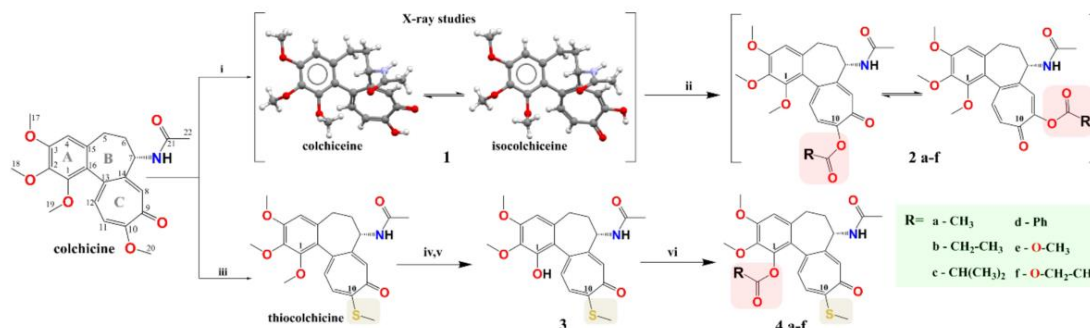
For this reason, we carried out a series of regioselective demethylation reactions of colchicine and thiocolchicine, in order to obtain colchiceine (1) and 1-demethylthiocolchicine (3). On the basis of 1 and 3, we synthesized a series of 12 mono- and double-modified colchicine analogs. Within these analogs, we present 5 novel esters (2b–c and 4b–d) and 4 novel carbonates (2e–f and 4e–f). The pathways used for these syntheses are depicted in Scheme 1.

We also evaluated the antiproliferative activity of these derivatives using three human cancer cell lines, human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma cell line (LoVo), and one normal murine embryonic fibroblast cell line (BALB/3T3). Moreover, on the basis of the *in silico* calculations, we were able to predict the physicochemical properties of the obtained compounds and check their compliance with Lipiński's rule of 5, permitting evaluation of its drug-likeness.

2. Results and Discussion

2.1. Chemistry

A series of colchicine esters and carbonates substituted at C1 and C10 positions in colchicine were synthesized by performing regioselective demethylation reactions of C1 and C10 methoxy groups, respectively. At first, we performed regioselective demethylation of the colchicine C10 methoxy group by treating colchicine with a mixture of glacial acetic acid and 0.1N hydrochloric acid (i). Reaction resulted in precipitation of yellow solid [41], which was further recrystallized in acetone, leading to growth of good quality, large single crystals. Performed X-ray diffraction analysis proved them to be a colchiceine (1, Scheme 1) hemihydrate of the same structure as that reported by Mackay et al. [33], in which the unit-cell accommodates two tautomeric forms: colchiceine and isocolchiceine.



Scheme 1. Synthesis of novel colchicine analogs. The obtained crystal structure of **1** was identical to the one proposed by Mackey et al. [33]. Reagents and conditions: (i) glacial acetic acid, hydrochloric acid, 100 °C, 6h; (iii) MeOH/H₂O, CH₃SNa, RT, 24h; (iv) DCM, acetyl chloride, SnCl₄, 0 °C → RT, 48h; (v) MeOH/H₂O, LiOH, RT, 1h; (ii,vi) DCM, Et₃N, respective acyl chloride/chloroformate, 0 °C → RT, 24h.

Colchicine was also treated with sodium methanethiolate (iii) to obtain thiolcolchicine [42]. By using a slightly modified method proposed by Bladé-Font [43] (iv,v), thiolcolchicine was regioselectively demethylated at the C1 methoxy group, leading to 1-demethylthiolcolchicine (**3**) with 40% yield. This regioselective demethylation was confirmed by X-ray analysis (Figure 2). Finally, compounds **1** and **3** were treated with respective acyl chlorides or chloroformates (ii, vi) to give single modified derivatives **2a–f** and double modified derivatives **4a–f** with 38%–58% yields (Scheme 1).

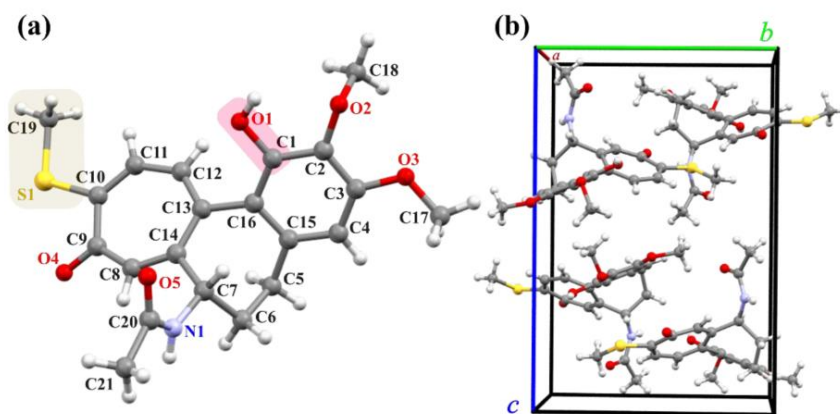


Figure 2. (a) Molecule of 1-demethylthiolcolchicine (**3**) and (b) its crystal structure projected along the crystal direction [100].

The purity and structure of synthesized colchicine derivatives **1**, **2a–f**, **3** and **4a–f** were determined on the basis of ESI-MS, NMR and X-ray analysis. The specific data can be found in Section 3 and in Supplementary Materials. The disappearance of the signal at about 56.5 ppm in the ¹³C NMR spectrum of colchicine, assigned to the C10 methoxy group, is the evidence of the formation of colchiceine (**1**). Moreover, in the spectra of derivatives **2a–2f**, an additional signal from the carbonyl group can be observed in the range 164.1–174.3 ppm for esters **2a–2d**, and 151.9–152.5 ppm for carbonates **2e–f**. A shift of the signal corresponding to the C10 methoxy group from 56.5 to 15.1 ppm is evidence for the introduction of the thiomethyl group at position C10, and the absence of one of the signals around 61 ppm assigned to the C1 methoxy group is evidence of 1-demethylthiolcolchicine (**3**) formation. An additional signal corresponding to the carbonyl carbon atom can be observed in the range 168.3–174.7 ppm for esters **4a–4d** and in the range 152.8–152.9 ppm for carbonates **4e–f**.

2.2. In Silico Calculations of the Physicochemical Properties

We used the Molinspiration online database (<http://www.molinspiration.com>, free of charge) to predict the physicochemical properties of all synthesized compounds and collected them in Table 1 [44].

Table 1. Physicochemical properties of the synthesized compounds based on the Molinspiration database [44].

Compound	MW	clogP	tPSA	n(O,N)	N(OH,NH)	Rotb	MV
1	385.42	0.83	94.10	7	2	4	346.63
2a	427.45	0.60	100.18	8	1	6	383.14
2b	441.48	1.27	100.18	8	1	7	399.94
2c	455.51	1.51	100.18	8	1	7	416.53
2d	489.52	2.92	100.18	8	1	7	437.99
2e	443.45	0.95	109.41	9	1	7	392.12
2f	457.48	1.33	109.41	9	1	8	408.93
3	401.48	1.89	84.86	6	2	4	355.77
4a	443.52	1.66	90.94	7	1	6	392.28
4b	457.55	2.33	90.94	7	1	7	409.08
4c	471.57	2.57	90.94	7	1	7	425.67
4d	505.59	3.98	90.94	7	1	7	447.13
4e	459.52	2.02	100.18	8	1	7	401.27
4f	473.55	2.39	100.18	8	1	8	418.07
colchicine	399.44	1.10	83.11	7	1	5	364.15

MW: Molecular weight; clogP: calculated log octanol/water partition coefficient; tPSA: total polar surface area; n(O,N): number of hydrogen acceptors; n(OH,NH): number of hydrogen donors; Rotb: rotatable bonds; MV: molecular volume.

According to Lipinski's rule of 5, most "drug-like" molecules should have $\log P \leq 5$, molecular weight ≤ 500 , number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5 . Additionally, it was demonstrated that a total polar surface area (tPSA) < 140 and number of rotatable bonds < 10 , in combination with $\log P < 5$, are crucial for both good bioavailability and permeability through biological membranes [45].

The demethylation of the C1 and C10 position of colchicine, both in **1** and **3**, increases the number of H-donor sites while the substitution of colchicine's C10 methoxy group with thiomethyl results in a decreased number of H-acceptor sites in **3**. Additionally, the demethylation of the C1 and C10 position of colchicine reduces the number of rotatable bonds. Substitution of a hydroxyl group, both in **1** and **3**, with an ester or carbonate substituent, increases the number of H-acceptor sites and reduces the number of H-donor sites to that of colchicine. According to the performed calculations, all synthesized compounds **2a–e** and **4a–e** have a rather lipophilic character, with the clogP values between 0.60 and 3.98. Conducted syntheses allowed us to obtain compounds with less lipophilic character than that of colchicine for compounds **1**, **2a** and **2e**, with clogP values 0.83, 0.60 and 0.95, respectively. Interestingly, esters and carbonates in the C10 position (**2a–e**) are in general less lipophilic than corresponding esters and carbonates in the C1 position (**4a–e**). Except for **4d**, whose molecular weight is above 500, the calculations for all colchicine analogs are in agreement with Lipinski's descriptors. Importantly, in order to be active, a drug should not have more than one violation [46]. This, in combination with the number of rotatable bonds between 4 and 8, as well as tPSA between 84.86 and 100.18, confirm good physicochemical profiles of all obtained compounds.

2.3. X-ray Analysis

The structures of compounds **3**, **4a** and **4e** were additionally confirmed by X-ray diffraction. Selected crystallographic data are collected in Table 2, and more detailed information can be found in Supplementary Information. The single crystals of **3**, **4a**, **4e** were obtained by slow evaporation from an ethyl acetate mixture. The 1-Demethylthiocolchicine (**3**) crystallizes in the orthorhombic space group $P2_12_12_1$ (Figure 2) while the crystals of **4a** and **4e** are monoclinic, space group $P2_1$. The substitution

at the C1 atom with acetyl ester (**4a**) and methyl carbonate (**4e**), and the following change in the ratio between H-bond acceptors and donors, increases the tendency of these colchicine derivatives to co-crystallize with water; hence, monohydrate **4a**·H₂O and hemihydrate **4e**·½H₂O were obtained.

Molecules **4a** and **4e** significantly differ in conformation (Figure 3). The arrangement of the C2 methoxy group, which can be described by the C1-C2-O2-C18 torsion angle, changed from −122.36° to 108.75° in **4a** and **4e**, respectively. Together with increasing mass of the C1 moiety, the twist between the planar phenyl and tropolone rings around the C13-C16 bond increases, altering the C1-C16-C13-C12 angle from 45.43° in **3**, through 58.54° in **4a**, to 60.11° in **4e**.

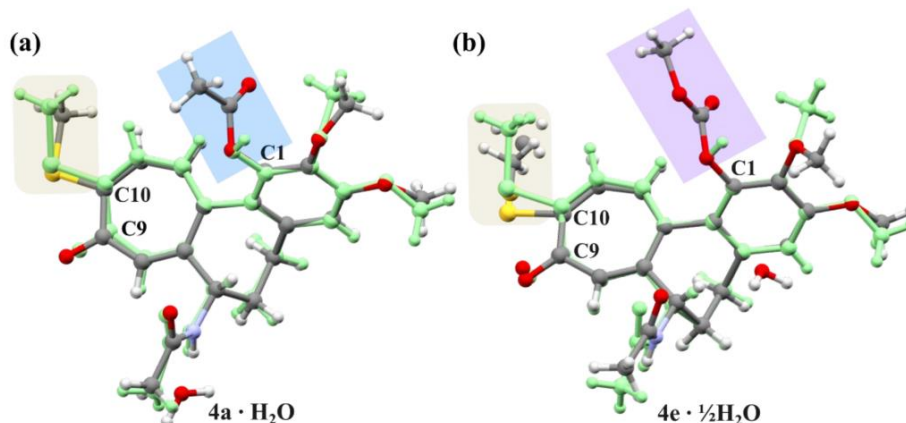


Figure 3. Comparison of the molecular conformation from the X-ray analysis studies between (a) molecule **4a** and (b) **4e**, superimposed with respect to the benzene ring on the 1-demethylthiocolchicine (molecule **3**), highlighted with a light-green color.

Table 2. Selected crystallographic data for compounds **3**, **4a**·H₂O and **4e**·½H₂O.

Label	3	4a ·H ₂ O	4e ·½H ₂ O
Formula	C ₂₁ H ₂₂ NO ₅ S	C ₂₃ H ₂₅ NO ₆ S · H ₂ O	C ₂₃ H ₂₅ NO ₇ S · ½H ₂ O
CCDC number	1966196	1966194	1966195
Crystal system	orthorhombic	monoclinic	monoclinic
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	P2 ₁
Unit cell dimensions	<i>a</i> (Å)	9.1005(17)	10.600(2)
	<i>b</i> (Å)	11.866(2)	6.9635(11)
	<i>c</i> (Å)	17.881(4)	16.712(3)
	β (°)	90	107.07(2)
Volume (Å³)	1930.8(7)	1179.2(4)	1170.80(18)
Z/Z'	4/1	2/1	2/1
D_x (g/cm³)	1.378	1.300	1.329

The presence of water significantly alters the pattern of H-bonds between **3** and **4a**·H₂O and **4e**·½H₂O. Although in all structures amide nitrogen participates in the formation of intermolecular contacts, only in **3** are the NH···S, of 2.793(2) Å, and NH···O4, of 2.370(3) Å, bonds formed. In a monohydrate crystal **4a**·H₂O, water interrupts the NH···O4 interaction observed in the crystal structure of **3**, accepting NH amide proton, of 2.086(1) Å and further donating its H-atoms to the HO-H···O4', of 2.007(3) Å, and HO-H···O5', of 1.994(3) Å bond. Much weaker H-bonds are present in the crystal structure of **4e**·½H₂O. Despite the presence of a water molecule in the crystal structure of **4e**·½H₂O, the strong NH···O5' bond, of 2.033(2) Å is present again. The disordered by location at half-occupied sites water molecule is involved in formation of a weak HO-H···O5'' interaction of 2.538(1) Å and the H-bond at the disordered terminal carbonyl at C9. Due to the HO-H···O4' of 2.241(1) Å bond, the occupational disorder of H₂O is passed onto the orientation of **4e** molecules as illustrated in Figure 3.

Depending on the presence or absence of this water molecule, its adjacent molecule **4e** is slightly shifted in the crystal structure. This disorder is most apparent for the terminal carbonyl at C9 (dislocated by 0.358 Å) and thiomethyl at C10 (dislocated by 0.625 Å).

2.4. Antiproliferative Activity

The antiproliferative activity of all synthesized colchicine analogs **1**, **2a–f**, **3**, **4a–f** was tested *in vitro* against three human cancer cell lines: human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma cell line (LoVo). For a more accurate evaluation of cytotoxic activity, the effect on normal murine embryonic fibroblast cell line (BALB/3T3) was also tested according to the previously published procedure [12]. Detailed information concerning antiproliferation assay can be found in Supplementary Materials. The mean values of $IC_{50} \pm SD$ of the tested compounds are collected in Table 3.

Table 3. Antiproliferative activity (IC_{50}) of 10-demethylcolchicine (colchicine, **1**), 1-demethylthiocolchicine (**3**) and their derivatives (**2a–f**, **4a–f**) compared to the antiproliferative activity of colchicine and standard anticancer drugs doxorubicin and cisplatin and the calculated values of selectivity index (SI).

Compound	A549	SI	MCF-7	SI	LoVo	SI	BALB/3T3
	IC_{50} (μM)		IC_{50} (μM)		IC_{50} (μM)		IC_{50} (μM)
1	12.99 $\pm$ 1.79	0.79	11.23 $\pm$ 2.52	0.91	6.00 $\pm$ 1.88	1.71	10.25 $\pm$ 0.96
2a	12.47 $\pm$ 2.77	0.68	10.03 $\pm$ 2.18	0.85	6.18 $\pm$ 1.48	1.37	8.50 $\pm$ 0.50
2b	9.97 $\pm$ 0.70	0.75	7.56 $\pm$ 2.84	0.99	2.39 $\pm$ 1.20	3.13	7.47 $\pm$ 0.50
2c	20.39 $\pm$ 11.30	0.41	11.95 $\pm$ 1.54	0.70	6.47 $\pm$ 0.35	1.30	8.42 $\pm$ 1.14
2d	8.75 $\pm$ 0.33	1.02	8.21 $\pm$ 3.35	1.09	4.87 $\pm$ 2.17	1.83	8.92 $\pm$ 3.93
2e	0.98 $\pm$ 0.16	0.84	0.98 $\pm$ 0.33	0.84	0.48 $\pm$ 0.32	1.70	0.82 $\pm$ 0.11
2f	9.67 $\pm$ 0.84	0.86	9.07 $\pm$ 1.90	0.92	6.41 $\pm$ 1.57	1.30	8.36 $\pm$ 0.17
3	0.82 $\pm$ 0.02	0.74	0.12 $\pm$ 0.05	4.92	0.11 $\pm$ 0.03	5.38	0.61 $\pm$ 0.14
4a	0.48 $\pm$ 0.15	1.36	0.10 $\pm$ 0.02	6.85	0.11 $\pm$ 0.02	5.92	0.65 $\pm$ 0.12
4b	0.97 $\pm$ 0.04	0.77	0.96 $\pm$ 0.13	0.78	0.50 $\pm$ 0.19	1.53	0.75 $\pm$ 0.10
4c	0.89 $\pm$ 0.07	0.80	0.86 $\pm$ 0.10	0.83	0.56 $\pm$ 0.06	1.27	0.71 $\pm$ 0.03
4d	0.60 $\pm$ 0.31	0.75	0.78 $\pm$ 0.22	0.58	0.10 $\pm$ 0.08	4.71	0.45 $\pm$ 0.23
4e	0.90 $\pm$ 0.09	0.87	1.02 $\pm$ 0.10	0.77	0.43 $\pm$ 0.19	1.84	0.78 $\pm$ 0.06
4f	0.96 $\pm$ 0.13	1.08	0.95 $\pm$ 0.20	1.09	0.79 $\pm$ 0.16	1.31	1.03 $\pm$ 0.20
colchicine	0.07 $\pm$ 0.01	0.63	0.01 $\pm$ 0.01	4.30	0.01 $\pm$ 0.01	5.38	0.04 $\pm$ 0.01
cisplatin	3.60 $\pm$ 0.25	0.81	3.05 $\pm$ 0.67	0.95	3.80 $\pm$ 0.28	0.77	2.91 $\pm$ 1.83
doxorubicin	0.16 $\pm$ 0.03	0.25	0.15 $\pm$ 0.05	0.26	0.08 $\pm$ 0.03	0.49	0.04 $\pm$ 0.03

The IC_{50} value is defined as the concentration of a compound at which 50% growth inhibition is observed. Human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line (LoVo) and normal murine embryonic fibroblast cell line (BALB/3T3). The SI (Selectivity Index) was calculated for each compound, using the formula $SI = IC_{50}$ for normal cell line BALB/3T3/ IC_{50} for the respective cancerous cell line. A beneficial SI > 1.0 indicates a drug with efficacy against tumor cells greater than the toxicity against normal cells.

All colchicine analogs showed stronger antiproliferative activity against all tested cancer cell lines than the conventional chemotherapeutic, cisplatin. In general, the IC_{50} values are better for colchicines doubly modified at C1 and C10 positions (**3**, **4a–f**) than for the analogs singly modified at position C10 only (**1**, **2a–f**). Compound **4a**, with a thiomethyl group at position C10 and an acetyl ester substituent at C1, showed the highest activity against all tested cell lines ($IC_{50} = 0.48 \pm 0.15$, 0.10 ± 0.02 , $0.11 \pm 0.02 \mu M$ for A549, MCF-7, LoVo cancer cell lines, respectively).

All tested colchicine analogs exhibit higher SI values than doxorubicin, which indicates the therapeutic potential of the synthesized compounds. Compound **3**, 1-demethylthiocolchicine, as well as its acylated analog **4a**, showed high SI values against MCF-7 (SI = 4.92, SI = 6.88) as well as LoVo cell lines (SI = 5.36, SI = 5.95). From among analogs modified exclusively at position C10, compound **2b** with propionyl ester substituent revealed a higher SI value towards LoVo cell line (SI = 3.13).

3. Materials and Methods

3.1. General

Information concerning reagents and solvents, as well as equipment used for measurements, can be found in the Supplementary Materials.

3.1.1. Synthesis and Characterization of 10-Demethylcolchicine (colchicine, **1**), Thiocolchicine and 1-Demethylthiocolchicine (**3**)

Information concerning the synthesis of 10-demethylcolchicine (colchicine, **1**), thiocolchicine and 1-demethylthiocolchicine (**3**) can be found in Supplementary Materials.

3.1.2. General Route for Synthesis of Compounds 2a–f and 4a–f

To a solution of **1** or **3** (100 mg, 0.25 mmol) in dichloromethane (DCM, 10 mL) cooled to 0 °C, Et₃N (1 mL, 7 mmol) and respective acyl chloride or chloroformate were added (excess). The mixture was first stirred at 0 °C for 30 min and then for the next 24 h at RT. Reaction time was determined by TLC. After that time the solution was filtered to remove triethylamine hydrochloride, the DCM was evaporated under reduced pressure and the residue was purified by CombiFlash® (chloroform/acetone, increasing concentration gradient) to give respective compounds as amorphous yellow solids.

3.1.3. Characterization of Acetyl Ester of Colchicine 2a

Amorphous yellow solid, yield 49%, m.p 120–122 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.50 (1H, s), 7.31 (1H, d, *J* = 11.3 Hz), 7.22 (1H, d, *J* = 11.3 Hz), 6.53 (1H, s), 4.60 (1H, dt, *J* = 12.7, 6.5 Hz), 3.91 (3H, s), 3.88 (3H, s), 3.66 (3H, s), 2.50 (1H, dd, *J* = 13.6, 6.3 Hz), 2.35 (3H, s), 2.26–2.17 (1H, m), 1.96 (3H, s), 1.87 (1H, td, *J* = 12.1, 6.9 Hz). ¹³C NMR (CDCl₃, 101 MHz) δ 169.9, 168.2, 153.9, 151.0, 148.6, 141.8, 141.5, 137.0, 134.5, 131.2, 130.3, 125.2, 107.4, 61.6, 61.3, 56.0, 52.1, 36.8, 29.7, 22.7, 20.7. ESI-MS (*m/z*): [M + H]⁺ 428, [M + Na]⁺ 450, [M + K]⁺ 466, [2M + Na]⁺ 877.

3.1.4. Characterization of Propionyl Ester of Colchicine 2b

Amorphous yellow solid, yield 52%, m.p 133–135 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.50 (1H, s), 7.30 (1H, d, *J* = 11.3 Hz), 7.22 (1H, d, *J* = 11.2 Hz), 6.52 (1H, s), 4.66–4.57 (1H, m), 3.91 (3H, s), 3.88 (3H, s), 3.66 (3H, s), 2.66 (2H, q, *J* = 7.4 Hz), 2.50 (1H, dd, *J* = 13.5, 6.3 Hz), 2.35 (1H, ddd, *J* = 12.7, 10.3, 6.0 Hz), 2.24–2.16 (1H, m), 1.97 (3H, s), 1.87 (2H, td, *J* = 12.0, 6.7 Hz), 1.26 (3H, t, *J* = 7.6 Hz). ¹³C NMR (CDCl₃, 101 MHz) δ 171.8, 169.9, 153.9, 151.1, 148.9, 141.7, 141.6, 136.7, 134.5, 130.9, 130.5, 125.2, 107.4, 61.7, 61.4, 56.1, 52.1, 36.9, 29.8, 27.4, 22.8, 9.0. ESI-MS (*m/z*): [M + Na]⁺ 464, [2M + Na]⁺ 905.

3.1.5. Characterization of Isobutyryl Ester of Colchicine 2c

Yellowish brown oil, yield 55%; ¹H NMR (CDCl₃, 400 MHz) δ 7.56 (1H, s), 7.28 (1H, d, *J* = 11.0 Hz), 7.23 (1H, d, *J* = 10.6 Hz), 6.52 (1H, s), 4.64 (1H, dt, *J* = 12.2, 6.8 Hz), 3.91 (3H, s), 3.88 (3H, s), 3.65 (3H, s), 2.90–2.82 (1H, m), 2.54–2.47 (1H, m), 2.36 (1H, td, *J* = 13.2, 6.9 Hz), 2.24–2.12 (2H, m), 1.96 (3H, s), 1.92–1.85 (1H, m), 1.35 (3H, d, *J* = 1.4 Hz), 1.33 (3H, d, *J* = 1.3 Hz). ¹³C NMR (CDCl₃, 101 MHz) δ 174.3, 170.0, 153.9, 151.1, 150.3, 142.1, 141.5, 136.0, 134.5, 131.6, 130.1, 125.2, 107.4, 61.7, 61.3, 56.0, 52.1, 36.6, 34.0, 29.8, 26.4, 22.7, 19.0. ESI-MS (*m/z*): [M + Na]⁺ 478, [2M + Na]⁺ 933.

3.1.6. Characterization of Benzyl Ester of Colchicine 2d

Amorphous yellow solid, yield 41%, m.p 123–125 °C; ¹H NMR (CDCl₃, 400 MHz) δ 8.18 (2H, d, *J* = 7.4 Hz), 7.65–7.60 (2H, m), 7.49 (1H, ddd, *J* = 4.8, 3.1, 1.1 Hz), 7.34 (2H, s), 6.53 (1H, s), 4.66–4.58 (1H, m), 3.93 (3H, d, *J* = 0.9 Hz), 3.90 (3H, d, *J* = 0.7 Hz), 3.68 (3H, d, *J* = 1.1 Hz), 2.49 (1H, dd, *J* = 13.4, 4.9 Hz), 2.42–2.32 (1H, m), 2.17–2.06 (1H, m), 1.86 (3H, d, *J* = 4.0 Hz), 1.84–1.78 (1H, m). ¹³C NMR

(CDCl₃, 101 MHz) δ 169.9, 164.1, 153.9, 151.1, 142.0, 141.6, 134.5, 133.8, 130.4, 128.9, 128.5, 125.3, 107.4, 61.7, 61.4, 56.1, 52.0, 36.9, 29.8, 22.7. ESI-MS (*m/z*): [M + H]⁺ 490, [M + Na]⁺ 512, [M + K]⁺ 528.

3.1.7. Characterization of Methyl Carbonate of Colchicine 2e

Amorphous yellowish brown solid, yield 58%, m.p 115–118 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.57 (1H, s), 7.32 (1H, d, *J* = 20.5 Hz), 6.54 (1H, s), 4.62 (1H, dt, *J* = 11.8, 6.0 Hz), 3.92 (3H, s)*, 3.92 (3H, s)*, 3.89 (3H, s), 3.68 (3H, s), 2.52 (1H, dd, *J* = 13.4, 6.1 Hz), 2.37 (1H, td, *J* = 13.0, 6.7 Hz), 2.29–2.18 (1H, m), 1.99 (3H, s), 1.91 (1H, dd, *J* = 19.0, 12.2 Hz). ¹³C NMR (CDCl₃, 101 MHz) δ 169.9, 154.1, 152.5, 151.1, 141.6, 134.5, 125.1, 107.4, 61.7, 61.3, 56.1, 55.8, 52.2, 37.2, 29.8, 22.8. ESI-MS (*m/z*): [M + H]⁺ 444, [M + Na]⁺ 466, [M + K]⁺ 482, [2M + Na]⁺ 909.

3.1.8. Characterization of Ethyl Carbonate of Colchicine 2f

Yellowish brown oil, yield 55%; ¹H NMR (CDCl₃, 400 MHz) δ 7.46 (1H, s), 7.40–7.32 (1H, m, *J* = 6.2 Hz), 7.30–7.21 (1H, m), 6.55 (1H, s), 4.62 (1H, dt, *J* = 12.6, 6.4 Hz), 4.36 (2H, q, *J* = 7.1 Hz), 3.93 (3H, s, *J* = 2.9 Hz), 3.90 (3H, s), 3.68 (3H, s), 2.54 (1H, dd, *J* = 13.6, 6.4 Hz), 2.41 (1H, td, *J* = 13.1, 7.1 Hz), 2.32–2.21 (1H, m), 2.01 (3H, s), 1.94–1.84 (1H, m), 1.41 (3H, t, *J* = 7.1 Hz). ¹³C NMR (CDCl₃, 101 MHz) δ 169.9, 154.0, 151.9, 151.1, 141.6, 134.5, 125.1, 107.4, 65.4, 61.7, 61.3, 56.1, 52.1, 37.2, 29.8, 22.8, 14.1. ESI-MS (*m/z*): [M + H]⁺ 458, [M + Na]⁺ 480, [M + K]⁺ 496, [2M + Na]⁺ 937.

3.1.9. Characterization of Acetyl Ester of 1-demethylthiocolchicine 4a

Amorphous yellow solid, yield 45%, m.p 168–172 °C; ¹H NMR (DMSO-d₆, 400 MHz) δ 8.51 (1H, d, *J* = 7.7 Hz), 7.23 (1H, d, *J* = 10.8 Hz), 7.02 (1H, s), 6.97 (1H, s), 6.92 (1H, d, *J* = 10.3 Hz), 3.89 (3H, s), 3.74 (3H, s), 2.65 (1H, dd, *J* = 13.5, 6.0 Hz), 2.41 (3H, s), 2.32–2.24 (1H, m), 2.22 (3H, s), 2.12–2.01 (1H, m), 1.85 (3H, s, *J* = 6.3 Hz), 1.84–1.77 (1H, m, *J* = 12.1, 6.8 Hz). ¹³C NMR (DMSO-d₆, 101 MHz) δ 181.2, 168.9, 168.2, 157.7, 152.8, 150.4, 140.8, 139.3, 136.4, 134.4, 132.7, 128.2, 126.4, 124.6, 109.9, 60.1, 56.0, 50.7, 36.2, 29.1, 22.5, 20.3, 14. ESI-MS (*m/z*): [M + Na]⁺ 466, [2M + Na]⁺ 909.

3.1.10. Characterization of Propionyl Ester of 1-demethylthiocolchicine 4b

Amorphous yellow solid, yield 47%, m.p 136–140 °C; ¹H NMR (DMSO-d₆, 400 MHz) δ 8.52 (1H, d, *J* = 7.7 Hz), 7.23 (1H, d, *J* = 10.7 Hz), 7.03 (1H, s), 6.98 (1H, s), 6.92 (1H, d, *J* = 10.2 Hz), 4.34 (1H, dt, *J* = 11.8, 7.1 Hz), 3.90 (3H, s), 3.76–3.71 (3H, m), 2.66 (1H, dd, *J* = 13.2, 6.4 Hz), 2.41 (3H, s), 2.33–2.19 (2H, m), 2.08 (2H, ddd, *J* = 11.4, 9.8, 5.4 Hz), 1.84–1.78 (1H, m), 1.11 (3H, t, *J* = 7.5 Hz). ¹³C NMR (DMSO-d₆, 101 MHz) δ 181.2, 172.3, 168.3, 157.7, 152.8, 150.5, 140.9, 139.2, 136.4, 134.4, 132.9, 128.3, 126.4, 124.7, 109.9, 60.1, 56.0, 50.7, 36.2, 29.1, 26.7, 22.6, 14.5, 9.2. ESI-MS (*m/z*): [M + H]⁺ 458, [M + Na]⁺ 480, [M + K]⁺ 496, [2M + Na]⁺ 937.

3.1.11. Characterization of Isobutyryl Ester of 1-demethylthiocolchicine 4c

Amorphous yellow solid, yield 49%, m.p 165–167 °C; ¹H NMR (DMSO-d₆, 101 MHz) δ 8.50 (1H, d, *J* = 7.8 Hz), 7.22 (1H, d, *J* = 10.6 Hz), 7.03 (1H, s), 6.98 (1H, s), 6.91 (1H, d, *J* = 8.8 Hz), 4.35 (1H, dt, *J* = 11.7, 7.3 Hz), 3.90 (3H, s), 3.72 (3H, s), 2.74 (1H, dt, *J* = 13.9, 6.9 Hz), 2.66 (1H, dd, *J* = 13.6, 6.1 Hz), 2.39 (3H, s), 2.34–2.25 (1H, m), 2.09 (1H, td, *J* = 12.7, 5.9 Hz), 1.86 (3H, s), 1.81 (1H, dd, *J* = 12.0, 6.9 Hz), 1.16 (3H, d, *J* = 3.5 Hz), 1.14 (3H, d, *J* = 3.5 Hz). ¹³C NMR (DMSO-d₆, 101 MHz) δ 181.2, 174.7, 168.3, 157.7, 152.7, 150.4, 140.8, 139.2, 136.4, 134.4, 132.9, 128.3, 126.3, 124.8, 109.9, 60.1, 56.1, 50.7, 36.2, 33.3, 29.1, 22.6, 18.8, 14.6. ESI-MS (*m/z*): [M + Na]⁺ 494, [2M + Na]⁺ 965.

3.1.12. Characterization of Benzyl Ester of 1-demethylthiocolchicine 4d

Amorphous yellow solid, yield 38%, m.p 166–170 °C; ¹H NMR (DMSO-d₆, 101 MHz) δ 8.52 (1H, s), 8.07 (2H, d, *J* = 7.3 Hz), 7.77–7.71 (1H, m), 7.63–7.56 (2H, m), 7.22–7.12 (1H, m), 7.06 (1H, s), 7.02 (1H, s), 4.49–4.40 (1H, m), 3.94 (3H, s), 3.74 (3H, s), 2.72 (1H, dd, *J* = 13.0, 5.9 Hz), 2.36 (1H, dd, *J* = 7.4,

5.5 Hz), 2.31 (3H, s), 2.13 (2H, ddd, $J = 19.4, 12.8, 6.9$ Hz), 1.87 (3H, s). ^{13}C NMR (DMSO- d_6 , 101 MHz) δ 181.1, 168.3, 157.7, 152.9, 150.6, 141.0, 139.4, 136.3, 134.6, 134.2, 129.8, 129.1, 128.4, 128.3, 126.2, 124.9, 110.2, 60.3, 56.1, 50.8, 36.3, 29.2, 22.6, 14.5. ESI-MS (m/z): $[\text{M} + \text{Na}]^+ 528$, $[2\text{M} + \text{Na}]^+ 1033$.

3.1.13. Characterization of Methyl Carbonate of 1-demethylthiocolchicine 4e

Amorphous yellow solid, yield 53%, m.p 158–162 °C; ^1H NMR (DMSO- d_6 , 101 MHz) δ 8.55 (1H, d, $J = 7.6$ Hz), 7.26 (1H, d, $J = 10.6$ Hz), 7.05–6.99 (3H, m), 4.34 (1H, dt, $J = 11.8, 7.0$ Hz), 3.91 (3H, s), 3.82 (3H, s), 3.79 (3H, s), 2.69 (1H, dd, $J = 13.5, 6.2$ Hz), 2.43 (3H, s), 2.29 (1H, ddd, $J = 18.6, 12.3, 5.4$ Hz), 2.09 (1H, ddd, $J = 18.7, 12.5, 6.2$ Hz), 1.88 (3H, s), 1.86–1.80 (1H, m). ^{13}C NMR (DMSO- d_6 , 101 MHz) δ 181.2, 168.4, 157.9, 152.9, 152.8, 150.5, 140.8, 139.3, 136.0, 134.5, 133.2, 128.2, 126.4, 124.3, 110.2, 60.4, 56.14, 56.07, 50.8, 36.1, 29.1, 22.6, 14.4. ESI-MS (m/z): $[\text{M} + \text{H}]^+ 460$, $[\text{M} + \text{Na}]^+ 482$, $[\text{M} + \text{K}]^+ 498$, $[2\text{M} + \text{Na}]^+ 941$.

3.1.14. Characterization of Ethyl Carbonate of 1-demethylthiocolchicine 4f

Amorphous yellow solid, yield 48%, m.p 165–167 °C; ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.52 (1H, d, $J = 7.6$ Hz), 7.23 (1H, d, $J = 10.7$ Hz), 7.01 (3H, t, $J = 10.3$ Hz), 4.33 (1H, dt, $J = 12.6, 7.5$ Hz), 4.27–4.20 (2H, m), 3.90 (3H, s), 3.78 (3H, s), 2.67 (1H, dd, $J = 13.2, 6.6$ Hz), 2.41 (3H, s), 2.34–2.23 (1H, m), 2.08 (3H, s), 1.87 (3H, s), 1.85–1.78 (1H, m), 1.22 (3H, t, $J = 7.1$ Hz). ^{13}C NMR (DMSO- d_6 , 101 MHz) δ 181.2, 168.4, 157.9, 152.8, 152.4, 150.5, 140.8, 139.3, 136.2, 134.5, 133.1, 128.2, 126.3, 124.4, 110.2, 65.1, 60.3, 56.1, 50.8, 36.1, 29.1, 22.6, 14.4, 14.0. ESI-MS (m/z): $[\text{M} + \text{Na}]^+ 496$, $[\text{M} + \text{K}]^+ 512$, $[2\text{M} + \text{Na}]^+ 969$.

3.2. Antiproliferative Activity

Detailed information concerning antiproliferative activity assay is given in Supplementary Materials.

3.3. X-ray Measurements

X-ray measurements and detailed crystallographic data are given in Supplementary Materials.

4. Conclusions

On the basis of regioselectively demethylated colchicine analogs, colchicine (1) and 1-demethylthiocolchicine (3), we have designed and synthesized a series of 12 colchicine derivatives bearing ester and carbonate substituents, including 9 entirely novel derivatives (2b,c,e,f and 4b,c,d,e,f). Derivatives 4a· H_2O and 4e· $\frac{1}{2}\text{H}_2\text{O}$, with acetyl ester and methyl carbonate moieties, display reduced hydrophilic properties and crystallize as hydrates, which affects their biological activity and increases their solubility. The control over water co-crystallization from monohydrate to hemihydrate has been achieved through the number of H-donor and acceptor sites. These features can be invaluable for the regulation of the bioaccessibility and pharmaceutical processing of this group of compounds [47]. The synthesized derivatives exhibit a considerable in vitro antiproliferative activity against three human cancer cell lines. Compound 4a, carrying the thiomethyl group at position C10 and acetyl ester substituent at C1, showed the highest activity and selectivity index values. The biological evaluation has been supported by prediction of physicochemical properties, which are consistent with Lipinski's rule of five for all synthesized analogs.

Supplementary Materials: The following are available online: General procedures, experimental details, as well as an elaborate description of the X-ray diffraction studies and in vitro methods used.

Author Contributions: Conceptualization, D.C. and A.H.; methodology, D.C., A.H., S.S. and E.M. validation, A.H., A.K. and J.W.; investigation, D.C. S.S. and E.M. resources, A.H., A.K. and J.W. data curation, D.C., S.S. and E.M. writing—original draft preparation, D.C. and S.S. writing—review and editing, A.H., A.K. and J.W. visualization, D.C.; supervision, A.H.; project administration, A.H.; funding acquisition, A.H. All authors have read and agreed to the published version of the manuscript.

Funding: Financial support with a grant from the Polish National Science Centre (NCN)—No. 2016/21/B/ST5/00111 is gratefully acknowledged.

Acknowledgments: D.C. and S.S. acknowledge a scholarship (No. POWR. 03.02.00-00-I026/16 and POWR. 03.02.00-00-I023/17, respectively) co-financed by the European Union through the European Social Fund under the Operational Program Knowledge Education Development.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Slobodnick, A.; Shah, B.; Pillinger, M.H.; Krasnokutsky, S. Colchicine: Old and New. *Am. J. Med.* **2015**, *128*, 461–470. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Graham, W.; Roberts, J.B. Intravenous colchicine in the management of gouty arthritis. *Ann. Rheum. Dis.* **1953**, *12*, 16–19. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Khanna, D.; Fitzgerald, J.D.; Khanna, P.P.; Bae, S.; Singh, M.K.; Neogi, T.; Pillinger, M.H.; Merrill, J.; Lee, S.; Prakash, S.; et al. American college of rheumatology guidelines for management of gout. Part 1: Systematic nonpharmacologic and pharmacologic therapeutic approaches to hyperuricemia. *Arthritis Care Res.* **2012**, *64*, 1431–1446. [\[CrossRef\]](#)
4. Grattagliano, I.; Bonfrate, L.; Ruggiero, V.; Scaccianoce, G.; Palasciano, G.; Portincasa, P. Novel therapeutics for the treatment of familial mediterranean fever: From colchicine to biologics. *Clin. Pharmacol. Ther.* **2014**, *95*, 89–97. [\[CrossRef\]](#)
5. Imazio, M.; Gaita, F.; LeWinter, M. Evaluation and treatment of pericarditis: A systematic review. *J. Am. Med. Assoc.* **2015**, *314*, 1498–1506. [\[CrossRef\]](#)
6. Vindya, N.G.; Sharma, M.; Yadav, M.; Ethiraj, K.R. Tubulins—The Target for Anticancer Therapy. *Curr. Top. Med. Chem.* **2015**, *15*, 73–82. [\[CrossRef\]](#)
7. Seligmann, J.; Twelves, C. Tubulin: An example of targeted chemotherapy. *Future Med. Chem.* **2013**, *5*, 339–352. [\[CrossRef\]](#)
8. Katsetos, C.D.; Draber, P. Tubulins as Therapeutic Targets in Cancer: From Bench to Bedside. *Curr. Pharm. Des.* **2012**, *18*, 2778–2792. [\[CrossRef\]](#)
9. Cocco, G.; Chu, D.C.C.; Pandolfi, S. Colchicine in clinical medicine. A guide for internists. *Eur. J. Intern. Med.* **2010**, *21*, 503–508. [\[CrossRef\]](#)
10. Yang, L.P.H. Oral Colchicine (Colcris): In the treatment and prophylaxis of gout. *Drugs* **2010**, *70*, 1603–1613. [\[CrossRef\]](#)
11. Avendaño, C.; Menéndez, J.C. *Medicinal Chemistry of Anticancer Drugs*; Elsevier: Amsterdam, The Netherlands, 2008. [\[CrossRef\]](#)
12. Majcher, U.; Klejborowska, G.; Kaik, M.; Maj, E.; Wietrzyk, J.; Moshari, M.; Preto, J.; Tuszyński, J.; Huczyński, A. Synthesis and Biological Evaluation of Novel Triple-Modified Colchicine Derivatives as Potent Tubulin-Targeting Anticancer Agents. *Cells* **2018**, *7*, 216. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Klejborowska, G.; Urbaniak, A.; Preto, J.; Maj, E.; Moshari, M.; Wietrzyk, J.; Tuszyński, J.; Chambers, T.C.; Huczyński, A. Synthesis, biological evaluation and molecular docking studies of new amides of 4-bromothiocolchicine as anticancer agents. *Bioorg. Med. Chem.* **2019**, *23*, 115–144. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Nakagawa-Goto, K.; Chen, C.X.; Hamel, E.; Wu, C.C.; Bastow, K.F.; Brossi, A.; Lee, K.H. Antitumor agents. Part 236: Synthesis of water-soluble colchicine derivatives. *Bioorg. Med. Chem. Lett.* **2015**, *15*, 235–238. [\[CrossRef\]](#)
15. Shchegravina, E.S.; Maleev, A.A.; Ignatov, S.K.; Gracheva, I.A.; Stein, A.; Schmalz, H.G.; Gavryushin, A.E.; Zubareva, A.A.; Svirshchevskaya, E.V.; Fedorov, A.Y. Synthesis and biological evaluation of novel non-racemic indole-containing allocolchicinoids. *Eur. J. Med. Chem.* **2017**, *141*, 51–60. [\[CrossRef\]](#)
16. Yasobu, N.; Kitajima, M.; Kogure, N.; Shishido, Y.; Matsuzaki, T.; Nagaoka, M.; Takayama, H. Design, synthesis, and antitumor activity of 4-halocolchicines and their pro-drugs activated by cathepsin B. *ACS Med. Chem. Lett.* **2011**, *2*, 348–352. [\[CrossRef\]](#)
17. Huczyński, A.; Majcher, U.; Maj, E.; Wietrzyk, J.; Janczak, J.; Moshari, M.; Tuszyński, J.A.; Bartl, F. Synthesis, antiproliferative activity and molecular docking of Colchicine derivatives. *Bioorg. Chem.* **2016**, *64*, 103–112. [\[CrossRef\]](#)
18. Zhang, X.; Kong, Y.; Zhang, J.; Su, M.; Zhou, Y.; Zang, Y.; Li, J.; Chen, Y.; Fang, Y.; Zhang, X.; et al. Design, synthesis and biological evaluation of colchicine derivatives as novel tubulin and histone deacetylase dual inhibitors. *Eur. J. Med. Chem.* **2015**, *95*, 127–135. [\[CrossRef\]](#)

19. Nicolaou, K.C.; Valiulin, R.A.; Pokorski, J.K.; Chang, V.; Chen, J.S. Bio-inspired synthesis and biological evaluation of a colchicine-related compound library. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3776–3780. [\[CrossRef\]](#)
20. Chang, D.J.; Yoon, E.Y.; Lee, G.B.; Kim, S.O.; Kim, W.J.; Kim, Y.M.; Jung, J.W.; An, H.; Suh, Y.G. Design, synthesis and identification of novel colchicine-derived immunosuppressant. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4416–4420. [\[CrossRef\]](#)
21. Marzo-Mas, A.; Barbier, P.; Breuzard, G.; Allegro, D.; Falomir, E.; Murga, J.; Carda, M.; Peyrot, V.; Marco, J.A. Interactions of long-chain homologues of colchicine with tubulin. *Eur. J. Med. Chem.* **2017**, *126*, 526–535. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Johnson, L.; Goping, I.S.; Rieger, A.; Mane, J.Y.; Huzil, T.; Banerjee, A.; Luduena, R.; Hassani, B.; Winter, P.; Tuszyński, J.A. Novel Colchicine Derivatives and their Anti-cancer Activity. *Curr. Top. Med. Chem.* **2017**, *17*. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Kumar, A.; Sharma, P.R.; Mondhe, D.M. Potential anticancer role of colchicine-based derivatives: An overview. *Anticancer Drugs* **2016**, *28*, 250–262. [\[CrossRef\]](#)
24. Kozaka, T.; Nakagawa-Goto, K.; Shi, Q.; Lai, C.Y.; Hamel, E.; Bastow, K.F.; Brossi, A.; Lee, K.H. Antitumor agents 273. Design and synthesis of N-alkyl-thiocolchicinoids as potential antitumor agents. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4091–4094. [\[CrossRef\]](#)
25. Andreu, J.M.; Timasheff, S.N. Tubulin bound to colchicine forms polymers different from microtubules. *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 6753–6756. [\[CrossRef\]](#)
26. Cortese, F.; Bhattacharyya, B.; Wolff, J. Podophyllotoxin as a probe for the colchicine binding site of tubulin. *J. Biol. Chem.* **1977**, *252*, 1134–1140.
27. Chen, J.; Liu, T.; Dong, X.; Hu, Y. Recent Development and SAR Analysis of Colchicine Binding Site Inhibitors. *Mini Rev. Med. Chem.* **2009**, *9*, 1174–1190. [\[CrossRef\]](#)
28. Hastie, S.B.; Williams, R.C.; Puett, D.; Macdonald, T.L. The binding of isocolchicine to tubulin. Mechanisms of ligand association with tubulin. *J. Biol. Chem.* **1989**, *264*, 6682–6688.
29. Gohar, M.A.; Makkawi, M. The Antibacterial Action of Colchicine and Colchicine. *J. Pharm. Pharmacol.* **1951**, *3*, 415–419. [\[CrossRef\]](#)
30. Boyland, E.; Mawson, E.H. The conversion of colchicine into colchicine. *Biochem. J.* **1938**, *32*, 1204–1206. [\[CrossRef\]](#)
31. Klein, A.E.; Davis, P.J. Determination of Colchicine and Colchicine in Microbial Cultures by High-Performance Liquid Chromatography. *Anal. Chem.* **1980**, *52*, 2432–2435. [\[CrossRef\]](#)
32. Elguero, J.; Muller, R.N.; Blade-Font, A.; Faure, R.; Vincent, E.J. Carbon-13 Magnetic Resonance Spectroscopy. A Study Of Colchicine And Related Compounds. *Bull. Soc. Chim. Belges* **1980**, *89*, 193–204. [\[CrossRef\]](#)
33. Mackay, M.F.; Morrison, J.D.; Gulbis, J.M. Crystal Structure of Triclinic Colchicine Hemihydrate. *Aust. J. Phys.* **1985**, *38*, 413. [\[CrossRef\]](#)
34. Kurek, J.; Barczynski, P. Colchicine complexes with lithium, sodium and potassium salts-spectroscopic studies. *Croat. Chem. Acta* **2016**, *89*, 297–308. [\[CrossRef\]](#)
35. Shi, Q.; Verdier-Pinard, P.; Brossi, A.; Hamel, E.; Lee, K.H. Antitumor Agents-CLXXV. Anti-tubulin action of (+)-thiocolchicine prepared by partial synthesis. *Bioorg. Med. Chem.* **1997**, *5*, 2277–2282. [\[CrossRef\]](#)
36. Prajapati, P.B.; Bodiwala, K.B.; Marolia, B.P.; Bhingradiya, N.; Shah, S. Oxidative Degradation Kinetic Study of Thiocolchicoside using Stability Indicating High Performance Thin Layer Chromatographic Method. *Pharm. Methods* **2014**, *5*, 69–78. [\[CrossRef\]](#)
37. Banerjee, A.; Kasmala, L.T.; Hamel, E.; Sun, L.; Lee, K.H. Interaction of novel thiocolchicine analogs with the tubulin isoforms from bovine brain. *Biochem. Biophys. Res. Commun.* **1999**, *254*, 334–337. [\[CrossRef\]](#)
38. Kerekes, P.; Sharma, P.N.; Brossi, A.; Chignell, C.F.; Quinn, F.R. Synthesis and Biological Effects of Novel Thiocolchicines. 3. Evaluation of N-Acyldeacetylthiocolchicines, N-(Alkoxyacetyl)deacetylthiocolchicines, and O-Ethyldeacetylthiocolchicines. New Synthesis of Thiodemecolchicine and Antileukemic Effects of 2-Demeth. *J. Med. Chem.* **1985**, *28*, 1204–1208. [\[CrossRef\]](#)
39. Alkadi, H.; Khubeiz, M.J. Colchicine: A Review About Chemical Structure and Clinical Using. *Infect. Disord. Drug Targets* **2017**, *17*. [\[CrossRef\]](#)
40. Shi, Q.; Chen, K.; Morris-Natschke, S.L.; Lee, K.H. Recent progress in the development of tubulin inhibitors as antimitotic antitumor agents. *Curr. Pharm. Des.* **1998**, *4*, 219–248.

41. Dumont, R.; Brossi, A.; Chignell, C.F.; Quinn, F.R.; Suffness, M. A Novel Synthesis of Colchicine and Analogues from Thiocolchicine and Congeners: Reevaluation of Colchicine as a Potential Antitumor Agent. *J. Med. Chem.* **1987**, *30*, 732–735. [[CrossRef](#)] [[PubMed](#)]
42. Voitovich, Y.V.; Shegravina, E.S.; Sitnikov, N.S.; Faerman, V.I.; Fokin, V.V.; Schmalz, H.G.; Combes, S.; Allegro, D.; Barbier, P.; Beletskaya, I.P.; et al. Synthesis and biological evaluation of furanoalcolchicinoids. *J. Med. Chem.* **2015**, *58*, 692–704. [[CrossRef](#)] [[PubMed](#)]
43. Blade-Font, A. New chemistry of colchicine and related compounds IV. Selective demethylation of colchicine with Lewis acids and the structure of Zeisel's dimethylcolchicinic acid. *Afinidad* **1979**, *36*, 329.
44. Molinspiration Property Calculation Service. Available online: <http://www.molinspiration.com> (accessed on 15 January 2020).
45. Lipinski, C. Lead-and drug-like compounds: The rule-of-five revolution. *Drug Discov. Today Technol.* **2004**, *4*, 337–341. [[CrossRef](#)]
46. Lipinski, C.; Lombardo, F.; Dominy, B.W.; Feeney, P.J. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Deliv. Rev.* **1997**, *23*, 3–25. [[CrossRef](#)]
47. Khankari, R.K.; Grant, D.J.W. Pharmaceutical hydrates. *Thermochim. Acta* **1995**, *248*, 61–79. [[CrossRef](#)]

Sample Availability: Samples of the compounds **1**, **2a–f**, **3** and **4a–f** are available from the authors.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine

Dominika Czerwonka ^a, Szymon Sobczak ^b, Ewa Maj ^c, Joanna Wietrzyk ^c, Andrzej Katrusiak ^b, Adam Huczyński ^{a,*}

^a Department of Bioorganic Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland.

^b Department of Materials Chemistry, Faculty of Chemistry Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland

^c Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland

Supplementary materials

Index

General procedures.....	1
The antiproliferative activity	2
X-ray measurements.....	4
Synthesis and characterization of 10-demethylcolchicine (colchicine, 1).....	7
Synthesis and characterization of thiocolchicine	7
Synthesis and characterization of 1-demethylthiocolchicine (3).....	7
¹ H, ¹³ C NMR and ESI-MS spectra of compounds 2a-f and 4a-f	8

General procedures

All precursors for the synthesis of colchicine derivatives and solvents were obtained from Merck or Fluka and were used as received without further purification. CDCl₃ and DMSO-*d*₆ spectral-grade solvents were stored over 3 Å molecular sieves for several days. All the solvents used in flash chromatography were of HPLC grade (CHROMASOLV from Merck) and were used as received. Reaction mixtures were stirred using Teflon-coated magnetic stir bars. Reactions were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60F254). TLC plates were visualized in UV-light (254 nm). The ¹H, ¹³C spectra were recorded on a Varian VNMR-S 400 MHz spectrometer using TMS as the internal standard in both cases. No window function or zero filling was used. ¹H NMR measurements of 1-14 (0.07 mol dm⁻³) in CDCl₃ were carried out at the operating frequency 402.64 MHz.

The error of the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at the operating frequency 101.25 MHz. The error of chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of CDCl_3 . The ESI (Electrospray Ionisation) mass spectra were recorded on a Waters/Micromass (Waters Corporation, Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples were prepared in dry acetonitrile ($5 \times 10^{-5} \text{ mol dm}^{-3}$). The sample was infused into the ESI source using a Harvard pump at a flow rate of 20 ml min^{-1} . The ESI source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages: 10 and 30 V. The source temperature was 120°C and the desolvation temperature was 300°C . Nitrogen was used as the nebulizing and desolvation gas at flow-rates of $100 \text{ dm}^3 \text{ h}^{-1}$. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from $m/z = 100$ to $m/z = 1000$, as well as from $m/z = 200$ to $m/z = 1500$.

The antiproliferative activity

Four human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of colchicine and its derivatives: human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo), respectively, and normal murine embryonic fibroblast cell line (BALB/3T3). The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), A549 and MCF-7 cell lines—from European Collection of Authenticated Cell Cultures (Salisbury, UK), LoVo cell line was purchased from the ATCC (ATCC, Manassas, VA, USA). All the cell lines are maintained in the Institute of Immunology and Experimental Therapy (IET), Wrocław, Poland. Human lung adenocarcinoma cell line was cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM L-glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human breast adenocarcinoma cell line was cultured in mixture of Eagle medium (IET, Wrocław, Poland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 8 $\mu\text{g/mL}$ insulin and 1% amino-acids (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human colon adenocarcinoma cell lines were cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan UT, USA), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Murine embryonic fibroblast cells were cultured in

Dulbecco medium (Life Technologies Limited, Paisley, UK), supplemented with 10% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). All culture media contained antibiotics: 100 U/mL penicillin and 100 µg/mL streptomycin (Polfa-Tarchomin, Warsaw, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO₂. Cells were tested for mycoplasma contamination by mycoplasma detection kit for conventional PCR: Venor GeM Classic (Minerva Biolabs GmbH, Berlin, Germany) and negative results was obtained. The procedure is repeated every year or in the case of less frequently used lines: after thawing.

The Antiproliferative Assays *In Vitro*

Twenty-four hours before adding the tested compounds, all cell lines were seeded in 384-well plates (Sarstedt, Nümbrecht, Germany) in appropriate media with 1×10^3 or $1,5 \times 10^3$ or 3×10^3 cells per well. All cell lines were exposed to each tested agent at four different concentrations in the range 100–0.001 µg/mL for 72 h. Cells were also exposed to the reference drug cisplatin (Teva Pharmaceuticals Polska, Warsaw, Poland) and doxorubicin (Accord Healthcare Limited, Middlesex, UK). Additionally, all cell lines were exposed to DMSO (solvent used for tested compounds) (POCH, Gliwice, Poland) at concentrations corresponding to those present in tested agents' dilutions. After 72 h sulforhodamine B assay (SRB) was performed¹.

SRB

After 72 h of incubation with the tested compounds, cells were fixed in situ by gently adding of 30 µL per well of cold 50% trichloroacetic acid TCA (POCH, Gliwice, Poland) and were incubated at 4 °C for one hour. Following, wells were washed four times with water and air dried. Next, 25 µL of 0.1% solution of sulforhodamine B (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA) in 1% acetic acid (POCH, Gliwice, Poland) were added to each well and plates were incubated at RT for 0.5 h. After incubation time, unbound dye was removed by washing plates four times with 1% acetic acid whereas stain bound to cells was solubilized with 10 mM Tris base (Sigma-Aldrich, Steinheim, Germany). Absorbance of each solution was read at Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) at the 540 nm wavelength.

Results are presented as mean IC₅₀ (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC₅₀ values were calculated in Cheburator 0.4, Dmitry Nevozhay software (version 1.2.0 software by Dmitry Nevozhay, 2004–

2014, <http://www.cheburator.nevozhay.com>, freely available) for each experiment². Compounds at each concentration were tested in triplicates in single experiment and each experiment was repeated at least three times independently.

X-ray measurements

The X-ray diffraction measurements at **RT** of **3**, **4a** and **4e**, were carried out on two different diffractometers. First one equipped with MoK α (λ = 0.7108 Å) source, Eos Xcalibur, and the second one, with microfocus CuK α (λ = 1.5418 Å) radiation source, SuperNova Agilent diffractometer. For data collections and their preliminary reduction, CrysAlisPro 171.37.31 software was used³. Crystal structure was solved by direct methods with Shelxs and further refined with the Shelxl^{4,5}; with the Olex2 interface⁶. The position of hydrogen atoms were located at their best geometrical positions. In the structure **4e** disorder on C9 carbonyl group and C10 thiomethoxy group was refined with half occupancy in two positions accordingly. The details of crystal data, data collection, structure solution and refinement are given in Table S2. Details on data collection and refinement, fractional atomic coordinates, anisotropic displacement parameters and full list of bond lengths and angles in the crystallographic-information-file (CIF) format has been deposited with the Cambridge Crystallographic Database Centre as a supplementary Publication No. 1966195, 1966194 and 1966196 for compound **3**, **4a** and **4e** respectively. Copies of this information may be obtained free of charge from <http://www.ccdc.cam.ac.uk>

Table S1. Length and angle values of H-bonds present in molecules **3**, **4a** and **4e**. The asterisk marks the intermolecular H-bond.

Compound	H-bond	Length	Angle Value	Symmetric code
3	N–H $\cdots$ S	2.793(2) Å	137.77(1)°	-x, ½+y, -z
	N–H $\cdots$ O4	2.370(3) Å	159.17(3)°	-x, 1/2+y, -z
	O1–H $\cdots$ O4	1.974(4) Å	146.64(1)°	1+x, y, z
4a	N–H $\cdots$ OH ₂	2.086(1) Å	167.13(2)°	x, y, z
	HO–H $\cdots$ O4	1.994(3) Å	151.10(5)°	x, 1+y, z
	HO–H $\cdots$ O5	2.007(3) Å	157.67(3)°	2-x, ½+y, -z
4e	N–H $\cdots$ O5	2.033(2) Å	158.76(1)°	-x, ½+y, -z
	HO–H $\cdots$ O4	2.555(1) Å	149.46(3)°	-x, -½+y, -z
	HO–H $\cdots$ O5	2.538(1) Å	146.10(2)°	x, y, z

Table S2. Detailed crystallographic data of **3**, **4a** and **4e**.

		3	4a	4e
Formula		C ₂₁ H ₂₂ NO ₅ S	C ₂₃ H ₂₅ NO ₆ S · H ₂ O	C ₂₃ H ₂₅ NO ₇ S · ½H ₂ O
Wavelength (Å)		0.71073	1.54184	1.54184
Crystal system		orthorhombic	monoclinic	monoclinic
Space group		<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Unit cell dimensions	<i>a</i> (Å)	9.1005(17)	10.600(2)	10.8835(10)
	<i>b</i> (Å)	11.866(2)	6.9635(11)	9.2158(6)
	<i>c</i> (Å)	17.881(4)	16.712(3)	12.0632(12)
	α (°)	90	90	90
	β (°)	90	107.07(2)	104.613(9)
	γ (°)	90	90	90
Volume (Å ³)		1930.8(7)	1179.2(4)	1170.80(18)
<i>Z</i> / <i>Z'</i>		4/1	2/1	2/1
<i>D_x</i> (g/cm ³)		1.378	1.300	1.329
Absorption (mm ⁻¹)		0.201	1.587	1.624
F(000)		844.0	488.0	494.0
Crystal size (mm)		0.403 × 0.155 × 0.038	0.636 × 0.141 × 0.089	0.517 × 0.173 × 0.05
2 θ -range (°)		5.642 to 52.742	11.926 to 152.97	7.574 to 152.97
Min/max indices: <i>h</i> , <i>k</i> , <i>l</i>		-10 ≤ <i>h</i> ≤ 11, -14 ≤ <i>k</i> ≤ 14, -21 ≤ <i>l</i> ≤ 22	-10 ≤ <i>h</i> ≤ 10, -6 ≤ <i>k</i> ≤ 6, -16 ≤ <i>l</i> ≤ 16	-13 ≤ <i>h</i> ≤ 12, -11 ≤ <i>k</i> ≤ 6, -15 ≤ <i>l</i> ≤ 14
Ref. collected/unique		3875/2540	4653/1011	4646/3124
<i>R</i> _{int}		0.1442	0.0811	0.0757
Completeness (%)		100%	93%	92%
Data/restraints/parameter		3875/126/258	1011/277/300	3124/129/357
Goodness-of-fit on F ²		0.889	1.028	1.014
Final <i>R</i> ₁ / <i>wR</i> ₂ (<i>I</i> > 2 σ 1)		0.0789/0.0835	0.0887/0.2070	0.0575/0.1551
<i>R</i> ₁ / <i>wR</i> ₂ (all data)		0.2254/0.1254	0.1045/0.2270	0.0731/0.1724
Largest diff. peak/hole (e.Å ⁻³)		0.23/-0.26	0.26/-0.29	0.23/-0.21

Synthesis and characterization of 10-demethylcolchicine (colchicine, 1)

A mixture of colchicine (500 mg, 1.25 mmol), glacial acetic acid (0.5 mL), and hydrochloric acid (0.1N, 30 mL) was stirred for 6h at 100 °C. After that time crystals start to precipitate, and X-ray analysis, which was in consistence with results obtained by Mackey et al.[33], confirmed them to be the desired products. **1** was obtained as yellow-brown crystals (435 mg, 90%) and directly used for next steps.

^1H NMR (CDCl_3 , 400 MHz) δ 7.65 (1H, s), 7.59 (1H, d, $J = 11.5$ Hz), 7.49 (1H, s), 7.34 (1H, d, $J = 11.5$ Hz), 6.57 (1H, s), 4.71 – 4.64 (1H, m), 3.94 (3H, s), 3.91 (3H, s), 3.64 (3H, s), 2.53 – 2.48 (1H, m), 2.36 – 2.27 (2H, m), 2.00 (3H, s), 1.97 – 1.89 (1H, m). ^{13}C NMR (CDCl_3 , 400 MHz) δ 170.13, 170.07, 169.9, 153.6, 151.5, 150.8, 141.5, 141.3, 136.3, 134.3, 125.9, 122.2, 119.4, 107.3, 61.3, 61.25, 56.0, 52.7, 37.4, 29.7, 22.7. ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ 408, $[\text{M}+\text{K}]^+$ 424, $[2\text{M}+\text{Na}]^+$ 793.

Synthesis and characterization of thiocolchicine

To a mixture of colchicine (500 mg, 1.25 mmol) in MeOH/water (1/1, v/v, 5 mL), the sodium methanethiolate (solution 21% in H_2O , 0.83 mL, 2.5 mmol) was added. The mixture was stirred in at RT for 24 h. Reaction was controlled by TLC. After that time, the reaction mixture was quenched by the addition of water (150 mL). The whole mixture was extracted four times with CH_2Cl_2 , and the combined organic layers were dried over MgSO_4 , filtered, and evaporated under reduced pressure. The residue was purified by CombiFlash® (hexane/EtOAc (1/1), then EtOAc/MeOH, increasing concentration gradient) to give thiocolchicine as amorphous yellow solid with yield 78%. ^1H , ^{13}C NMR and ESI-MS analysis were in agreement with the one obtained by Shi *et al*⁷.

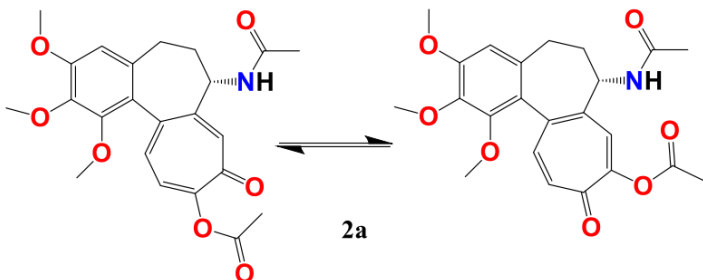
^1H NMR (CDCl_3 , 400 MHz) δ 7.92 (1H, s), 7.46 (1H, s), 7.36 – 7.26 (1H, m), 7.10 (1H, d, $J = 10.5$ Hz), 6.55 (1H, s), 4.73 – 4.63 (1H, m), 3.95 (3H, s), 3.91 (3H, s), 3.67 (3H, s), 2.54 (1H, dd, $J = 13.0, 5.8$ Hz), 2.45 (3H, s), 2.33 (2H, dtd, $J = 31.1, 12.8, 6.5$ Hz), 1.99 (3H, s), 1.96 – 1.85 (1H, m). ^{13}C NMR (CDCl_3 , 400 MHz) δ 182.4, 170.0, 158.1, 153.6, 151.8, 151.1, 141.6, 138.6, 134.8, 134.4, 128.3, 126.7, 125.6, 107.3, 61.6, 61.4, 56.1, 52.3, 36.4, 29.9, 22.8, 15.1. ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 416, $[\text{M}+\text{Na}]^+$ 438, $[\text{M}+\text{K}]^+$ 454, $[2\text{M}+\text{Na}]^+$ 853, $[3\text{M}+\text{Na}]^+$ 1268.

Synthesis and characterization of 1-demethylthiocolchicine (3)

To the solution of thiocolchicine (1.0 g, 2.41 mmol) in dichloromethane (DCM, 50 mL) cooled to the 0 °C temperature, an acetyl chloride (3 mL) and tin tetrachloride (1mL) were added. The mixture was first stirred at 0 °C temperature for 1h, and then for the next 48h at RT. After that time TLC analysis showed no presence of thiocolchicine, and the DCM was evaporated to dryness under reduced pressure. The residue was dissolved in MeOH/water (5/1, v/v, 60 mL), then lithium hydroxide (excess) was added and the mixture was stirred at RT for 1h. Reaction time was determined by TLC. The solvent was evaporated to dryness under reduced pressure and the residue was purified by CombiFlash® (chloroform/acetone, increasing concentration gradient) to give product as amorphous yellow solid with 40% yield.

^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ 9.09 (1H, s), 8.52 (1H, d, $J = 7.8$ Hz), 7.29 (1H, d, $J = 10.4$ Hz), 7.24 (1H, d, $J = 10.9$ Hz), 7.04 (1H, s), 6.50 (1H, s), 3.83 (3H, s), 3.74 (3H, s), 2.58 – 2.53 (1H, m), 2.43 – 2.39 (3H, m), 2.26 – 2.15 (1H, m), 2.09 – 1.99 (1H, m), 1.86 (3H, s), 1.84 – 1.77 (1H, m). ^{13}C NMR ($\text{DMSO}-d_6$, 101 MHz) δ 181.1, 168.3, 156.6, 152.5, 151.3, 147.0, 138.2, 135.2, 134.4, 134.2, 128.1, 126.7, 119.2, 103.2, 60.4, 55.7, 50.8, 36.4, 29.5, 22.6, 14.4. ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 402, $[\text{M}+\text{Na}]^+$ 424, $[\text{M}+\text{K}]^+$ 440, $[2\text{M}+\text{Na}]^+$ 825.

¹H, ¹³C NMR and ESI-MS spectra of compounds 2a-f and 4a-f



Chemical formula: C₂₃H₂₅NO₇, MW = 427,5 g/mol

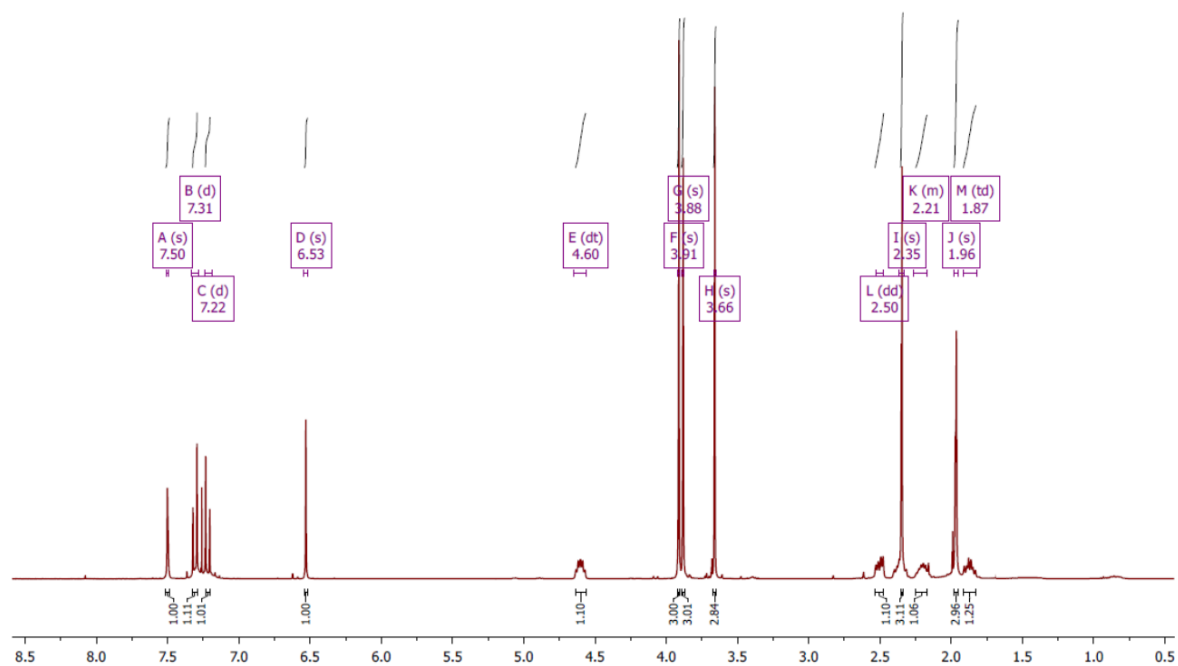


Figure S1. The ¹H NMR spectrum of **2a** in CDCl₃.

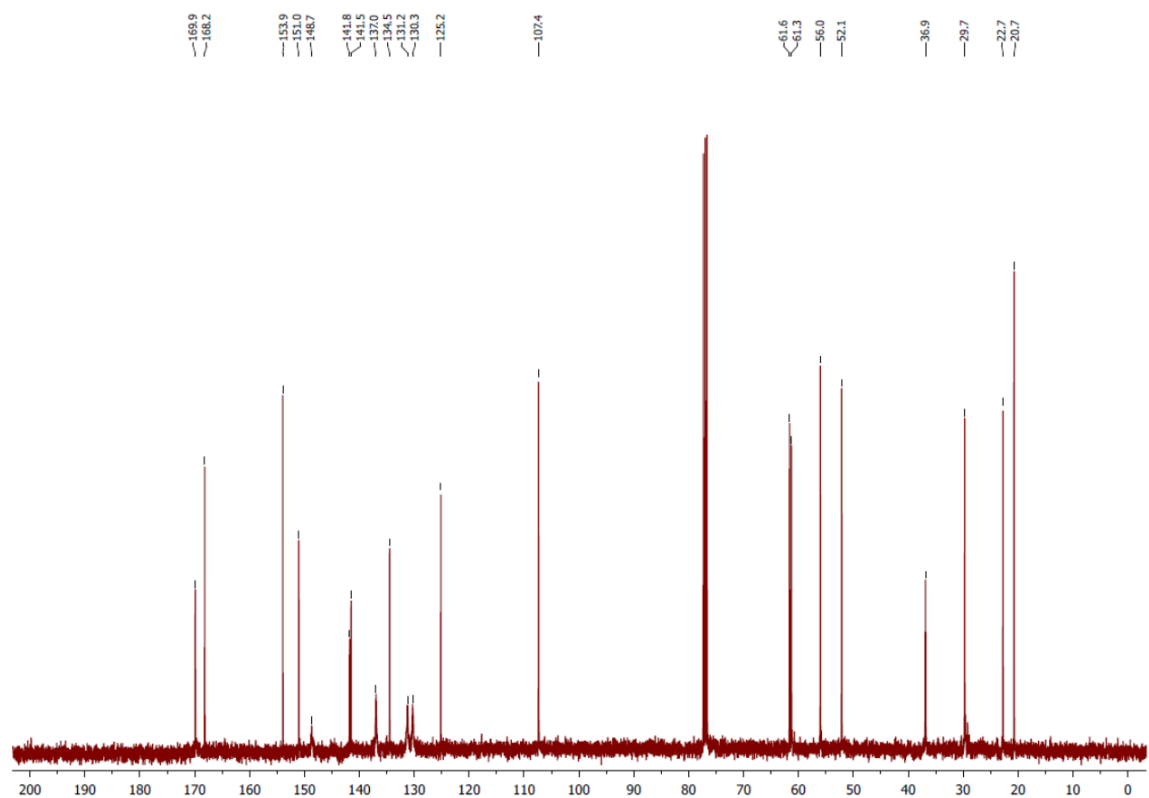


Figure S2. The ^{13}C NMR spectrum of **2a** in CDCl_3 .

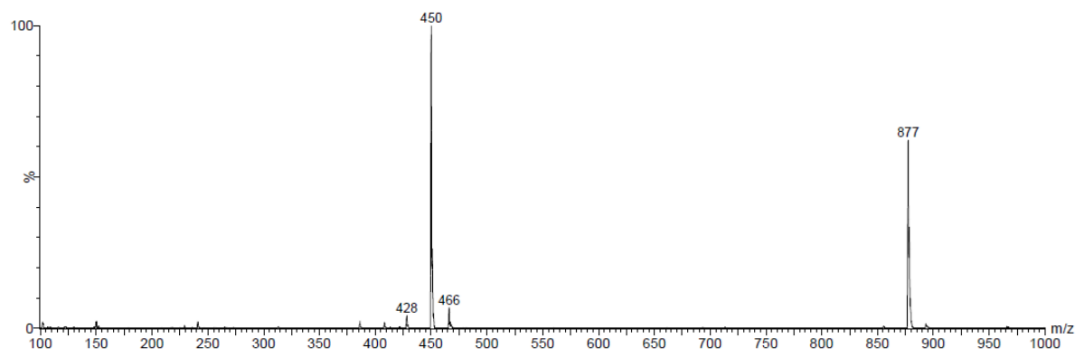
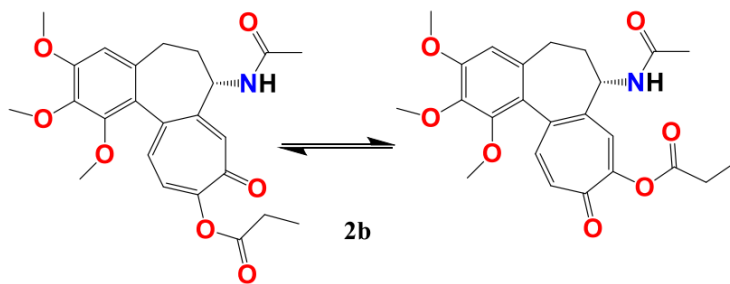


Figure S3. The ESI-MS spectrum of **2a**.



Chemical formula: $\text{C}_{24}\text{H}_{27}\text{NO}_7$, MW = 441,5 g/mol

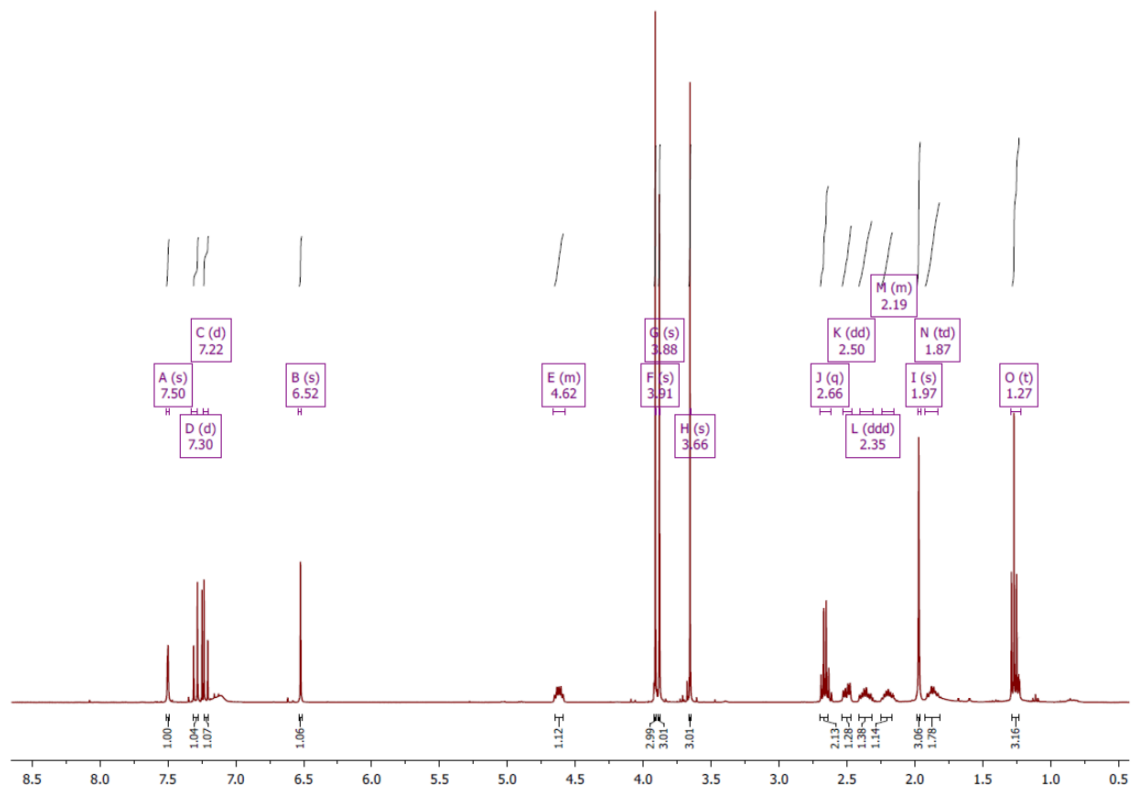


Figure S4. The ¹H NMR spectrum of **2b** in CDCl₃.

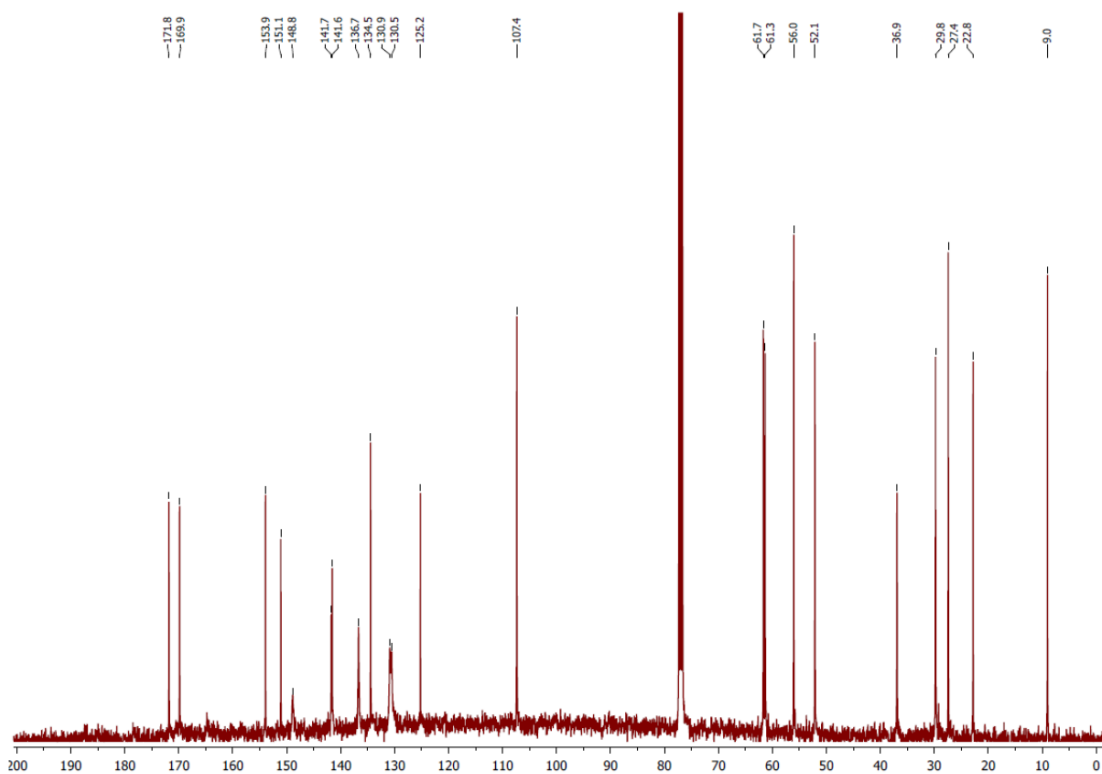


Figure S5. The ¹³C NMR spectrum of **2b** in CDCl₃.

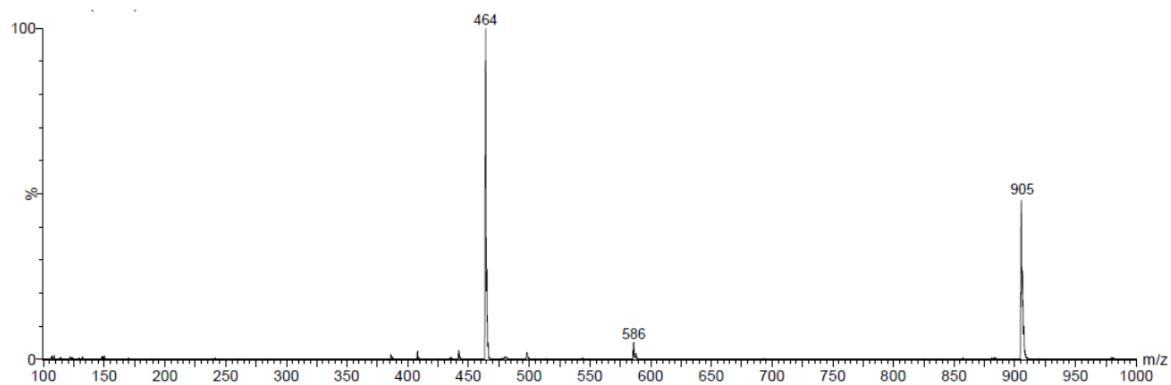
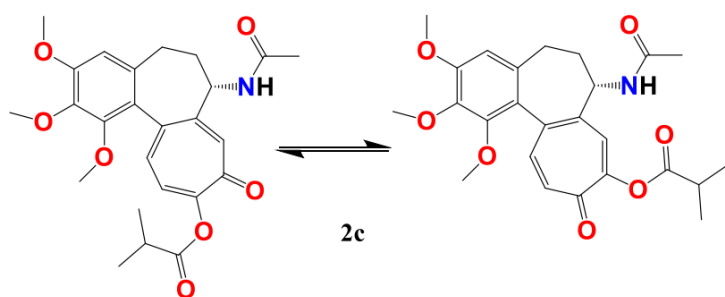


Figure S6. The ESI-MS spectrum of **2b**.



Chemical formula: $C_{25}H_{29}NO_7$, MW = 455,5 g/mol

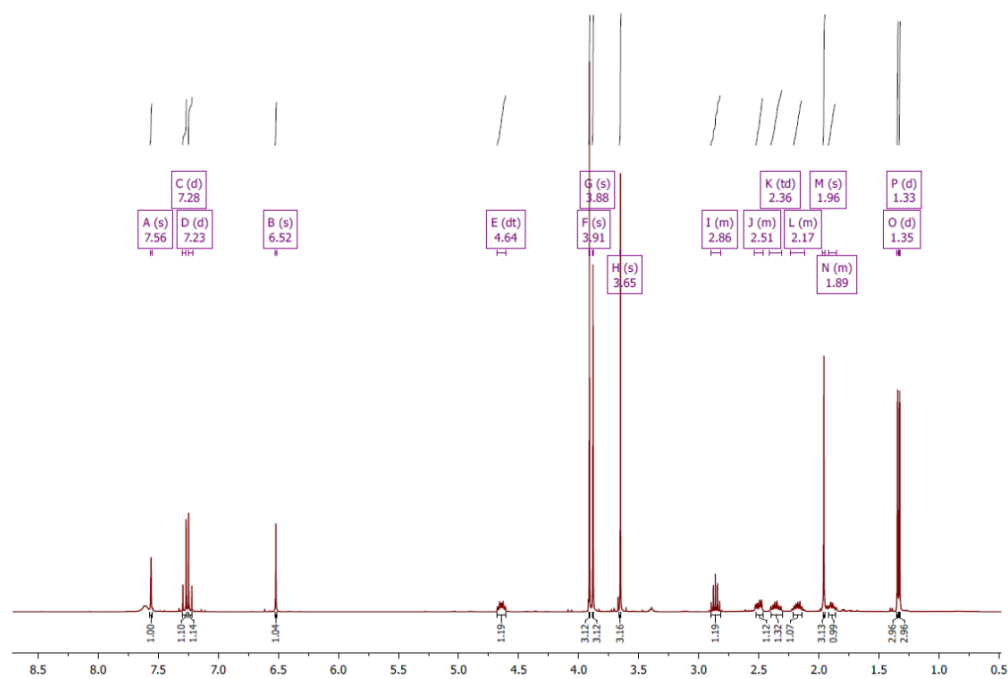


Figure S7. The 1H NMR spectrum of **2c** in $CDCl_3$.

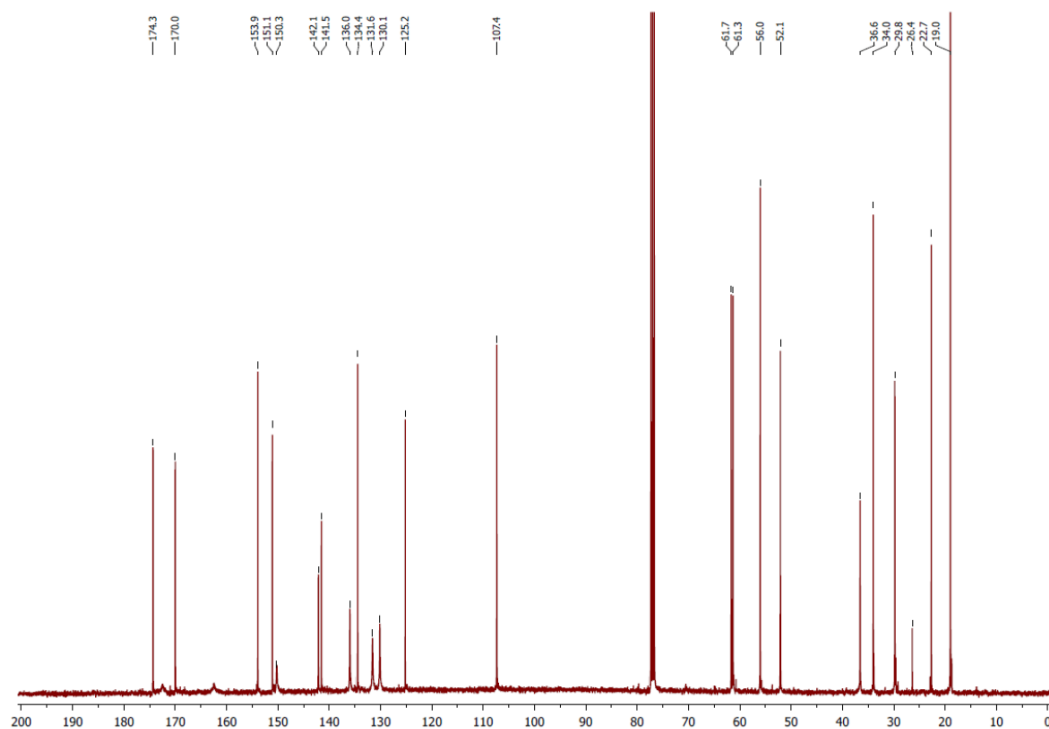


Figure S8. The ^{13}C NMR spectrum of **2c** in CDCl_3 .

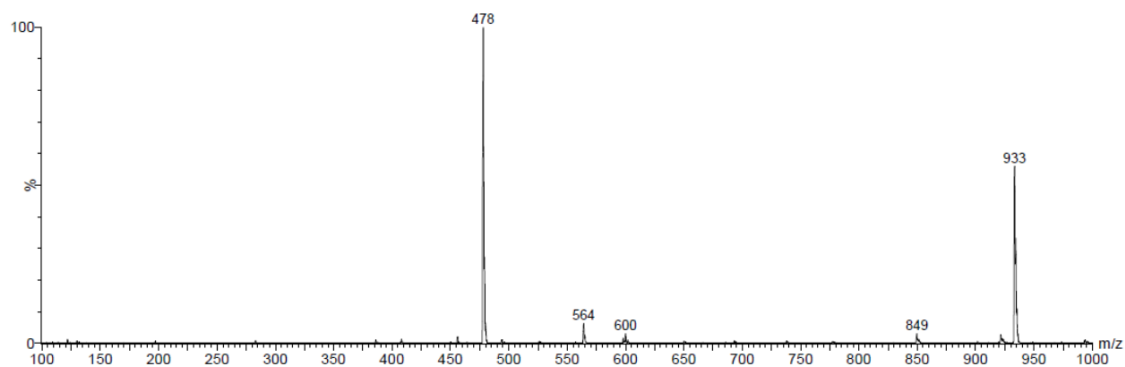
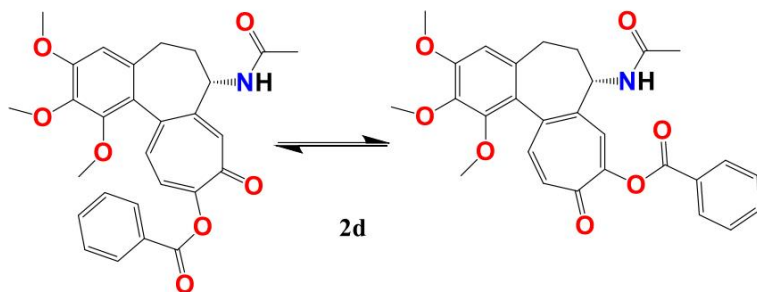


Figure S9. The ESI-MS spectrum of **2c**.



Chemical formula: $C_{28}H_{27}NO_7$, MW = 489,5 g/mol

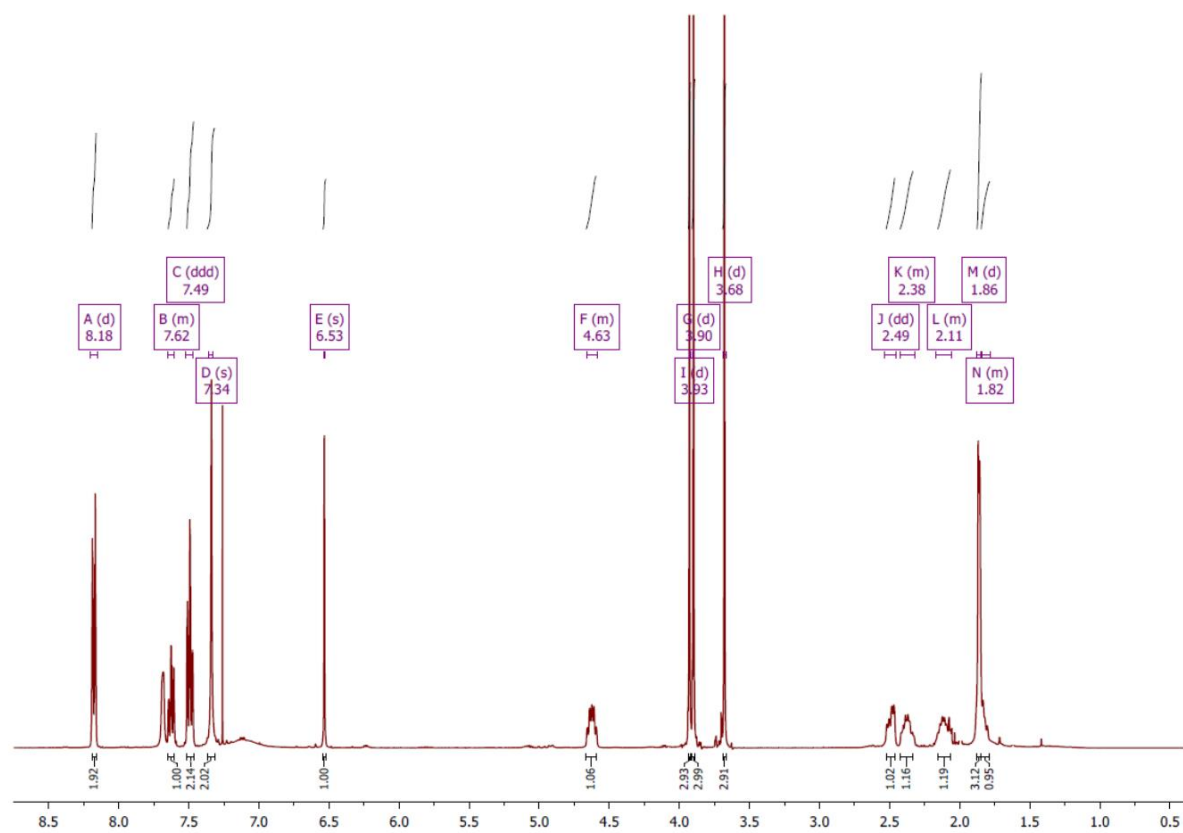


Figure S10. The ^1H NMR spectrum of **2d** in CDCl_3 .

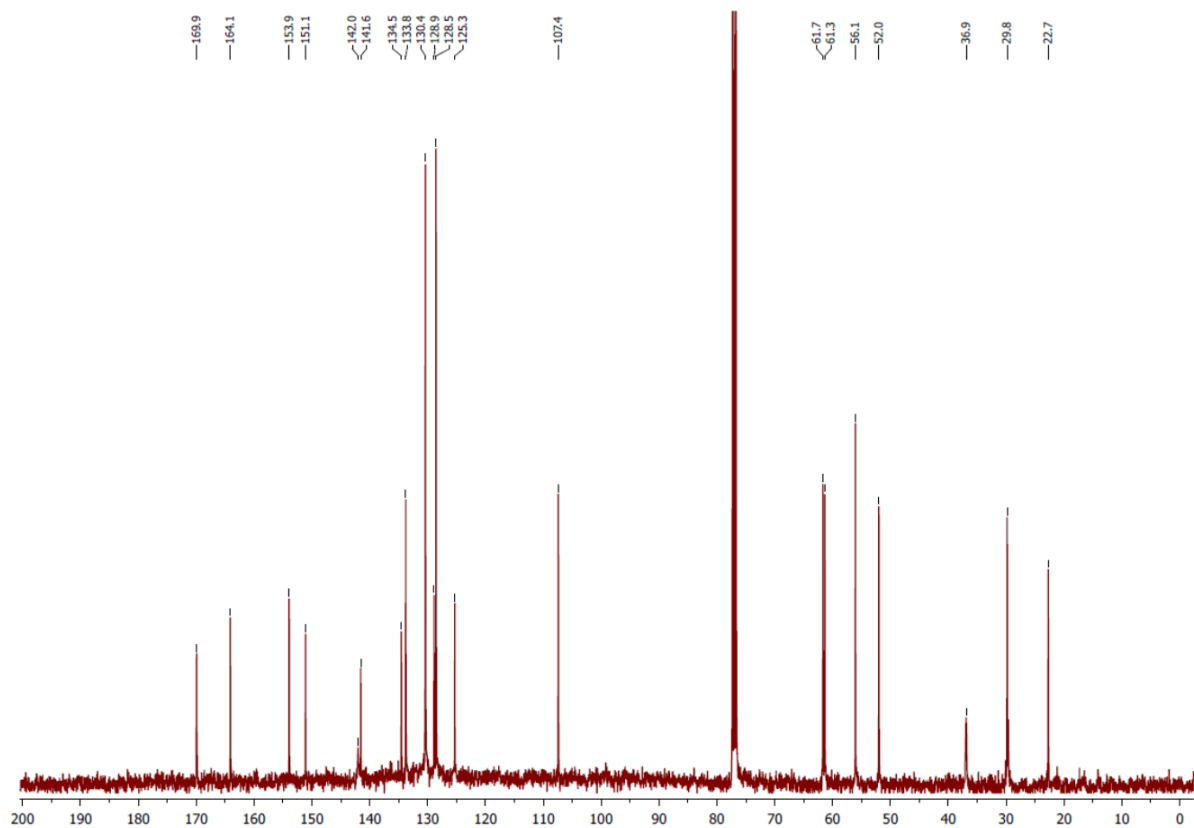


Figure S11. The ^{13}C NMR spectrum of **2d** in CDCl_3 .

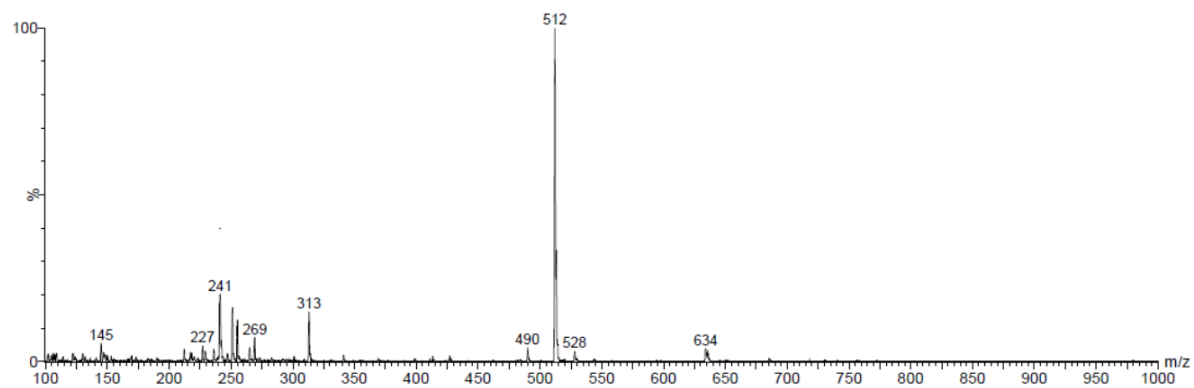
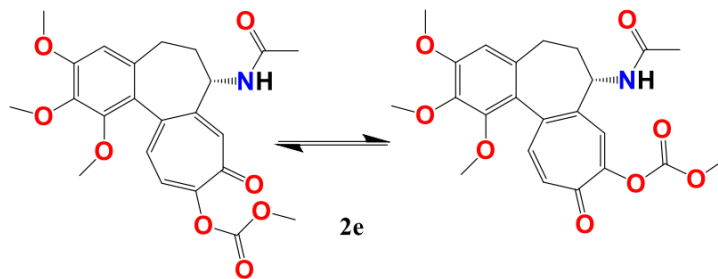


Figure S12. The ESI-MS spectrum of **2d**.



Chemical formula: $C_{23}H_{25}NO_8$, MW = 443,5 g/mol

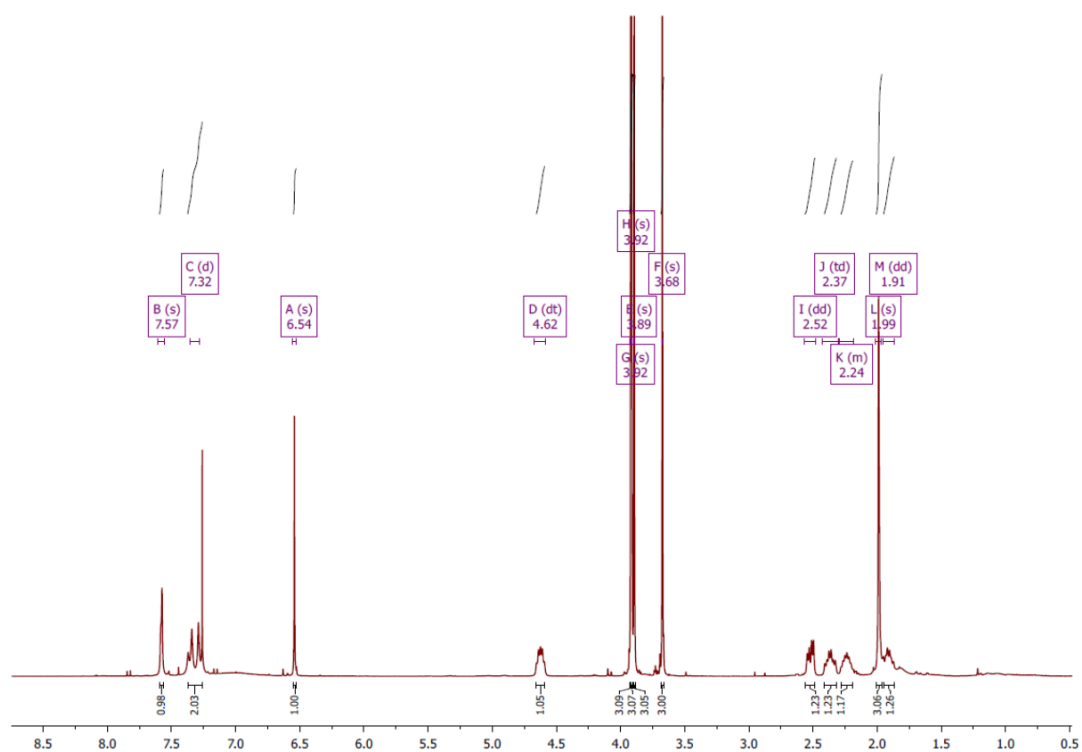


Figure S13. The 1H NMR spectrum of **2e** in $CDCl_3$.

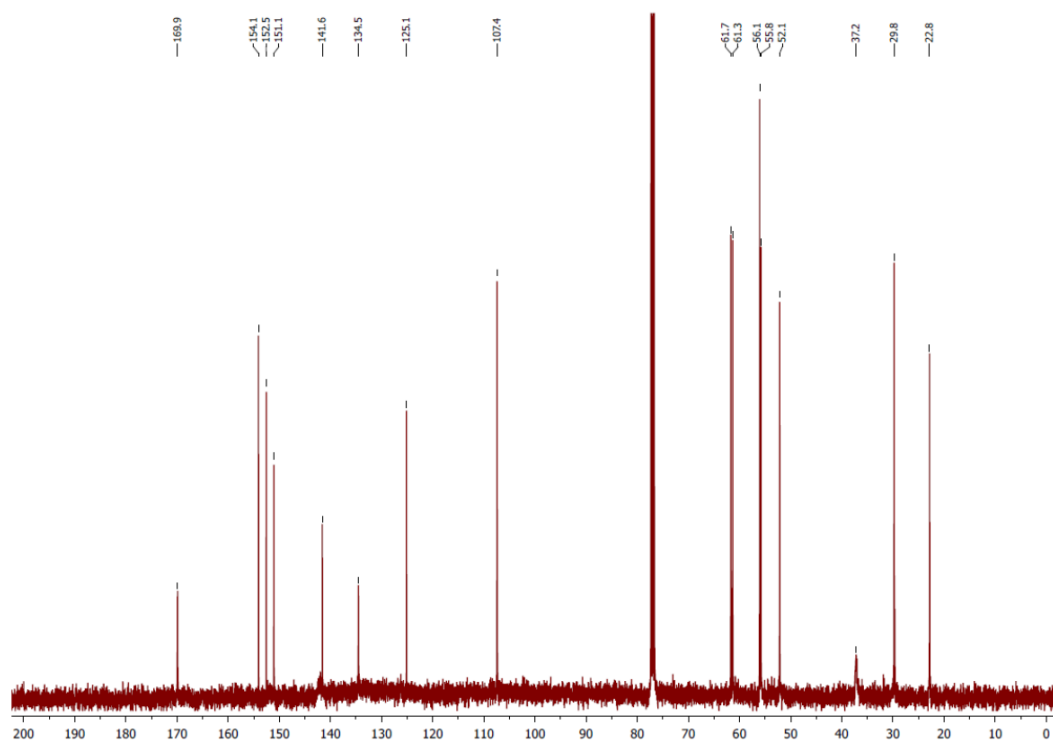


Figure S14. The ^{13}C NMR spectrum of **2e** in CDCl_3 .

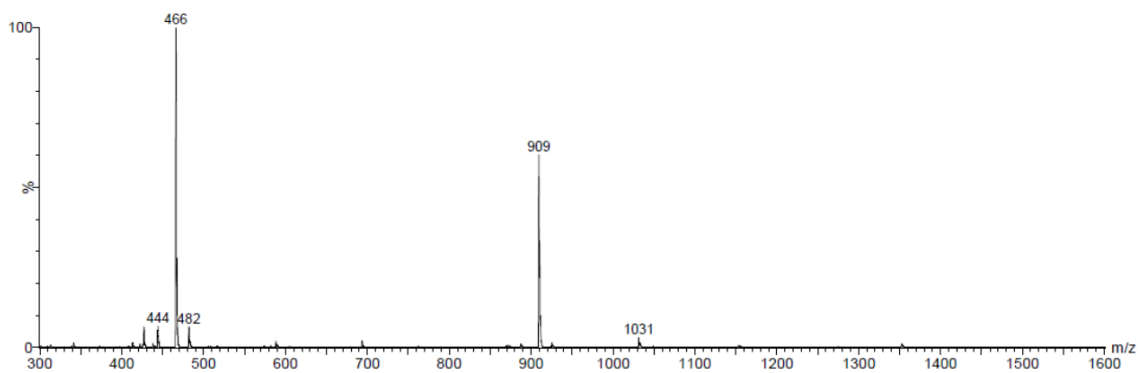
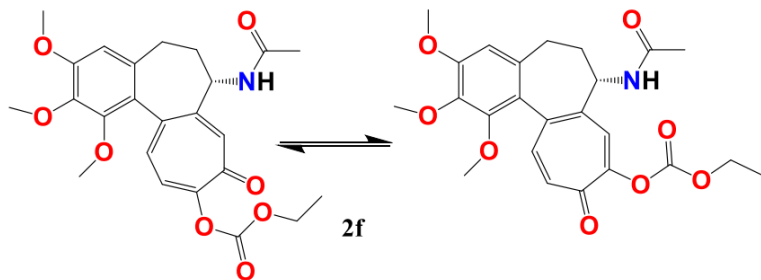


Figure S15. The ESI-MS spectrum of **2e**.



Chemical formula: $\text{C}_{24}\text{H}_{27}\text{NO}_8$, MW = 457,5 g/mol

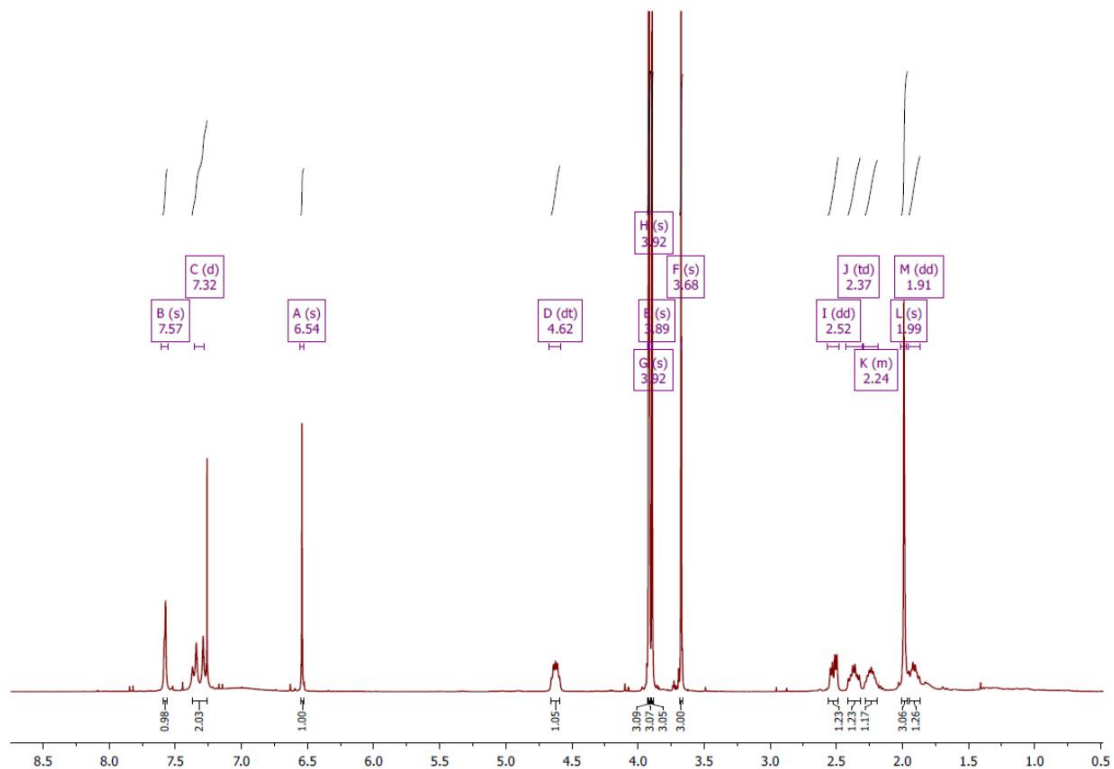


Figure S16. The ^1H NMR spectrum of **2f** in CDCl_3 .

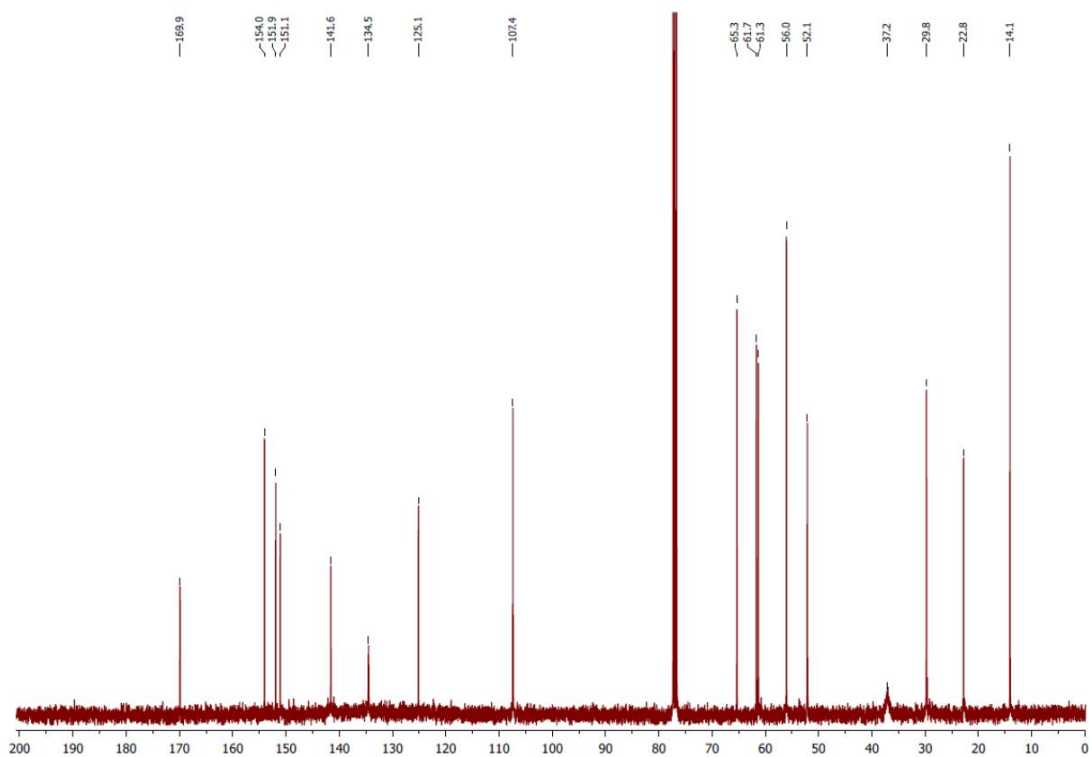


Figure S17. The ^{13}C NMR spectrum of **2f** in CDCl_3 .

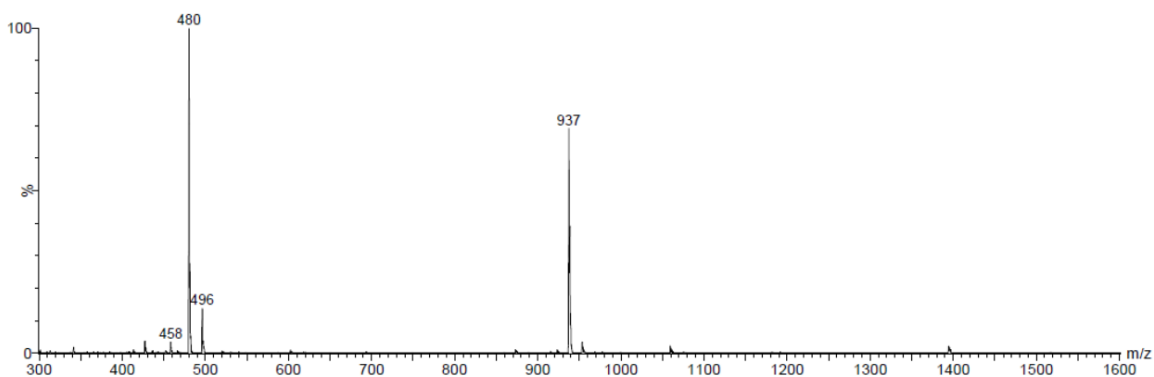
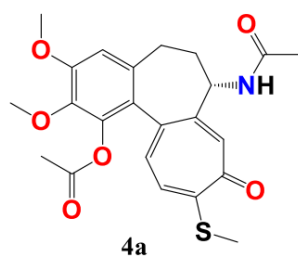


Figure S18. The ESI-MS spectrum of **2f**.



Chemical formula: $C_{23}H_{25}NO_6S$, MW = 443,5 g/mol

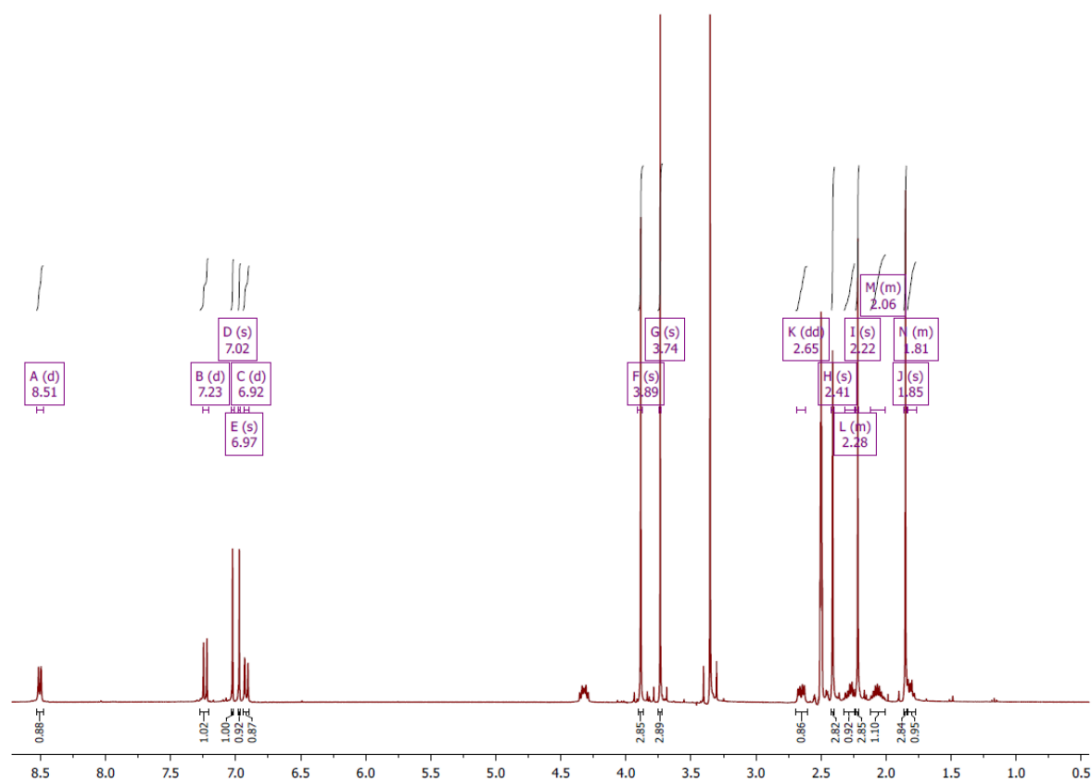


Figure S19. The 1H NMR spectrum of **4a** in $DMSO-d_6$.

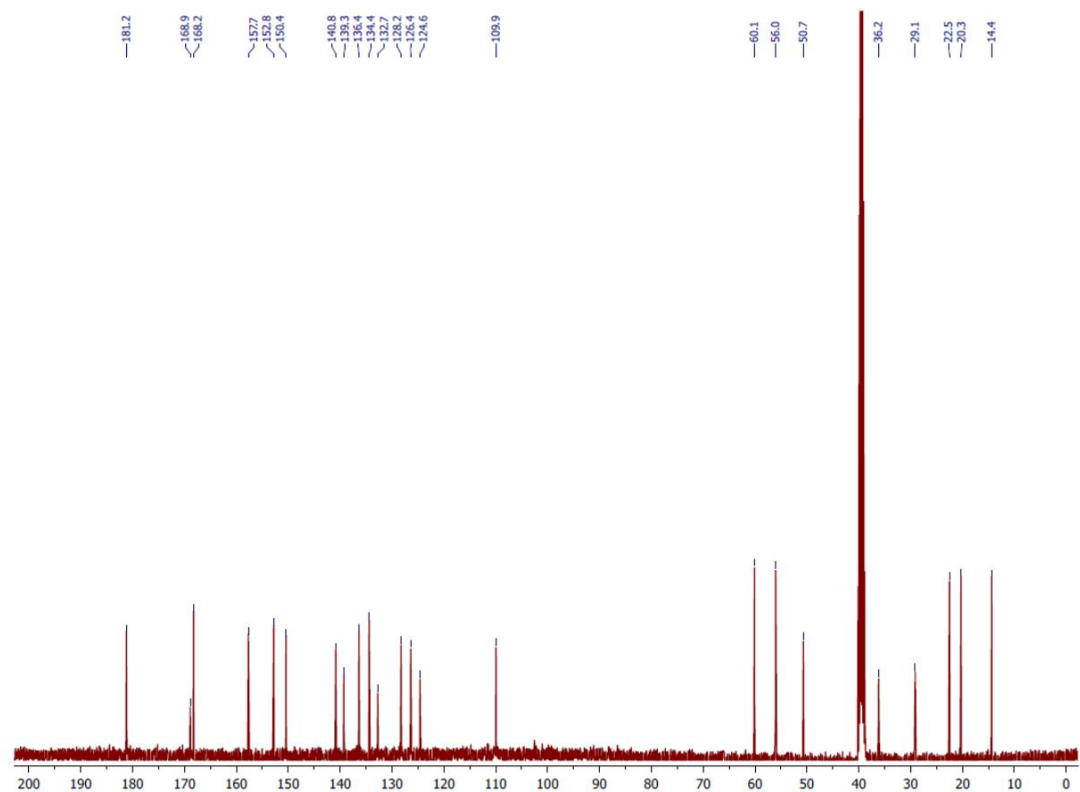


Figure S20. The ^{13}C NMR spectrum of **4a** in $\text{DMSO}-d_6$.

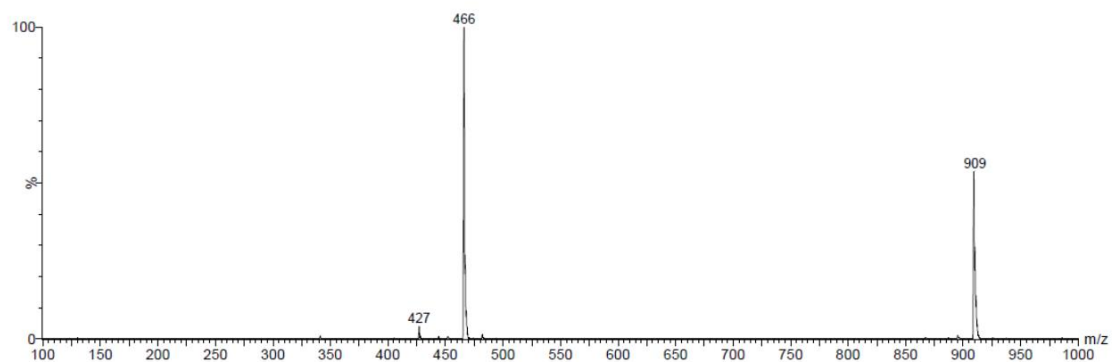
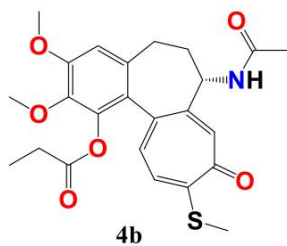


Figure S21. The ESI-MS spectrum of **4a**.



Chemical formula: $\text{C}_{24}\text{H}_{27}\text{NO}_6\text{S}$, MW = 457,5 g/mol

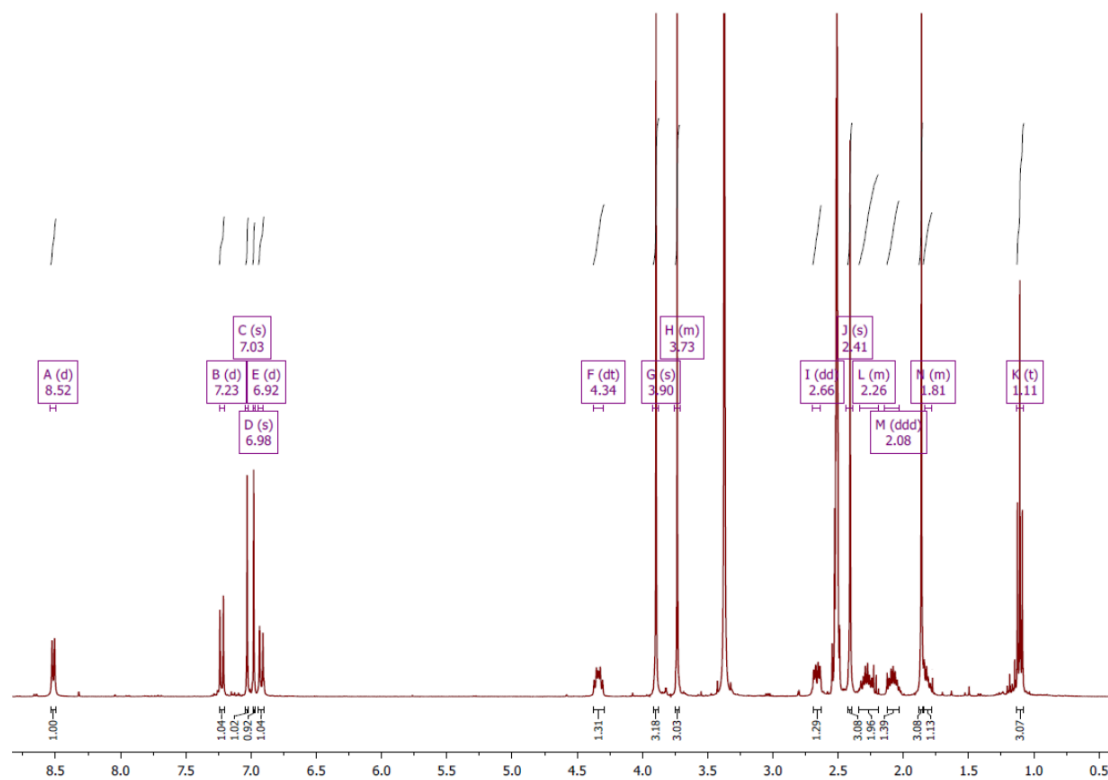


Figure S22. The ^1H NMR spectrum of **4b** in $\text{DMSO}-d_6$.

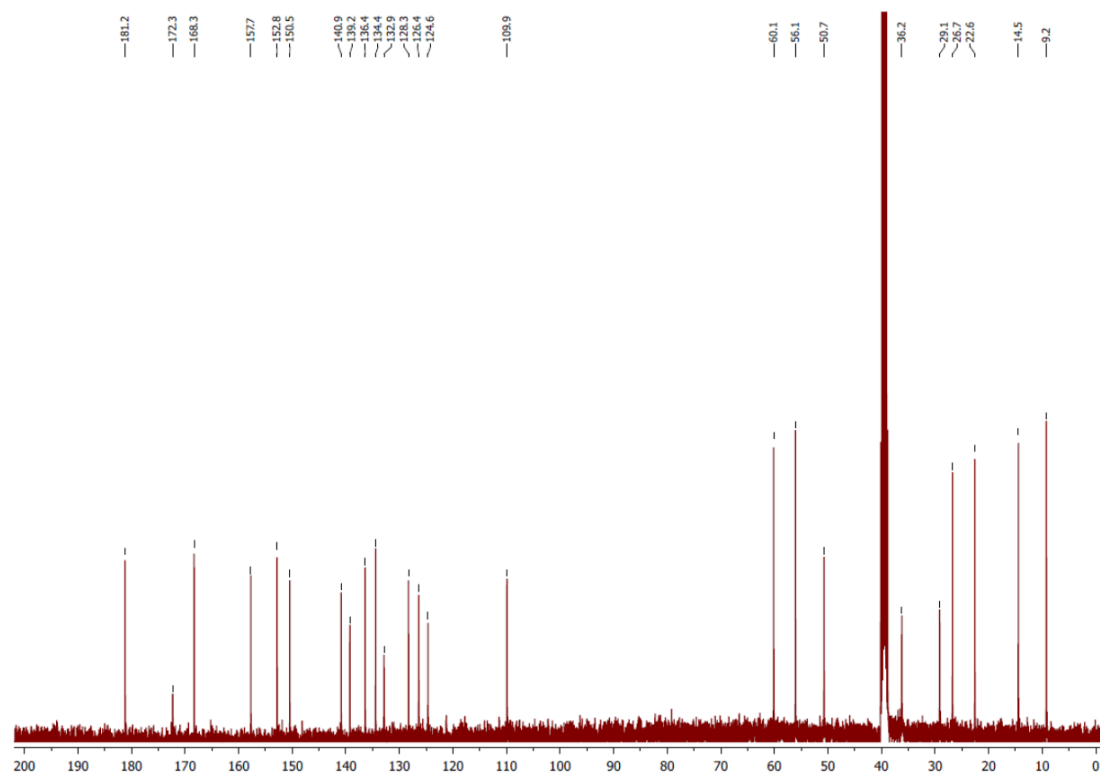


Figure S23. The ^{13}C NMR spectrum of **4b** in $\text{DMSO-}d_6$.

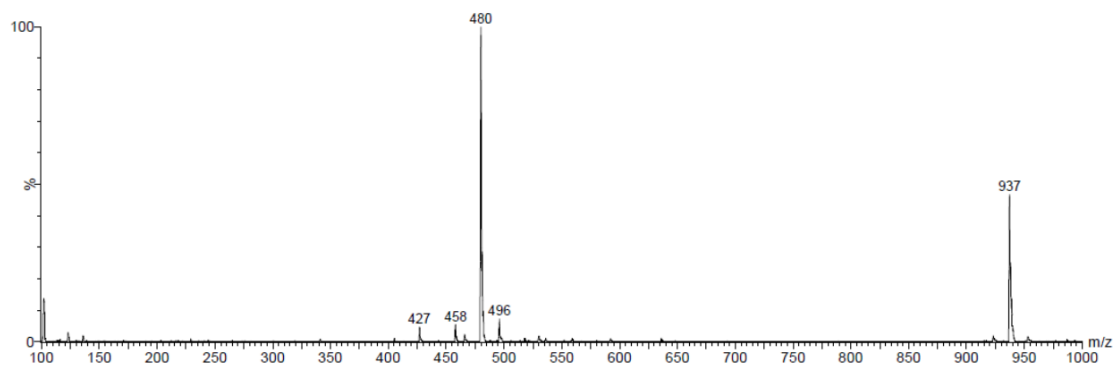
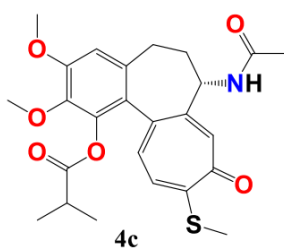


Figure S24. The ESI-MS spectrum of **4b**.



Chemical formula: $\text{C}_{25}\text{H}_{29}\text{NO}_6\text{S}$, MW = 471,6 g/mol

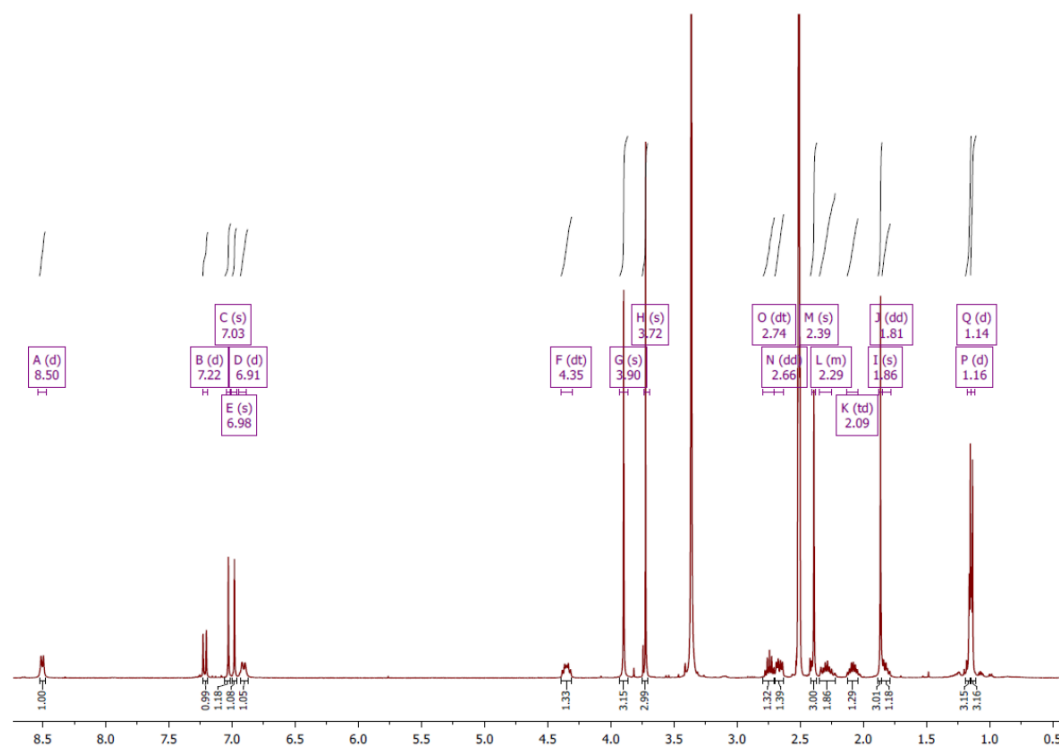


Figure S25. The ^1H NMR spectrum of **4c** in $\text{DMSO-}d_6$.

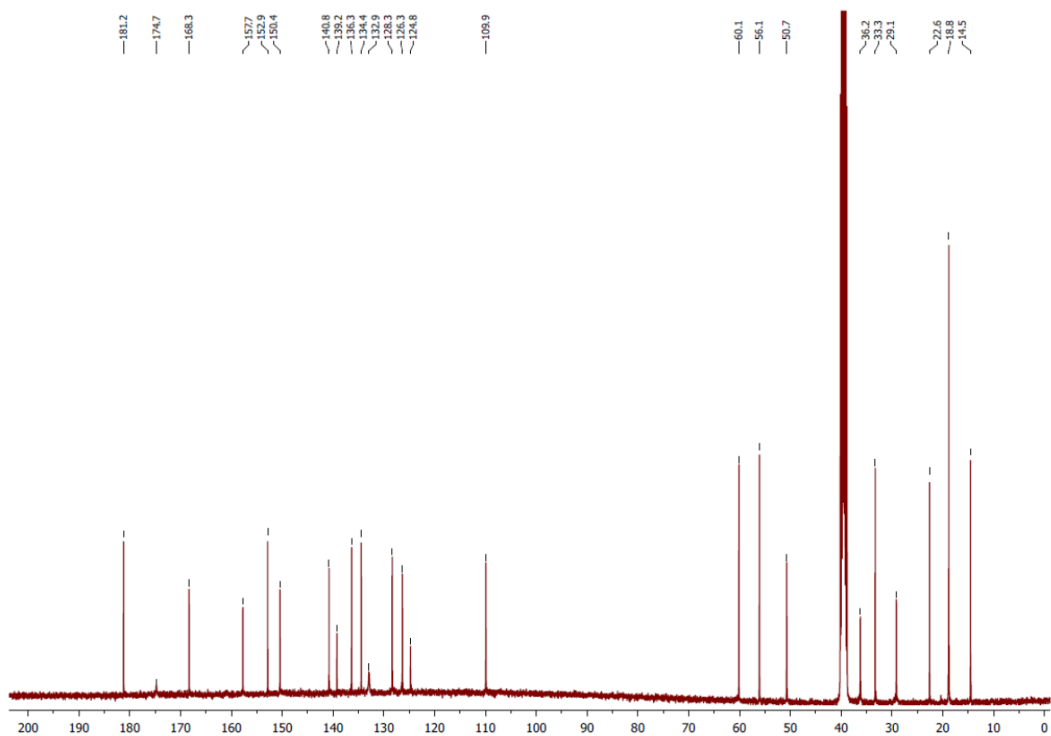


Figure S26. The ^{13}C NMR spectrum of **4c** in $\text{DMSO-}d_6$.

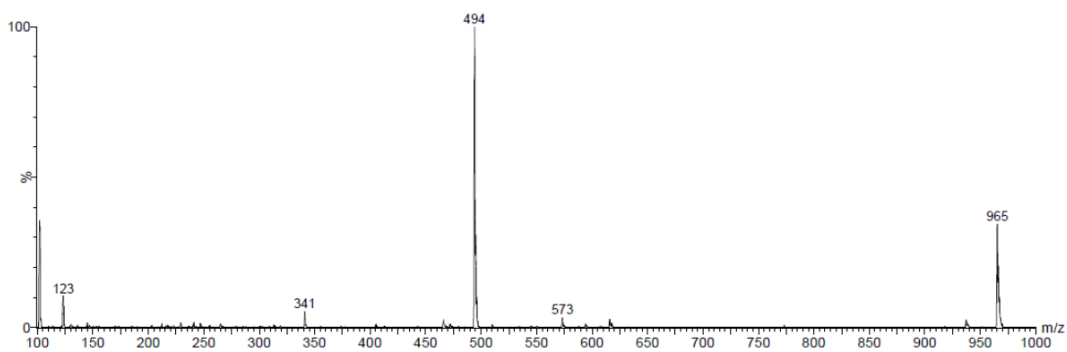
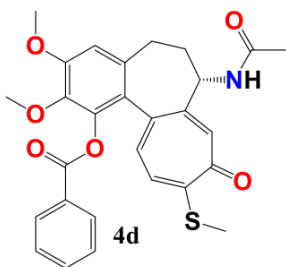


Figure S27. The ESI-MS spectrum of **4c**.



Chemical formula: $\text{C}_{28}\text{H}_{27}\text{NO}_6\text{S}$, MW = 505,6 g/mol

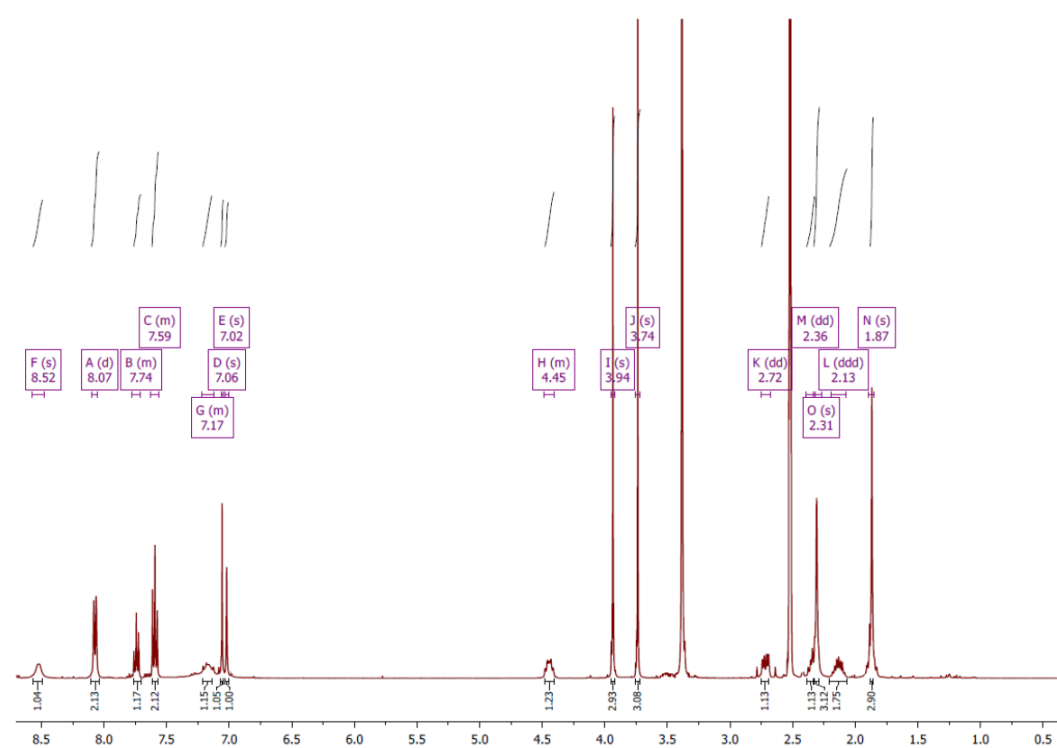


Figure S28. The ^1H NMR spectrum of **4d** in $\text{DMSO}-d_6$.

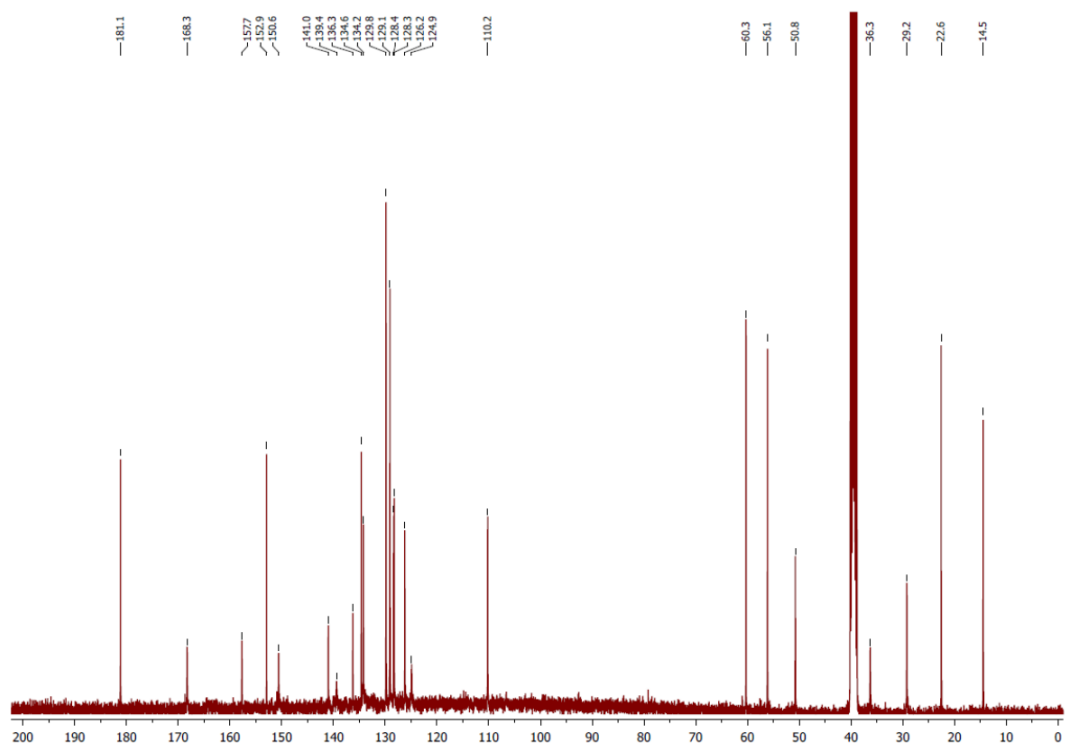


Figure S29. The ^{13}C NMR spectrum of **4d** in $\text{DMSO}-d_6$.

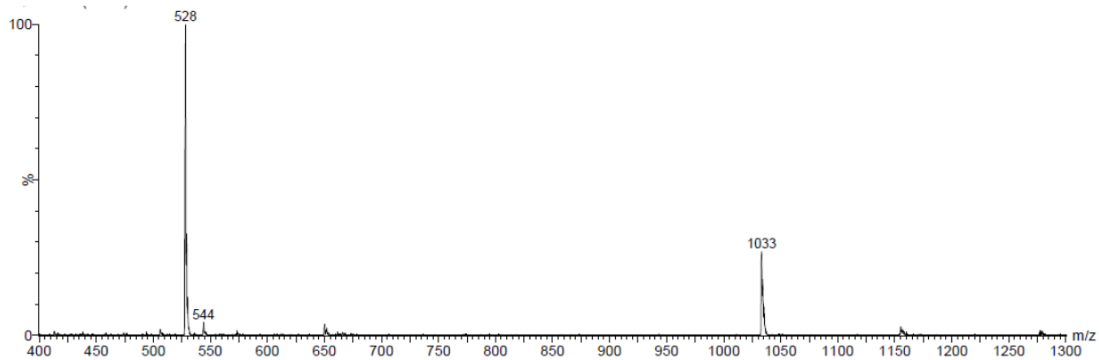
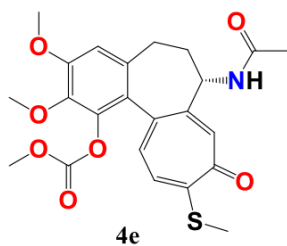


Figure S30. The ESI-MS spectrum of **4d**.



Chemical formula: $C_{23}H_{25}NO_7S$, MW = 459,5 g/mol

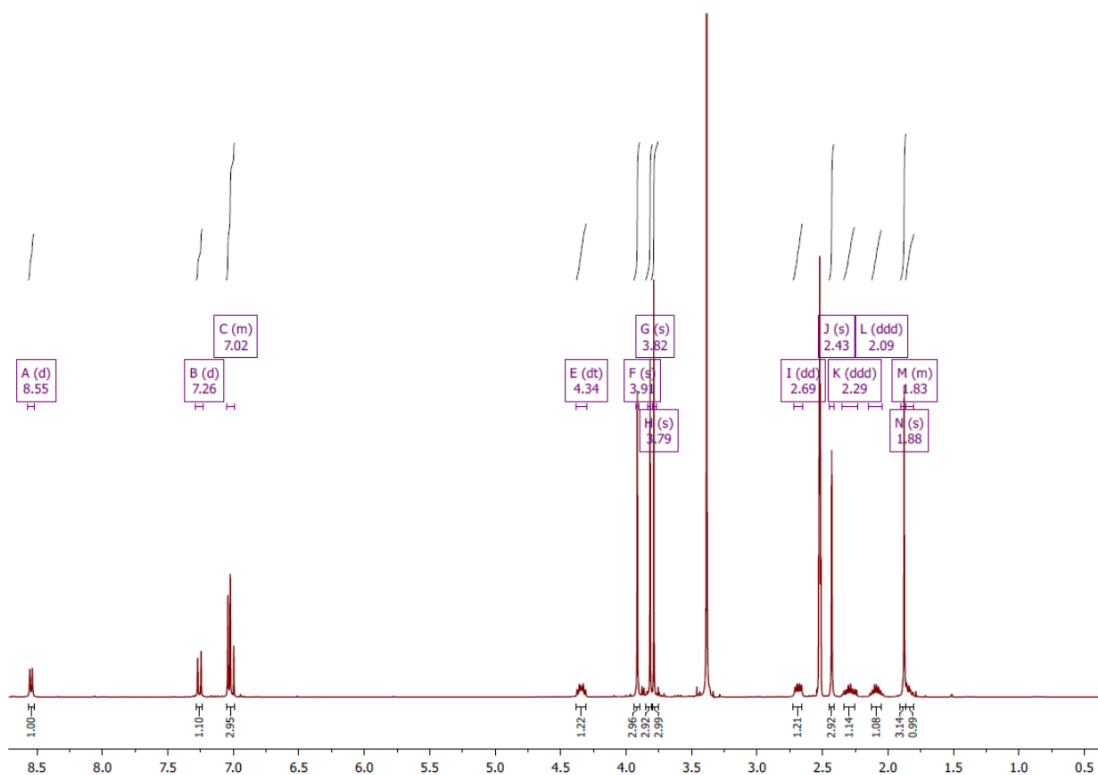


Figure S31. The 1H NMR spectrum of **4e** in $DMSO-d_6$.

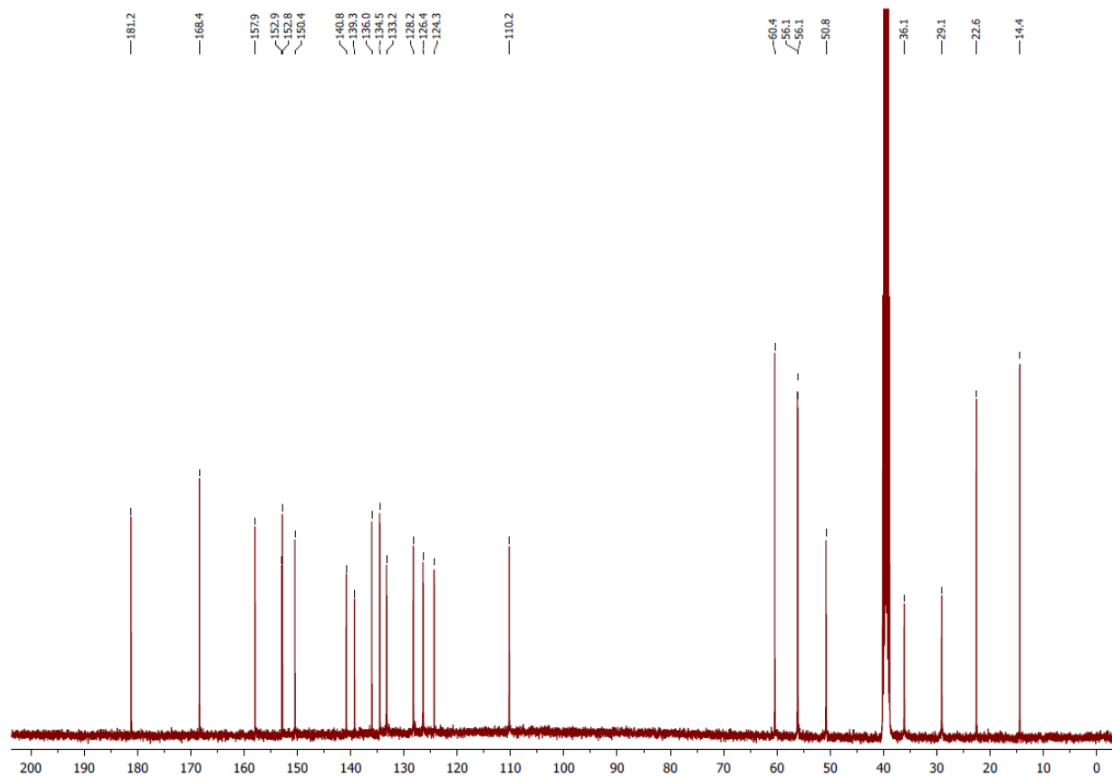


Figure S32. The ^{13}C NMR spectrum of **4e** in $\text{DMSO}-d_6$.

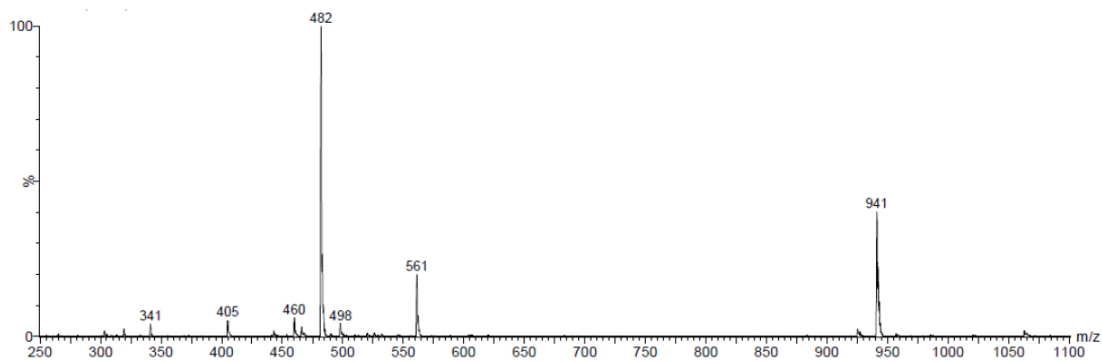
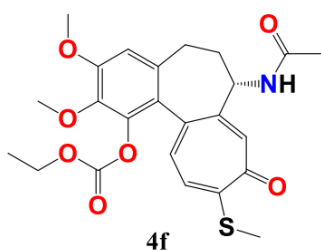


Figure S33. The ESI-MS spectrum of **4e**.



Chemical formula: $\text{C}_{24}\text{H}_{27}\text{NO}_7\text{S}$, MW = 473,5 g/mol

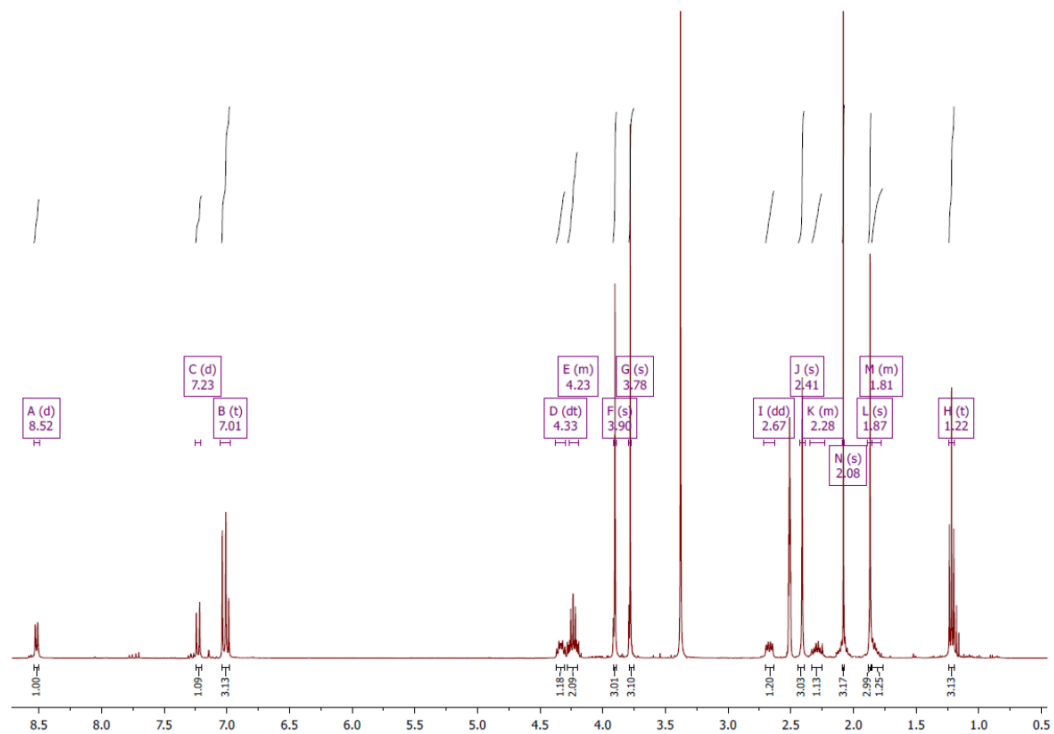


Figure S34. The ^1H NMR spectrum of **4f** in $\text{DMSO}-d_6$.

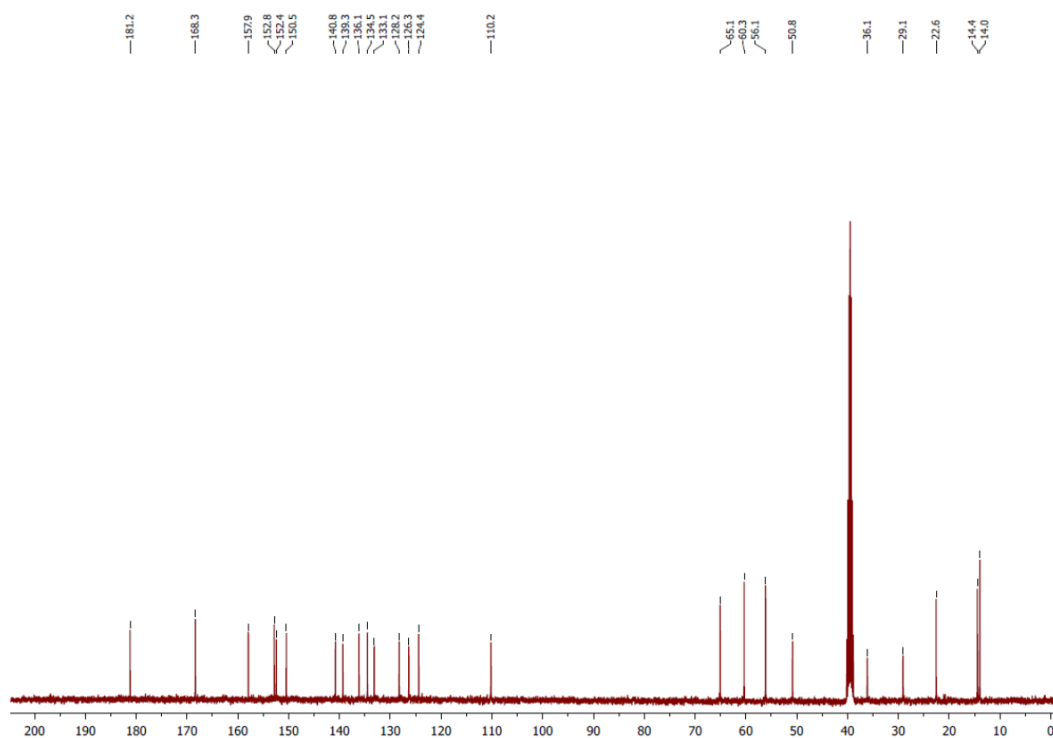


Figure S35. The ^{13}C NMR spectrum of **4f** in $\text{DMSO}-d_6$.

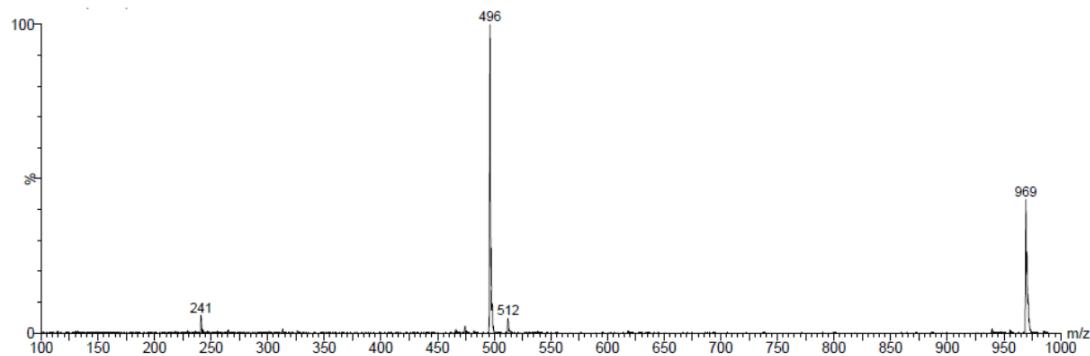


Figure S36. The ESI-MS spectrum of **4f**.

1. Skehan, P. *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**, 1107–1112 (1990).
2. Nevozhay, D. Cheburator software for automatically calculating drug inhibitory concentrations from in vitro screening assays. *PLoS One* **9**, (2014).
3. Xcalibur CCD System. CrysAlisPro Software System, version 1.171.33; Oxford Diffraction Ltd.: Wrocław, Poland. (2009).
4. Sheldrick, G. M. A short history of SHELX. *Acta Crystallographica Section A: Foundations of Crystallography* **64**, 112–122 (2008).
5. Sheldrick, G. M. Crystal structure refinement with SHELXL. *Acta Crystallogr. Sect. C Struct. Chem.* **71**, 3–8 (2015).
6. Dolomanov, O. V., Bourhis, L. J., Gildea, R. J., Howard, J. A. K. & Puschmann, H. OLEX2 : a complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.* **42**, 339–341 (2009).
7. Shi, Q., Verdier-Pinard, P., Brossi, A., Hamel, E. & Lee, K. H. Antitumor Agents-CLXXV. Anti-tubulin action of (+)-thiocolchicine prepared by partial synthesis. *Bioorganic Med. Chem.* **5**, 2277–2282 (1997).

PUBLIKACJA V wraz z suplementem

Dominika Czerwonka, Szymon Sobczak, Tomasz Pędziński, Ewa Maj, Joanna
Wietrzyk, Lech. Celewicz, Andrzej Katrusiak, Adam Huczyński

“Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives”

Journal of Organic Chemistry 2021, 86, 16, 11029–11039

Reprinted with permission from *J. Org. Chem.* 2021, 86, 16, 11029–11039, Publication Date: December 22, 2020,
<https://doi.org/10.1021/acs.joc.0c02507>. Copyright © 2020 American Chemical Society

Photoinduced Skeletal Rearrangement of *N*-Substituted Colchicine Derivatives

Dominika Czerwonka, Szymon Sobczak, Tomasz Pędziński, Ewa Maj, Joanna Wietrzyk, Lech Celewicz, Andrzej Katrusiak, and Adam Huczyński*

Cite This: *J. Org. Chem.* 2021, 86, 11029–11039

Read Online

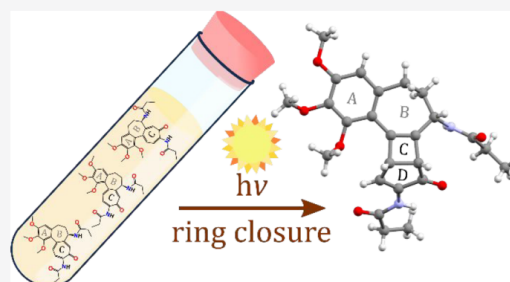
ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Colchicine is an active pharmaceutical ingredient widely used for treating gout, pericarditis, and familial Mediterranean fever with high antimitotic activity. The photoisomerization of colchicine deactivates its anti-inflammatory and antimitotic properties. However, despite numerous reports on colchicine derivatives, their photostability has not been investigated in detail. This report reveals the effects of UV-induced rearrangement on the structure and reports the biological activity of new *N*-substituted colchicine derivatives.



Many organic compounds, including drugs, exhibit low photostability. Some compounds decompose upon exposition to light in minutes, while it takes weeks or months for others. The ultraviolet component of sunlight or of artificial light affects the chemical structure of compounds and the physicochemical properties of the products, such as their color, solubility, or viscosity.¹ Most importantly, the photodecomposed drugs lose therapeutic activity and can cause severe side effects.² Transparent plastic packages or amber and brown glass vials offer only a little protection from UV radiation in the case of some highly photolabile compounds.³ The photodegradation often limits the application or even eliminates highly active drugs, so photostability has become an important parameter in drug characterization.

Colchicine (Figure 1), a pseudoalkaloid produced by *Colchicum autumnale*, is used as the first-line therapy for gout, pericarditis, and familial Mediterranean fever.^{4–7} Colchicine is also known for its high capability of binding to the tubulin in rapidly dividing cancer cells, which inhibits microtubule polymerization leading to mitosis arrest and finally to cell apoptosis.^{8–10} However, the severe toxicity and photosensitivity limit the applications of this compound,^{11–13} although its numerous new derivatives are reported each year.^{12–27} When colchicine is exposed to sunlight, it transforms to a mixture of α -, β -, and γ -lumicolchicine, where β - and γ -lumicolchicine are stereoisomers and α -lumicolchicine is a cyclobutane dimer of β -lumicolchicine (Figure 1).^{28,29} This process highly depends on the solvent polarity and can be connected to the formation of a complex with a solvent in the ground state.^{30,31}

Colchicine photoisomers differ from the parent compound in physical and biological properties. The rearrangement of methyl

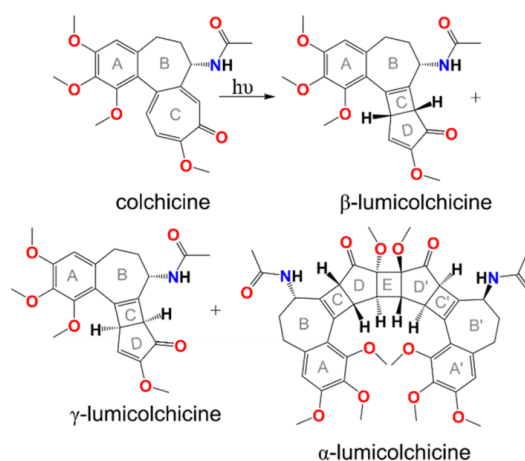


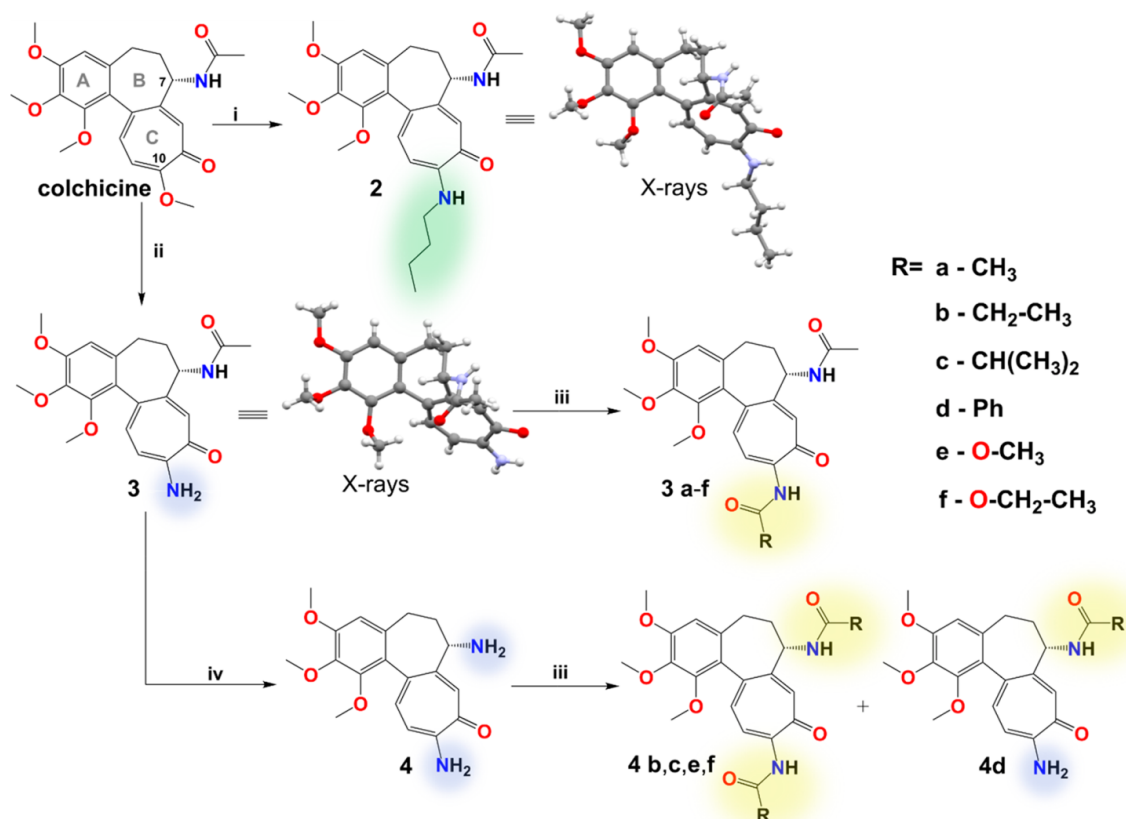
Figure 1. Structural formula of colchicine and its photoisomers: α -, β and γ -lumicolchicines.

Special Issue: Natural Products: An Era of Discovery in Organic Chemistry

Received: October 25, 2020

Published: December 22, 2020



Scheme 1. Syntheses of Novel Colchicine Derivatives^a

^aReagents and conditions: (i) *n*-butylamine, CH₃CN, reflux, 24 h; (ii) ammonia solution, CH₃CN, reflux, 24 h; (iii) DCM, Et₃N, respective acyl chloride/chloroformate, 0 °C → room temperature, 24 h; (iv) 4M HCl, MeOH, 90 °C, 72 h; the molecular structures of 2 and 3 are illustrated by their ball-and-stick models determined by X-ray diffraction, and denoted "X-rays".

tropolone ring C of colchicine to a conjugated 4- and 5-membered ring system prevents tubulin binding and mitosis arrest, which deactivates the therapeutic potency.^{32,33} This is important from a clinical point of view, as considerable amounts of lumicolchicine were found in clear-glass ampules filled with colchicine for intravenous injections.³² Studies on colchicine photoisomerization have been reported,^{30,32–34} but the influence of different substituents on colchicine photosensitivity has not been investigated. It has been reported that the colchicine (C10-demethylated colchicine analogue) undergoes photoisomerization similarly as colchicine, whereas thiocolchicine (C10-thiomethyl analogue) is resistant to UV irradiation. This information suggests that photostability of colchicine derivatives depends on the C-ring substituents.^{29,34–36}

So far, the chemical modifications of colchicine have been mostly aimed at reducing its toxicity and increasing its bioavailability, while the improvement of its photostability has been neglected. Herein, we present the mechanism of the sun or/and UV irradiation induced rearrangement for a series of mono- and double-modified *N*-substituted colchicines at the C7 and C10 positions, as well as the analysis of biological activity of these new derivatives. Furthermore, we provide for the first time molecular structures of β -lumicolchicine and two *N*-substituted β -lumiderivatives, determined by single-crystal X-ray diffraction.

RESULTS AND DISCUSSION

The synthetic route for novel *N*-substituted colchicine derivatives is presented in Scheme 1. Compounds 2 and 3 were obtained quantitatively by refluxing colchicine with an excess of *n*-butylamine or aqueous ammonia solution (i, ii), respectively. The structures of these compounds were verified by the disappearance of the characteristic signal assigned to the C10 methoxy group at 56.5 ppm in the ¹³C NMR spectrum of colchicine as well as by the X-ray analysis of the obtained single crystals from the solutions of 2 and 3 in chloroform/acetone 1:1 mixture (Scheme 1). The hydrolysis of 3 with 4 M HCl (iv) yielded compound 4 with two free amino groups at C7 and C10, traced by the disappearance of C7 acetyl group signals at 168.4 and 22.6 ppm present in the ¹³C NMR spectra of colchicine. Single-modified derivatives 3a–f and 4d, as well as double-modified derivatives 4b,c,e,f, were obtained by treating compounds 3 and 4 with appropriate acyl chlorides or chloroformates (iii). In the case of 4d, the substituent was introduced only at the C7 position, while the amino group at the C10 remained free. The synthesized compounds were characterized by the ¹³C NMR spectra, where one additional signal assigned to the carbonyl atom was observed for 3a–f and 4d, while two additional signals were noted for compounds 4b,c,e,f.

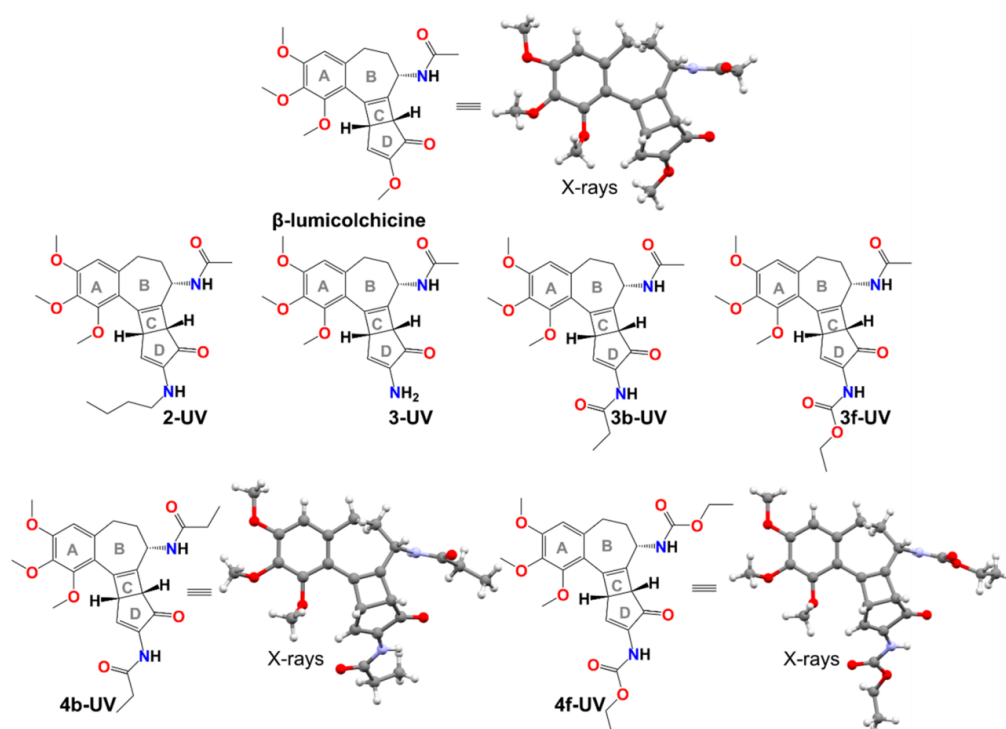


Figure 2. Structures of obtained β -lumicolchicine and its N-substituted derivatives (2-UV, 3-UV, 3b-UV, 3f-UV, 4b-UV, 4f-UV); the molecular structures of β -lumicolchicine, 4b-UV, and 4f-UV are illustrated by their ball-and-stick models, determined by X-ray diffraction, and denoted “X-rays”. The single crystals were obtained from the solutions of β -lumicolchicine, 4b-UV, and 4f-UV in chloroform/acetone 1:1 mixture.

Single crystals suitable for X-ray diffraction were obtained from the solutions of 4b and 4f in a chloroform/acetone 1:1 mixture. Surprisingly, the 5-day long crystallizations in transparent glass tubes led to photoproduct precipitation. The structures of the recovered crystals were no longer those of compounds 4b and 4f, but their photoisomers 4b-UV and 4f-UV (Figure 2). Remarkably, indirect sunlight was sufficient to induce migration of the C8–C14 and C12–C13 double bond and subsequent rearrangement of the tropolone ring C into 4- and 5-membered rings. Compounds 4b-UV and 4f-UV remain chiral and crystallize in acentrosymmetric space groups $P2_12_12_1$ and $P2_1$ respectively.

Photoinduced rearrangement of colchicine can lead to two possible stereochemical forms, β - and γ -lumicolchicine (Figure 1). Two stereoisomeric forms are possible, with the C7-substituent and the five-membered ring either in cis or trans configuration to each other, across the system of the four-membered ring nearly coplanar with rings A and B.³⁷ Only in the cis isomer of β -lumicolchicine, the intramolecular H-bond can link the carbonyl group of ring D with the amide or carbamate group of ring B in 4b-UV or 4f-UV.³⁷ In γ -lumicolchicine, this intramolecular H-bond is not possible. In the crystal structure of 4b-UV, the H-bond between carbonyl O4 and amide N1 is 3.309 Å long; in 4f-UV the H-bond of carbonyl O4 involves both N1 and O6 atoms from the carbamate substituent, and the O4...N1 and O4...O6 distances are 3.309 and 3.264 Å, respectively. As expected, the light-induced rearrangement retains the S-configuration at C7 but differentiates the photoproducts due to the bridging modes in tropolone ring C.

In order to obtain more quantitative description of photo-rearrangement of colchicine derivatives, we applied a high-pressure mercury lamp for illuminating the methanol solutions of compounds 4b and 4f. Reactions were carried out until the disappearance of the starting material (SM) on the TLC plate, followed by a change in the illuminated solution color from light-yellow to dark-brown or green. This method is convenient for measuring the time required for the full decay of the starting material. Hence, the exposure times in Table 1 correspond to the full conversions. The full conversion of 4b into 4b-UV was observed after 9 h, while the reaction from 4f to 4f-UV took only 4 h.

To confirm our observations, we decided to further illuminate the methanol solutions of derivatives 2, 3, 3b, and 3f. The illumination required for the photoisomerization of mono-modified 3b and 3f, bearing the same substituents at C10 as 4b and 4f, but with the acetamide group at C7, differed significantly.

Table 1. Illumination Times and Isolation Yields of Novel β -Lumidervatives of Colchicine

starting material	exposure time (h)	isolation yield (%)
colchicine	4	44
2	6	37
3	6	61
3b	12	41
3f	4	52
4b	9	33
4f	4	31

The conversion of **3b** to **3b-UV** required 12 h, while the reaction of **3f** to **3f-UV** took only 4 h, similarly as the **4f** $\rightarrow$ **4f-UV** reaction. The 3-times longer reaction time and longer irradiation for **3b** $\rightarrow$ **3b-UV**, compared to **3f** $\rightarrow$ **3f-UV**, is likely to be related to the effect of the amide group at C10 stabilizing the tropolone ring C. The irradiation of compounds **2** and **3**, with *n*-butylamine and free amine group at C10, respectively, took 6 h in both cases, in contrast to the required conversion time of 4 h for unmodified colchicine. The exposure times of starting materials and isolation yields for all the above-mentioned reactions are collected in Table 1.

To elucidate the mechanism of the rearrangement of *N*-substituted colchicine derivatives, we conducted additional time-resolved photochemical studies. The femtosecond flash-photolysis technique allowed observation of excited singlet-state transient absorption spectra ($S_1 \rightarrow S_n$, $S_2 \rightarrow S_n$) and excited-state dynamics ($S_1 \rightarrow S_0$) of colchicine derivatives in MeOH. The transient absorption spectra of MeOH solutions of **2** and **3** were recorded at room temperature, pumped at 330 nm, and probed by a delayed femtosecond pulsed light continuum (see Supporting Information for the experimental details). As can be seen in Figure 3, the spectra of compound **2** at different time delays were found to be a combination of ground-state bleaching, excited state $S_1 \rightarrow S_n$ and $S_2 \rightarrow S_n$ transient absorption, with some minor contribution of stimulated emission. The excited-state dynamics have been analyzed in different spectral regions as discussed in details below.

After a pulse excitation (330 nm, 80 fs) of MeOH solution of **2**, the broad absorption band at around 600 nm was observed. As can be seen in Figure 3, this initially formed absorption rapidly decays with a lifetime of $\tau_{1a} = 2.1 \pm 0.5$ ps, and a new, much stronger absorption band at 430 nm builds up with the same kinetics (growth fitting gives $\tau_1 = 1.9 \pm 0.6$ ps) followed by its decay with a lifetime of $\tau_2 = 29.1 \pm 2.0$ ps. The ground-state absorption bleach monitored at 356 nm was found to recover with exactly the same kinetics as visualized in Figure 3. After about 200 ps, the transient absorption signals decay to the initial signal level proving that no long-lived species (e.g., triplet excited state or free radicals) are generated and the ground state of **2** is completely recovered. The set of transient absorption spectra taken at different time delays (from subpicoseconds to 150 ps) is shown in Figure 3. The initial short-lived transient signal observed at around 600 nm can be assigned to the $S_2 \rightarrow S_n$ absorption of **2** quickly relaxing and populating a more stable S_1 state. Since in our experiment we used a relatively short excitation wavelength (330 nm), it is quite likely to excite directly to state S_2 or higher, any of which rapidly converts back to S_1 .

The transient absorption spectra of compound **3** taken at different time delays following the 330 nm excitation pulse are shown in Figure 4. The most immediate broad absorption peaked around 530 nm decays with lifetime $\tau_1 = 1.7 \pm 0.5$ ps, and this feature can be assigned to the $S_2 \rightarrow S_n$ transient absorption. This ultrafast decay is followed by the growth of another absorption band ($\tau_{1a} = 1.6 \pm 0.7$ ps) peaking around 430 nm, subsequently decaying with lifetime $\tau_2 = 15.4 \pm 1.2$ ps, and this absorption can be assigned to $S_1 \rightarrow S_n$ absorption. The negative signal is attributed to the ground state bleach and, as can be seen in Figure 4, both the negative (ground state S_0 depletion) and positive (S_1 transient absorption) signals are fully recovered after ca. 100 ps and they both decay with the same kinetics. Moreover, there are no absorption signals for these decays, which would suggest a triplet formation.

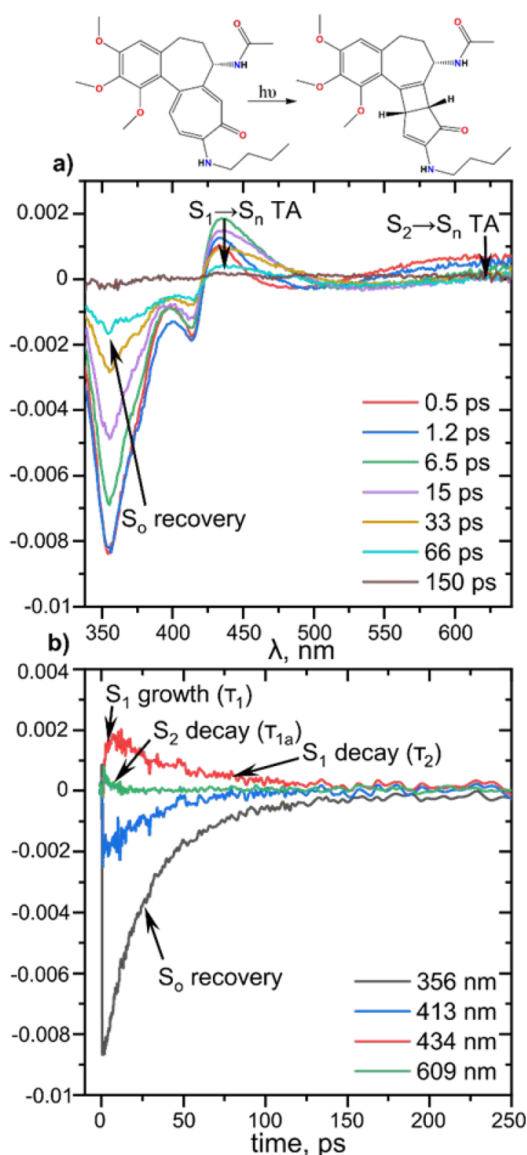


Figure 3. Absorption dynamics of **2** after 330 nm pulse laser excitation. Kinetic traces at selected wavelengths (b) and the set of transient absorption spectra (a) monitored at different time intervals after laser excitation of **2** in MeOH. $\tau_1 = 1.9 \pm 0.6$ ps (growth of S_1 TA); $\tau_{1a} = 2.1 \pm 0.6$ ps (decay of S_2 TA); $\tau_2 = 29.1 \pm 2.0$ ps.

The fast decay of the lowest excited singlet state S_1 (29 and 15 ps for **2** and **3**, respectively) can be attributed to the partly reversible excited-state intramolecular proton transfer (ESIPT) process occurring from the singlet excited state, followed by the photoisomerization of the compound. There was no indication of the triplet-state formation, as the ground-state absorption was recovered in less than 100 ps after the excitation pulse. In the ESIPT process, a proton is transferred from a donor to the proton acceptor and hydrogen-bonding interaction is the driving force for the reaction.^{38–40} Thus, the H-bond strength plays a crucial role in the energetics and dynamics of the proton-transfer processes. In the investigated systems of *N*-substituted

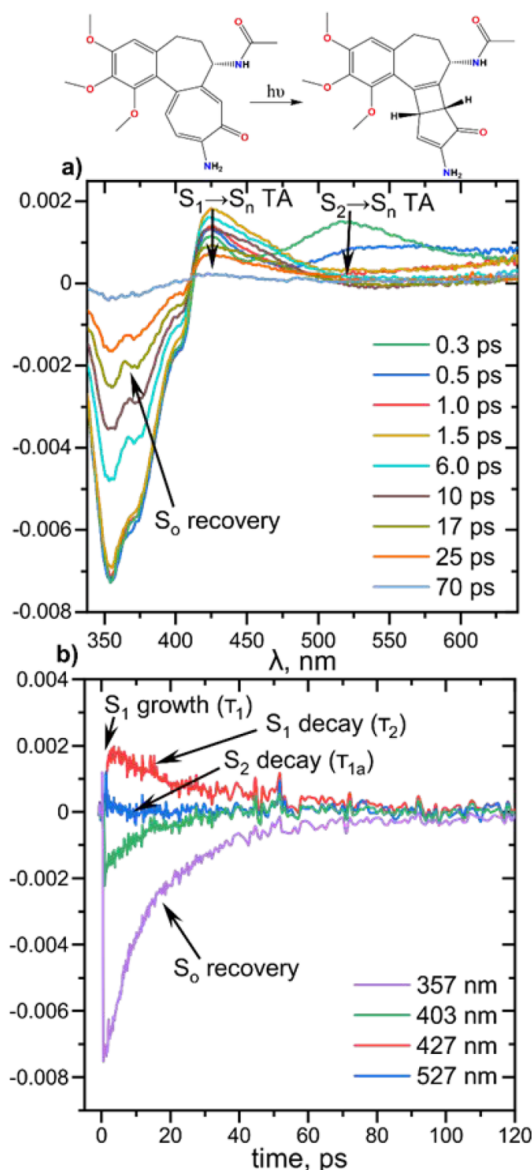


Figure 4. Absorption dynamics of **3** after 330 nm pulse laser excitation. Kinetic traces at selected wavelengths (b) and the set of transient absorption spectra (a) monitored at different time delays following 330 nm excitation of **3** in MeOH. Lifetimes $\tau_1 = 1.7 \pm 0.5$ ps; $\tau_{1a} = 1.6 \pm 0.7$ ps; $\tau_2 = 15.4 \pm 1.2$ ps.

colchicines, such intramolecular proton transfer in 5-membered-ring hydrogen-bonded system can be formed at C9 carbonyl atom, ultimately leading to photoisomerization (Figure 5).⁴⁰ This reasoning clearly explains the lack of photoreactivity of thiocolchicine, which forms weak or no hydrogen bonds.^{29,34,36}

The novel β -lumiderivatives were fully characterized by spectrometric and spectroscopic methods. As expected, there were no differences in ESI-MS spectra, as the lumiderivatives have the same mass as the unrearranged derivatives. There are clear differences between lumiderivatives and unrearranged derivatives in the IR and UV-vis spectra. In the UV-vis spectra,

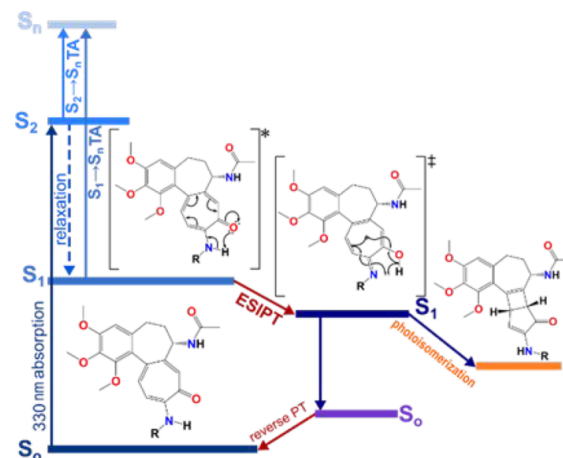


Figure 5. Photoisomerization mechanism scheme of N-substituted colchicines (TA, transient absorption; PT, proton transfer).

the disappearance of the tropolonic maximum absorption band around 350 nm was observed for all compounds and accompanied by the appearance of a new band around 280 nm, which confirmed the rearrangement of tropolone ring C.

Additionally, to observe how the UV-vis spectra evolve in time during irradiation, we exposed the methanol solution of **2** to UV radiation on an irradiation bench system (355 nm, CW laser, 20 mW). The absorption spectrum significantly changed after few minutes of exposure, suggesting a relatively fast photoisomerization of the sample under UV light. As can be seen in Figure 6, at least three clearly defined isosbestic points are

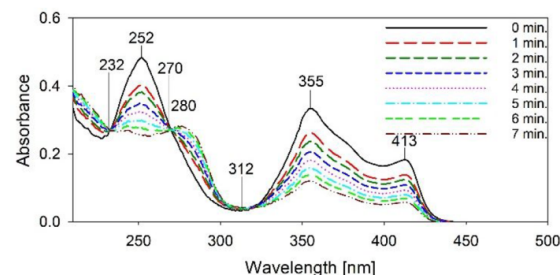


Figure 6. Cumulative effect of irradiation (355 nm) on absorption spectra of MeOH solution of **2** ($c = 2 \times 10^{-5}$ M). The quantum yield for photoisomerization of **2** is 0.004.

observed at 232, 270, and 312 nm, suggesting no secondary photochemical reactions (i.e., further photodegradation of the irradiation product). This is consistent with the proposed reaction scheme where no significant absorption at 355 nm is expected from the final isomeric photoproduct due to the lower number of aromatic π electrons in the conjugated system as compared to the starting reactant resulting in the blue-shift of the spectrum.

The most pronounced differences, which provided the clearest evidence for obtaining novel lumiderivatives (apart from the X-ray results), were observed in the NMR spectra. We used one- as well as two-dimensional NMR techniques (¹H-¹H COSY, ¹H-¹³C HSQC, and ¹H-¹³C HMB) for assignment of signals of compounds **2** and **2**-UV. Almost all signals were

shifted both in the ^1H and ^{13}C spectra when the rearrangement of ring C occurred. The most significant changes can be observed for the signal assigned to ring C, which shifted significantly due to the loss of aromaticity; e.g., the signal of C9 carbonyl atom shifted from 175.5 to 202.5 ppm. In Figure 7, we

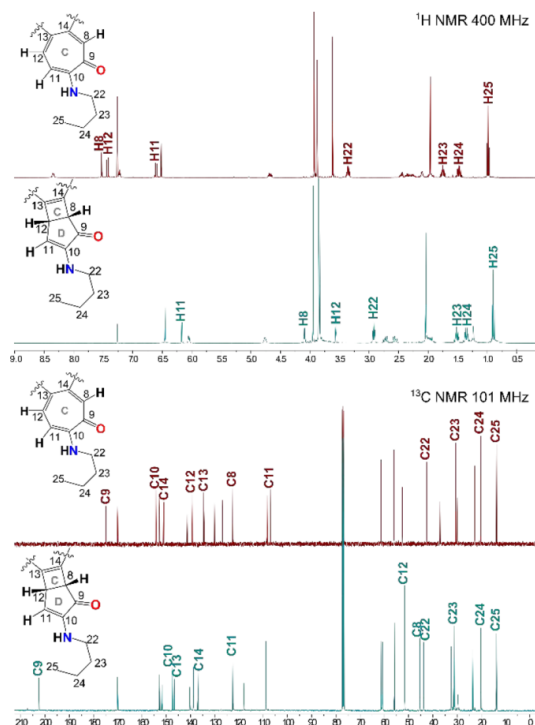


Figure 7. Stacked ^1H and ^{13}C NMR spectra of compounds **2** and **2-UV** in CDCl_3 with the signals assigned to rings C and D.

present the stacked spectra of compounds **2** and **2-UV** with the signals assigned to ring C. The copies of all spectra (^1H and ^{13}C NMR, IR, UV–vis) of all compounds studied are collected in the Supporting Information.

The biological activity of synthesized compounds was tested *in vitro* against three human cancer cell lines, human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), and human colon adenocarcinoma cell line (LoVo), as well as against normal murine embryonic fibroblast cell line (BALB/3T3) according to the previously published procedure.⁴¹ The mean values of $\text{IC}_{50} \pm \text{SD}$ of the tested compounds are collected in Table 2. The structure–activity relationship of the synthesized series of mono- and double-modified colchicine derivatives at C7 and C10 position (Scheme 1) showed the strongest antiproliferative activity of compounds with small substituents at C10 position, e.g., **3**, **3a**, and **4d**. In the case of compounds **3**, **4**, and **4d**, which share a free amino group at the C10, their further modification and introduction of the diversified substituent at C7 influences their activity significantly. Compound **3** has an identical acetyl substituent at C7 as colchicine, and it exhibits comparable activity against all tested cell lines. Compound **4d**, with benzyl amide at C7, appears to be slightly less active than colchicine, which suggests that expanded substituent at C7 may affect interaction with the colchicine binding site (CBS) on the β subunit of tubulin. Compound **4**

with additional free amino group at the C7 is almost 100 times less active than **3**. In this case, high polarity of two free amine groups will improve water solubility but may hinder solubility in membranes and their penetration. This result is consistent with the previously reported derivatives deacylated at C7, which also exhibited low biological activity.^{25,27} All β -lumiderivatives (β -lumicolchicine, **2-UV**, **3-UV**, **3b-UV**, **3f-UV**, **4b-UV**, **4f-UV**, Figure 2) are much less active than the regular derivatives. The activity of compound **3** and **3-UV** toward the A549 cell line deteriorated almost 10000 times, illustrating the unfavorable effects of light on the activity of colchicine derivatives. The rearrangement of ring C to conjugated 4- and 5-membered rings system in lumicolchicines most likely changes their position in colchicine binding site (CBS) and reduces their tubulin binding potency, which deactivates the biological activity of this class of colchicine derivatives.

CONCLUSIONS

We have developed an efficient method for the synthesis of mono- and double-modified *N*-substituted colchicine derivatives. Their recrystallization revealed the low stability of novel analogues **4b** and **4f** to sunlight, which prompted us to reproduce the photoeffects of sunlight on methanol solutions of **2**, **3**, **3b**, **3f**, **4b**, and **4f** using a high-pressure mercury lamp. The exposure time and irradiation doses varied depending on the character of the substituent at C7 and C10. Six novel β -lumiderivatives were obtained with isolation yields from 31 to 61% of compounds **4f-UV** and **3**, respectively. Detailed photochemical studies suggest that photoisomerization is associated with the partly reversible ESIPT process, during which intramolecular proton transfer in 5-membered-ring hydrogen-bonded system can occur at C9 carbonyl atom. The antiproliferative activity of the β -lumiderivatives decreased drastically when compared to those of the nonrearranged analogues, among which the most active derivatives were **3** and **4d**, with the free amino group at C10. These results indicate a new alley for controlling and improving the stability of colchicine APIs (active pharmaceutical ingredients) and in particular their resistance to the photoinduced conversions.

EXPERIMENTAL SECTION

General Methods. All precursors for the synthesis of colchicine derivatives and solvents were obtained from Merck or Fluka and were used as received without further purification. CDCl_3 and $\text{DMSO}-d_6$ spectral-grade solvents were stored over 3 Å molecular sieves for several days. All of the solvents used in flash chromatography were of HPLC grade (CHROMASOLV from Merck) and were used as received. Reaction mixtures were stirred using Teflon-coated magnetic stir bars. For reactions that require heating, a temperature-controlled heating block on a hot plate stirrer (Heidolph, MR-Hei Standard) was used. Reactions were monitored by thin-layer chromatography (TLC) using aluminum-backed plates (Merck 60 F254). TLC plates were visualized in UV light (254 nm). The ^1H , ^{13}C spectra were recorded on a Varian VNMR-S 400 MHz spectrometer using TMS as the internal standard in both cases. No window function or zero filling was used. ^1H NMR measurements of **1–14** (0.07 mol dm^{-3}) in CDCl_3 were carried out at an operating frequency of 402.64 MHz. The error of the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at an operating frequency 101.25 MHz. The error of chemical shift value was 0.1 ppm. All spectra were locked to a deuterium resonance of CDCl_3 . The ESI (electrospray ionization with the single quadrupole detection) mass spectra were recorded on a Waters/Micromass (Waters Corp., Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples were prepared in dry acetonitrile ($5 \times 10^{-5} \text{ mol dm}^{-3}$). The sample was infused into the ESI source using

Table 2. Antiproliferative Activity (IC_{50}) of Colchicine, Its Derivatives (2, 3, 3a–f, 4, and 4b–f) as Well as β -Lumiderivatives (β -Lumicolchicine, 2-UV, 3-UV, 3b-UV, 3f-UV, 4b-UV, and 4f-UV) Compared to the Antiproliferative Activity of Standard Anticancer Drugs Doxorubicin and Cisplatin and the Calculated Values of Selectivity Index (SI)^a

Compound	A549	SI	MCF-7	SI	LoVo	SI	BALB/3T3	
	IC ₅₀ (μM)		IC ₅₀ (μM)		IC ₅₀ (μM)		IC ₅₀ (μM)	
2	0.85 ± 0.29	1.05	0.69 ± 0.30	1.29	0.63 ± 0.30	1.41	0.89 ± 0.35	
3	0.04 ± 0.03	0.73	0.01 ± 0.01	3.00	0.02 ± 0.01	1.50	0.03 ± 0.02	
3a	0.09 ± 0.01	0.78	0.09 ± 0.01	0.78	0.06 ± 0.01	1.17	0.07 ± 0.01	
3b	1.05 ± 0.22	1.09	0.93 ± 0.31	1.23	0.73 ± 0.35	1.57	1.14 ± 0.38	
3c	9.85 ± 0.55	0.67	6.96 ± 1.95	0.95	2.11 ± 1.12	3.14	6.62 ± 1.93	
3d	6.99 ± 1.20	0.30	5.99 ± 2.69	0.35	0.72 ± 0.19	2.91	2.10 ± 0.76	
3e	2.16 ± 0.94	0.60	1.12 ± 0.19	1.16	0.79 ± 0.08	1.64	1.30 ± 0.19	
3f	9.72 ± 0.22	0.83	6.06 ± 1.36	1.32	3.95 ± 0.81	2.03	8.03 ± 0.32	
4	1.68 ± 0.21	0.83	1.11 ± 0.17	1.26	0.99 ± 0.29	1.40	1.39 ± 0.49	
4b	5.69 ± 2.53	1.16	1.23 ± 0.60	5.35	1.56 ± 0.60	4.22	6.58 ± 1.53	
4c	12.64 ± 2.41	0.59	7.30 ± 1.53	1.03	6.36 ± 0.39	1.18	7.49 ± 2.80	
4d	0.15 ± 0.12	0.53	0.12 ± 0.08	0.67	0.08 ± 0.01	1.00	0.08 ± 0.01	
4e	6.91 ± 2.19	0.74	2.29 ± 1.00	2.23	0.92 ± 0.14	5.56	5.12 ± 0.98	
4f	9.50 ± 0.54	0.88	7.94 ± 2.23	1.05	5.01 ± 2.50	1.66	8.32 ± 4.08	
β-lumiderivatives	2-UV	74.87 ± 3.58	1.53	73.00 ± 7.30	1.57	70.49 ± 7.64	1.62	114.41 ± 12.31
	3-UV	99.60 ± 7.50	1.08	95.66 ± 7.86	1.13	82.44 ± 8.60	1.31	107.80 ± 2.32
	3b-UV	107.37 ± 14.44	1.26	95.47 ± 7.67	1.41	85.17 ± 9.93	1.58	134.77 ± 20.06
	3f-UV	83.82 ± 14.48	1.26	78.93 ± 9.56	1.34	94.63 ± 16.14	1.12	105.79 ± 11.59
	4b-UV	85.71 ± 6.27	1.42	78.93 ± 0.75	1.54	76.01 ± 8.01	1.60	121.79 ± 20.71
	4f-UV	15.71 ± 1.45	*	NA	*	15.86 ± 2.01	*	NA
	β-lumicolch	61.10 ± 24.12	1.09	65.28 ± 18.06	1.02	20.65 ± 3.49	3.22	66.53 ± 34.55
colchicine	0.07 ± 0.01	0.63	0.01 ± 0.01	4.30	0.01 ± 0.01	5.38	0.04 ± 0.01	
cisplatin	3.60 ± 0.25	0.81	3.05 ± 0.67	0.95	3.80 ± 0.28	0.77	2.91 ± 1.83	
doxorubicin	0.16 ± 0.03	0.25	0.15 ± 0.05	0.26	0.08 ± 0.03	0.49	0.04 ± 0.03	

^aThe IC_{50} value is defined as the concentration of a compound at which 50% growth inhibition of cancer cells is observed. Human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line (LoVo), and normal murine embryonic fibroblast cell line (BALB/3T3). The SI (selectivity index) was calculated for each compound, using the formula $SI = IC_{50}$ for normal cell line BALB/3T3/ IC_{50} for the respective cancerous cell line. A beneficial SI > 1.0 indicates a drug with efficacy against tumor cells greater than the toxicity against normal cells. NA, compound inactive; at the highest concentration tested the inhibition of proliferation does not exceed 50%. *SI values were not calculated for inactive compounds.

a Harvard pump at a flow rate of 20 mL min⁻¹. The ESI source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages: 10 and 30 V. The source temperature was 120 °C and the desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 dm³/h. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from m/z = 100 to m/z = 1000, as well as from m/z = 200 to m/z = 1500. The elemental analysis of compounds was carried out on Vario ELIII (Elementar, Langensfeld, Germany).

Synthesis and Characterization of 2. To a solution of colchicine (400 mg; 1 mmol) in CH₃CN (10 mL) was added *n*-butylamine (0.5 mL, 5 mmol). The reaction mixture was stirred under reflux for 24 h (using a temperature controlled heating block on a hot plate-stirrer) while the reaction progress was determined by TLC. After that time, the solvent was evaporated under reduced pressure. The residue was purified by CombiFlash (0 $\rightarrow$ 10% methanol/EtOAc) to give 2. Amorphous yellow solid, yield 94% (415 mg). ¹H NMR (400 MHz, CDCl₃): δ 8.35 (d, J = 6.9 Hz, 1H), 7.53 (s, 1H), 7.43 (d, J = 11.2 Hz, 1H), 7.23 (t, J = 5.7 Hz, 1H), 6.60 (d, J = 11.3 Hz, 1H), 6.52 (s, 1H),

4.74–4.65 (m, 1H), 3.93 (s, 3H), 3.89 (s, 3H), 3.62 (s, 3H), 3.35 (dd, J = 13.0, 7.0 Hz, 2H), 2.46 (dd, J = 12.4, 5.6 Hz, 1H), 2.41–2.32 (m, 1H), 2.32–2.21 (m, 1H), 2.18–2.07 (m, 1H), 1.98 (s, 3H), 1.81–1.70 (m, 2H), 1.54–1.43 (m, 2H), 0.98 (t, J = 7.3 Hz, 3H). ¹³C{H} NMR (400 MHz, CDCl₃): δ 175.5, 169.9, 154.3, 152.8, 151.1, 151.0, 141.5, 139.4, 134.6, 130.3, 126.8, 122.6, 108.7, 107.1, 61.4, 61.3, 56.1, 52.6, 42.5, 37.2, 30.5, 30.0, 22.8, 20.3, 13.7. ESI-MS (m/z): [M + H]⁺ 441, [M + Na]⁺ 463, [2M + Na]⁺ 903. Anal. Calcd for C₂₃H₃₂N₂O₅: C, 68.16; H, 7.32; N, 6.36. Found: C, 68.11; H 7.41; N, 6.29.

Synthesis and Characterization of 3. To a solution of 1 (400 mg; 1 mmol) in CH₃CN (10 mL) ammonia (25% aqueous solution, 0.5 mL, 7.35 mmol) was added. The reaction mixture was stirred under reflux for 24 h (using a temperature controlled heating block on a hot plate-stirrer), while the reaction progress was determined by TLC. After that time the solvent was evaporated under reduced pressure. The residue was purified by CombiFlash (0 $\rightarrow$ 50% acetone/chloroform) to give 3. Amorphous yellow solid, yield 96% (370 mg). ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.56 (d, J = 7.7 Hz, 1H), 7.13–7.06 (m, 2H), 6.89 (d, J = 11.1 Hz, 1H), 6.74 (s, 1H), 4.40–4.32 (m, 1H), 3.82 (s, 3H), 3.78 (s, 3H), 3.48 (s, 3H), 3.37 (s, 3H), 2.23–2.11 (m, 1H), 2.08–1.96 (m, 1H), 1.86 (s, 3H), 1.85–1.80 (m, 1H). ¹³C{H} NMR (101 MHz,

DMSO- d_6): δ 174.3, 168.4, 156.9, 152.3, 150.3, 149.4, 140.7, 138.0, 134.4, 128.8, 126.7, 123.6, 110.16, 107.5, 60.7, 60.5, 55.8, 51.4, 36.7, 29.4, 22.6. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.57; H 6.37; N, 7.24.

General Route for Synthesis of Compounds 3a–f and 4b–f. To a solution of 3 or 4 (100 mg, 0.25 mmol) in dichloromethane (DCM, 10 mL) cooled to 0 °C, Et_3N (0.5 mL, 3.5 mmol) and respective acyl chloride or chloroformate were added (3.5 mmol). The mixture was first stirred at 0 °C for 30 min and then for the next 24 h at rt. Reaction progress was determined by TLC. Subsequently, the solvent was evaporated under reduced pressure and the residue was purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform) to give respective compounds as amorphous yellow solids.

Characterization of 3a. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3a. Amorphous yellow solid, yield 55% (61 mg). 1H NMR (400 MHz, $CDCl_3$): δ 9.35 (s, 1H), 9.03 (d, J = 10.9 Hz, 1H), 7.60 (s, 1H), 7.46 (d, J = 10.9 Hz, 1H), 7.23 (d, J = 6.9 Hz, 1H), 6.52 (s, 1H), 4.71–4.56 (m, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.62 (s, 3H), 2.52 (dd, J = 13.0, 6.1 Hz, 1H), 2.31 (s, 3H), 2.30–2.20 (m, 1H), 1.98 (s, 3H), 1.90–1.79 (m, 1H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.7, 170.0, 169.7, 153.7, 152.8, 151.2, 145.4, 141.7, 139.6, 138.4, 133.9, 129.9, 125.6, 120.9, 107.2, 61.4, 61.3, 56.1, 52.5, 36.7, 29.8, 25.6, 22.8. ESI-MS (m/z): [M + Na] $^+$ 449, [M + K] $^+$ 465, [2M + Na] $^+$ 875, [2M + K] $^+$ 891. Anal. Calcd for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.15; N, 6.57. Found: C, 64.81; H 6.10; N, 6.62.

Characterization of 3b. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3b. Amorphous yellow solid, yield 58% (66 mg). 1H NMR (400 MHz, $CDCl_3$): δ 9.38 (s, 1H), 9.05 (d, J = 10.9 Hz, 1H), 7.60 (s, 1H), 7.46 (d, J = 10.9 Hz, 1H), 6.52 (s, 1H), 4.69–4.62 (m, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.62 (s, 3H), 2.55 (q, J = 7.5 Hz, 2H), 2.38–2.30 (m, 2H), 2.28–2.20 (m, 1H), 2.01–1.97 (m, 3H), 1.98 (s, 3H), 1.88–1.79 (m, 1H), 1.26 (t, 3H). ^{13}C {H} NMR (400 MHz, $CDCl_3$): δ 177.7, 173.7, 169.8, 153.6, 152.9, 151.2, 145.4, 141.6, 139.5, 138.5, 134.0, 129.8, 125.6, 120.9, 107.2, 61.4, 61.3, 56.1, 52.5, 36.6, 31.7, 29.7, 22.8. ESI-MS (m/z): [M + H] $^+$ 441, [M + Na] $^+$ 463, [M + K] $^+$ 479, [2M + Na] $^+$ 903, [2M + K] $^+$ 919. Anal. Calcd $C_{24}H_{28}N_2O_6$ for C, 65.44; H, 6.41; N, 6.36. Found: C, 65.40; H 6.49; N, 6.31.

Characterization of 3c. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3c. Amorphous yellow solid, yield 63% (74 mg). 1H NMR (400 MHz, $CDCl_3$): δ 9.46 (s, 1H), 9.05 (d, J = 10.9 Hz, 1H), 7.59 (s, 1H), 7.46 (d, J = 10.9 Hz, 1H), 6.52 (s, 1H), 4.74–4.61 (m, 1H), 3.93 (s, 3H), 3.89 (s, 3H), 3.62 (s, 3H), 2.70 (dt, J = 13.8, 6.9 Hz, 1H), 2.51 (dd, J = 13.1, 5.9 Hz, 1H), 2.40–2.31 (m, 1H), 2.29–2.20 (m, 1H), 1.99 (s, 3H), 1.88–1.79 (m, 2H), 1.28 (d, J = 6.9 Hz, 6H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.9, 177.0, 169.6, 153.6, 152.7, 151.2, 145.6, 141.6, 139.3, 138.4, 133.9, 129.7, 125.7, 120.8, 107.2, 61.4, 61.3, 56.1, 52.5, 37.5, 36.7, 29.7, 22.9, 19.5, 19.4. ESI-MS (m/z): [M + H] $^+$ 455, [M + Na] $^+$ 477, [M + K] $^+$ 493, [2M + Na] $^+$ 931, [2M + K] $^+$ 947. Anal. Calcd for $C_{25}H_{30}N_2O_6$: C, 66.06; H, 6.65; N, 6.16. Found: C, 66.16; H 6.71; N, 6.07.

Characterization of 3d. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3d. Amorphous yellow solid, yield 35% (44 mg). 1H NMR (400 MHz, $CDCl_3$): δ 10.33 (s, 1H), 9.23 (d, J = 10.9 Hz, 1H), 8.02–7.97 (m, 2H), 7.67 (s, 1H), 7.63–7.58 (m, 1H), 7.57–7.53 (m, 1H), 7.52 (d, J = 1.7 Hz, 2H), 6.54 (s, 1H), 4.74–4.67 (m, 1H), 3.95 (s, 3H), 3.90 (s, 3H), 3.66 (s, 3H), 2.54 (dd, J = 13.4, 6.0 Hz, 1H), 2.45–2.35 (m, 1H), 2.33–2.23 (m, 1H), 2.02 (s, 3H), 1.90–1.82 (m, 1H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 178.1, 169.6, 166.4, 153.7, 152.8, 151.3, 145.7, 141.8, 139.7, 138.4, 133.9, 132.5, 129.8, 128.9, 128.3, 127.5, 125.7, 121.0, 107.3, 61.5, 61.3, 56.1, 52.5, 36.9, 29.8, 23.0. ESI-MS (m/z): [M + Na] $^+$ 511. Anal. Calcd for $C_{28}H_{28}N_2O_6$: C, 68.84; H, 5.78; N, 5.73. Found: C, 68.75; H 5.80; N, 5.69.

Characterization of 3e. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3e. Amorphous yellow solid, yield 49% (56 mg).

1H NMR (400 MHz, $CDCl_3$): δ 8.93 (s, 1H), 8.67 (d, J = 10.9 Hz, 1H), 7.63 (d, J = 3.9 Hz, 1H), 7.48 (d, J = 11.0 Hz, 1H), 6.54 (s, 1H), 4.71–4.63 (m, 1H), 3.93 (s, 3H), 3.89 (s, 3H), 3.84 (s, 3H), 3.63 (s, 3H), 2.52 (dd, J = 12.7, 5.5 Hz, 1H), 2.39–2.22 (m, 2H), 1.96 (s, 3H), 1.93–1.84 (m, 2H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.1, 169.8, 153.6, 153.1, 153.0, 151.2, 145.9, 141.6, 138.7, 138.2, 134.1, 129.2, 125.7, 119.2, 107.2, 61.5, 61.3, 56.1, 52.8, 52.6, 36.5, 29.8, 22.8. ESI-MS (m/z): [M + H] $^+$ 443, [M + Na] $^+$ 465, [M + K] $^+$ 481, [2M + Na] $^+$ 907, [2M + K] $^+$ 923. Anal. Calcd for $C_{23}H_{26}N_2O_7$: C, 62.43; H, 5.92; N, 6.33. Found: C, 62.36; H 5.98; N, 6.21.

Characterization of 3f. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3f. Amorphous yellow solid, yield 61% (72 mg). 1H NMR (400 MHz, $CDCl_3$): δ 8.89 (s, 1H), 8.68 (d, J = 10.9 Hz, 1H), 7.64–7.59 (m, 1H), 7.47 (d, J = 10.9 Hz, 1H), 6.52 (s, 1H), 4.67 (m, 1H), 4.28 (q, J = 7.1, 1.2 Hz, 2H), 3.93 (s, 3H), 3.89 (s, 3H), 3.63 (s, 3H), 2.52 (dd, J = 12.6, 5.4 Hz, 1H), 2.41–2.22 (m, 3H), 1.96 (s, 3H), 1.91–1.83 (m, 2H), 1.35 (t, J = 7.1 Hz, 3H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.2, 169.8, 153.6, 153.2, 152.9, 151.2, 146.1, 141.6, 138.5, 138.2, 134.1, 129.2, 125.7, 119.1, 107.2, 61.8, 61.5, 61.3, 56.1, 52.6, 36.6, 29.8, 22.8, 14.4. ESI-MS (m/z): [M + H] $^+$ 457, [M + Na] $^+$ 479, [M + K] $^+$ 495, [2M + Na] $^+$ 935, [2M + K] $^+$ 951. Anal. Calcd for $C_{24}H_{28}N_2O_7$: C, 63.15; H, 6.18; N, 6.14. Found: C, 63.07; H 6.22; N, 6.17.

Synthesis and Characterization of 4. To a solution of 3 (500 mg, 1.11 mmol) in MeOH (3 mL) was added 4 N HCl solution (5 mL). The mixture was stirred at 90 °C for 72 h. Reaction progress was determined by TLC. Subsequently the solvent was evaporated under reduced pressure. The residue was purified by CombiFlash (0 $\rightarrow$ 10% Methanol/ $EtOAc$) to give 4 with yield 92%. 1H NMR (401 MHz, DMSO- d_6): δ 8.78 (s, 2H), 7.15 (d, J = 11.2 Hz, 1H), 7.05 (s, 1H), 6.98 (d, J = 11.2 Hz, 1H), 6.79 (s, 1H), 3.83 (s, 3H), 3.76 (s, 3H), 3.55 (s, 3H), 2.65–2.57 (m, 2H), 2.41–2.31 (m, 1H), 2.27–2.13 (m, 1H), 2.04–1.94 (m, 1H). ^{13}C {H} NMR (101 MHz, DMSO- d_6): δ 173.9, 157.3, 152.7, 150.2, 143.0, 140.8, 138.9, 133.6, 127.8, 125.8, 123.0, 110.5, 107.6, 60.5, 55.9, 52.8, 35.7, 30.7, 28.8. ESI-MS (m/z): [M + H] $^+$ 343, [M + Na] $^+$ 356. Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.70; H 6.59; N, 8.15.

Characterization of 4b. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4b. Amorphous yellow solid, yield 43% (57 mg). 1H NMR (400 MHz, $CDCl_3$): δ 9.38 (s, 1H), 9.02 (d, J = 11.9, 1H), 7.51 (s, 1H), 7.44 (d, J = 11.9 Hz, 1H), 6.84–6.71 (m, 1H), 6.51 (s, 1H), 4.73–4.60 (m, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.62 (s, 3H), 2.54 (q, J = 7.4, 2H), 2.40–2.30 (m, 1H), 2.25 (q, J = 7.6 Hz, 2H), 1.84–1.76 (m, 2H), 1.26 (t, J = 7.5 Hz, 3H), 1.10 (t, J = 7.6 Hz, 3H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.8, 173.7, 173.2, 153.6, 152.5, 151.3, 145.4, 141.7, 139.2, 138.3, 133.9, 129.8, 125.7, 120.6, 107.2, 61.5, 61.3, 56.1, 52.2, 36.9, 31.7, 29.8, 29.3, 9.5, 9.3. ESI-MS (m/z): [M + H] $^+$ 455, [M + Na] $^+$ 477, [M + K] $^+$ 493. Anal. Calcd for $C_{25}H_{30}N_2O_6$: C, 66.06; H, 6.65; N, 6.16. Found: C, 66.01; H 6.61; N, 6.22.

Characterization of 4c. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4c. Amorphous yellow solid, yield 57% (80 mg). 1H NMR (400 MHz, $CDCl_3$): δ 9.47 (s, 1H), 9.03 (d, J = 10.9 Hz, 1H), 7.54 (d, J = 6.1 Hz, 1H), 7.45 (d, J = 10.9 Hz, 1H), 6.85–6.62 (m, 1H), 6.51 (s, 1H), 4.71–4.61 (m, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.63 (s, 3H), 2.68 (hept, J = 6.9 Hz, 1H), 2.54–2.43 (m, 2H), 2.43–2.30 (m, 1H), 2.29–2.18 (m, 1H), 1.83–1.73 (m, 1H), 1.28 (d, J = 6.9 Hz, 6H), 1.13 (d, J = 7.0 Hz, 6H). ^{13}C {H} NMR (101 MHz, $CDCl_3$): δ 177.9, 176.9, 176.4, 153.6, 152.5, 151.3, 145.5, 141.7, 139.2, 138.3, 133.9, 129.8, 125.7, 120.6, 107.2, 61.5, 61.3, 56.1, 51.8, 37.5, 37.0, 35.3, 29.8, 19.49, 19.46, 19.44, 19.35. ESI-MS (m/z): [M + Na] $^+$ 505, [M + K] $^+$ 521, [2M + Na] $^+$ 987. Anal. Calcd for $C_{27}H_{34}N_2O_6$: C, 67.20; H, 7.10; N, 5.81; C, 67.29; H 7.07; N, 5.88.

Characterization of 4d. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4d. Amorphous yellow solid, yield 33% (43 mg). 1H NMR (400 MHz, $CDCl_3$): δ 8.28 (s, 1H), 7.81 (d, J = 7.6 Hz, 2H),

7.71 (s, 1H), 7.34 (d, $J = 11.0$ Hz, 1H), 7.22–7.15 (m, 2H), 6.88 (d, $J = 11.0$ Hz, 1H), 6.52 (s, 1H), 6.16 (s, 1H), 4.99–4.89 (m, 1H), 3.94 (s, 3H), 3.88 (s, 3H), 3.68 (s, 3H), 2.54–2.44 (m, 1H), 2.48 (dd, $J = 12.9$, 6.1 Hz, 1H), 2.13–2.01 (m, 1H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 174.9, 167.1, 155.7, 153.0, 151.1, 150.8, 141.5, 138.9, 134.5, 133.7, 132.0, 131.3, 128.3, 127.1, 126.7, 125.4, 112.2, 107.3, 61.5, 61.4, 56.1, 52.8, 37.1, 30.1. ESI-MS (m/z): $[\text{M} + \text{H}]^+$ 447, $[\text{M} + \text{Na}]^+$ 469, $[\text{M} + \text{K}]^+$ 485, $[\text{2M} + \text{Na}]^+$ 915. Anal. Calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_5$: C, 69.94; H, 5.87; N, 6.27. Found: C, 70.01; H 5.79; N, 6.35.

Characterization of 4e. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4e. Amorphous yellow solid, yield 56% (75 mg). ^1H NMR (400 MHz, CDCl_3): δ 8.95 (s, 1H), 8.62 (d, $J = 10.9$ Hz, 1H), 7.54 (s, 1H), 7.43 (d, $J = 10.9$ Hz, 1H), 6.53 (s, 1H), 5.26 (s, 1H), 4.52–4.40 (m, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.83 (s, 3H), 3.61 (s, 3H), 3.60 (s, 3H), 2.52 (dd, $J = 12.9$, 5.8 Hz, 1H), 2.44–2.22 (m, 3H), 1.80–1.69 (m, 1H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 177.4, 155.8, 153.8, 153.6, 151.7, 151.2, 146.0, 141.7, 137.8, 134.0, 129.7, 125.7, 118.6, 107.2, 61.4, 61.3, 56.1, 53.9, 52.7, 52.3, 37.4, 29.8, 29.7. ESI-MS (m/z): $[\text{M} + \text{H}]^+$ 459, $[\text{M} + \text{Na}]^+$ 481, $[\text{M} + \text{K}]^+$ 497. Anal. Calcd for $\text{C}_{23}\text{H}_{26}\text{N}_2\text{O}_8$: C, 60.26; H, 5.72; N, 6.11. Found: C, 60.20; H 5.64; N, 6.18.

Characterization of 4f. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4f. Amorphous yellow solid, yield 62% (88 mg). ^1H NMR (400 MHz, CDCl_3): δ 8.91 (s, 1H), 8.61 (d, $J = 10.9$ Hz, 1H), 7.60 (s, 1H), 7.41 (d, $J = 10.9$ Hz, 1H), 6.52 (s, 1H), 5.64 (d, $J = 7.1$ Hz, 1H), 4.51–4.40 (m, 1H), 4.26 (q, $J = 7.1$ Hz, 2H), 3.96 (m, q, $J = 6.9$ Hz, 2H), 3.92 (s, 3H), 3.88 (s, 3H), 3.59 (s, 3H), 2.56 (dd, $J = 12.6$, 5.4 Hz, 1H), 2.41–2.22 (m, 2H), 1.83–1.73 (m, 1H), 1.33 (t, $J = 7.1$ Hz, 3H), 1.12 (t, $J = 7.1$ Hz, 3H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 177.4, 155.4, 153.5, 153.3, 152.0, 151.2, 146.0, 141.6, 137.8, 134.1, 129.8, 125.7, 118.6, 107.2, 61.7, 61.3, 61.2, 61.0, 56.1, 53.7, 37.4, 29.8, 14.4, 14.3. ESI-MS (m/z): $[\text{M} + \text{H}]^+$ 487, $[\text{M} + \text{Na}]^+$ 509, $[\text{M} + \text{K}]^+$ 525, $[\text{2M} + \text{Na}]^+$ 995. Anal. Calcd for $\text{C}_{25}\text{H}_{30}\text{N}_2\text{O}_8$: C, 61.72; H, 6.22; N, 5.76. Found: C, 61.83; H 6.29; N, 5.72.

General Route for Photoisomers β -Lumicolchicine, 2-UV, 3-UV, 3b-UV, 3f-UV, 4b-UV, and 4f-UV. A solution of starting material (100 mg) in 5.5 mL of MeOH was irradiated in Pyrex glass tube, using a cylindrical photoreactor with an internal radiation source, equipped with a medium-pressure mercury lamp TQ 150W (Heraeus, Hanau, Germany), placed inside a cylindrical filter made of Pyrex glass with a wall thickness of 1.5 mm. The lamp with the filter was inside a quartz water jacket. The reaction was conducted until the disappearance of starting material on a TLC plate. After that the solvent was evaporated, and the crude product was purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform) to give specifically β -isomers. The photograph of the photoreaction setup is shown in Figure S62.

Characterization of Photoisomer β -Lumicolchicine. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give β -lumicolchicine. Amorphous yellow solid, yield 44% (44 mg). ^1H NMR (400 MHz, CDCl_3): δ 6.65 (d, $J = 3.2$ Hz, 1H), 6.47 (s, 1H), 6.00 (d, $J = 7.0$ Hz, 1H), 4.84–4.76 (m, 1H), 4.10 (q, $J = 2.9$ Hz, 1H), 3.95 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.69 (s, 3H), 3.61 (dd, $J = 2.6$, 1.8 Hz, 1H), 2.75 (dd, $J = 15.4$, 9.1 Hz, 1H), 2.56 (dd, $J = 15.4$, 9.4 Hz, 1H), 2.05 (s, 3H), 2.03–1.96 (m, 1H), 1.96–1.88 (m, 1H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 200.9, 170.3, 157.7, 153.1, 151.7, 145.1, 140.2, 138.8, 137.2, 128.8, 117.6, 109.1, 61.4, 60.8, 56.8, 55.9, 51.42, 51.39, 43.2, 32.5, 31.3, 23.5. ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ 422, $[\text{2M} + \text{Na}]^+$ 821. Anal. Calcd for $\text{C}_{22}\text{H}_{25}\text{NO}_6$: C, 66.15; H, 6.31; N, 3.51. Found: C, 66.03; H 6.45; N, 3.24.

Characterization of Photoisomer 2-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 2-UV. Amorphous green solid, yield 37% (37 mg). ^1H NMR (400 MHz, CDCl_3): δ 6.45 (s, 1H), 6.17 (d, $J = 3.3$ Hz, 1H), 6.05 (d, $J = 6.8$ Hz, 1H), 4.78–4.73 (m, 1H), 4.09 (dd, $J = 5.7$, 2.9 Hz, 1H), 3.95 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.58–3.54 (m, 1H), 2.91 (t, $J = 1.1$ Hz, 2H), 2.72 (dd, $J = 15.3$, 8.8 Hz, 1H), 2.55 (dd, $J = 15.3$, 9.2 Hz, 1H), 2.04 (s, 3H), 2.01–1.86 (m, 2H), 1.56–

1.47 (m, 2H), 1.41–1.29 (m, 2H), 0.89 (t, $J = 7.3$ Hz, 3H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 202.5, 170.1, 152.8, 151.7, 147.4, 146.7, 140.21, 138.7, 137.0, 122.5, 118.1, 108.9, 61.3, 60.7, 55.8, 51.6, 45.3, 43.8, 32.5, 31.3, 31.1, 23.5, 20.1, 13.9. ESI-MS (m/z): $[\text{M} + \text{H}]^+$ 441, $[\text{M} + \text{Na}]^+$ 463, $[\text{2M} + \text{Na}]^+$ 903. Anal. Calcd for $\text{C}_{23}\text{H}_{32}\text{N}_2\text{O}_5$: C, 68.16; H, 7.32; N, 6.36. Found: C, 68.08; H 7.45; N, 6.22.

Characterization of Photoisomer 3-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3-UV. Amorphous brown solid, yield 61% (61 mg). ^1H NMR (101 MHz, $\text{DMSO}-d_6$): δ 7.78 (d, $J = 9.1$ Hz, 1H), 6.68 (s, 1H), 6.24 (d, $J = 3.2$, 0.8 Hz, 1H), 4.72 (dd, $J = 6.9$, 1.8 Hz, 1H), 3.94 (dd, $J = 5.5$, 2.8 Hz, 1H), 3.86 (s, 3H), 3.79 (s, 3H), 3.74 (s, 3H), 3.37 (s, 3H), 3.35 (dd, $J = 2.8$, 1.5 Hz, 1H), 2.69 (dd, $J = 7.0$, 3.6 Hz, 2H), 1.81 (s, 3H), 1.81–1.67 (m, 2H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, $\text{DMSO}-d_6$): δ 200.3, 168.3, 152.2, 151.3, 146.6, 144.6, 139.8, 139.6, 138.6, 123.7, 118.0, 109.3, 61.1, 60.3, 55.7, 50.8, 48.6, 44.2, 31.9, 31.6, 22.9 ppm. ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ 407, $[\text{2M} + \text{Na}]^+$ 791. Anal. Calcd for $\text{C}_{21}\text{H}_{24}\text{N}_2\text{O}_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.68; H 6.24; N, 7.32.

Characterization of Photoisomer 3b-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3b-UV. Amorphous orange solid, yield 41% (41 mg). ^1H NMR (400 MHz, CDCl_3): δ 8.02 (d, $J = 3.1$ Hz, 1H), 7.7 (s, 1H), 6.38 (s, 1H), 5.82–5.76 (m, 1H), 4.81–4.74 (m, 1H), 4.21 (dd, $J = 5.3$, 2.7 Hz, 1H), 3.93 (s, 3H), 3.78 (s, 3H), 3.77 (s, 3H), 3.52 (dd, $J = 2.6$, 1.6 Hz, 1H), 2.69 (dd, $J = 15.4$, 9.2 Hz, 1H), 2.57–2.49 (m, 2H), 2.34–2.26 (m, 2H), 1.99 (s, 3H), 1.91–1.82 (m, 2H), 1.13 (t, $J = 7.5$ Hz, 3H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 201.2, 172.3, 169.9, 153.2, 152.0, 145.3, 141.5, 140.2, 138.5, 137.3, 136.7, 117.5, 108.8, 61.1, 60.7, 55.9, 51.2, 50.4, 46.8, 32.4, 31.3, 29.9, 29.6, 23.5, 9.2. ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ 463, $[\text{2M} + \text{Na}]^+$ 903. Anal. Calcd for $\text{C}_{24}\text{H}_{28}\text{N}_2\text{O}_6$: C, 65.44; H, 6.41; N, 6.36. Found: C, 65.48; H 6.52; N, 6.27.

Characterization of Photoisomer 3f-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 3f-UV. Amorphous beige solid, yield 52% (52%). ^1H NMR (400 MHz, CDCl_3): δ 7.71–7.60 (m, 1H), 6.75 (s, 1H), 6.38 (s, 1H), 5.82 (d, $J = 7.2$ Hz, 1H), 4.80–4.70 (m, 1H), 4.18 (dd, $J = 5.5$, 2.7 Hz, 1H), 4.11 (q, $J = 7.1$ Hz, 2H), 3.92 (s, 3H), 3.77 (s, 3H), 3.81 (s, 3H), 3.54–3.51 (m, 1H), 2.67 (dd, $J = 15.4$, 9.1 Hz, 1H), 2.50 (dd, $J = 15.4$, 9.5 Hz, 1H), 1.97 (s, 3H), 1.89–1.80 (m, 2H), 1.27–1.21 (m, 3H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 200.4, 169.9, 153.3, 153.1, 151.9, 145.3, 140.1, 138.6, 138.5, 137.7, 136.8, 117.5, 108.8, 61.6, 61.1, 60.7, 55.8, 51.1, 50.6, 46.3, 32.4, 31.3, 23.4, 14.3. ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ 479, $[\text{2M} + \text{Na}]^+$ 935. Anal. Calcd for $\text{C}_{24}\text{H}_{28}\text{N}_2\text{O}_7$: C, 63.15; H, 6.18; N, 6.14. Found: C, 63.11; H, 6.27; N, 6.10.

Characterization of Photoisomer 4b-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% acetone/chloroform), to give 4b-UV. Amorphous yellow solid, yield 33% (33 mg). ^1H NMR (400 MHz, CDCl_3): δ 8.07 (d, $J = 3.1$ Hz, 1H), 7.40 (s, 1H), 6.43 (s, 1H), 5.74 (d, $J = 7.3$ Hz, 1H), 4.88–4.80 (m, 1H), 4.27 (dd, $J = 5.5$, 2.8 Hz, 1H), 3.98 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.57 (dd, $J = 2.7$, 1.7 Hz, 1H), 2.74 (dd, $J = 15.3$, 9.2 Hz, 1H), 2.59–2.50 (m, 1H), 2.35 (q, $J = 7.5$ Hz, 2H), 2.30–2.21 (m, 2H), 2.07–2.00 (m, 1H), 1.94–1.85 (m, 1H), 1.19 (m, 6H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 201.1, 173.5, 172.3, 153.2, 152.0, 145.3, 141.5, 140.2, 138.6, 137.3, 136.8, 117.5, 108.7, 61.1, 60.7, 55.9, 51.1, 50.4, 46.8, 32.4, 31.4, 29.9, 29.8, 9.5, 9.2. ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ 477, $[\text{2M} + \text{Na}]^+$ 931. Anal. Calcd for $\text{C}_{25}\text{H}_{30}\text{N}_2\text{O}_6$: C, 66.06; H, 6.65; N, 6.16. Found: C, 65.98; H 6.72; N, 6.20.

Characterization of Photoisomer 4f-UV. The compound was prepared according to the general procedure, purified by CombiFlash (0 $\rightarrow$ 30% Acetone/Chloroform), to give 4f-UV. Amorphous beige solid, yield 31% (31 mg). ^1H NMR (400 MHz, CDCl_3): δ 7.74–7.64 (m, 1H), 6.83 (s, 1H), 6.44 (s, 1H), 4.95 (d, $J = 5.6$ Hz, 1H), 4.58–4.52 (m, 1H), 4.25 (dd, $J = 5.5$, 2.7 Hz, 1H), 3.97 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.63–3.57 (m, 1H), 2.71 (dd, $J = 15.3$, 8.4 Hz, 1H), 2.52 (dd, $J = 15.3$, 9.3 Hz, 1H), 2.06–1.90 (m, 2H), 1.33–1.22 (m, 6H). $^{13}\text{C}\{\text{H}\}$ NMR (101 MHz, CDCl_3): δ 200.1, 155.9, 153.4, 153.1, 152.0, 145.5,

140.2, 138.9, 137.8, 137.7, 137.0, 117.6, 108.7, 61.5, 61.1, 61.0, 60.7, 55.9, 52.3, 50.7, 46.3, 32.0, 31.8, 14.6, 14.4. ESI-MS (m/z): $[M + Na]^+$ 509, $[2M + Na]^+$ 995. Anal. Calcd for $C_{25}H_{30}N_2O_8$: C, 61.72; H, 6.22; N, 5.76. Found: C, 61.66; H 6.18; N, 5.83.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.0c02507>.

Detailed description of conducted antiproliferative assay, X-ray measurements with thermal ellipsoid plot for each crystal structure and crystallographic data, photochemical measurements, a photograph of the photoreaction setup, copies of 1H and ^{13}C NMR, IR, and UV-vis spectra, a photograph of the reaction setup (PDF)

Accession Codes

CCDC 2033154–2033158 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

■ AUTHOR INFORMATION

Corresponding Author

Adam Huczyński – Faculty of Chemistry, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0003-4770-215X; Phone: +48618291673; Email: adhucz@amu.edu.pl

Authors

Dominika Czerwonka – Faculty of Chemistry, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0002-8098-5975

Szymon Sobczak – Faculty of Chemistry, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0001-8234-2503

Tomasz Pędziński – Faculty of Chemistry and Center for Advanced Technology, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0002-4765-6264

Ewa Maj – Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wrocław, Poland; orcid.org/0000-0001-5205-8712

Joanna Wietrzyk – Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wrocław, Poland; orcid.org/0000-0003-4980-6606

Lech Celewicz – Faculty of Chemistry, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0001-6156-2523

Andrzej Kątrusiak – Faculty of Chemistry, Adam Mickiewicz University, 61-614 Poznań, Poland; orcid.org/0000-0002-1439-7278

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.joc.0c02507>

Author Contributions

Conceptualization: D.C. and A.H.. Methodology: D.C., A.H., T.P., S.S., and E.M.. Validation: A.H., A.K., and J.W.. Investigation: D.C., T.P., S.S., and E.M. Resources: L.C., T.P., A.H., A.K., and J.W.. Data curation: D.C., S.S., and E.M. Writing—original draft preparation as well as TOC design: D.C. and S.S.. Writing—review and editing: D.C., A.H., A.K., and J.W.

Visualization: D.C. Supervision: A.H.. Project administration: A.H.. Funding acquisition: A.H. All authors have read and agreed to the published version of the manuscript.

Funding

Financial support from grant of the Polish National Science Centre (NCN) (No. 2016/21/B/ST5/00111) is gratefully acknowledged. D.C. and S.S. acknowledge a scholarship (No. POWR. 03.02.00-00-1026/16 and POWR. 03.02.00-00-1023/17, respectively) cofinanced by the European Union through the European Social Fund under the Operational Program Knowledge Education Development. D.C. acknowledges the NCN for a doctoral scholarship ETIUDA (2020/36/T/ST4/00041).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Greta Klejborowska for valuable suggestions.

■ REFERENCES

- (1) Ahmad, I.; Ahmed, S.; Anwar, Z.; Sheraz, M. A.; Sikorski, M. Photostability and Photostabilization of Drugs and Drug Products. *International Journal of Photoenergy*; Hindawi Publishing Corp., 2016. DOI: 10.1155/2016/8135608.
- (2) *Photostability of Drugs and Drug Formulations*, 2nd ed.; Hanne, HJ, Ed.; <https://www.routledge.com/Photostability-of-Drugs-and-Drug-Formulations/Tonnesen/p/book/9780367394103> (accessed 2020-09-21).
- (3) Tonnesen, H. H. Formulation and Stability Testing of Photolabile Drugs. *Int. J. Pharm.* **2001**, 225 (1–2), 1–14.
- (4) Slobodnick, A.; Shah, B.; Pillinger, M. H.; Krasnokutsky, S. Colchicine: Old and New. *Am. J. Med.* **2015**, 128, 461–470.
- (5) Khanna, D.; Fitzgerald, J. D.; Khanna, P. P.; Bae, S.; Singh, M. K.; Neogi, T.; Pillinger, M. H.; Merrill, J.; Lee, S.; Prakash, S.; Kaldas, M.; Gogia, M.; Perez-Ruiz, F.; Taylor, W.; Lioté, F.; Choi, H.; Singh, J. A.; Dalbeth, N.; Kaplan, S.; Niyyar, V.; Jones, D.; Yarows, S. A.; Roessler, B.; Kerr, G.; King, C.; Levy, G.; Furst, D. E.; Edwards, N. L.; Mandell, B.; Schumacher, H. R.; Robbins, M.; Wenger, N.; Terkeltaub, R. 2012 American College of Rheumatology Guidelines for Management of Gout. Part 1: Systematic Nonpharmacologic and Pharmacologic Therapeutic Approaches to Hyperuricemia. *Arthritis Care Res.* **2012**, 64 (10), 1431–1446.
- (6) Grattagliano, I.; Bonfrate, L.; Ruggiero, V.; Scaccianoce, G.; Palasciano, G.; Portincasa, P. Novel Therapeutics for the Treatment of Familial Mediterranean Fever: From Colchicine to Biologics. *Clin. Pharmacol. Ther.* **2013**, 95 (1), 89–97.
- (7) Imazio, M.; Gaita, F.; Le Winter, M. Evaluation and Treatment of Pericarditis: A Systematic Review. *JAMA - Journal of the American Medical Association.* **2015**, 314, 1498–1506.
- (8) Vindya, N. G.; Sharma, N.; Yadav, M.; Ethiraj, K. R. Tubulins - The Target for Anticancer Therapy. *Curr. Top. Med. Chem.* **2015**, 15 (1), 73–82.
- (9) Seligmann, J.; Twelves, C. Tubulin: An Example of Targeted Chemotherapy. *Future Med. Chem.* **2013**, 5, 339–352.
- (10) Katsetos, C. D.; Draber, P. Tubulins as Therapeutic Targets in Cancer: From Bench to Bedside. *Curr. Pharm. Des.* **2012**, 18 (19), 2778–2792.
- (11) Cocco, G.; Chu, D. C. C.; Pandolfi, S. Colchicine in Clinical Medicine. A Guide for Internists. *Eur. J. Intern. Med.* **2010**, 21, 503–508.
- (12) Yang, L. P. H. Oral Colchicine (Colcris®): In the Treatment and Prophylaxis of Gout. *Drugs* **2010**, 70, 1603–1613.
- (13) Avendaño, C.; Menéndez, J. C. *Medicinal Chemistry of Anticancer Drugs*; Elsevier, 2008. DOI: 10.1016/B978-0-444-52824-7.X0001-7.
- (14) Kozaka, T.; Nakagawa-Goto, K.; Shi, Q.; Lai, C. Y.; Hamel, E.; Bastow, K. F.; Brossi, A.; Lee, K. H. Antitumor Agents 273. Design and

Synthesis of N-Alkyl-Thiocolchicinoids as Potential Antitumor Agents. *Bioorg. Med. Chem. Lett.* **2010**, *20* (14), 4091–4094.

(15) Kumar, A.; Sharma, P. R.; Mondhe, D. M. Potential Anticancer Role of Colchicine-Based Derivatives: An Overview. *Anti-Cancer Drugs* **2017**, *28*, 250–262.

(16) Johnson, L.; Goping, I. S.; Rieger, A.; Mane, J. Y.; Huzil, T.; Banerjee, A.; Luduena, R.; Hassani, B.; Winter, P.; Tuszynski, J. A. Novel Colchicine Derivatives and Their Anti-Cancer Activity. *Curr. Top. Med. Chem.* **2017**, DOI: 10.2174/1568026617666170104143618.

(17) Marzo-Mas, A.; Barbier, P.; Breuzard, G.; Allegro, D.; Falomir, E.; Murga, J.; Carda, M.; Peyrot, V.; Marco, J. A. Interactions of Long-Chain Homologues of Colchicine with Tubulin. *Eur. J. Med. Chem.* **2017**, *126*, 526–535.

(18) Chang, D. J.; Yoon, E. Y.; Lee, G. B.; Kim, S. O.; Kim, W. J.; Kim, Y. M.; Jung, J. W.; An, H.; Suh, Y. G. Design, Synthesis and Identification of Novel Colchicine-Derived Immunosuppressant. *Bioorg. Med. Chem. Lett.* **2009**, *19* (15), 4416–4420.

(19) Nicolaou, K. C.; Valiulin, R. A.; Pokorski, J. K.; Chang, V.; Chen, J. S. Bio-Inspired Synthesis and Biological Evaluation of a Colchicine-Related Compound Library. *Bioorg. Med. Chem. Lett.* **2012**, *22* (11), 3776–3780.

(20) Zhang, X.; Kong, Y.; Zhang, J.; Su, M.; Zhou, Y.; Zang, Y.; Li, J.; Chen, Y.; Fang, Y.; Zhang, X.; Lu, W. Design, Synthesis and Biological Evaluation of Colchicine Derivatives as Novel Tubulin and Histone Deacetylase Dual Inhibitors. *Eur. J. Med. Chem.* **2015**, *95*, 127–135.

(21) Yasobu, N.; Kitajima, M.; Kogure, N.; Shishido, Y.; Matsuzaki, T.; Nagaoka, M.; Takayama, H. Design, Synthesis, and Antitumor Activity of 4-Halocolchicines and Their pro-Drugs Activated by Cathepsin B. *ACS Med. Chem. Lett.* **2011**, *2* (5), 348–352.

(22) Shchegravina, E. S.; Maleev, A. A.; Ignatov, S. K.; Gracheva, I. A.; Stein, A.; Schmalz, H. G.; Gavryushin, A. E.; Zubareva, A. A.; Svirshchevskaya, E. V.; Fedorov, A. Y. Synthesis and Biological Evaluation of Novel Non-Racemic Indole-Containing Alcolchicinoids. *Eur. J. Med. Chem.* **2017**, *141*, 51–60.

(23) Nakagawa-Goto, K.; Chen, C. X.; Hamel, E.; Wu, C. C.; Bastow, K. F.; Brossi, A.; Lee, K. H. Antitumor Agents. Part 236: Synthesis of Water-Soluble Colchicine Derivatives. *Bioorg. Med. Chem. Lett.* **2005**, *15* (1), 235–238.

(24) Czerwonka, D.; Sobczak, S.; Maj, E.; Wietrzyk, J.; Katrusiak, A.; Huczyński, A. Synthesis and Antiproliferative Screening Of Novel Analogs of Regioselectively Demethylated Colchicine and Thiocolchicine. *Molecules* **2020**, *25* (5), 1180.

(25) Majcher, U.; Urbaniak, A.; Maj, E.; Moshari, M.; Delgado, M.; Wietrzyk, J.; Bartl, F.; Chambers, T. C.; Tuszynski, J. A.; Huczyński, A. Synthesis, Antiproliferative Activity and Molecular Docking of Thiocolchicine Urethanes. *Bioorg. Chem.* **2018**, *81*, 553–566.

(26) Huczyński, A.; Majcher, U.; Maj, E.; Wietrzyk, J.; Janczak, J.; Moshari, M.; Tuszynski, J. A.; Bartl, F. Synthesis, Antiproliferative Activity and Molecular Docking of Colchicine Derivatives. *Bioorg. Chem.* **2016**, *64*, 103–112.

(27) Klejborowska, G.; Urbaniak, A.; Preto, J.; Maj, E.; Moshari, M.; Wietrzyk, J.; Tuszynski, J. A.; Chambers, T. C.; Huczyński, A. Synthesis, Biological Evaluation and Molecular Docking Studies of New Amides of 4-Bromothiocolchicine as Anticancer Agents. *Bioorg. Med. Chem.* **2019**, *27*, 115144.

(28) Sabnis, D. D. Lumicolchicine as a Tool in the Study of Plant Microtubules: Some Biological Effects of Sequential Products Formed during Phototransformations of Colchicine. *J. Exp. Bot.* **1981**, *32* (1), 271–278.

(29) Bussotti, L.; Caccioli, I.; D'Auria, M.; Foggi, P.; Lesma, G.; Silvani, A.; Villani, V. Photochemical Isomerization of Colchicine and Thiocolchicine. *J. Phys. Chem. A* **2003**, *107* (43), 9079–9085.

(30) Roigt, H.; Leblanc, R. M. Processus Photophysiques Dans La Molécule de Colchicine. *Can. J. Chem.* **1973**, *51* (17), 2821–2827.

(31) Ghanem, R.; Baker, H.; Seif, M. A.; Al-Qawasmeh, R. A.; Mataneh, A. A.; Al-Gharabli, S. I. Photochemical Transformation of Colchicine: A Kinetic Study. *J. Solution Chem.* **2010**, *39* (4), 441–456.

(32) Sagorin, C.; Ertel, N. H.; Wallace, S. L. Photoisomerization of Colchicine. Loss of Significant Antimitotic Activity in Human Lymphocytes. *Arthritis Rheum.* **1972**, *15* (2), 213–217.

(33) Malawista, S. E.; Chang, Y. -H.; Wilson, L. Lumicolchicine: Lack of Antiinflammatory Effect. *Arthritis Rheum.* **1972**, *15* (6), 641–643.

(34) Bussotti, L.; D'Auria, M.; Foggi, P.; Lesma, G.; Righini, R.; Silvani, A. The Photochemical Behavior of Colchicone and Thiocolchicone. *Photochem. Photobiol.* **2000**, *71* (1), 29.

(35) Singh, S. P.; Stenberg, V. I.; Parmar, S. S. Photochemistry of Alkaloids. *Chem. Rev.* **1980**, *80* (3), 269–282.

(36) Caccioli, I.; D'Auria, M.; Villani, V. Why Thiocolchicine Does Not Undergo Photochemical Isomerization: A Theoretical Study. *Chem. Phys. Lett.* **2008**, *459* (1–6), 167–171.

(37) Forbes, E. J. Colchicine and Related Compounds. Part XIV. Structure of β - and γ -Lumicolchicine. *J. Chem. Soc.* **1955**, *0* (0), 3864–3870.

(38) Yuan, H.; Guo, X.; Zhang, J. Ab Initio Insights into the Mechanism of Excited-State Intramolecular Proton Transfer Triggered by the Second Excited Singlet State of a Fluorescent Dye: An Anti-Kasha Behavior. *Mater. Chem. Front.* **2019**, *3* (6), 1225–1230.

(39) Ignasiak, M. T.; Houée-Levin, C.; Kciuk, G.; Marciniak, B.; Pedzinski, T. A Reevaluation of the Photolytic Properties of 2-Hydroxybenzophenone-Based UV Sunscreens: Are Chemical Sunscreens Inoffensive? *ChemPhysChem* **2015**, *16* (3), 628–633.

(40) Han, K.; Zhao, G.; Pradhan, R.; Lourderaj, U. Computational Studies on the Excited-State Intramolecular Proton Transfer in Five-Membered-Ring Hydrogen-Bonded Systems. In *Hydrogen-Bonding Research in Photochemistry, Photobiology, and Optoelectronic Materials*; World Scientific, 2019; pp 155–178. DOI: 10.1142/9781786346087_0007.

(41) Majcher, U.; Klejborowska, G.; Moshari, M.; Maj, E.; Wietrzyk, J.; Bartl, F.; Tuszynski, J. A.; Huczyński, A. Antiproliferative Activity and Molecular Docking of Novel Double-Modified Colchicine Derivatives. *Cells* **2018**, *7* (11), 192.

(42) Kerkes, P.; Sharma, P. N.; Brossi, A.; Chignell, C. F.; Quinn, F. R. Synthesis and Biological Effects of Novel Thiocolchicines. 3. Evaluation of N-Acyldeacetylthiocolchicines, N-(Alkoxy carbonyl)-Deacetylthiocolchicines, and O-Ethyldeacetylthiocolchicines. New Synthesis of Thiodemecolcine and Antileukemic Effects of 2-Demeth. *J. Med. Chem.* **1985**, *28* (9), 1204–1208.

Photoinduced skeletal rearrangement of *N*-substituted colchicine derivatives

Dominika Czerwonka¹, Szymon Sobczak¹, Tomasz Pędziński^{1,2}, Ewa Maj³, Joanna Wietrzyk³, Lech Celewicz¹, Andrzej Katrusiak¹, Adam Huczyński^{1*}

¹ Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland;

² Center for Advanced Technology, Adam Mickiewicz University, Uniwersytetu Poznańskiego 10, 61-614 Poznań, Poland

³ Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland;

* Correspondence: adhucz@amu.edu.pl; Tel.: +48618291673

Supplementary Information

INDEX

The antiproliferative activity	S4
The Antiproliferative Assays <i>In Vitro</i>	S4
SRB	S5
Photochemical measurements	S5
X-ray measurements	S6
Figure S1. Crystallographically determined X-ray structure representations of β -lumicolchicine.	S7
Figure S2. Crystallographically determined X-ray structure representations of 4f.	S8
Figure S3. Crystallographically determined X-ray structure representations of 4b.	S9
Figure S4. Crystallographically determined X-ray structure representations of 3·0.5 Ac.....	S10
Figure S5. Crystallographically determined X-ray structure representations of 2·Ac.....	S11
Table S1. Detailed crystallographic data compounds β -lumicolchicine, 2, 3 and 4b-UV and 4f-UV.....	S12
¹ H and ¹³ C NMR spectra of compounds 2, 3, 3a-f and 4, 4b-f.....	S13
Figure S6. The ¹ H (400 MHz) NMR spectrum of 2 in CDCl ₃	S13
Figure S7. The ¹³ C (101 MHz) NMR spectrum of 2 in CDCl ₃	S14
Figure S8. The ¹ H (400 MHz) NMR spectrum of 3 in DMSO- <i>d</i> ₆	S15
Figure S9. The ¹³ C (101 MHz) NMR spectrum of 3 in DMSO- <i>d</i> ₆	S16
Figure S10. The ¹ H (400 MHz) NMR spectrum of 3a in CDCl ₃	S17
Figure S11. The ¹³ C (101 MHz) NMR spectrum of 3a in CDCl ₃	S18

Figure S12. The ^1H (400 MHz) NMR spectrum of 3b in CDCl_3 .	S19
Figure S13. The ^{13}C (101 MHz) NMR spectrum of 3b in CDCl_3 .	S20
Figure S14. The ^1H (400 MHz) NMR spectrum of 3c in CDCl_3 .	S21
Figure S15. The ^{13}C (101 MHz) NMR spectrum of 3c in CDCl_3 .	S22
Figure S16. The ^1H (400 MHz) NMR spectrum of 3d in CDCl_3 .	S23
Figure S17. The ^{13}C (101 MHz) NMR spectrum of 3d in CDCl_3 .	S24
Figure S18. The ^1H (400 MHz) NMR spectrum of 3e in CDCl_3 .	S25
Figure S19. The ^{13}C (101 MHz) NMR spectrum of 3e in CDCl_3 .	S26
Figure S20. The ^1H (400 MHz) NMR spectrum of 3f in CDCl_3 .	S27
Figure S21. The ^{13}C (101 MHz) NMR spectrum of 3f in CDCl_3 .	S28
Figure S22. The ^1H (400 MHz) NMR spectrum of 4 in $\text{DMSO}-d_6$.	S29
Figure S23. The ^{13}C (101 MHz) NMR spectrum of 4 in $\text{DMSO}-d_6$.	S30
Figure S24. The ^1H (400 MHz) NMR spectrum of 4b in CDCl_3 .	S31
Figure S25. The ^{13}C (101 MHz) NMR spectrum of 4b in CDCl_3 .	S32
Figure S26. The ^1H (400 MHz) NMR spectrum of 4c in CDCl_3 .	S33
Figure S27. The ^{13}C (101 MHz) NMR spectrum of 4c in CDCl_3 .	S34
Figure S28. The ^1H (400 MHz) NMR spectrum of 4d in CDCl_3 .	S35
Figure S29. The ^{13}C (101 MHz) NMR spectrum of 4d in CDCl_3 .	S36
Figure S30. The ^1H (400 MHz) NMR spectrum of 4e in CDCl_3 .	S37
Figure S31. The ^{13}C (101 MHz) NMR spectrum of 4e in CDCl_3 .	S38
Figure S32. The ^1H (400 MHz) NMR spectrum of 4f in CDCl_3 .	S39
Figure S33. The ^{13}C (101 MHz) NMR spectrum of 4f in CDCl_3 .	S40
^1H and ^{13}C NMR spectra of β -lumiderivatives: β -lumicolchicine, 2-UV, 3-UV, 3b-UV, 3f-UV, 4b-UV, 4f-UV)	S41
Figure S34. The ^1H (400 MHz) NMR spectrum of β -lumicolchicine in CDCl_3 .	S41
Figure S35. The ^{13}C (101 MHz) NMR spectrum of β -lumicolchicine in CDCl_3 .	S42
Figure S36. The ^1H (400 MHz) NMR spectrum of 2-UV in CDCl_3 .	S43
Figure S37. The ^{13}C (101 MHz) NMR spectrum of 2-UV in CDCl_3 .	S44
Figure S38. The ^1H (400 MHz) NMR spectrum of 3-UV in $\text{DMSO}-d_6$.	S45
Figure S39. The ^{13}C (101 MHz) NMR spectrum of 3-UV in $\text{DMSO}-d_6$.	S46
Figure S40. The ^1H (400 MHz) NMR spectrum of 3b-UV in CDCl_3 .	S47
Figure S41. The ^{13}C (101 MHz) NMR spectrum of 3b-UV in CDCl_3 .	S48
Figure S42. The ^1H (400 MHz) NMR spectrum of 3f-UV in CDCl_3 .	S49
Figure S43. The ^{13}C (101 MHz) NMR spectrum of 3f-UV in CDCl_3 .	S50
Figure S44. The ^1H (400 MHz) NMR spectrum of 4b-UV in CDCl_3 .	S51
Figure S45. The ^{13}C (101 MHz) NMR spectrum of 4b-UV in CDCl_3 .	S52

Figure S46. The ^1H (400 MHz) NMR spectrum of 4f-UV in CDCl_3 .	S53
Figure S47. The ^{13}C (101 MHz) NMR spectrum of 4f-UV in CDCl_3 .	S54
Table S2. The ^1H and ^{13}C NMR chemical shifts δ (ppm) of 2 and 2-UV in chloroform-d, assigned based on 2D NMR.	S55
IR spectra	S56
Figure S48. The stacked FT-IR spectra of colchicine (blue solid line) and β -lumicolchicine (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S56
Figure S49. The stacked FT-IR spectra of 2 (blue solid line) and 2-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S57
Figure S50. The stacked FT-IR spectra of 3 (blue solid line) and 3-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S58
Figure S51. The stacked FT-IR spectra of 3b (blue solid line) and 3b-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S59
Figure S52. The stacked FT-IR spectra of 3f (blue solid line) and 3f-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S60
Figure S53. The stacked FT-IR spectra of 4b (blue solid line) and 4b-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S61
Figure S54. The stacked FT-IR spectra of 4f (blue solid line) and 4f-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .	S62
UV-VIS spectra	S63
Figure S55. The stacked UV-VIS spectra of colchicine (blue solid line) and β -lumicolchicine (red dashed line) in methanol.	S63
Figure S56. The stacked UV-VIS spectra of 2 (blue solid line) and 2-UV (red dashed line) in methanol.	S63
Figure S57. The stacked UV-VIS spectra of 3 (blue solid line) and 3-UV (red dashed line) in methanol.	S64
Figure S58. The stacked UV-VIS spectra of 3b (blue solid line) and 3b-UV (red dashed line) in methanol.	S64
Figure S59. The stacked UV-VIS spectra of 3f (blue solid line) and 3f-UV (red dashed line) in methanol.	S65
Figure S60. The stacked UV-VIS spectra of 4b (blue solid line) and 4b-UV (red dashed line) in methanol.	S65
Figure S61. The stacked UV-VIS spectra of 4f (blue solid line) and 4f-UV (red dashed line) in methanol.	S66
Photoreaction Setup	S67
Figure S62. Photograph of a photoreaction setup.	S67
Figure S63. Emission spectrum of the Hanau TQ 150 W lamp after passing through the standard Pyrex filter.	S68

The antiproliferative activity

Three human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of colchicine and its derivatives: human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line (LoVo), and normal murine embryonic fibroblast cell line (BALB/3T3). The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), A549 and MCF-7 cell lines—from European Collection of Authenticated Cell Cultures (Salisbury, UK), LoVo cell line was purchased from the ATCC (ATCC, Manassas, VA, USA). All the cell lines are maintained in the Institute of Immunology and Experimental Therapy (IET), Wrocław, Poland. Human lung adenocarcinoma cell line was cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM L-glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human breast adenocarcinoma cell line was cultured in mixture of Eagle medium (IET, Wrocław, Poland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 8 µg/mL insulin and 1% amino-acids (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human colon adenocarcinoma cell line was cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan UT, USA), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Murine embryonic fibroblast cells were cultured in Dulbecco medium (Life Technologies Limited, Paisley, UK), supplemented with 10% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). All culture media contained antibiotics: 100 U/mL penicillin and 100 µg/mL streptomycin (Polfa-Tarchomin, Warsaw, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO₂. Cells were tested for mycoplasma contamination by mycoplasma detection kit for conventional PCR: Venor GeM Classic (Minerva Biolabs GmbH, Berlin, Germany) and negative results was obtained. The procedure is repeated every year or in the case of less frequently used lines: after thawing.

The Antiproliferative Assays *In Vitro*

Twenty-four hours before adding the tested compounds, all cell lines were seeded in 384-well plates (Sarstedt, Nümbrecht, Germany) in appropriate media with 1×10^3 or $1,5 \times 10^3$

or 3×10^3 cells per well. All cell lines were exposed to each tested agent at four different concentrations in the range 100–0.001 $\mu\text{g/mL}$ for 72 h. Cells were also exposed to the reference drug cisplatin (Teva Pharmaceuticals Polska, Warsaw, Poland) and doxorubicin (Accord Healthcare Limited, Middlesex, UK). Additionally, all cell lines were exposed to DMSO (solvent used for tested compounds) (POCh, Gliwice, Poland) at concentrations corresponding to those present in tested agents' dilutions. After 72 h sulforhodamine B assay (SRB) was performed.¹

SRB

After 72 h of incubation with the tested compounds, cells were fixed in situ by gently adding of 30 μL per well of cold 50% trichloroacetic acid TCA (POCh, Gliwice, Poland) and were incubated at 4 °C for one hour. Following, wells were washed four times with water and air dried. Next, 25 μL of 0.1% solution of sulforhodamine B (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA) in 1% acetic acid (POCh, Gliwice, Poland) were added to each well and plates were incubated at room temperature for 0.5 h. After incubation time, unbound dye was removed by washing plates four times with 1% acetic acid whereas stain bound to cells was solubilized with 10 mM Tris base (Sigma-Aldrich, Steinheim, Germany). Absorbance of each solution was read at Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) at the 540 nm wavelength.

Results are presented as mean IC_{50} (concentration of the tested compound, that inhibits cell proliferation by 50%) $\pm$ standard deviation. IC_{50} values were calculated in Cheburator 0.4, Dmitry Nevozhay software (version 1.2.0 software by Dmitry Nevozhay, 2004–2014, <http://www.cheburator.nevozhay.com>, freely available) for each experiment.² Compounds at each concentration were tested in triplicates in single experiment and each experiment was repeated at least three times independently.

Photochemical measurements

Femtosecond transient absorption (TA) measurements were carried out using the Solstice Ti:Sapphire regenerative amplifier from Spectra Physics and an optical detection system provided by Ultrafast Systems (Helios). The source for the pump and probe pulses was the fundamental emission at 800 nm. The fundamental output was split into two beams: a pump (95%) and a probe (5%). The pump beam was directed through the TOPAS-Prime automated optical parametric amplifier (from Light Conversion) to tune the desired excitation

wavelength (e.g. 330 nm). The probe continuum beam was directed to the Helios, a CCD-based pump–probe TA spectrometer from Ultrafast Systems LLC with an optical delay line allowing delays between the pump and probe up to 3 ns. For the detection of the transients, a white light continuum was used, which was generated from 5% of the fundamental beam by passing it through a sapphire or calcium fluoride crystal. The setup is described in more detail in ref. [3].³

X-ray measurements

The single crystals were obtained from the solutions of β -lumicolchicine, **2**, **3**, 4b-UV and 4f-UV in chloroform:acetone 1:1 mixture. The X-ray diffraction measurements at room temperature of above mentioned compounds were carried out on two different diffractometers. First one equipped with MoK α (λ = 0.7108 Å) source, Eos Xcalibur, and the second one, with microfocus CuK α (λ = 1.5418 Å) radiation source, SuperNova Agilent diffractometer. For data collections and their preliminary reduction, CrysAlisPro 171.37.31 software was used.⁴ The crystal structures were solved by direct methods in Shelxs and refined by full-matrix least squares by Shelxl;^{5,6} implemented in the Olex2.⁷ The position of hydrogen atoms were located at their best geometrical positions. Molecules **2** and **3** crystallizes as a acetone solvates with one and half disordered acetone molecule per formula unit respectively. The final crystal data are summarized in Table S1 and have been deposited in the Cambridge Structural Database as a supplementary publications CCSD No. 2033156, 2033158, 2033157, 2033154 and 2033155 for compound β -lumicolchicine, **2**, **3**, 4b-UV and 4f-UV, respectively. Copies of this information may be obtained free of charge from <http://www.ccdc.cam.ac.uk>

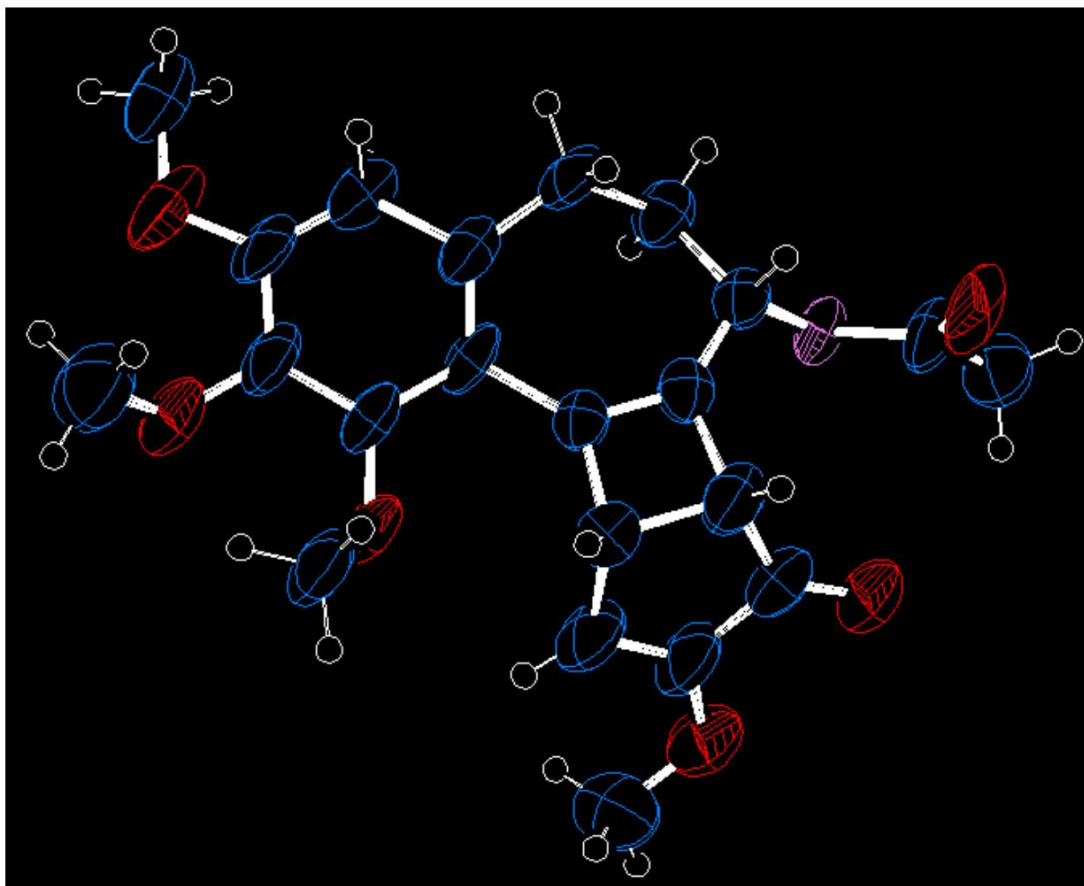


Figure S1. Crystallographically determined X-ray structure representations of β -lumicolchicine. The thermal ellipsoid plot were drawn at 50% probability level. Atom colour code: carbon, blue; nitrogen, magenta; oxygen, red; hydrogen, white.

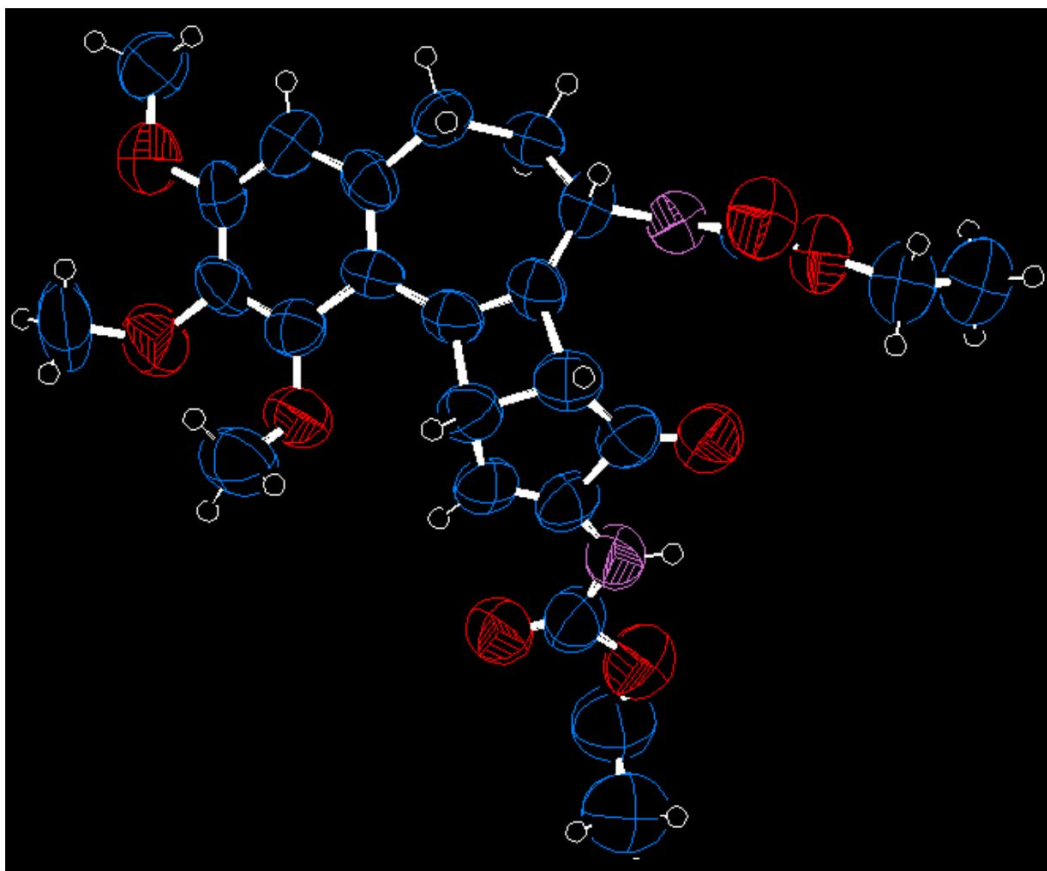


Figure S2. Crystallographically determined X-ray structure representations of **4f**. The thermal ellipsoid plot were drawn at 50% probability level. Atom colour code: carbon, blue; nitrogen, magenta; oxygen, red; hydrogen, white.

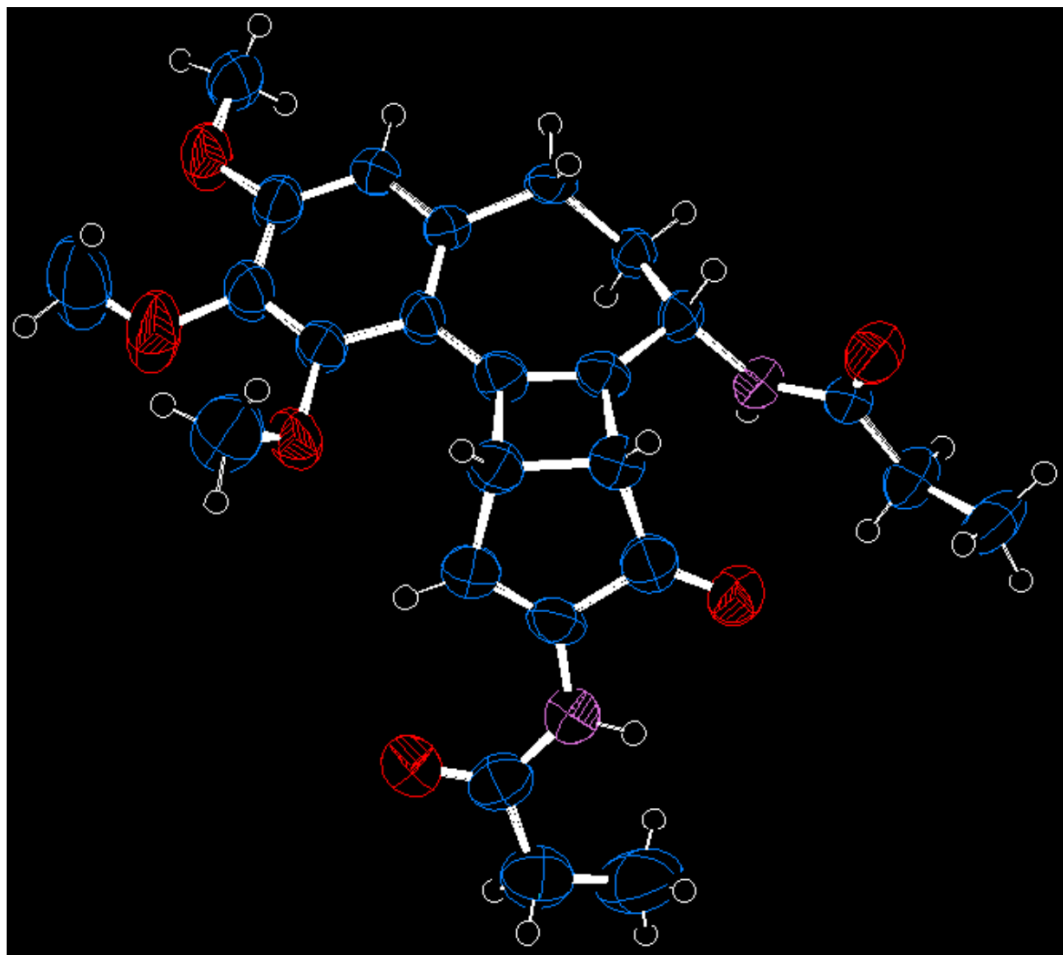


Figure S3. Crystallographically determined X-ray structure representations of **4b**. The thermal ellipsoid plot were drawn at 50% probability level. Atom colour code: carbon, blue; nitrogen, magenta; oxygen, red; hydrogen, white.

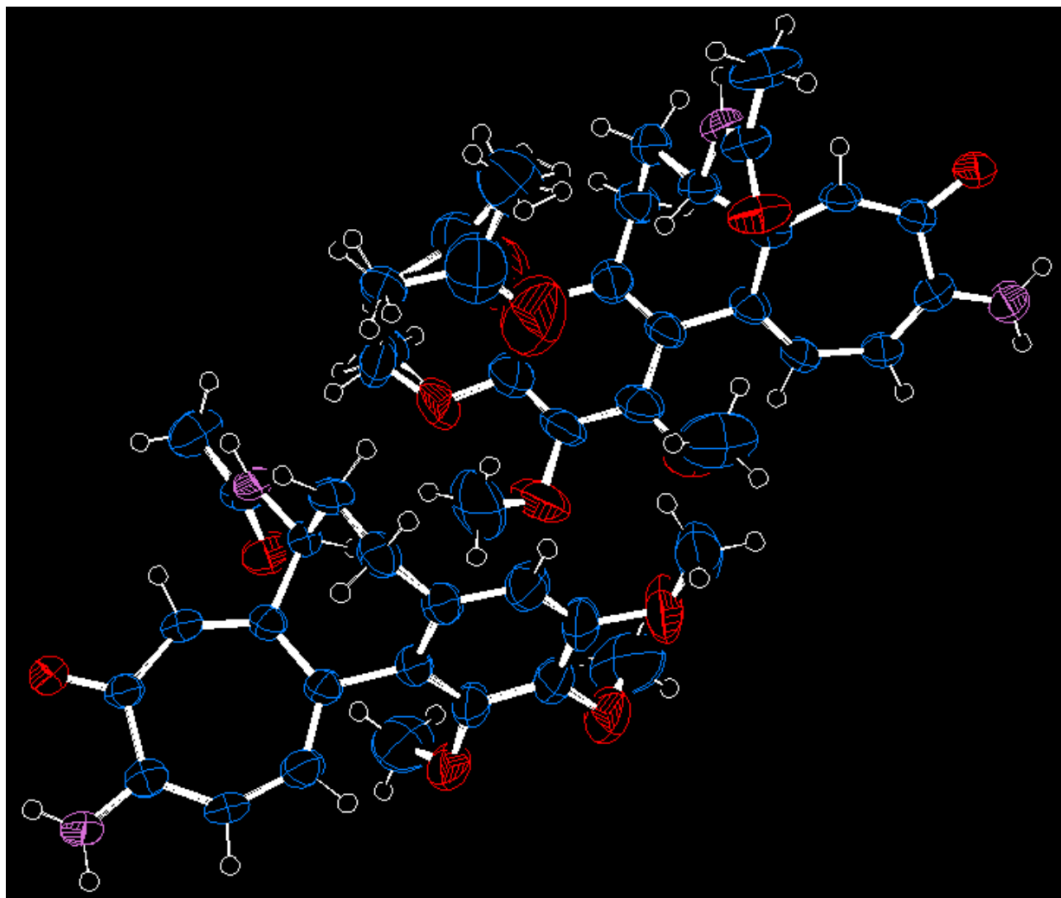


Figure S4. Crystallographically determined X-ray structure representations of **3·0.5 Ac**. The thermal ellipsoid plot were drawn at 50% probability level. Atom colour code: carbon, blue; nitrogen, magenta; oxygen, red; hydrogen, white.

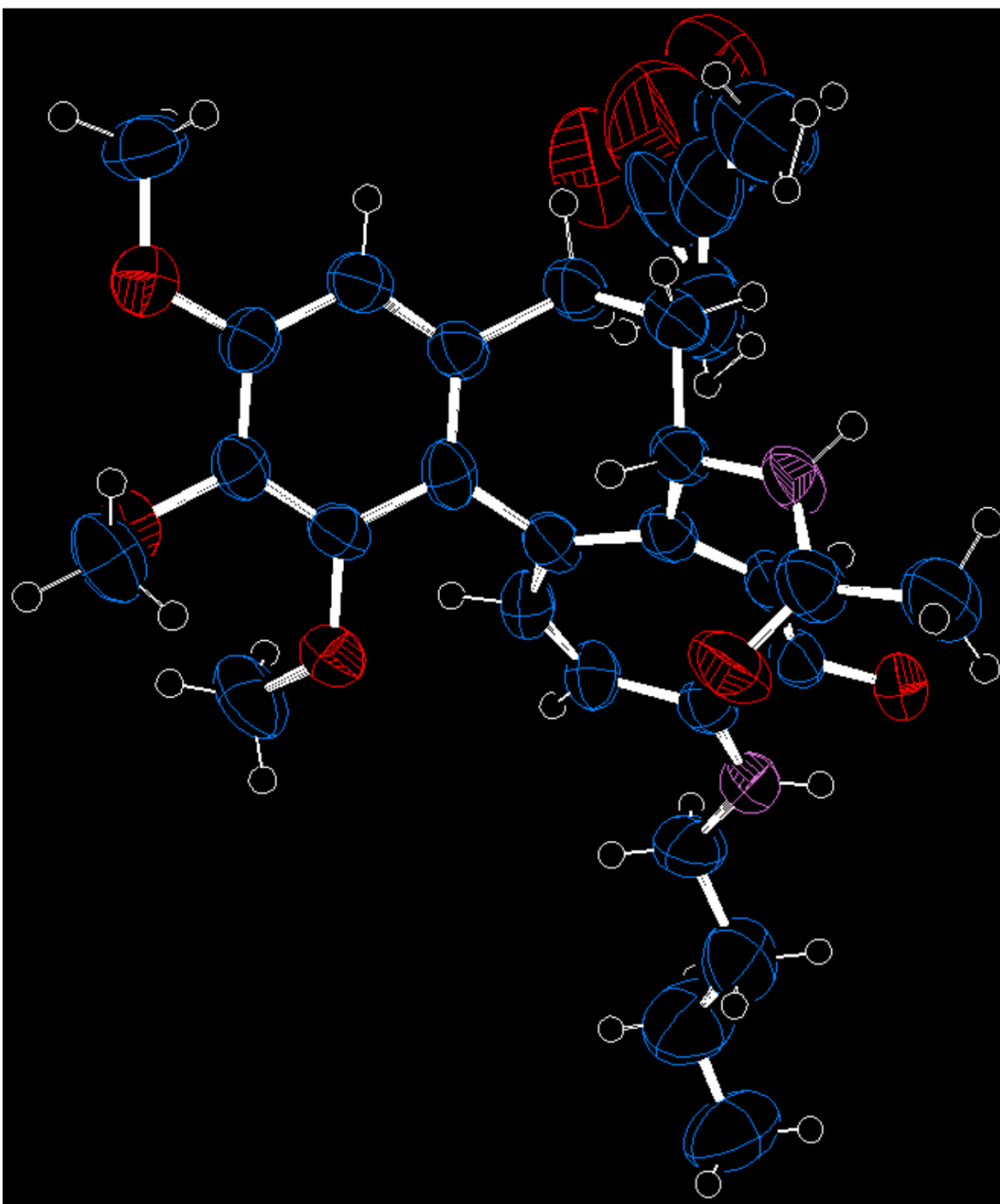


Figure S5. Crystallographically determined X-ray structure representations of 2·Ac. The thermal ellipsoid plot were drawn at 50% probability level. Atom colour code: carbon, blue; nitrogen, magenta; oxygen, red; hydrogen, white.

Table S1. Detailed crystallographic data compounds β -lumicolchicine, 2, 3 and 4b-UV and 4f-UV.

Name	β -lumicolchicine	2	3	4b-UV	4f-UV
Formula	$C_{22}H_{25}NO_6$	$C_{25}H_{32}N_2O_5 \cdot Ac$	$C_{21}H_{24}N_2O_5 \cdot 0.5 Ac$	$C_{25}H_{30}N_2O_6$	$C_{25}H_{30}N_2O_6$
Crystal system	orthorhombic	tetragonal	monoclinic	orthorhombic	monoclinic
Space group	$P2_12_12_1$	$P4_12_12$	$P2_1$	$P2_12_12_1$	$P2_1$
a (Å)	5.0076(15)	9.0814(4)	8.827(17)	5.1856(4)	15.6750(15)
b (Å)	12.3940(14)	9.0814(4)	27.10(3)	18.4774(9)	5.0498(5)
c (Å)	32.017(19)	61.5763(18)	10.21(2)	23.8235(15)	16.5812(17)
Unit cell dimensions	α (°)	90	90	90	90
	β (°)	90	115.3(3)	90	106.585(10)
	γ (°)	90	90	90	90
Volume (Å ³)	1987.1(13)	5078.3(4)	2207(8)	2282.7(3)	1257.9(2)
Z/Z'	4/1	8/1	4/2	4/1	2/1
Calculated density (g/cm ³)	1.335	1.223	1.245	1.323	1.282
Radiation type	Cu K α ($\lambda = 1.54184$)	Cu K α ($\lambda = 1.54184$)	Mo K α ($\lambda = 0.71073$)	Cu K α ($\lambda = 1.54184$)	Cu K α ($\lambda = 1.54184$)
Absorption (mm ⁻¹)	0.803	0.697	0.089	0.778	0.802
$F(000)$	848.0	2000.0	880	968.0	515.9
Crystal size (mm)	$0.088 \times 0.03 \times 0.017$	$0.25 \times 0.25 \times 0.1$	$0.622 \times 0.389 \times 0.171$	$0.05 \times 0.024 \times 0.01$	$0.4 \times 0.15 \times 0.05$
2 θ -range for data collection (°)	5.52 to 118.104	5.926 to 117.818	5.926 to 51.362	6.054 to 117.85	11.5 to 117.86
Min/max indices: h, k, l	-5 $\leq$ h $\leq$ 4, -13 $\leq$ k $\leq$ 11, -31 $\leq$ l $\leq$ 34	-10 $\leq$ h $\leq$ 7, -5 $\leq$ k $\leq$ 9, -68 $\leq$ l $\leq$ 42	-10 $\leq$ h $\leq$ 10, -33 $\leq$ k $\leq$ 33, -12 $\leq$ l $\leq$ 12	-5 $\leq$ h $\leq$ 5, -20 $\leq$ k $\leq$ 20, -26 $\leq$ l $\leq$ 24	-17 $\leq$ h $\leq$ 17, -5 $\leq$ k $\leq$ 5, -18 $\leq$ l $\leq$ 18
Reflect. Collected/unique	4834/2698	4188/2520	77577/8349	4384/2788	13112/1509
Data/restraints/parameters	2698/293/265	2520/80/337	8349/183/581	2788/0/303	1509/139/322
Goodness-of-fit on F ²	0.850	1.079	1.028	0.992	1.158
Final R1/wR2 ($>2\sigma$)	0.1176/0.1831	0.0396/0.1016	0.0638/0.1658	0.0641/0.0923	0.0876/0.2038
R1/wR2 (all data)	0.3972/0.3767	0.0453/0.1053	0.0743/0.1769	0.1221/0.1184	0.0970/0.2161
Largest diff. peak/hole (e.Å ⁻³)	0.20/-0.26	0.18/-0.17	0.42/-0.35	0.19/-0.23	0.23/-0.20

$$w = 1/(\sigma^2 F_o^2 + w_1^2 \Delta P^2 + w_2^2 P), \text{ where } P = (\text{Max}(F_o^2, 0) + 2^*F_o^2)$$

S12

1H and ^{13}C NMR spectra of compounds 2, 3, 3a-f and 4, 4b-f

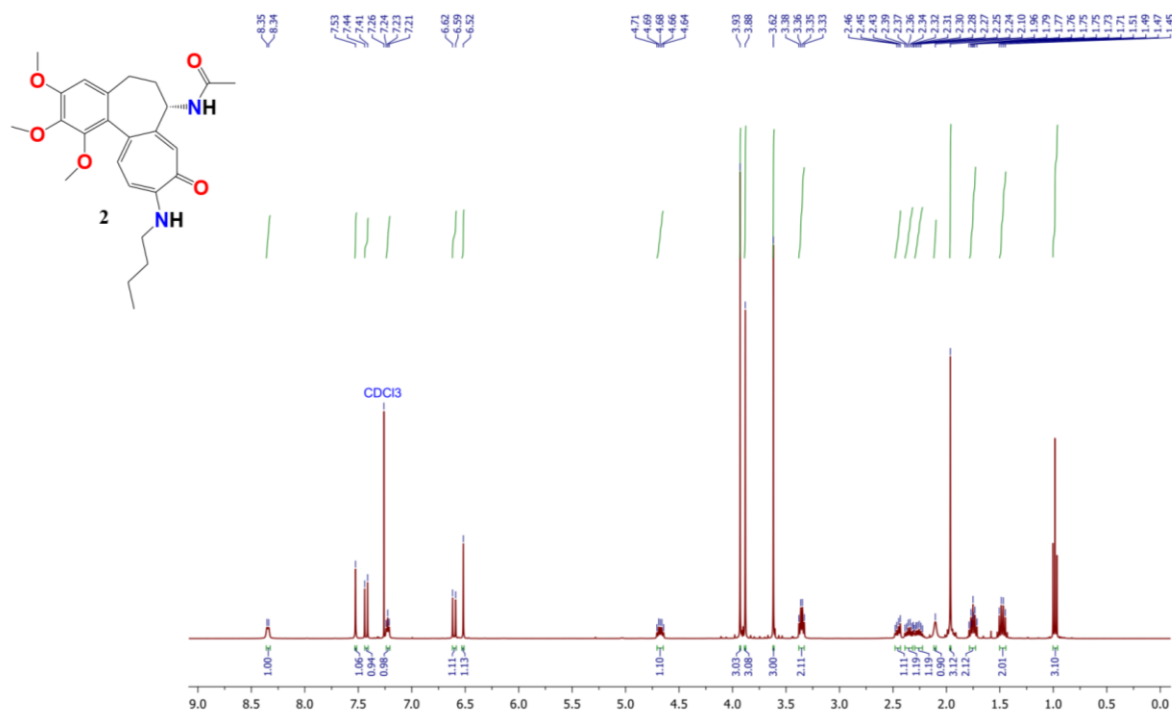


Figure S6. The 1H (400 MHz) NMR spectrum of 2 in $CDCl_3$.

S13

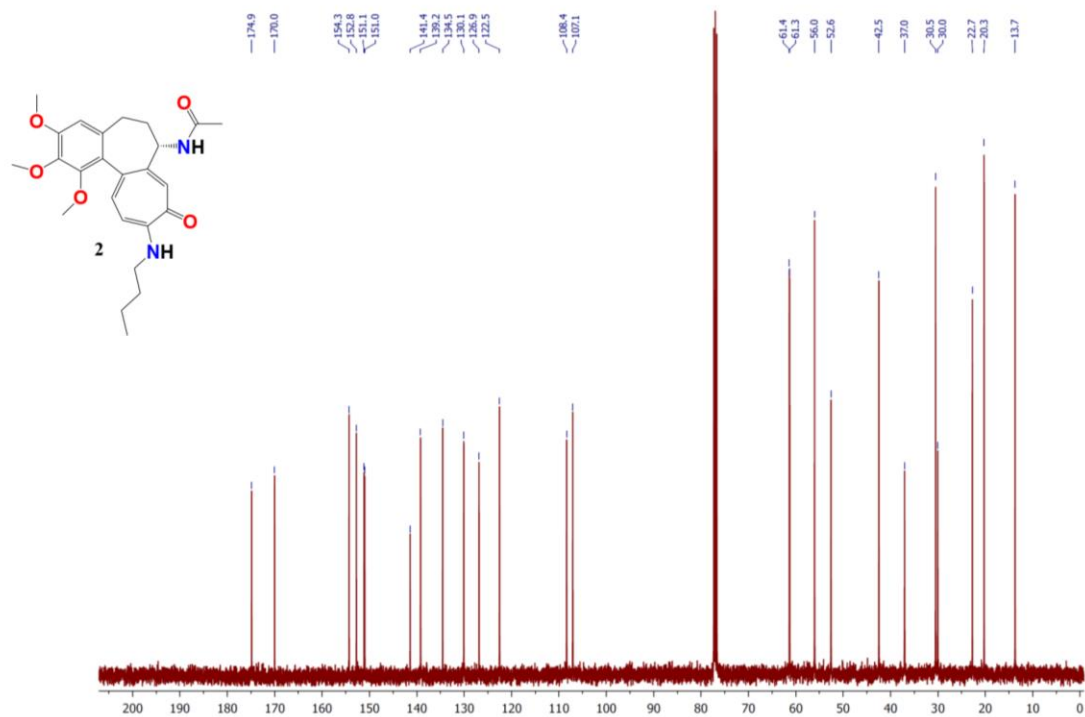


Figure S7. The ^{13}C (101 MHz) NMR spectrum of 2 in CDCl_3 .

S14

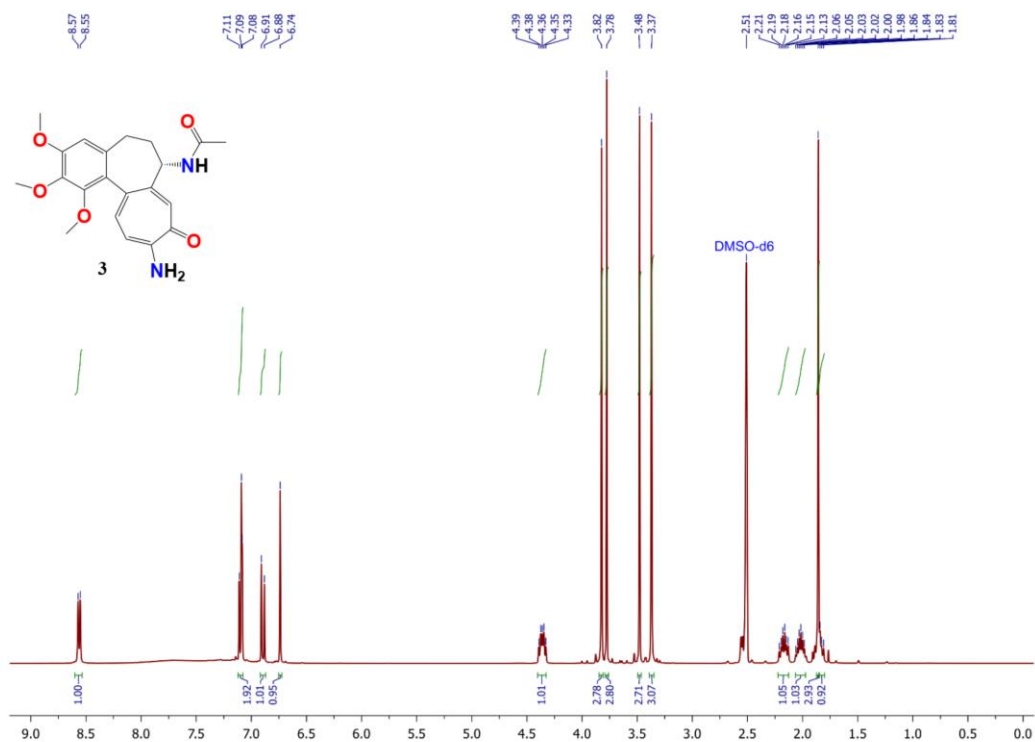


Figure S8. The ^1H (400 MHz) NMR spectrum of 3 in $\text{DMSO}-d_6$.

S15

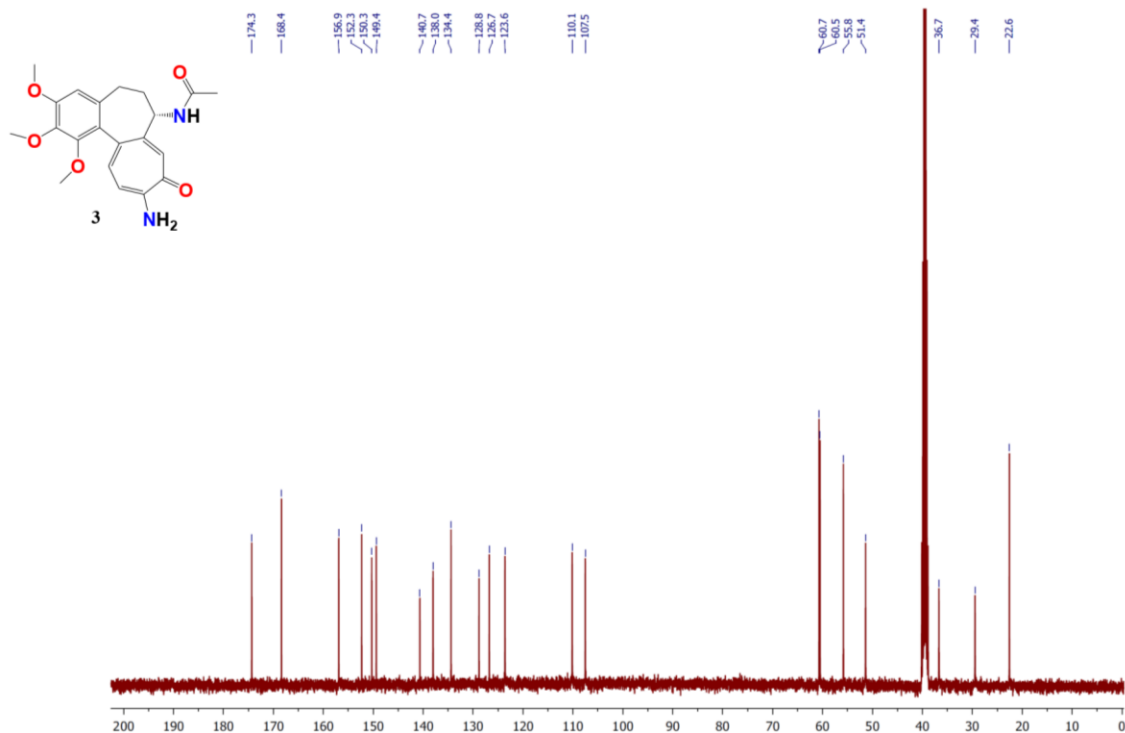


Figure S9. The ¹³C (101 MHz) NMR spectrum of 3 in DMSO-*d*₆.

S16

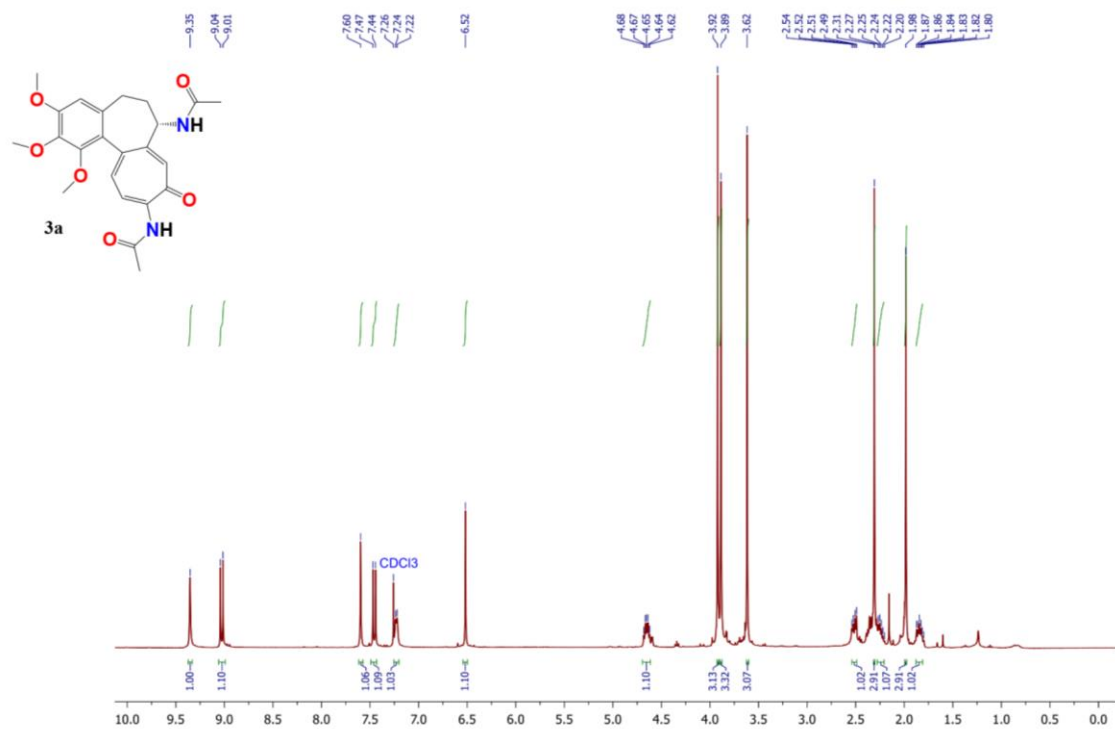


Figure S10. The ¹H (400 MHz) NMR spectrum of 3a in CDCl₃.

S17

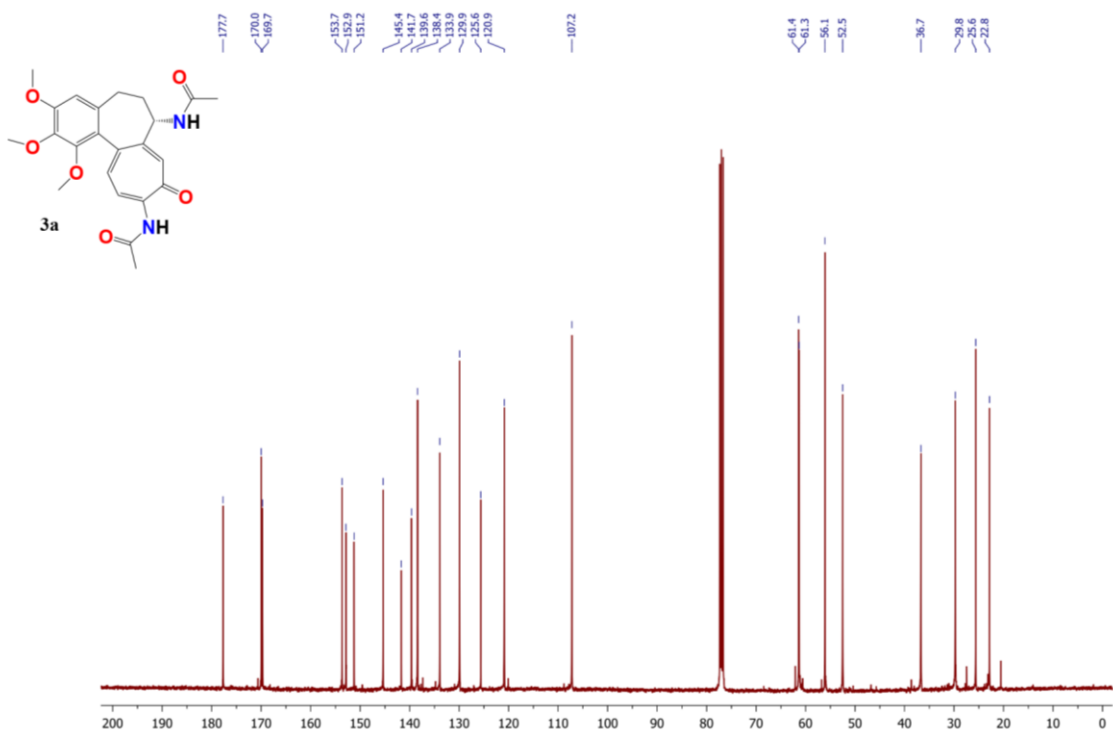


Figure S11. The ^{13}C (101 MHz) NMR spectrum of **3a** in CDCl_3 .

S18

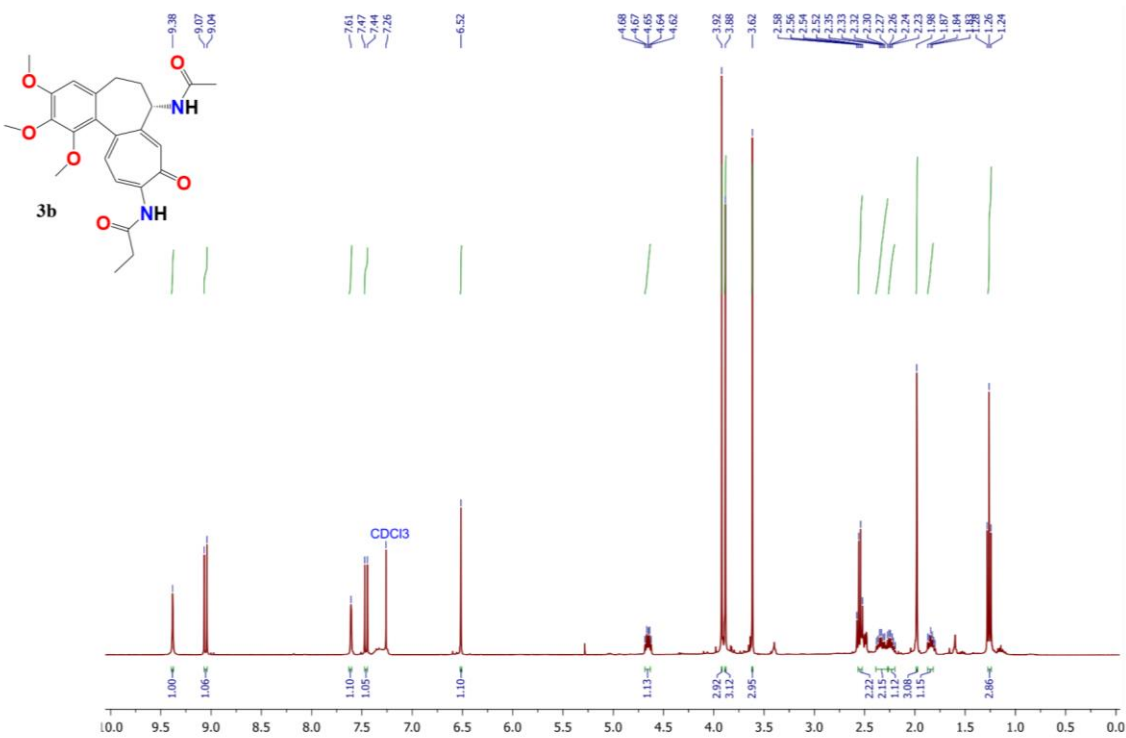


Figure S12. The ^1H (400 MHz) NMR spectrum of **3b** in CDCl_3 .

S19

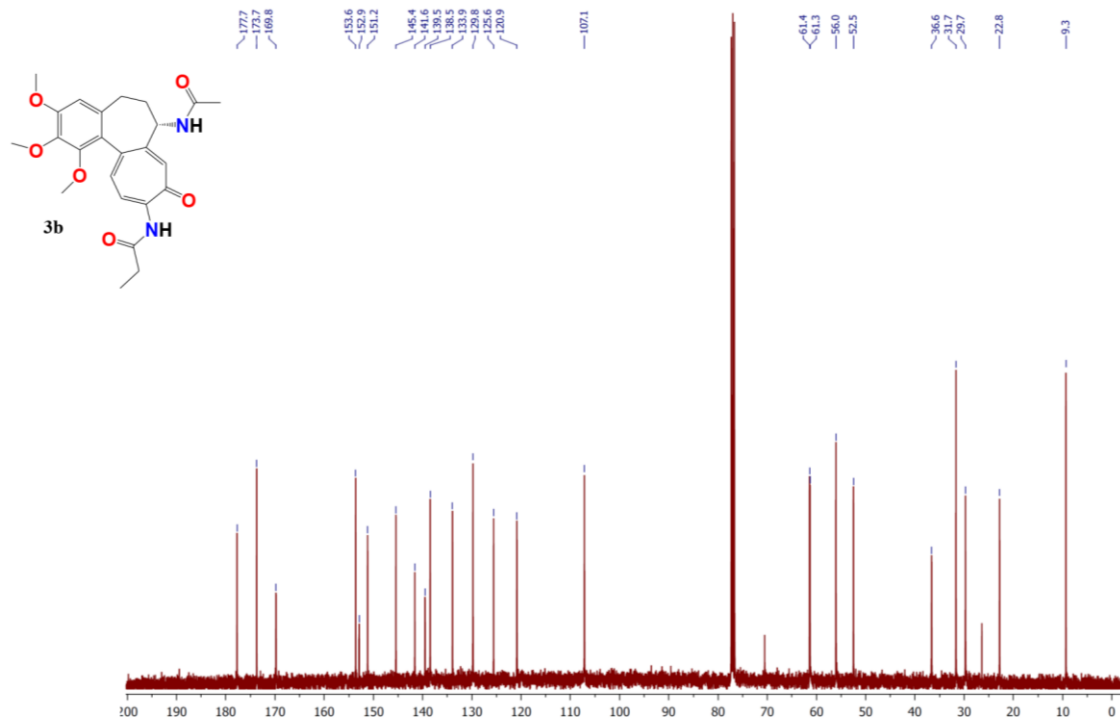


Figure S13. The ^{13}C (101 MHz) NMR spectrum of **3b** in CDCl_3 .

S20

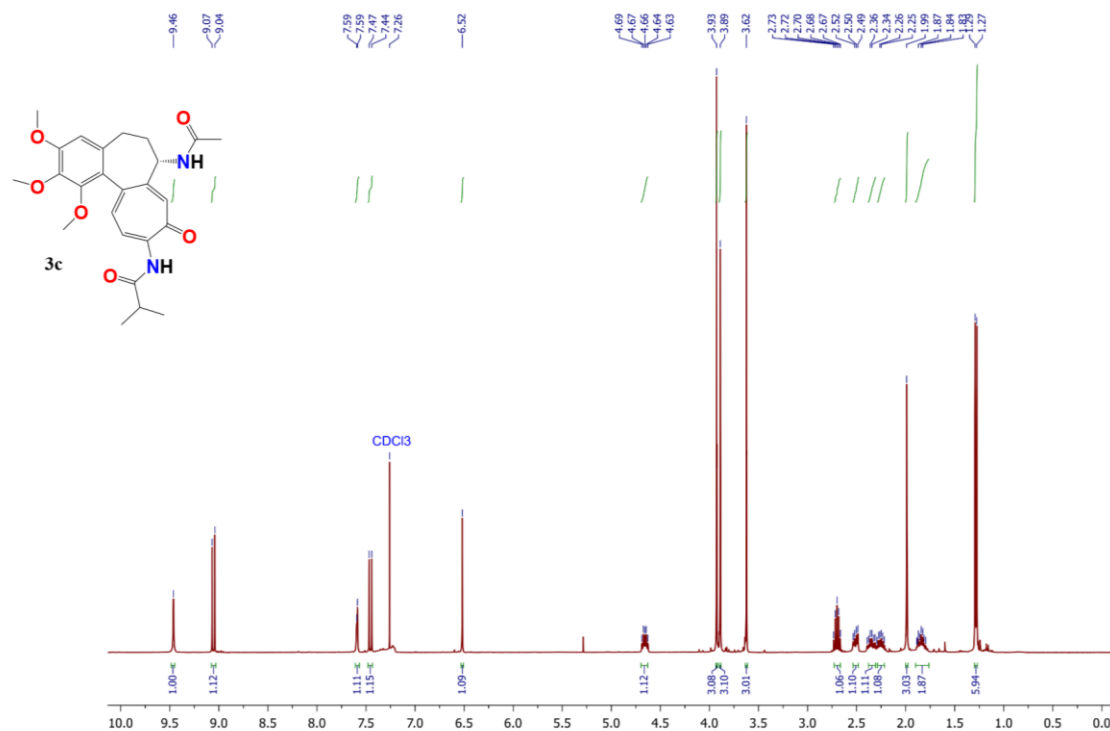


Figure S14. The ^1H (400 MHz) NMR spectrum of **3c** in CDCl_3 .

S21

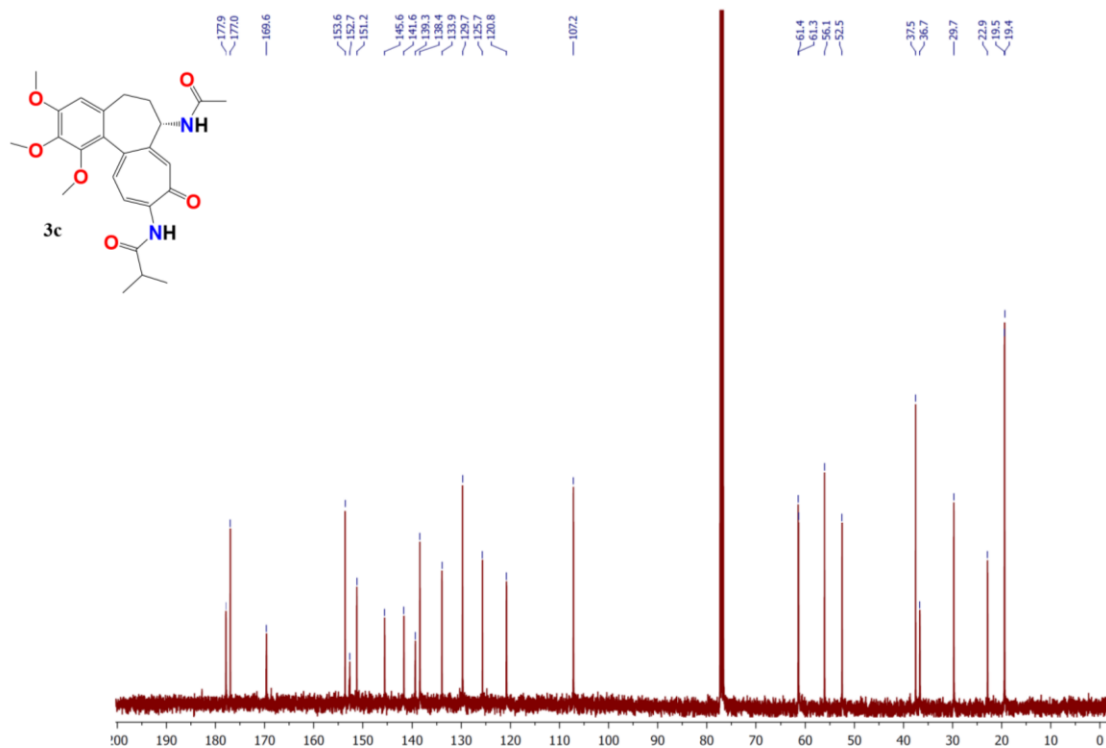


Figure S15. The ¹³C (101 MHz) NMR spectrum of 3c in CDCl₃.

S22

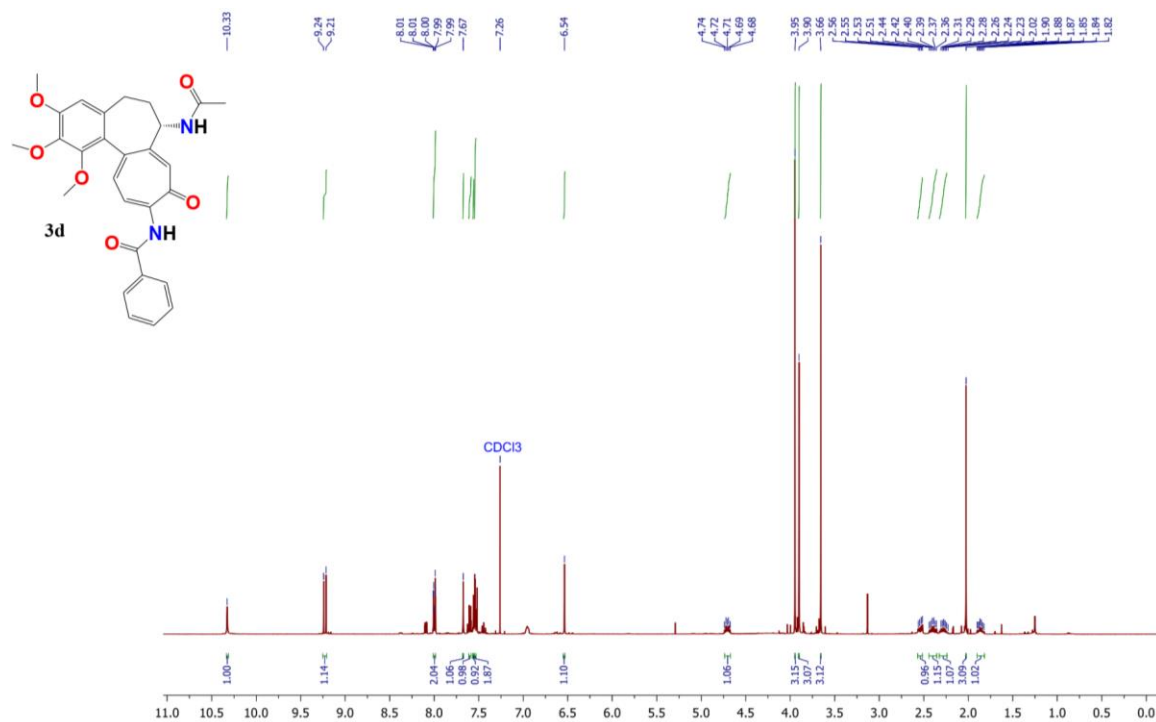


Figure S16. The ¹H (400 MHz) NMR spectrum of 3d in CDCl₃.

S23

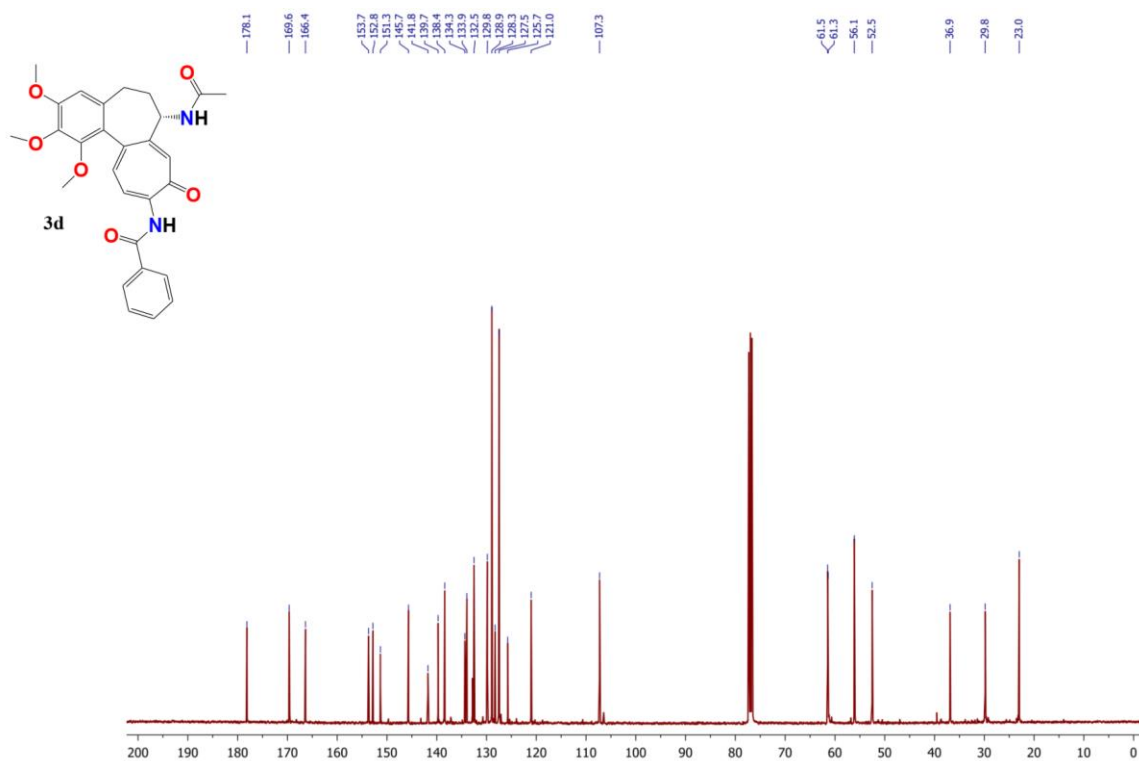


Figure S17. The ¹³C (101 MHz) NMR spectrum of 3d in CDCl₃.

S24

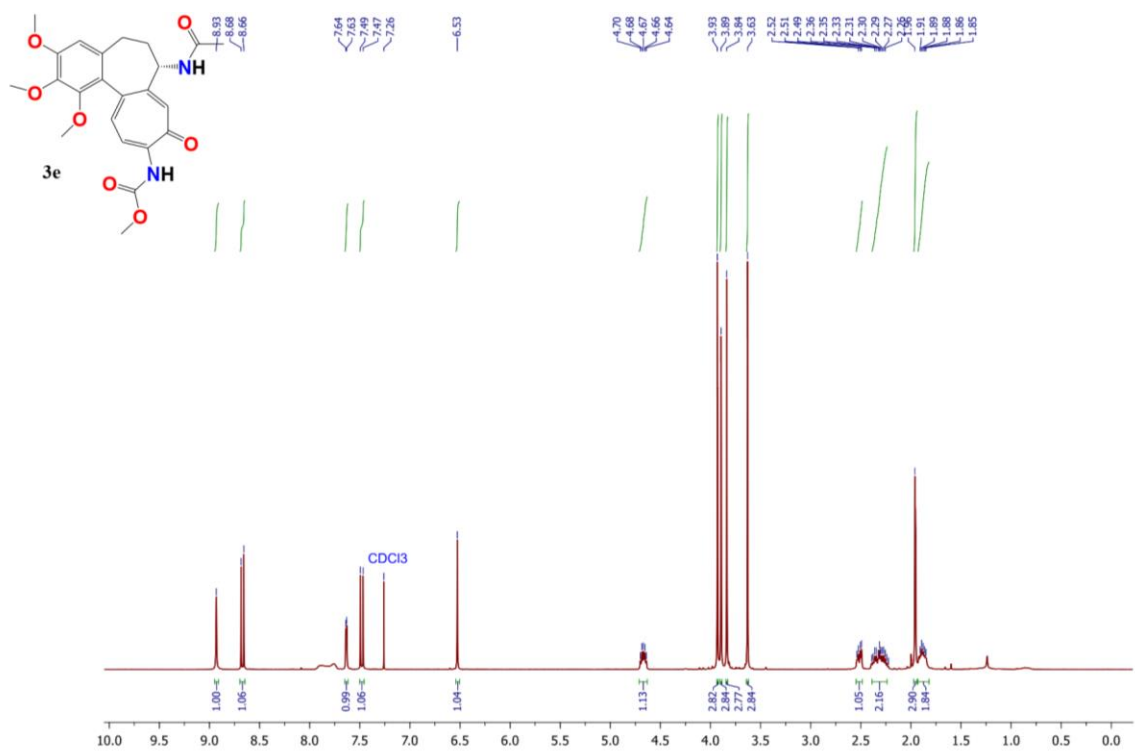


Figure S18. The ¹H (400 MHz) NMR spectrum of 3e in CDCl₃.

S25

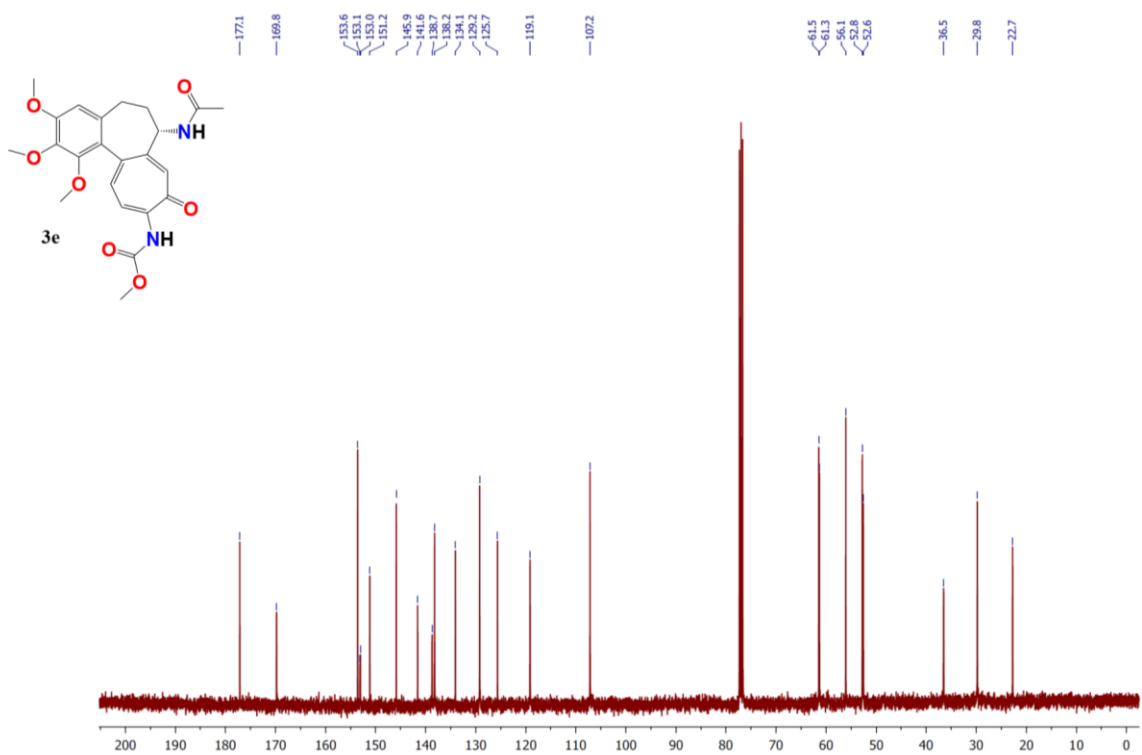


Figure S19. The ^{13}C (101 MHz) NMR spectrum of **3e** in CDCl_3 .

S26

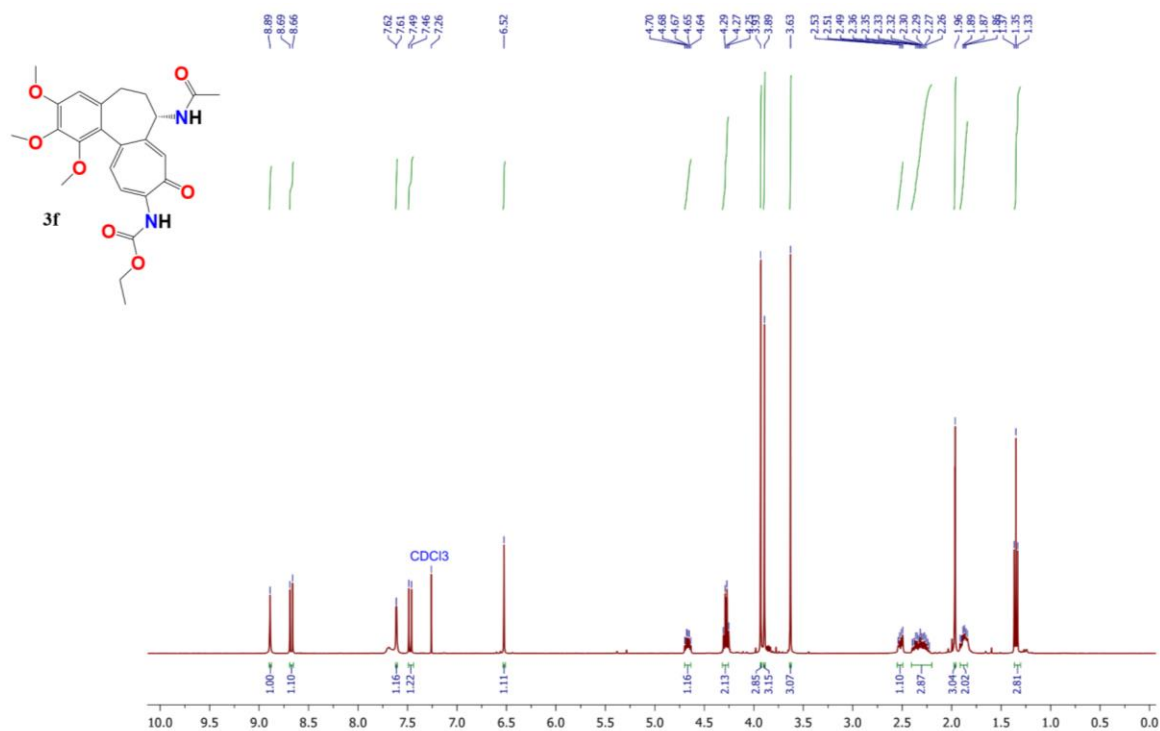


Figure S20. The ^1H (400 MHz) NMR spectrum of **3f** in CDCl_3 .

S27

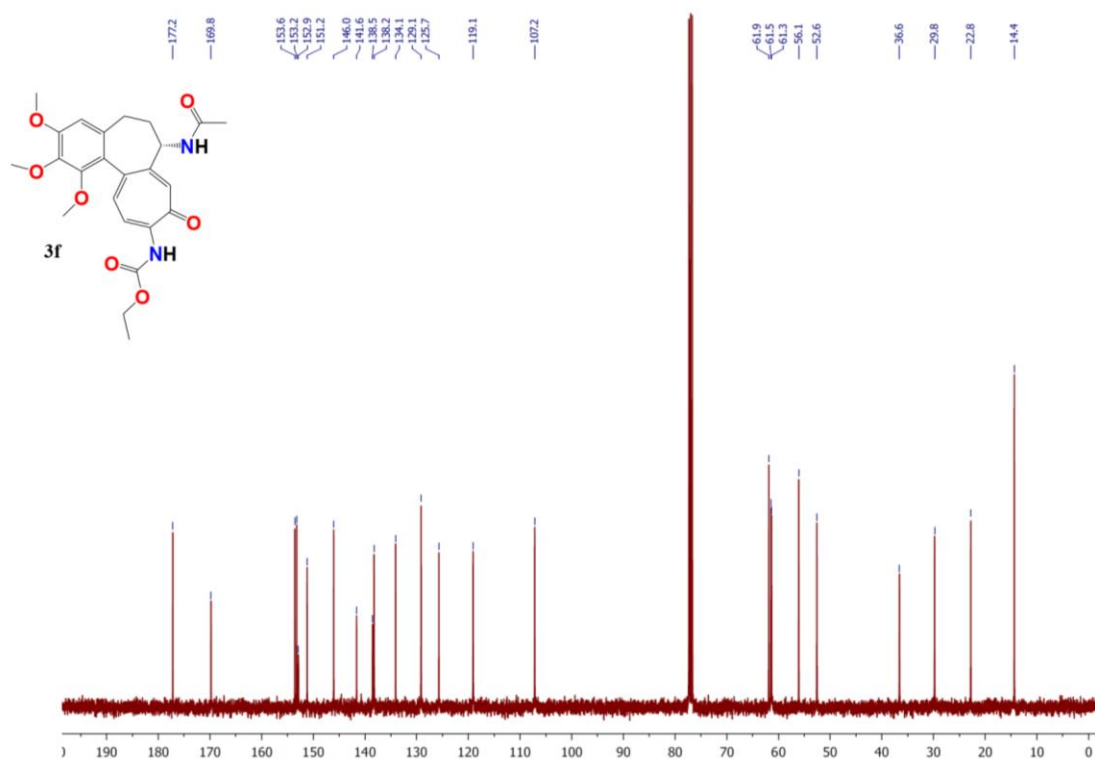


Figure S21. The ^{13}C (101 MHz) NMR spectrum of **3f** in CDCl_3

S28

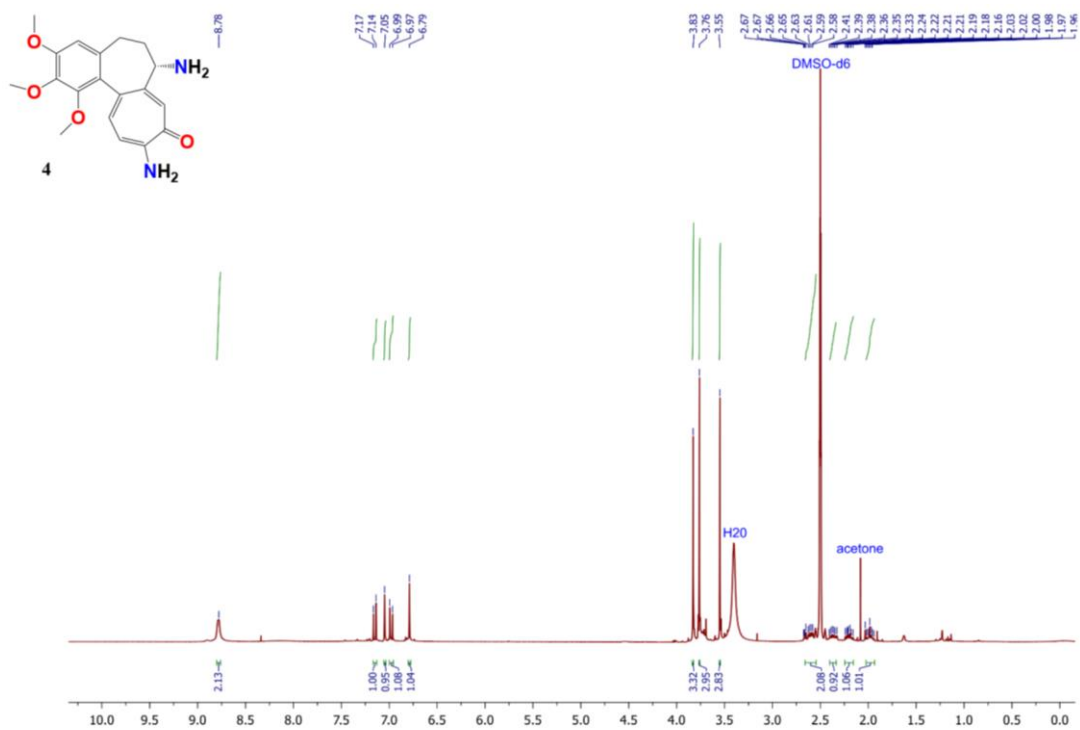


Figure S22. The ^1H (400 MHz) NMR spectrum of **4** in $\text{DMSO}-d_6$.

S29

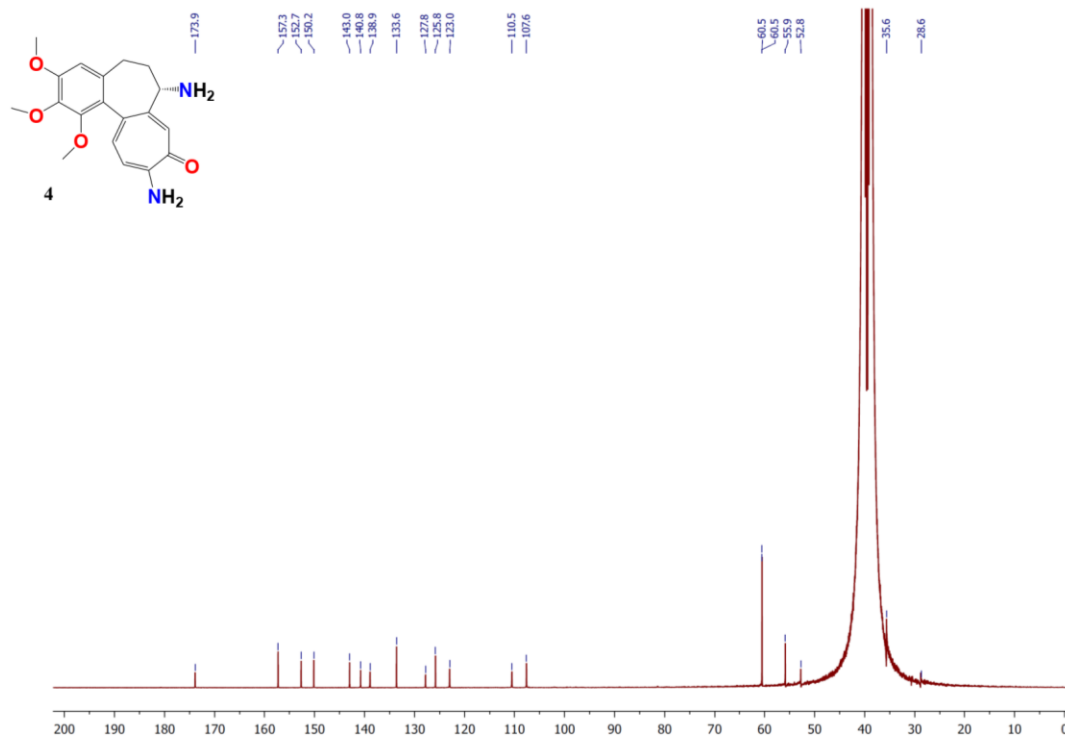


Figure S23. The ^{13}C (101 MHz) NMR spectrum of 4 in $\text{DMSO-}d_6$.

S30

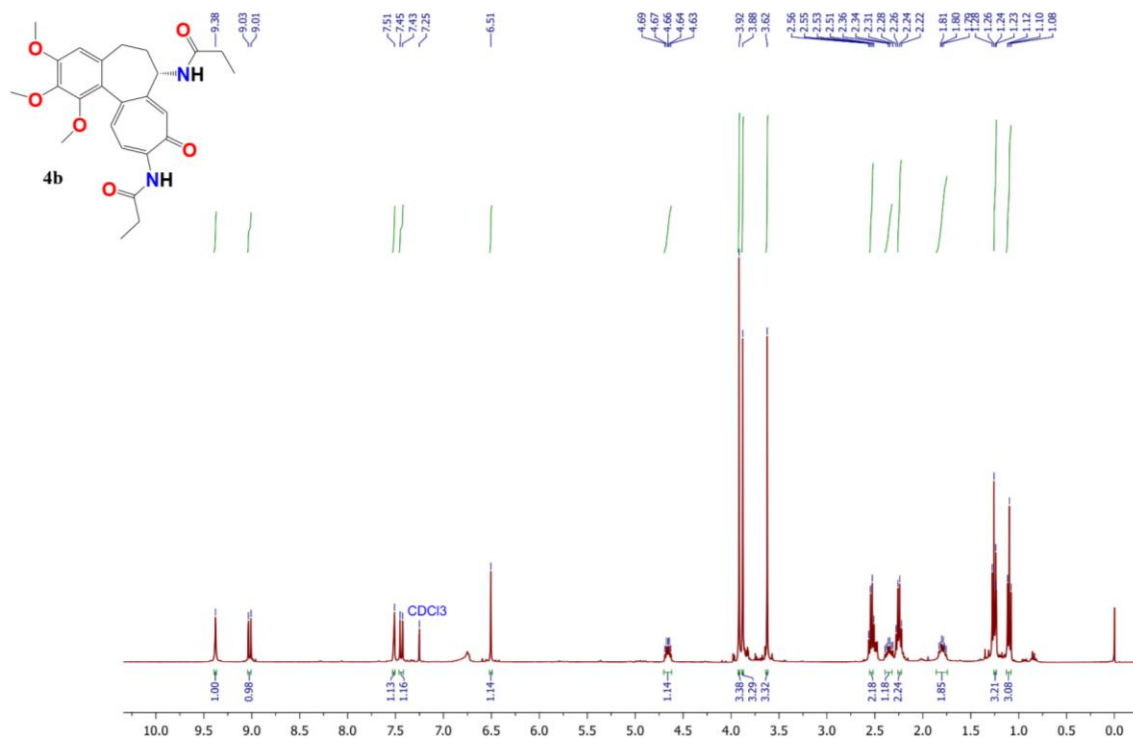


Figure S24. The ^1H (400 MHz) NMR spectrum of 4b in CDCl_3 .

S31

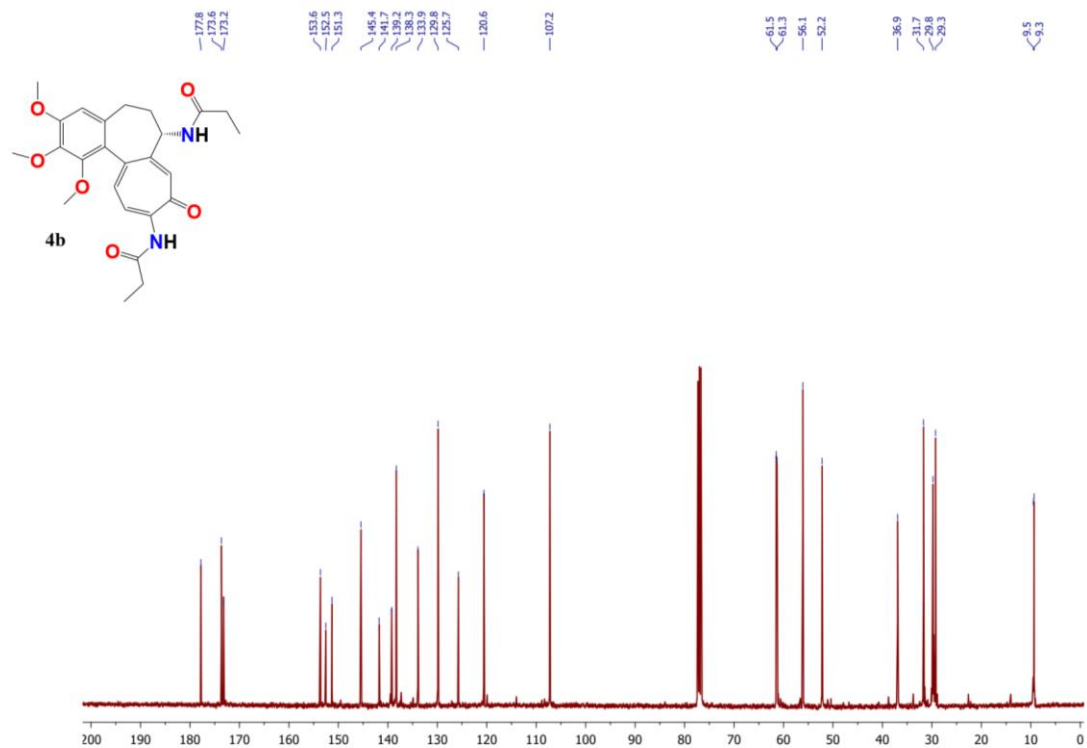


Figure S25. The ^{13}C (101 MHz) NMR spectrum of 4b in CDCl_3 .

S32

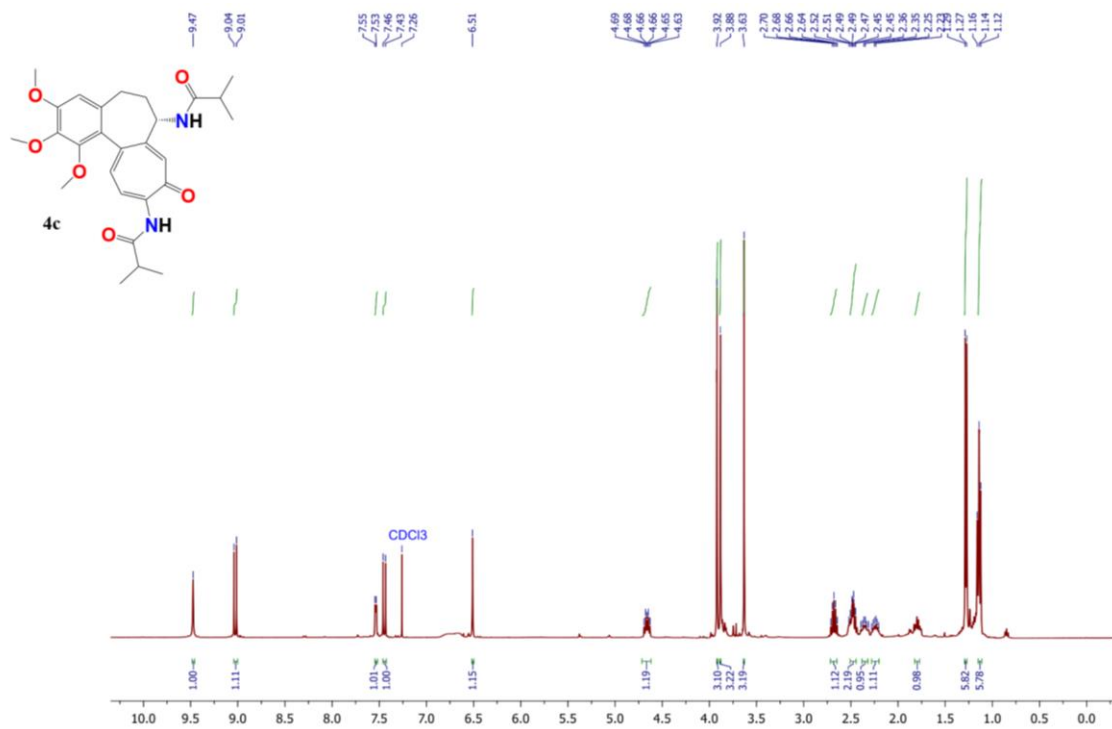


Figure S26. The ^1H (400 MHz) NMR spectrum of 4c in CDCl_3 .

S33

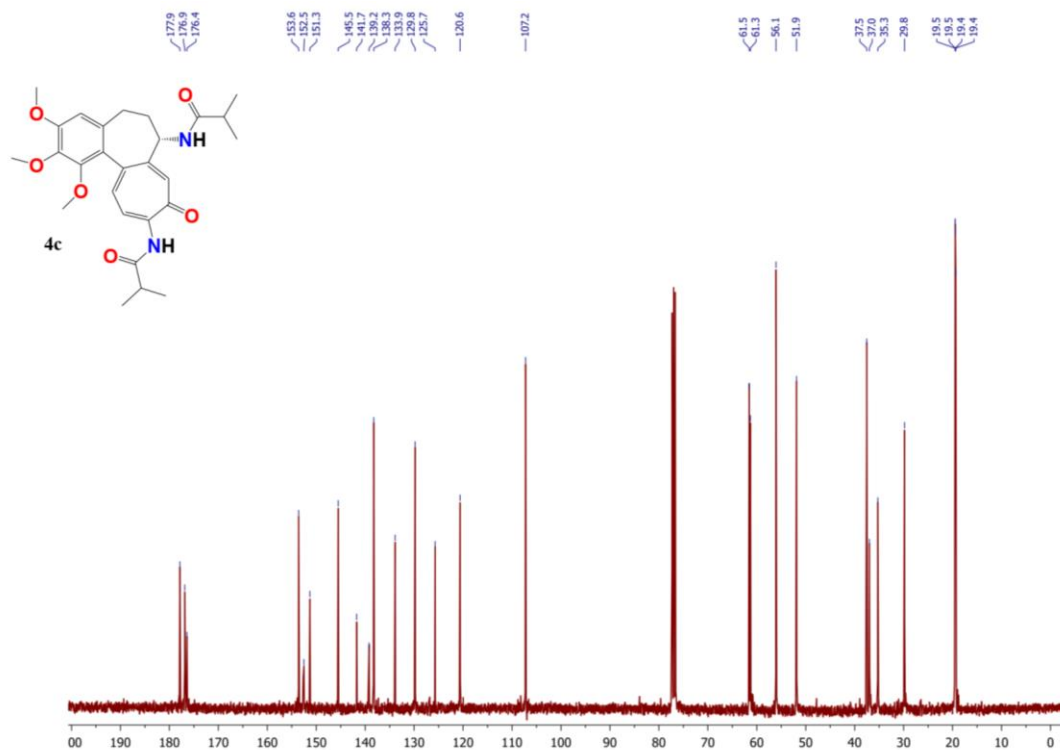


Figure S27. The ¹³C (101 MHz) NMR spectrum of 4c in CDCl₃.

S34

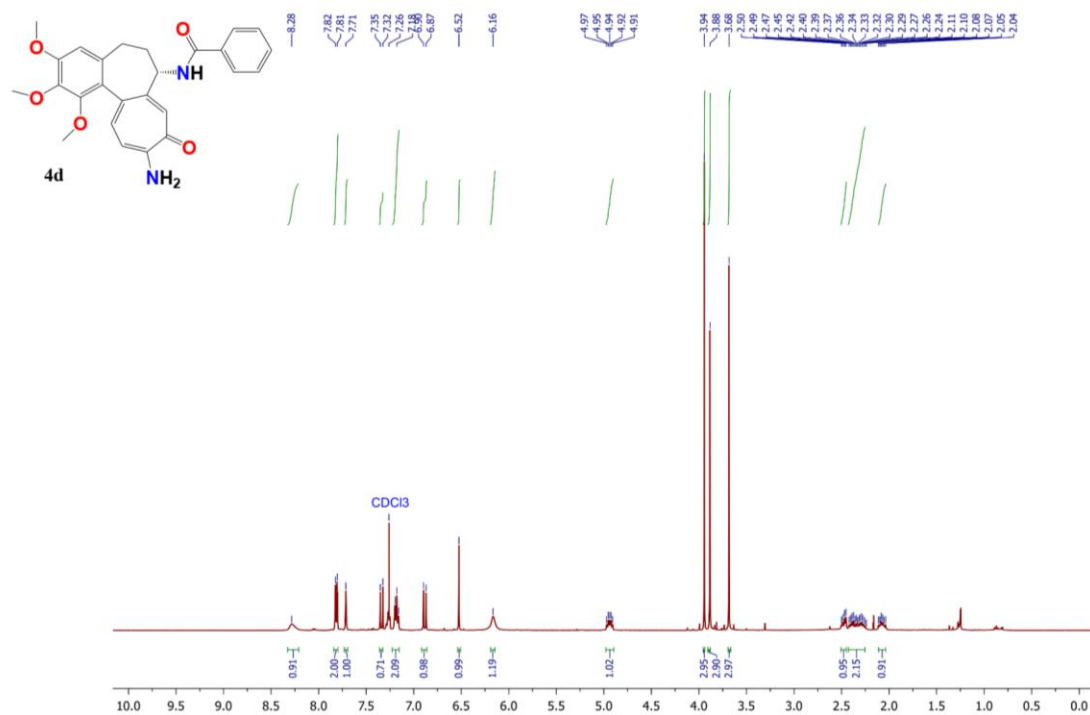


Figure S28. The ¹H (400 MHz) NMR spectrum of 4d in CDCl₃.

S35

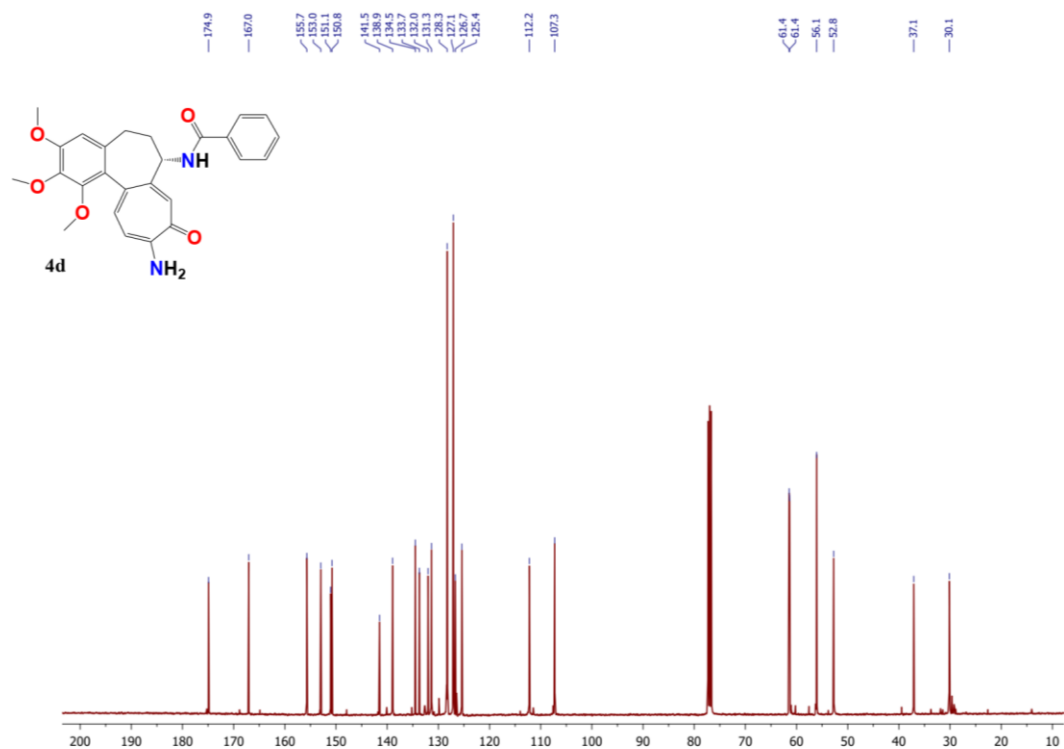


Figure S29. The ¹³C (101 MHz) NMR spectrum of 4d in CDCl₃.

S36

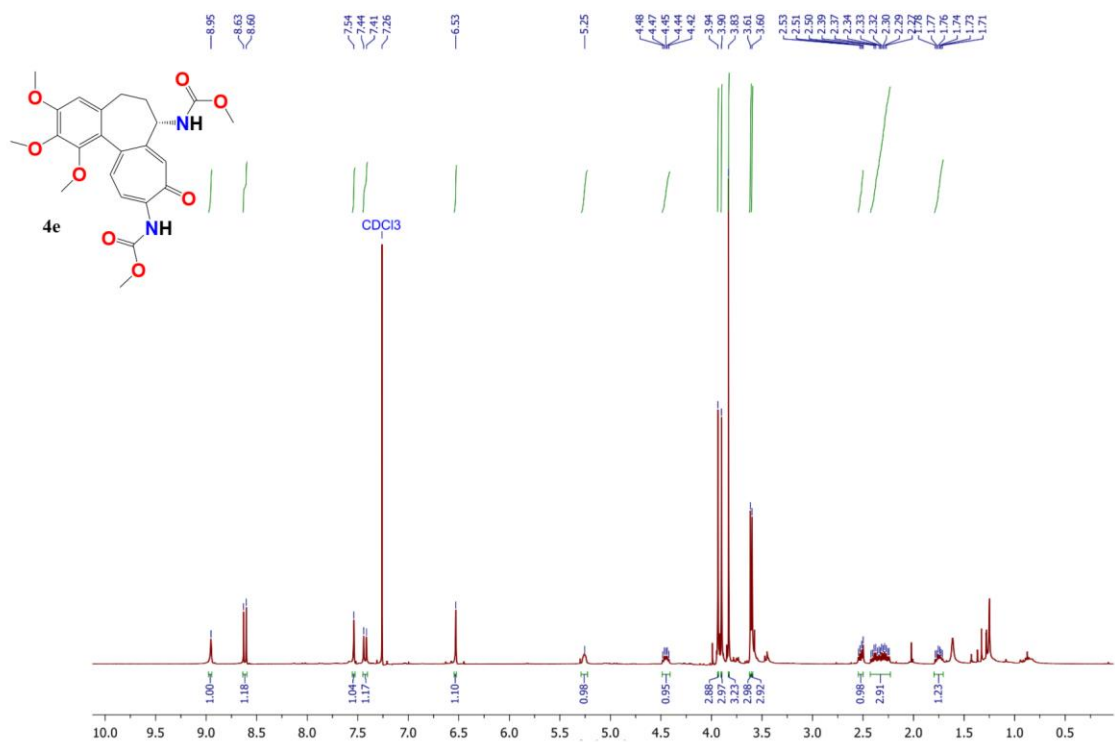


Figure S30. The ¹H (400 MHz) NMR spectrum of 4e in CDCl₃.

S37

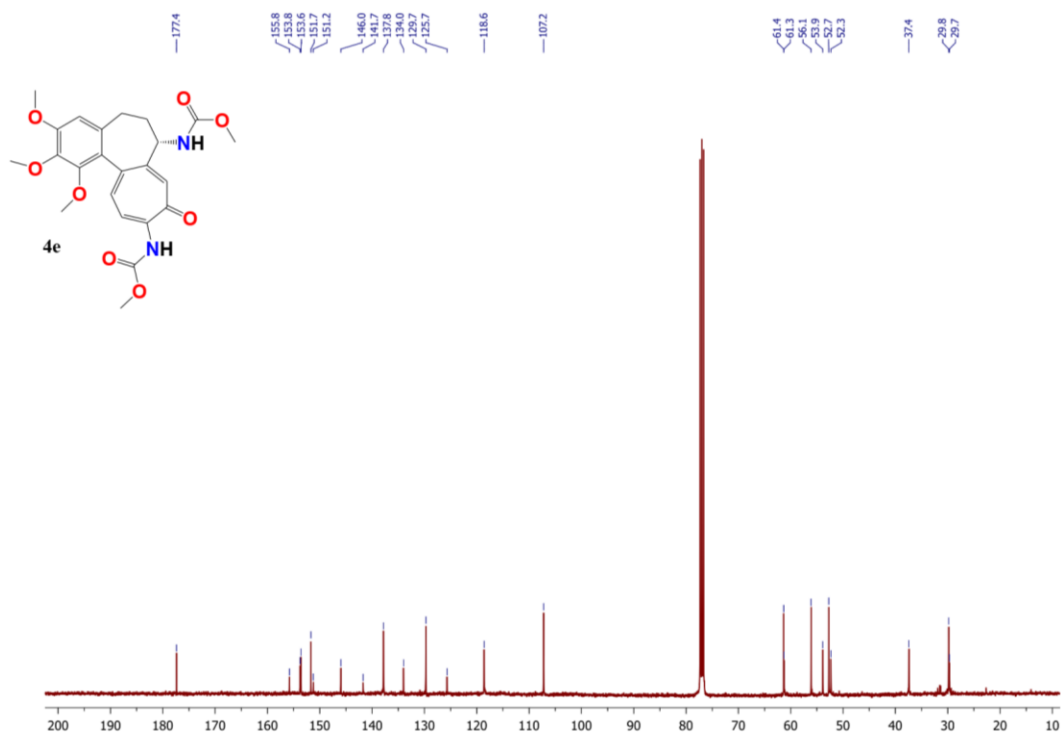


Figure S31. The ¹³C (101 MHz) NMR spectrum of 4e in CDCl₃.

S38

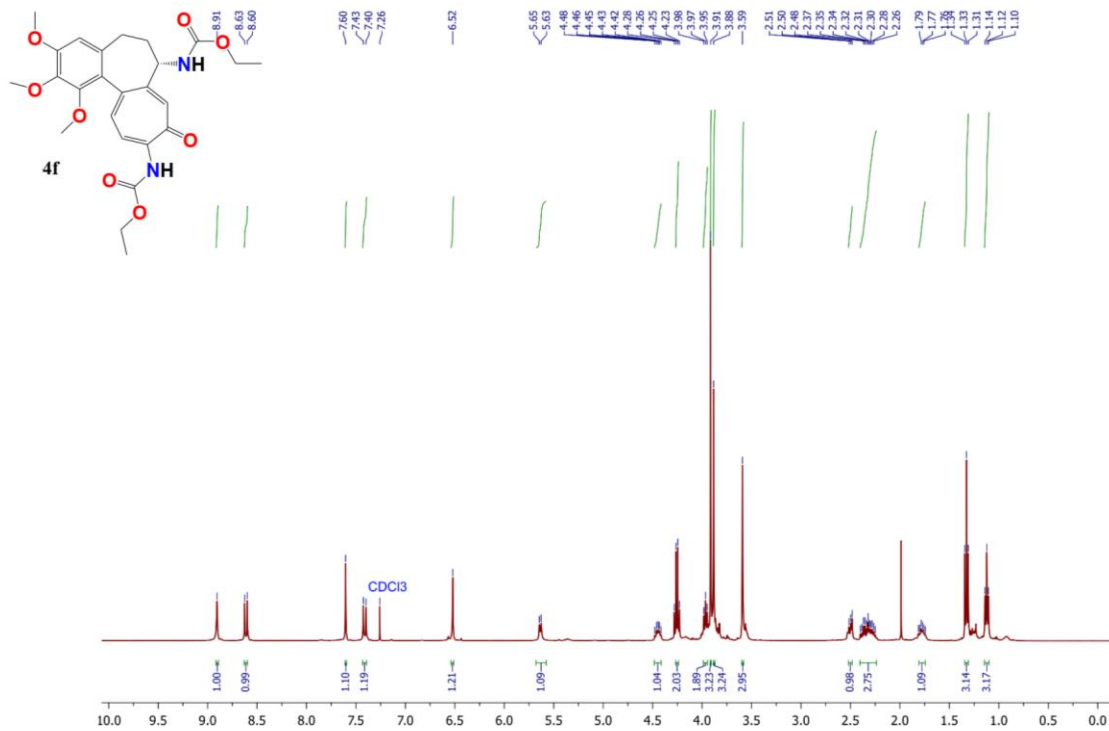


Figure S32. The ¹H (400 MHz) NMR spectrum of 4f in CDCl₃.

S39

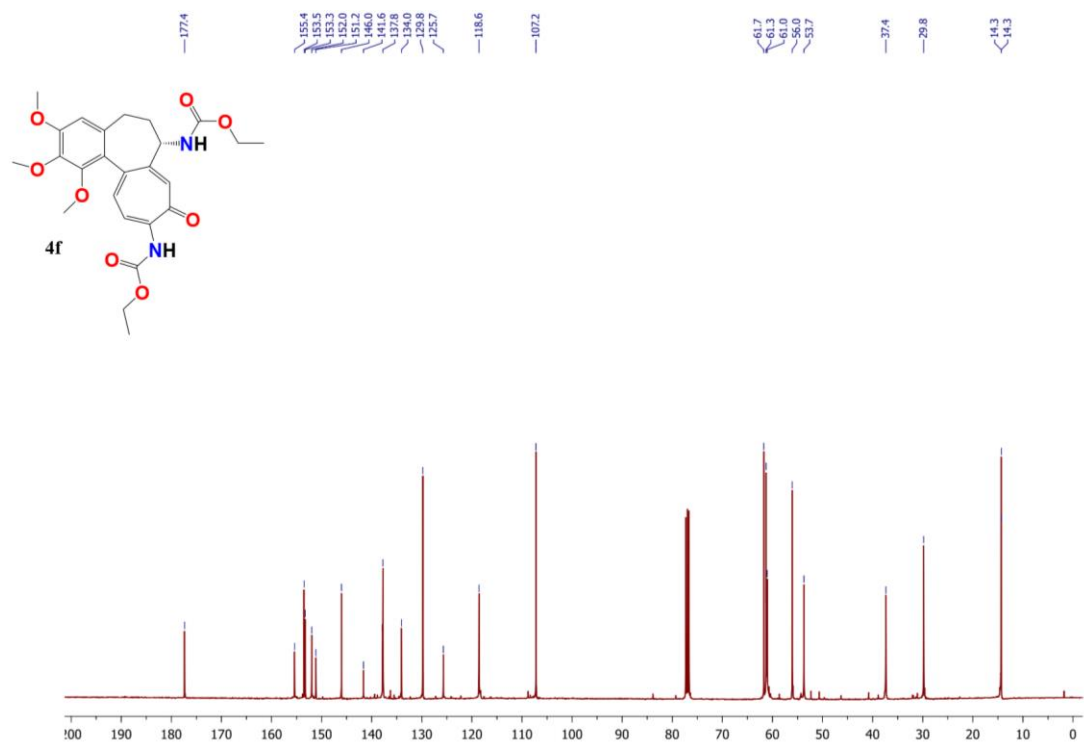


Figure S33. The ^{13}C (101 MHz) NMR spectrum of **4f** in CDCl_3 .

S40

^1H and ^{13}C NMR spectra of β -lumiderivatives: β -lumicolchicine, **2-UV**, **3-UV**, **3b-UV**, **3f-UV**, **4b-UV**, **4f-UV**)

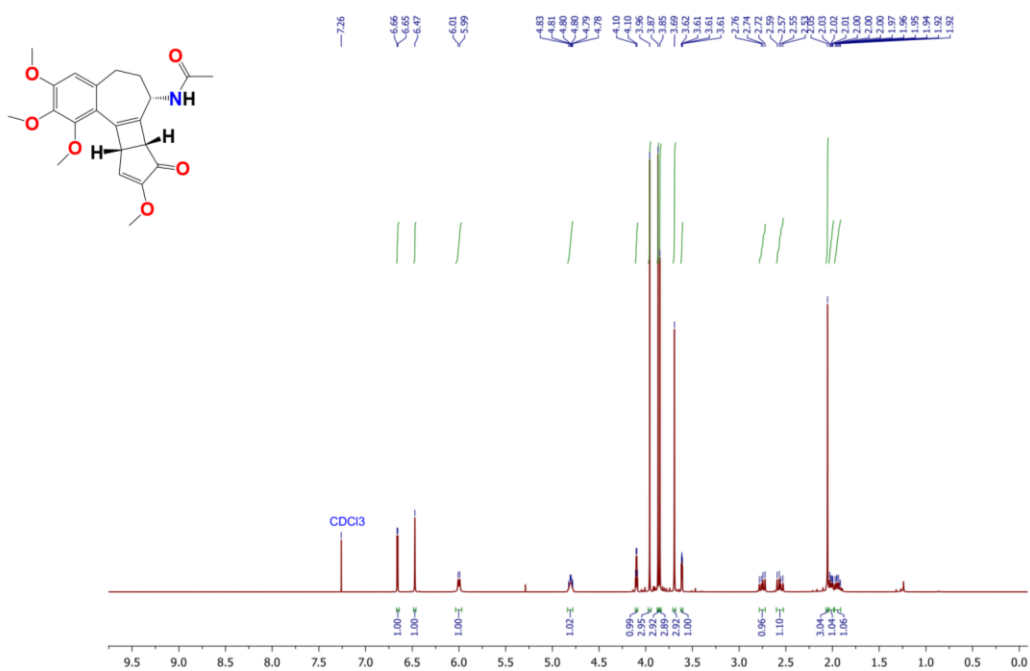


Figure S34. The ^1H (400 MHz) NMR spectrum of β -lumicolchicine in CDCl_3 .

S41

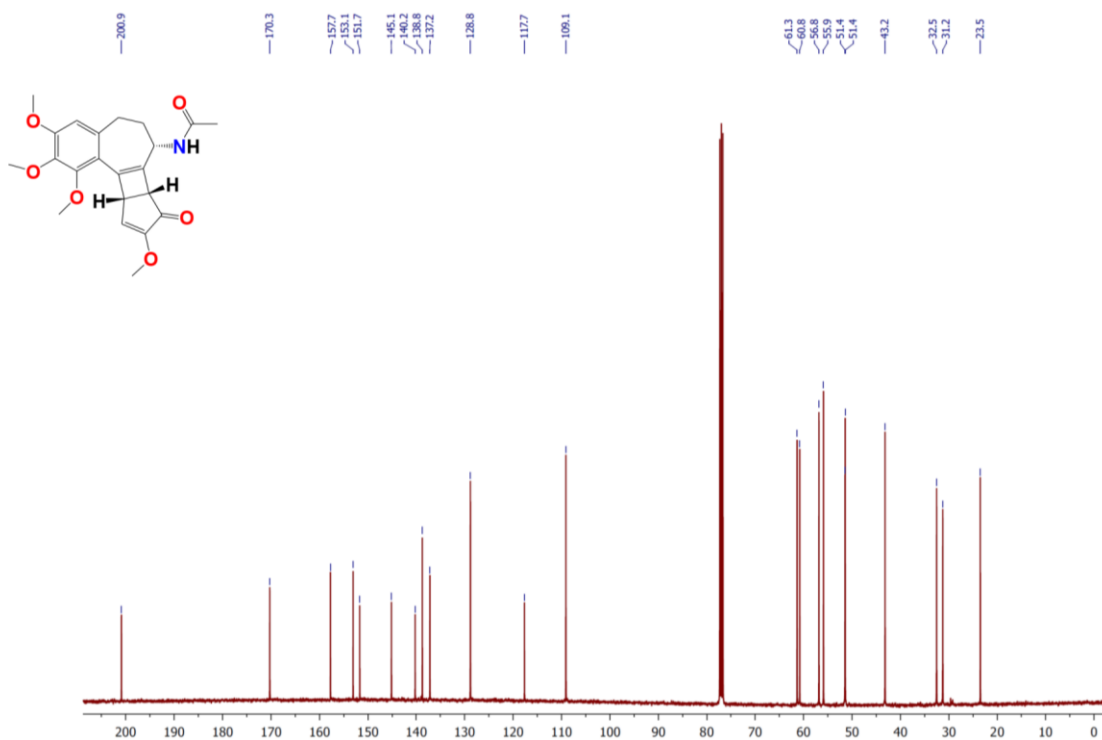


Figure S35. The ^{13}C (101 MHz) NMR spectrum of β -lumicolchicine in CDCl_3 .

S42

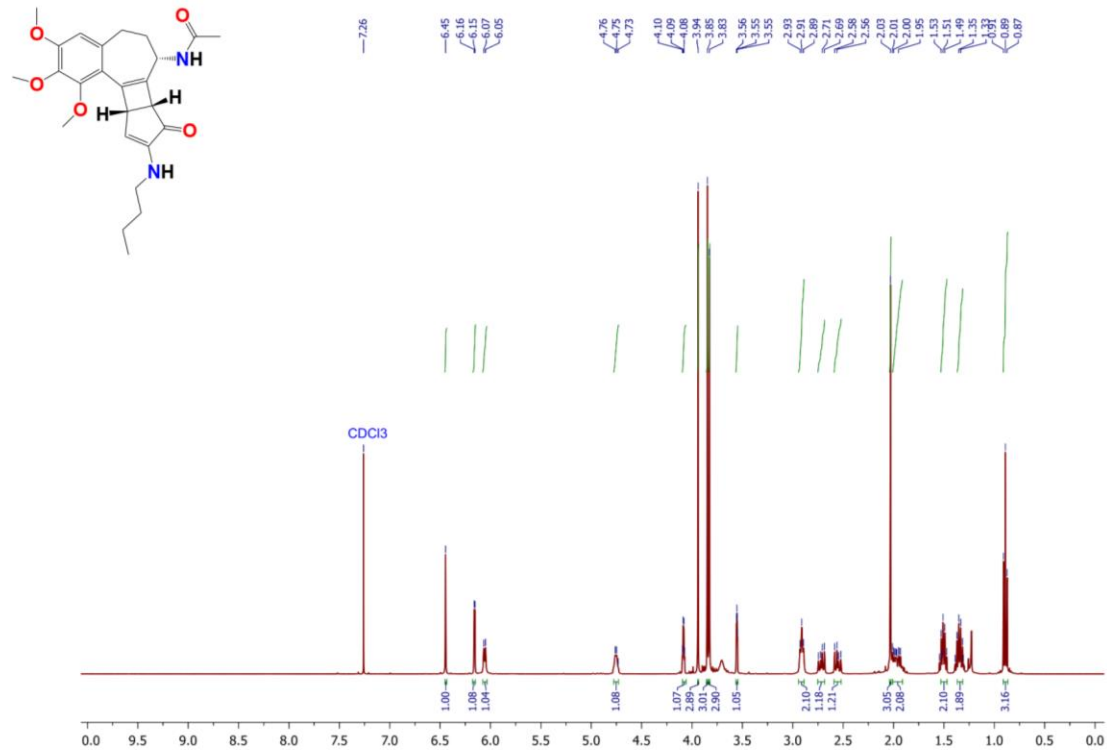


Figure S36. The ^1H (400 MHz) NMR spectrum of 2-UV in CDCl_3 .

S43

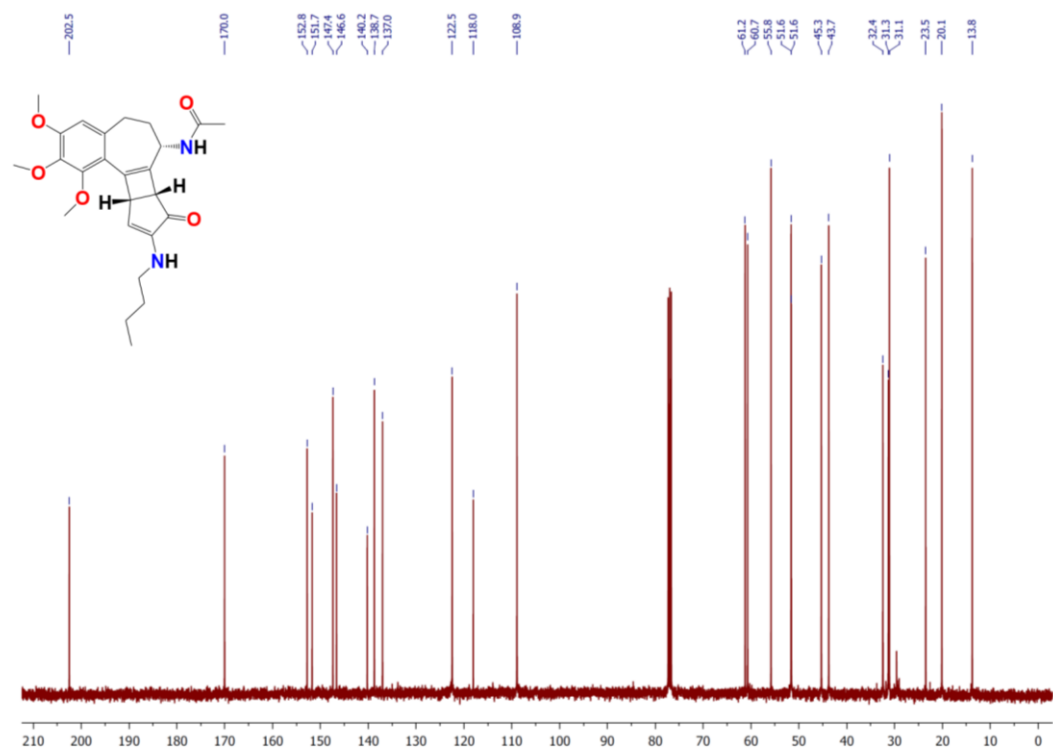


Figure S37. The ^{13}C (101 MHz) NMR spectrum of 2-UV in CDCl_3 .

S44

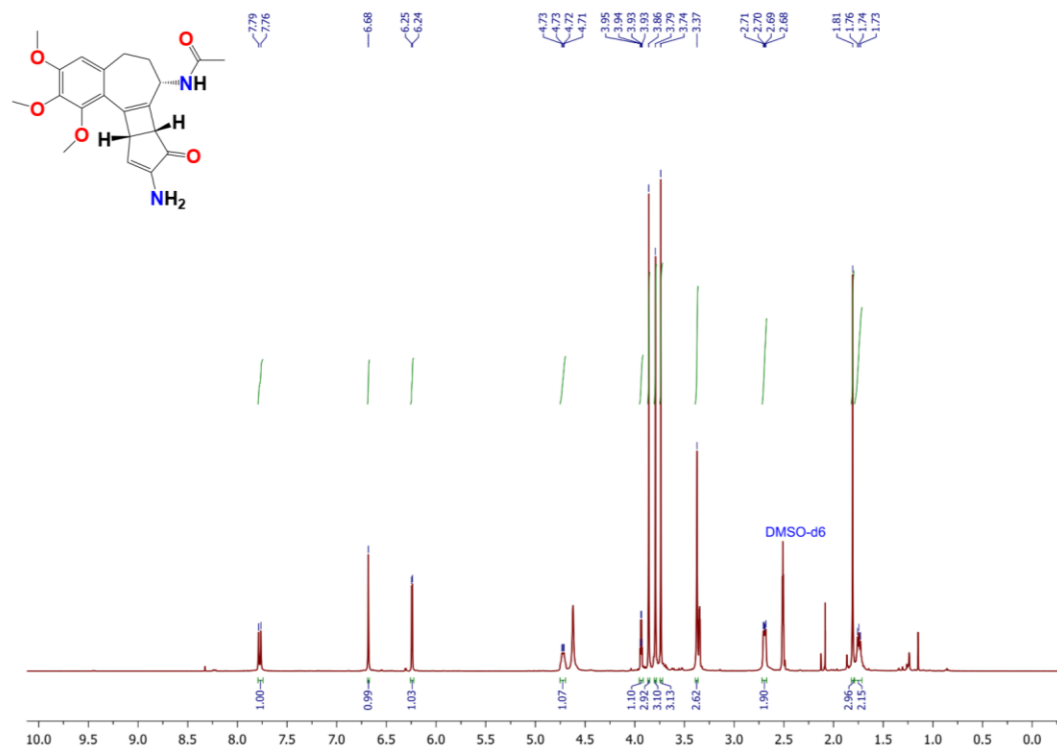


Figure S38. The ^1H (400 MHz) NMR spectrum of 3-UV in DMSO-d_6 .

S45

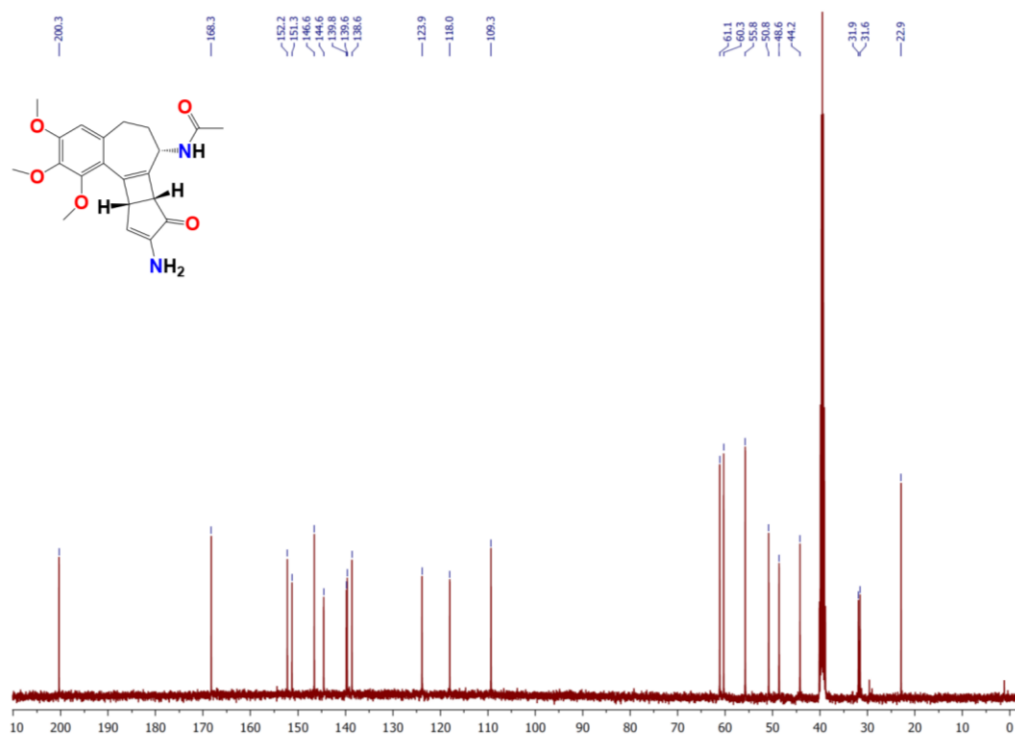


Figure S39. The ^{13}C (101 MHz) NMR spectrum of 3-UV in DMSO-d_6 .

S46

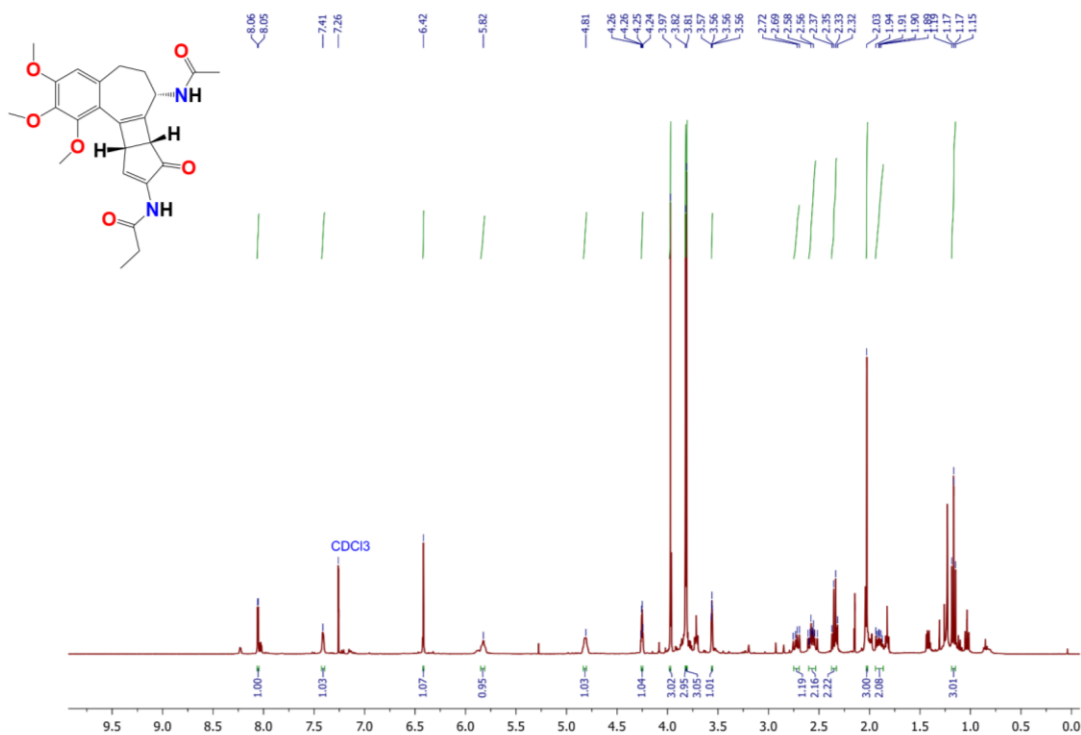


Figure S40. The ^1H (400 MHz) NMR spectrum of 3b-UV in CDCl_3 .

S47

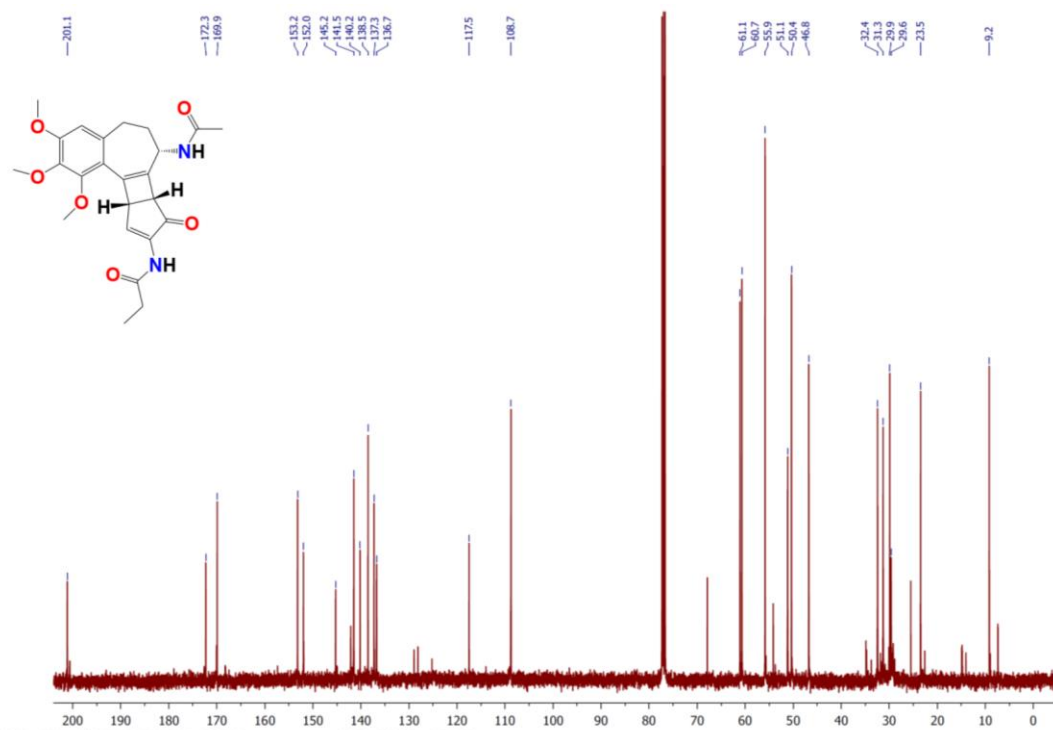


Figure S41. The ¹³C (101 MHz) NMR spectrum of 3b-UV in CDCl₃.

S48

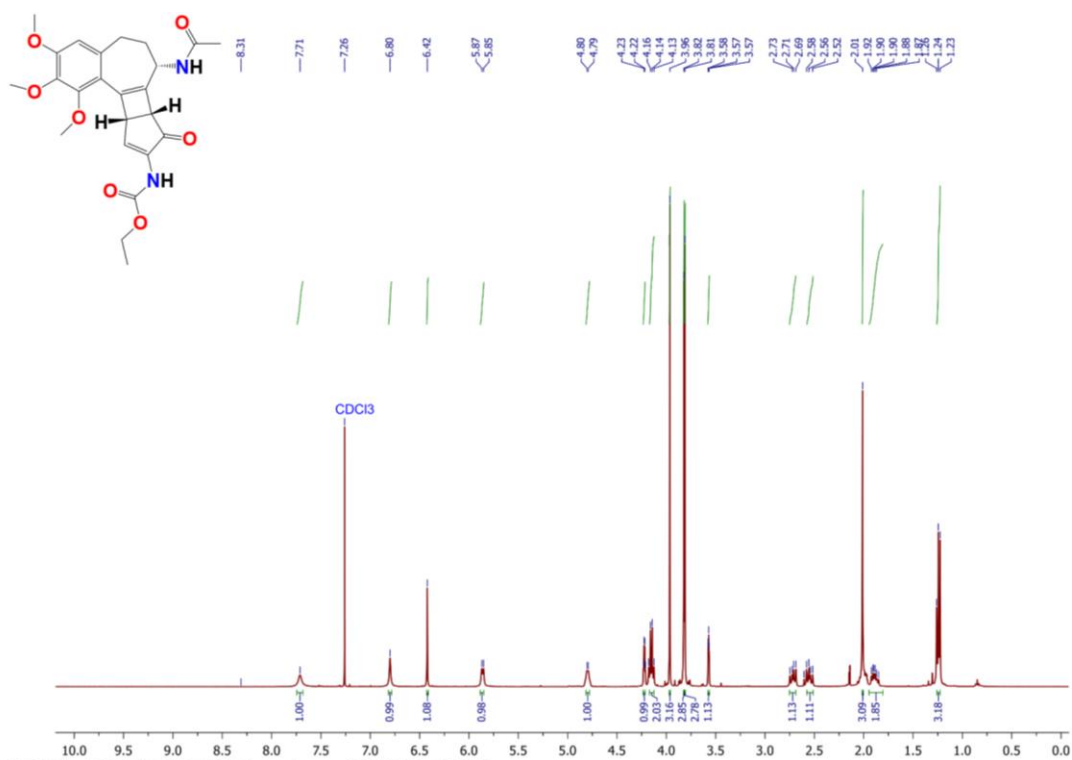


Figure S42. The ¹H (400 MHz) NMR spectrum of 3f-UV in CDCl₃.

S49

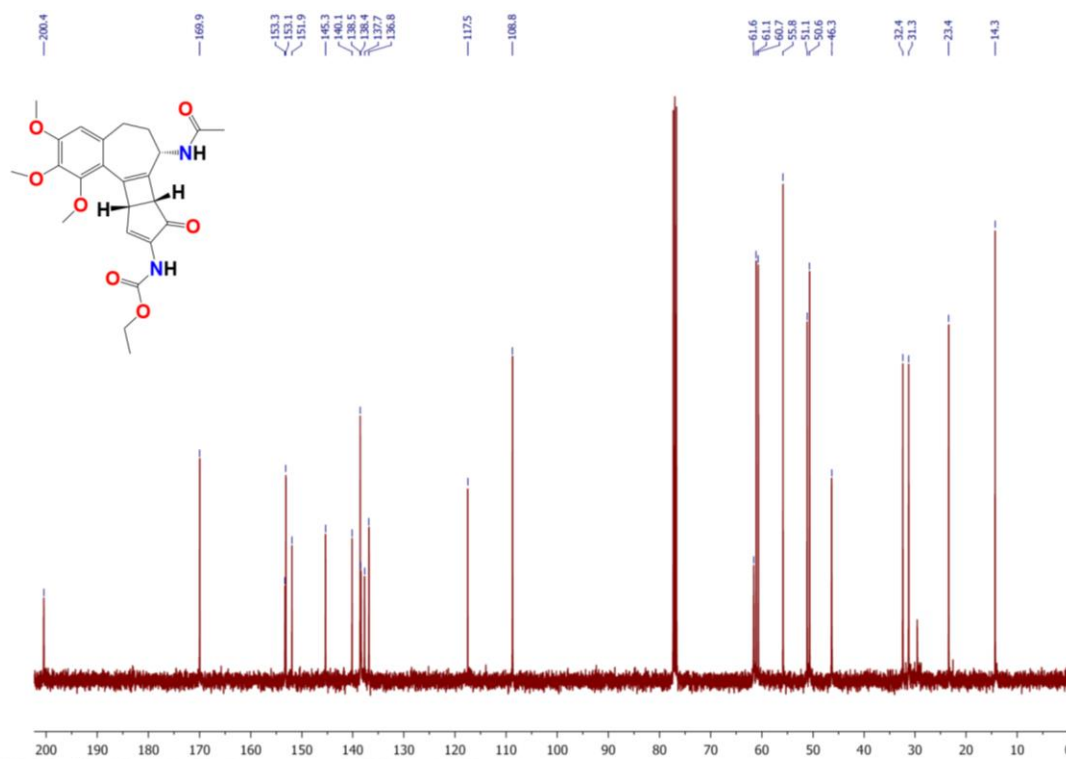


Figure S43. The ^{13}C (101 MHz) NMR spectrum of 3f-UV in CDCl_3 .

S50

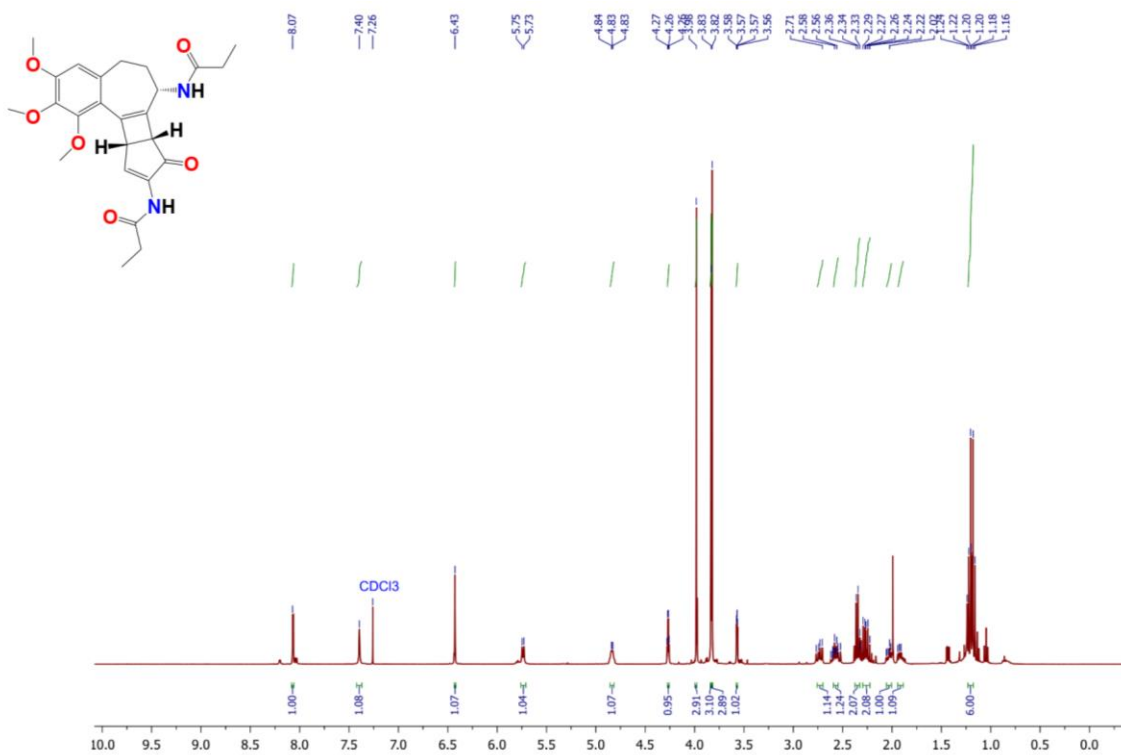


Figure S44. The ^1H (400 MHz) NMR spectrum of 4b-UV in CDCl_3 .

S51

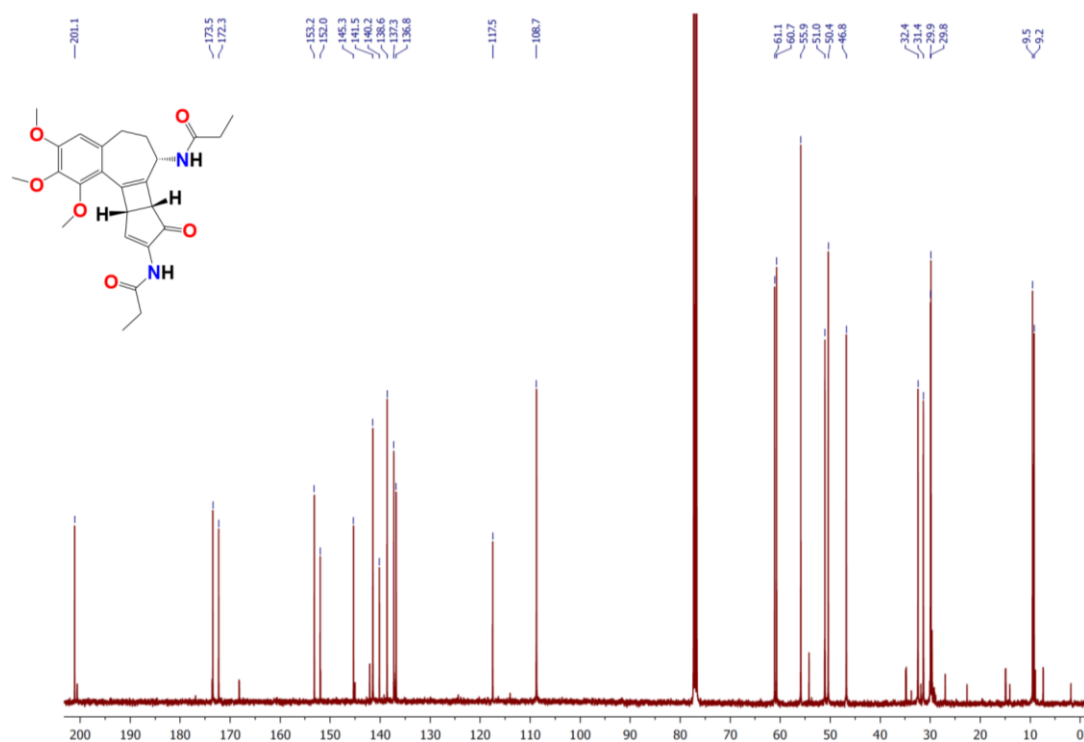


Figure S45. The ^{13}C (101 MHz) NMR spectrum of 4b-UV in CDCl_3 .

S52

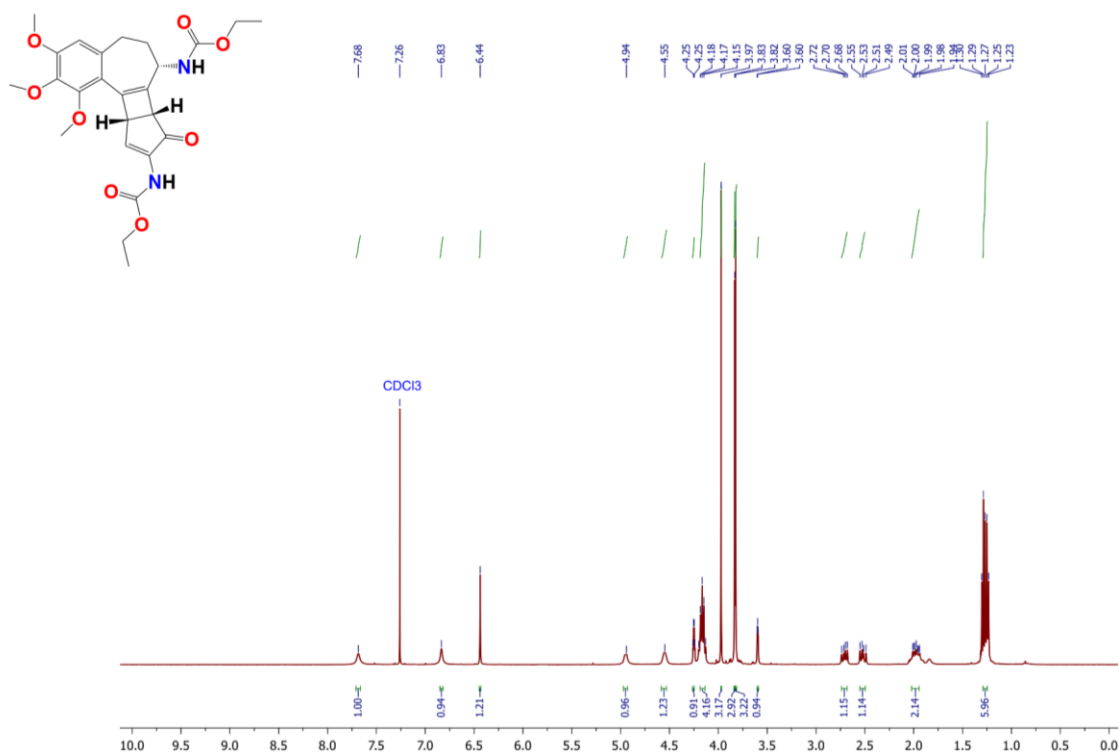


Figure S46. The ^1H (400 MHz) NMR spectrum of 4f-UV in CDCl_3 .

S53

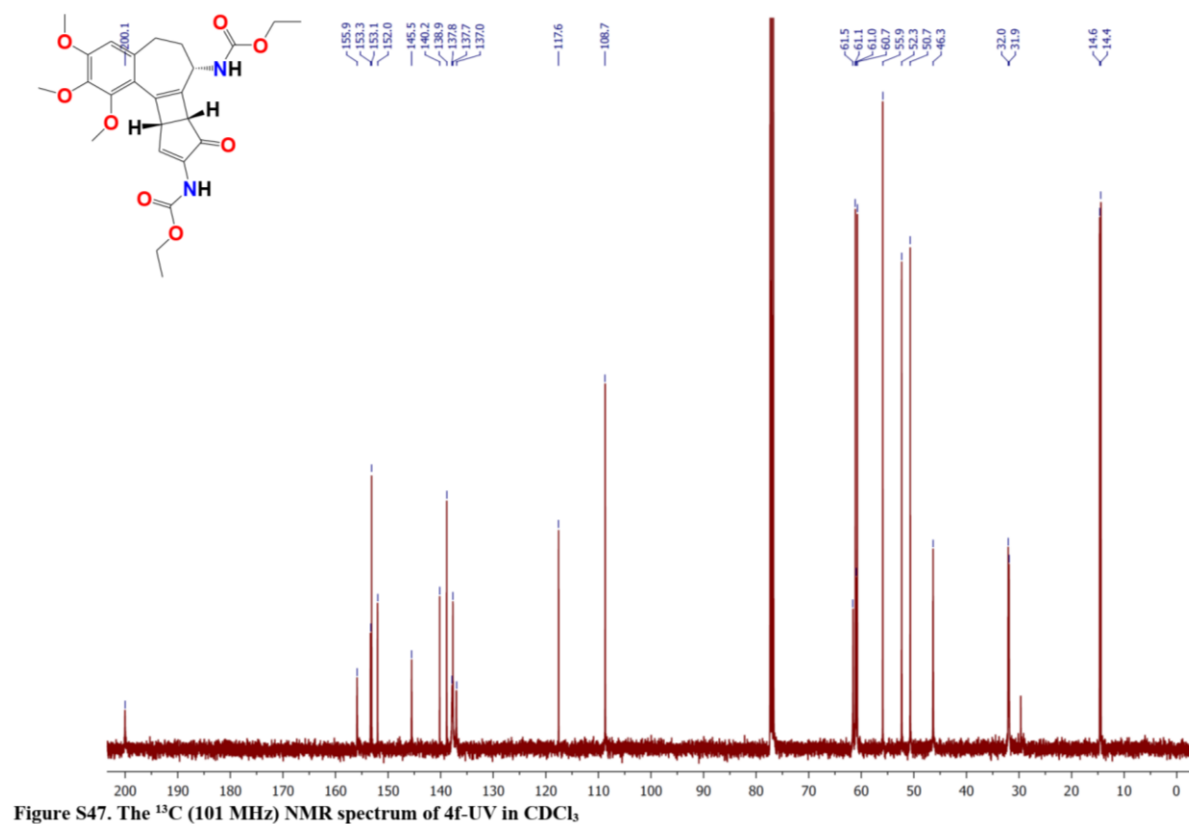


Figure S47. The ^{13}C (101 MHz) NMR spectrum of 4f-UV in CDCl_3

S54

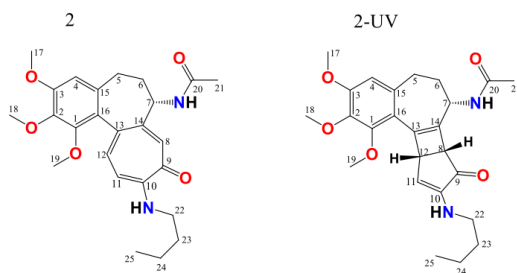


Table S2. The ^1H and ^{13}C NMR chemical shifts δ (ppm) of 2 and 2-UV in chloroform- d , assigned based on 2D NMR.

No.	2	2-UV		Δ ^1H NMR	Δ ^{13}C NMR	
atoms	$\delta^1\text{H}$ NMR	$\delta^{13}\text{C}$ NMR	$\delta^1\text{H}$ NMR	$\delta^{13}\text{C}$ NMR		
1	-	151.1	-	151.7	-	0.6
2	-	141.5	-	140.2	-	-1.3
3	-	152.8	-	152.8	-	0
4	6.53 (s, 1H)	107.1	6.45 (s, 1H)	108.9	-0.08	1.8
5	2.47 (dd, $J = 12.4, 5.6$ Hz, 1H) 2.41 – 2.32 (m, 1H)	30.1	2.72 (dd, $J = 15.3, 8.8$ Hz, 1H)	32.4	0.25	1.3
6	2.32 – 2.21 (m, 1H)	37.2	2.56 (dd, $J = 15.3, 9.2$ Hz, 1H) 2.01 – 1.86 (m, 2H)	31.3	0.15 -0.33	-5.9
7	1.97 – 1.91 (m, 1H) 4.74 – 4.65 (m, 1H)	52.6	4.78 – 4.73 (m, 1H)	51.6*	0.005 006	-1.0
8	7.54 (s, 1H)	122.6	4.09 (dd, $J = 5.7, 2.9$ Hz, 1H)	45.4	-3.45	-77.2
9	-	175.5	-	202.5	-	27.0
10	-	154.3	-	147.4	-	-6.9
11	6.62 (d, $J = 11.3$ Hz, 1H)	108.7	6.17 (d, $J = 3.3$ Hz, 1H)	122.5	-0.45	13.8
12	7.45 (d, $J = 11.2$ Hz, 1H)	139.4	3.58 – 3.54 (m, 1H)	51.6*	-3.89	-87.8
13	-	134.6	-	146.7	-	12.1
14	-	151.0	-	137.0	-	-14.0
15	-	130.3	-	138.7	-	8.4
16	-	126.8	-	118.1	-	-8.7
17	3.89 (s, 3H)	56.1	3.84 (s, 3H)	55.8	-0.05	-0.3
18	3.94 (s, 3H)	61.4	3.86 (s, 3H)	60.7	-0.08	-0.7
19	3.62 (s, 3H)	61.3	3.95 (s, 3H)	61.3	0.33	0
20	-	169.9	-	170.1	-	0.2
21	1.98 (s, 3H)	22.8	2.04 (s, 3H)	23.5	0.06	0.7
22	3.36 (td, $J = 7.2, 5.6$ Hz, 2H)	42.5	2.92 (t, $J = 7.1$ Hz, 2H)	43.8	-0.44	1.3
23	1.81 – 1.70 (m, 2H)	30.5	1.56 – 1.47 (m, 2H)	31.1	-0.24	0.6
24	1.54 – 1.43 (m, 2H)	20.3	1.41 – 1.29 (m, 2H)	21.1	-0.135	0.8
25	0.99 (t, $J = 7.3$ Hz, 3H)	13.7	0.90 (t, $J = 7.3$ Hz, 3H)	13.8	-0.09	0.1

IR spectra

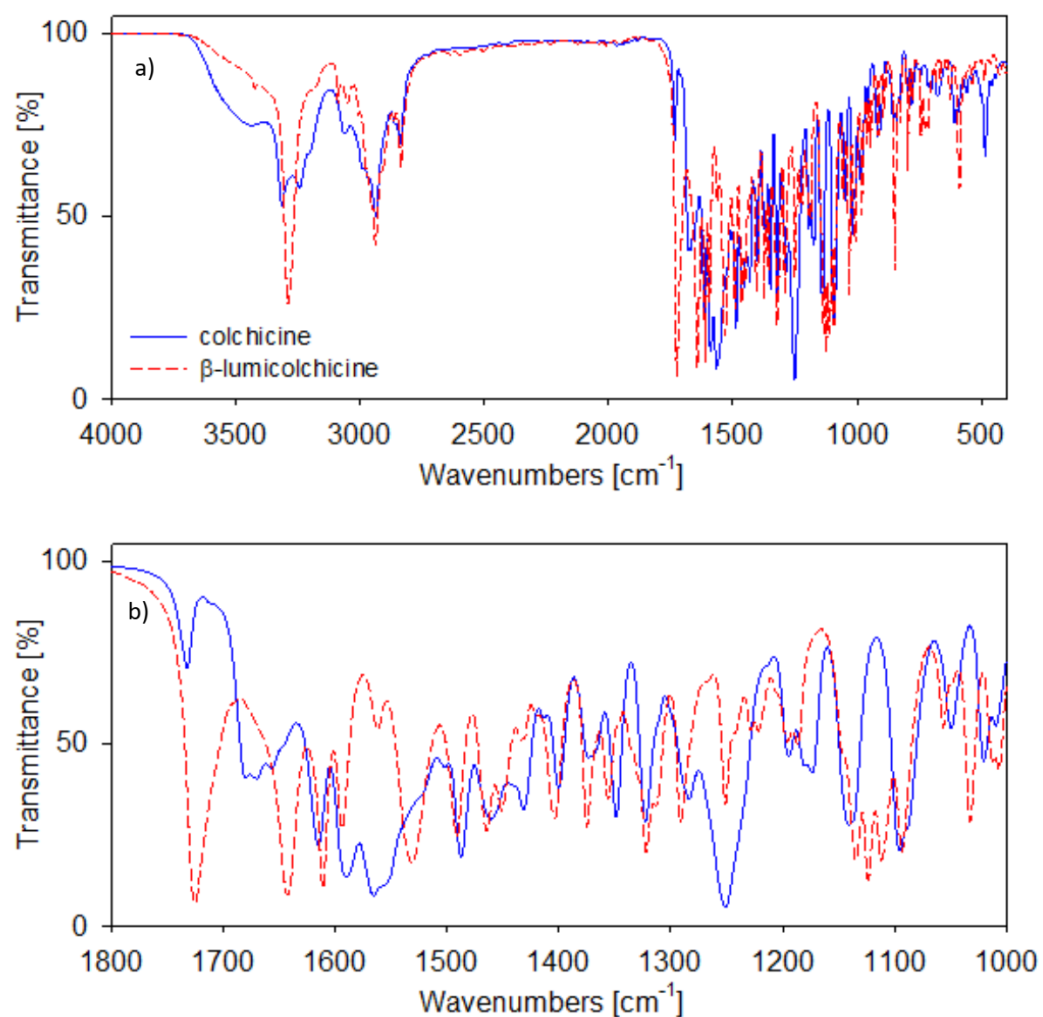


Figure S48. The stacked FT-IR spectra of colchicine (blue solid line) and β -lumicolchicine (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .

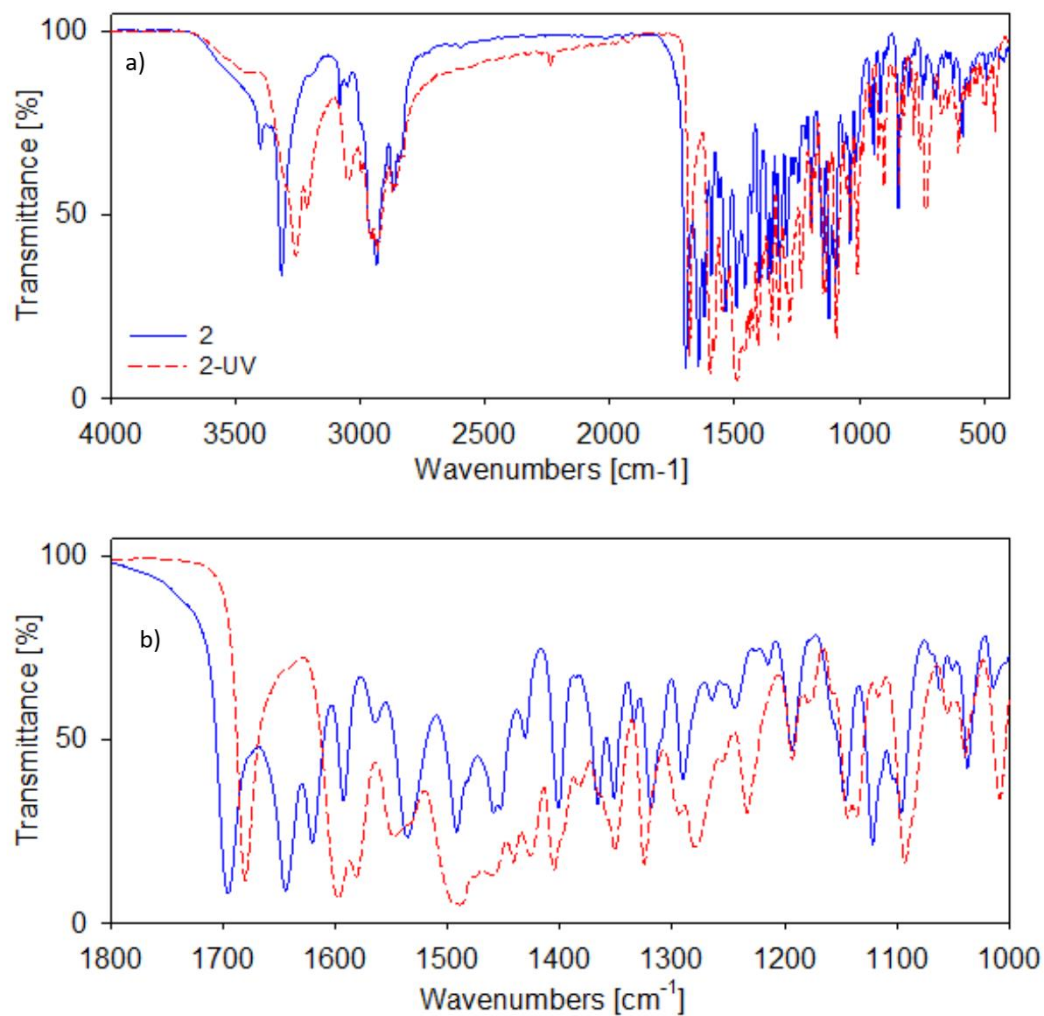


Figure S49. The stacked FT-IR spectra of **2** (blue solid line) and **2-UV** (red dashed line) in KBr: (a) 4000-500 cm⁻¹, (b) 1800-1000 cm⁻¹.

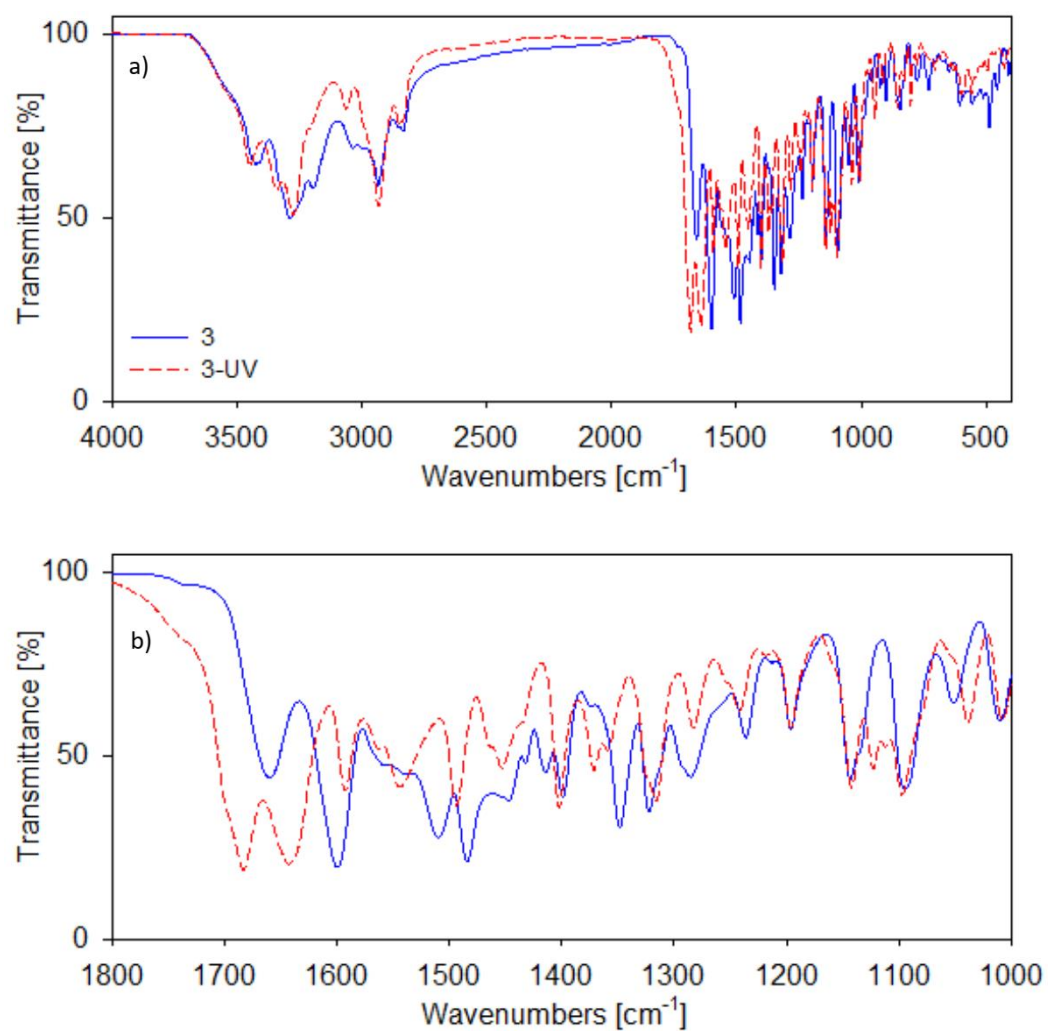


Figure S50. The stacked FT-IR spectra of 3 (blue solid line) and 3-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .

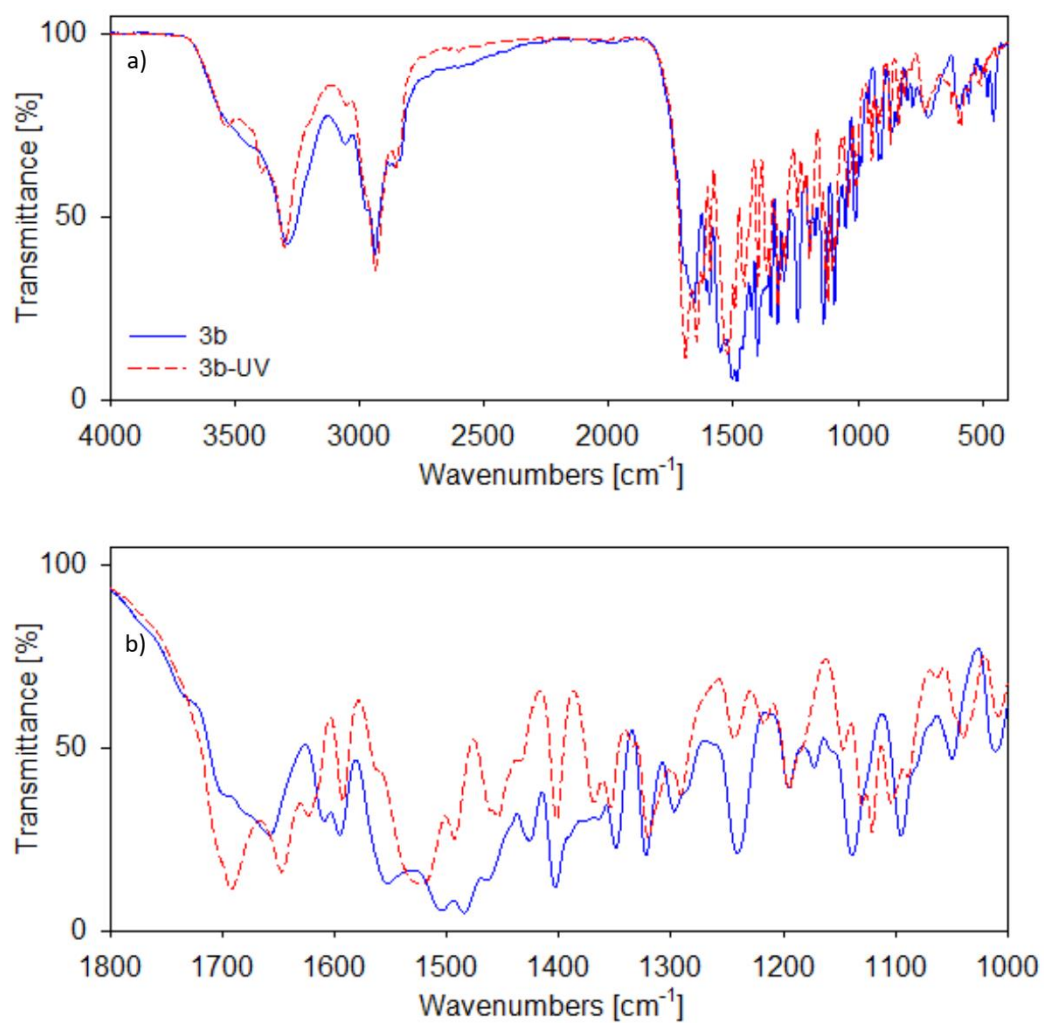


Figure S51. The stacked FT-IR spectra of 3b (blue solid line) and 3b-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .

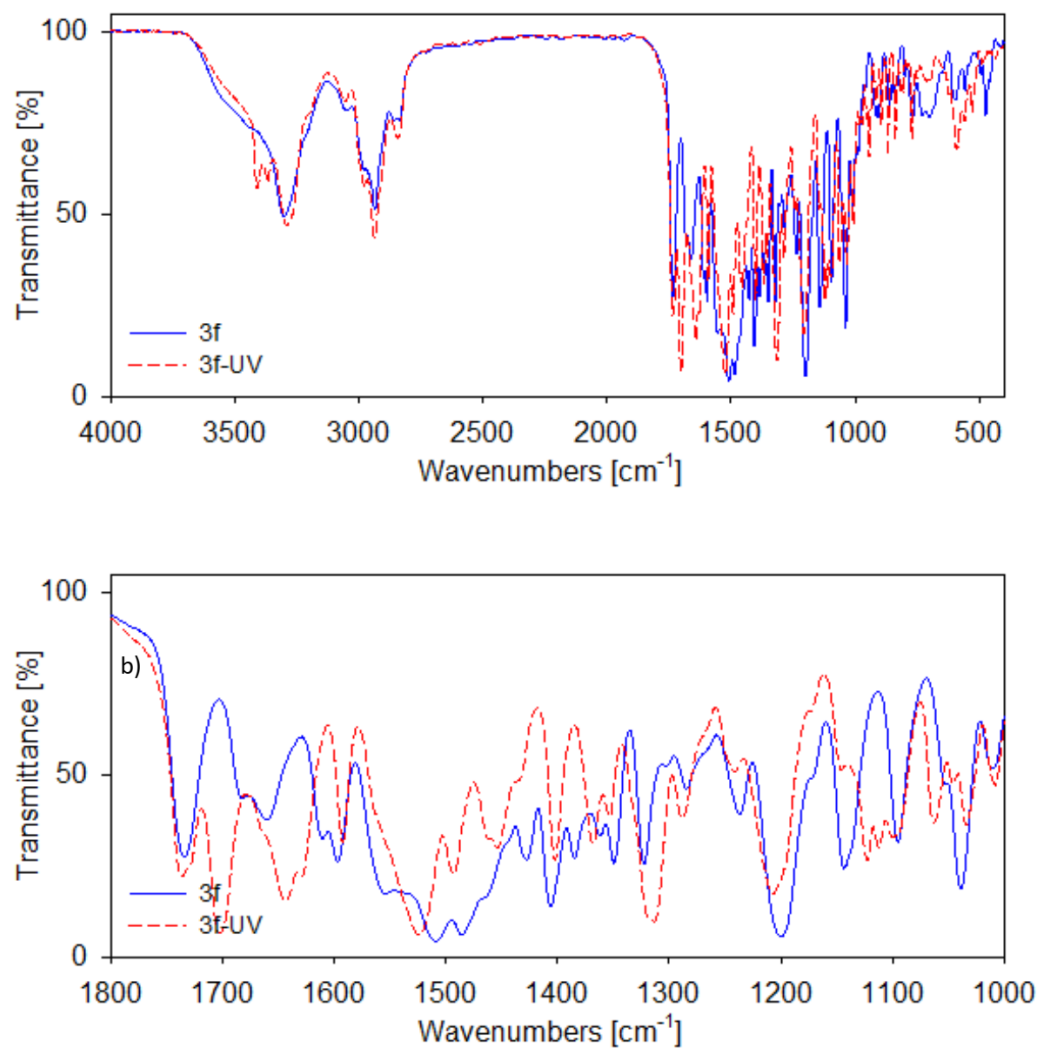


Figure S52. The stacked FT-IR spectra of 3f (blue solid line) and 3f-UV (red dashed line) in KBr: (a) 4000-500 cm^{-1} , (b) 1800-1000 cm^{-1} .

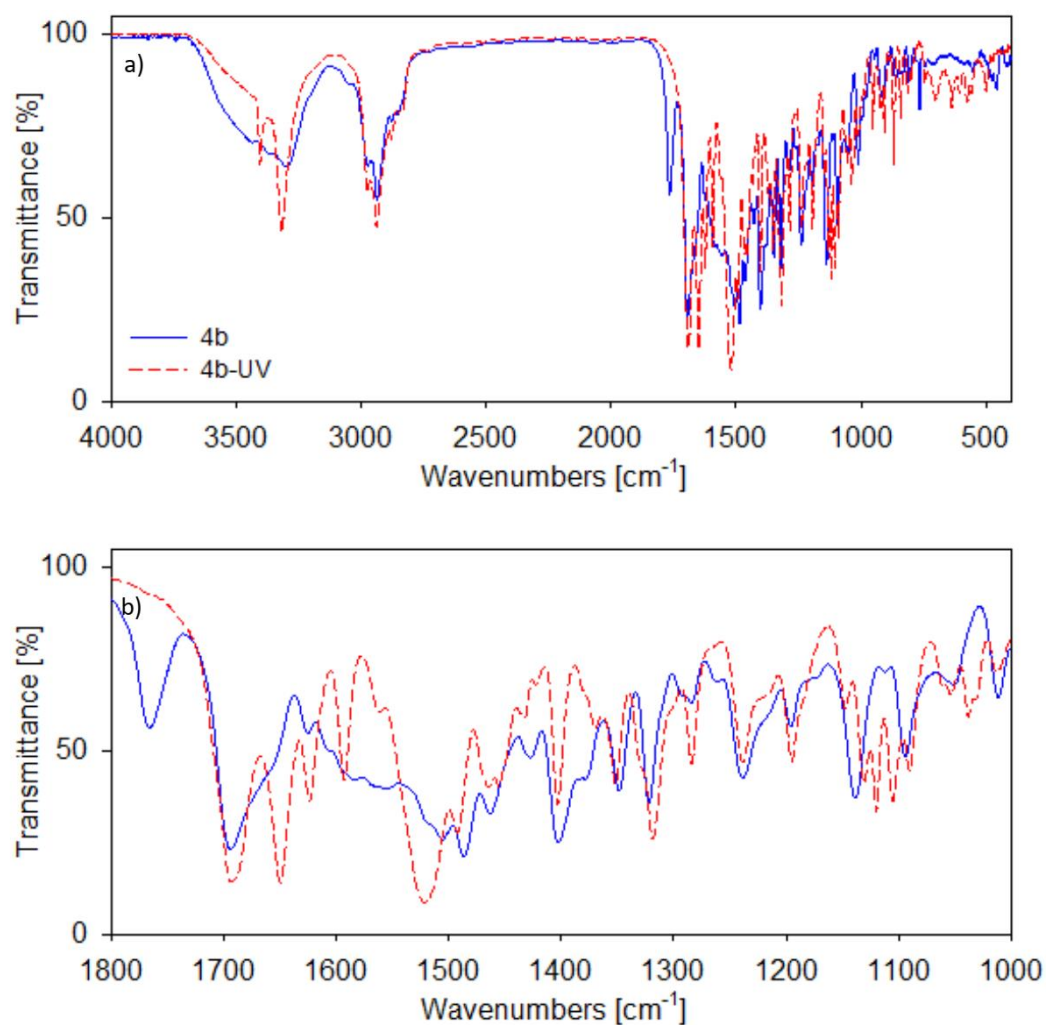


Figure S53. The stacked FT-IR spectra of 4b (blue solid line) and 4b-UV (red dashed line) in KBr: (a) 4000-500 cm⁻¹, (b) 1800-1000 cm⁻¹.

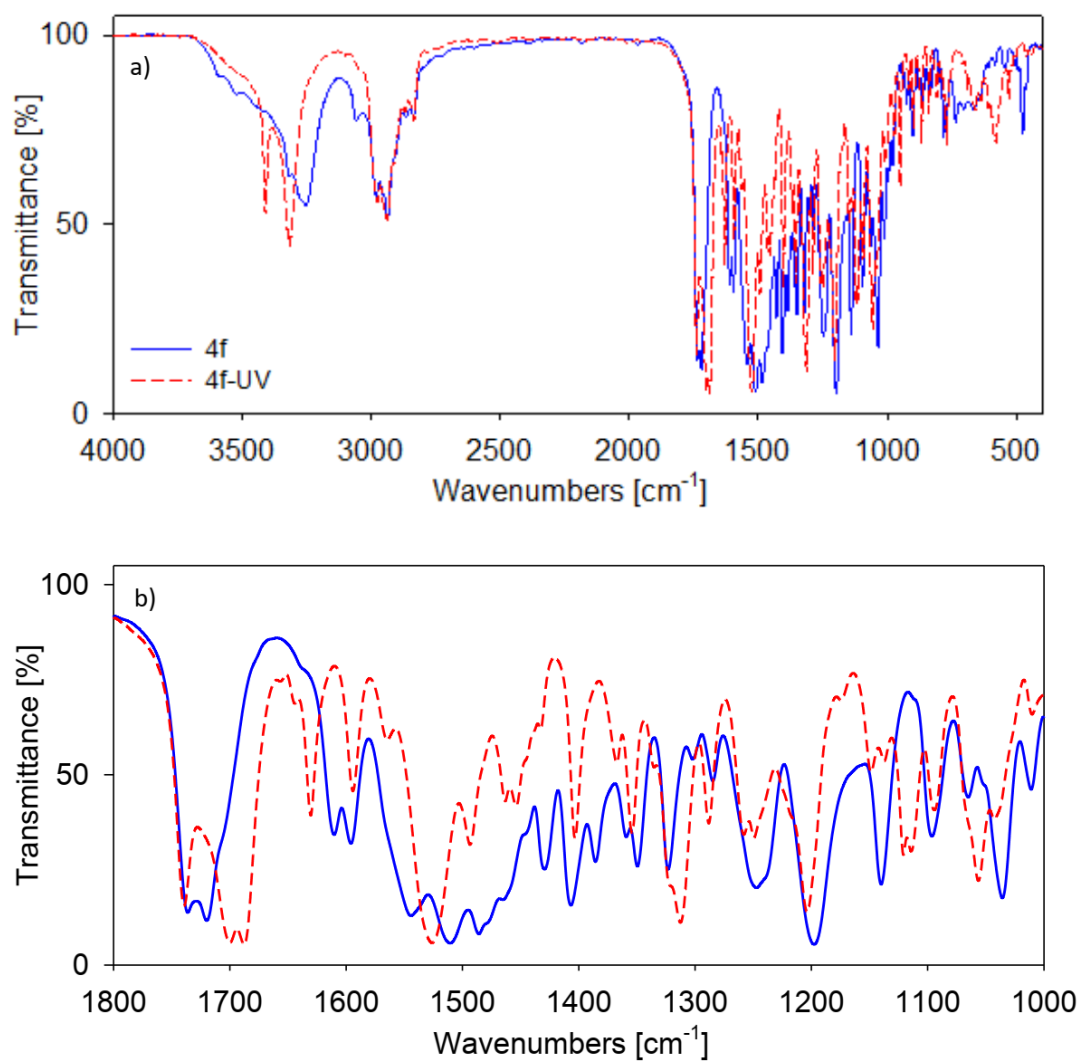


Figure S54. The stacked FT-IR spectra of 4f (blue solid line) and 4f-UV (red dashed line) in KBr: (a) 4000-500 cm⁻¹, (b) 1800-1000 cm⁻¹.

UV-VIS spectra

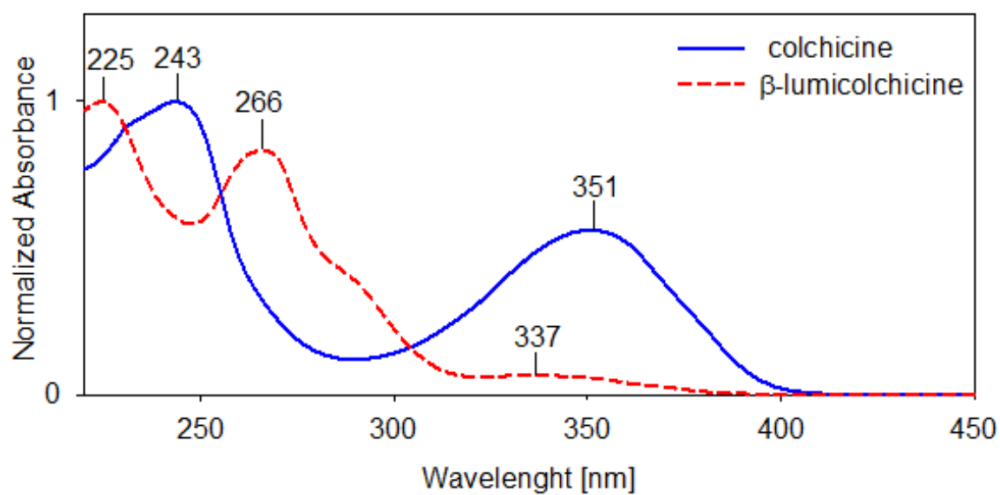


Figure S55. The stacked UV-VIS spectra of colchicine (blue solid line) and β -lumicolchicine (red dashed line) in methanol.

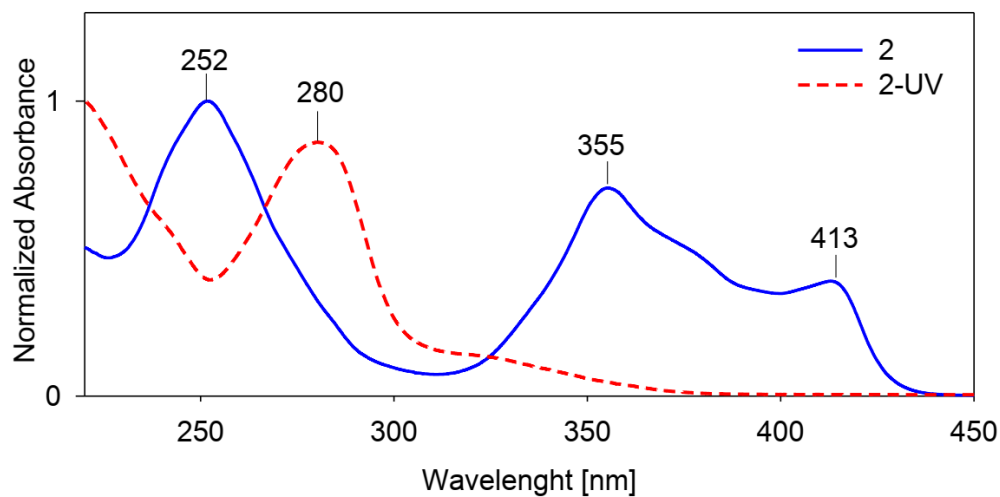


Figure S56. The stacked UV-VIS spectra of 2 (blue solid line) and 2-UV (red dashed line) in methanol.

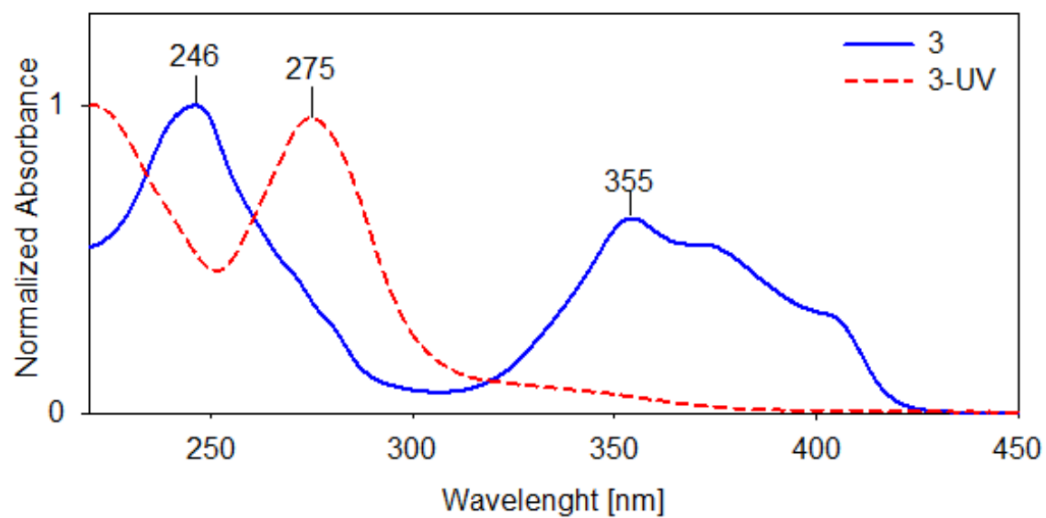


Figure S57. The stacked UV-VIS spectra of 3 (blue solid line) and 3-UV (red dashed line) in methanol.

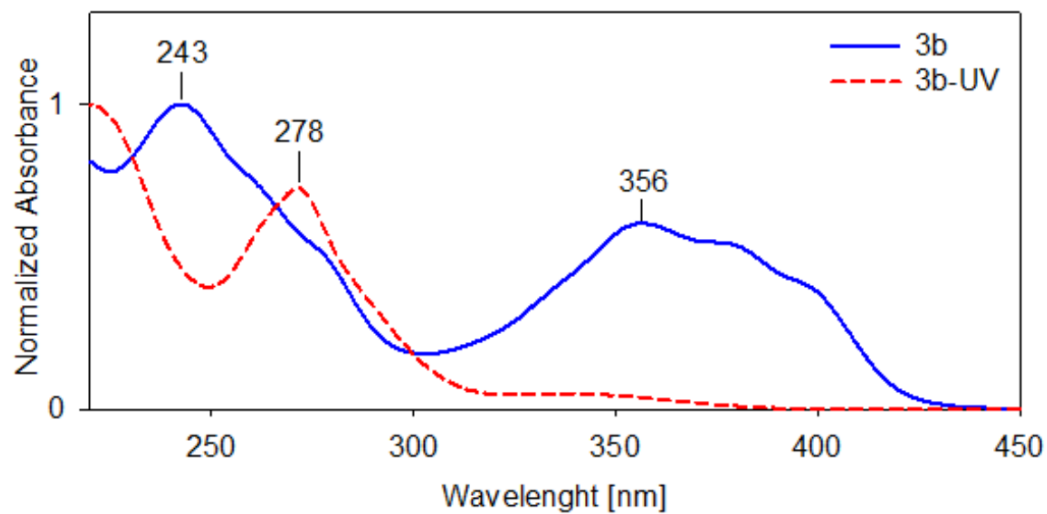


Figure S58. The stacked UV-VIS spectra of 3b (blue solid line) and 3b-UV (red dashed line) in methanol.

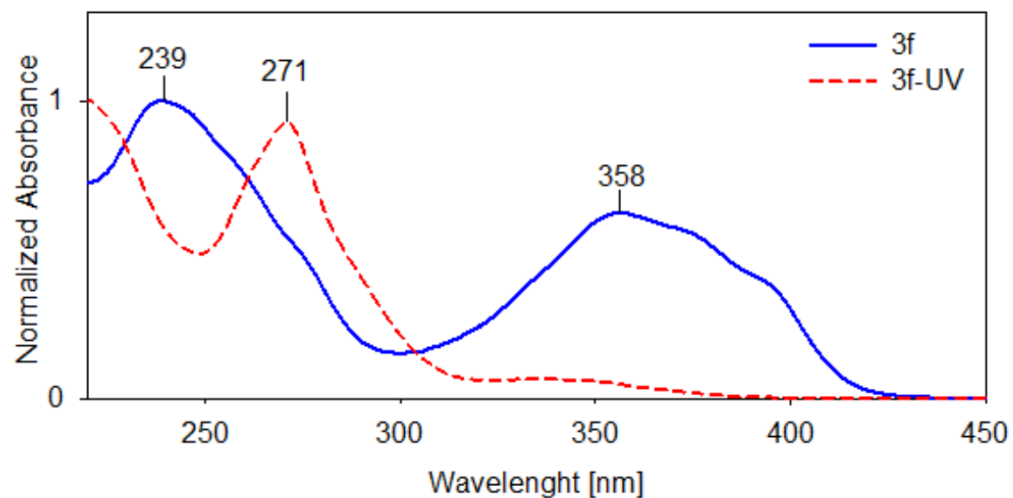


Figure S59. The stacked UV-VIS spectra of 3f (blue solid line) and 3f-UV (red dashed line) in methanol.

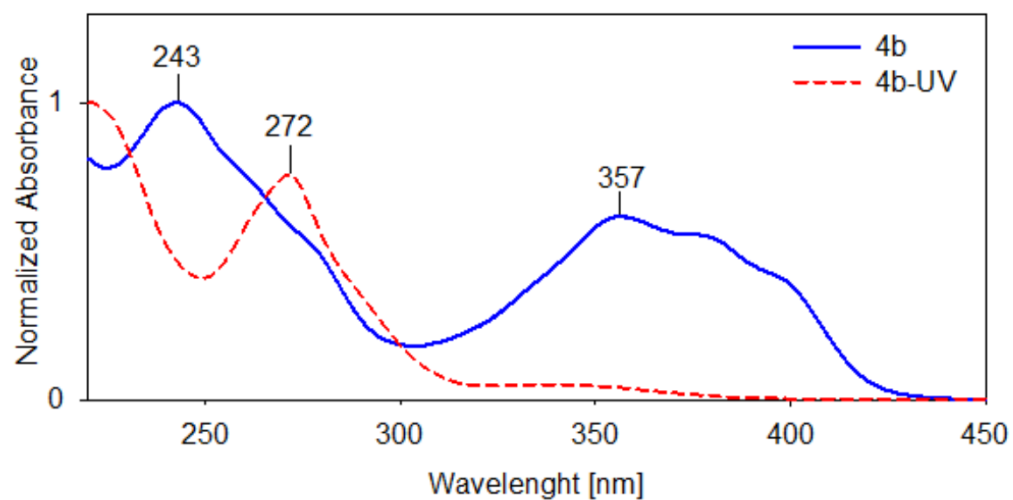


Figure S60. The stacked UV-VIS spectra of 4b (blue solid line) and 4b-UV (red dashed line) in methanol.

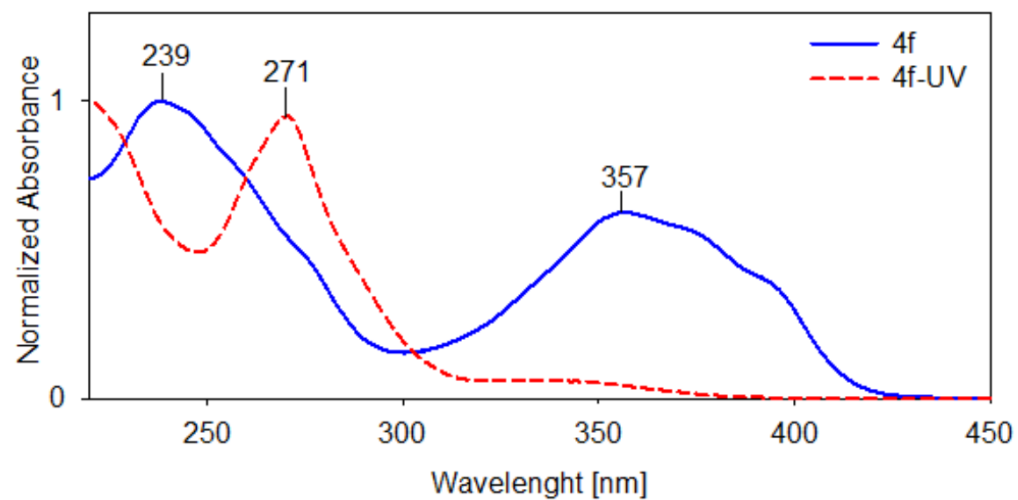


Figure S61. The stacked UV-VIS spectra of 4f (blue solid line) and 4f-UV (red dashed line) in methanol.

Photoreaction Setup

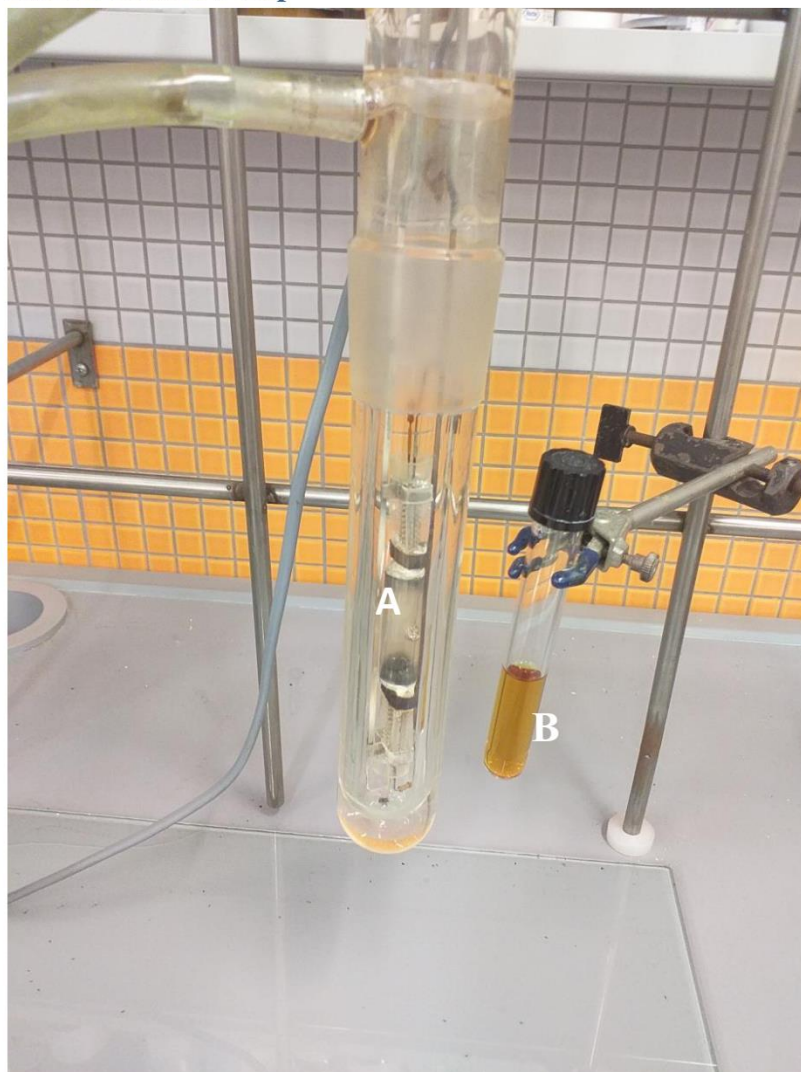


Figure S62. Photograph of a photoreaction setup. Reaction was carried out using a cylindrical photoreactor with an internal radiation source, equipped with a medium-pressure mercury lamp TQ 150W (Heraeus, Hanau, Germany), placed inside a cylindrical filter made of pyrex glass with a wall thickness of 1.5 mm (A). The lamp with the filter was inside a quartz glass cooler and was cooled with water. A solution of starting material was irradiated in Pyrex glass tube (B). During irradiation the whole setup was wrapped with aluminum foil and the irradiated solution was stirred.

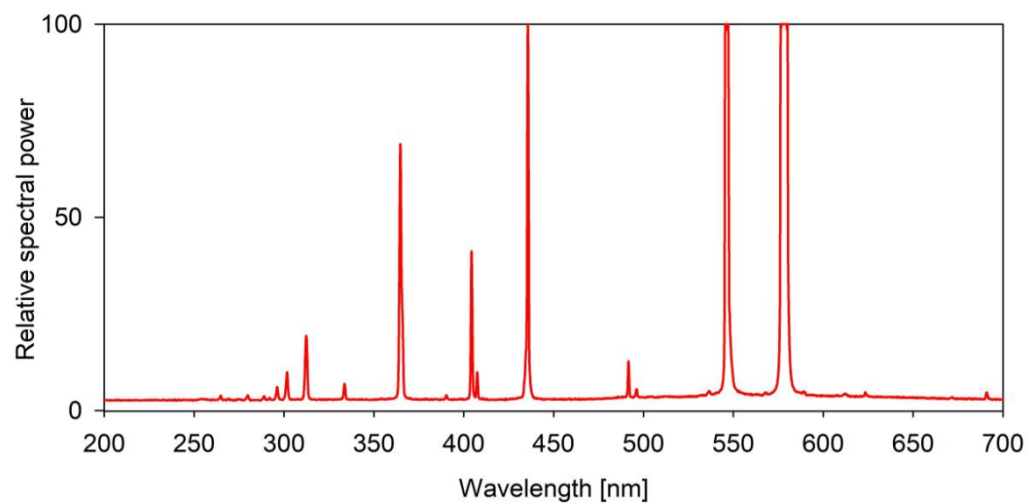


Figure S63. Emission spectrum of the Hanau TQ 150 W lamp after passing through the standard Pyrex filter.

The spectrum was acquired by the hand-held, fiber-coupled CCD spectrometer (Edmund Optics)

PUBLIKACJA VI wraz z suplementem

Dominika Czerwonka, Ewa Maj, Joanna Wietrzyk, Adam Huczyński

„Synthesis of thiocolchicine amine derivatives and evaluation of their
antiproliferative activity”

Bioorganic & Medicinal Chemistry Letters 2021, 52, 128382.



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity

Dominika Czerwonka^a, Ewa Maj^b, Joanna Wietrzyk^b, Adam Huczynski^{a,*}^a Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland^b Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland

ARTICLE INFO

Keywords:

Colchicine analogs
Thiocolchicine
Natural products
Antiproliferative activity
Structure–activity relationship
Reductive amination

ABSTRACT

A series of 22 amine analogs of thiocolchicine were synthesized using the reductive amination reaction. The antiproliferative activities of these compounds were tested against four tumor cell lines as well as one normal cell line. The tested analogs exhibited IC₅₀ values in the nanomolar range accompanied by high selectivity indexes, and most importantly, they were able to break the drug resistance of the human colon adenocarcinoma resistant cell line (LoVo/DX). Also, a correlation between the antiproliferative activity and physicochemical properties of the novel compounds has been found.

Colchicine (COLCH, [scheme 1](#)), a pseudoalkaloid produced by *Colchicum autumnale*, has been used clinically since 2009 but its beneficial properties have been known for centuries.^{1,2} Because of its strong anti-inflammatory effect, colchicine is a cure for many different diseases, such as familial Mediterranean fever, Behcet's disease, gout and pericarditis.^{3–5} Despite the strong antiproliferative activity, related to high-binding affinity to tubulin and inhibition of microtubule polymerization, Colchicine cannot be used in the treatment of cancer due to its high toxicity towards normal cells.^{6–8} Over the years, much effort has been put into surpassing this disadvantage by developing numerous novel colchicine derivatives.^{9–25}

According to the broad structure–activity relationship (SAR) studies, the substitution of colchicine's C10-methoxy group with a thiomethyl group improves the binding affinity to tubulin, as well as increases the molecular stability of the resulting thiocolchicine (TCOLCH, [scheme 1](#)).^{22–28} Moreover, the vast majority of thiocolchicine's derivatives exhibit enhanced antiproliferative activity compared to that of colchicine.^{29–36} With this in view, we used thiocolchicine as a starting point for further modifications.

In this report we describe the synthesis of twenty-two thiocolchicine's amine derivatives, modified at C7 position, *via* reductive amination reaction. We also present the physicochemical properties of the obtained compounds, predicted on the basis of *in silico* calculations. This, together with the evaluation of antiproliferative activity against four human cancer cell lines, allows us to present a comprehensive analysis of the structure–activity relationship.

To obtain our new derivatives, we needed to get access to the free amino group at C7 position. At first colchicine was transformed into thiocolchicine using the previously reported procedures.³⁵ Subsequently, because of the high stability of thiocolchicine, we were able to perform the deacetylation reaction with 5M HCl aqueous solution and obtain *N*-deacetylthiocolchicine (DTCOLCH, [scheme 1](#)).³⁴

Finally, a series of reductive amination reactions were performed, using various aliphatic and aromatic aldehydes and cyanoborohydride as a reducing agent. In this way, ten colchicine derivatives with aliphatic chains (1–10), and eleven derivatives with aromatic substituents (11–21) were obtained. Also, by using pyridine-2,6-dicarbaldehyde, one dimeric analog (22) was synthesized. Detailed descriptions of synthetic procedures can be found in [Supplementary Materials](#). The structures of all products 1–22 ([Scheme 1](#)) were determined using the ESI-MS as well as ¹H and ¹³C NMR methods. The spectra, included in [Supplementary Materials](#) (Figs. S1–S44), confirmed the structures of the studied compounds.

In a previously conducted study, the structurally related benzyl analogs of thiocolchicine were found potent against various tumor cell lines.³⁷ Thus, we decided to evaluate the *in vitro* anti-proliferative activity of our compounds. The inhibition of cell growth *in vitro* was tested on four human cancer cell lines: human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/DX) respectively. To evaluate the derivatives cytotoxicity, the effect of our compounds on normal murine embryonic fibroblast cell line (BALB/

* Corresponding author.

E-mail address: adhucz@amu.edu.pl (A. Huczynski).<https://doi.org/10.1016/j.bmcl.2021.128382>

Received 28 June 2021; Received in revised form 15 September 2021; Accepted 22 September 2021

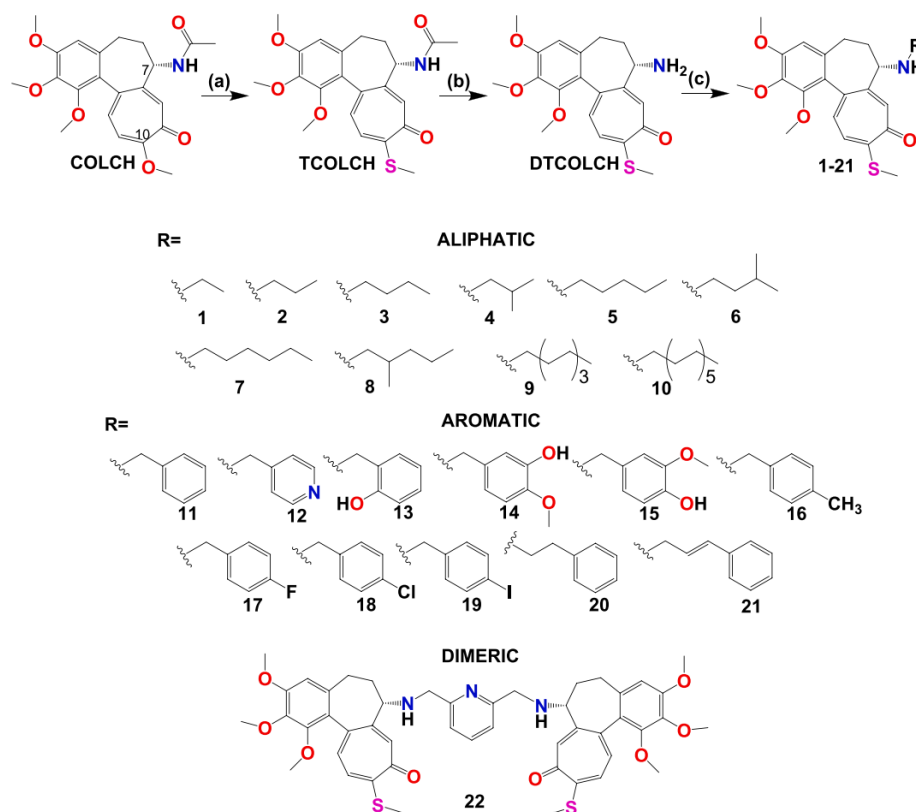
Available online 27 September 2021

0960-894X/© 2021 The Author(s).

Published by Elsevier Ltd.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).



Scheme 1. Synthesis of amino analogs of thiocolchicine (1–22), modified at C7 position via reductive amination reaction. Reagents and conditions: (a) MeOH/H₂O, CH₃SNa, room temperature, 24 h; (b) 5 M HCl, reflux, 72 h; (c) RCHO, NaBH₃CN, DCM/MeOH, room temperature, 24 h.

3T3) was also tested. The antiproliferative activity was assessed according to the previously reported procedures,³⁰ detailed information can be found in [Supplementary Materials](#). The mean values of IC₅₀ ± SD of the tested compounds are collected in [Table 1](#).

In general, the vast majority of the tested compounds showed higher antiproliferative activity than that of conventional chemotherapeutics cisplatin and doxorubicin. The compounds most active against the A549 cancer cell line were: 1–4, 6, 8, 11–15, 17, 20 (IC₅₀ < 13 nM); those most active against the MCF-7 cell line were: 1–4, 6, 8, 11–15, 17, 20 and 21 (IC₅₀ < 18 nM), those most active against the LoVo cell line were: 1–4, 6, 8, 11–14, 17, 20 (IC₅₀ < 12 nM), and those most active against the LoVo/DX cell line were: 4, 6 and 11 (IC₅₀ < 14 nM). Importantly, it should be noted that two compounds with aliphatic substituents (4 and 6) and one with an aromatic ring (11) were found the most active against all tested cell lines.

Comparison of the data obtained for the set of compounds with aliphatic side chains (1–10) revealed a drastic change in their activity against all tested cell lines with the elongation of the *n*-alkyl chain, the most potent antiproliferative activity was observed for the analogs with a short *n*-alkyl chain (1–3) or branched substituents (4, 6, 8). For compounds with longer *n*-alkyl chains, like *n*-pentyl, *n*-hexyl, *n*-octyl and *n*-dodecyl (5, 7, 9, 10) the activity drops, even up to 100-times for the substituent with the longest chain. For the analogs with an aromatic substituent at C7 position, introduction of benzyl, salicyl or 4-pyridine motif (11–13) resulted in comparable activity against A549, MCF-7 and LoVo cells. Interestingly, compounds 14 and 15, with isovanillin and vanillin moieties, showed similar IC₅₀ values against lines A549 and MCF-7, whereas compound 14 exhibited 6-time higher activity towards

the LoVo cell line than 15 (IC₅₀ = 8.8 ± 4.6 and 53.3 ± 16.5 nM, respectively). There are also significant differences in the activities of halogenated analogs 17–19, bearing benzyl rings substituted in the *para* position with fluorine, chlorine and iodine, respectively. The activities of these analogs decrease with increasing atomic weight of the halogen. It is also worth noting that the only dimeric analog 22, showed a potent activity towards the MCF-7 cell line (IC₅₀ = 7.7 ± 2.0 nM).

Novel analogs 1–22 were found to be less effective in inhibiting the growth of the doxorubicin-resistant subline LoVo/DX than the sensitive LoVo cell line. However, the calculated RI values ([Fig. 1](#)) revealed that all tested compounds, except dimeric analog 22, were able to break the drug resistance of the LoVo/DX line (RI < 10) and ten analogs (3–7, 9, 11, 15, 16, 18) exhibited the outstanding values of RI < 2. Moreover, the values of the resistance indexes of our derivatives exceeded those obtained for doxorubicin. The lowest RI of 0.8 and 0.9 were obtained for analogs 9 and 7, respectively. These analogs showed one of the weakest IC₅₀ inhibition rates toward all tested cancer cell lines.

Unfortunately, favorable antiproliferative activity against cancer cells can be accompanied by cytotoxic effects towards normal cells. Indeed, a perfect drug candidate should affect normal cells as little as possible. Calculations of the selectivity index (SI) values revealed a few compounds with beneficial SI ([Fig. 2](#)). Analog 10, bearing dodecyl chain at C7, was characterized with a surprising selectivity of 7.0 despite one of the poorest antiproliferative activities towards the A549 cell line (IC₅₀ = 151.8 ± 36.4 nM). The potent action of the dimeric compound 22 towards the MCF-7 cell line (IC₅₀ = 7.7 ± 2.0 nM) was also followed by good SI = 6.5. Two of the tested analogs, one with a 2-methylvaleric chain (8) and one with the phenylethyl motif (20) at C7 position,

Table 1

Antiproliferative activity (IC_{50}) [nM] of novel thiocolchicine's analogs (1–22) in comparison to those of COLCH, TCOLCH, DTCOLCH as well as standard anticancer drugs doxorubicin (DX) and cisplatin (CP).

	Compound	A549 IC_{50} (nM)	MCF-7 IC_{50} (nM)	LoVo IC_{50} (nM)	LoVo/DX IC_{50} (nM)	BALB/3T3 IC_{50} (nM)
aliphatic	1	10.8 ± 1.4	10.3 ± 0.4	6.5 ± 1.9	54.9 ± 22.0	10.2 ± 1.9
	2	11.6 ± 1.3	12.0 ± 0.4	8.5 ± 0.5	31.1 ± 13.7	14.3 ± 3.5
	3	10.9 ± 0.5	12.2 ± 1.3	8.8 ± 0.8	17.9 ± 5.0	11.7 ± 0.8
	4	10.5 ± 0.9	11.3 ± 1.4	8.5 ± 0.9	10.2 ± 1.4	11.0 ± 1.7
	5	89.5 ± 7.5	92.7 ± 10.5	52.8 ± 16.4	77.8 ± 7.1	99.4 ± 9.2
	6	13.1 ± 5.1	12.0 ± 2.1	8.4 ± 0.5	9.4 ± 5.0	13.3 ± 1.5
	7	104.0 ± 4.1	109.3 ± 10.2	83.0 ± 11.8	72.7 ± 7.3	106.7 ± 27.2
	8	11.0 ± 5.5	17.8 ± 4.7	12.0 ± 2.6	70.2 ± 3.9	78.5 ± 8.3
	9	1007.3 ± 88.2	1124.4 ± 158.9	902.9 ± 189.0	692.7 ± 100.5	1036.3 ± 236.3
	10	151.8 ± 36.4	719.2 ± 193.1	453.0 ± 177.0	1984.1 ± 214.4	1068.4 ± 230.5
aromatic	11	9.6 ± 0.4	10.3 ± 0.6	7.9 ± 0.7	13.9 ± 6.2	9.8 ± 0.9
	12	10.9 ± 1.5	9.7 ± 1.0	7.8 ± 1.1	56.2 ± 9.4	10.9 ± 1.3
	13	9.3 ± 1.2	10.3 ± 1.6	9.4 ± 4.4	41.7 ± 23.0	8.8 ± 3.7
	14	10.8 ± 4.0	10.3 ± 4.3	8.8 ± 4.6	69.4 ± 27.5	10.8 ± 4.9
	15	7.1 ± 2.5	8.2 ± 1.8	53.3 ± 16.5	85.6 ± 15.1	385.2 ± 101.3
	16	83.4 ± 22.6	100.1 ± 14.7	47.6 ± 23.2	52.3 ± 22.0	85.4 ± 13.4
	17	9.6 ± 0.9	11.2 ± 0.8	7.6 ± 0.3	33.2 ± 26.5	11.7 ± 1.6
	18	70.5 ± 27.4	73.4 ± 27.1	37.0 ± 20.5	64.9 ± 10.4	88.2 ± 19.1
	19	101.2 ± 36.8	84.2 ± 2.0	72.4 ± 9.8	233.9 ± 142.6	118.9 ± 47.6
	20	10.7 ± 3.5	11.4 ± 5.1	10.7 ± 4.8	66.9 ± 7.8	61.2 ± 29.1
	21	95.0 ± 6.0	102.3 ± 6.3	79.2 ± 10.1	210.5 ± 80.7	98.6 ± 30.7
	22	47.3 ± 4.0	7.7 ± 2.0	27.9 ± 90.2	399.6 ± 63.2	50.3 ± 60.2
reference	COLCH	19.3 ± 4.1	10.1 ± 3.3	12.7 ± 3.8	966.8 ± 272.0	82.3 ± 17.5
	TCOLCH	0.9 ± 0.4	1.1 ± 0.2	1.0 ± 0.5	157.8 ± 65.5	9.6 ± 2.5
	DTCOLCH	11.0 ± 3.8	9.5 ± 3.8	10.3 ± 2.6	77.8 ± 33.8	15.4 ± 5.1
	DX	149.0 ± 55.0	146.0 ± 56.0	71.0 ± 32.0	3957.0 ± 257.0	315.0 ± 107.0
	CP	4227.0 ± 607.0	5579.0 ± 361.0	7784.0 ± 1102.0	4473.0 ± 2936.0	7300.0 ± 1614.0

The IC_{50} value is defined as the concentration of a compound at which 50% growth inhibition is observed. The IC_{50} values shown are means ± SD. Human lung carcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line (LoVo) and doxorubicin-resistant subline (LoVo/DX), normal murine embryonic fibroblast cell line (BALB/3T3).

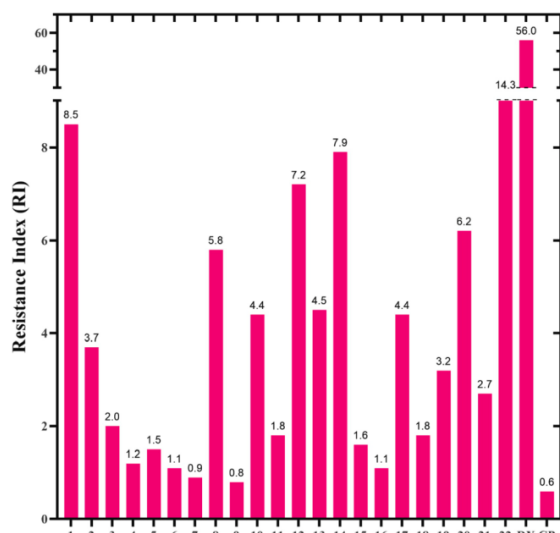


Fig. 1. Comparison of resistance index (RI) values of tested compounds. RI indicates how many times more chemoresistant is a resistant subline relative to its parental cell line. The RI was calculated for each compound using the formula: $RI = (IC_{50} \text{ for LoVo/DX}) / (IC_{50} \text{ for LoVo})$ cell line. When RI is 0–2, the cells are sensitive to the compound tested, RI in the range 2–10 means that the cell shows moderate sensitivity to the drug tested, RI above 10 indicates strong drug resistance. DX- doxorubicin, CP- cisplatin.

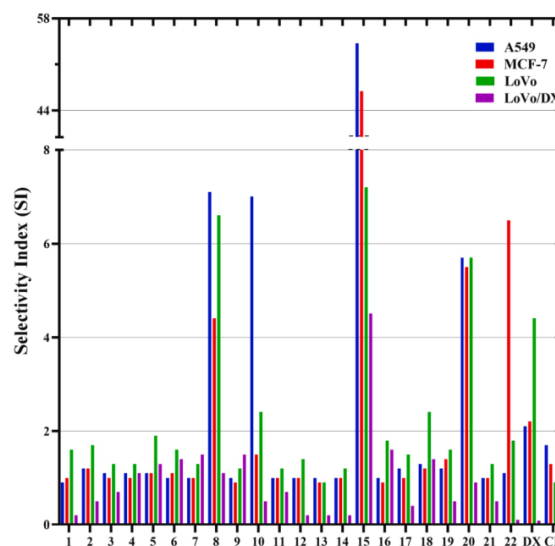


Fig. 2. Comparison of selectivity index (SI) values of the tested compounds. The SI was calculated for each compound using the formula: $SI = (IC_{50} \text{ for normal cell line BALB/3T3}) / (IC_{50} \text{ for respective cancerous cell line})$. A beneficial SI greater than 1.0 indicates a drug with efficacy against tumor cells greater than the toxicity against normal cells. DX- doxorubicin, CP- cisplatin.

exhibited excellent selectivity (SI from 4.4 to 7.1) towards three out of four tested cancer cell lines (A549, MCF-7 and LoVo). Importantly, these attractive SI values were also accompanied by low IC_{50} values between 8.4 and 13.1 nM. Eventually, compound 15 with the vanillin motif,

Table 2

Physicochemical properties of the novel analogs calculated using the Molinspiration database.³⁸ The violated parameters are marked in bold.

	Compound	MW	clogP	tPSA	n(O,N)	n(OH,NH)	Rotb	MV
reference	TCOLCH	415.51	2.71	73.87	6	1	5	373.30
aliphatic	1	401.53	3.18	56.80	5	1	6	371.12
	2	415.56	3.68	56.80	5	1	7	387.92
	3	429.58	4.24	56.80	5	1	8	404.72
	4	429.58	3.93	56.80	5	1	7	404.51
	5	443.61	4.75	56.80	5	1	9	421.52
	6	443.61	4.46	56.80	5	1	8	421.31
	7	457.64	5.25	56.80	5	1	10	438.32
	8	457.64	4.99	56.80	5	1	9	438.11
	9	485.69	6.26	56.80	5	1	12	471.93
	10	541.80	8.25	56.80	5	1	16	539.13
aromatic	11	463.60	4.20	56.80	5	1	7	425.96
	12	464.59	2.91	69.69	6	1	7	421.81
	13	479.60	4.14	77.03	6	2	7	433.98
	14	509.62	3.54	86.26	7	2	8	459.53
	15	509.62	3.54	86.26	7	2	8	459.53
	16	477.63	4.65	56.80	5	1	7	442.52
	17	481.59	4.37	56.80	5	1	7	430.90
	18	498.04	4.88	56.80	5	1	7	439.50
	19	589.50	5.29	56.80	5	1	7	449.95
	20	477.63	4.61	56.80	5	1	8	442.77
	21	489.64	5.16	56.80	5	1	8	453.38
	22	850.07	5.37	126.49	11	2	14	763.73

MW: Molecular weight; clogP: calculated log octanol/water partition coefficient; tPSA: total polar surface area; n(O,N): number of hydrogen acceptors; n(OH,NH): number of hydrogen donors; Rotb: rotatable bonds; MV: molecular volume.

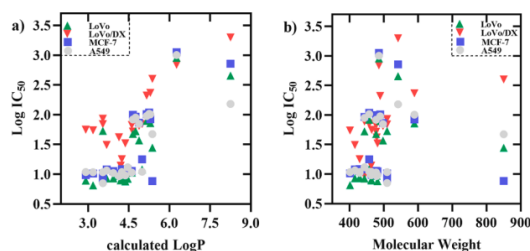


Fig. 3. a) Correlation between the calculated LogP values of novel analogs 1–22 and their LogIC₅₀ values against the used cancer cell lines; b) Correlation between the molecular weight of novel analogs 1–22, and LogIC₅₀ values against used cancer cell lines.

showed outstanding selectivity towards all tested cell lines: SI values of 54.2, 46.9, 7.2 and 4.5 towards A549, MCF-7, LoVo and LoVo/DX, respectively. These SI values together with the activity between 7.1 and 85.6 nM and RI = 1.6, illustrate the undoubted potential of analog 15.

To check whether it would be possible to find some relationship between the biological activity and the physicochemical properties of synthesized molecules, we used the Molinspiration online database (<http://www.molinspiration.com>, free of charge).³⁸ “The rule of 5” (RO5), established by Christopher Lipinski in 1997, is a set of rules that should be followed during drug discovery.^{39,40} For a compound to be an orally active drug in humans, it should have <5H-bond donors, 10H-bond acceptors, the molecular weight (MW) smaller than 500 and the calculated Log P (cLogP) smaller than 5. Besides the above-mentioned parameters, if the total polar surface area (tPSA) is smaller than 140 and there are <10 rotatable bonds (Rotb), the compound is expected to have good oral bioavailability.⁴¹ To be active, a proposed drug should not violate more than one of the above-mentioned rules.

In general, novel analogs are characterized by good physicochemical properties, summarized in Table 2. All obtained compounds exhibited clogP values in the range 2.91 – 8.25, which are higher than that calculated for TCOLCH (clogP = 2.71). Six out of twenty-two synthesized compounds (7, 9, 10, 19, 21 and 22) showed clogP values higher than 5. Also, the number of rotatable bonds was found to increase with

elongation or branching of the side chain at the C7 position. Analogs 9, 10 and 22 violated the rule about the number of Rotb, exhibiting twelve, sixteen and fourteen rotatable bonds, respectively. Five new compounds (10, 14, 15, 19, 22) had molecular weight greater than 500.

Knowing the physicochemical properties of our compounds and their activities, we searched for a correlation between them. At first the values of LogP and LogIC₅₀ were analyzed (Fig. 3a) to find that the least active analogs (LogIC₅₀ > 2.1) showed cLogP values greater than 5, which is in agreement with one of the Lipinski’s rules. A comparison of the molecular weight values with LogIC₅₀ (Fig. 3b) showed that the majority of the most active compounds (LogIC₅₀ < 2.1) were characterized by MW lower than 500. This, again, was in line with Lipinski’s “rule of 5”.

In this paper we reported the synthesis of twenty-two amine analogs of thiocolchicine, modified at C7 position. Many of the obtained compounds showed antiproliferative potency higher than that of colchicine, doxorubicin and cisplatin. Importantly, the obtained compounds were able to break the drug resistance of the LoVo/DX line, exhibiting RI values in the range of 8.5 – 0.8 (with exception of compound 22, RI = 14.3). The physicochemical properties of the analogs indicated their good oral bioavailability. A correlation was also found between the antiproliferative activity and the physicochemical properties of the novel compounds. The most active analogs were those characterized by MW lower than 500 and cLogP lower than 5, which is in agreement with the Lipinski’s “rule of 5”. The most potent compound 15, exhibited very good activity in the range of 7.1 and 85.6 nM against all tested cancer cell lines (A549, MCF-7, LoVo and LoVo/DX), followed by outstanding selectivity between 4.5 and 54.2, and resistance index equal 1.6. The unquestionable potential of derivative 15 makes it a highly promising candidate for further evaluation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Financial support by grant of the Polish National Science Centre (NCN) - No. 2016/21/B/ST5/00111 is gratefully acknowledged. D.C.

wishes to acknowledge the scholarship no. POWR.03.02.00e00-1026/16, co-financed by the European Union through the European Social Fund under the Operational Program Knowledge Education Development. D.C. also wishes to acknowledge the NCN for the doctoral scholarship ETIUDA (2020/36/T/ST4/00041).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmcl.2021.128382>.

References

- Slobodnick A, Shah B, Pillinger MH, Krasnokutsky S. Colchicine: Old and New. *Am J Med*. 2015;128(5):461–470. <https://doi.org/10.1016/j.amjmed.2014.12.010>.
- Graham W, Roberts JB. Intravenous colchicine in the management of gouty arthritis. *Ann Rheum Dis*. 1953;12(1):16–19. <https://doi.org/10.1136/ard.12.1.16>.
- Khanna D, FitzGerald JD, Khanna PP, et al. 2012 American college of rheumatology guidelines for management of gout. Part 1: Systematic nonpharmacologic and pharmacologic therapeutic approaches to hyperuricemia. *Arthritis Care Res*. 2012;64(10):1431–1446. <https://doi.org/10.1002/acr.21772>.
- Grattagliano I, Bonfrate L, Ruggiero V, Scaccianoce G, Portincasa P. Novel therapeutics for the treatment of familial mediterranean fever: From colchicine to biologics. *Clin Pharmacol Ther*. 2014;95(1):89–97. <https://doi.org/10.1038/clpt.2013.148>.
- Imazio M, Gaita F, LeWinter M. Evaluation and treatment of pericarditis: A systematic review. *JAMA - J Am Med Assoc*. 2015;314(14):1498–1506. <https://doi.org/10.1001/jama.2015.12763>.
- Cocco G, Chu DCC, Pandolfi S. Colchicine in clinical medicine. A guide for internists. *Eur J Intern Med*. 2010;21(6):503–508. <https://doi.org/10.1016/j.ejim.2010.09.010>.
- Yang LPH. Oral Colchicine (colcryst®). In: The treatment and prophylaxis of gout. *Drugs*. 2010;70(12):1603–1613. <https://doi.org/10.2165/11205470-000000000-00000>.
- Avendaño C, Menéndez JC. Medicinal Chemistry of Anticancer Drugs. *Elsevier*. 2008 <https://doi.org/10.1016/B978-0-444-52824-7.X0001-7>.
- Krzywick J, Aminpour M, Maj E, et al. New series of double-modified colchicine derivatives: Synthesis, cytotoxic effect and molecular docking. *Molecules*. 2020;25(15):3540. <https://doi.org/10.3390/molecules25153540>.
- Krzywick J, Aminpour M, Janczak J, et al. An insight into the anticancer potential of carbamates and thiocarbamates of 10-demethoxy-10-methylaminocolchicine. *Eur J Med Chem*. 2021;215:113282. <https://doi.org/10.1016/j.ejmech.2021.113282>.
- Krzywick J, Mozga W, Aminpour M, et al. Synthesis, antiproliferative activity and molecular docking studies of novel doubly modified colchicine amides and sulfonamides as anticancer agents. *Molecules*. 2020;25(8):1789. <https://doi.org/10.3390/molecules25081789>.
- Yasobu N, Kitajima M, Kogure N, et al. Design, synthesis, and antitumor activity of 4-halocolchicines and their pro-drugs activated by cathepsin B. *ACS Med Chem Lett*. 2011;2(5):348–352. <https://doi.org/10.1021/ml100287y>.
- Nakagawa-Goto K, Chen CX, Hamel E, et al. Antitumor agents. Part 236: Synthesis of water-soluble colchicine derivatives. *Bioorganic Med Chem Lett*. 2005;15(1):235–238. <https://doi.org/10.1016/j.bmcl.2004.07.098>.
- Shchegryavina ES, Maleev AA, Ignatov SK, et al. Synthesis and biological evaluation of novel non-racemic indole-containing allicolchicinoids. *Eur J Med Chem*. 2017;141:51–60. <https://doi.org/10.1016/j.ejmech.2017.09.055>.
- Zhang X, Kong Y, Zhang J, et al. Design, synthesis and biological evaluation of colchicine derivatives as novel tubulin and histone deacetylase dual inhibitors. *Eur J Med Chem*. 2015;95:127–135. <https://doi.org/10.1016/j.ejmech.2015.03.035>.
- Nicolaou KC, Valiulin RA, Pokorski JK, Chang V, Chen JS. Bio-inspired synthesis and biological evaluation of a colchicine-related compound library. *Bioorganic Med Chem Lett*. 2012;22(11):3776–3780. <https://doi.org/10.1016/j.bmcl.2012.04.007>.
- Chang D-J, Yoon E-Y, Lee G-B, et al. Design, synthesis and identification of novel colchicine-derived immunosuppressant. *Bioorganic Med Chem Lett*. 2009;19(15):4416–4420. <https://doi.org/10.1016/j.bmcl.2009.05.054>.
- Marzo-Mas A, Barbier P, Breuzard G, et al. Interactions of long-chain homologues of colchicine with tubulin. *Eur J Med Chem*. 2017;126:526–535. <https://doi.org/10.1016/j.ejmech.2016.11.049>.
- Johnson L, Goping IS, Rieger A, et al. Novel Colchicine Derivatives and their Anticancer Activity. *Curr Top Med Chem*. 2017;17(22). <https://doi.org/10.2174/1568026617666170104143618>.
- Ghawanmeh AA, Al-Bajalan HM, Mackeen MM, Alali FQ, Chong KF. Recent developments on (–)-colchicine derivatives: Synthesis and structure-activity relationship. *Eur J Med Chem*. 2020;185:111788. <https://doi.org/10.1016/j.ejmech.2019.111788>.
- Gracheva IA, Shchegryavina ES, Schmalz HG, Beletskaya IP, Fedorov AY. Colchicine Alkaloids and Synthetic Analogues: Current Progress and Perspectives. *J Med Chem*. 2020;63(19):10618–10651. <https://doi.org/10.1021/acs.jmedchem.0c00222>.
- Medrano FJ, Andreu JM, Gorbunoff MJ, Timasheff SN. Roles of Colchicine Rings B and C in the Binding Process to Tubulin. *Biochemistry*. 1989;28(13):5589–5599. <https://doi.org/10.1021/bi00439a038>.
- Banerjee A, Kasmala LT, Hamel E, Sun L, Lee KH. Interaction of novel thiocolchicine analogs with the tubulin isoforms from bovine brain. *Biochem Biophys Res Commun*. 1999;254(2):334–337. <https://doi.org/10.1006/bbrc.1998.9943>.
- Cacelli I, D'Auria M, Villani V. Why thiocolchicine does not undergo photochemical isomerization: A theoretical study. *Chem Phys Lett*. 2008;459(1–6):167–171. <https://doi.org/10.1016/j.cplett.2008.05.048>.
- Bussotti L, D'Auria M, Foggi P, Lesma G, Righini R, Silvani A. The Photochemical Behavior of Colchicine and Thiocolchicine. *Photochem Photobiol*. 2000;71(1):29. [https://doi.org/10.1562/0031-8655\(2000\)071<0029:tpboca>2.0.co;2](https://doi.org/10.1562/0031-8655(2000)071<0029:tpboca>2.0.co;2).
- Prajapati PB, Bodiwala KB, Marolia BP, Bhingradiya N, Shah S. Oxidative Degradation Kinetic Study of Thiocolchicine using Stability Indicating High Performance Thin Layer Chromatographic Method. *Pharm Methods*. 2014;5(2):69–78. <https://doi.org/10.5530/phm.2014.2.5>.
- Czerwonka D, Sobczak S, Pedziński T, et al. Photoinduced skeletal rearrangement of n-substituted colchicine derivatives. *J Org Chem*. 2021;86(16):11029–11039. <https://doi.org/10.1021/acs.joc.0c02507>.
- Pallante L, Rocca A, Klejborowska G, et al. In silico Investigations of the Mode of Action of Novel Colchicine Derivatives Targeting β -Tubulin Isoforms: A Search for a Selective and Specific β -III Tubulin Ligand. *Front Chem*. 2020;8. <https://doi.org/10.3389/fchem.2020.00108>.
- Kozaka T, Nakagawa-Goto K, Shi Q, et al. Antitumor agents 273. Design and synthesis of N-alkyl-thiocolchicinoids as potential antitumor agents. *Bioorganic Med Chem Lett*. 2010;20(14):4091–4094. <https://doi.org/10.1016/j.bmcl.2010.05.081>.
- Majcher U, Klejborowska G, Kaik M, et al. Synthesis and Biological Evaluation of Novel Triple-Modified Colchicine Derivatives as Potent Tubulin-Targeting Anticancer Agents. *Cells*. 2018;7(11):216. <https://doi.org/10.3390/cells7110216>.
- Majcher U, Klejborowska G, Moshari M, et al. Antiproliferative activity and molecular docking of novel double-modified colchicine derivatives. *Cells*. 2018;7(11):192. <https://doi.org/10.3390/cells7110192>.
- Bartusik D, Tomanek B, Lattová E, Perreault H, Tuszynski J, Fallone G. Derivatives of thiocolchicine and its applications to CEM cells treatment using 19F Magnetic Resonance ex vivo. *Bioorg Chem*. 2010;38(1):1–6. <https://doi.org/10.1016/j.bioorg.2009.10.002>.
- Czerwonka D, Sobczak S, Maj E, Wietrzyk J, Katrusiak A, Huczynski A. Synthesis and Antiproliferative Screening Of Novel Analogs of Regioselectively Demethylated Colchicine and Thiocolchicine. *Mol*. 2020, Vol 25, Page 1180. 2020;25(5):1180. doi: 10.3390/MOLECULES25051180.
- Kerekes P, Sharma PN, Brossi A, Chignell CF, Quinn FR. Synthesis and Biological Effects of Novel Thiocolchicines. 3. Evaluation of N-Acyldeacetylthiocolchicines, N-(Alkoxyacetyl)deacetylthiocolchicines, and O-Ethyldeacetylthiocolchicines. New Synthesis of Thiocolchicine and Antileukemic Effects of 2-Demeth. *J Med Chem*. 1985;28(9):1204–1208. <https://doi.org/10.1021/jm00147a014>.
- Shi Q, Verdier-Pinard P, Brossi A, Hamel E, Lee KH. Antitumor Agents-CLXXV. Antitubulin action of (+)-thiocolchicine prepared by partial synthesis. *Bioorganic Med Chem*. 1997;5(12):2277–2282. doi:10.1016/S0968-0896(97)00171-5.
- Urbaniak Alicja, Jousheghany Fariba, Piña-Oviedo Sergio, et al. Carbamate derivatives of colchicine show potent activity towards primary acute lymphoblastic leukemia and primary breast cancer cells—in vitro and ex vivo study. *J Biochem Mol Toxicol*. 2020;34(6). <https://doi.org/10.1002/jbt.v34.6>.
- Sun Li, Hamel Ernest, Lin Chii M, Hastie Susan B, Pyluck Amy, Lee Kuo Hsiung. 141. Synthesis and biological evaluation of novel thiocolchicine analogs: N-acyl, N-aryl-, and N-(substituted benzyl)deacetylthiocolchicines as potent cytotoxic and antimetabolic compounds. *J Med Chem*. 1993;36(10):1474–1479. <https://doi.org/10.1021/jm00062a021>.
- Molinspiration Property Calculation Service [Http://Www.Molinspiration.Com]. <https://www.molinspiration.com/services/index.html>. Accessed November 14, 2019.
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv Drug Deliv Rev*. 1997;23:3–25. Accessed April 23, 2017.
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001;46(1–3):3–26. [https://doi.org/10.1016/S0169-409X\(00\)00129-0](https://doi.org/10.1016/S0169-409X(00)00129-0).
- Lipinski C. Lead-and drug-like compounds: the rule-of-five revolution. *Drug Discov Today Technol*. 2004. Accessed April 23, 2017.

Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity

Dominika Czerwonka ¹, Ewa Maj ², Joanna Wietrzyk ² and Adam Huczyński ^{1*}

¹ Department of Medical Chemistry, Faculty of Chemistry, Adam Mickiewicz University, Uniwersytetu Poznańskiego 8, 61-614 Poznań, Poland; dominika.czerwonka@amu.edu.pl;

² Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland; ewa.maj@hirsfeld.pl (E.M.); joanna.wietrzyk@hirsfeld.pl (J.W.);

* Correspondence: adhucz@amu.edu.pl

Supplementary Materials

Index

General precedures.....	2
Synthetic procedures	3
Synthesis and characterization of thiocolchicine	3
Synthesis and characterization of <i>N</i> -deacetylthiocolchicine.....	3
General procedures for obtaining compounds 1 – 22	3
The antiproliferative activity.....	3
The Antiproliferative Assays In Vitro	4
SRB	4
¹ H and ¹³ C NMR spectra of compounds 1-22	5
Figure S1 The ¹³ C NMR spectrum of 1 in CDCl ₃	6
Figure S2 The ¹ H NMR spectrum of 1 in CDCl ₃	7
Figure S3 The ¹³ C NMR spectrum of 2 in CDCl ₃	9
Figure S4 The ¹ H NMR spectrum of 2 in CDCl ₃	10
Figure S5 The ¹³ C NMR spectrum of 3 in CDCl ₃	12
Figure S6 The ¹ H NMR spectrum of 3 in CDCl ₃	13
Figure S7 The ¹³ C NMR spectrum of 4 in CDCl ₃	15
Figure S8 The ¹ H NMR spectrum of 4 in CDCl ₃	16
Figure S9 The ¹³ C NMR spectrum of 5 in CDCl ₃	18
Figure S10 The ¹ H NMR spectrum of 5 in CDCl ₃	19
Figure S11 The ¹³ C NMR spectrum of 6 in CDCl ₃	21
Figure S12 The ¹ H NMR spectrum of 6 in CDCl ₃	22
Figure S13 The ¹³ C NMR spectrum of 7 in CDCl ₃	24
Figure S14 The ¹ H NMR spectrum of 7 in CDCl ₃	25
Figure S15 The ¹³ C NMR spectrum of 8 in CDCl ₃	27
Figure S16 The ¹ H NMR spectrum of 8 in CDCl ₃	28
Figure S17 The ¹³ C NMR spectrum of 9 in CDCl ₃	30
Figure S18 The ¹ H NMR spectrum of 9 in CDCl ₃	31
Figure S19 The ¹³ C NMR spectrum of 10 in CDCl ₃	33
Figure S20 The ¹ H NMR spectrum of 10 in CDCl ₃	34
Figure S21 The ¹³ C NMR spectrum of 11 in CDCl ₃	36
Figure S22 The ¹ H NMR spectrum of 11 in CDCl ₃	37
Figure S23 The ¹³ C NMR spectrum of 12 in CDCl ₃	39
Figure S24 The ¹ H NMR spectrum of 12 in CDCl ₃	40
Figure S25 The ¹³ C NMR spectrum of 13 in CDCl ₃	42

Figure S26 The ^1H NMR spectrum of 13 in CDCl_3 .	43
Figure S27 The ^{13}C NMR spectrum of 14 in CDCl_3 .	45
Figure S28 The ^1H NMR spectrum of 14 in CDCl_3 .	46
Figure S29 The ^{13}C NMR spectrum of 15 in CDCl_3 .	48
Figure S30 The ^1H NMR spectrum of 15 in CDCl_3 .	49
Figure S31 The ^{13}C NMR spectrum of 16 in CDCl_3 .	51
Figure S32 The ^1H NMR spectrum of 16 in CDCl_3 .	52
Figure S33 The ^{13}C NMR spectrum of 17 in CDCl_3 .	54
Figure S34 The ^1H NMR spectrum of 17 in CDCl_3 .	55
Figure S35 The ^{13}C NMR spectrum of 18 in CDCl_3 .	57
Figure S36 The ^1H NMR spectrum of 18 in CDCl_3 .	58
Figure S37 The ^{13}C NMR spectrum of 19 in CDCl_3 .	60
Figure S38 The ^1H NMR spectrum of 19 in CDCl_3 .	61
Figure S39 The ^{13}C NMR spectrum of 20 in CDCl_3 .	63
Figure S40 The ^1H NMR spectrum of 20 in CDCl_3 .	64
Figure S41 The ^{13}C NMR spectrum of 21 in CDCl_3 .	66
Figure S42 The ^1H NMR spectrum of 21 in CDCl_3 .	68
Figure S43 The ^{13}C NMR spectrum of 22 in CDCl_3 .	69
Figure S44 The ^1H NMR spectrum of 22 in CDCl_3 .	70

General procedures

All precursors for the synthesis of colchicine derivatives and solvents were obtained from TriMen Chemicals, Merck or Fluka and were used as received without further purification. CDCl_3 spectral-grade solvent was stored over 3 Å molecular sieves for several days. All the solvents used in flash chromatography were of HPLC grade (CHROMASOLV from Merck) and were used as received. Reaction mixtures were stirred using Teflon-coated magnetic stir bars. Reactions were monitored by thin layer chromatography (TLC) using aluminum-backed plates (Merck 60F254). TLC plates were visualized in UV-light (254 nm). The ^1H , ^{13}C spectra were recorded on a Varian VNMR-S 400 MHz spectrometer using TMS as the internal standard in both cases. No window function or zero filling was used. ^1H NMR measurements of 1-14 (0.07 mol dm^{-3}) in CDCl_3 were carried out at the operating frequency 402.64 MHz. The error of the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at the operating frequency 101.25 MHz. The error of chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of CDCl_3 . The ESI (Electrospray Ionisation) mass spectra were recorded on a Waters/Micromass (Waters Corporation, Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples were prepared in dry acetonitrile ($5 \times 10^{-5} \text{ mol dm}^{-3}$). The sample was infused into the ESI source using a Harvard pump at a flow rate of 20 ml min^{-1} . The ESI source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages: 10 and 30 V. The source temperature was 120°C and the desolvation temperature was 300°C . Nitrogen was used as the nebulizing and desolvation gas at flow-rates of $100 \text{ dm}^3 \text{ h}^{-1}$. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from $m/z = 100$ to $m/z = 1000$, as well as from $m/z = 200$ to $m/z = 1500$.

Synthetic procedures

Synthesis and characterization of thiocolchicine

To a mixture of colchicine (500 mg, 1.25 mmol) in MeOH/water (1/1, v/v, 5 mL), the sodium methanethiolate (solution 21% in H₂O, 0.83 mL, 2.5 mmol) was added. The mixture was stirred in at room temperature for 24 h. Reaction was controlled by TLC. After that time, the reaction mixture was quenched by the addition of water (150 mL). The whole mixture was extracted four times with CH₂Cl₂, and the combined organic layers were dried over MgSO₄, filtered, and evaporated under reduced pressure. The residue was purified by CombiFlash® (EtOAc/MeOH, increasing concentration gradient) to give thiocolchicine as amorphous yellow solid with yield 78%.

¹H NMR (403 MHz, CDCl₃) δ 7.92 (s, 1H), 7.46 (s, 1H), 7.36 – 7.26 (m, 1H), 7.10 (d, *J* = 10.5 Hz, 1H), 6.55 (s, 1H), 4.73 – 4.63 (m, 1H), 3.95 (s, 3H), 3.91 (s, 3H), 3.67 (s, 3H), 2.54 (dd, *J* = 13.0, 5.8 Hz, 1H), 2.45 (s, 3H), 2.33 (dtd, *J* = 31.1, 12.8, 6.5 Hz, 2H), 1.99 (s, 3H), 1.96 – 1.85 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 182.4, 170.0, 158.1, 153.6, 151.8, 151.1, 141.6, 138.6, 134.8, 134.4, 128.3, 126.7, 125.6, 107.3, 61.6, 61.4, 56.1, 52.3, 36.4, 29.9, 22.8, 15.1. ESI-MS (*m/z*): [M+H]⁺ 416, [M+Na]⁺ 438, [M+K]⁺ 454, [2M+Na]⁺ 853, [3M+Na]⁺ 1268.

Synthesis and characterization of *N*-deacetylthiocolchicine

To the solution of thiocolchicine (500 mg, 1.18 mmol) in MeOH (3 mL) 4 N HCl solution was added (5 mL). The mixture was stirred at 90°C for 72 h. Reaction progress was determined by TLC. Subsequently the solvent was evaporated under reduced pressure. The residue was purified by CombiFlash (EtOAc/MeOH, increasing concentration gradient)) to give *N*-deacetylthiocolchicine with yield 82%.

¹H NMR (401 MHz, DMSO-*d*₆): δ 8.78 (s, 2H), 7.15 (d, *J* = 11.2 Hz, 1H), 7.05 (s, 1H), 6.98 (d, *J* = 11.2 Hz, 1H), 6.79 (s, 1H), 3.83 (s, 3H), 3.76 (s, 3H), 3.55 (s, 3H), 2.65–2.57 (m, 2H), 2.41–2.31 (m, 1H), 2.27 – 2.13 (m, 1H), 2.04–1.94 (m, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 173.9, 157.3, 152.7, 150.2, 143.0, 140.8, 138.9, 133.6, 127.8, 125.8, 123.0, 110.5, 107.6, 60.5, 55.9, 52.8, 35.7, 30.7, 28.8. ESI-MS (*m/z*): [M+H]⁺ 343, [M+Na]⁺ 356.

General procedures for obtaining compounds 1 – 22

To the stirred solution of *N*-deacetylthiocolchicine (100 mg, 0.26 mmol, 1.0 equiv.) in 5 mL of CH₂Cl₂ the corresponding aldehyde was added (1.00 mmol, 4.0 equiv.). The solution was stirred for 24h at room temperature, and after this time the solution of NaBH₃CN (20 mg, 0.32 mmol, 1.2 equiv.) in 2 mL of MeOH was added dropwise. The reaction mixture was further stirred for additional 30 minutes. After that, the solvent was evaporated under reduced pressure. Purification of the filtrated residue on silica gel using the CombiFlash system (hexane/EtOAc, increasing concentration gradient) gave the corresponding products of the reactions as the white amorphous solids.

The antiproliferative activity

Four human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of colchicine and its derivatives: human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line sensitive and resistant to doxorubicin (LoVo and LoVo/DX, respectively), and normal murine embryonic fibroblast cell line (BALB/3T3). The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), A549 and MCF-7 cell lines—from European Collection of Authenticated Cell Cultures (Salisbury, UK), LoVo cell line was purchased from the ATCC (ATCC, Manassas, VA, USA) and LoVo/DX was a kind gift from Professor E. Borowski (Medical University of Gdańsk, Gdańsk, Poland). All the cell lines are maintained in the Institute of Immunology and Experimental Therapy (IIET), Wrocław, Poland.

5

Human lung adenocarcinoma cell line was cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wrocław, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM L-glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human breast adenocarcinoma cell line was cultured in mixture of Eagle medium (IET, Wrocław, Poland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 8 µg/mL insulin and 1% amino-acids (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human colon adenocarcinoma cell line was cultured in F-12K medium with L-glutamine (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (GE Healthcare, Logan UT, USA), and medium for LoVo/DX additionally contained 10 µg/100 mL doxorubicin (Accord Healthcare Limited, Middlesex, UK). Murine embryonic fibroblast cells were cultured in Dulbecco medium (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). All culture media contained antibiotics: 100 U/mL penicillin and 100 µg/mL streptomycin (Polfa-Tarchomin, Warsaw, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO₂. Cells were tested for mycoplasma contamination by mycoplasma detection kit for conventional PCR: Venor GeM Classic (Minerva Biolabs GmbH, Berlin, Germany) and negative results was obtained. The procedure is repeated every year or in the case of less frequently used lines: after thawing.

The Antiproliferative Assays In Vitro

Twenty-four hours before adding the tested compounds, all cell lines were seeded in 384-well plates (Sarstedt, Nümbrecht, Germany) in appropriate media with 1×10^3 or $1,5 \times 10^3$ or 3×10^3 cells per well. All cell lines were exposed to each tested agent at different concentrations in the range 100–0.001 µg/mL or lower for 72 h. Cells were also exposed to the reference drug cisplatin (Teva Pharmaceuticals Polska, Warsaw, Poland) and doxorubicin (Accord Healthcare Limited, Middlesex, UK). Additionally, all cell lines were exposed to DMSO (solvent used for tested compounds) (POCh, Gliwice, Poland) at concentrations corresponding to those present in tested agents' dilutions. After 72 h sulforhodamine B assay (SRB) was performed.¹

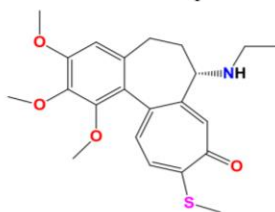
SRB

After 72 h of incubation with the tested compounds, cells were fixed in situ by gently adding of 30 µL per well of cold 50% trichloroacetic acid TCA (POCh, Gliwice, Poland) and were incubated at 4 °C for one hour. Following, wells were washed four times with water and air dried. Next, 25 µL of 0.1% solution of sulforhodamine B (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA) in 1% acetic acid (POCh, Gliwice, Poland) were added to each well and plates were incubated at room temperature for 0.5 h. After incubation time, unbound dye was removed by washing plates four times with 1% acetic acid whereas stain bound to cells was solubilized with 10 mM Tris base (Sigma-Aldrich, Steinheim, Germany). Absorbance of each solution was read at Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) at the 540 nm wavelength.

Results are presented as mean IC₅₀ (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC₅₀ values were calculated in Cheburator 0.4, Dmitry Nevozhay software (version 1.2.0 software by Dmitry Nevozhay, 2004–2014, <http://www.cheburator.nevozhay.com>, freely available) for each experiment.² Compounds at each concentration were tested in triplicates in single experiment and each experiment was repeated at least three times independently

¹H and ¹³C NMR spectra of compounds 1-22

1



Chemical Formula: C₂₂H₂₇NO₄S
Molecular Weight: 401,52

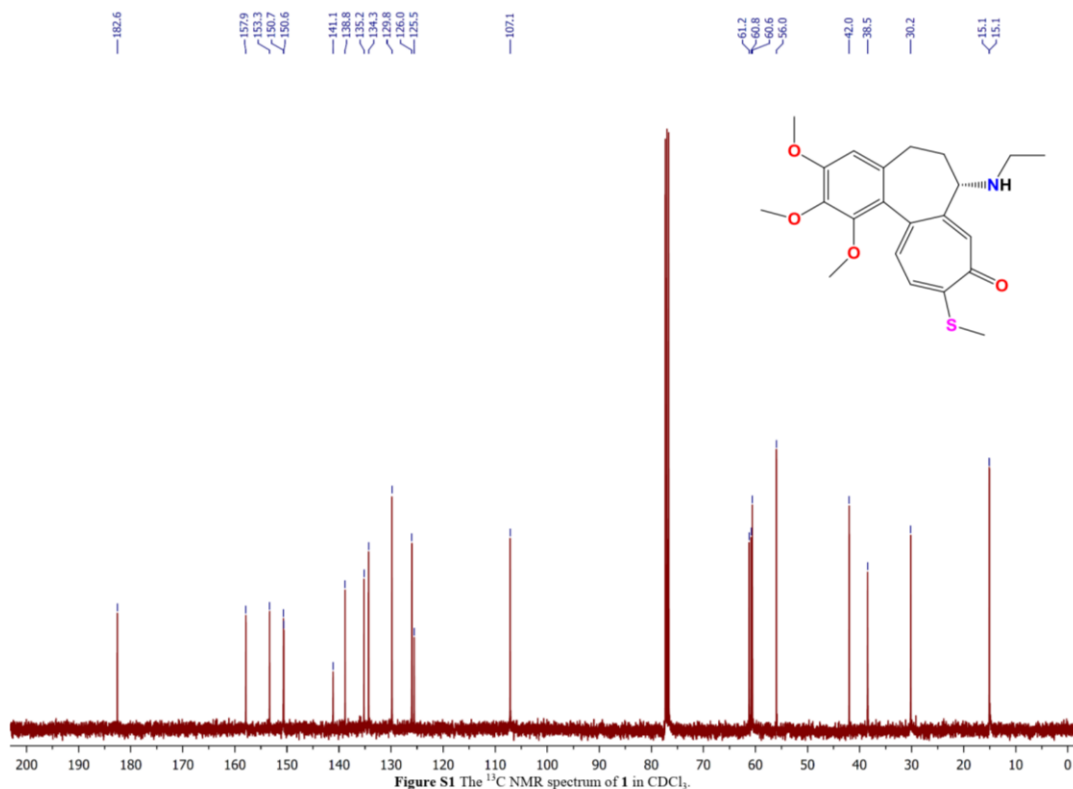
Yield: 57.6 %

¹³C NMR (101 MHz, CDCl₃) δ 182.6, 157.9, 153.3, 150.7, 150.6, 141.1, 138.3, 135.2, 134.3, 129.8, 126.0, 125.6, 107.1, 61.2, 60.8, 60.6, 56.0, 42.0, 38.5, 30.2, 15.10, 15.07 ppm.

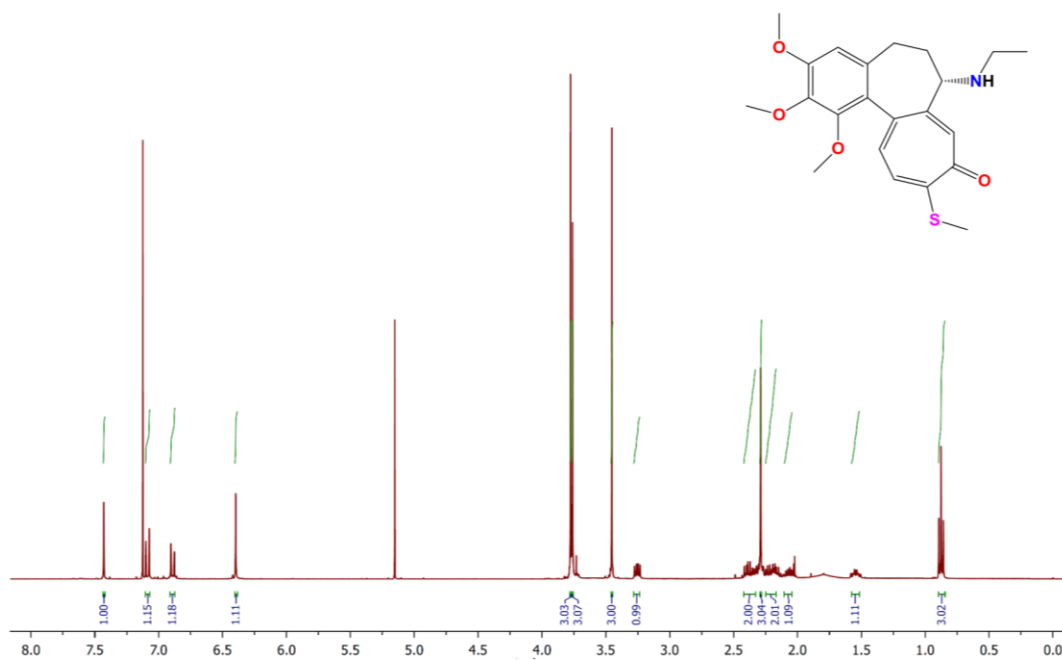
¹H NMR (400 MHz, CDCl₃) δ 7.57 (s, 1H), 7.22 (dd, *J* = 10.2, 0.5 Hz, 1H), 7.03 (dd, *J* = 10.3, 0.7 Hz, 1H), 6.53 (s, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 3.59 (s, 3H), 3.44 – 3.34 (m, 2H), 2.58 – 2.48 (m, 1H), 2.50 – 2.44 (m, 1H), 2.42 (s, 3H), 2.38 (dd, *J* = 13.1, 7.0 Hz, 1H), 2.31 (ddd, *J* = 11.2, 7.0, 3.6 Hz, 1H), 2.24 – 2.15 (m, 1H), 1.01 (t, *J* = 7.1 Hz, 3H) ppm.

ESI-MS (*m/z*): [M+H]⁺ 402.

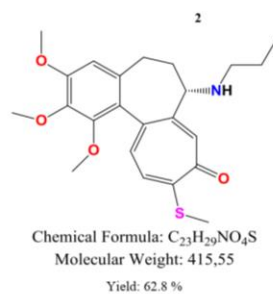
5



6



7

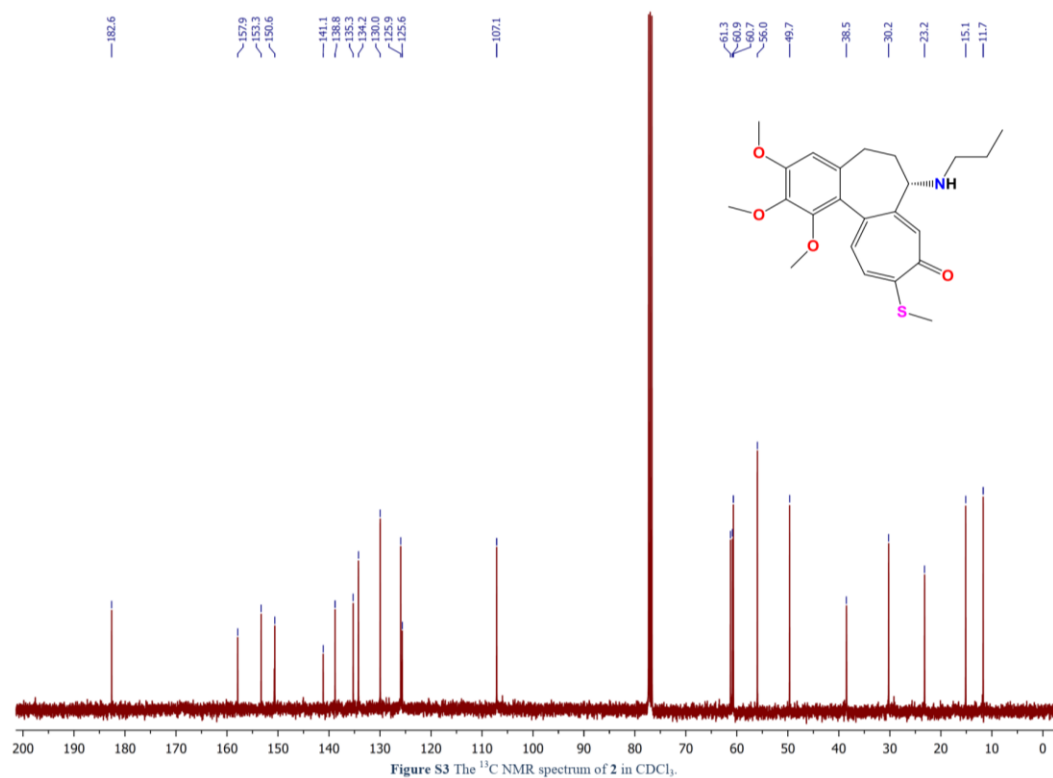


^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 157.9, 153.3, 150.7, 150.6, 141.1, 138.8, 135.3, 134.2, 130.0, 125.9, 125.6, 107.1, 61.3, 60.9, 60.7, 56.0, 49.7, 38.5, 30.2, 23.2, 15.1, 11.7 ppm.

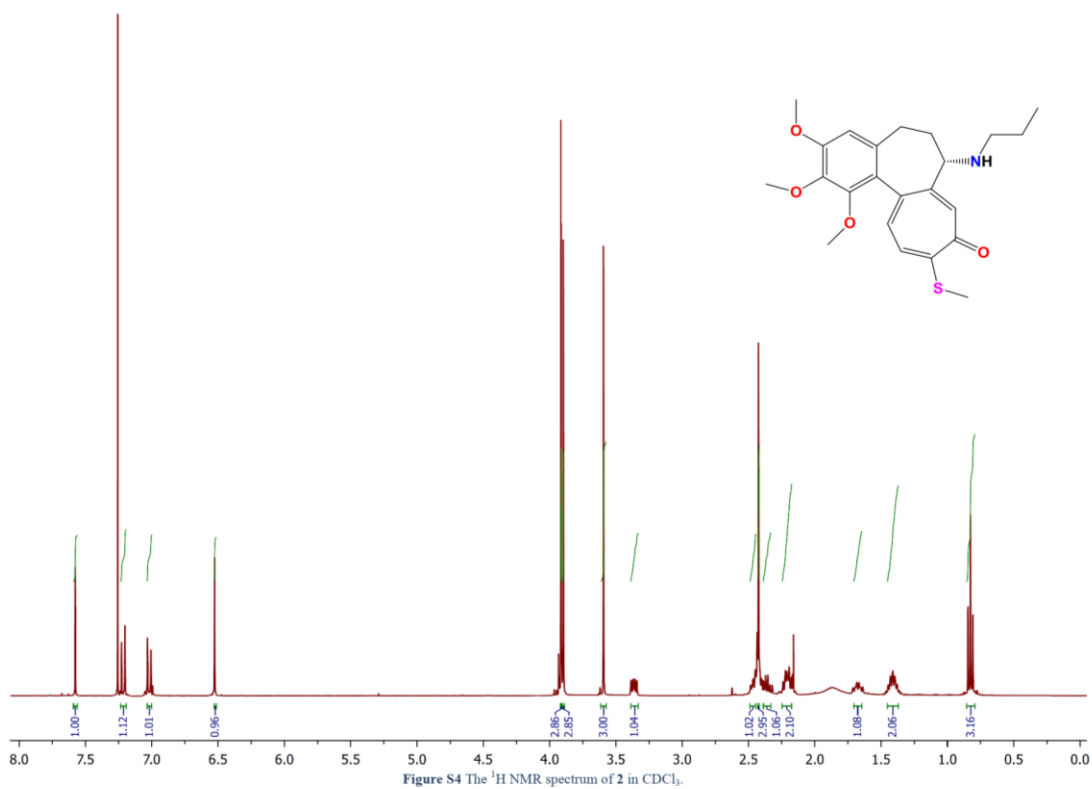
^1H NMR (400 MHz, CDCl_3) δ 7.58 (s, 1H), 7.22 (d, $J = 10.2$ Hz, 1H), 7.02 (d, $J = 10.3$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.37 (ddd, $J = 11.1, 6.3, 1.3$ Hz, 2H), 2.43 (s, 3H), 2.37 (td, $J = 13.2, 12.7, 6.5$ Hz, 2H), 2.25 – 2.17 (m, 2H), 1.68 (td, $J = 11.4, 6.6$ Hz, 1H), 1.49 – 1.37 (m, 2H), 0.83 (t, $J = 7.4$ Hz, 3H) ppm.

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 416, $[\text{M}+\text{Na}]^+$ 438.

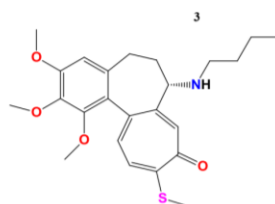
8



9



10



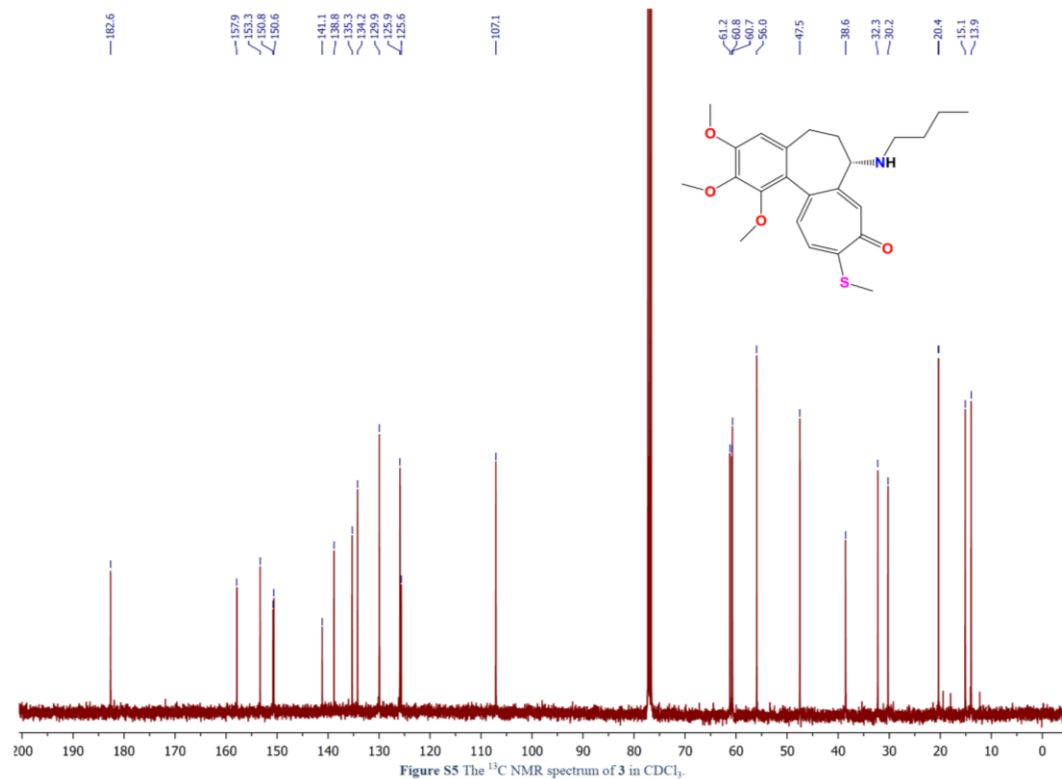
Chemical Formula: $C_{24}H_{31}NO_4S$
Molecular Weight: 429.57

Yield: 67.7 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 157.9, 153.3, 150.8, 150.6, 141.1, 138.8, 135.3, 134.2, 130.0, 125.9, 125.6, 107.1, 61.2, 60.8, 60.7, 56.0, 47.5, 38.6, 32.3, 30.2, 20.4, 15.1, 13.9 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.57 (s, 1H), 7.21 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 11.0$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.36 (dd, $J = 10.9, 6.2$ Hz, 2H), 2.50–2.44 (m, 1H), 2.43 (s, 3H), 2.36 (m, 1H), 2.27–2.13 (m, 2H), 1.68 (dd, $J = 11.5, 6.5$ Hz, 2H), 1.44–1.32 (m, 2H), 1.30–1.19 (m, 2H), 0.97–0.92 (m, 1H), 0.82 (t, $J = 7.2$ Hz, 3H) ppm.

ESI-MS (m/z): $[M+H]^+$ 430.



11

12

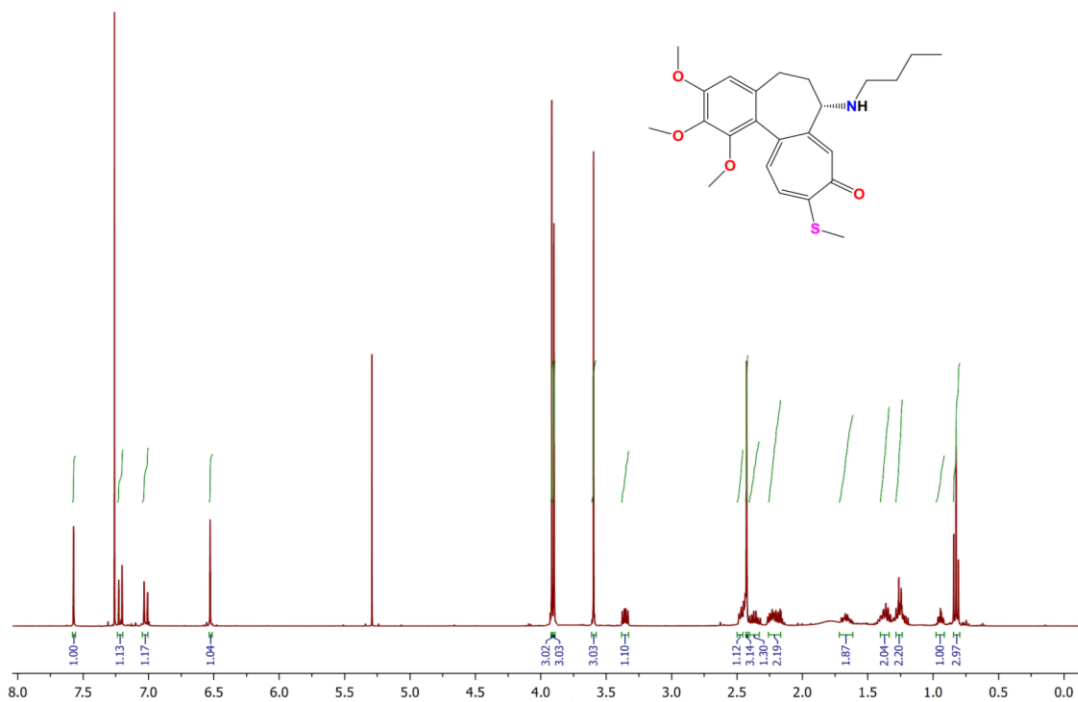
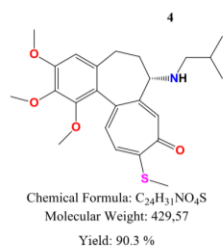


Figure S6 The ^1H NMR spectrum of **3** in CDCl_3 .

13

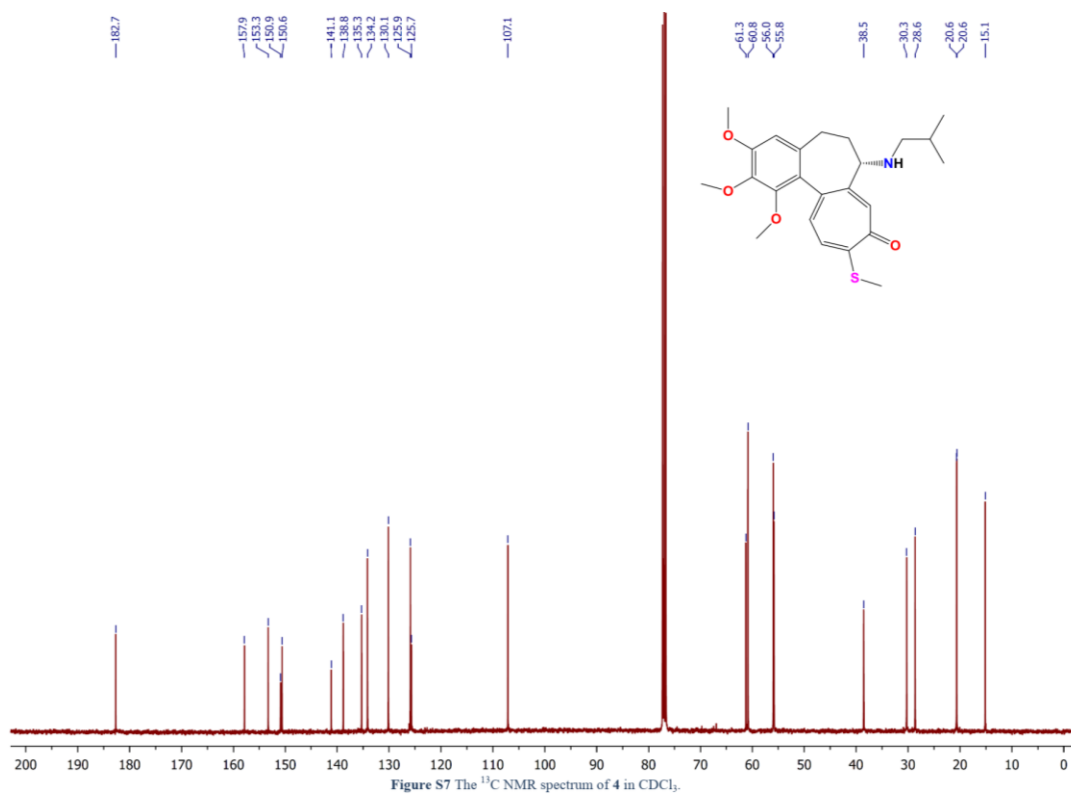


^{13}C NMR (101 MHz, CDCl_3) δ 182.7 157.8, 153.3, 150.9, 150.6, 141.1, 138.8, 135.3, 134.2, 130.1, 125.9, 125.7, 107.1, 61.3, 60.8*, 60.8*, 56.0, 55.8, 38.5, 30.3, 28.6, 20.64, 20.61, 15.1 ppm.

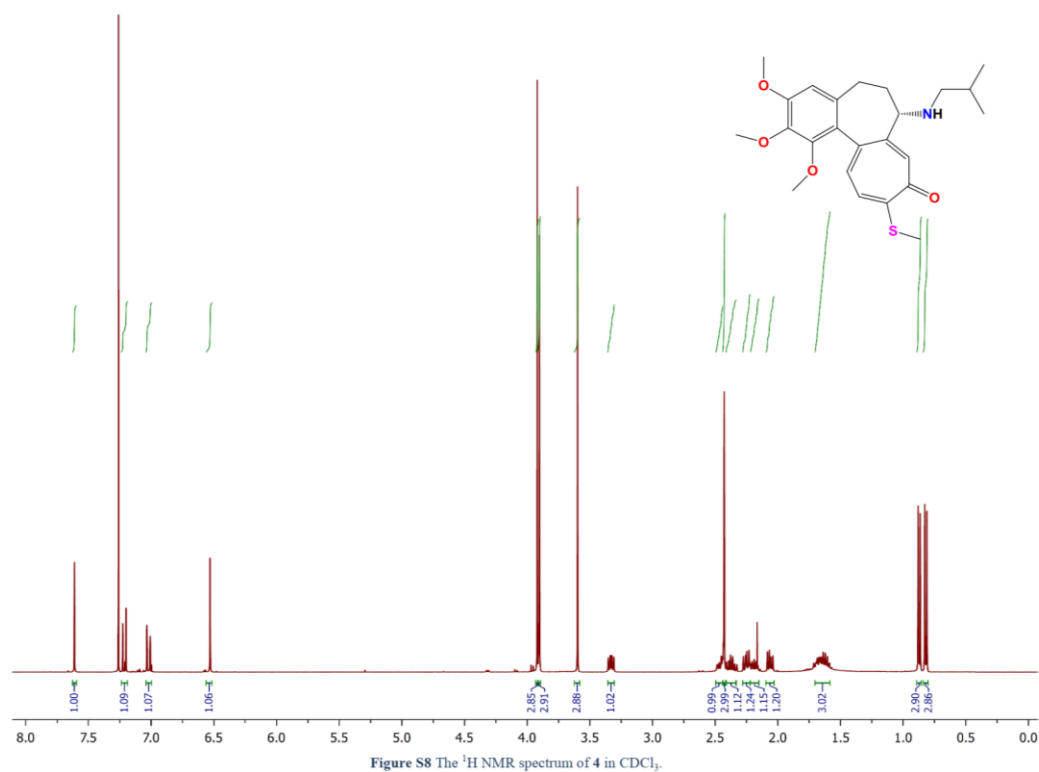
^1H NMR (400 MHz, CDCl_3) δ 7.61 (s, 1H), 7.21 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 10.3$ Hz, 1H), 6.53 (s, 1H), 3.92 (d, $J = 0.4$ Hz, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.33 (dd, $J = 10.9, 6.2$ Hz, 2H), 2.50 – 2.46 (m, 1H), 2.43 (s, 3H), 2.37 (dd, $J = 13.2, 6.2$ Hz, 1H), 2.25 (dd, $J = 11.2, 7.0$ Hz, 1H), 2.22 – 2.15 (m, 1H), 2.06 (dd, $J = 11.2, 6.4$ Hz, 1H), 1.72 – 1.58 (m, 3H), 0.87 (d, $J = 6.6$ Hz, 3H), 0.82 (d, $J = 6.6$ Hz, 3H) ppm.

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 430, $[\text{M}+\text{Na}]^+$ 452.

14

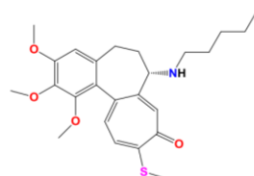


15



5

16



Chemical Formula: $C_{25}H_{33}NO_4S$

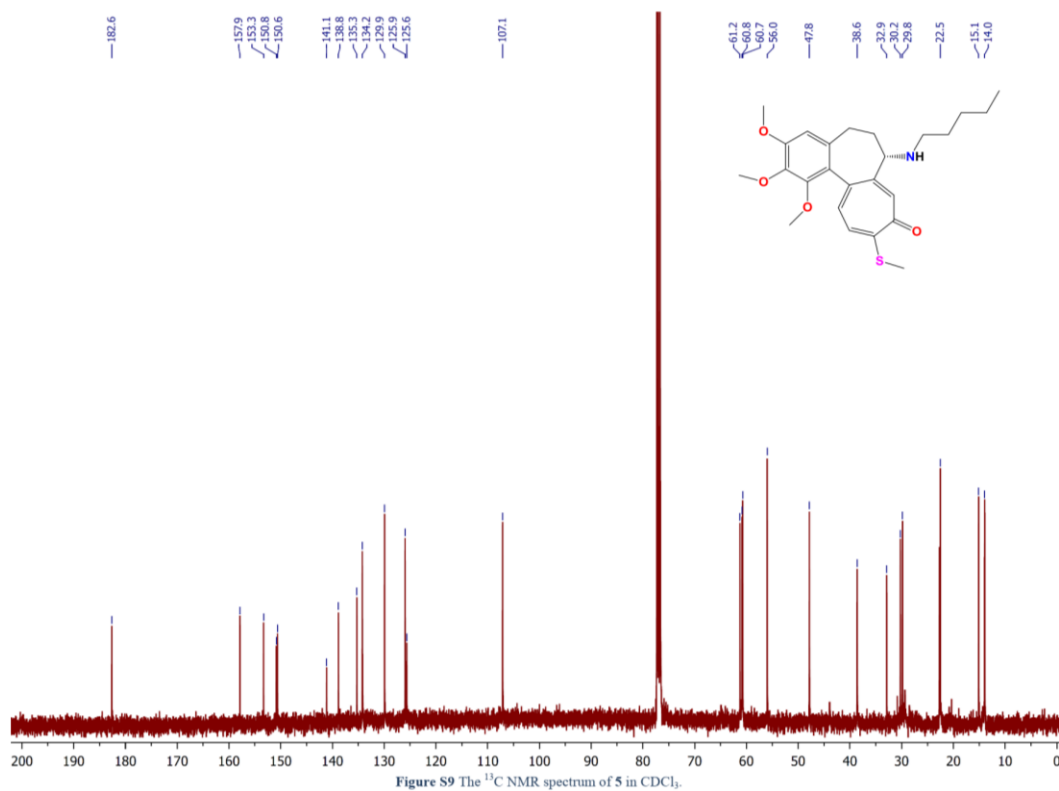
Molecular Weight: 443,60

Yield: 40.3 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 157.9, 153.3, 150.8, 150.6, 141.1, 138.9, 135.3, 134.2, 129.9, 125.9, 125.6, 107.1, 61.3, 60.9, 60.7, 56.0, 47.8, 38.6, 32.9, 30.3, 29.8, 22.5, 15.1, 14.0 ppm.

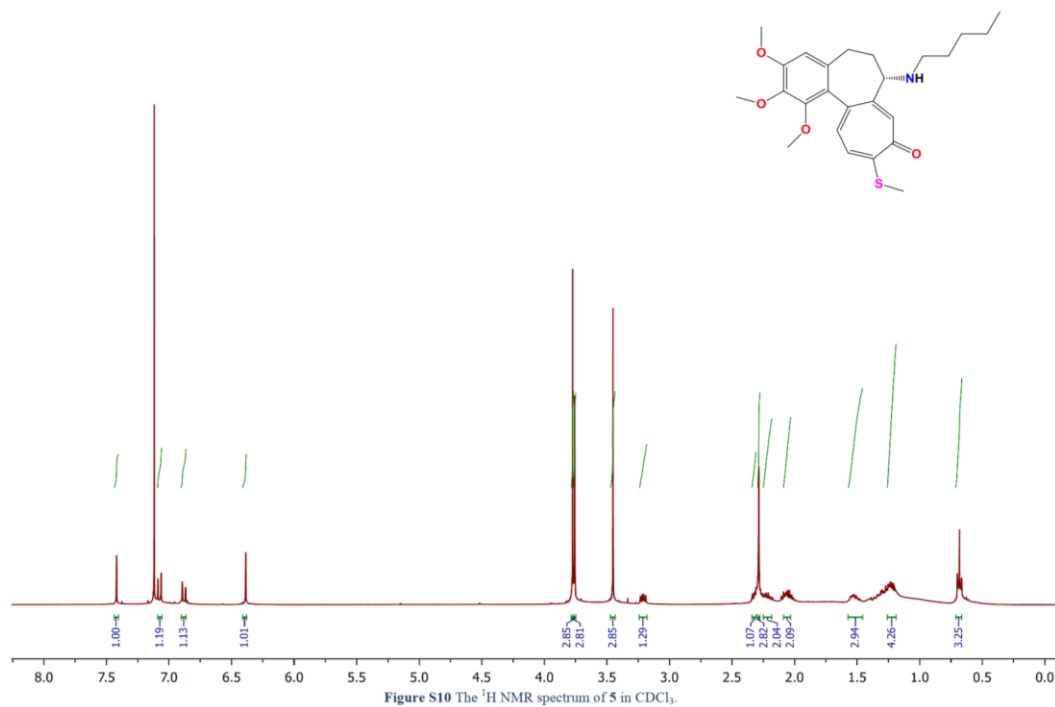
1H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 11.0$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.59 (s, 3H), 3.35 (dd, $J = 10.9, 6.2$ Hz, 2H), 2.43 (s, 3H), 2.35 (dt, $J = 13.2, 6.4$ Hz, 1H), 2.28 – 2.13 (m, 3H), 1.72 – 1.61 (m, 2H), 1.52 – 1.17 (m, 3H), 0.96 – 0.88 (m, 2H), 0.68 (t, $J = 6.9$ Hz, 3H) ppm.

ESI-MS (m/z): $[M+H]^+$ 444.

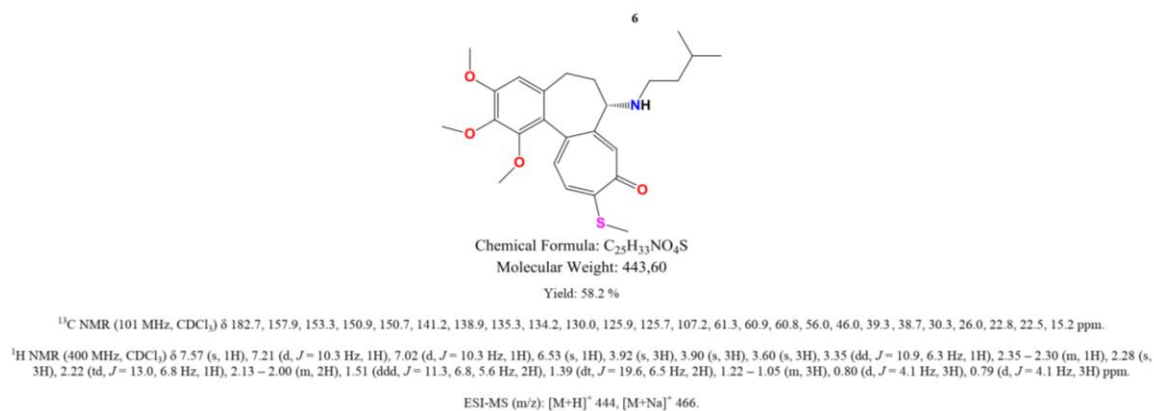


17

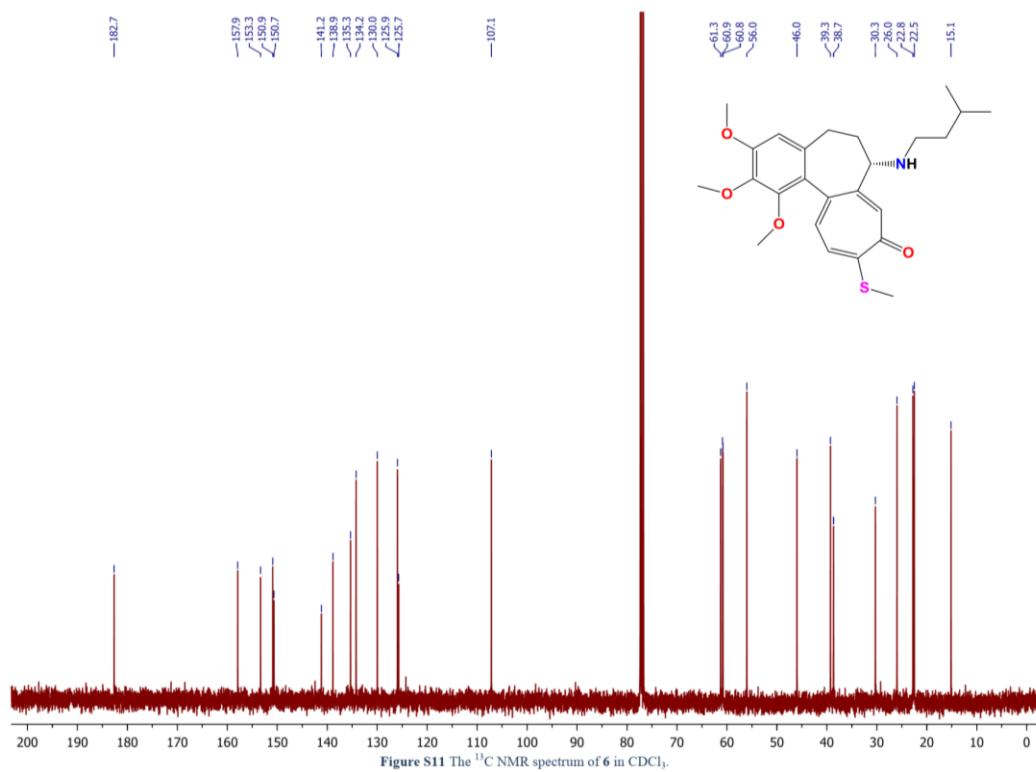
18



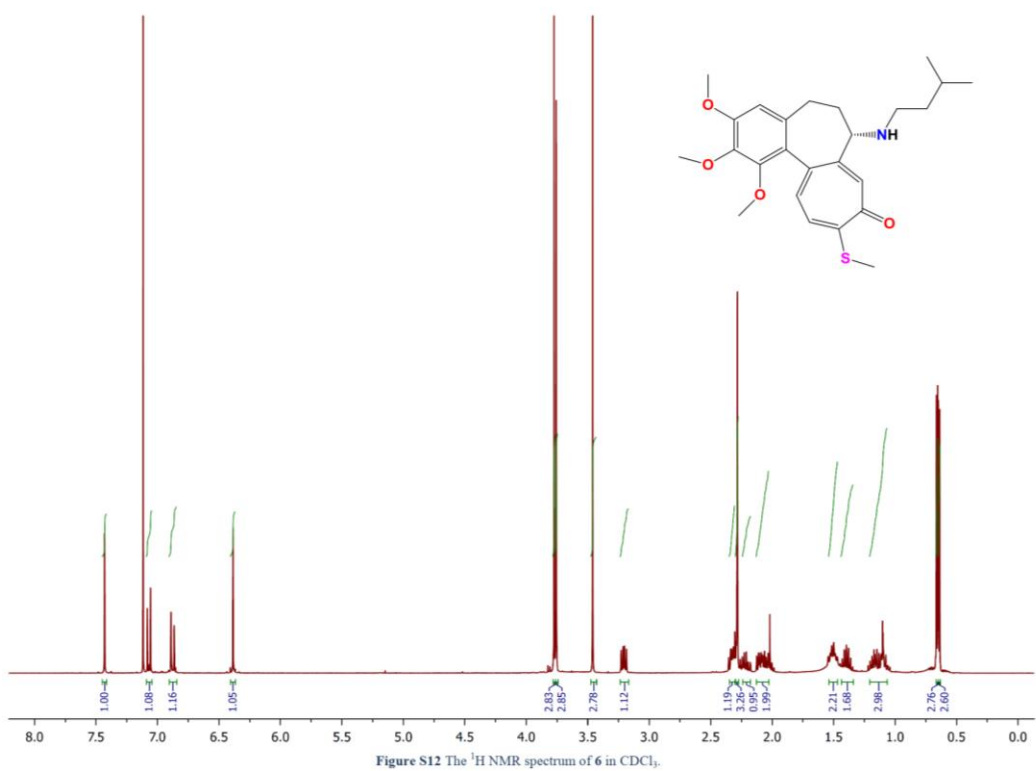
19



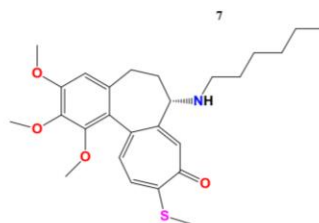
20



21



22



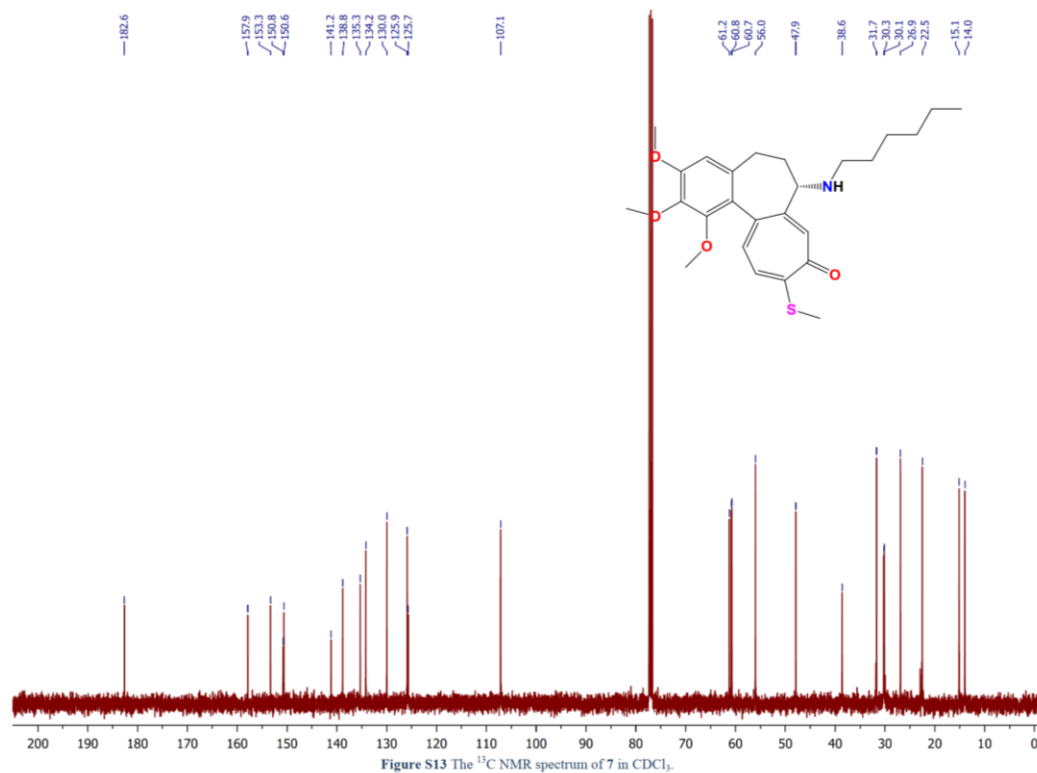
Chemical Formula: $C_{26}H_{35}NO_4S$
Molecular Weight: 457.63

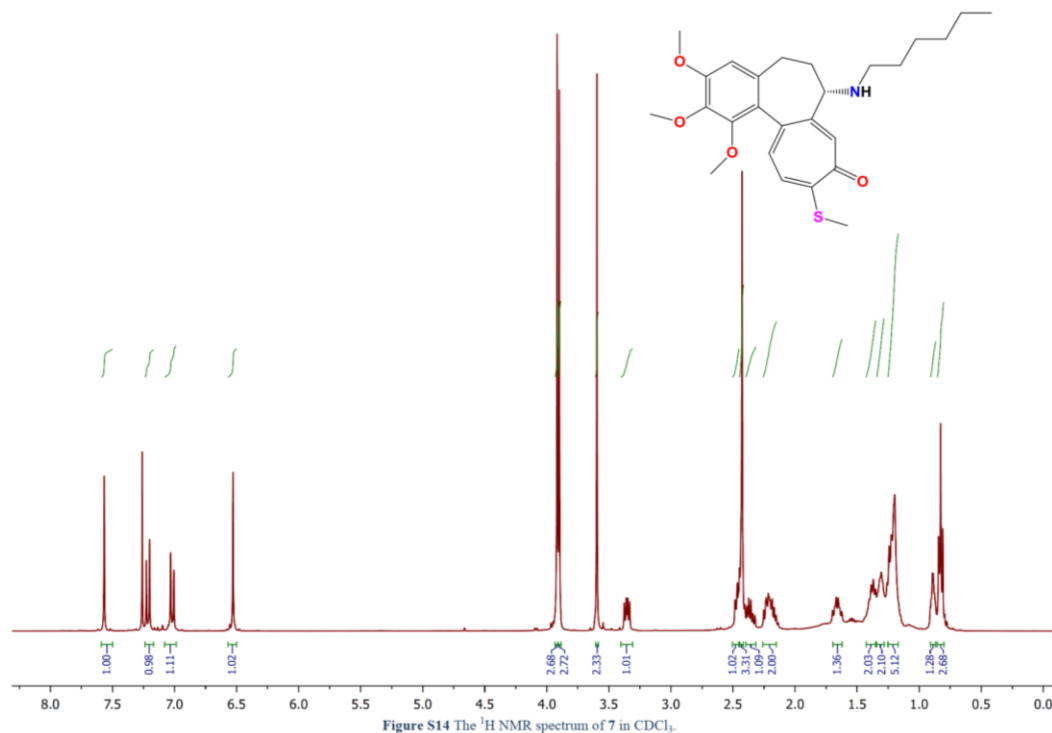
Yield: 60.4 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 157.9, 153.3, 150.8, 150.6, 141.2, 138.8, 135.3, 134.2, 130.0, 125.9, 125.7, 107.1, 61.3, 60.9, 60.7, 56.0, 47.9, 38.6, 31.7, 30.3, 30.1, 26.9, 22.5, 15.1, 14.0 ppm.

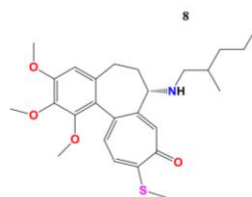
1H NMR (400 MHz, $CDCl_3$) δ 7.57 (s, 1H), 7.21 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 10.7$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.35 (dd, $J = 10.9, 6.3$ Hz, 2H), 2.50 – 2.44 (m, 1H), 2.43 (s, 3H), 2.36 (td, $J = 13.0, 6.8$ Hz, 1H), 2.26 – 2.12 (m, 2H), 1.71 – 1.61 (m, 2H), 1.42 – 1.35 (m, 2H), 1.34 – 1.28 (m, 2H), 1.26 – 1.16 (m, 5H), 1.43 – 1.14 (m, 5H), 0.92 – 0.86 (m, 1H), 0.87 – 0.78 (m, 3H) ppm.

ESI-MS (m/z): $[M+H]^+$ 458, $[M+Na]^+$ 480.





25



Chemical Formula: $\text{C}_{26}\text{H}_{35}\text{NO}_4\text{S}$
Molecular Weight: 457.63

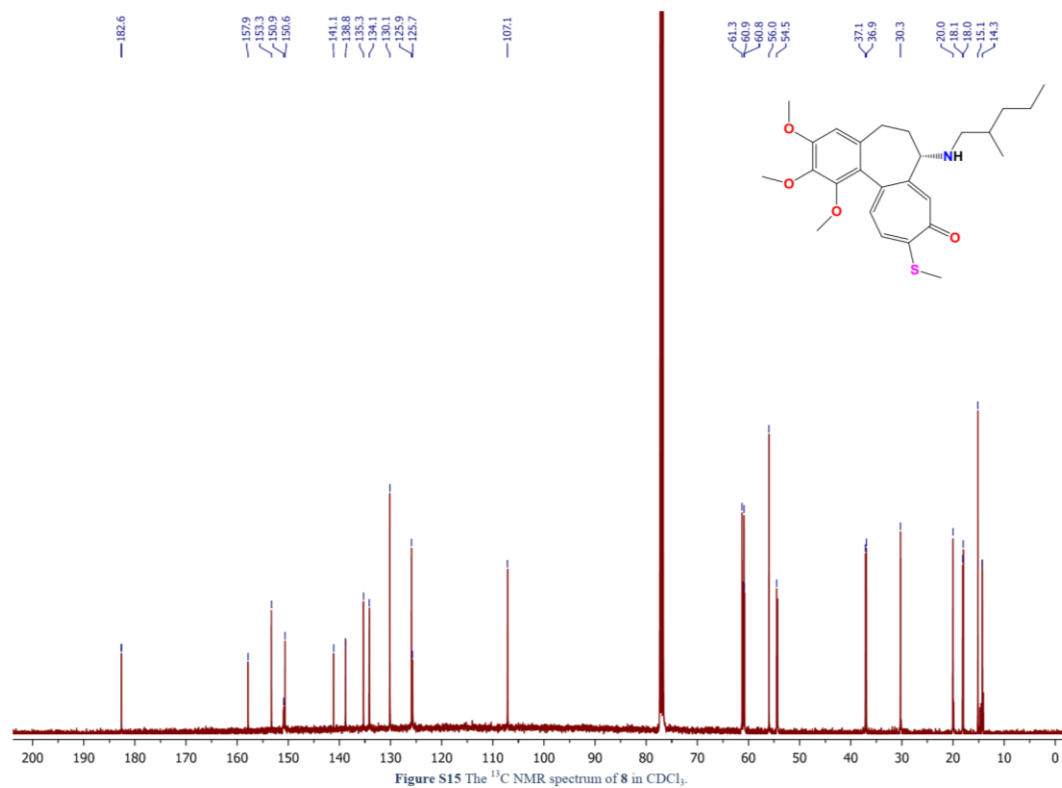
Yield: 70.1 %

^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 157.9, 153.3, 150.9, 150.6, 141.1, 138.8, 135.3, 134.1, 130.1, 125.9, 125.7, 107.1, 61.3, 61.0, 60.9, 56.0, 54.5, 37.1, 36.9, 30.3, 20.0, 18.1, 18.0, 15.1, 14.3 ppm.

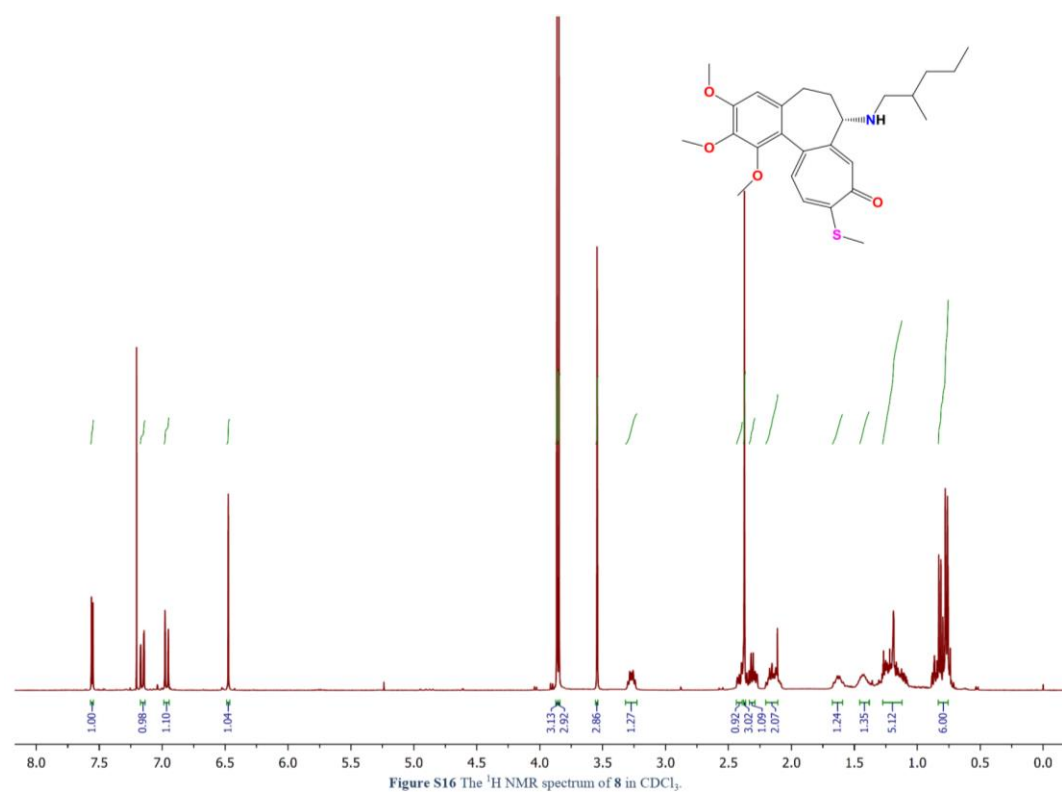
^1H NMR (400 MHz, CDCl_3) δ 7.62 (s, 1H), 7.22 (d, $J = 9.1$ Hz, 1H), 7.03 (d, $J = 10.4$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.36 – 3.28 (m, 1H), 2.45 – 2.39 (m, 1H), 2.37 (s, 3H), 2.35 – 2.27 (m, 1H), 2.21 – 2.10 (m, 2H), 1.66 – 1.59 (m, 1H), 1.47 – 1.38 (m, 1H), 1.27 – 1.12 (m, 5H), 0.83 – 0.75 (m, 6H) ppm.

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 458, $[\text{M}+\text{Na}]^+$ 480.

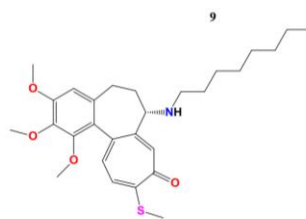
26



27



28



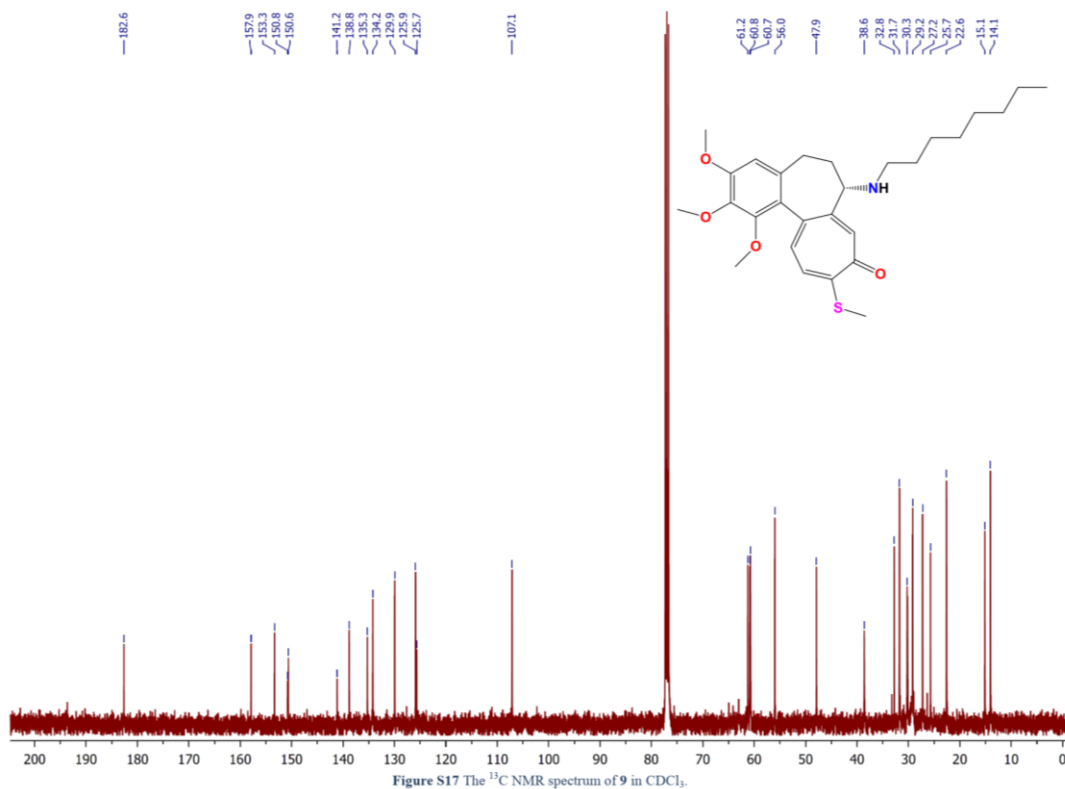
Chemical Formula: $C_{28}H_{30}NO_4S$
Molecular Weight: 485.68

Yield: 55.4 %

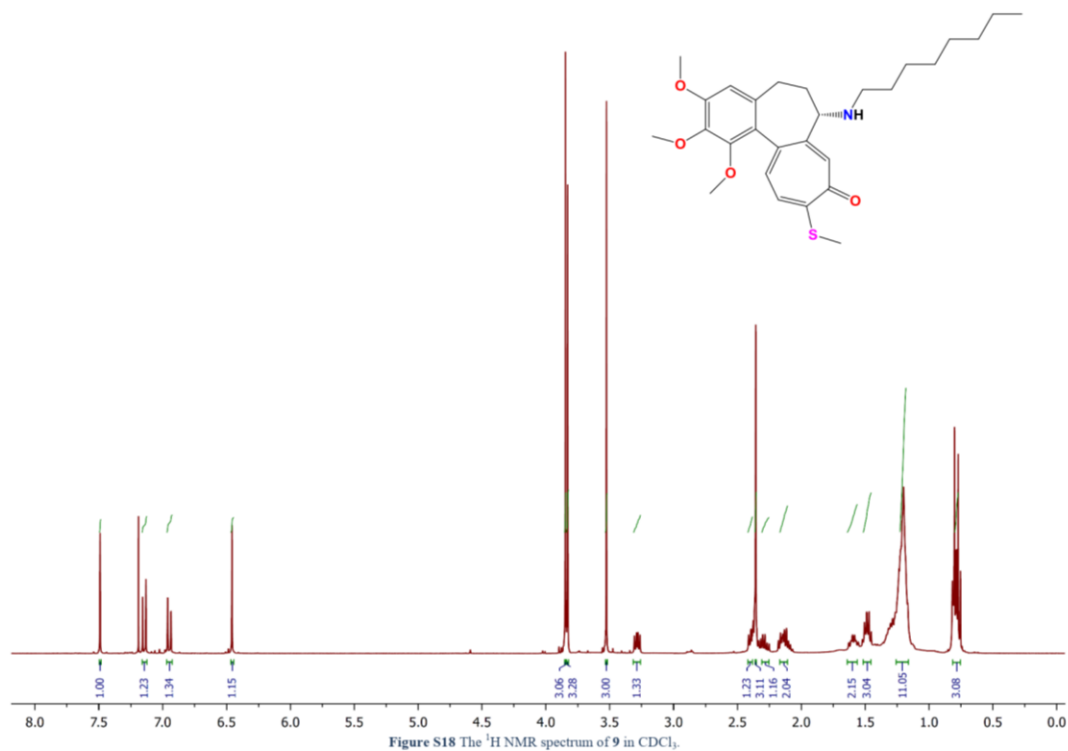
^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 157.9, 153.3, 150.8, 150.6, 141.2, 138.8, 135.3, 134.2, 129.9, 125.9, 125.7, 107.1, 61.3, 60.9, 60.7, 56.0, 47.9, 38.6, 32.8, 31.7, 30.3, 30.2, 27.2, 25.7, 22.6, 15.1, 14.1 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.21 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 10.9$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.36 (dd, $J = 10.9, 6.2$ Hz, 1H), 2.42 – 2.37 (m, 1H), 2.36 (s, 3H), 2.28 (dd, $J = 13.3, 6.8$ Hz, 1H), 2.17 – 2.10 (m, 2H), 1.64 – 1.56 (m, 2H), 1.53 – 1.45 (m, 3H), 1.26 – 1.18 (m, 11H), 0.81 – 0.75 (m, 3H) ppm.

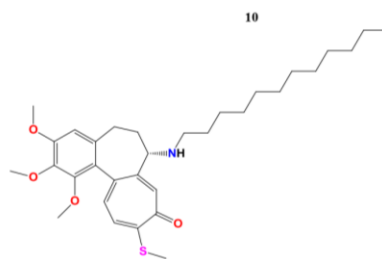
ESI-MS (m/z): $[M+H]^+$ 486, $[M+Na]^+$ 508.



30



31



Chemical Formula: $\text{C}_{32}\text{H}_{47}\text{NO}_4\text{S}$
Molecular Weight: 541.78

Yield: 89.5 %

^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.0, 153.4, 150.70, 150.66, 141.2, 138.8, 135.3, 134.3, 130.0, 125.9, 125.7, 107.2, 61.3, 60.9, 60.8, 56.0, 47.9, 38.6, 32.8, 31.9, 30.3, 29.7, 29.65, 29.63, 29.61, 29.59, 29.4, 29.34, 27.28, 25.7, 22.7, 15.2, 14.1 ppm.

^1H NMR (400 MHz, CDCl_3) δ 7.56 (s, 1H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.02 (d, $J = 10.4$ Hz, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.60 (s, 3H), 3.37 (dd, $J = 10.9, 6.2$ Hz, 1H), 2.50 – 2.46 (m, 1H), 2.43 (s, 3H), 2.41 – 2.31 (m, 1H), 2.27 – 2.17 (m, 2H), 1.72 – 1.63 (m, 2H), 1.61 – 1.51 (m, 5H), 1.30 – 1.20 (m, 16H), 0.88 – 0.84 (m, 3H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 542.

32

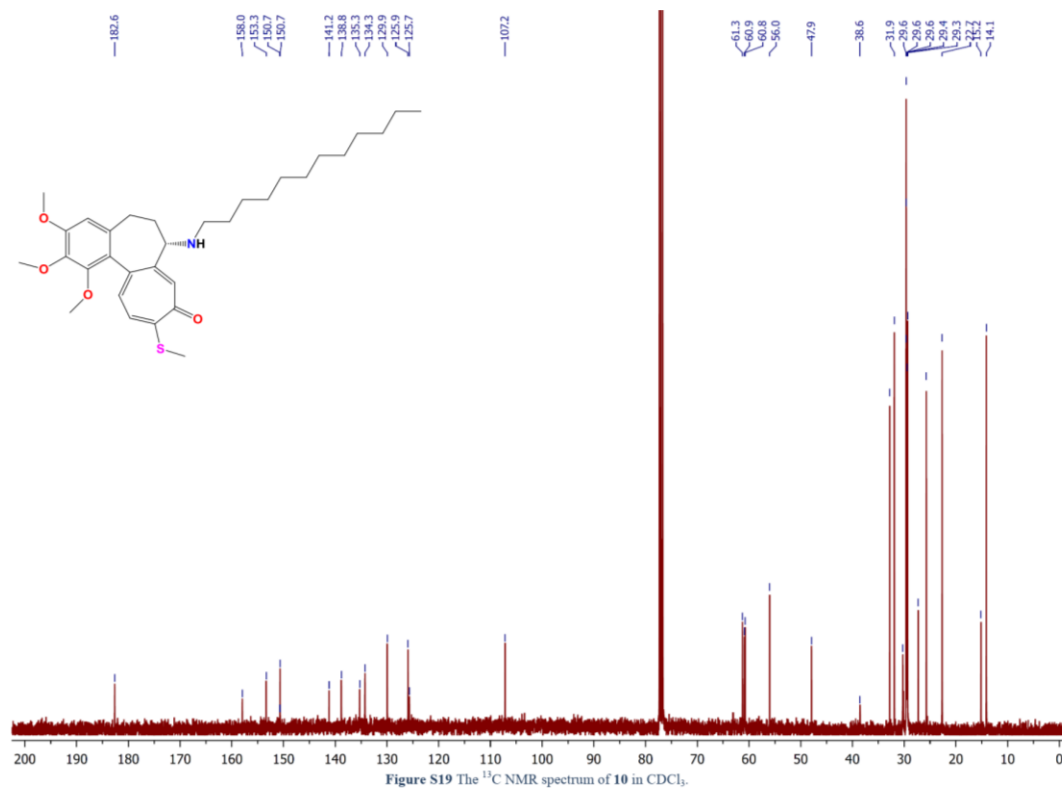


Figure S19 The ^{13}C NMR spectrum of **10** in CDCl_3 .

33

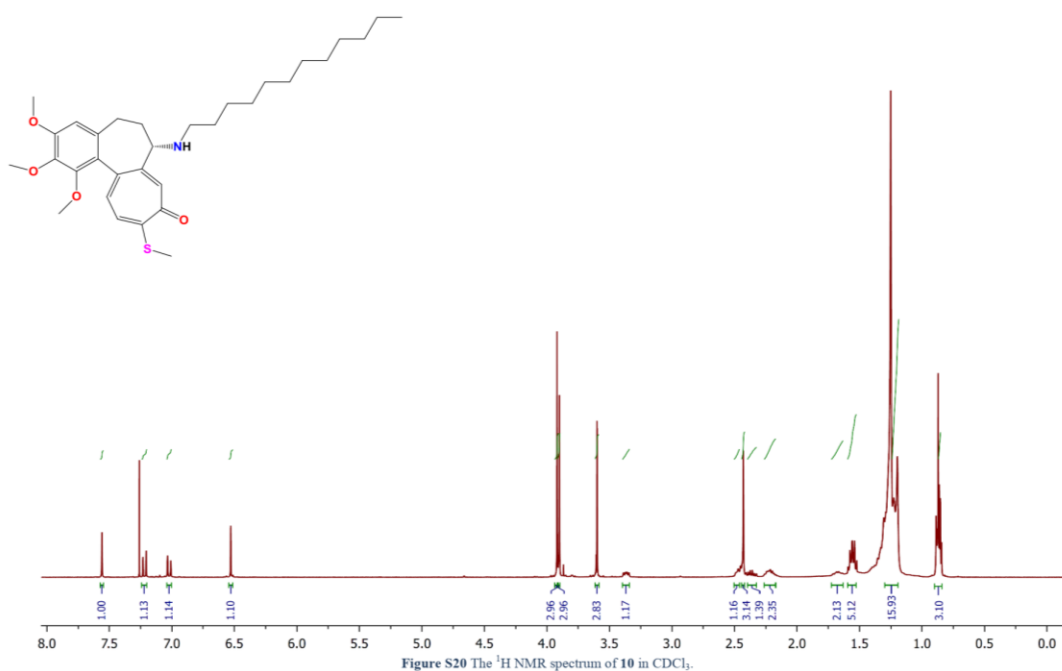
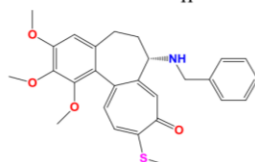


Figure S20 The ^1H NMR spectrum of **10** in CDCl_3 .

34

11

Chemical Formula: $C_{27}H_{29}NO_4S$

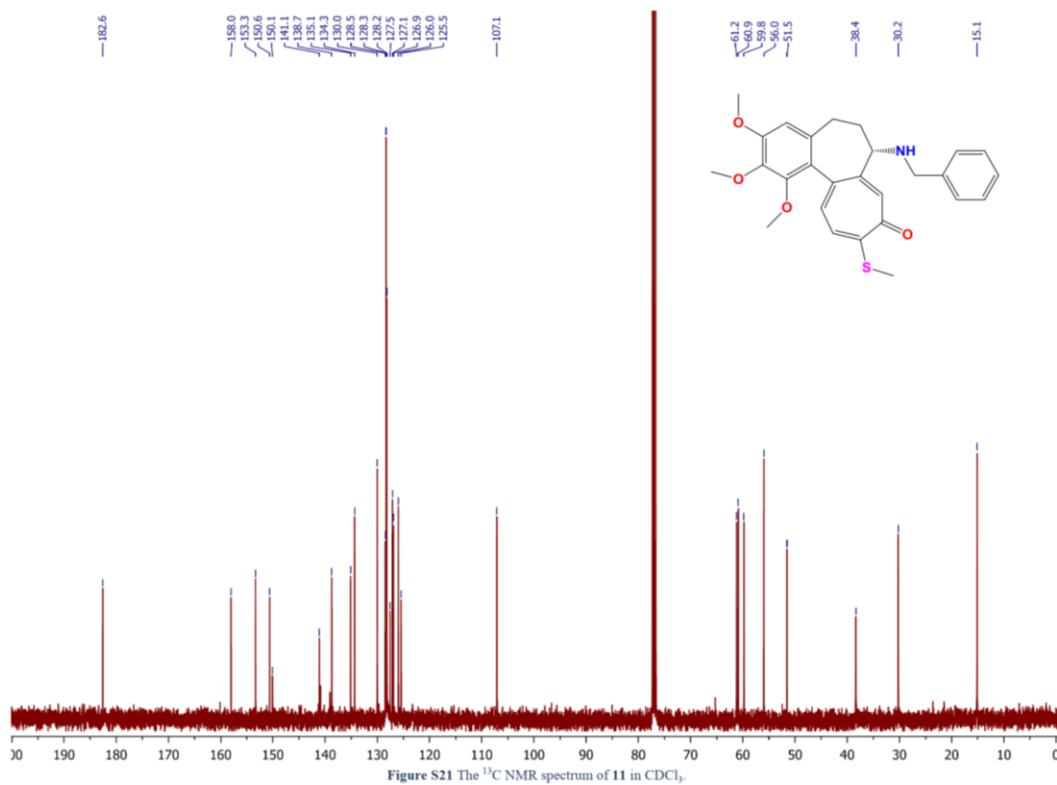
Molecular Weight: 463.59

Yield: 48.3 %

 ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 158.0, 153.3, 150.6, 150.1, 141.1, 138.7, 135.1, 134.4, 130.0, 128.5, 128.3, 128.2, 127.6, 127.1, 126.9, 126.0, 125.5, 107.1, 61.2, 60.9, 59.8, 56.0, 51.5, 38.5, 30.3, 15.2 ppm.

 1H NMR (400 MHz, $CDCl_3$) 7.75 (s, 1H), 7.37 (s, 1H), 7.35 (d, $J = 2.9$ Hz, 1H), 7.24–7.20 (m, 4H), 7.04 (d, $J = 10.6$ Hz, 1H), 6.51 (s, 1H), 4.70 (s, 1H), 3.90 (s, 3H), 3.90 (s, 3H), 3.73 (d, $J = 13.0$ Hz, 1H), 3.53 (s, 3H), 3.46 (dd, $J = 11.4, 6.4$ Hz, 2H), 2.50–2.45 (m, 1H), 2.44 (s, 3H), 2.42–2.33 (m, 1H), 2.22 (ddd, $J = 22.8, 11.6, 5.2$ Hz, 2H), 1.75 (td, $J = 11.2, 6.3$ Hz, 1H).
ESI-MS (m/z): $[M+H]^+$ 464, $[M+Na]^+$ 486, $[M+K]^+$ 502.

35

Figure S21 The ^{13}C NMR spectrum of 11 in $CDCl_3$.

36

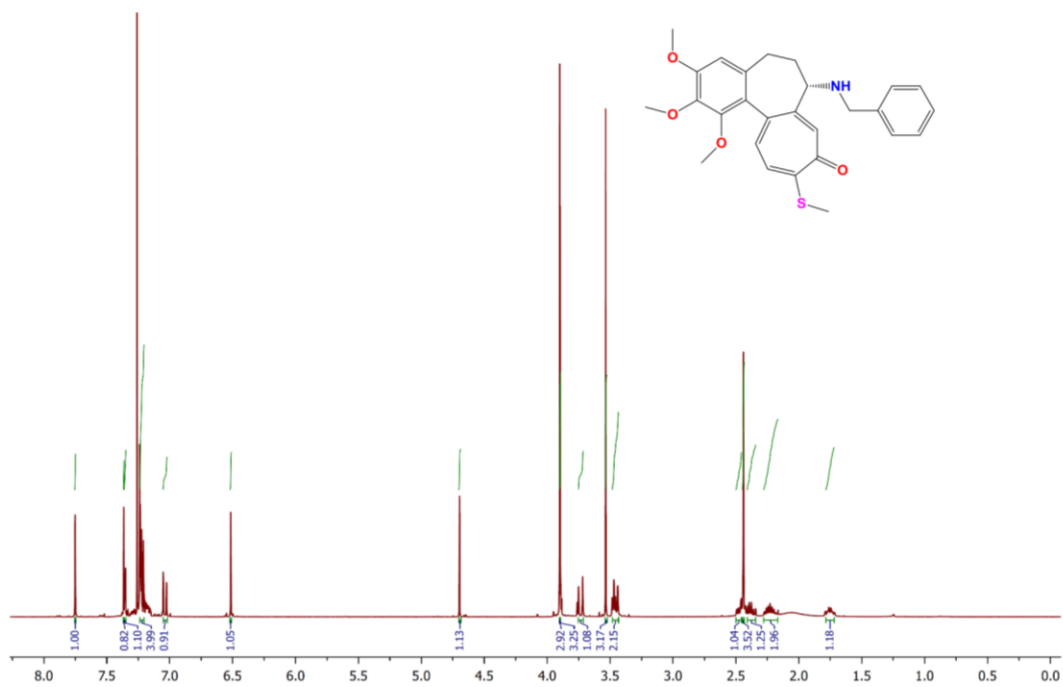
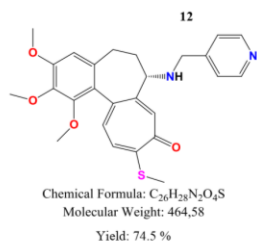


Figure S22 The ^1H NMR spectrum of 11 in CDCl_3 .

37

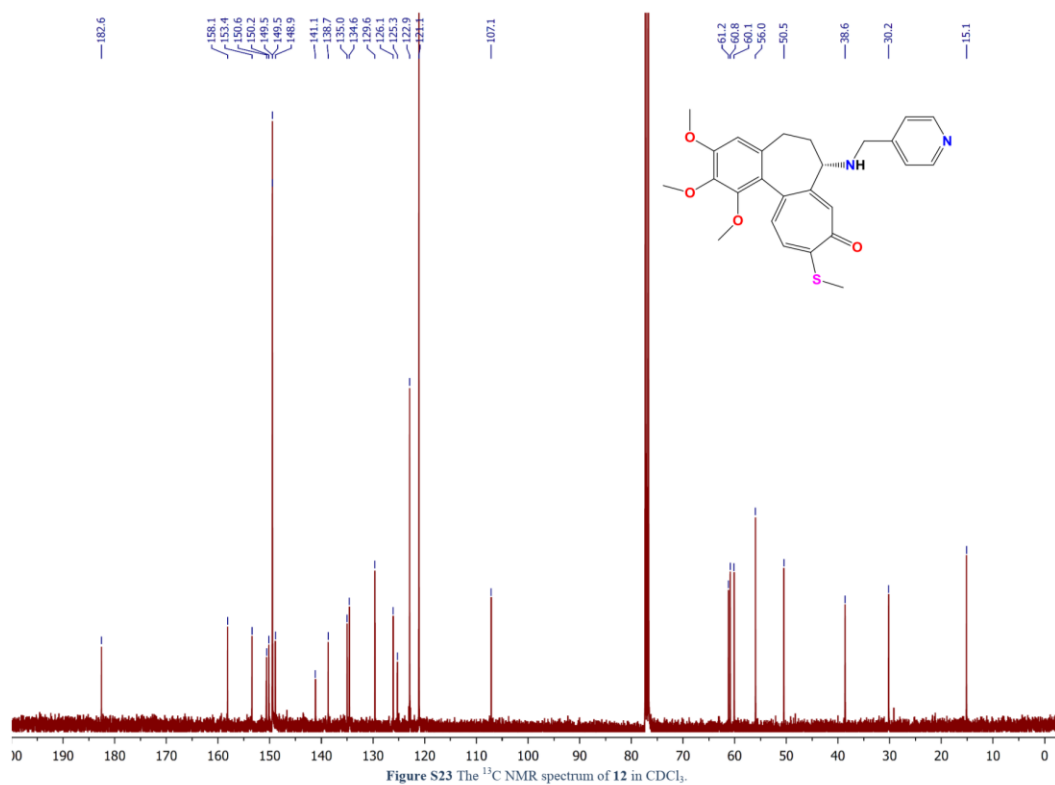


^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.2, 153.4, 150.6, 150.2, 149.48, 149.46, 148.9, 141.2, 138.7, 135.0, 134.6, 129.7, 126.1, 125.3, 122.9, 121.1, 107.1, 61.2, 60.8, 60.1, 56.0, 50.5, 38.6, 30.2, 15.1 ppm.

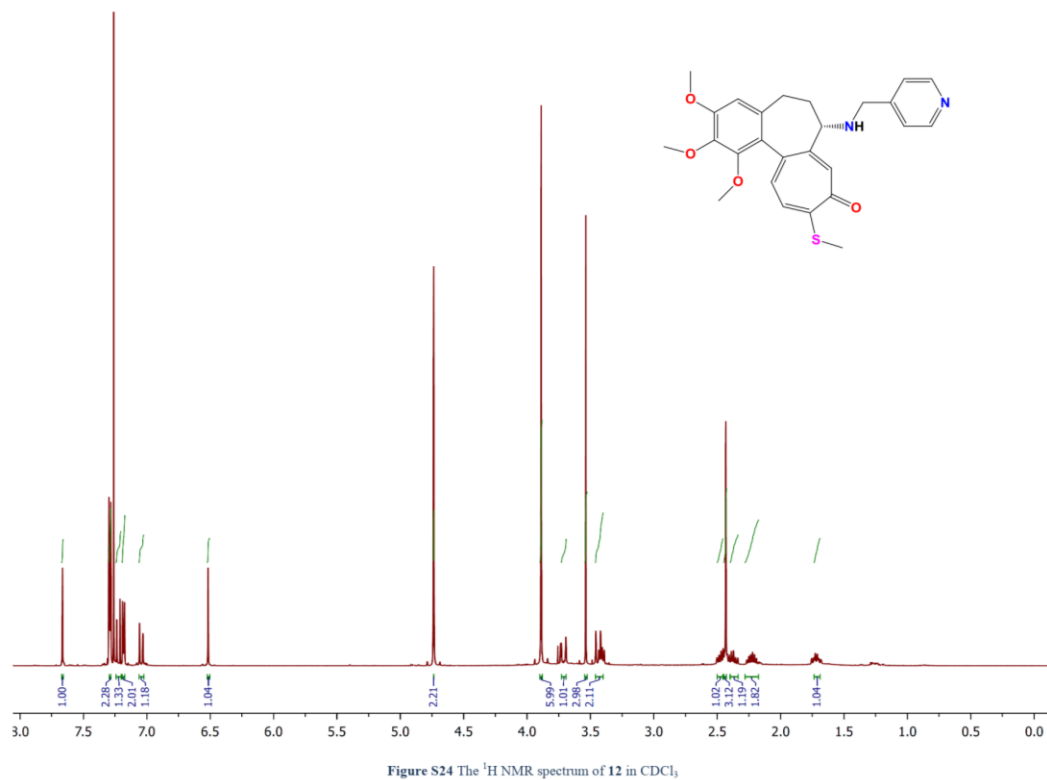
^1H NMR (400 MHz, CDCl_3) δ 7.66 (s, 1H), 7.29 (dd, $J = 5.3, 0.7$ Hz, 2H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.18 (dd, $J = 4.5, 1.5$ Hz, 2H), 7.04 (d, $J = 10.6$ Hz, 1H), 6.52 (s, 1H), 4.74 (s, 2H), 3.89 (s, 6H), 3.71 (d, $J = 14.3$ Hz, 1H), 3.54 (s, 3H), 3.46 – 3.39 (m, 2H), 2.52 – 2.45 (m, 1H), 2.44 (s, 3H), 2.41 – 2.33 (m, 1H), 2.27 – 2.18 (m, 2H), 1.72 (td, $J = 11.0, 5.7$ Hz, 1H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 465, $[\text{M}+\text{Na}]^+$ 487, $[2\text{M}+\text{Na}]^+$ 951.

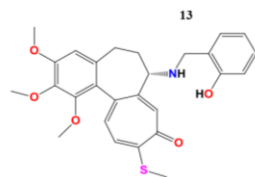
38



39



40



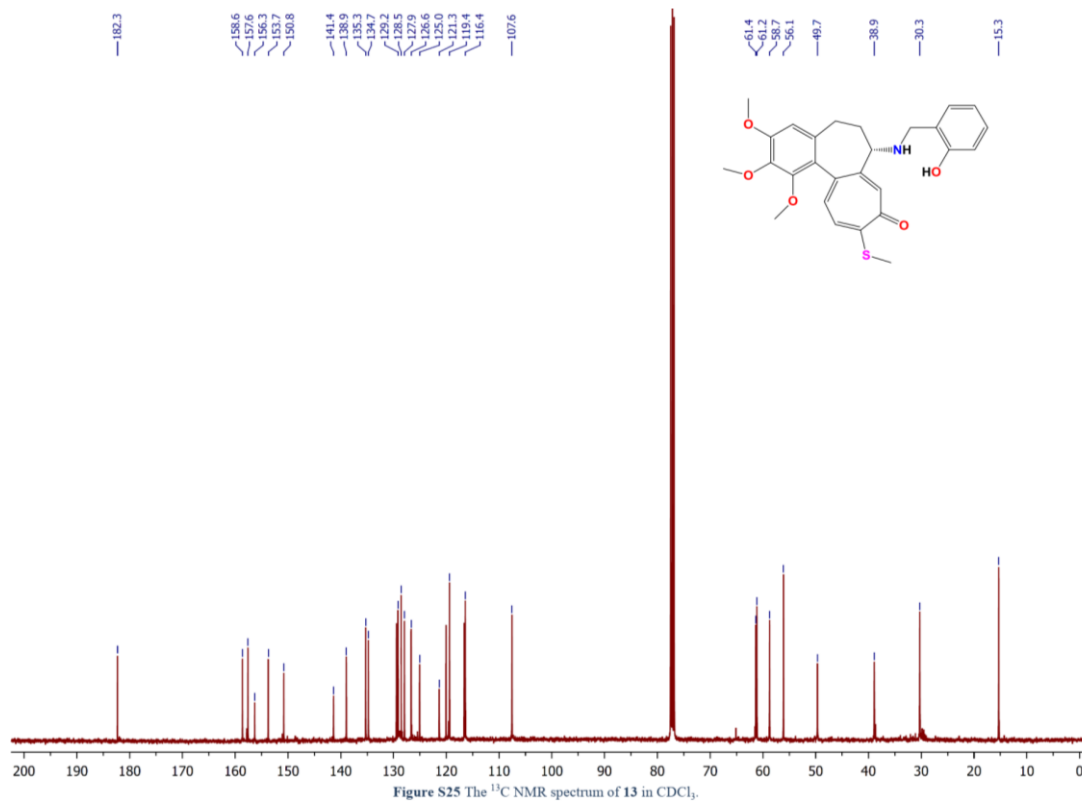
Chemical Formula: $C_{27}H_{29}NO_2S$
Molecular Weight: 479,59

Yield: 33.3 %

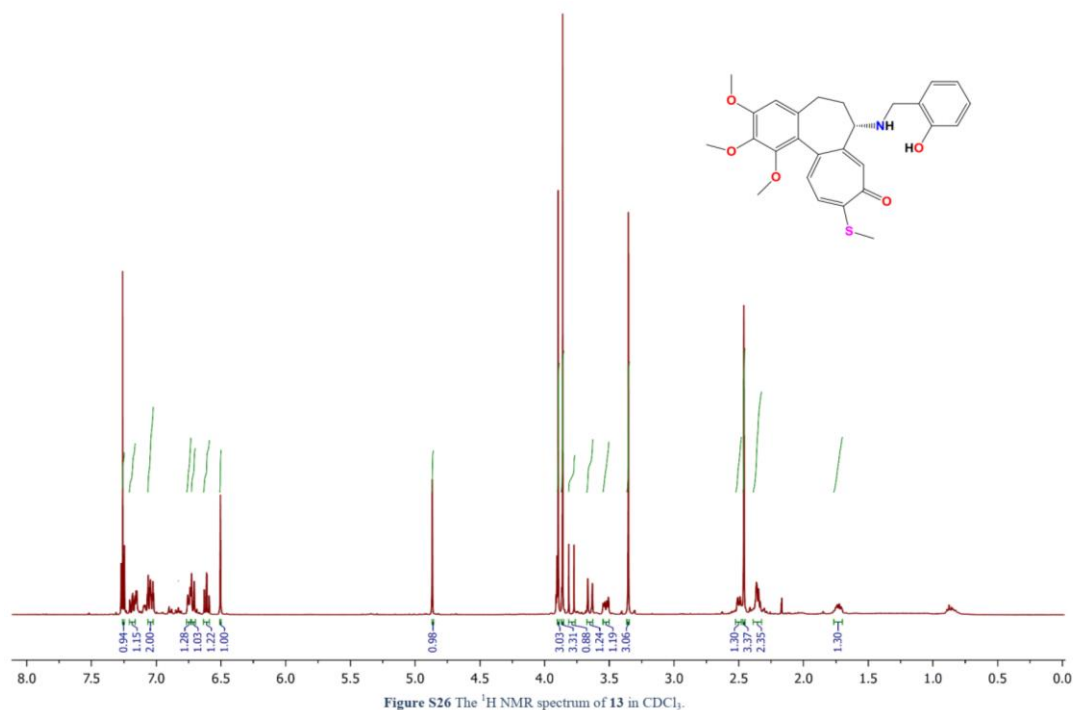
^{13}C NMR (101 MHz, $CDCl_3$) δ 182.3, 158.6, 157.6, 156.3, 153.7, 150.8, 141.4, 139.0, 135.3, 134.8, 129.2, 128.5, 127.9, 126.7, 125.0, 121.3, 119.4, 116.4, 107.6, 61.4, 61.2, 58.8, 56.1, 49.7, 38.9, 30.3, 15.3 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.26 (d, J = 10.3 Hz, 1H), 7.20 – 7.16 (m, 1H), 7.08 – 7.02 (m, 1H), 6.74 (d, J = 3.2 Hz, 1H), 6.72 (d, J = 9.0 Hz, 1H), 6.61 (td, J = 7.4, 1.2 Hz, 1H), 6.50 (s, 1H), 4.87 (s, 1H), 3.90 (s, 3H), 3.86 (s, 3H), 3.79 (d, J = 16.1 Hz, 1H), 3.65 (d, J = 14.0 Hz, 1H), 3.53 (dd, J = 11.1, 5.1 Hz, 1H), 3.35 (s, 3H), 2.53 – 2.48 (m, 1H), 2.46 (s, 3H), 2.39 – 2.32 (m, 2H), 1.78 – 1.69 (m, 1H).

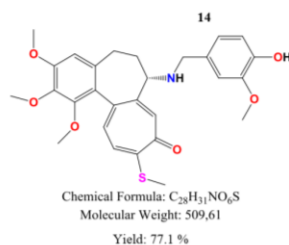
ESI-MS (m/z): $[M+H]^+$ 480, $[M+Na]^+$ 502, $[M+K]^+$ 518.



42



43

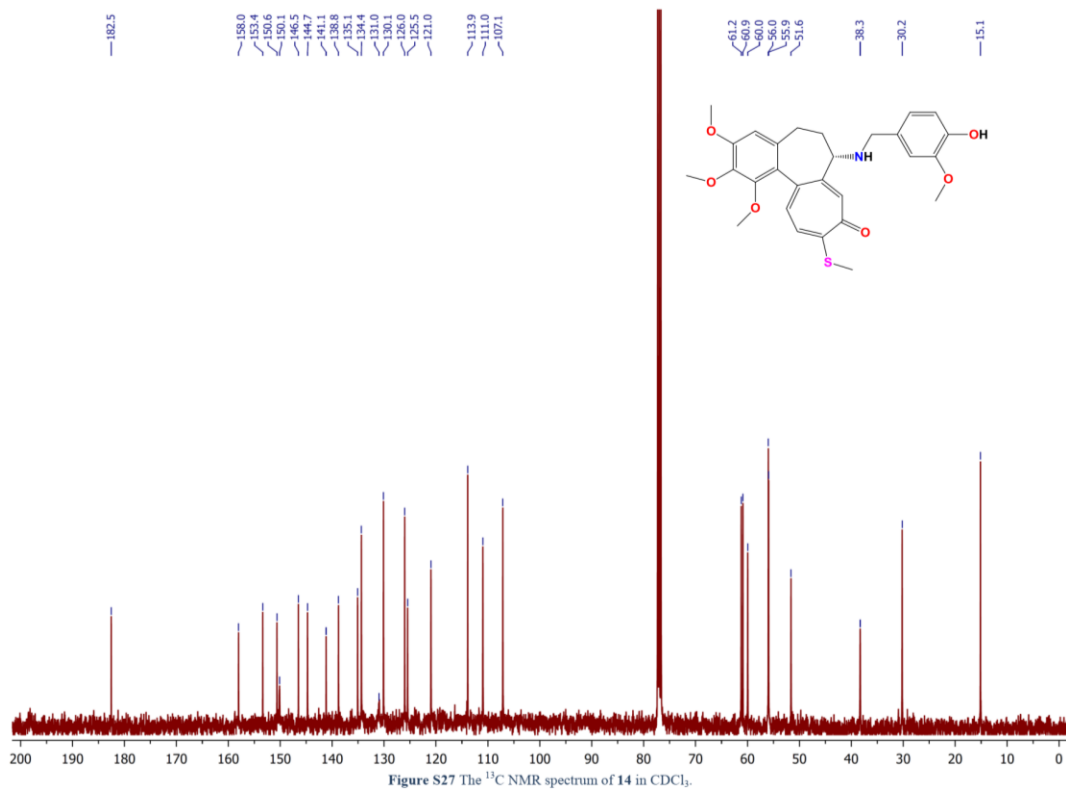


^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.0, 153.4, 150.6, 150.1, 146.5, 144.7, 141.2, 138.8, 135.1, 134.4, 131.0, 130.1, 126.0, 125.5, 121.0, 113.9, 111.0, 107.1, 61.2, 60.9, 60.0, 56.0, 55.9, 51.6, 38.3, 30.2, 15.1 ppm.

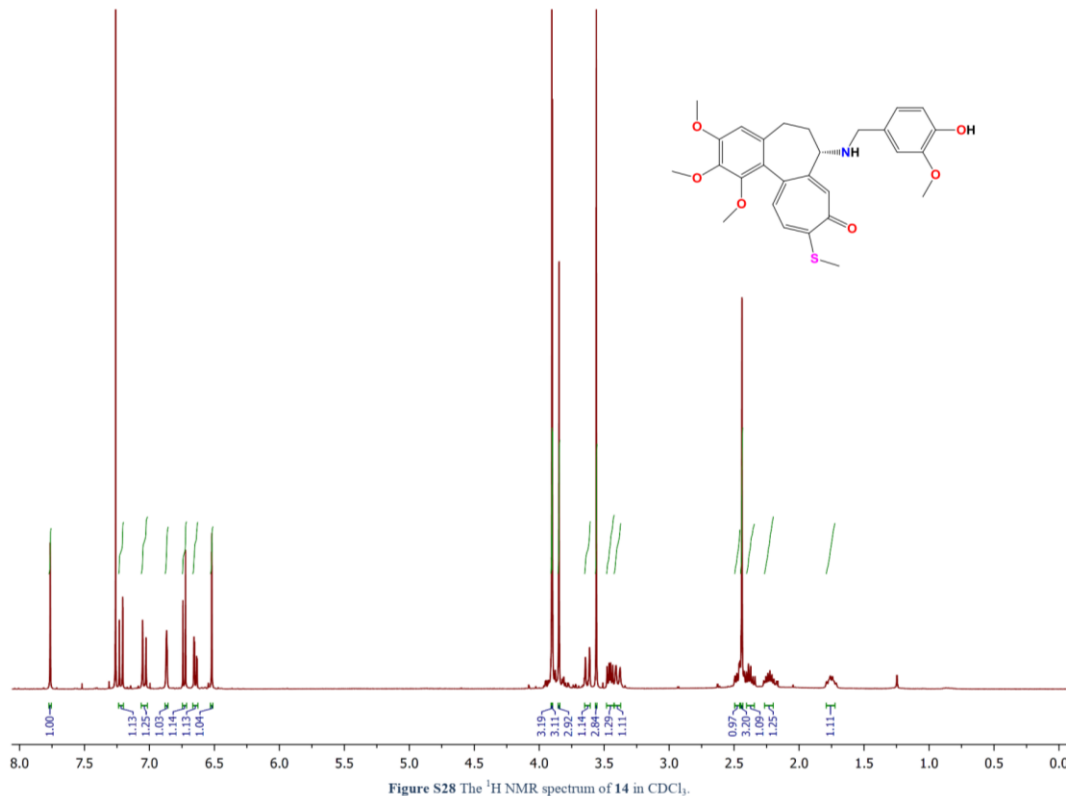
^1H NMR (400 MHz, CDCl_3) δ 7.76 (s, 1H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.04 (d, $J = 10.5$ Hz, 1H), 6.87 (s, $J = 9.8$ Hz, 1H), 6.73 (d, $J = 8.0$ Hz, 1H), 6.65 (dd, $J = 8.0, 1.9$ Hz, 1H), 6.52 (s, 1H), 3.90 (s, 3H), 3.85 (s, 3H), 3.65 – 3.61 (m, 1H), 3.56 (s, 3H), 3.46 (dd, $J = 10.9, 6.3$ Hz, 1H), 3.39 (d, $J = 13.0$ Hz, 1H), 2.51 – 2.45 (m, 1H), 2.44 (s, 3H), 2.43 – 2.32 (m, 1H), 2.28 – 2.18 (m, 1H), 1.80 – 1.70 (m, 1H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 510, $[\text{M}+\text{Na}]^+$ 532, $[\text{M}+\text{K}]^+$ 548, $[2\text{M}+\text{Na}]^+$ 1041.

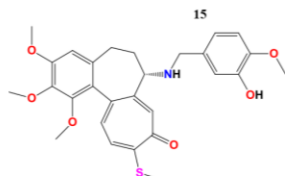
44



45



46



Chemical Formula: $C_{28}H_{31}NO_6S$
Molecular Weight: 509.61

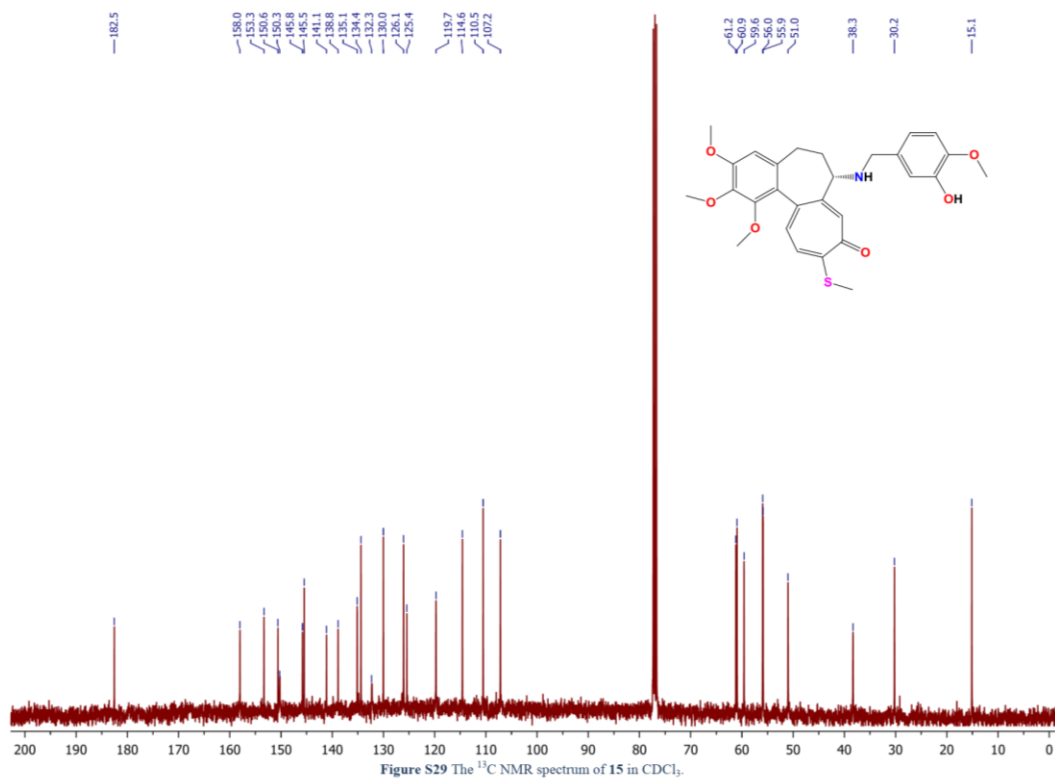
Yield: 70.4 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 158.0, 153.3, 150.6, 150.2, 145.8, 145.5, 141.1, 138.9, 135.1, 134.4, 132.2, 130.0, 126.1, 125.4, 119.7, 114.6, 110.5, 107.2, 61.2, 61.0, 59.6, 56.0, 55.9, 51.0, 38.3, 30.2, 15.1 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.04 (d, $J = 10.4$ Hz, 1H), 6.80 (s, 1H), 6.69 (s, $J = 1.5$ Hz, 2H), 6.51 (s, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.80 (s, 3H), 3.64 – 3.59 (m, 1H), 3.56 (s, 3H), 3.47 – 3.42 (m, 1H), 3.39 – 3.31 (m, 1H), 2.49 – 2.45 (m, 1H), 2.44 (s, 3H), 2.42 – 2.33 (m, 1H), 2.28 – 2.18 (m, 1H), 1.79 – 1.68 (m, 1H).

ESI-MS (m/z): $[M+H]^+$ 510, $[M+Na]^+$ 532, $[2M+Na]^+$ 1041.

47



48

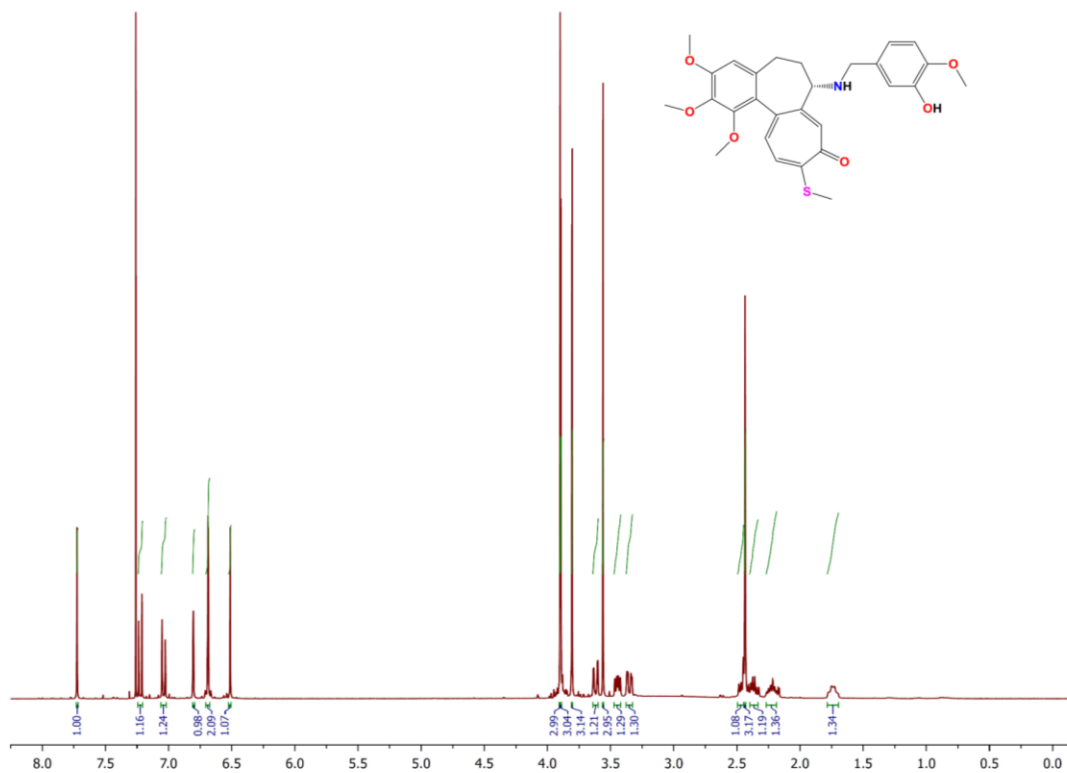
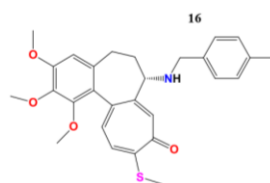


Figure S30 The ^1H NMR spectrum of **15** in CDCl_3 .

49



Chemical Formula: $\text{C}_{28}\text{H}_{31}\text{NO}_4\text{S}$

Molecular Weight: 477.62

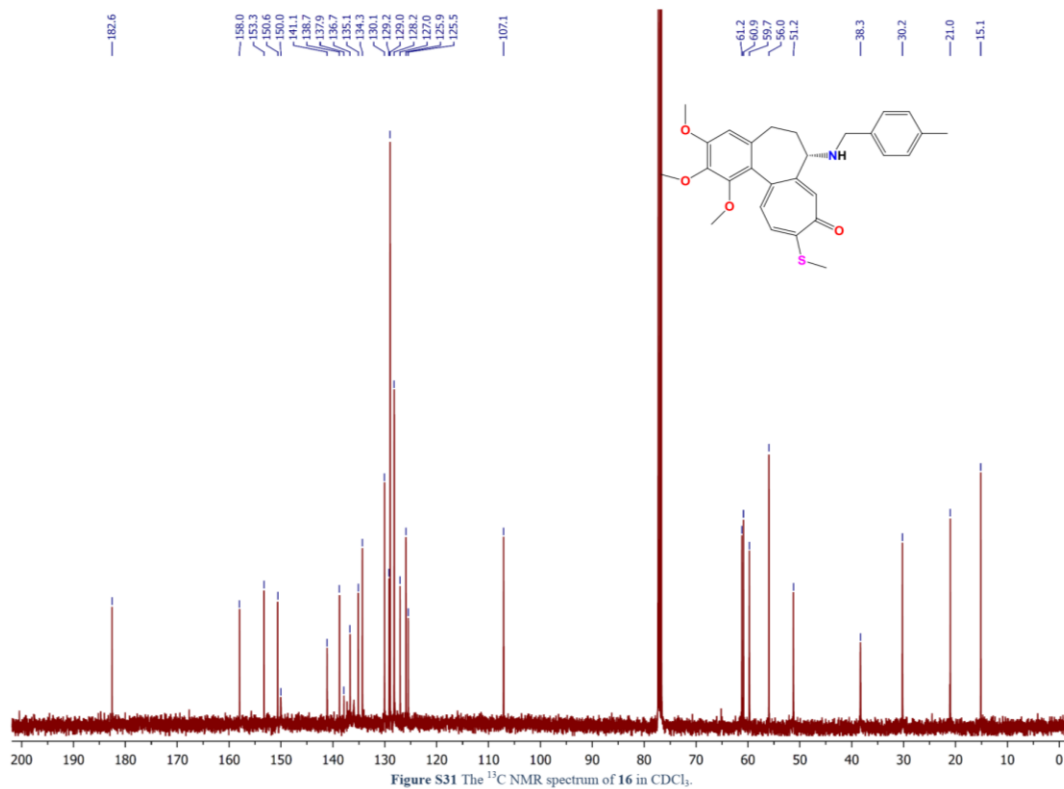
Yield: 80.4 %

^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.0, 153.3, 150.6, 150.0, 141.1, 138.7, 136.7, 135.1, 134.3, 130.1, 129.2, 129.0, 128.2, 127.0, 125.9, 125.5, 107.1, 61.2, 60.9, 59.7, 56.0, 51.2, 38.3, 30.2, 21.0, 15.3 ppm.

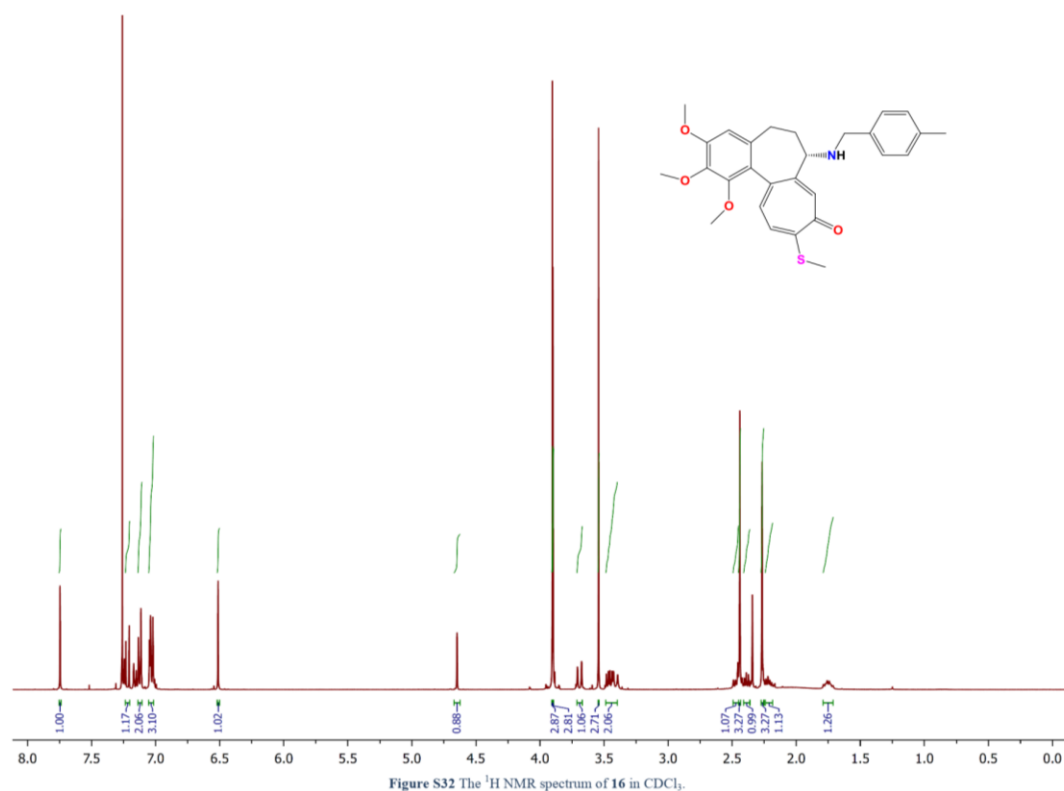
^1H NMR (400 MHz, CDCl_3) δ 7.75 (s, 1H), 7.22 (d, $J = 10.3$ Hz, 1H), 7.12 (d, $J = 8.0$ Hz, 2H), 7.05 – 7.01 (m, 3H), 6.51 (s, 1H), 4.65 (s, 1H), 3.90 (s, 3H), 3.90 (s, 3H), 3.69 (d, $J = 12.8$ Hz, 1H), 3.54 (s, 3H), 3.49 – 3.39 (m, 2H), 2.50 – 2.45 (m, 1H), 2.44 (s, 3H), 2.42 – 2.36 (m, 1H), 2.27 (s, 3H), 2.21 (dd, $J = 12.3, 6.2$ Hz, 1H), 1.79 – 1.72 (m, 1H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 478, $[\text{M}+\text{Na}]^+$ 500.

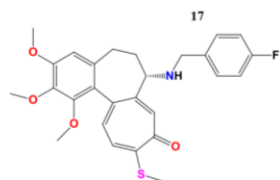
50



51



52



Chemical Formula: $C_{27}H_{29}FNO_4S$
Molecular Weight: 481,58

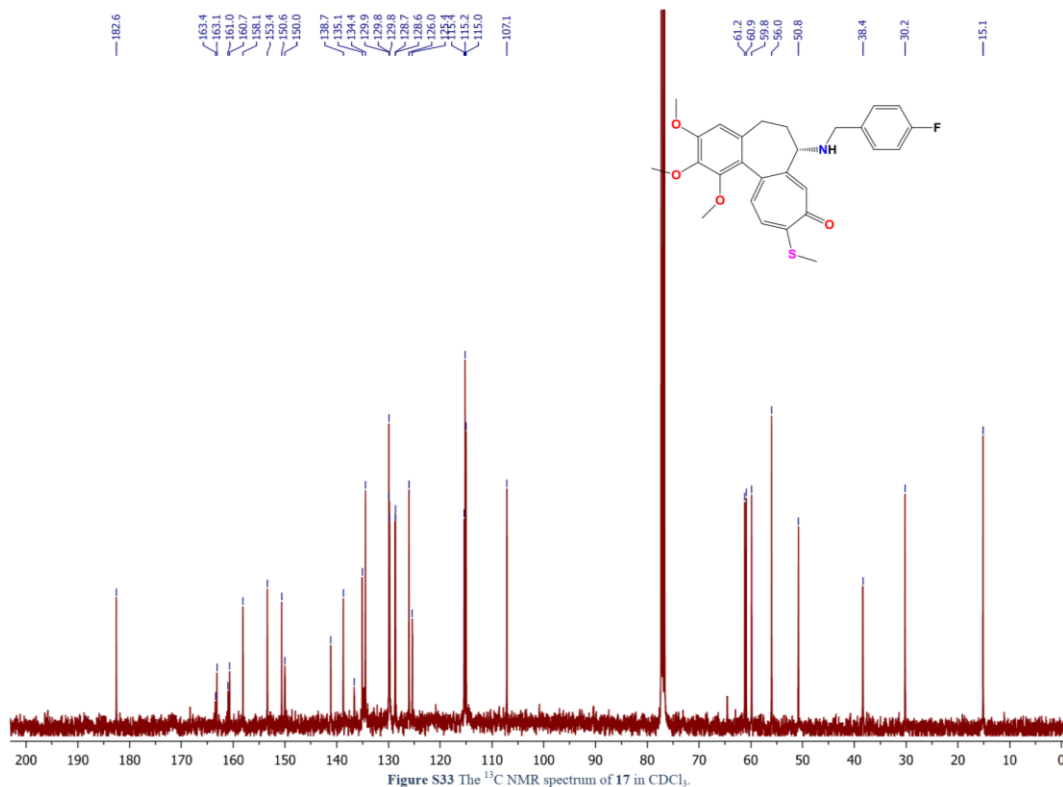
Yield: 90.8 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 163.1, 160.7, 158.1, 153.4, 150.6, 150.0, 141.2, 138.7, 135.1, 134.4, 129.9, 128.7, 126.0, 125.4, 115.2, 115.0, 107.13, 61.2, 60.9, 59.9, 56.0, 50.8, 38.4, 30.2, 15.1 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.71 (s, 1H), 7.35 – 7.31 (m, 2H), 7.23 – 7.19 (m, 2H), 7.06 – 7.00 (m, 3H), 6.95 – 6.88 (m, 2H), 6.52 (s, 1H), 4.66 (s, 2H), 3.90 (s, 3H), 3.90 (s, 3H), 3.68 (d, J = 12.9 Hz, 1H), 3.57 (s, 3H), 3.46 – 3.41 (m, 2H), 2.50 – 2.46 (m, 1H), 2.44 (s, 3H), 2.41 – 2.34 (m, 1H), 2.28 – 2.15 (m, 1H), 1.74 (td, J = 11.4, 6.7 Hz, 1H).

ESI-MS (m/z): $[M+H]^+$ 482, $[M+Na]^+$ 504, $[M+K]^+$ 520.

53



54

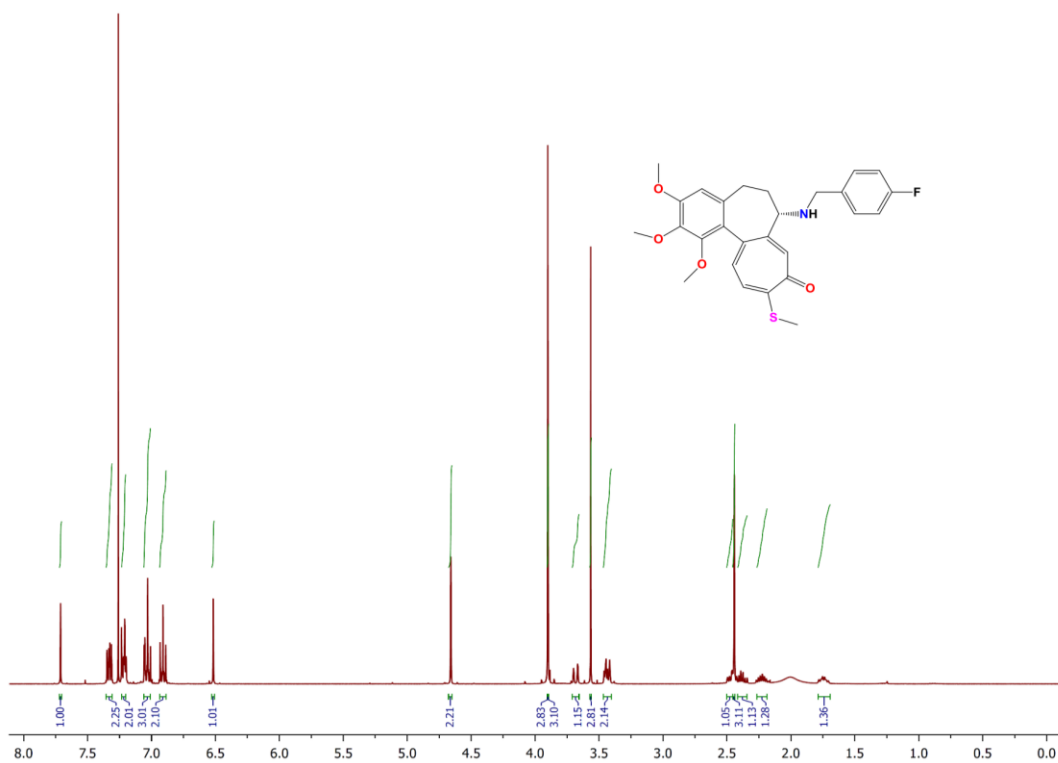
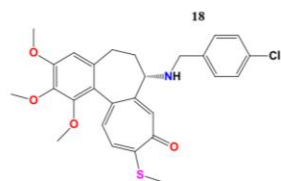


Figure S34 The ^1H NMR spectrum of 17 in CDCl_3 .

55



Chemical Formula: $\text{C}_{27}\text{H}_{28}\text{ClNO}_4\text{S}$

Molecular Weight: 498.03

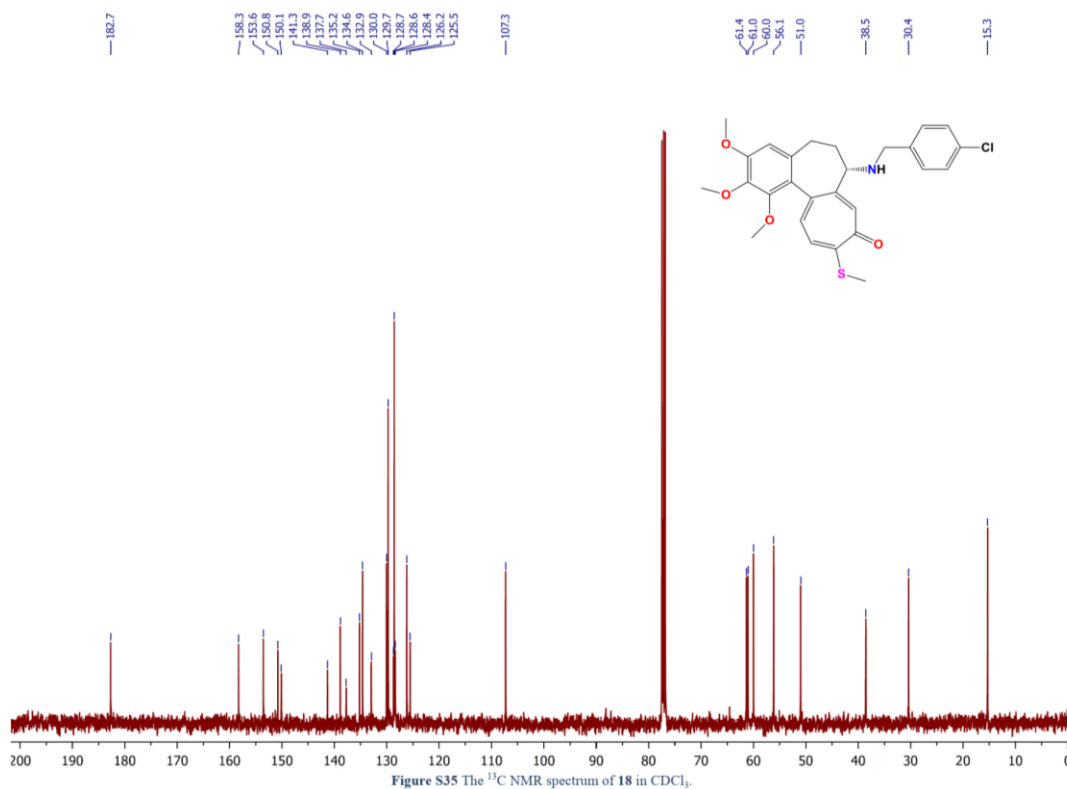
Yield: 42.8 %

^{13}C NMR (101 MHz, CDCl_3) δ 182.5, 158.1, 153.4, 150.6, 150.0, 141.1, 138.7, 137.6, 135.0, 134.4, 132.8, 129.9, 129.6, 128.6, 128.4, 128.2, 126.0, 125.4, 107.1, 61.2, 60.9, 59.8, 56.0, 50.8, 38.4, 30.2, 15.1 ppm.

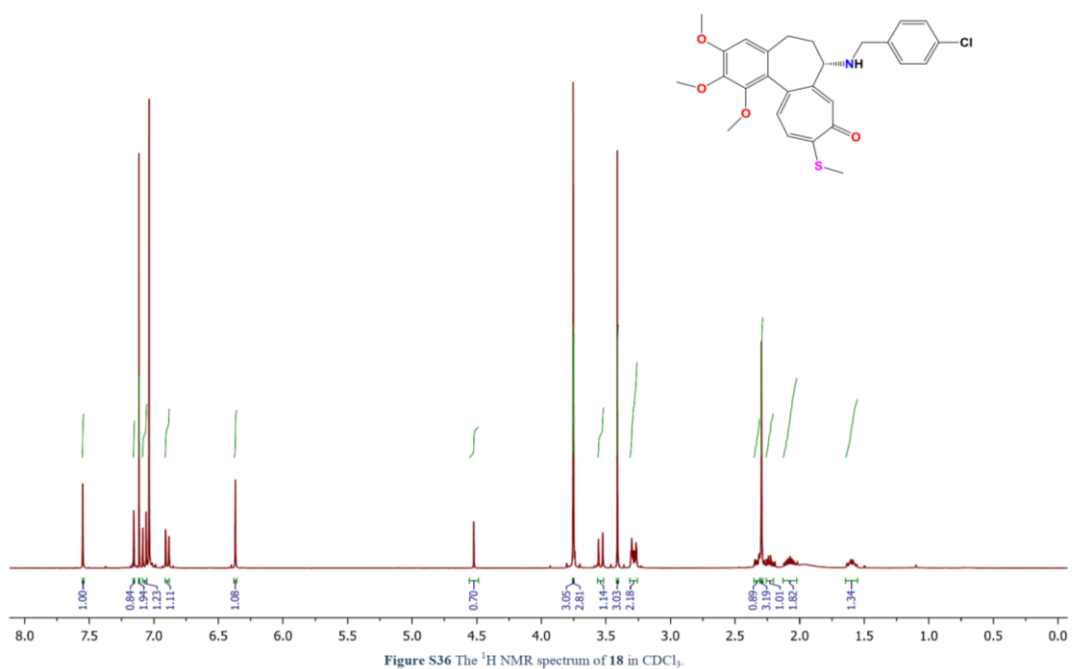
^1H NMR (400 MHz, CDCl_3) δ 7.55 (s, 1H), 7.16 (d, $J = 0.8$ Hz, 1H), 7.11 (s, 2H), 7.07 (d, $J = 10.3$ Hz, 1H), 6.90 (d, $J = 10.6$ Hz, 1H), 6.37 (s, 1H), 4.52 (s, 1H), 3.75 (s, 3H), 3.75 (s, 3H), 3.57–3.51 (m, 1H), 3.41 (s, 3H), 3.32–3.25 (m, 2H), 2.36–2.32 (m, 1H), 2.30 (s, 3H), 2.27–2.16 (m, 1H), 2.12–1.97 (m, 2H), 1.60 (td, $J = 11.2, 5.9$ Hz, 1H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 498, $[\text{M}+\text{Na}]^+$ 520, $[2\text{M}+\text{H}]^+$ 996.

56



57



58



Chemical Formula: $C_{27}H_{28}INO_4S$
Molecular Weight: 589.48

Yield: 49.9 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.5, 158.1, 153.4, 150.6, 150.0, 141.1, 138.7, 137.3, 135.1, 134.4, 130.2, 129.9, 128.7, 126.0, 125.4, 107.1, 92.4, 61.2, 60.9, 59.8, 56.0, 51.0, 38.4, 30.2, 15.2 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, $J = 3.5$ Hz, 1H), 7.54 (d, $J = 8.3$ Hz, 2H), 7.21 (d, $J = 10.3$ Hz, 1H), 7.04 (d, $J = 10.5$ Hz, 1H), 6.99 (d, $J = 8.3$ Hz, 2H), 6.52 (s, 1H), 4.65 (s, 1H), 3.90 (s, 3H), 3.90 (s, 3H), 3.66 (d, $J = 13.2$ Hz, 1H), 3.55 (s, 3H), 3.45 – 3.37 (m, 2H), 2.50 – 2.45 (m, 1H), 2.44 (s, 3H), 2.42 – 2.32 (m, 1H), 2.21 (ddd, $J = 13.0, 9.6, 4.6$ Hz, 1H), 1.77 – 1.67 (m, 1H).

ESI-MS (m/z): $[M+H]^+$ 590, $[M+Na]^+$ 612, $[M+K]^+$ 628, $[2M+H]^+$ 1179.

59

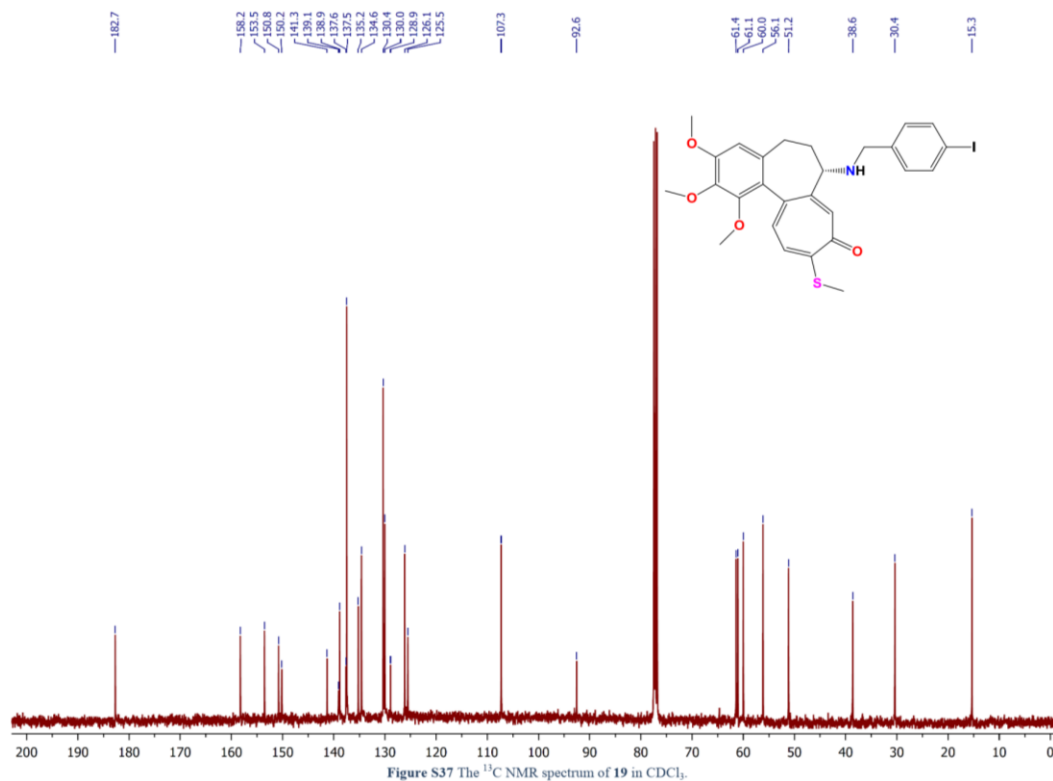


Figure S37 The ^{13}C NMR spectrum of 19 in $CDCl_3$.

60

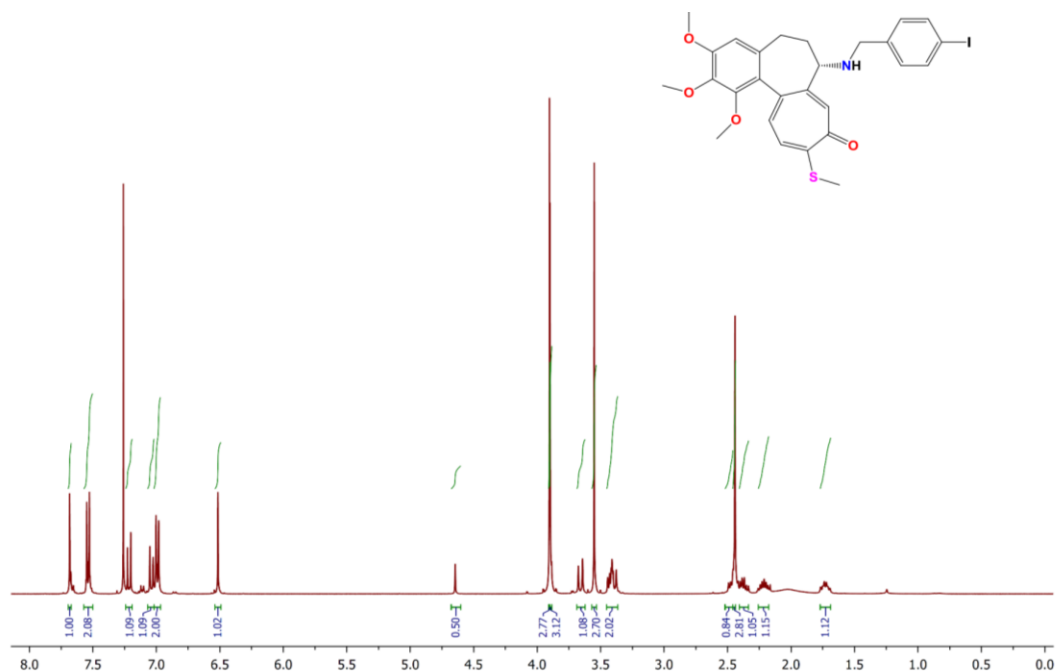
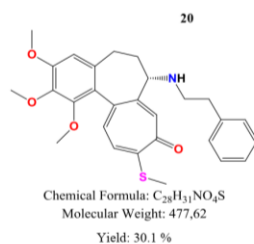


Figure S38 The ^1H NMR spectrum of 19 in CDCl_3 .

61



^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.0, 153.3, 150.6, 150.4, 141.2, 139.5, 138.7, 135.2, 134.3, 129.9, 128.6, 128.4, 126.2, 125.9, 125.5, 107.1, 61.2, 60.9, 60.6, 56.0, 49.0, 38.3, 36.3, 30.2, 15.1 ppm.

^1H NMR (400 MHz, cdcl_3) δ 7.61 (s, 1H), 7.22 (dd, J = 8.8, 7.2 Hz, 3H), 7.14 – 7.10 (m, 2H), 7.02 (d, J = 10.6 Hz, 1H), 6.51 (s, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 3.59 (s, 3H), 3.41 – 3.33 (m, 1H), 2.81 – 2.70 (m, 3H), 2.55 – 2.45 (m, 2H), 2.43 (s, 3H), 2.41 – 2.32 (m, 1H), 2.16 – 2.09 (m, 1H), 1.71 – 1.62 (m, 2H).

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 478, $[\text{M}+\text{Na}]^+$ 500, $[\text{M}+\text{K}]^+$ 516, $[2\text{M}+\text{Na}]^+$ 977.

62

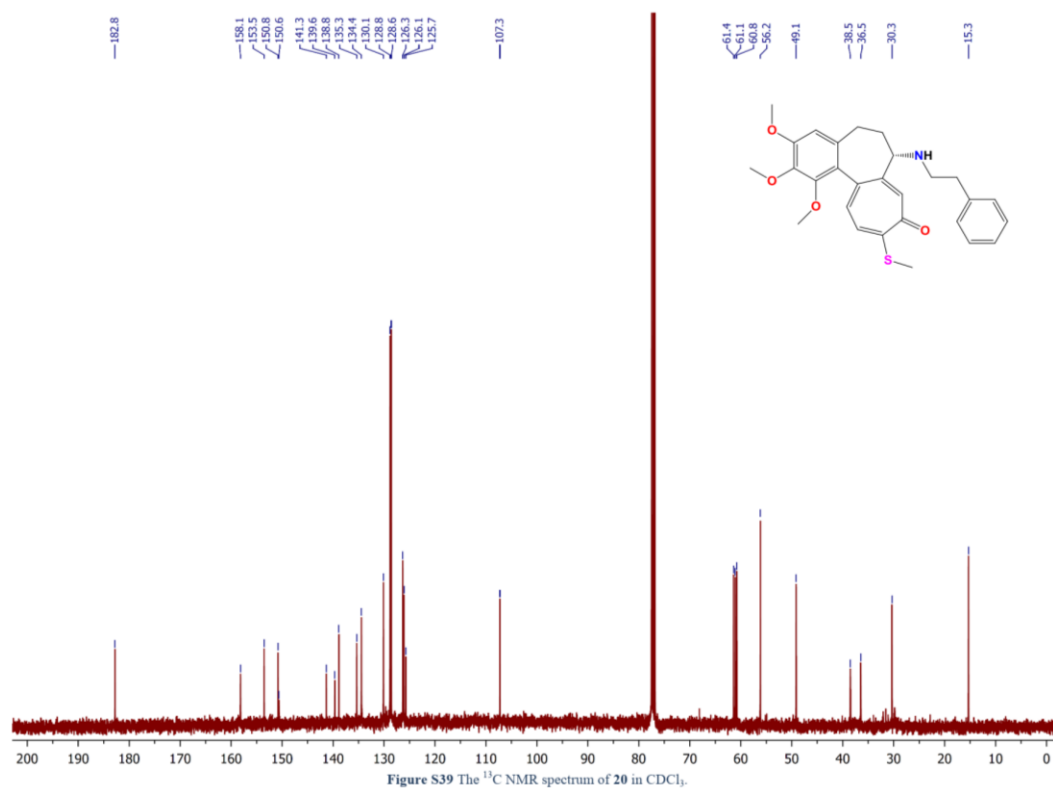


Figure S39 The ¹³C NMR spectrum of 20 in CDCl₃.

63

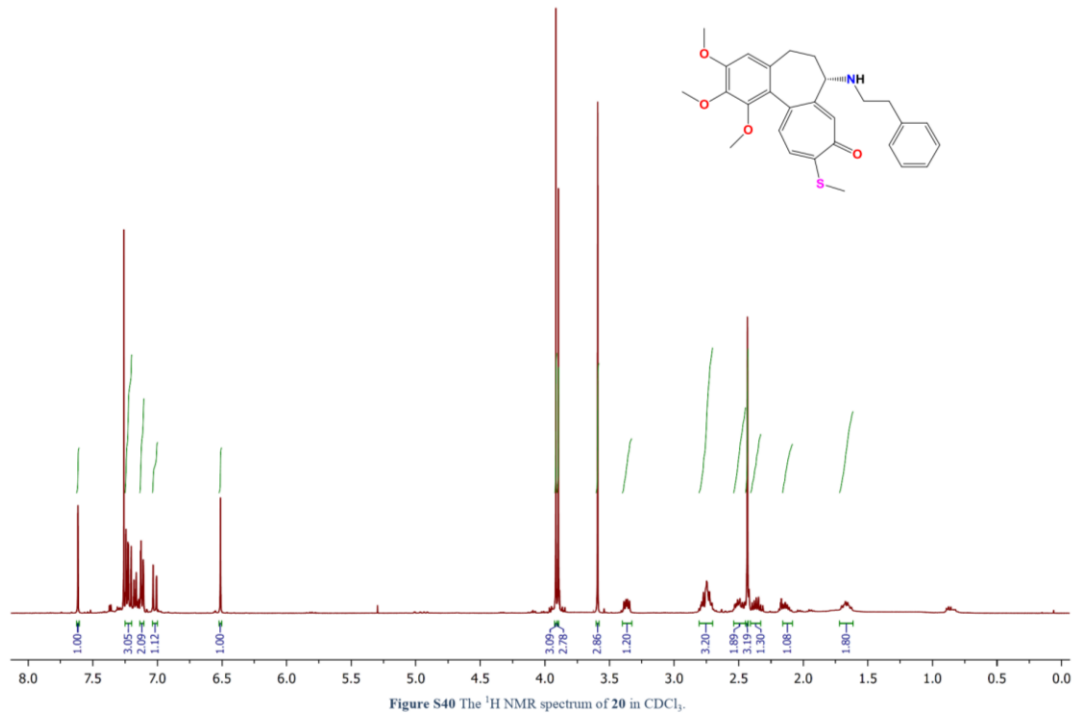
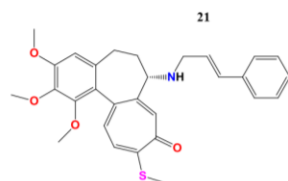


Figure S40 The ¹H NMR spectrum of 20 in CDCl₃.

64



Chemical Formula: $C_{29}H_{31}NO_4S$
Molecular Weight: 489.63

Yield: 93.9 %

^{13}C NMR (101 MHz, $CDCl_3$) δ 182.6, 157.8, 153.3, 150.7, 150.4, 141.2, 138.8, 136.7, 135.9, 135.2, 134.3, 131.6, 130.0, 128.4, 128.2, 127.7, 127.4, 126.2, 126.0, 125.5, 107.1, 61.2, 60.9, 59.7, 56.0, 49.7, 38.6, 30.3, 15.1 ppm.

1H NMR (400 MHz, $CDCl_3$) δ 7.64 (s, 1H), 7.25 – 7.23 (m, 4H), 7.21 – 7.16 (m, 2H), 7.03 (d, $J = 10.6$ Hz, 1H), 6.53 (s, 1H), 6.35 (d, $J = 15.9$ Hz, 1H), 6.15 (ddd, $J = 15.8, 7.0, 5.7$ Hz, 1H), 3.89 (s, 3H), 3.86 (s, 3H), 3.52 (s, 3H), 3.47 (dd, $J = 10.9, 6.3$ Hz, 1H), 3.34 (ddd, $J = 13.7, 5.6, 1.4$ Hz, 1H), 3.13 – 3.05 (m, 1H), 2.51 – 2.45 (m, 1H), 2.44 (s, $J = 4.2$ Hz, 3H), 2.41 – 2.33 (m, 1H), 2.28 – 2.18 (m, 1H), 1.72 (td, $J = 11.0, 6.2$ Hz, 2H).

ESI-MS (m/z): $[M+H]^+$ 490, $[M+Na]^+$ 512, $[2M+Na]^+$ 1001.

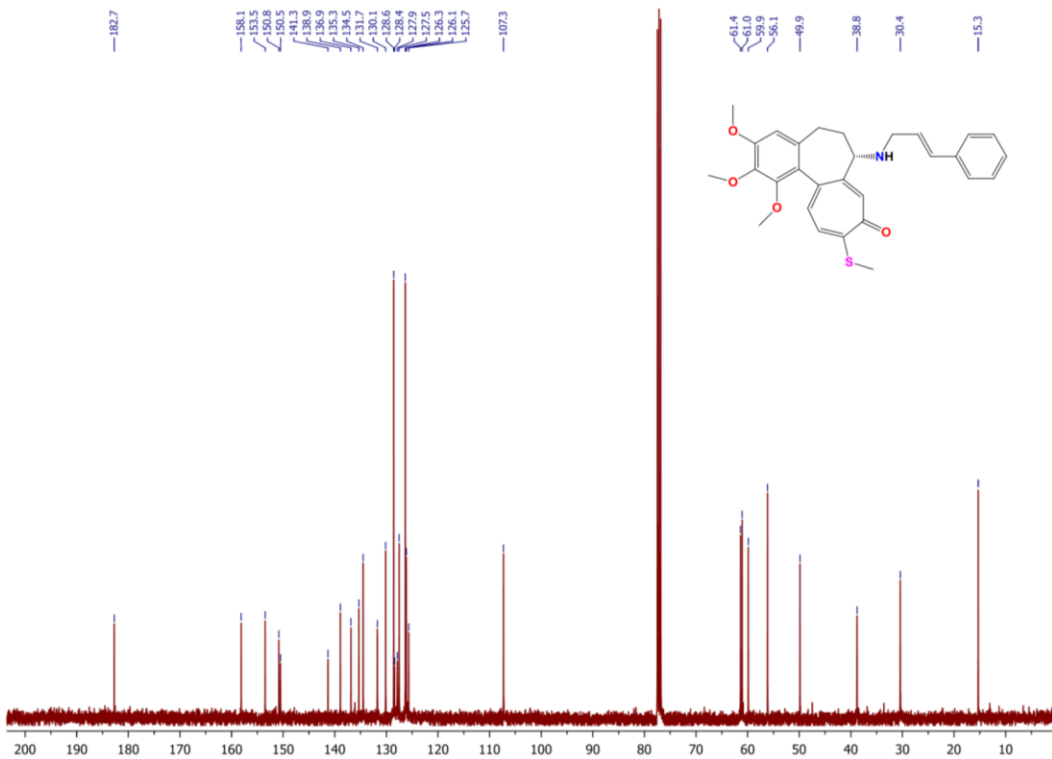
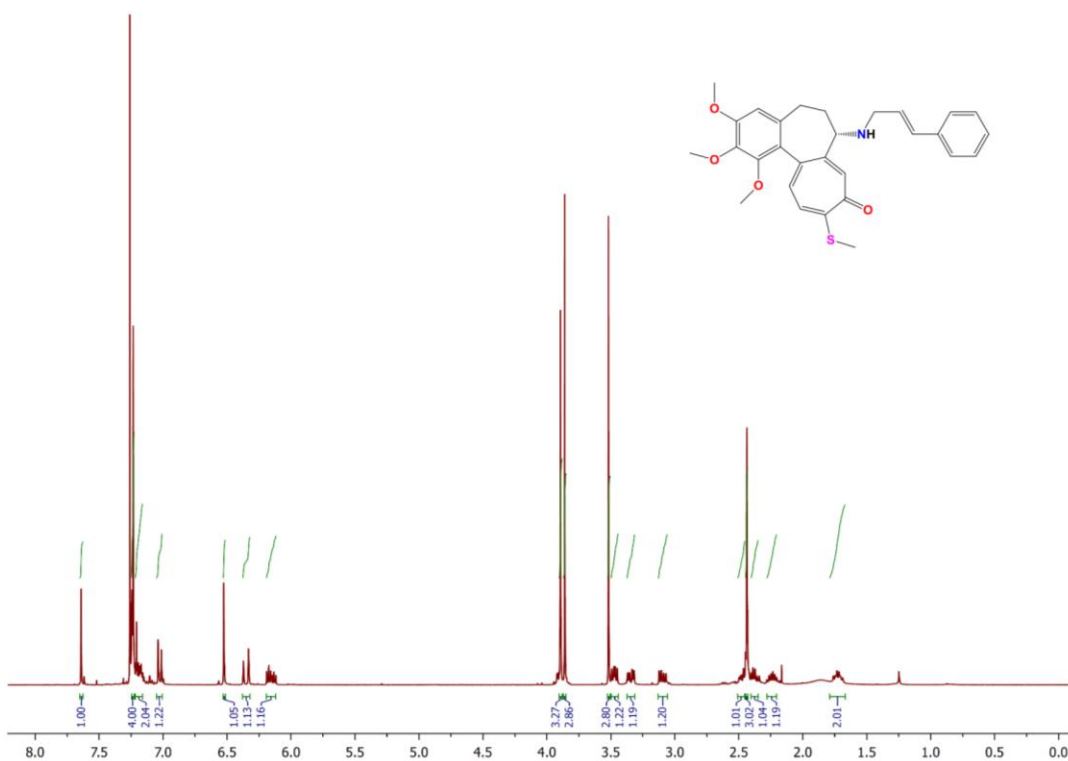


Figure S41 The ^{13}C NMR spectrum of **21** in $CDCl_3$.

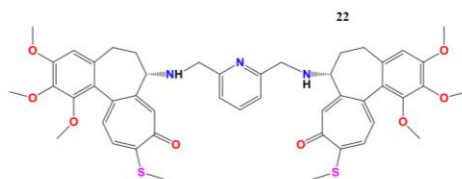
65

66



67

Figure S42 The ^1H NMR spectrum of 21 in CDCl_3 .



Chemical Formula: $\text{C}_{47}\text{H}_{51}\text{N}_3\text{O}_8\text{S}_2$

Molecular Weight: 850,05

Yield: 32.5 %

^{13}C NMR (101 MHz, CDCl_3) δ 182.6, 158.2, 157.9, 153.4, 150.7, 150.6, 141.1, 139.0, 137.0, 135.2, 134.4, 130.0, 126.2, 125.4, 120.7, 107.2, 61.2, 60.92, 60.85, 56.0, 53.2, 38.4, 30.2, 15.1 ppm.

^1H NMR (400 MHz, CDCl_3) δ 7.73 (s, 1H), 7.46 (t, $J = 7.7$ Hz, 1H), 7.23 (d, $J = 10.3$ Hz, 1H), 7.05 (d, $J = 5.4$ Hz, 1H), 7.02 (d, $J = 2.4$ Hz, 1H), 6.53 (s, $J = 3.9$ Hz, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.77 (d, $J = 13.9$ Hz, 1H), 3.57 (s, 3H), 3.51 – 3.44 (m, 2H), 2.50 – 2.46 (m, 1H), 2.42 (s, 3H), 2.39 – 2.24 (m, 4H), 1.83 – 1.76 (m, 1H) ppm.

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ 850.

68

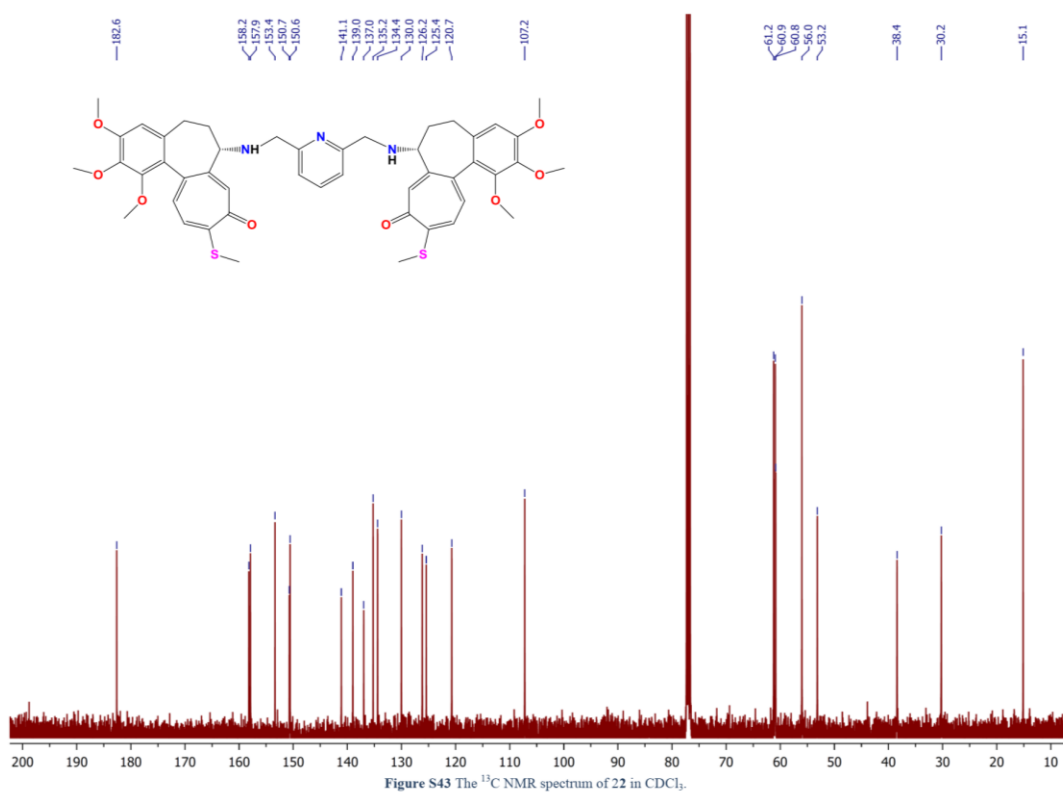


Figure S43 The ^{13}C NMR spectrum of 22 in CDCl_3 .

69

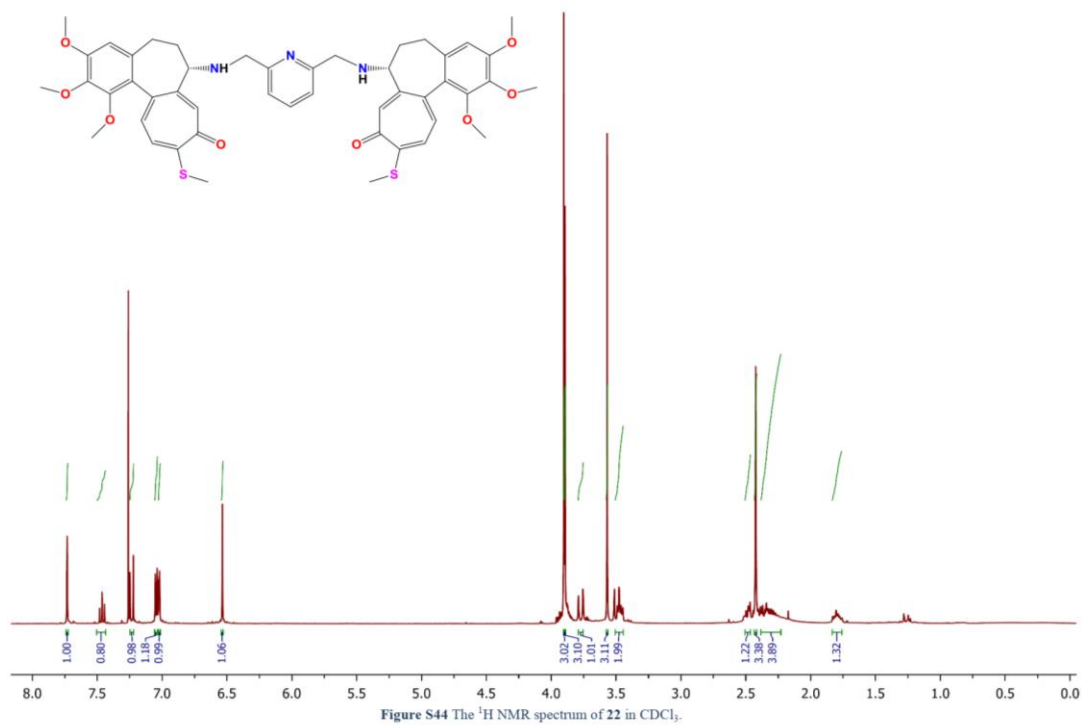


Figure S44 The ^1H NMR spectrum of 22 in CDCl_3 .

70

Oświadczenia współautorów

Poznań, 31 marca 2022 r.

Dominika Czerwona
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

Oświadczenie doktoranta

W związku z ubieganiem się o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

- **Dominika Czerwona**, Alicja Urbaniak, Szymon Sobczak, Sergio Piña-Oviedo, Timothy C. Chambers, Michał Antoszczak, Adam Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, **ChemMedChem** 2020, 15, 236–246.

Mój udział w publikacji obejmował syntezę oraz oczyszczenie 21 pochodnych salinomycyny (w tym 13 nowo otrzymanych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam także odpowiedzialna za ich krystalizację. Przygotowałam również wstęp oraz opis syntezy do manuskryptu, a także suplement. Mój udział procentowy stanowi 30%.

- **Dominika Czerwona**, Magdalena Mielczarek-Puta, Michał Antoszczak, Aleksandra Cioch, Marta Struga, Adam Huczyński, „*Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin*”, **European Journal of Pharmacology** 2021, 908, 174347.

Mój udział w publikacji obejmował syntezę oraz oczyszczenie 20 pochodnych salinomycyny (w tym 18 nowo otrzymanych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Przygotowałam również roboczą wersję manuskryptu oraz suplement. Mój udział procentowy stanowi 60%.



- **Dominika Czerwona**, Sebastian Müller, Tatiana Cañeque, Ludovic Colombeau, Adam Huczyński, Michał Antoszczak, Raphaël Rodriguez, „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*”, **ACS Organic & Inorganic Au** 2022, DOI: 10.1021/acsorginorgau.1c00046.³

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 18 nowych pochodnych salinomycyny. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za przeprowadzenie części badań biologicznych *in vitro* na liniach komórek nowotworowych w trakcie 3-miesięcznego stażu w Insytucie Curie w Paryżu, a także za analizę i opracowanie wyników przeprowadzonych badań. Przygotowałam manuskrypt, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement. Mój udział procentowy stanowi 50%.

- **Dominika Czerwona**, Szymon Sobczak, Ewa Maj, Joanna Wietrzyk, Andrzej Katrusiak, Adam Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, **Molecules** 2020, 25, 1180.

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 12 pochodnych kolchicyny (w tym 9 nowych pochodnych). Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za ich krystalizację. Przygotowałam kolejne wersje manuskryptu, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement, a także brałam aktywny udział w dyskusji z recenzentami. Mój udział procentowy stanowi 65%.

- **Dominika Czerwona**, Szymon Sobczak, Tomasz Pędziński, Ewa Maj, Joanna Wietrzyk, Lech Celewicz, Andrzej Katrusiak, Adam Huczyński, „*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, **Journal of Organic Chemistry** 2021, 86, 16, 11029–11039.

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 17 nowych pochodnych kolchicyny, w tym syntezę 6 nowych lumipochodnych. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Byłam również odpowiedzialna za ich krystalizację. Przygotowałam kolejne wersje manuskryptu, łącznie z opisem i dyskusją wyników badań biologicznych oraz

Pracownia
Huczyński

suplement, a także brałam aktywny udział w dyskusji z recenzentami. Mój udział procentowy stanowi 55%.

- **Dominika Czerwonka**, Ewa Maj, Joanna Wietrzyk, Adam Huczyński, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, **Bioorganic & Medicinal Chemistry Letters** 2021, 52, 128382.

Mój udział w publikacji obejmował zaprojektowanie, syntezę oraz oczyszczenie 22 nowych pochodnych kolchicyny. Przygotowywałam również próbki na analizy spektroskopowe i spektrometryczne (NMR, ESI-MS), a także przeprowadziłam analizę otrzymanych wyników w celu określenia struktury i czystości otrzymanych związków. Przygotowałam manuskrypt, łącznie z opisem i dyskusją wyników badań biologicznych oraz suplement, a także brałam aktywny udział w dyskusji z recenzentami. Mój udział procentowy stanowi 70%.





UNIwersytet
IM. ADAMA MICKIEWICZA
W POZNANIU

Prof. dr hab. Adam Huczyński
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 8, 61-614 Poznań
e-mail: adam.huczynski@amu.edu.pl
<https://adhucz.home.amu.edu.pl/>

Poznań, 21 marca 2022 roku

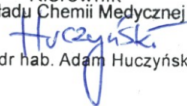
Oświadczenie promotora

W związku z ubieganiem się mgr Dominiki Czerwonki o uzyskanie stopnia doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

- D. Czerwonka, A. Urbaniak, S. Sobczak, S. Piña-Oviedo, T.C. Chambers, M. Antoszczak, **A. Huczyński**, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem, 2020, 15, 236–246. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, M. Mielczarek-Puta, M. Antoszczak, A. Cioch, M. Struga, **A. Huczyński**, „*Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin*” European Journal of Pharmacology 2021, 908, 174347. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, S. Müller, T. Cañeque, L. Colombeau, **A. Huczyński**, M. Antoszczak, R. Rodriguez, „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Organic & Inorganic Au 2022, DOI: 10.1021/acsorginorgau.1c00046. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.

str. 1

- D. Czerwonka, S. Sobczak, E. Maj, J. Wietrzyk, A. Katrusiak, **A. Huczyński**, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, *Molecules*, 2020, 25, 1180. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, S. Sobczak, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, **A. Huczyński**, „*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, *The Journal of Organic Chemistry*, 2021, 86, 16, 11029–11039. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, E. Maj, J. Wietrzyk, **A. Huczyński**, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, *Bioorganic & Medicinal Chemistry Letters* 2021, 52, 128382. Mój udział w przygotowaniu w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorem nad ich realizacją i współredakcją manuskryptu. Mój udział procentowy stanowi 10 %.

Kierownik
Zakładu Chemii Medycznej

prof. dr hab. Adam Huczyński

Poznań, 21 marca 2022 r.

dr Michał Antoszczak
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

Oświadczenie promotora pomocniczego

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

- D. Czerwonka, A. Urbaniak, S. Sobczak, S. Piña-Oviedo, T.C. Chambers, **M. Antoszczak**, A. Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem, 2020, 15, 236–246. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorze nad ich realizacją i współredakcji manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, M. Mielczarek-Put, **M. Antoszczak**, A. Cioch, M. Struga, A. Huczyński, „*Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin*” European Journal of Pharmacology 2021, 908, 174347. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorze nad ich realizacją i współredakcji manuskryptu. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, S. Müller, T. Cañeque, L. Colombeau, A. Huczyński, **M. Antoszczak**, R. Rodriguez, „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Organic & Inorganic Au 2022, DOI: 10.1021/acsorginorgau.1c00046. Mój udział w przygotowaniu tej publikacji polegał na współtworzeniu koncepcji badań, nadzorze nad ich realizacją i współredakcji manuskryptu. Mój udział procentowy stanowi 10 %.

Michał Antoszczak

Little Rock, 28 January 2022

Dr. Alicja Urbaniak
Department of Biochemistry and Molecular Biology,
University of Arkansas for Medical Sciences,
Little Rock

Co-author declaration

With reference to the Dominika Czerwionka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwionka, **A. Urbaniak**, S. Sobczak, S. Piña-Oviedo, T.C. Chambers, M. Antoszczak, A. Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem, 2020, 15, 236–246. My contribution to this paper concerns performance of: cell viability assays and *ex vivo* study, analysis of data associated with these experiments, and their description in the manuscript. My percentage contribution is 30%.



Little Rock, AR
January 31st, 2022

Dr. Sergio Piña-Oviedo,
Department of Pathology,
University of Arkansas for Medical Sciences,
Little Rock

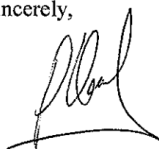
Co-author declaration

With reference to the Dominika Czerwinka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwinka, A. Urbaniak, S. Sobczak, **S. Piña-Oviedo**, T.C. Chambers, M. Antoszczak, A. Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem, 2020, 15, 236–246. My contribution to this paper concerns performance of microscopic interpretation of the effects in tumor tissue samples of the drugs mentioned above, as well as taking photographs of representative cases. My percentage contribution is 5%.

Please do not hesitate to contact me in case you have any questions.

Sincerely,



Sergio Pina Oviedo, MD

Little Rock, 27 January 2022

Dr. Timothy C. Chambers
Department of Biochemistry and Molecular Biology,
University of Arkansas for Medical Sciences,
Little Rock

Co-author declaration

With reference to the Dominika Czerwonka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwonka, A. Urbaniak, S. Sobczak, S. Piña-Oviedo, **T.C. Chambers**, M. Antoszczak, A. Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, ChemMedChem, 2020, 15, 236–246. My contribution to this paper concerns performance of experimental design, data interpretation, and writing and editing of the manuscript. My percentage contribution is 10%.

Signed: T. Chambers

Date: 27 January, 2022

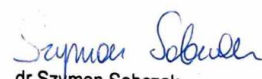
Poznań, 31 stycznia 2022 r.

dr Szymon Sobczak
Zakład Chemii Materiałów, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem wymienionych publikacji:

- D. Czerwonka, **S. Sobczak**, E. Maj, J. Wietrzyk, A. Katrusiak, A. Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, *Molecules*, 2020, 25, 1180. Mój wkład w przygotowanie tej publikacji polegał na wykonaniu i rozwiązaniu pomiarów krystalograficznych oraz na udziale w przygotowaniu wersji roboczej manuskryptu. Mój udział procentowy stanowi 5%.
- D. Czerwonka, **S. Sobczak**, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, *The Journal of Organic Chemistry*, 2021, 86, 16, 11029–11039. Mój wkład w przygotowanie tej publikacji polegał na wykonaniu i rozwiązaniu pomiarów krystalograficznych oraz na udziale w przygotowaniu wersji roboczej manuskryptu. Mój udział procentowy stanowi 5%.
- D. Czerwonka, A. Urbaniak, **S. Sobczak**, S. Piña-Oviedo, T.C. Chambers, M. Antoszczak, A. Huczyński, „*Synthesis and anticancer activity of tertiary amides of salinomycin and their C20-oxo analogs*”, *ChemMedChem*, 2020, 15, 236–246. Mój wkład w przygotowanie tej publikacji polegał na wykonaniu, rozwiązaniu oraz analizie wyników pomiarów krystalograficznych oraz na udziale w przygotowaniu wersji roboczej manuskryptu. Mój udział procentowy stanowi 5%.


dr Szymon Sobczak

Poznań, 24 stycznia, 2022 r.

Prof. dr hab. Andrzej Katrusiak
Zakład Chemii Materiałów, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem wymienionych publikacji:

- D. Czerwonka, S. Sobczak, E. Maj, J. Wietrzyk, **A. Katrusiak**, A. Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, *Molecules*, 2020, 25, 1180. Mój wkład w przygotowanie tej publikacji polegał na udziale w analizie pomiarów krystalograficznych oraz na udziale w redagowaniu ostatecznej wersji manuskryptu. Mój udział procentowy stanowi 5 %.
- D. Czerwonka, S. Sobczak, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, **A. Katrusiak**, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, *The Journal of Organic Chemistry*, 2021, 86, 16, 11029–11039. Mój wkład w przygotowanie tej publikacji polegał na udziale w analizie pomiarów krystalograficznych oraz na udziale w redagowaniu ostatecznej wersji manuskryptu. Mój udział procentowy stanowi 5 %.

Kierownik
Zakładu Chemii Materiałów

prof. dr hab. Andrzej Katrusiak



WARSZAWSKI
UNIwersytet
MEDYCZNY

KATEDRA I ZAKŁAD BIOCHEMII

Warszawa, 24 stycznia 2022 r.

Prof. dr n. med. Marta Struga
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

D. Czerwonka, M. Mielczarek-Puta, M. Antoszczak, A. Cioch, **M. Struga**, A. Huczyński, „Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin” European Journal of Pharmacology, 2021, 908, 174347. Mój wkład w przygotowanie tej publikacji polegał na zaplanowaniu części badań aktywności antyproliferacyjnej, analizie wyników tych badań oraz na redagowaniu ostatecznej wersji manuskryptu w części poświęconej badaniom biologicznym. Mój udział procentowy stanowi 5 %.

KIEROWNIK
Katedry i Zakładu Biochemii
Marta Struga
prof. dr hab. n. med. Marta Struga

ul. Banacha 1
02-097 Warszawa
www.biochemia.wum.edu.pl

tel.: +48 22 57 20 693; 57 20 639
fax: +48 22 57 20 679
biochemia@wum.edu.pl



WARSZAWSKI
UNIwersYTET
MEDYCZNY

KATEDRA I ZAKŁAD BIOCHEMII

Warszawa, 27.01.2021

dr Magdalena Mielczarek-Puta
Katedra i Zakład Biochemii, Wydział Lekarski
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

D. Czerwonka, **M. Mielczarek-Puta**, M. Antoszczak, A. Cioch, M. Struga, A. Huczyński, „Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin” *European Journal of Pharmacology*, 2021, 908, 174347. Mój udział w przygotowaniu tej publikacji polegał na ocenie cytotoksyczności badanych związków (test MTT) oraz ich mechanizmu działania (apoptoza i test IL-6), analizie uzyskanych wyników, a także na przygotowaniu roboczej wersji manuskryptu. Mój udział procentowy stanowi 10 %.

Magdalena Mielczarek-Puta

ul. Banacha 1
02-097 Warszawa
www.biochemia.wum.edu.pl

tel.: +48 22 57 20 693; 57 20 639
fax: + 48 22 57 20 679
biochemia@wum.edu.pl

Poznań, 26 stycznia 2022 r.

mgr Aleksandra Cioch-Biniaś
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorką publikacji:

D. Czerwonka, M. Mielczarek-Puta, M. Antoszczak, **A. Cioch**, M. Struga, A. Huczyński, „Evaluation of the anticancer activity of singly and doubly modified analogues of C20-epi-salinomycin” *European Journal of Pharmacology*, 908 (2021) 174347. Mój udział w przygotowaniu tej publikacji polegał na zsyntezowaniu i oczyszczeniu estru węglanowego salinomycyny w pozycji C20, a także jego 3 pochodnych modyfikowanych w pozycji C1. Przygotowałam również próbki na badania spektroskopowe i spektrometryczne. Mój udział procentowy stanowi 5%.

Aleksandra Cioch-Biniaś

Paris, 28 January 2022

Dr. Tatiana Cañeque
Department of Chemical Biology
Institut Curie, CNRS UMR 3666,
INSERM U1143, PSL Université,
75005 Paris, France

Co-author declaration

With reference to the Dominika Czerwanka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwanka, S. Müller, **T. Cañeque**, L. Colombeau, A. Huczyński, M. Antoszczak, R. Rodriguez: „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Org. Inorg. Au, 2022, DOI: 10.1021/acsorginorgau.1c00046. I was responsible performing the stability tests. My percentage contribution is 5%.



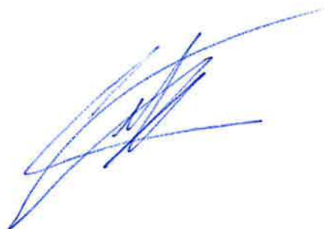
Paris, 27 January 2022

Dr. Ludovic Colombeau
Department of Chemical Biology
Institut Curie, CNRS UMR 3666,
INSERM U1143, PSL Université,
75005 Paris, France

Co-author declaration

With reference to the Dominika Czerwonka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwonka, S. Müller, T. Cañeque, **L. Colombeau**, A. Huczyński, M. Antoszczak, R. Rodriguez: „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Org. Inorg. Au, 2022, DOI: 10.1021/acsorginorgau.1c00046. I was responsible for editing the final version of the article. My percentage contribution is 5%.



Paris, 28 January 2022

Dr. Sebastian Müller
Department of Chemical Biology
Institut Curie, CNRS UMR 3666,
INSERM U1143, PSL Université,
75005 Paris, France

Co-author declaration

With reference to the Dominika Czerwanka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwanka, **S. Müller**, T. Cañeque, L. Colombeau, A. Huczyński, M. Antoszczak, R. Rodriguez: „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Org. Inorg. Au, 2022, DOI: 10.1021/acsorginorgau.1c00046. I was responsible for evaluating the compounds *in vitro*. My percentage contribution is 10%.



Paris, 28 January 2022

Dr. Raphaël Rodriguez
Department of Chemical Biology
Institut Curie, CNRS UMR 3666,
INSERM U1143, PSL Université,
75005 Paris, France

Co-author declaration

With reference to the Dominika Czerwonka's application for the Doctoral degree in Chemical Sciences at the Adam Mickiewicz University, I declare that I am a co-author of the publication:

D. Czerwonka, S. Müller, T. Cañeque, L. Colombeau, A. Huczyński, M. Antoszczak, **R. Rodriguez**: „*Expeditive Synthesis of Potent C20-epi-Amino Derivatives of Salinomycin against Cancer Stem-Like Cells*” ACS Org. Inorg. Au, 2022, DOI: 10.1021/acsorginorgau.1c00046. I was responsible for directing the research and writing the article. My percentage contribution is 10%.



Wrocław, 24 stycznia 2022 r.

dr Ewa Maj

Laboratorium Doświadczalnej Terapii Przeciwnowotworowej

Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda

Polskiej Akademii Nauk

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorką wymienionych publikacji:

- D. Czerwonka, S. Sobczak, E. Maj, J. Wietrzyk, A. Katrusiak, A. Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, *Molecules*, 2020, 25, 1180. Mój udział w przygotowaniu tej publikacji polegał na hodowli komórek *in vitro*, wykonaniu testów zahamowania proliferacji, analizie wyników SRB, obliczaniu parametru IC₅₀. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, S. Sobczak, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, *The Journal of Organic Chemistry*, 2021, 86, 16, 11029–11039. Mój udział w przygotowaniu tej publikacji polegał na hodowli komórek *in vitro*, wykonaniu testów zahamowania proliferacji, analizie wyników SRB, obliczaniu parametru IC₅₀. Mój udział procentowy stanowi 10 %.
- D. Czerwonka, E. Maj, J. Wietrzyk, A. Huczyński, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, *Bioorganic & Medicinal Chemistry Letters*, 2021, 52, 128382. Mój udział w przygotowaniu tej publikacji polegał na hodowli komórek *in vitro*, wykonaniu testów zahamowania proliferacji, analizie wyników SRB, obliczaniu parametru IC₅₀. Mój udział procentowy stanowi 15 %.

INSTYTUT IMMUNOLOGII
I TERAPII DOŚWIADCZALNEJ
im. Ludwika Hirszfelda
Polska Akademia Nauk
ZAKŁAD ONKOLOGII DOŚWIADCZALNEJ
ul. Rudolfa Weigla 12, 53-114 Wrocław
Tel. (71) 337-11-72, Fax (71) 337-13-82
NIP 896-000-56-96, REGON 000325383



Wrocław, 26 stycznia 2022 r.

Prof. dr hab. Joanna Wietrzyk
Laboratorium Doświadczalnej Terapii Przeciwnowotworowej
Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda
Polskiej Akademii Nauk

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorką wymienionych publikacji:

- D. Czerwonka, S. Sobczak, E. Maj, J. Wietrzyk, A. Katrusiak, A. Huczyński, „*Synthesis and antiproliferative screening of novel analogs of regioselectively demethylated colchicine and thiocolchicine*”, *Molecules*, 2020, 25, 1180. Mój wkład w przygotowanie tej publikacji polegał na udziale w zaplanowaniu badań aktywności antyproliferacyjnej, analizie wyników tych badań oraz na udziale w redagowaniu ostatecznej wersji manuskryptu. Mój udział procentowy stanowi 5%.
- D. Czerwonka, S. Sobczak, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, *The Journal of Organic Chemistry*, 2021, 86, 16, 11029–11039. Mój wkład w przygotowanie tej publikacji polegał na udziale w zaplanowaniu badań aktywności antyproliferacyjnej, analizie wyników tych badań oraz na udziale w redagowaniu ostatecznej wersji manuskryptu. Mój udział procentowy stanowi 5%.
- D. Czerwonka, E. Maj, J. Wietrzyk, A. Huczyński, „*Synthesis of thiocolchicine amine derivatives and evaluation of their antiproliferative activity*”, *Bioorganic & Medicinal Chemistry Letters*, 2021, 52, 128382. Mój wkład w przygotowanie tej publikacji polegał na udziale w zaplanowaniu badań aktywności antyproliferacyjnej, analizie wyników tych badań oraz na udziale w redagowaniu ostatecznej wersji manuskryptu. Mój udział procentowy stanowi 5%.


Prof. dr hab. Joanna Wietrzyk
Kierownik Laboratorium
Doświadczalnej Terapii Przeciwnowotworowej

Poznań, 25 stycznia 2022 r.

Prof. UAM dr hab. Lech Celewicz
Zakład Chemii Medycznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

D. Czerwonka, S. Sobczak, T. Pędziński, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, The Journal of Organic Chemistry, 2021, 86, 16, 11029–11039. Mój wkład w przygotowanie tej publikacji polegał na udziale w przeprowadzeniu pomiarów fotochemicznych oraz na pomocy w analizie wyników tych pomiarów. Mój udział procentowy stanowi 5%.

Lech Celewicz

Poznań, 24 stycznia 2022 r.

Prof. UAM dr hab. Tomasz Pędziński
Zakład Fizyki Chemicznej, Wydział Chemii
Uniwersytet im. Adama Mickiewicza

OŚWIADCZENIE

W związku z ubieganiem się mgr Dominiki Czerwonki o stopień doktora nauk chemicznych oświadczam, że jestem współautorem publikacji:

D. Czerwonka, S. Sobczak, **T. Pędziński**, E. Maj, J. Wietrzyk, L. Celewicz, A. Katrusiak, A. Huczyński, “*Photoinduced Skeletal Rearrangement of N-Substituted Colchicine Derivatives*”, The Journal of Organic Chemistry, 2021, 86, 16, 11029–11039.

Mój wkład w przygotowanie tej publikacji polegał na przeprowadzaniu i zanalizowaniu pomiarów laserowej femtosekundowej absorpcji przejściowej w celu ustalenia fotochemicznego mechanizmu przegrupowania *N*-podstawionych pochodnych kolchicyny. Mój udział procentowy szacuję na 5%.

Tomasz Pędziński