

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

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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 (IIET), Wrocław, Poland. Human lung adenocarcinoma cell line was cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IIET, 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 (IIET, 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 (IIET, 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 1.5×10^3 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.

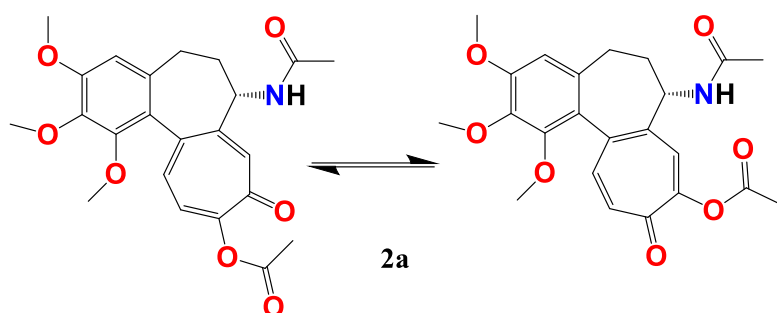
^1H NMR (CDCl_3 , 400 MHz) δ 7.92 (1H, s), 7.46 (1H, s), 7.36 – 7.26 (1H, m), 7.10 (1H, d, $J = 10.5$ Hz), 6.55 (1H, s), 4.73 – 4.63 (1H, m), 3.95 (3H, s), 3.91 (3H, s), 3.67 (3H, s), 2.54 (1H, dd, $J = 13.0, 5.8$ Hz), 2.45 (3H, s), 2.33 (2H, dtd, $J = 31.1, 12.8, 6.5$ Hz), 1.99 (3H, s), 1.96 – 1.85 (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

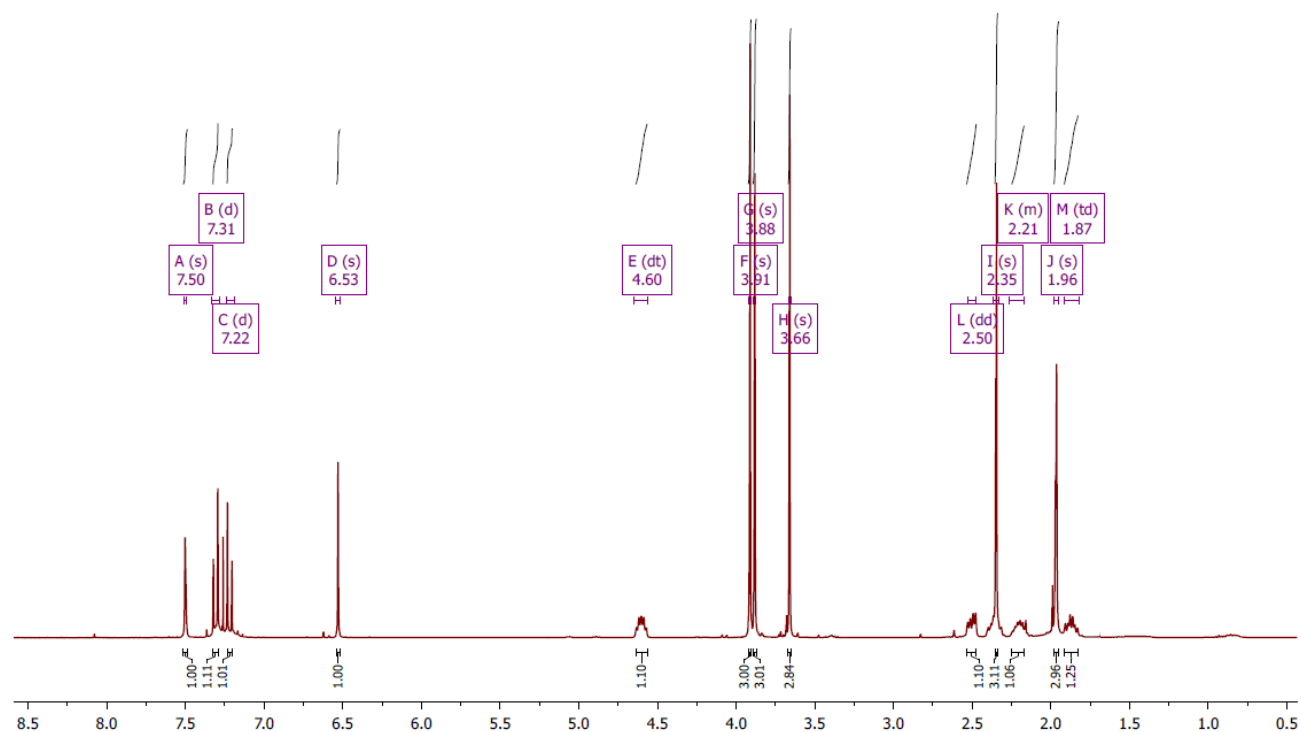


Figure S1. The ¹H NMR spectrum of **2a** in CDCl₃.

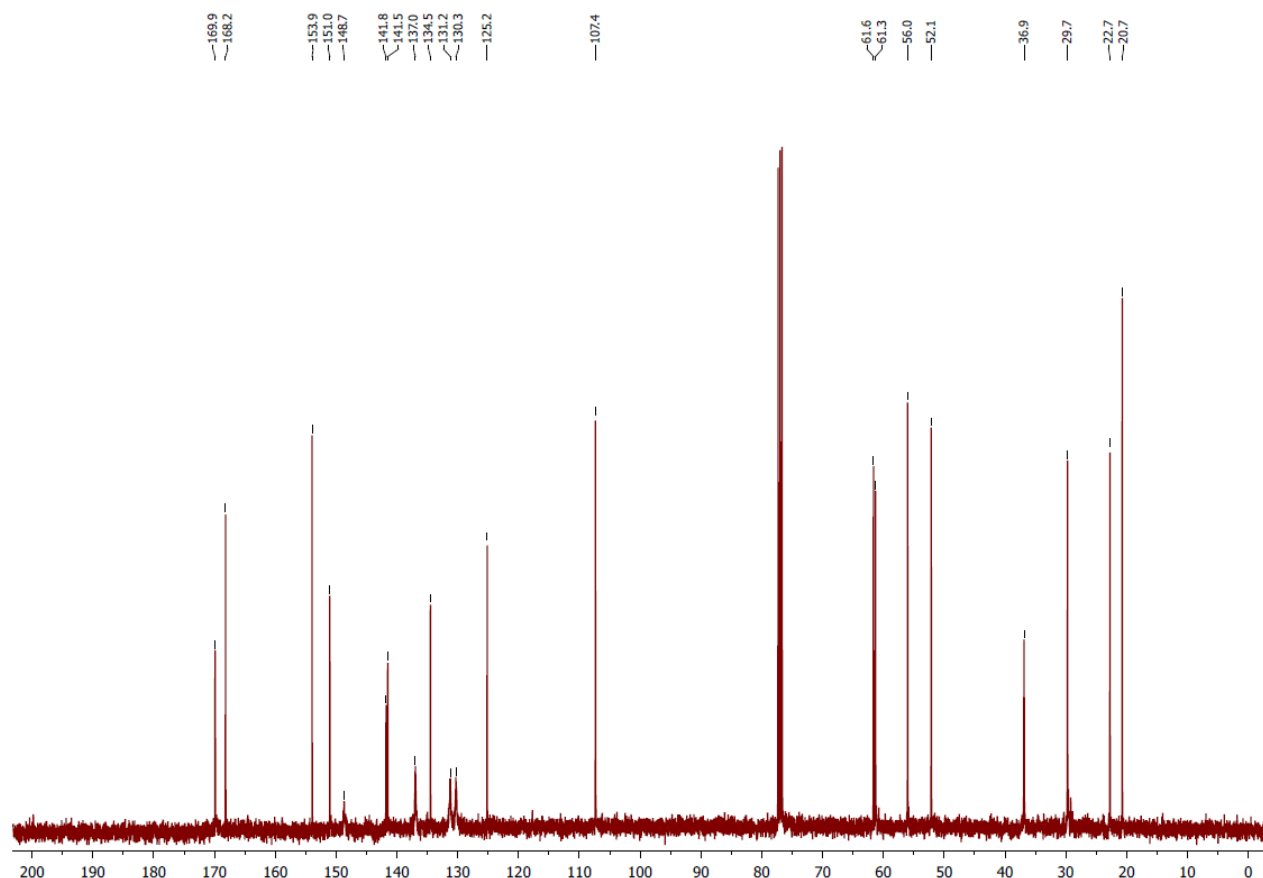


Figure S2. The ^{13}C NMR spectrum of **2a** in CDCl_3 .

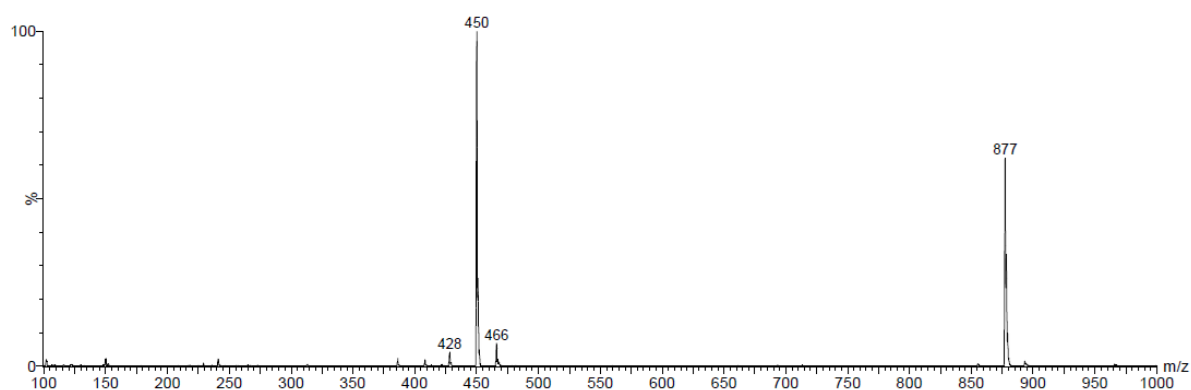
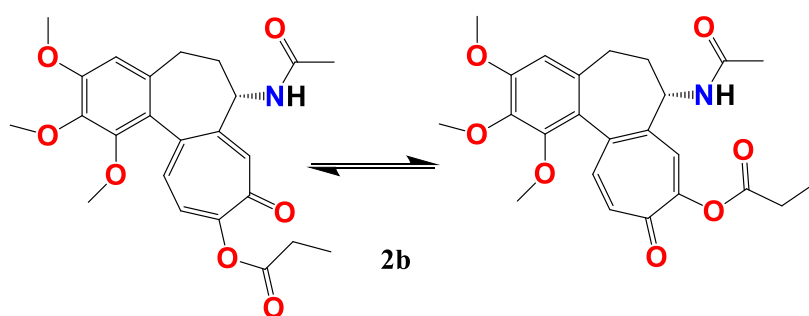


Figure S3. The ESI-MS spectrum of **2a**.



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

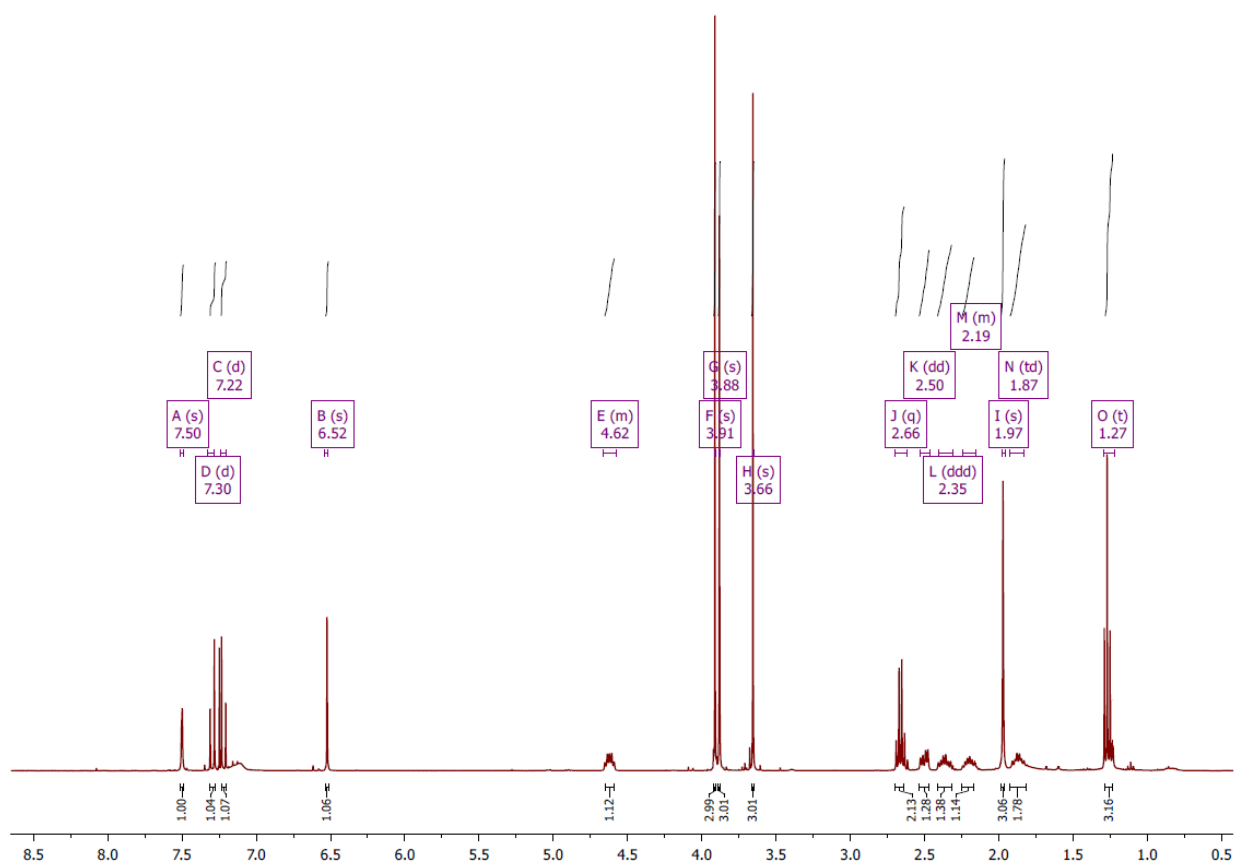


Figure S4. The ^1H NMR spectrum of **2b** in CDCl_3 .

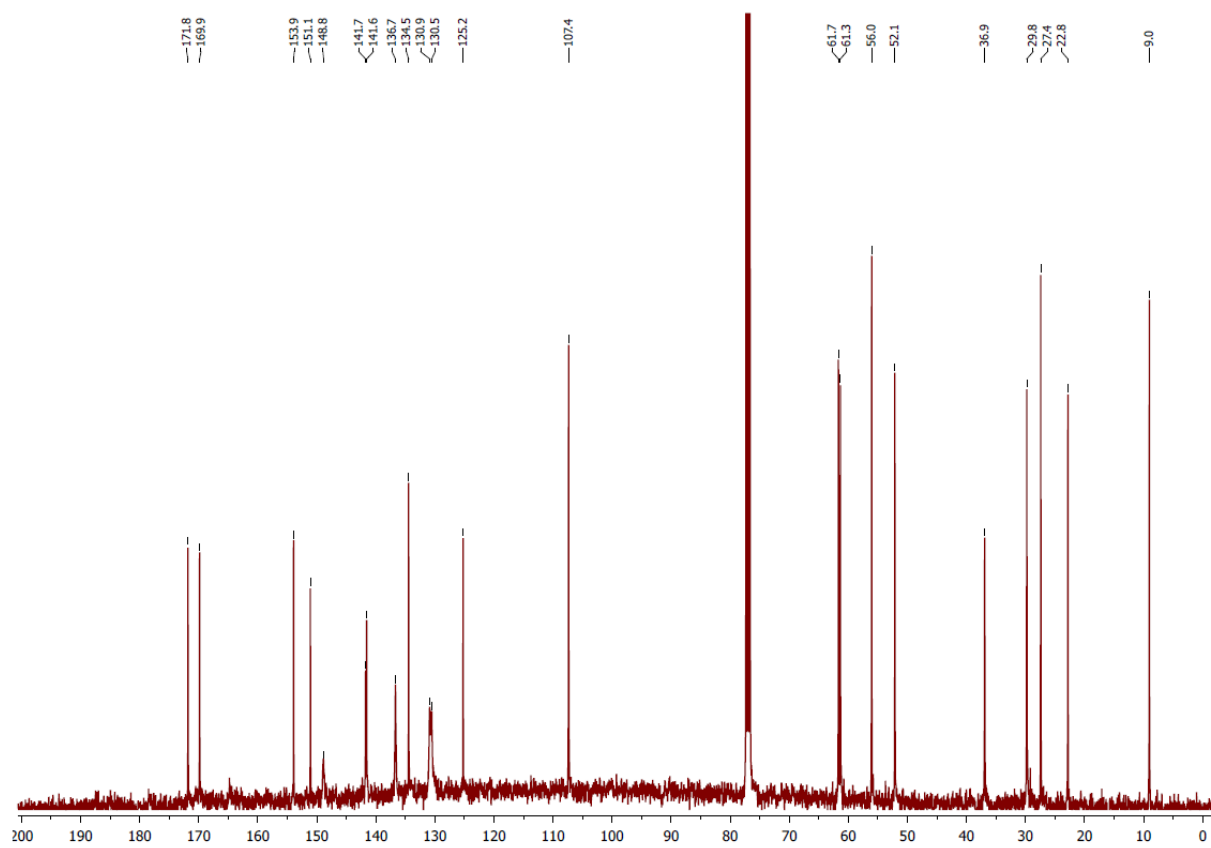


Figure S5. The ^{13}C NMR spectrum of **2b** in CDCl_3 .

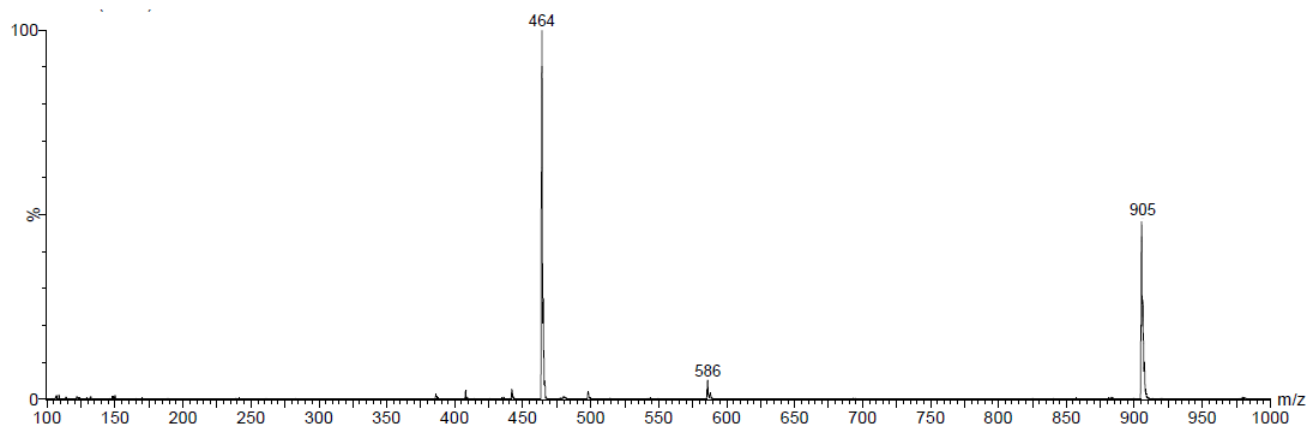
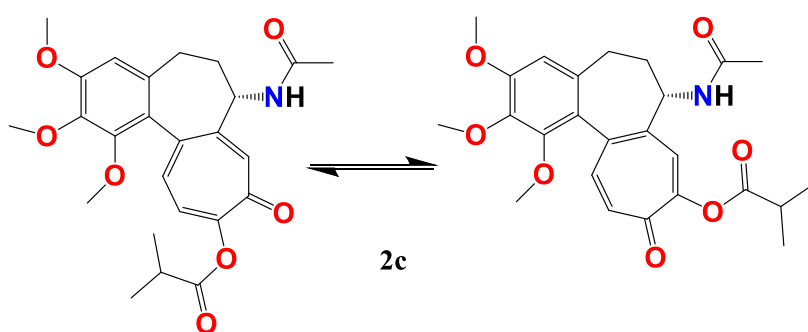


Figure S6. The ESI-MS spectrum of **2b**.



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

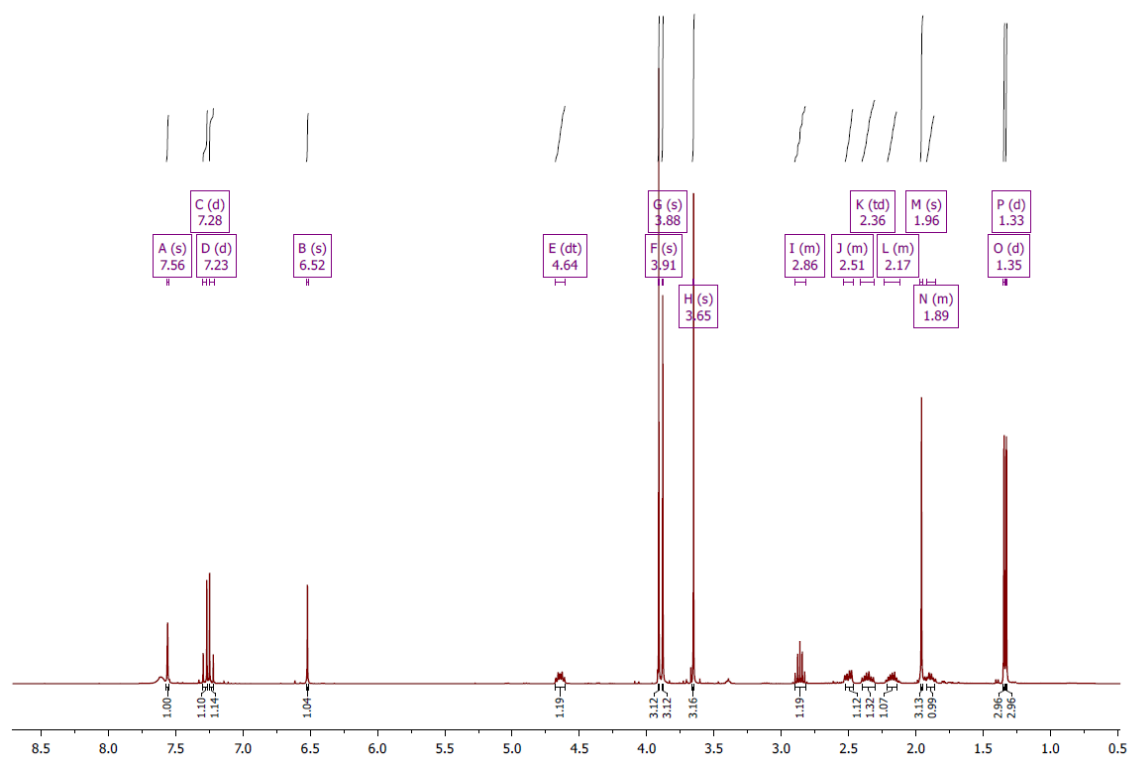


Figure S7. The 1H NMR spectrum of **2c** in $CDCl_3$.

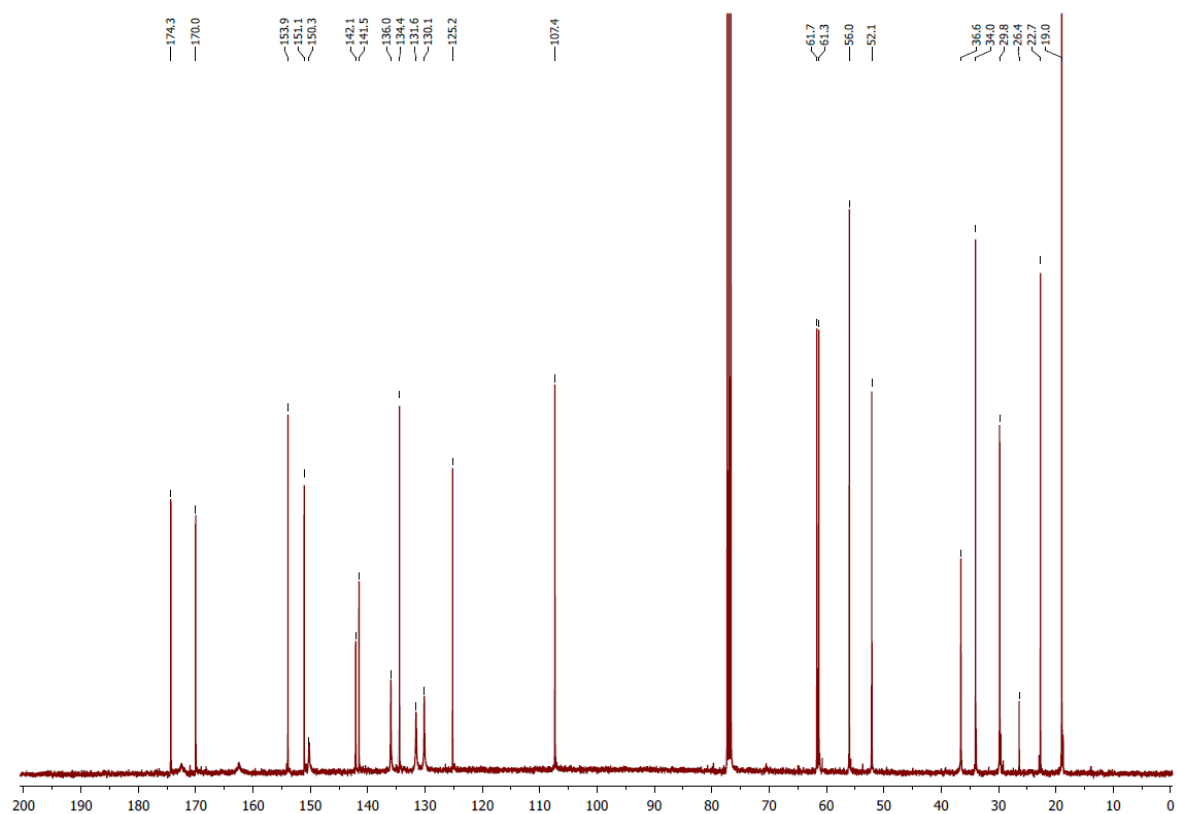


Figure S8. The ^{13}C NMR spectrum of **2c** in CDCl_3 .

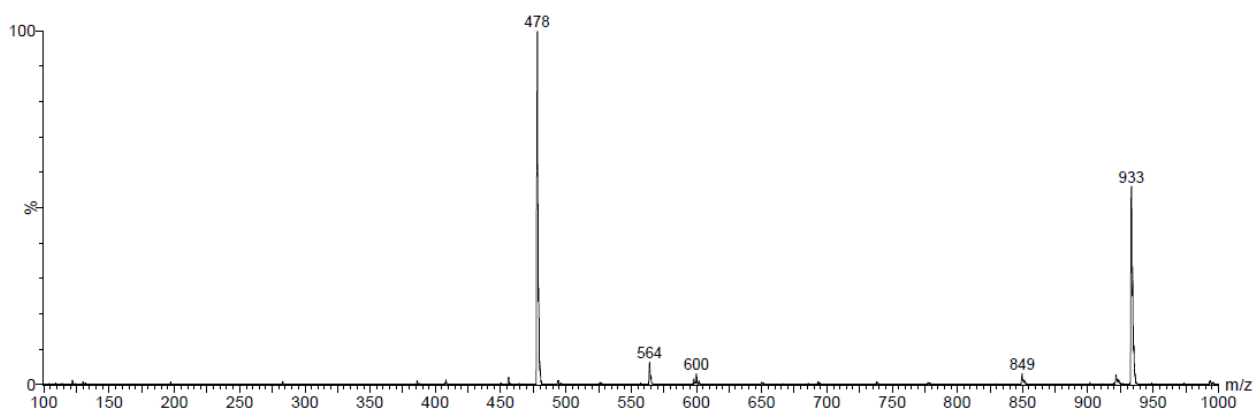
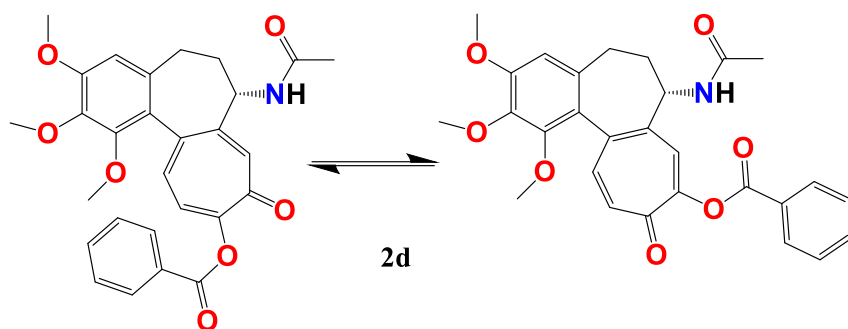


Figure S9. The ESI-MS spectrum of **2c**.



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

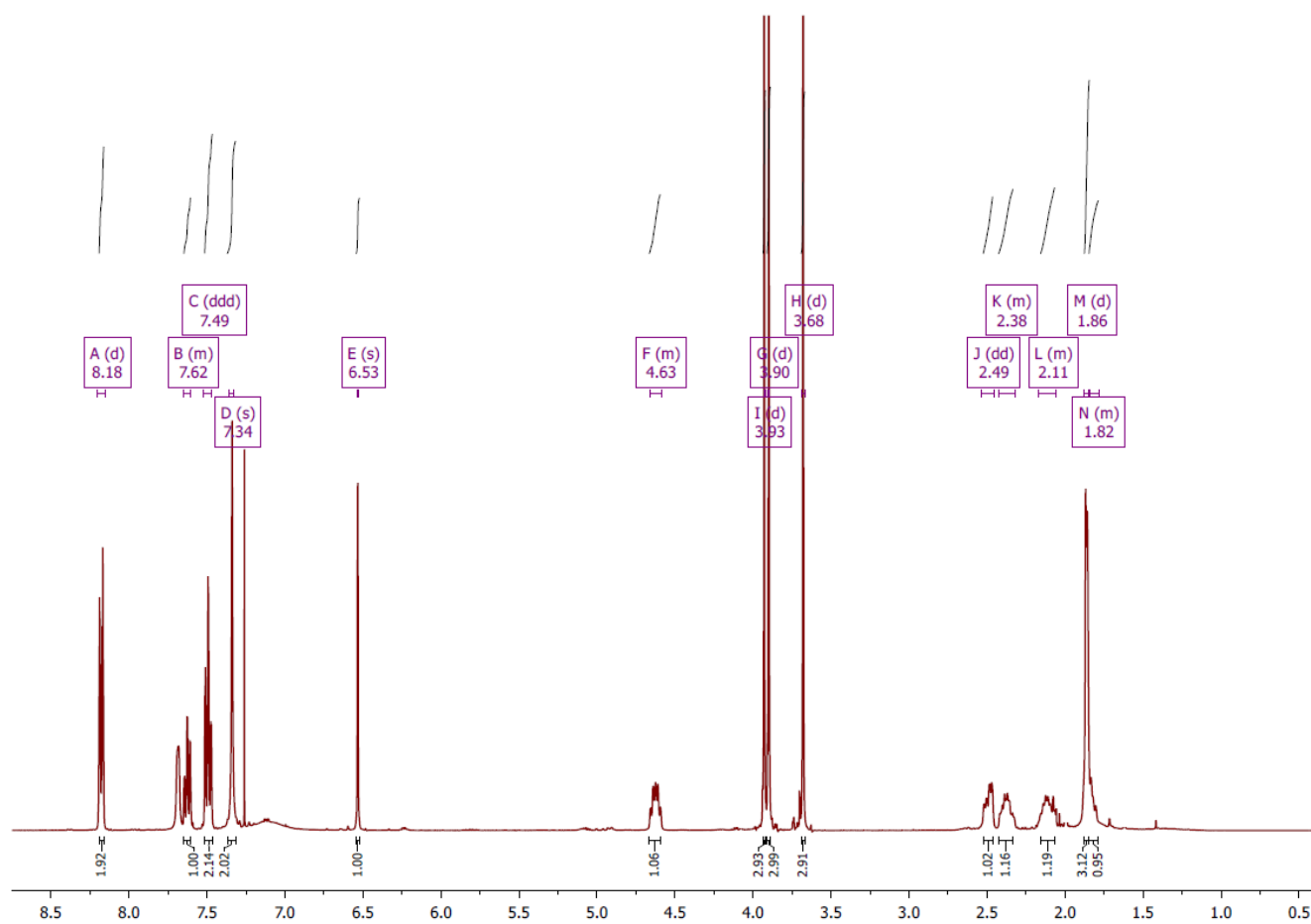


Figure S10. The 1H NMR spectrum of **2d** in $CDCl_3$.

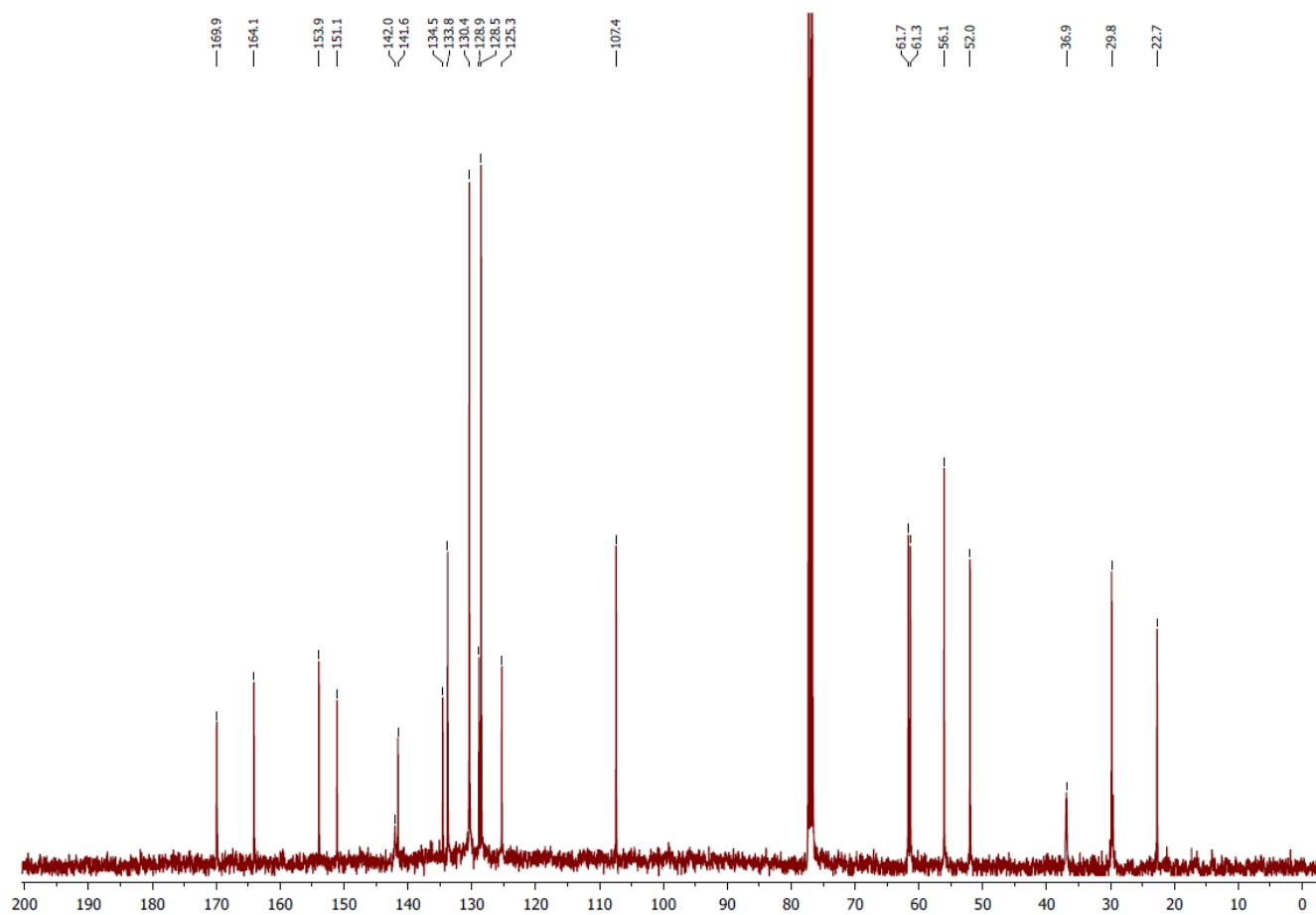


Figure S11. The ^{13}C NMR spectrum of **2d** in CDCl_3 .

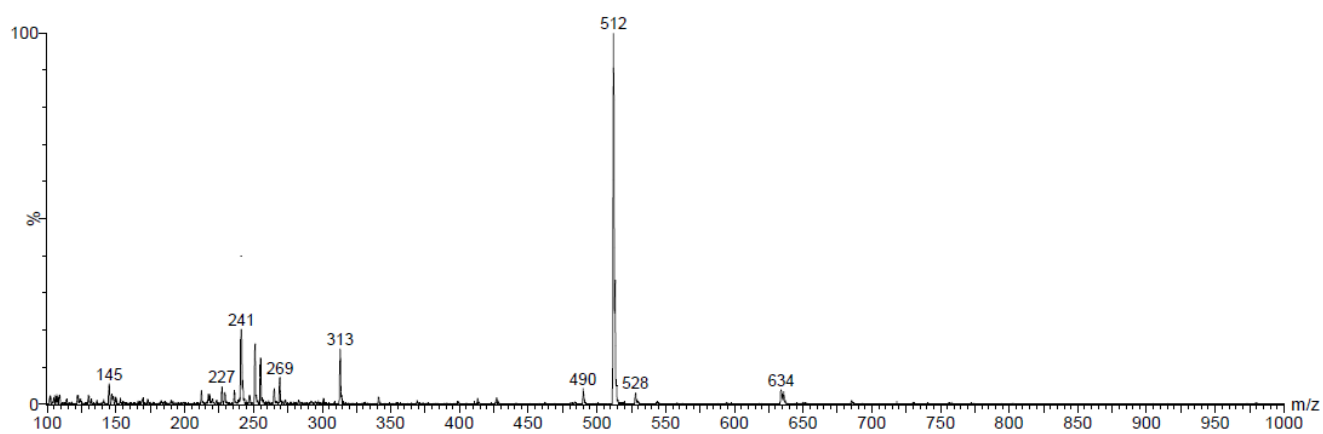
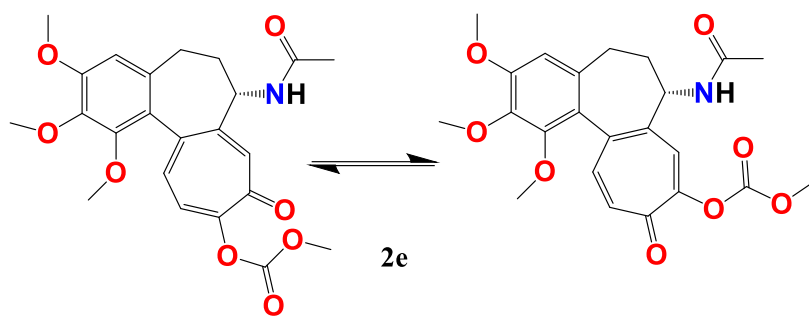


Figure S12. The ESI-MS spectrum of **2d**.



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

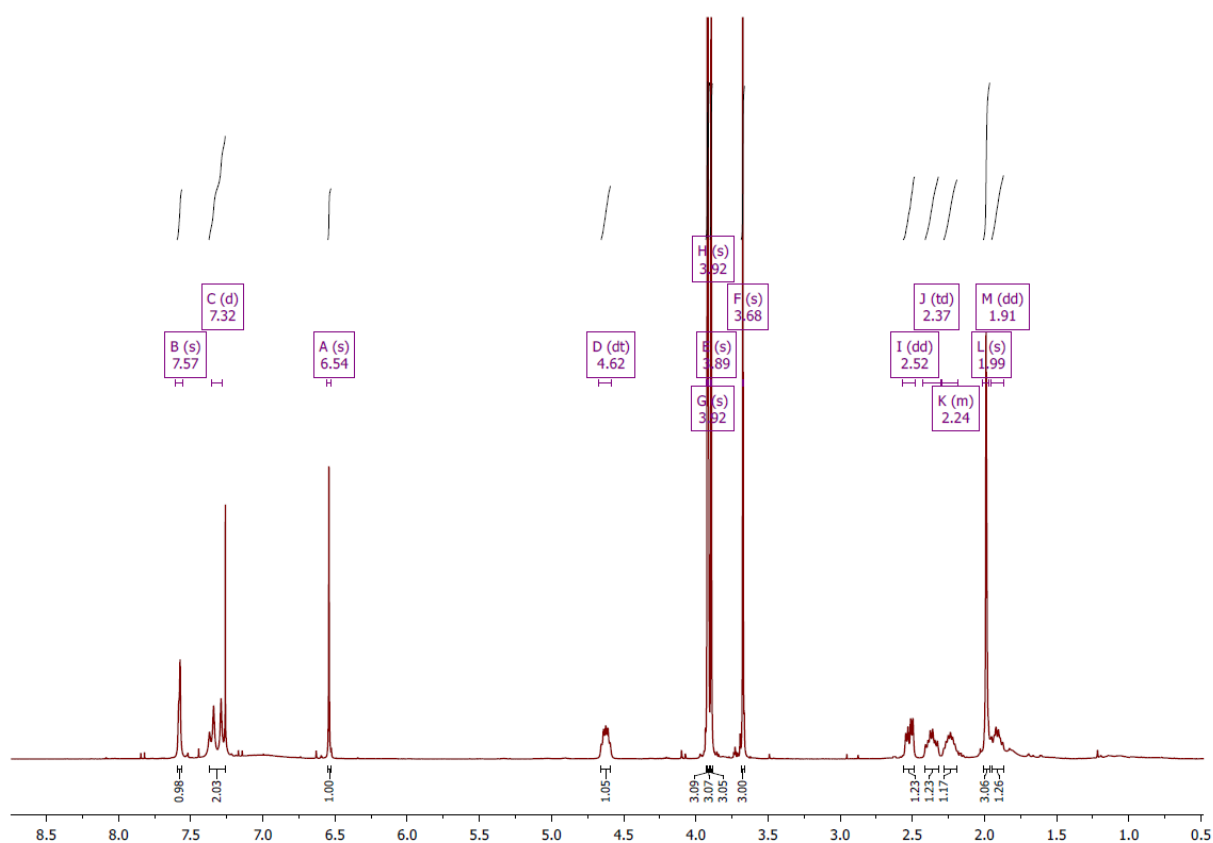


Figure S13. The 1H NMR spectrum of **2e** in $CDCl_3$.

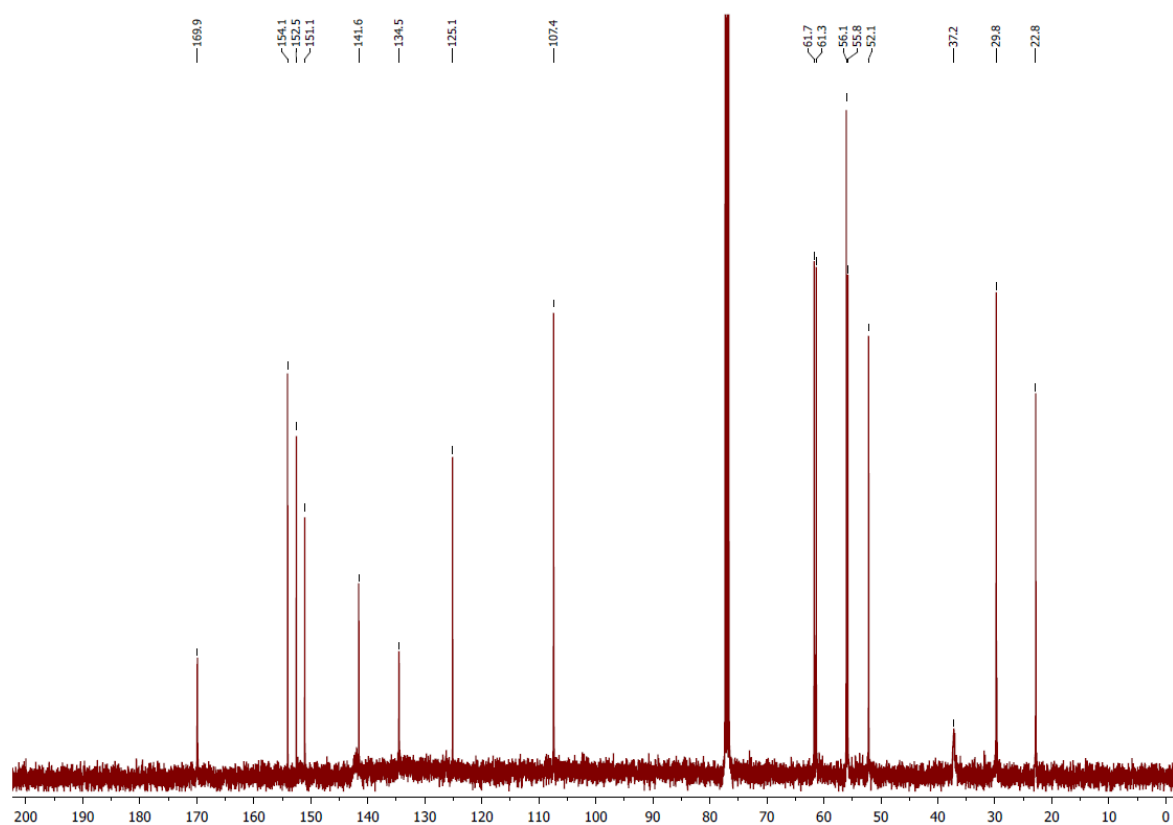


Figure S14. The ^{13}C NMR spectrum of **2e** in CDCl_3 .

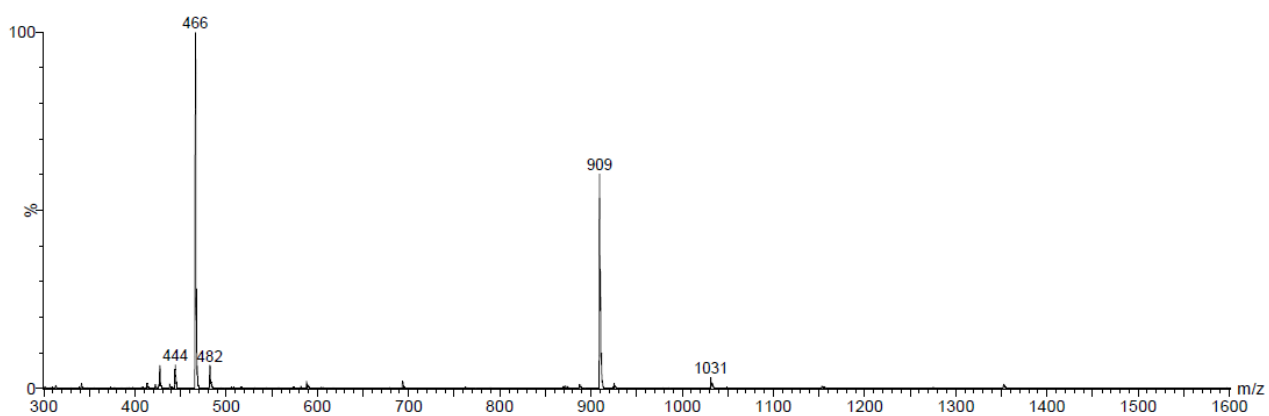
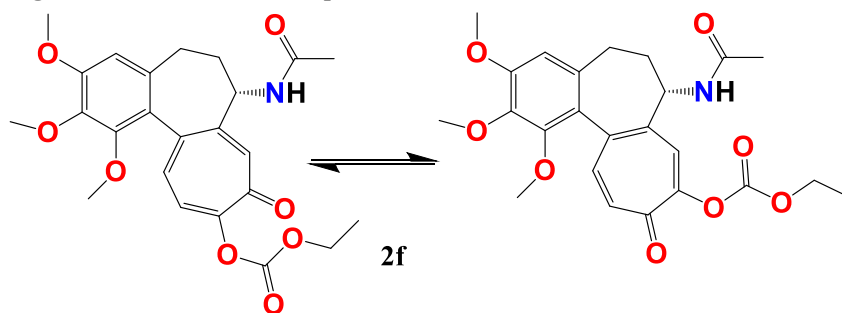


Figure S15. The ESI-MS spectrum of **2e**.



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

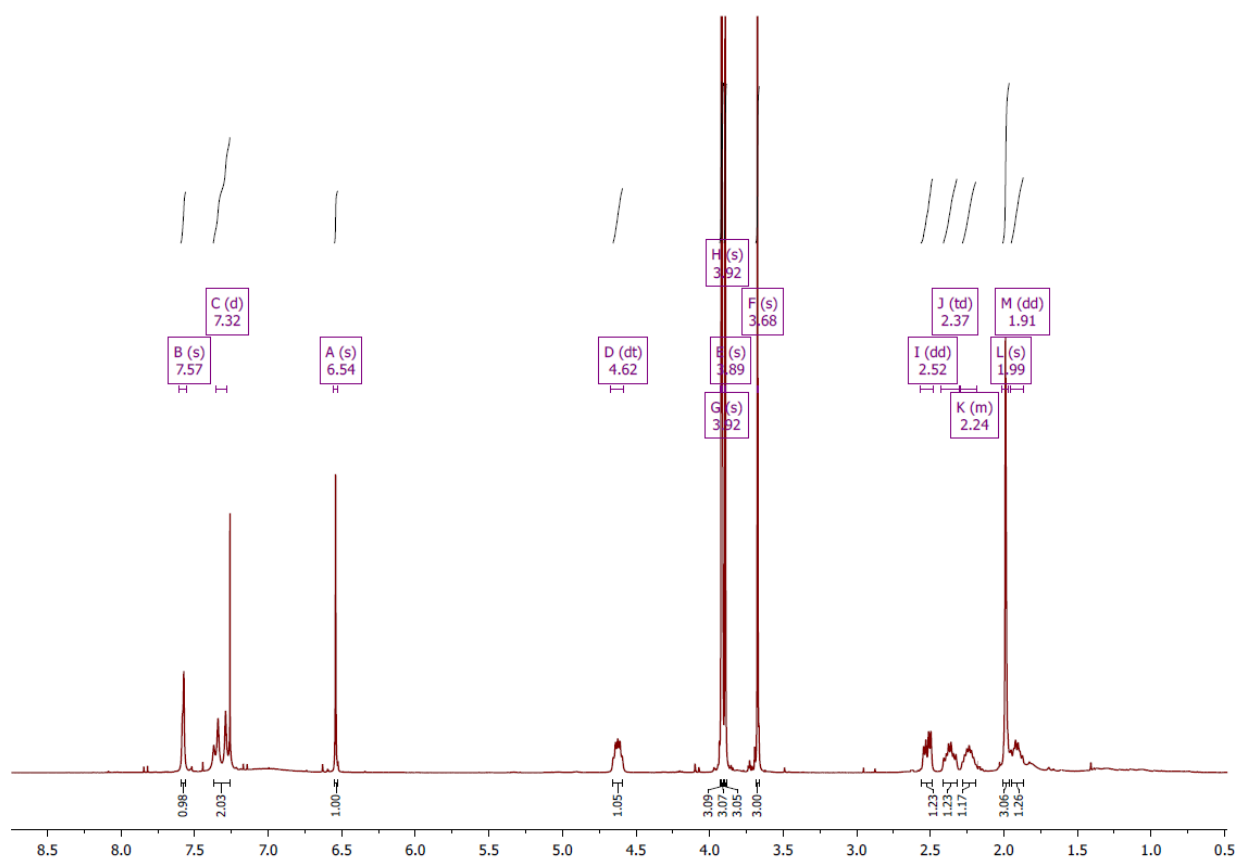


Figure S16. The ¹H NMR spectrum of **2f** in CDCl₃.

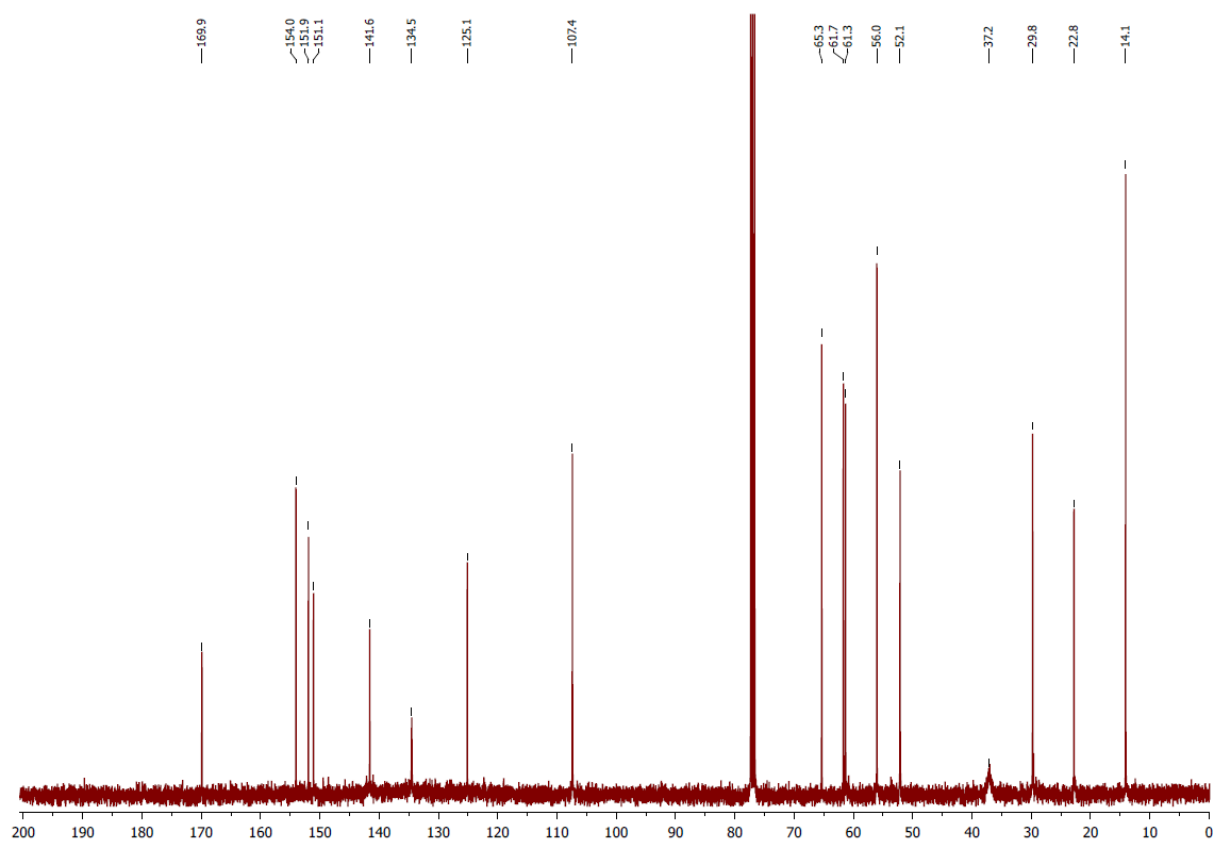


Figure S17. The ¹³C NMR spectrum of **2f** in CDCl₃.

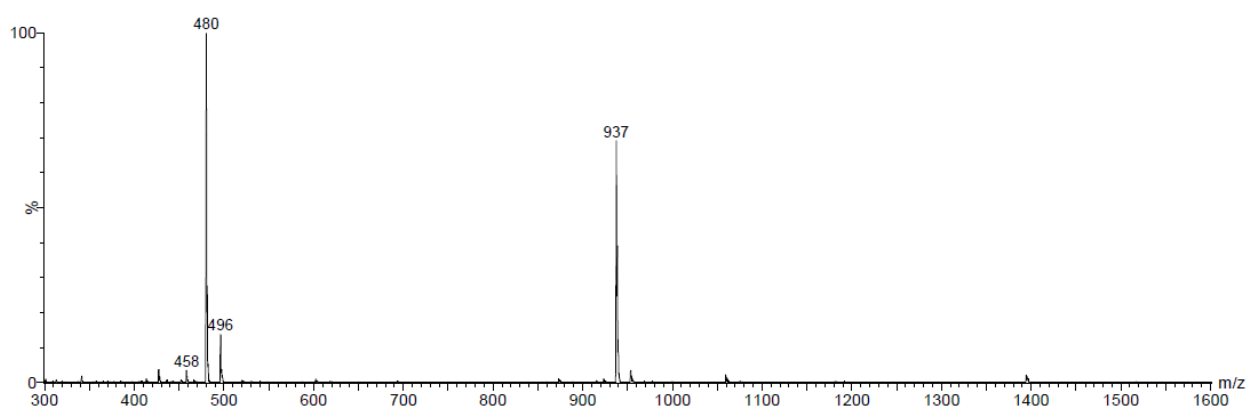
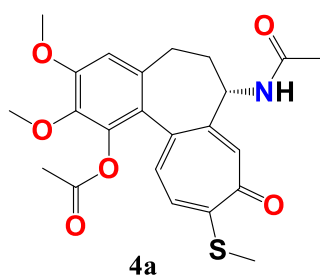


Figure S18. The ESI-MS spectrum of **2f**.



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

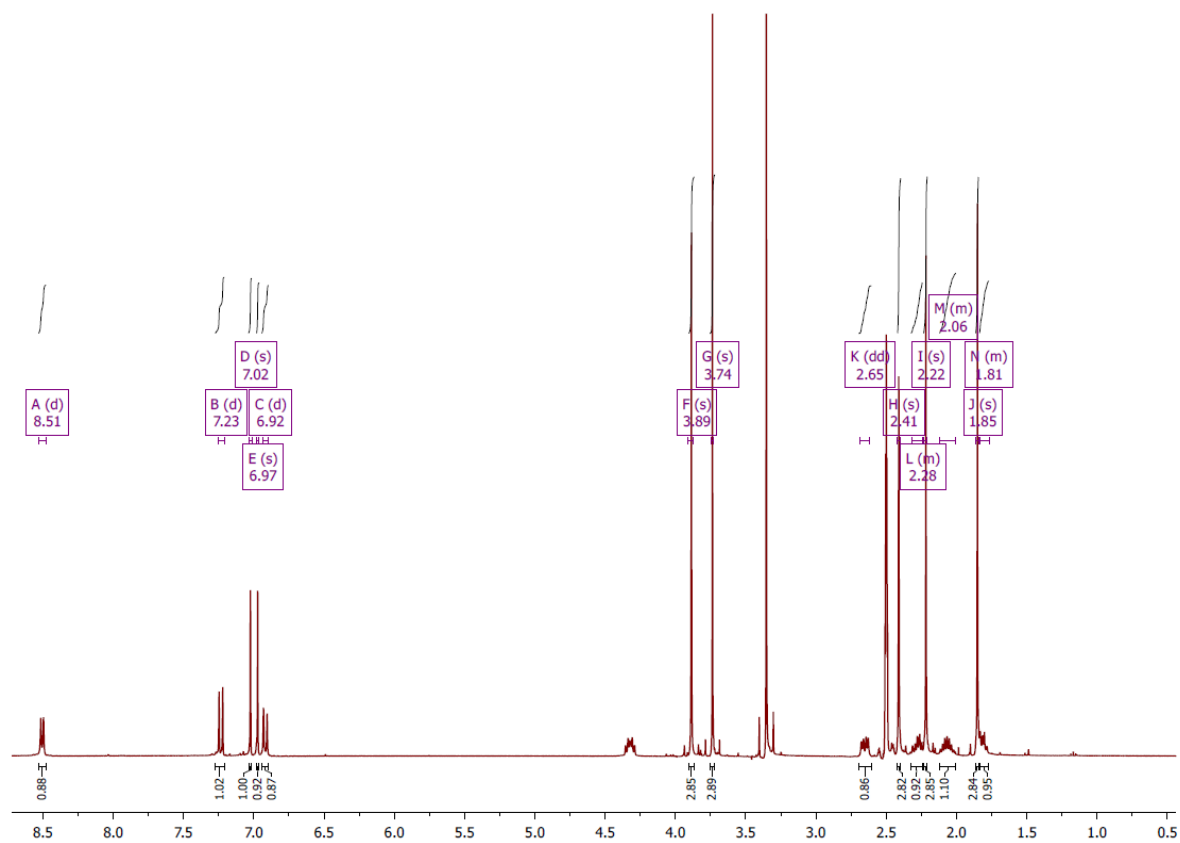


Figure S19. The 1H NMR spectrum of **4a** in $DMSO-d_6$.

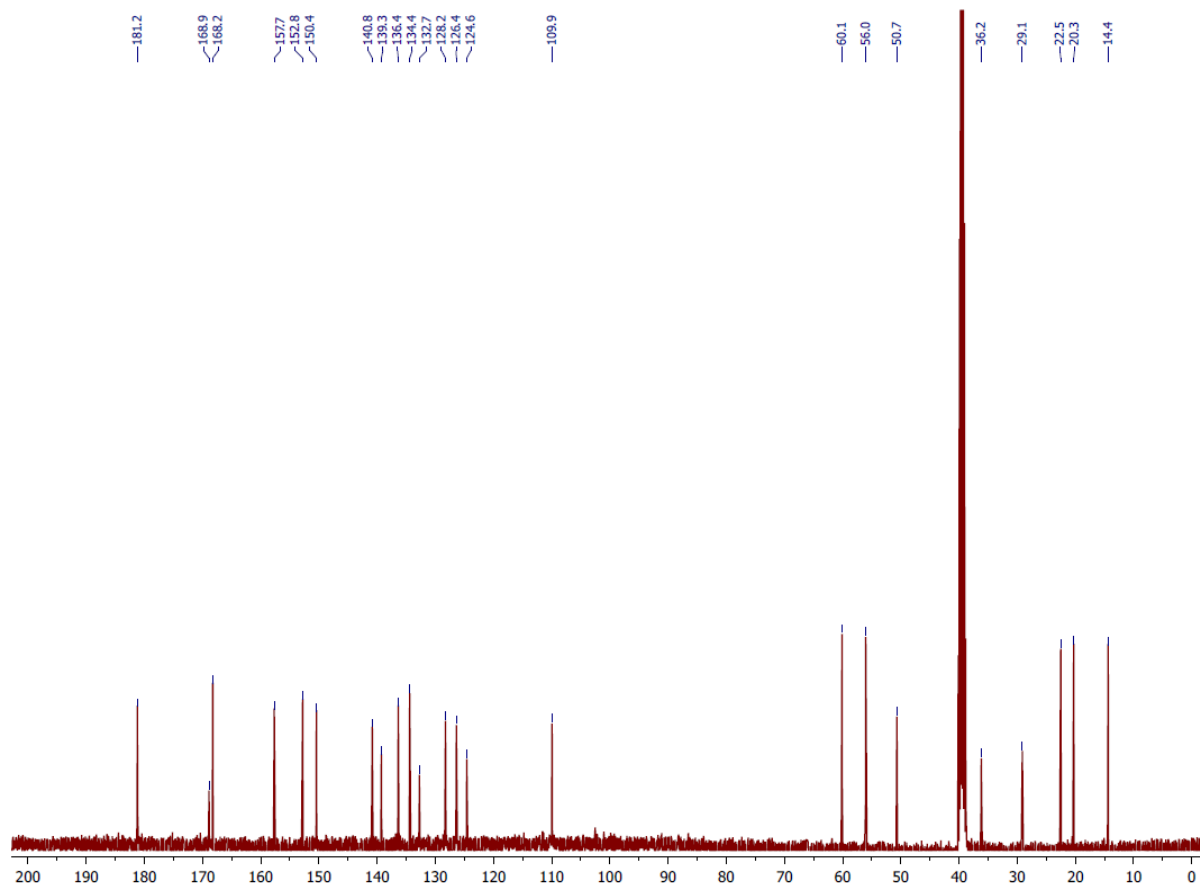


Figure S20. The ^{13}C NMR spectrum of **4a** in $\text{DMSO}-d_6$.

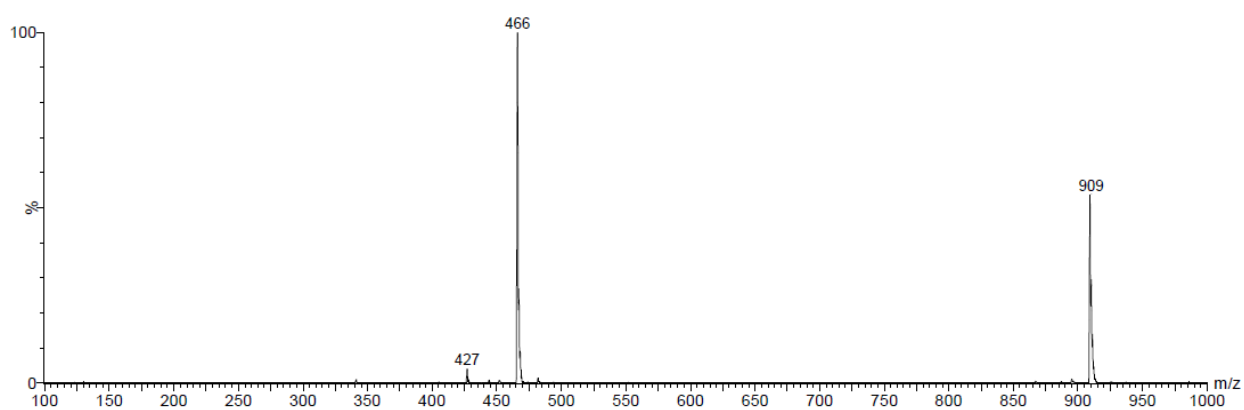
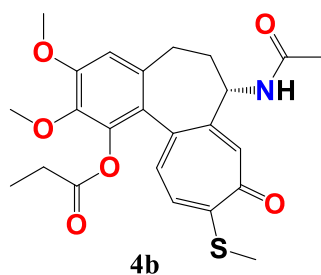


Figure S21. The ESI-MS spectrum of **4a**.



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

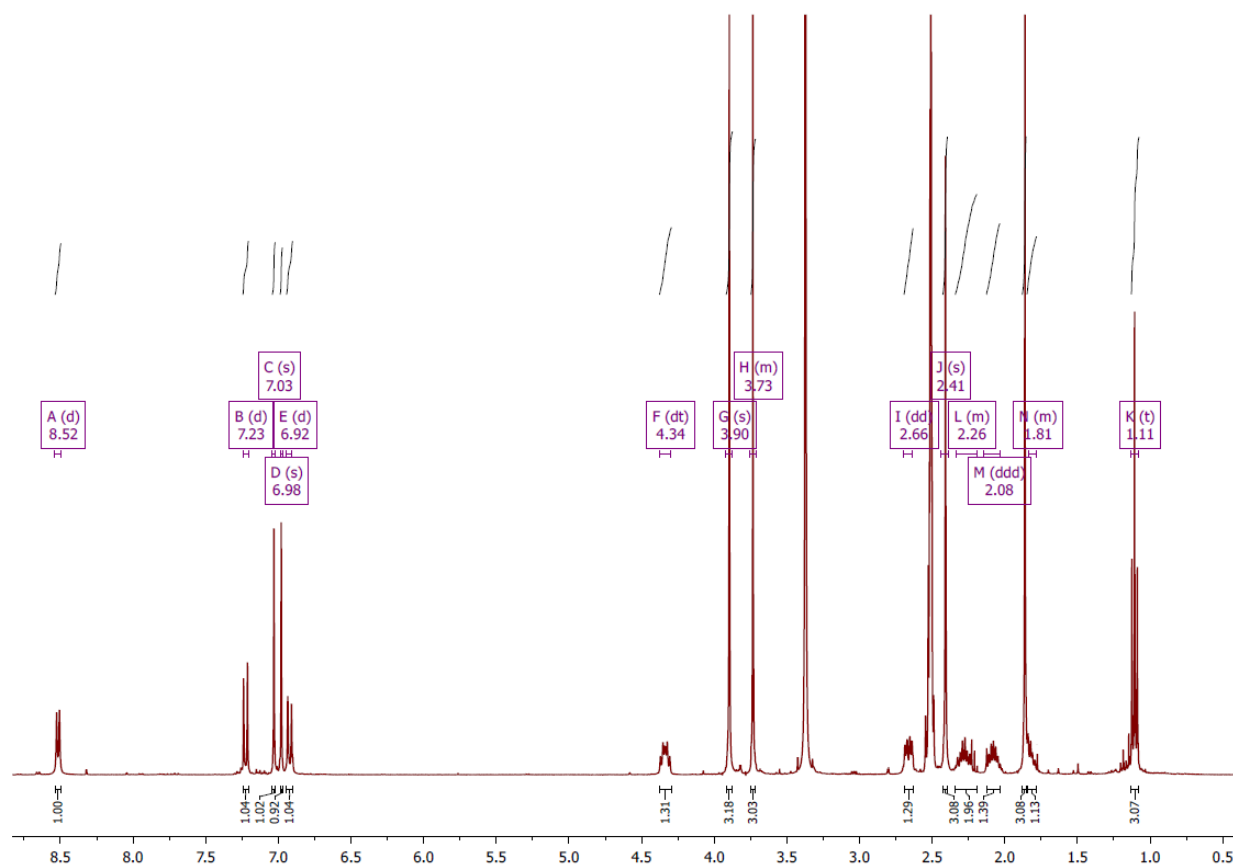


Figure S22. The ^1H NMR spectrum of **4b** in $\text{DMSO}-d_6$.

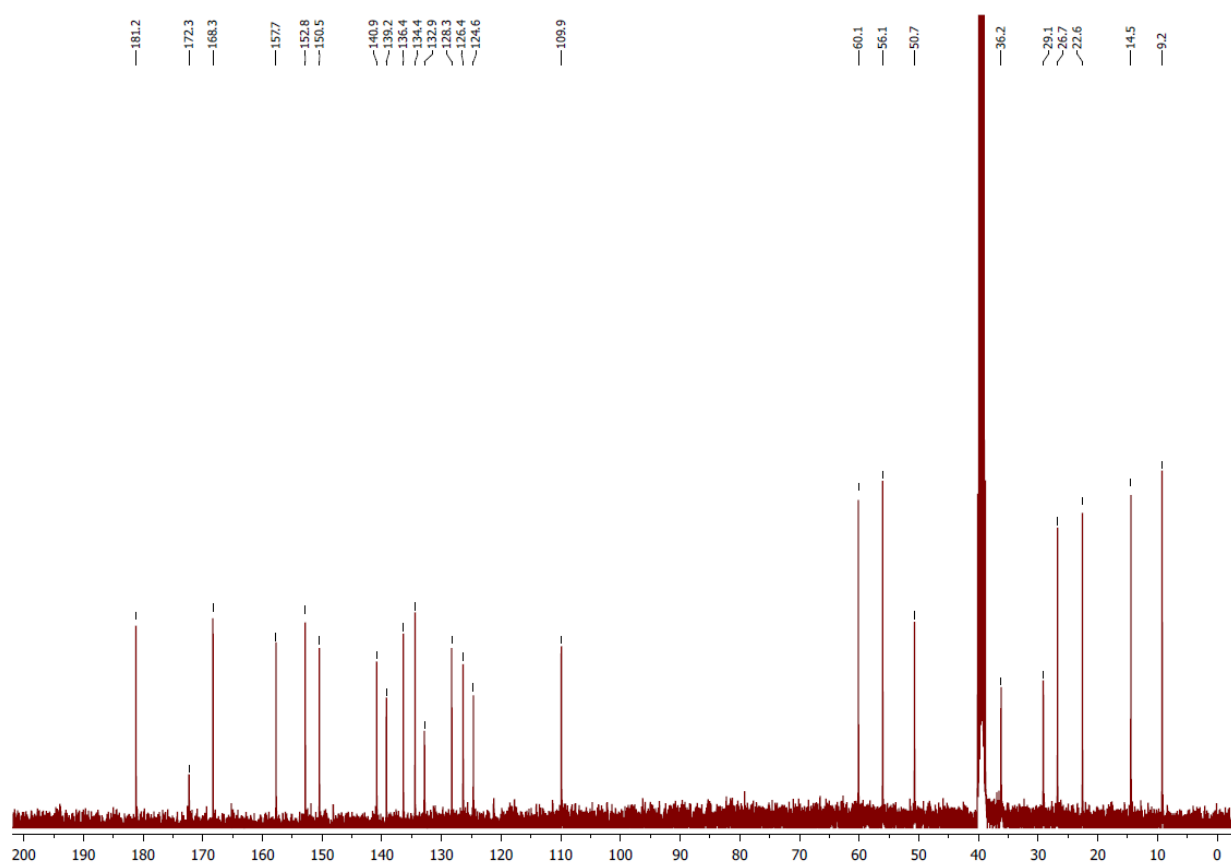


Figure S23. The ^{13}C NMR spectrum of **4b** in $\text{DMSO}-d_6$.

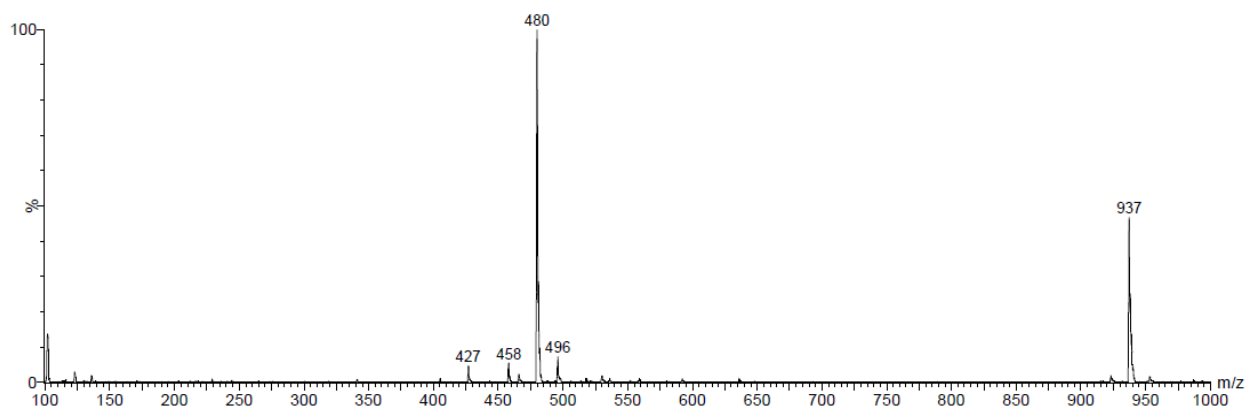
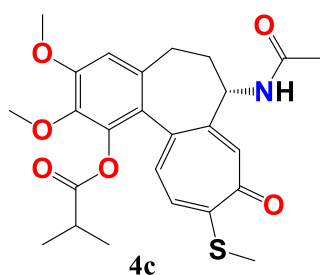


Figure S24. The ESI-MS spectrum of **4b**.



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

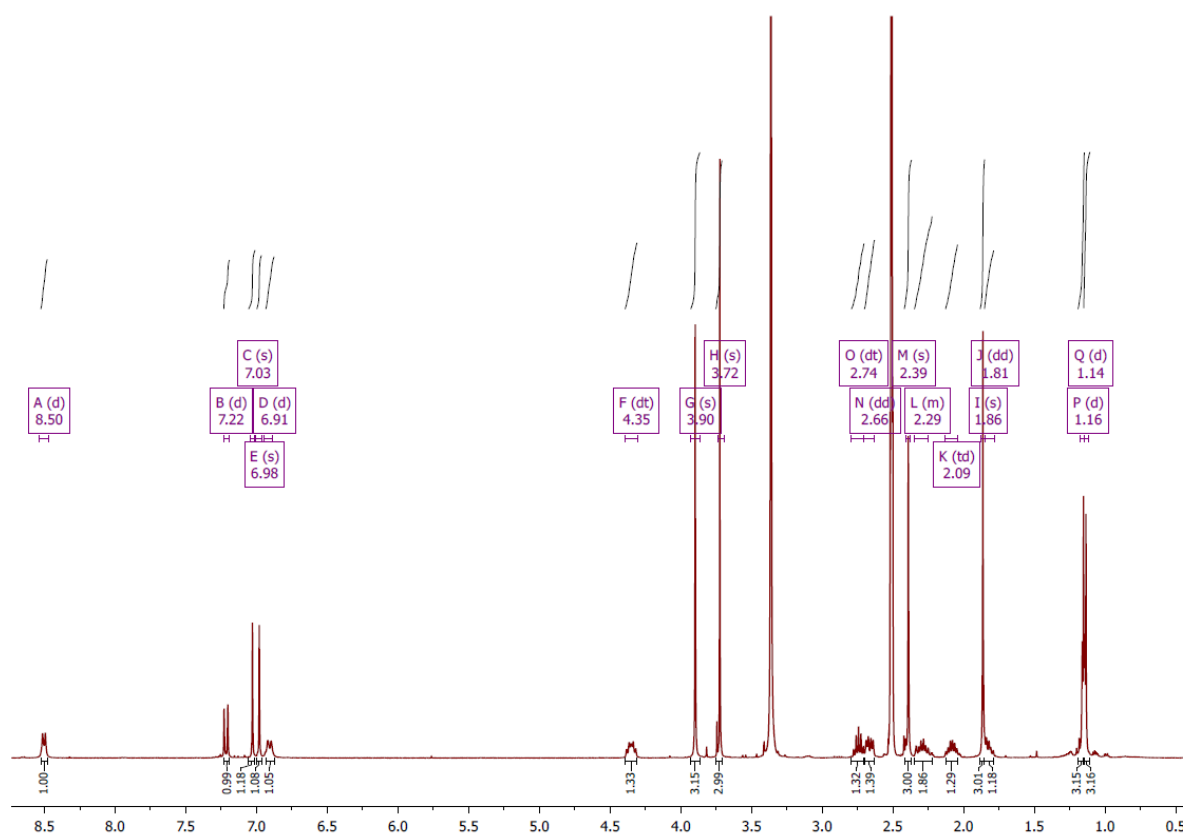


Figure S25. The ^1H NMR spectrum of **4c** in $\text{DMSO}-d_6$.

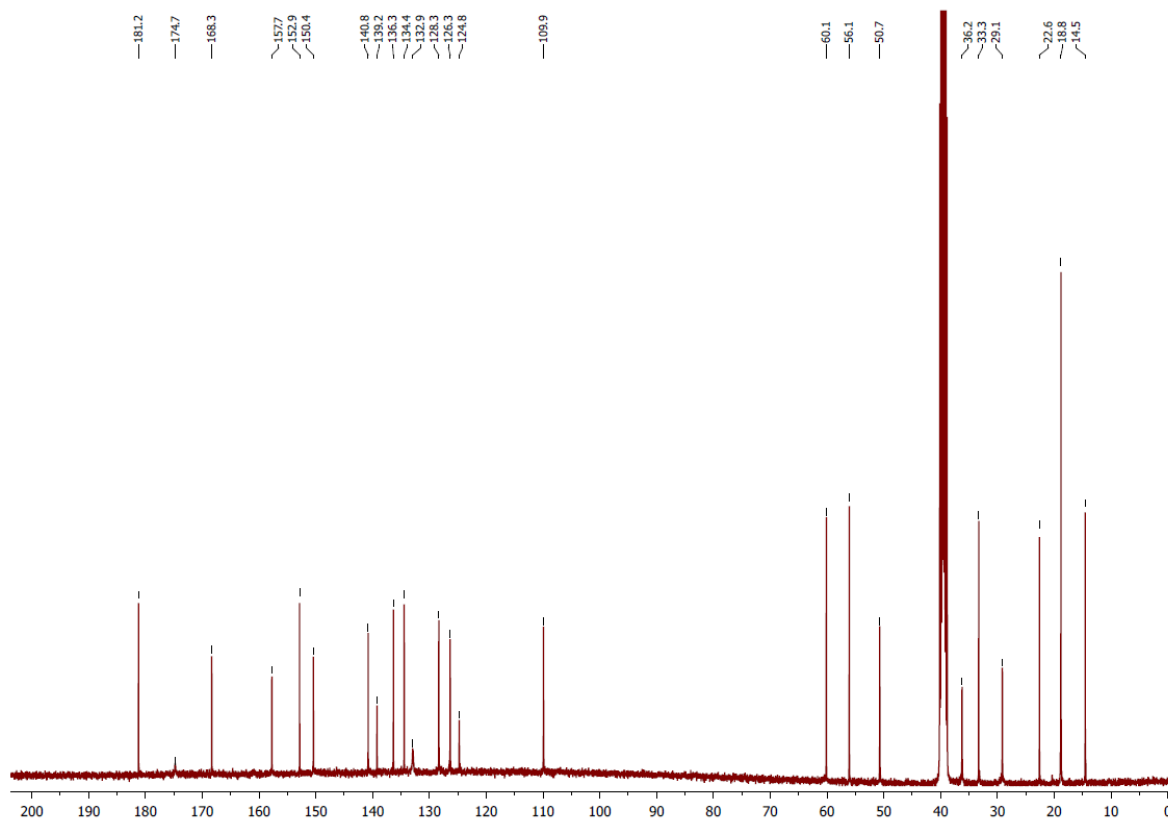


Figure S26. The ^{13}C NMR spectrum of **4c** in $\text{DMSO-}d_6$.

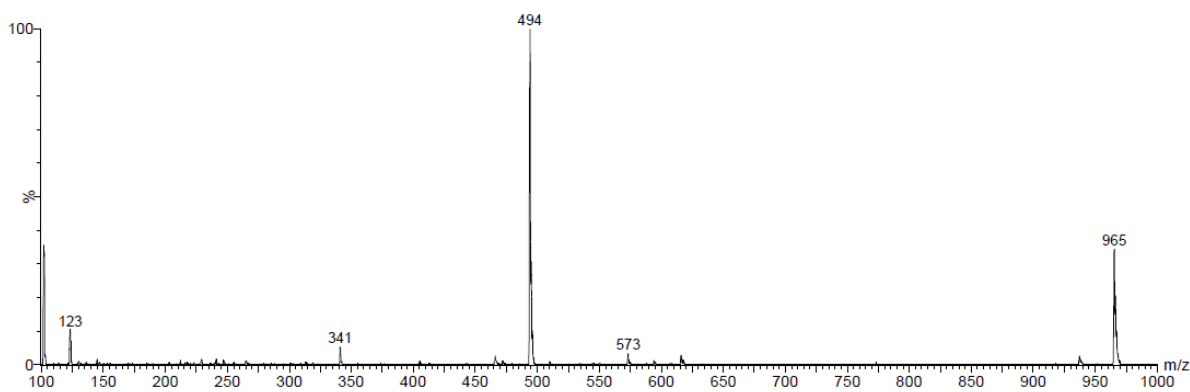
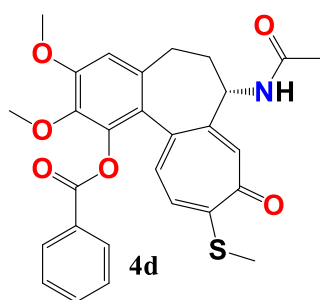


Figure S27. The ESI-MS spectrum of **4c**.



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

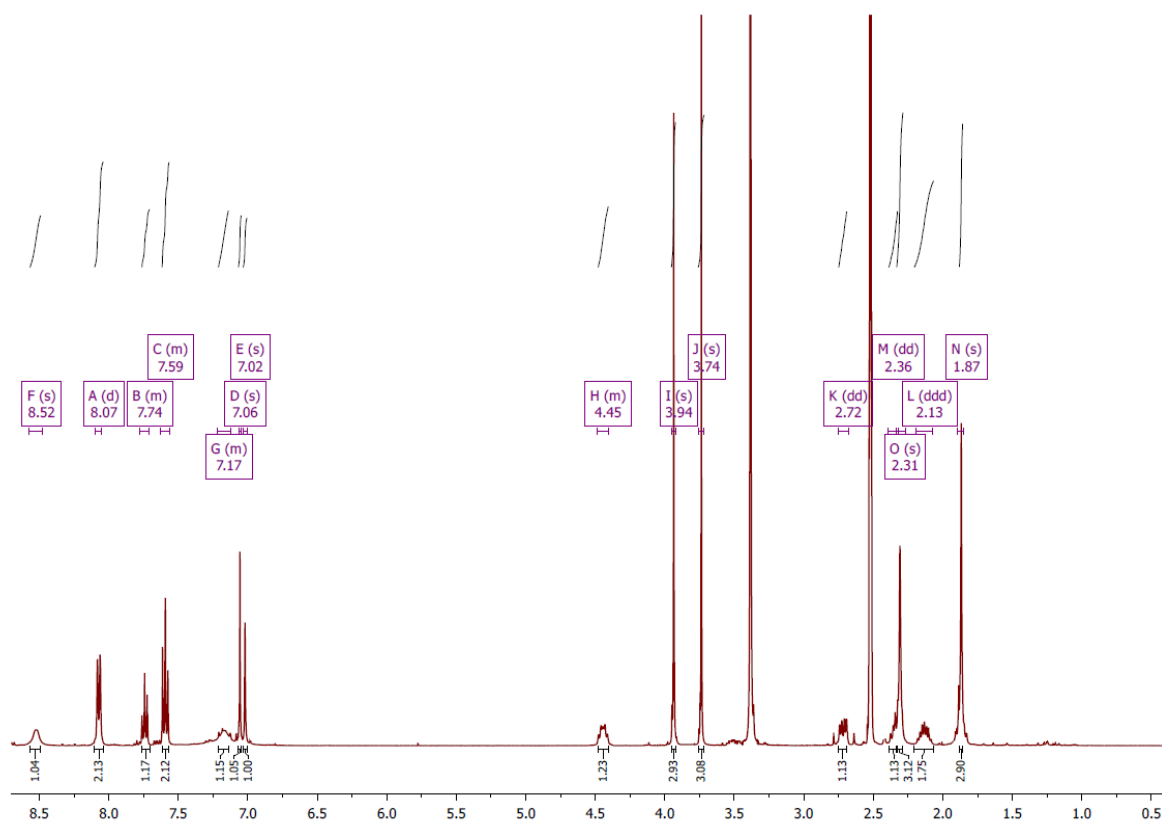


Figure S28. The ^1H NMR spectrum of **4d** in $\text{DMSO}-d_6$.

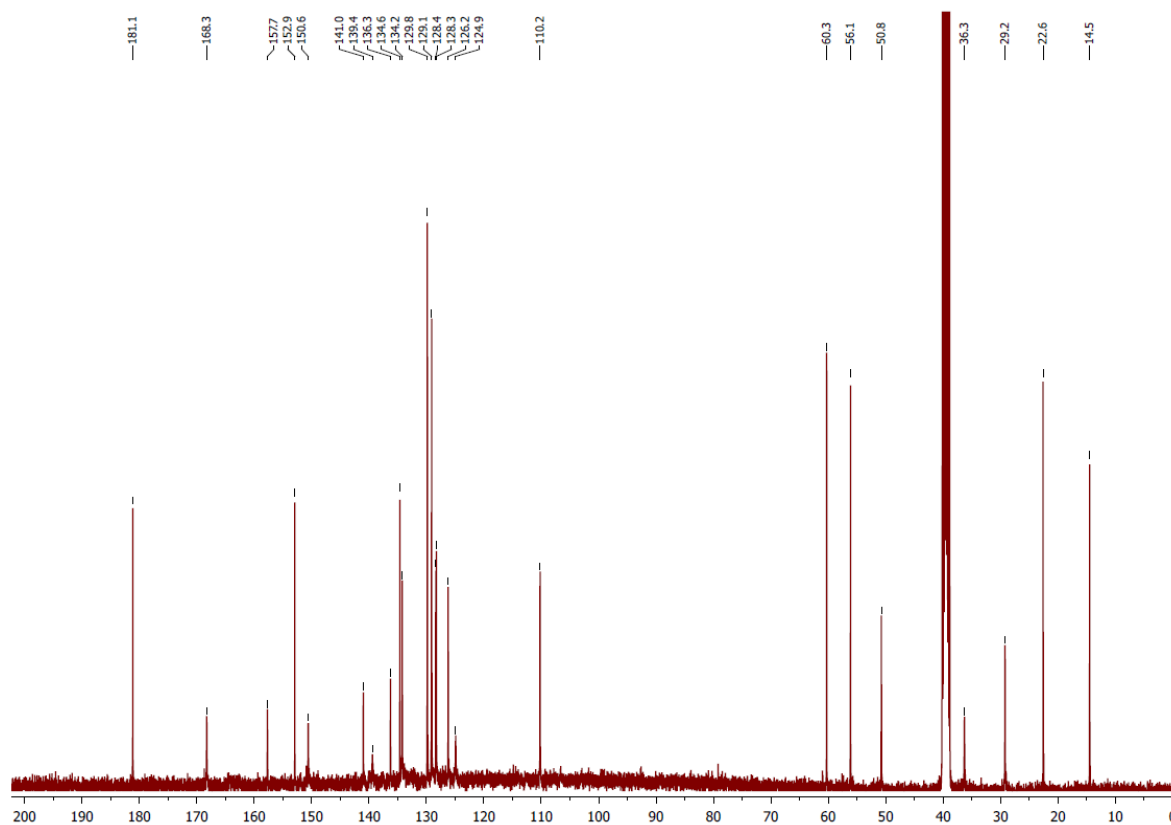


Figure S29. The ^{13}C NMR spectrum of **4d** in $\text{DMSO}-d_6$.

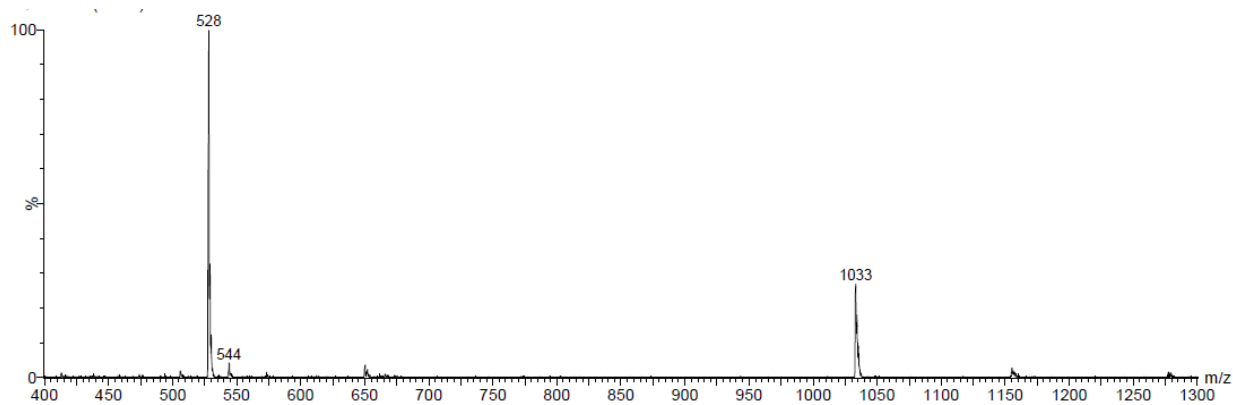
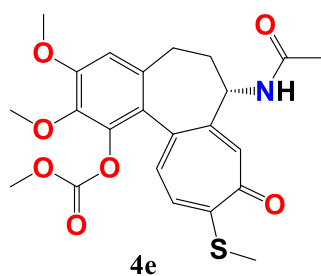


Figure S30. The ESI-MS spectrum of **4d**.



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

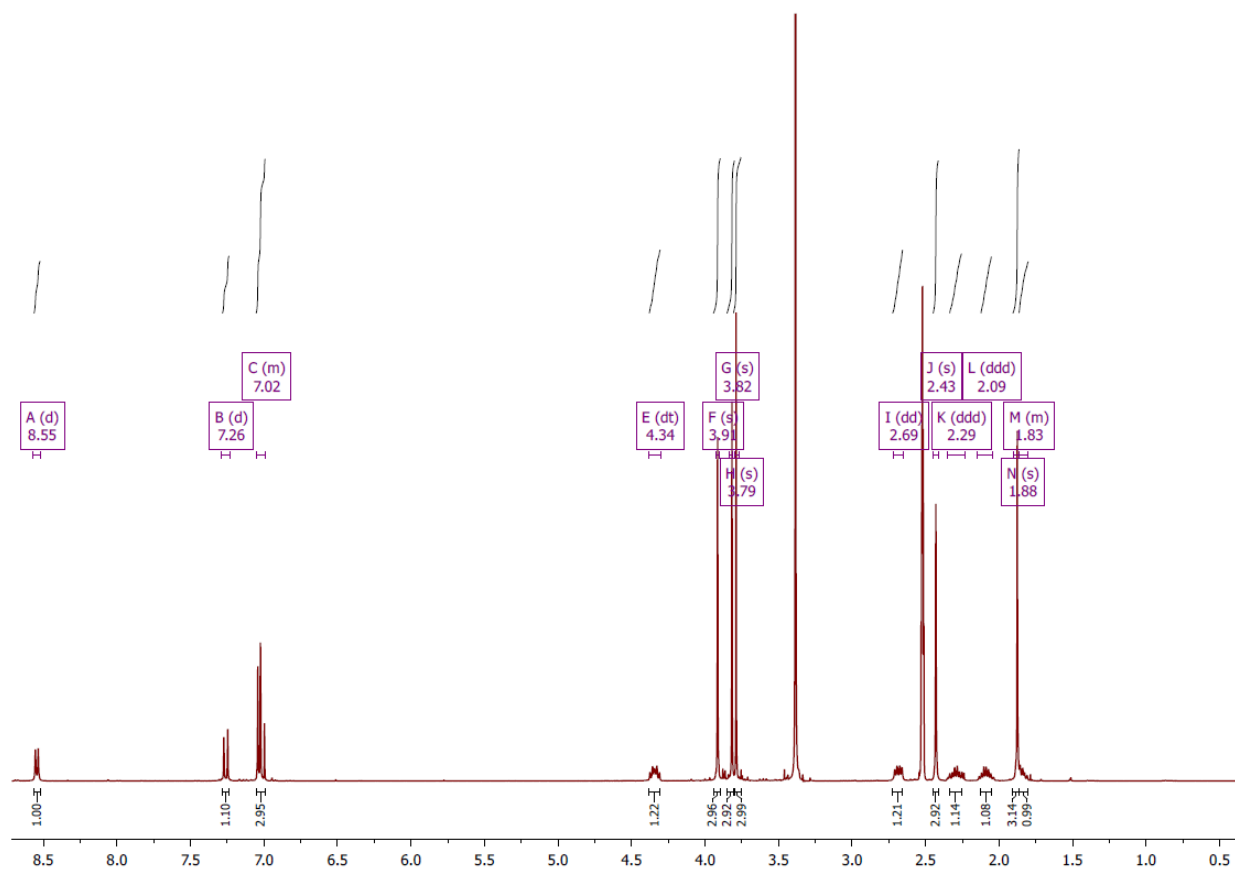


Figure S31. The 1H NMR spectrum of **4e** in $DMSO-d_6$.

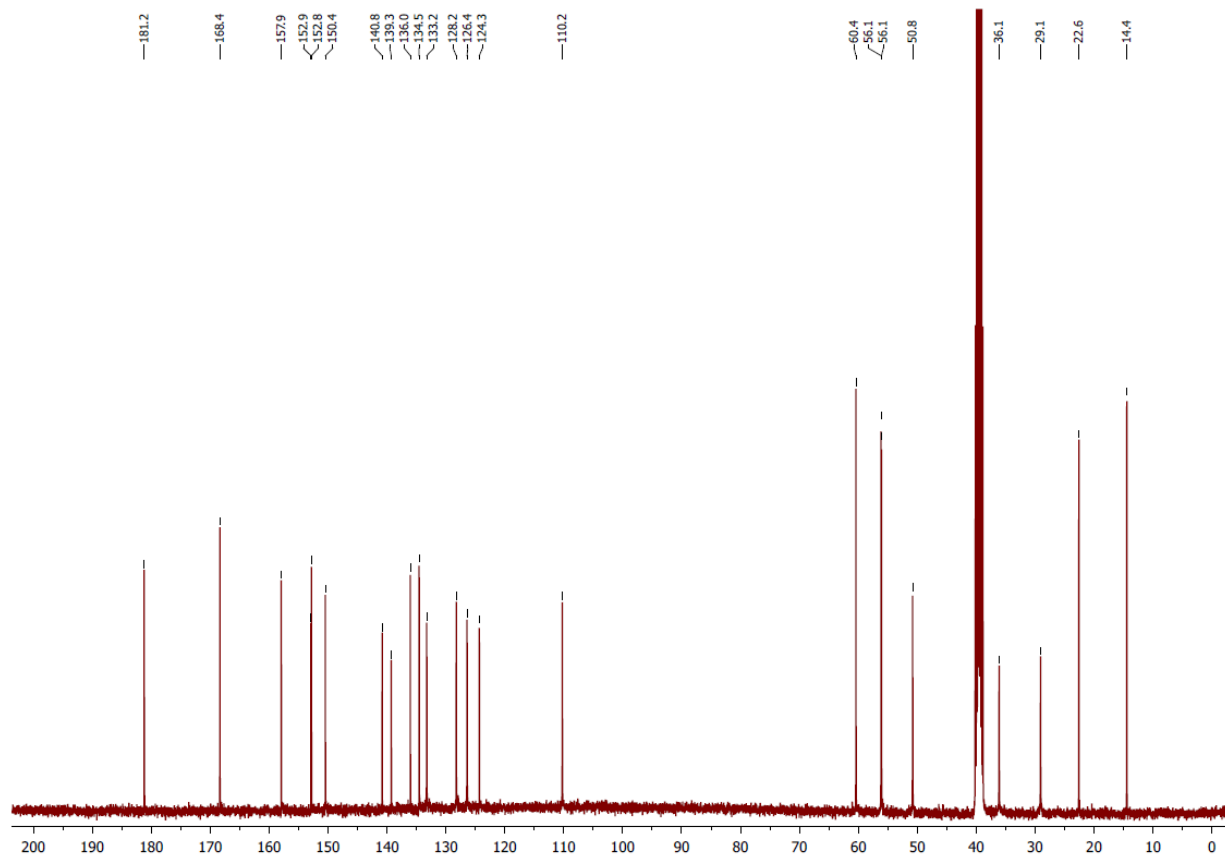


Figure S32. The ^{13}C NMR spectrum of **4e** in $\text{DMSO-}d_6$.

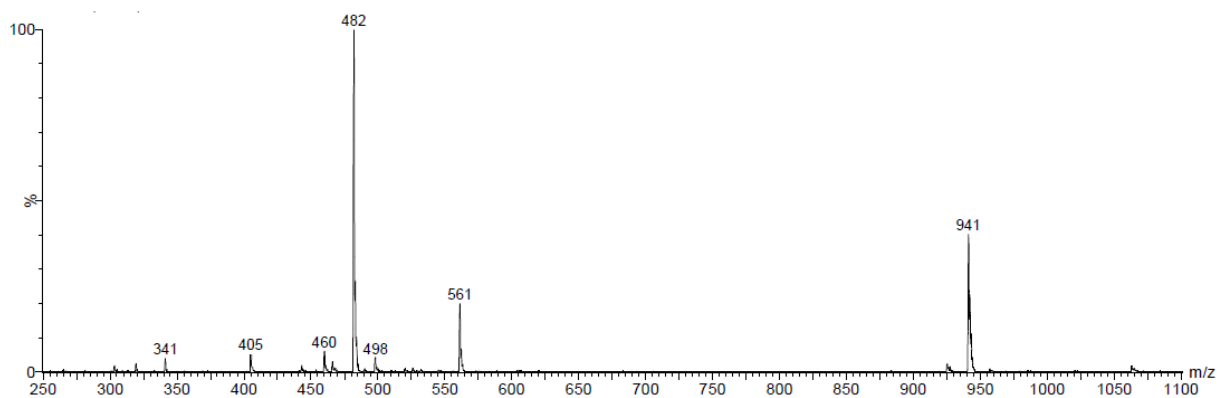
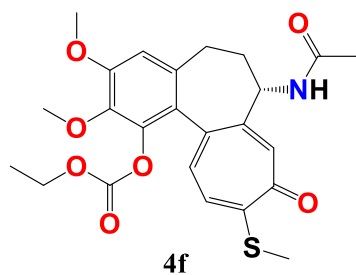


Figure S33. The ESI-MS spectrum of **4e**.



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

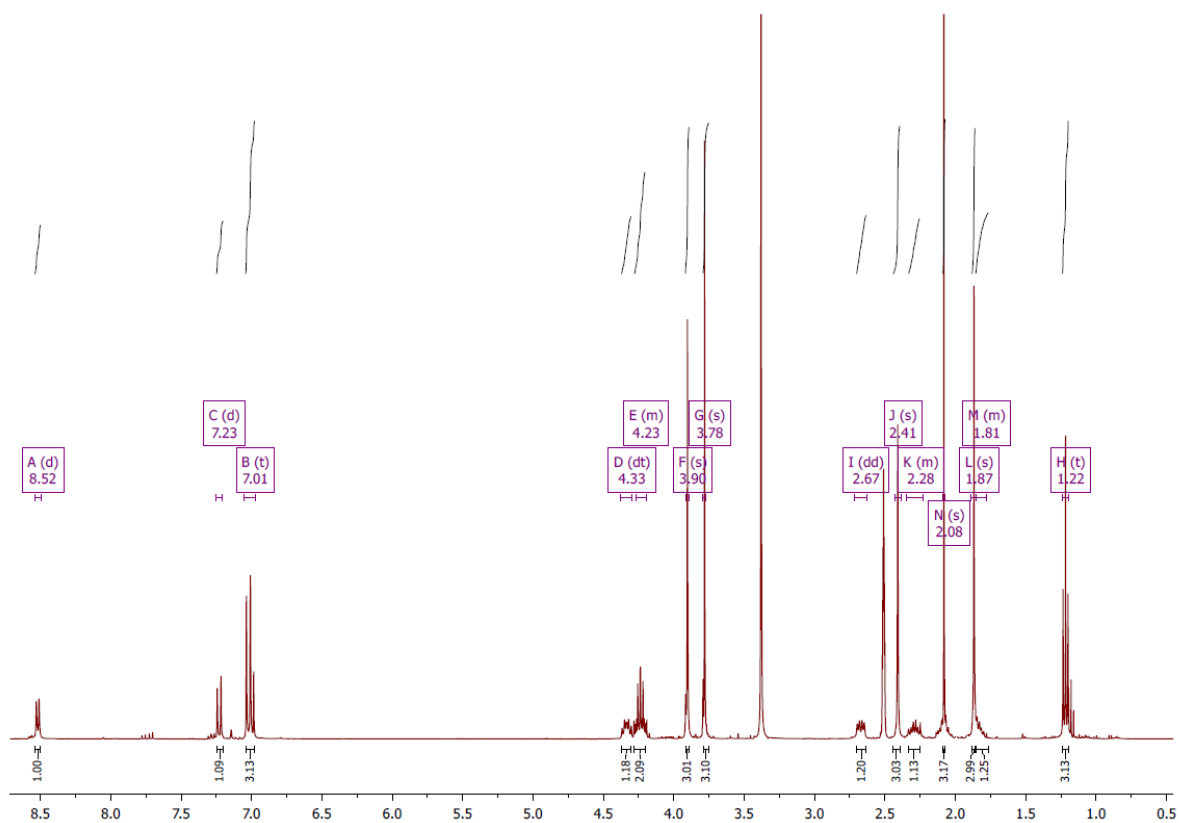


Figure S34. The ^1H NMR spectrum of **4f** in $\text{DMSO}-d_6$.

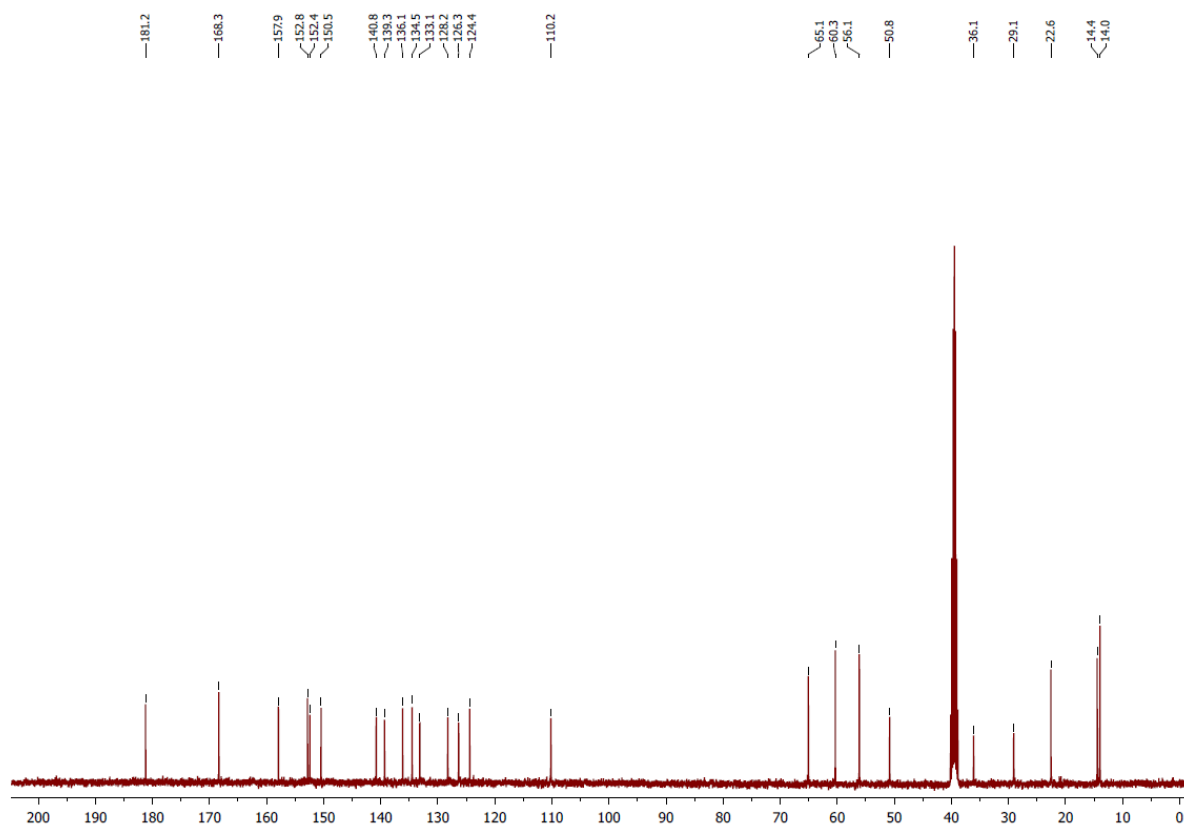


Figure S35. The ^{13}C NMR spectrum of **4f** in $\text{DMSO}-d_6$.

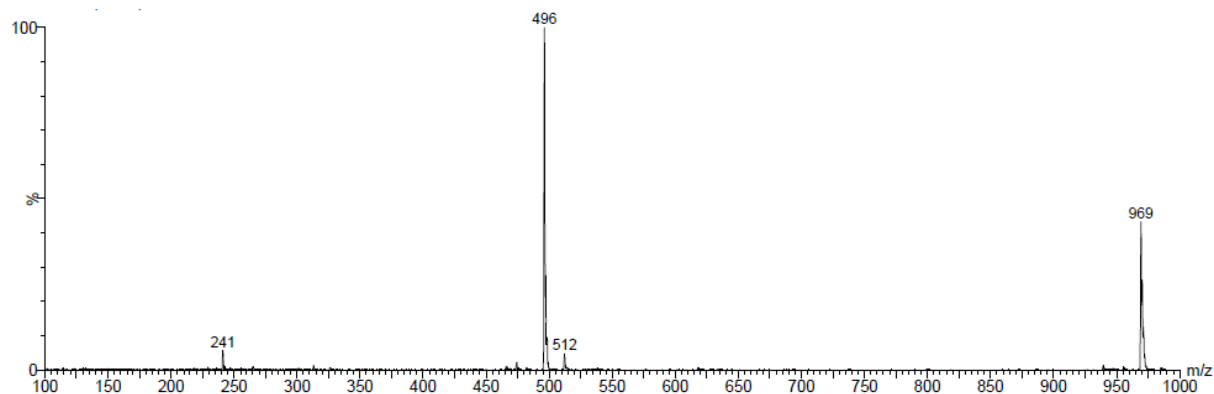


Figure S36. The ESI-MS spectrum of **4f**.

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