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# A novel molecular platform for co-delivery of monomethyl auristatin E and ispinesib to prostate cancer cells

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## General information about synthesis

All used solvents were purified according to described procedures.[S1] All starting compounds were commercially available reagents or were synthesized according to literature data.

$^{1}H$ and $^{13}C$ NMR spectra were registered on Bruker Avance 400 spectrometer (400 MHz for $^{1}H$ and 101 MHz for $^{13}C$) in $CDCl_3$ or DMSO-$d_6$. Preparative column chromatography was performed on INTERCHIM puriFlash 4250. For purification and analysis of samples we used Shimadzu Prominence LC-20 system with Phenomenex Luna 3µm C18 100A (150 x 4.6 mm) column in column oven at 40ºC and fraction collector coupled to single quadrupole mass-spectrometer Shimadzu LCMS-2020 with dual DUIS-ESI-APCI ionization source. Mobile phases: A - 0.1% formic acid in water, B - 10 mM ammonium formate in water, D - acetonitrile. LC parameters for analyses were: gradient flow of 1 mL/min (0-0.5 min - 5% D, 0.5 -10.5 min - 5% to 100% D, 10.5-12 min - 100% D, 12-14.5 min - 100% to 5% D) with optional UV detection for some compounds. MS parameters: drying gas 15.0 L/min, nebulizing gas 1.5 L/min, desolvation line temperature 250ºC, heat block temperature 400ºC, interface voltage -3.5 kV, corona needle voltage -3.5 kV. Positive (mass range 250-2000 Da, in some cases 155-2000 Da) and negative ions (mass range 100-2000 Da) were registered simultaneously. For purification we used identical LC parameters except gradient which was tailored for each compound (in some cases we used mobile phase B instead of A). Fractionation was based on UV detection only; fractions were collected based on UV signal level and slope. High resolution mass spectra were registered on Orbitrap Elite mass spectrometer (Thermo Scientific) with ESI ionization source. Compounds with concentration of 0.1-10 µg/ml (in 1% formic acid in acetonitrile) were directly infused into the ion source with syringe pump (5 µl/min). We didn't use auxiliary and sheath gases, spray voltage was +-3.5 kV, capillary temperature was set to 275ºC. MS spectra were registered by Orbitrap analyzer with 480000 resolution (1 microscan, AGC target value of 1×106, max inject time 1000 ms, averaged on 10 spectra, MS range 100-2000 Da, in some cases 200-4000 Da). We used DMSO and di-*iso*-octyl phthalate as internal calibration signals (*m/z* 157.03515 and 413.26623) in positive mode and dodecylsulfate (*m/z* 265.14790) in negative mode.

**Synthesis**

Synthesis of modified ispinesib (**2**)

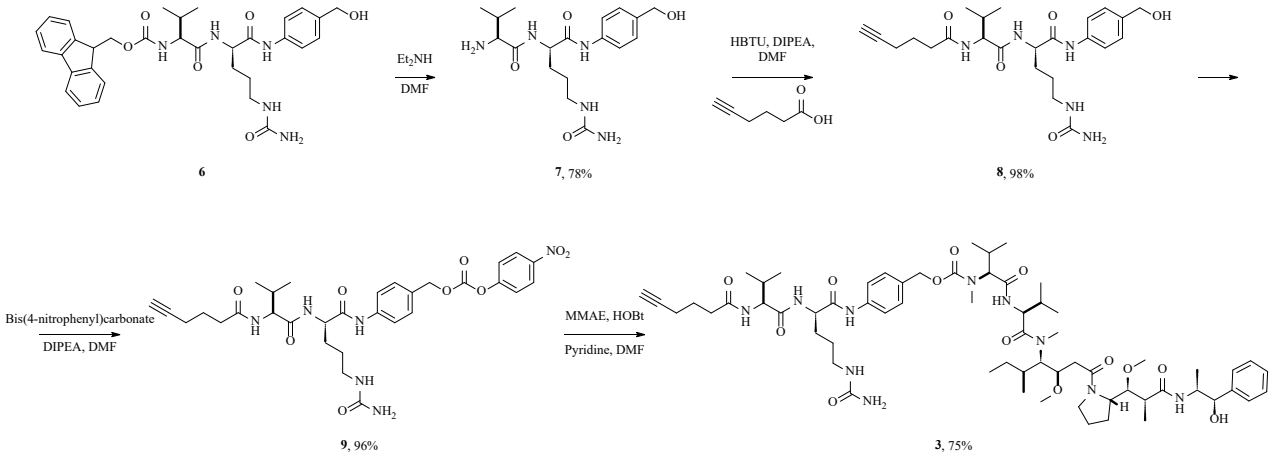
*Step 1. Compound* **1**. Ispinesib (25 mg, 48.4 μm, 1 eq.) was dissolved in 10 mL of DMF, 8 mg of succinic anhydride (79.9 μm, 1.65 eq.) and 40 μL of DIPEA (230 μm, 4.75 eq.) were added, and this was stirred until the reaction was complete. The reaction was monitored by TLC (elution system: 5% methanol in DCM). After completion of the reaction, the solvent was removed under reduced pressure, the resulting mixture was dissolved in 25 mL DCM, washed with 0.1 M HCl (2*20 mL), water (2*20 mL) and 20 mL brine. The organic fraction was dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure. Thus 28 mg of compound **1** (93% yield) was obtained as a white powder.

$^1$H NMR (400 MHz, $CDCl_3$, δ): 8.31 (d, *J*=8.55 Hz, 1H, Ar), 7.69 (d, *J*=1.53 Hz, 1H, Ar), 7.48 (d, *J*=8.50 Hz, 1H, Ar), 7.37−7.42 (m, 2H, Ar), 7.28−7.35 (m, 3H, Ar), 7.18−7.25 (m, 4H, Ar), 6.12 (d, *J*=15.57 Hz, 1H, $CH_2$ (benzyl fragment)), 5.70 (d, *J*=10.58 Hz, 1H, CH), 5.18 (d, *J*=15.68 Hz, 1H, $CH_2$ (benzyl fragment)), 3.29−3.45 (m, 2H, $CH_2$), 2.60−2.82 (m, 3H, $CH_2$+CH), 2.40−2.54 (m, 2H, $CH_2$), 2.36 (s, 3H, $CH_3$), 2.06−2.22 (m, 2H, $CH_2$), 1.20−1.36 (m, 2H, $CH_2$+$NH_2$), 0.95 (d, *J*=6.63 Hz, 3H, $CH_3$), 0.78−0.91 (m, 1H, $CH_2$), 0.39 (d, *J*=6.30 Hz, 3H, $CH_3$).

*Step 2. Compound* **2**. Precursor **1** (52 mg, 84.3 μmol, 1 eq.) was dissolved in 20 mL DCM, and 44 mg EDC*HCl (227.6 μmol, 2.7 eq.), 26 mg *N*-hydroxysuccinimide (227.6 μmol, 2.7 eq.) and 60 μL $Et_3N$ (0.43 mmol, 5.1 eq.) were added. The resulting mixture was stirred for 16 h under inert atmosphere. The solvent was removed under reduced pressure. The residue was dissolved in 40 mL DCM and washed with saturated $NaHCO_3$ solution (3*30 mL) and water (3*30 mL). The organic fraction was dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure. Compound **2** was isolated by preparative chromatography (InterchimPuriflash 14 g, 50μ, gradient 15 minutes with DCM, then from 0% methanol to 100% methanol in 5 minutes flow rate - 15 ml/min). Thus 18 mg of compound **2** (30% yield) was obtained as a white powder.

$^1$H NMR (400 MHz, $CDCl_3$, δ),: 8.31 (d, *J*=8.55 Hz, 1H, Ar), 7.71 (d, *J*=1.92 Hz, 1H, Ar), 7.50 (dd, *J*=8.55, 1.97 Hz, 1H, Ar), 7.37−7.42 (m, 2H, Ar), 7.27−7.35 (m, 3H, Ar), 7.19−7.26 (m, 4H, Ar), 6.11 (d, *J*=15.62 Hz, 1H, $CH_2$ (benzyl fragment)), 5.70 (d, *J*=10.58 Hz, 1H, CH), 5.19 (d, *J*=15.68 Hz, 1H, $CH_2$ (benzyl fragment)), 4.70−4.79 (m, 1H, NH), 3.29−3.48 (m, 2H, $CH_2$), 2.83−2.90 (m, 7H, CH+$CH_2$+$CH_3$), 2.65−2.82 (m, 3H, $CH_2$), 2.39 (s, 3H, $CH_3$), 2.19−2.34 (m, 2H, $CH_2$), 1.24−1.37 (m, 1H, $CH_2$), 0.96 (d, *J*=6.74 Hz, 3H, $CH_3$), 0.88 (dt, *J*=12.25, 5.93 Hz, 1H, $CH_2$), 0.38 (d, *J*=6.36 Hz, 3H, $CH_3$).

Synthesis of modified MMAE **3** was carried out according to Scheme S1 reported in the literature:[S2]




**Scheme S1**

*Synthesis of* **7**. Compound **6** (450 mg, 0.748 mmol) and diethylamine (3.7 mL, 0.020 mol) were dissolved in DMF (8 mL). The resulting solution was stirred for 3 h. Then, the solvent was removed under reduced pressure. The product was precipitated with EtOAc (5*13 mL), and the resulting gel was centrifuged and washed with diethyl ether (3*15 mL). The resulting gel was dried to obtain 220 mg (0.580 mmol) of a white amorphous powder, 78% yield.

$^{1}$H NMR (400 MHz, DMSO-d6) δ 10.02 (s, 1H), 8.10 (m, 1H), 7.53 (d, J = 8.2 Hz, 2H, Ar), 7.23 (d, J = 8.2 Hz, 2H), 5.95 (s, 1H), 5.39 (s, 2H), 5.09 (t, J = 5.5 Hz, 1H), 4.46 (m, 1H), 4.42 (d, J = 5.3 Hz, 2H), 3.00−3.01 (d, 2H), 2.91−2.98 (m, 2H), 1.90−1.96 (m, 2H), 1.36−1.39 (m, 6H, CH2), 0.87 (d, J = 6.8 Hz, 3H), 0.77 (d, J = 6.7 Hz, 3H).

$^{13}$C NMR (101 MHz, DMSO-d6) δ 175.31, 171.31, 159.68, 138.33, 138.27, 127.76, 119.76, 63.41, 60.50, 53.31, 32.18, 31.00, 27.54, 20.40, 17.74, 14.92.

Synthesis of **8**. Compound **7** (1000 mg, 2.635 mmol) was dissolved in 20 mL of DMF. To the solution were added DIPEA (505 µL, 2.899 mmol), HBTU (1.099 g, 2.899 mmol), and hex-5-ynoic acid (325 mg, 2.899 mmol). The resulting solution was stirred overnight. The crude product was purified by chromatography (water/acetonitrile; Puriflash C18 50 µ, 120 g, from 10% to 100% acetonitrile for 30 min), resulting in 1.223 mg (2.584 mmol) of a white amorphous powder, 98% yield.

$^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.89 (s, 1H), 8.06 (d, J = 7.6 Hz, 1H), 7.88 (d, J = 8.6 Hz, 1H), 7.53 (d, J = 8.3 Hz, 2H), 7.22 (d, J = 8.3 Hz, 2H), 5.97 (t, J = 5.5 Hz, 1H), 5.40 (s, 2H,), 5.09 (t, J = 5.6 Hz, 1H), 4.41 (d, J = 5.4 Hz, 2H), 4.34−4.37 (m, 1H), 4.17 (t, J = 7.6 Hz, 1H), 2.90−3.03 (m, 2H), 2.77 (t, J = 2.4 Hz, 1H), 2.21−2.31 (m, 2H), 2.12−2.16 (m, 2H), 1.92−1.99 (m,

1H), 1.56−1.69 (m, 4H), 1.32−1.44 (m, 2H), 1.11−1.22 (m, 2H), 0.85 (d, J = 6.7 Hz, 3H), 0.82 (d, J = 6.7 Hz, 3H).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.31, 171.70, 170.86, 159.35, 137.99, 137.86, 127.38, 119.29, 71.92, 63.05, 58.18, 53.55, 34.49, 30.78, 29.79, 27.28, 24.91, 19.70, 18.66, 17.85.

HRMS (ESI) m/z 474.2715 (calculated 474.2711 for $C_{24}H_{36}N_5O_5^+$ [M + H]$^+$); m/z 496.2536 (calculated 496.2530 for $C_{24}H_{35}N_5O_5Na^+$ [M + Na]$^+$); m/z 512.2283 (calculated 512.2270 for $C_{24}H_{35}N_5O_5K^+$ [M + K]$^+$).

*Synthesis of* **9**. Compound **8** (1.223 g, 2.582 mmol), bis(4-nitrophenyl) carbonate (2.357 g, 7.747 mmol), and DIPEA (494 μL, 2.840 mmol) were dissolved in 40 mL of DMF, and the mixture stirred for 5 h. The solvent was removed under reduced pressure, and the resulting substance was precipitated with ethyl acetate (150 mL). The precipitate was then filtered, washed additionally with ethyl acetate, and dried, resulting in 1.584 mg (2.482 mmol) of a brown amorphous powder, 96% yield.

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.06 (s, 1H,), 8.30 (d, J = 9 Hz, 2H) 8.11 (d, J = 7.3 Hz, 1H), 7.87 (d, J = 8.7 Hz, 1H,), 7.64 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 9.2 Hz, 2H), 7.22 (d, J = 8.6 Hz, 2H), 5.97 (t, J = 5.5 Hz, 1H), 5.41 (s, 2H), 5.23 (s, 2H), 4.35−4.40 (m, 1H), 4.18 (t, J = 7.6 Hz, 1H), 2.90−3.03 (m, 2H,), 2.76−2.78 (m, 1H), 2.22−2.32 (m, 2H), 2.07−2.16 (m, 2H), 1.92−2.01 (m, 1H), 1.58−1.69 (m, 4H), 1.33−1.44 (m, 2H), 0.86 (d, J = 6.8 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 171.83, 171.30, 170.77, 158.91, 155.29, 151.97, 145.17, 139.42, 129.51, 125.41, 122.64, 119.01, 84.14, 71.47, 70.26, 62.60, 57.67, 53.17, 34.04, 30.37, 29.22, 26.86, 24.47, 19.24, 18.22, 17.40.

HRMS (ESI) m/z 639.2787 (calculated 639.2773 for $C_{31}H_{39}N_6O_9^+$ [M + H]$^+$); m/z 661.2612 (calculated 661.2592 for $C_{31}H_{38}N_6O_9Na^+$ [M + Na]$^+$); m/z 677.2346 (calculated 677.2332 for $C_{31}H_{38}N_6O_9K^+$ [M + K]$^+$).

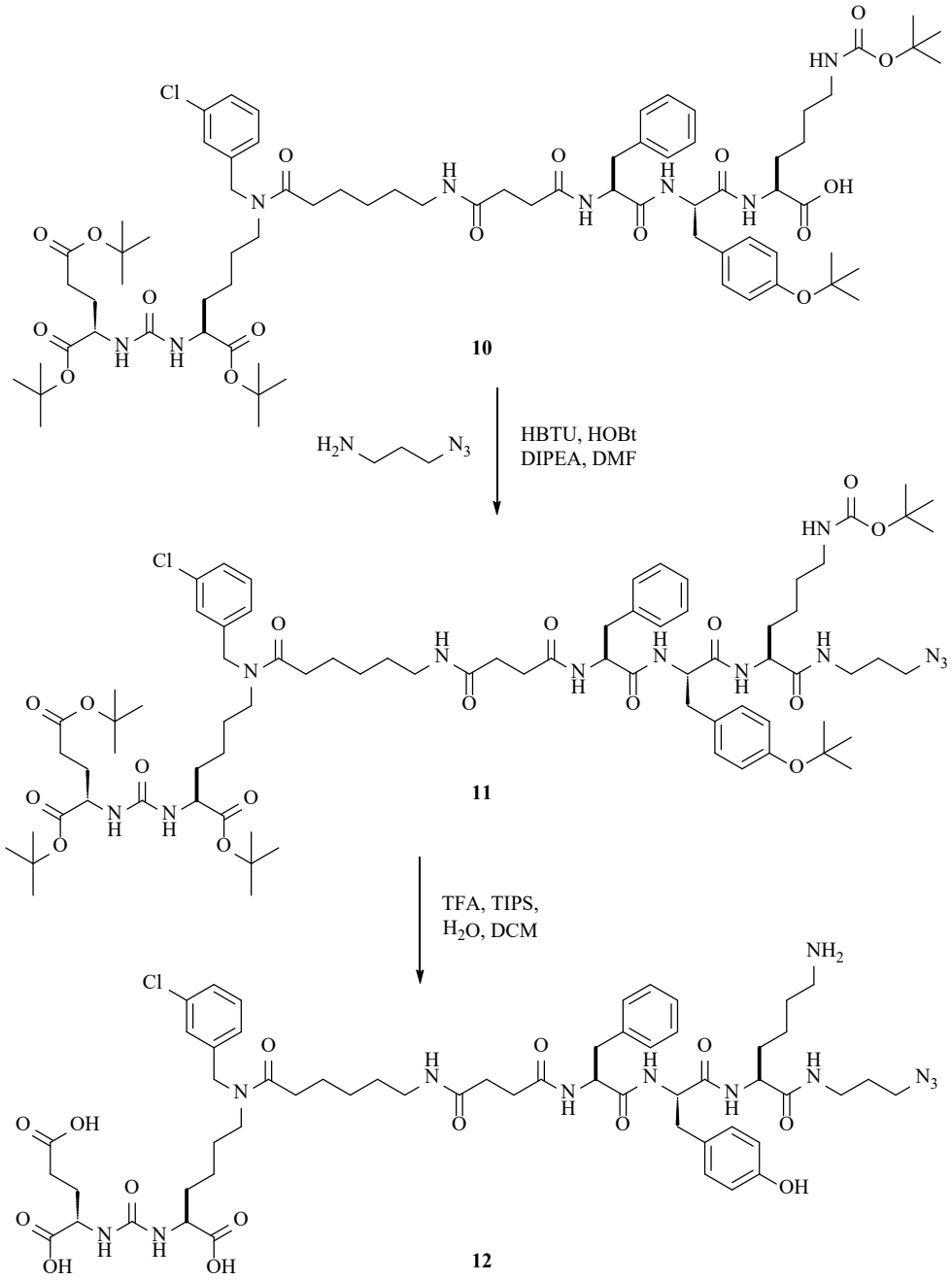
*Synthesis of* **3**. Compound **9** (934 mg, 1.436 mmol), monomethyl auristatin E (1000 mg, 1.393 mmol), HOBt (40 mg, 0.307 mmol), and pyridine (2.667 mL. 33.110 mmol) were dissolved in 20 mL of DMF. The resulting solution was stirred overnight. The reaction mixture was poured into ethyl acetate (100 mL). The precipitate was filtered and washed with ethyl acetate (3*30 mL). The final product was isolated using a preparative reversephase chromatography (gradient from 10:90 to 100:0 acetonitrile/water in 40 min, chromatographic column Puriflash C18-HP, 120 g, 15 μm). The final product was obtained as a white amorphous powder (1.276 g, 1.049 mmol), 75% yield.

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.97−9.99 (m, 1H), 8.29−8.31 (m,1H), 8.09−8.11 (m, 2H), 7.86−7.90 (m, 2H), 7.62−7.65 (d, J= 8.7 Hz, 1H), 7.56−7.58 (d, J = 6.4 Hz, 2H), 7.23−7.30 (m, 6H), 7.14−7.18 (m, 1H), 5.96−5.98 (m, 1H), 5.41−5.42 (m, 3H), 5.34−5.35 (m, 1H), 4.94−5.10

(m, 2H), 4.41−4.48 (m, 1H), 4.33−4.40 (m, 2H), 3.90−4.02 (m, 2H), 3.31−3.28 (m, 1H), 3.23−3.16 (m, 9H), 3.10−3.04 (m, 3H), 3.03−2.96 (m, 5H), 2.87−2.80 (m, 4H), 2.77−2.76 (m, 2H), 2.31−2.42 (m, 2H), 2.21−2.25 (m, 3H), 2.09−2.15 (m, 4H), 1.89−2.06 (m, 3H), 1.62−1.74 (m, 5H), 1.52−1.58 (m, 2H), 1.42−1.47 (m, 2H), 1.22−1.37 (m, 2H), 0.96−1.09 (m, 4H), 0.80−0.85 (m, 12H), 0.72−0.77 (m, 11H).

HRMS (ESI) m/z 1239.7361 (calculated 1239.7364 for $C_{64}H_{100}N_{10}O_{13}Na^+$ $[M + Na]^+$); m/z 1255.7090 (calculated 1255.7103 for $C_{64}H_{100}N_{10}O_{13}K^+$ $[M + K]^+$).

*Synthesis of ligand* **12** was carried out using literature methods according to the Scheme S2:[S3]




**Scheme S2**

*Synthesis of* **11**. Compound **10** (927 mg, 0.653 mmol, 1 eq.) was dissolved in 30 mL of DMF. The mixture was cooled down to 0 °C, and 131 mg of 3-azidopropylamine (1.306 mmol, 2 eq.), 132 mg of HOBt (0.979 mmol, 1.5 eq.), 227 µL of DIPEA (1.306 mmol, 2 eq.) and 371 mg of HBTU (0.979 mmol, 1.5 eq.) were added successively. The reaction mixture was stirred for 16 hours. The DMF was removed under reduced pressure, the dry residue was dissolved in dichloromethane and washed twice with water, twice with saturated sodium hydrogen carbonate solution and once with saturated sodium chloride solution. Then the organic fraction was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The product was isolated by column chromatography (InterchimPuriflash 120 g, 50µ, gradient from 0% methanol to 5% methanol in 30 min, then from 5% methanol to 10% in 15 min, then from 10% methanol to 100% in 10 min flow rate - 40 mL/min). Thus 796 mg (81% yield) of compound **11** was obtained as a yellow oily solid.

$^{1}$H NMR (400 MHz, $CDCl_3$) δ, ppm: 7.97 (t, J=6.11 Hz, 1H, NH), 7.79 (m, 1H, NH), 7.29-7.35 (m, 2H, Ar), 6.87-7.26 (m, 11H, Ar), 6.08-6.23 (m, 1H, NH), 4.41-4.57 (m, 3H, $CH_2$+CH), 4.21-4.41 (m, 4H, CH), 3.20-3.47 (m, 7H, $CH_2$), 2.85-3.20 (m, 6H, $CH_2$), 2.80 (m, 1H, $CH_2$), 2.53 (m, 1H, $CH_2$), 2.43 (m, 1H, $CH_2$), 2.13-2.39 (m, 6H, $CH_2$), 1.99-2.13 (m, 2H, $CH_2$), 1.79-1.91 (m, 3H, $CH_2$), 1.50-1.74 (m, 9H, $CH_2$), 1.36-1.50 (m, 40H, $CH_2$+$CH_3$), 1.19-1.36 (m, 17H, $CH_2$+$CH_3$).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ, ppm: 174.83, 172.85, 172.39, 172.11, 172.05, 172.00, 171.86, 171.67, 171.63, 156.58, 156.10, 154.94, 153.57, 139.58, 138.75, 135.18, 134.45, 133.92, 132.78, 129.82, 129.40, 128.99, 128.54, 128.24, 127.25, 126.96, 125.76, 125.51, 123.84, 81.46, 81.33, 81.06, 80.11, 80.04, 78.98, 77.95, 77.91, 76.89, 56.72, 56.64, 56.39, 53.49, 52.91, 52.70, 52.48, 52.38, 50.02, 48.33, 47.46, 46.79, 45.35, 40.29, 39.13, 36.49, 36.17, 34.62, 32.52, 32.22, 31.78, 31.17, 30.84, 30.35, 28.79, 28.43, 28.15, 27.98, 27.95, 27.76, 27.64, 27.59, 27.56, 26.28, 24.39, 23.78, 22.05.

Synthesis of **12**. Protected ligand **11** (795 mg) was dissolved in 15 ml mixture of trifluoroacetic acid (50% v/v), dichloromethane (40% v/v), triisopropylsilane (5% v/v) and water (5% v/v). The reaction mixture was stirred for 4 h. Then the solvent was removed under reduced pressure, after which the dry residue was precipitated with diethyl ether, decanted and the precipitate was washed three times with $Et_2O$. The product was isolated individually by reverse phase column chromatography (InterchimPuriflashC18 120 g, 15µ, gradient from 10% acetonitrile to 100% acetonitrile in 50 min, flow rate 40 mL/min). Thus 517 mg (76% yield) of compound **12** was obtained as a white amorphous powder.

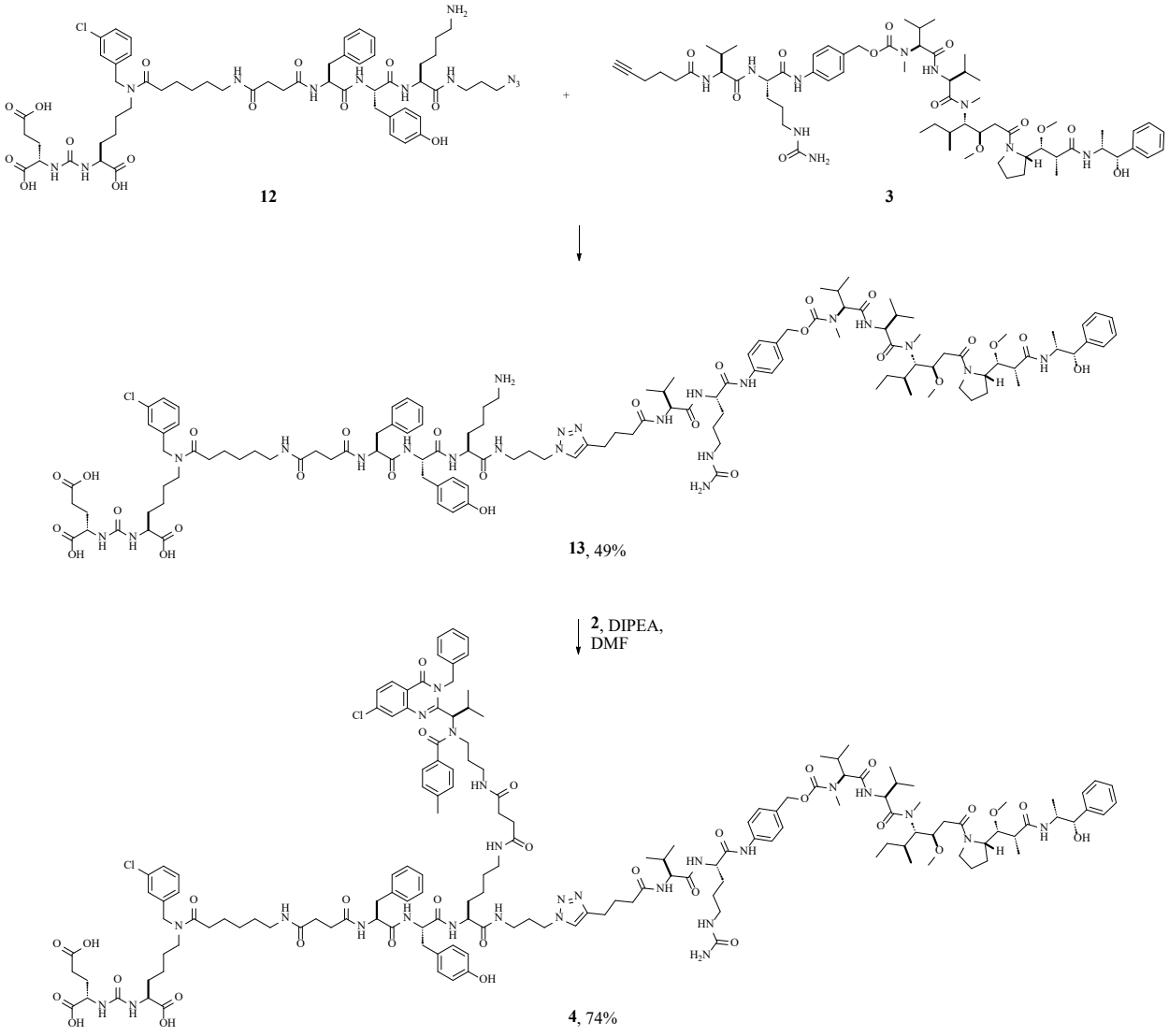
$^{1}$H NMR (400 MHz, DMSO-$d_6$) δ, ppm: 8.30-8.36 (m, 1H, NH), 8.12 (d, J=7.52 Hz, 1H, NH), 7.91-8.00 (m, 1H, NH), 7.74 (m, 1H, NH), 7.49-7.57 (m, 1H, NH), 7.24-7.39 (m, 2H, Ar), 7.07-7.24 (m, 7H, Ar), 7.03 (m, 2H, Ar), 6.65 (d, J=8.38 Hz, 2H, Ar), 6.28-6.38 (m, 2H, NH), 4.38-4.59 (m, 2H, $CH_2$), 4.22-4.35 (m, 2H, CH), 3.96-4.13 (m, 3H, CH), 3.25-3.33 (m, 2H, $CH_2$), 3.11-3.22 (m, 2H, $CH_2$), 3.07 (m, 2H, $CH_2$), 2.86-3.04 (m, 4H, $CH_2$), 2.76-2.86 (m, 1H, $CH_2$), 2.59-2.76 (m, 3H, $CH_2$), 2.11-2.39 (m, 8H, $CH_2$), 1.84-1.95 (m, 1H, $CH_2$), 1.55-1.75 (m, 5H, $CH_2$), 1.32-1.55 (m, 10H, $CH_2$), 1.07-1.32 (m, 7H, $CH_2$).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ, ppm: 174.87, 174.58, 174.15, 173.07, 172.56, 171.95, 171.62, 171.46, 157.70, 156.28, 141.58, 138.89, 138.32, 133.44, 130.98, 130.62, 130.42, 129.40, 128.47, 128.22, 127.57, 127.24, 126.67, 126.45, 125.34, 115.38, 58.52, 55.38, 52.99, 52.55, 52.06, 48.63, 47.29, 45.72, 39.07, 37.41, 37.28, 36.25, 32.16, 31.57, 31.16, 30.98, 30.31, 29.47, 29.37, 28.64, 27.95, 27.01, 26.67, 25.10, 22.68.

HPLC-MS: target compound content – 99.9%, $t_R$=11.05 min.

ESI-HRMS: for $C_{56}H_{77}ClN_{12}O_{14}$: m/z calculated for $[M+H]^+$ 1177.5371, found: 1177.5443; m/z calculated for $[M+Na]^+$ 1199.5269, found: 1199.5263.

Synthesis of bimodal conjugate **4** was carried out using adopted literature methods according to Scheme S3:[S3]




**Scheme S3**

Synthesis of **13**. Ligand **12** (104 mg, 80.5 μmol, 1.1 eq.) and compound **3** (89 mg, 73.2 μmol, 1 eq.) were dissolved in 13 mL of DMF/water mixture (3:1). The flask was filled with argon, then aqueous solutions 17 mg of sodium ascorbate (87.8 μmol, 1.2 eq.) and 6 mg of copper sulfate pentahydrate (29.3 μmol, 0.4 eq.) were added. The reaction mixture was stirred for 18 hours. Afterwards 17 mg of EDTA (58.6 μmol, 0.8 eq.) was added, and this was stirred for 3 hours in contact with air oxygen. The solvent was removed under reduced pressure, then the dry residue was precipitated with acetonitrile and decanted. The product was isolated by reverse phase column chromatography (InterchimPuriflash C18 40 g, 15μ, gradient from 10% acetonitrile to 100% acetonitrile in 30 min, flow rate 20 mL/min). Thus 86 mg (49% yield) of compound **13** was obtained as a white amorphous powder.

$^{1}$H NMR (400 MHz, DMSO-$d_6$) δ, ppm: 12.51 (br. s, 3H, COOH), 10.00 (m, 1H, NH), 9.22 (br. s, 1H, OH), 8.28 (m, 2H, NH), 8.13 (m, 3H, NH), 7.84-7.96 (m, 3H, NH), 7.82 (s, 2H, Triazole+NH), 7.52-7.74 (m, 6H, Ar+NH), 7.09-7.40 (m, 16H, Ar), 7.00-7.07 (m, 2H, Ar), 6.65 (d, J=7.82Hz, 2H, Ar), 6.31 (m, 2H, NH), 5.99 (br. s, 1H, NH), 5.42 (br. s, 2H, $NH_2$), 4.90-5.13 (m, 2H, $CH_2$), 4.59-4.77 (m, 1H, CH), 4.44-4.59 (m, 3H, $CH_2$+CH), 4.18-4.43 (m, 8H, CH+$CH_2$), 3.90-4.17 (m, 5H, CH), 3.30 (m, 2H, $CH_2$), 3.14-3.26 (m, 9H, $CH_2$+$CH_3$), 3.10 (s, 2H, $CH_2$), 2.91-3.07 (m, 9H, $CH_2$), 2.70-2.90 (m, 7H, $CH_2$), 2.61-2.69 (m, 1H, $CH_2$), 2.56 (t, J=7.49 Hz, 2H, $CH_2$), 2.30-2.44 (m, 3H, $CH_2$), 2.14-2.29 (m, 9H, $CH_2$), 2.11 (m, 1H, $CH_2$), 1.84-2.01 (m, 5H, $CH_2$), 1.56-1.84 (m, 10H, $CH_2$), 1.34-1.56 (m, 13H, $CH_2$+$CH_3$), 1.10-1.34 (m, 8H, $CH_2$), 0.94-1.07 (m, 6H, $CH_2$), 0.67-0.89 (m, 23H, $CH_2$+$CH_3$).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ, ppm: 174.50, 174.20, 173.77, 172.52, 172.17, 172.11, 171.71, 171.50, 171.34, 171.10, 170.62, 169.85, 168.75, 158.96, 157.29, 155.88, 146.46, 143.69, 141.22, 137.88, 133.41, 133.05, 130.62, 130.26, 130.05, 129.03, 128.18, 128.08, 127.83, 127.77, 127.19, 126.86, 126.68, 126.43, 126.29, 126.09, 124.97, 121.96, 118.81, 114.98, 114.84, 81.64, 74.82, 66.11, 63.29, 60.95, 58.18, 57.56, 57.14, 54.92, 53.18, 52.57, 52.15, 51.66, 50.28, 49.75, 49.18, 47.23, 46.86, 46.26, 43.77, 43.21, 38.71, 37.14, 36.97, 35.81, 35.06, 34.67, 32.28, 31.81, 31.57, 31.21, 30.79, 30.59, 30.47, 29.91, 29.84, 29.34, 29.10, 27.79, 27.54, 26.83, 26.65, 26.29, 25.43, 24.66, 24.37, 23.14, 22.52, 22.26, 19.28, 18.95, 18.77, 18.56, 18.40, 18.24, 15.91, 15.48, 15.33, 15.05, 10.32.

HPLC-MS: target compound content – 99.9%, $t_R$=11.47 min.

ESI-HRMS: for $C_{120}H_{177}ClN_{22}O_{27}$: m/z calculated for $[M+2H]^{2+}$ 1197.64938, found: 1197.645

Synthesis of **4**. Monoconjugate **13** (54 mg, 22.5 μmol, 1 eq.) was dissolved in 5 ml DMF, compound **2** (19 mg, 27.1 μmol, 1.2 eq.) and 24 μl DIPEA (135.2 μmol, 6 eq.) were added. The reaction mixture was stirred for 20 hours, after which the solvent was removed under reduced

pressure. The dry residue was precipitated with acetonitrile, the resulting precipitate was decanted and washed three times with acetonitrile. The solvent residue was removed under reduced pressure. Thus 50 mg (74% yield) of compound **4** was obtained as a white amorphous powder.

$^1$H NMR (400 MHz, DMSO-$d_6$) δ, ppm: 9.95−10.20 (m, 1H, NH), 8.47−9.03 (m, 1H, NH), 8.17−8.41 (m, 3H, Ar+NH), 7.97−8.13 (m, 2H), 7.82−7.96 (m, 3H, Ar+NH), 7.80 (s, 1H, Triazole), 7.53−7.69 (m, 3H, Ar), 7.40−7.50 (m, 1H, NH), 7.07−7.39 (m, 25H, Ar), 7.04 (d, *J*=7.46 Hz, 2H, Ar), 6.66 (d, *J*=8.01 Hz, 2H, Ar), 6.06−6.43 (m, 2H, NH), 5.87 (d, *J*=16.51 Hz, 1H, $CH_2$), 5.35−5.57 (m, 3H, CH+$CH_2$), 4.90−5.15 (m, 3H, $CH_2$), 4.58−4.79 (m, 1H, CH), 4.07−4.56 (m, 13H, CH+$CH_2$), 3.74−4.07 (m, 6H, CH+$CH_2$), 2.78−3.08 (m, 19H, $CH_2$), 2.64−2.78 (m, 3H, $CH_2$), 2.06−2.41 (m, 19H, $CH_2$+$CH_3$), 1.13−2.04 (m, 42H, $CH_2$+$CH_3$+CH), 0.92−1.12 (m, 10H, $CH_2$+$CH_3$), 0.69−0.91 (m, 28H, $CH_2$+$CH_3$), 0.46 (d, *J*=5.75 Hz, 3H, $CH_3$).

HPLC-MS: target compound content – 99.9%, $t_R$=8.69 min.

ESI-HRMS: for $C_{154}H_{212}Cl_2N_{26}O_{31}$: m/z calculated for $[M+3H]^{3+}$ 998.18024, found: 998.180; m/z calculated for $[M+2H]^{2+}$ 1496.76672, found: 1496.766; m/z calculated for $[M-2H]^{2-}$ 1494.75217, found: 1494.757

**General information about *in vitro* studies**

Cytotoxicity tests against human cells (MTT test)

Cytotoxicity of each sample was tested in vitro against human cells in 72 hours for a series of dilutions of the sample in the Mosmann MTT test.[S4]

Tests were performed against tumor adhesion cells of breast adenocarcinoma MCF7, lung adenocarcinoma A549, androgen-independent prostate cancer PC3, androgen-sensitive prostate cancer LNCaP, androgen-responding prostate cancer 22Rv1, etiologically non-tumor adhesion cells of human embryonic kidney HEK293T and immortalized lung fibroblasts VA13. A drug with known cytotoxicity, doxorubicin, was used as a test control.

5000 VA13 cells, or 3000 A549 cells, or 6000 MCF7 cells, or 2500 HEK293T cells per well were sown on 96-well plates in a DMEM-F12 medium containing 10% FBS, 50 U/ml penicillin, 0.05 mg/ml of streptomycin. Medium DMEM-F12 for MCF7 cells additionally contains 0.01 mg/ml of insulin.

2500 PC3 cells, or 4000 LNCaP cells, or 3000 22Rv1 cells per well were sown on 96-well plates in a RPMI medium containing 10% FBS, 50 U/ml penicillin, 0.05 mg/ml of streptomycin.

After 18 hours of growth, samples diluted in DMSO were added to the cells (8-14 concentrations in 3x increments, the highest concentration in cells was 11111 nmol for ispinesib and 100000 nmol for other compounds). Cells with added compounds were incubated for 72 hours at 37°C and 5% $CO_2$. 1 g of MTT reagent was dissolved in 200 ml of PBS in an ultrasonic bath and then filtered (0,45 μm filter) (concentration of MTT solution 5 mg/ml). After that MTT was added to the cells with a final concentration of 0.5 mg/ml and incubated for 2-4 hours at 37°C in the incubator, in the atmosphere of 5% $CO_2$.

Then the MTT solution was removed and 140 μl of DMSO per well was added. The tablets were shaken for 10 minutes on a shaker (80 rpm) to dissolve the formazane. Absorption was measured using a tablet reader (VICTOR X5 Light Plate Reader, PerkinElmer, USA) at a wavelength of 565 nm. The results were processed in GraphPad Software (San Diego, California), $CC_{50}$ values were obtained.

When processing the test results, a concentration leading to a decrease in the number of cells by 50% ($CC_{50}$) was noted.

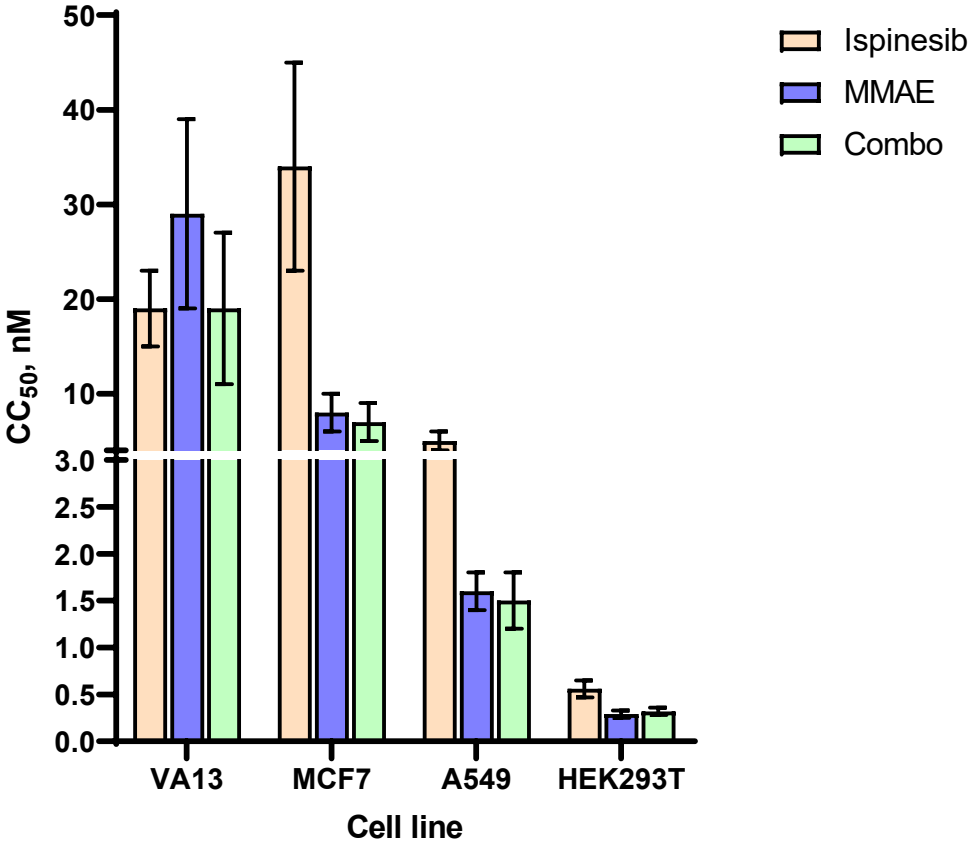
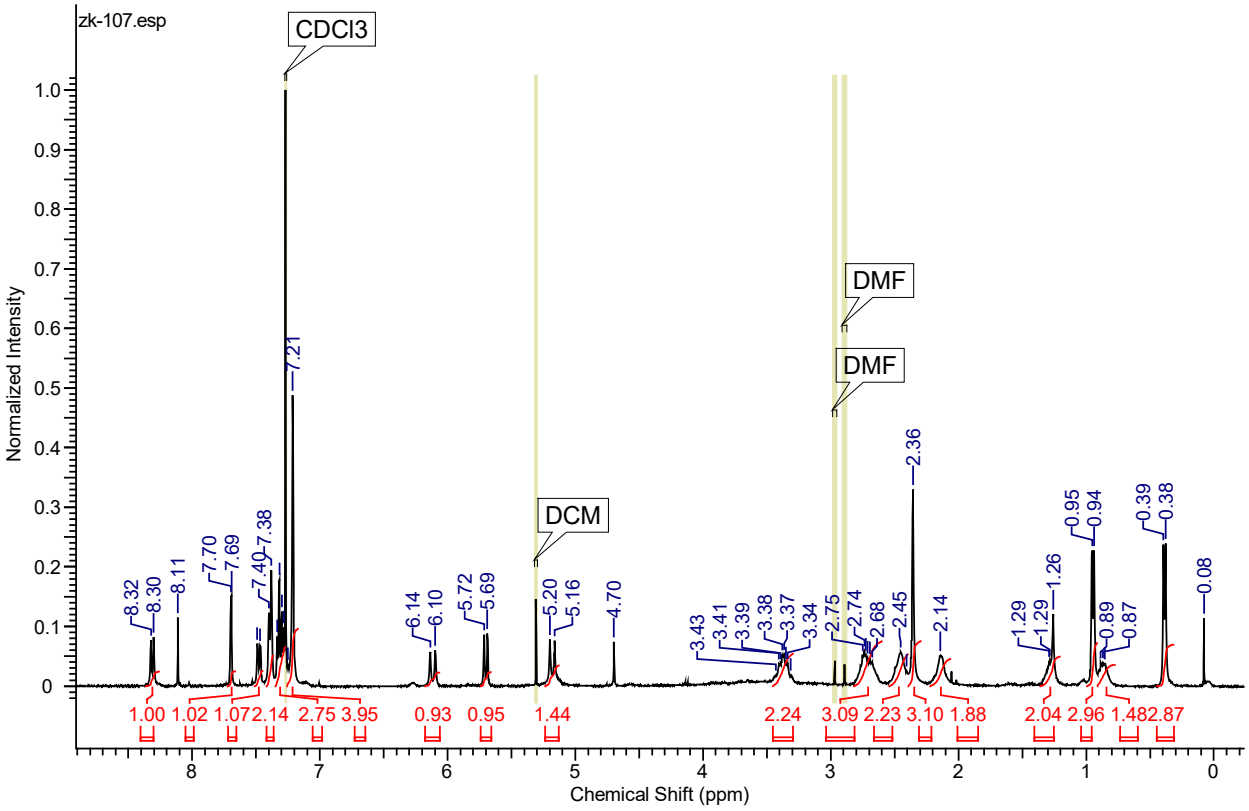


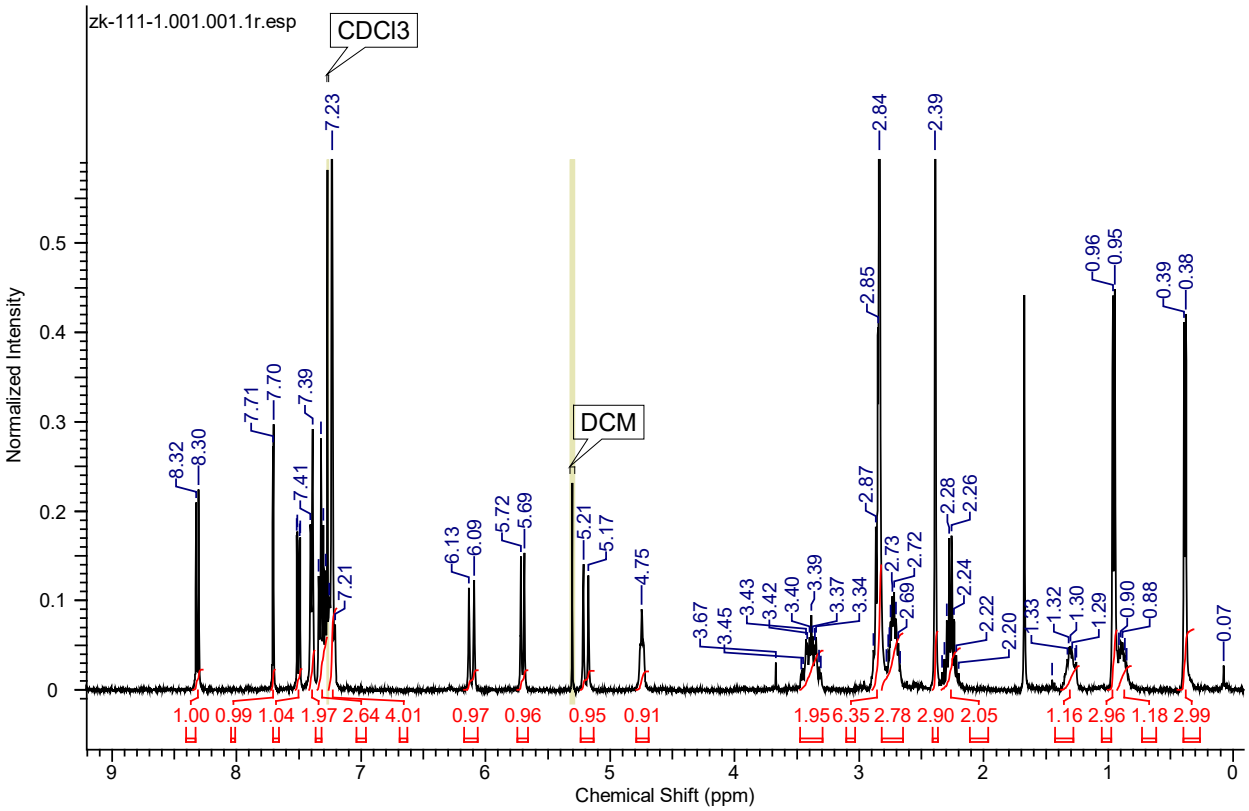

**Figure S1.** $CC_{50}$ values obtained for MMAE, ispinesib and their equimolar mixture

## Spectral data of the synthesized compounds

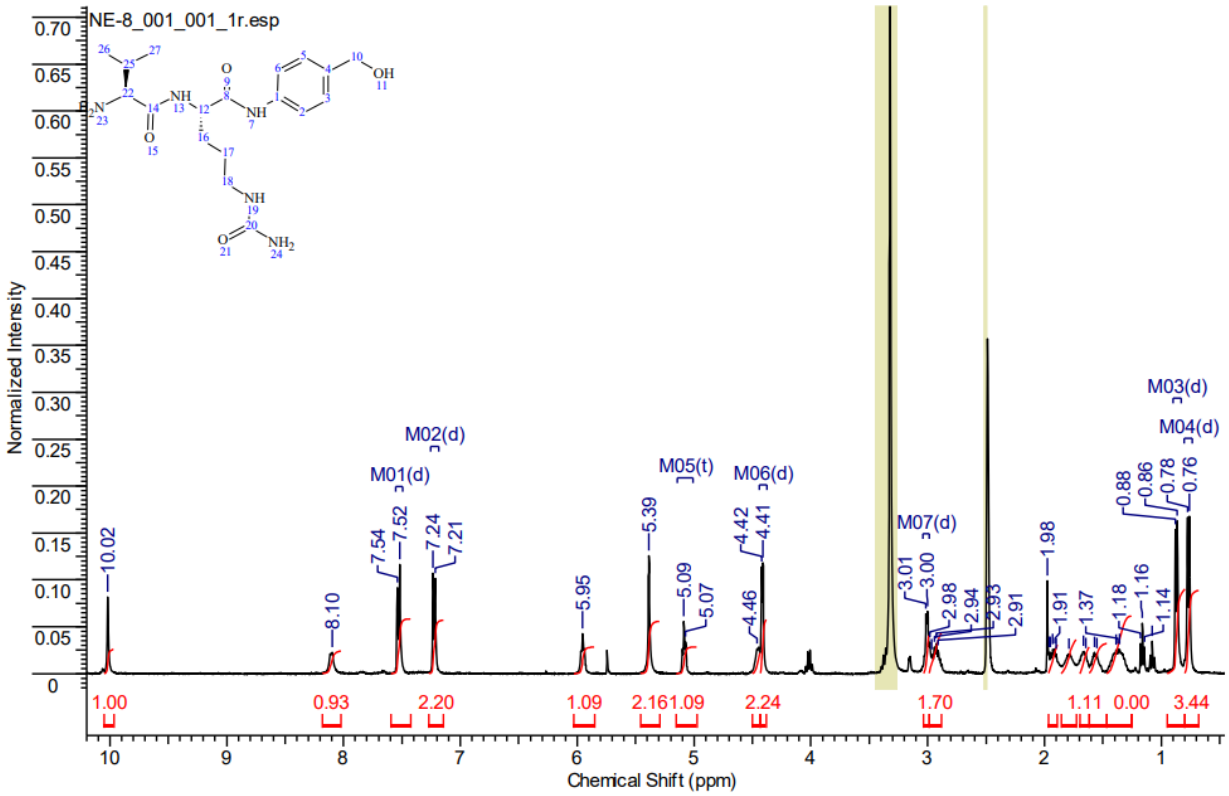
$^1$H NMR spectra for compound **1**



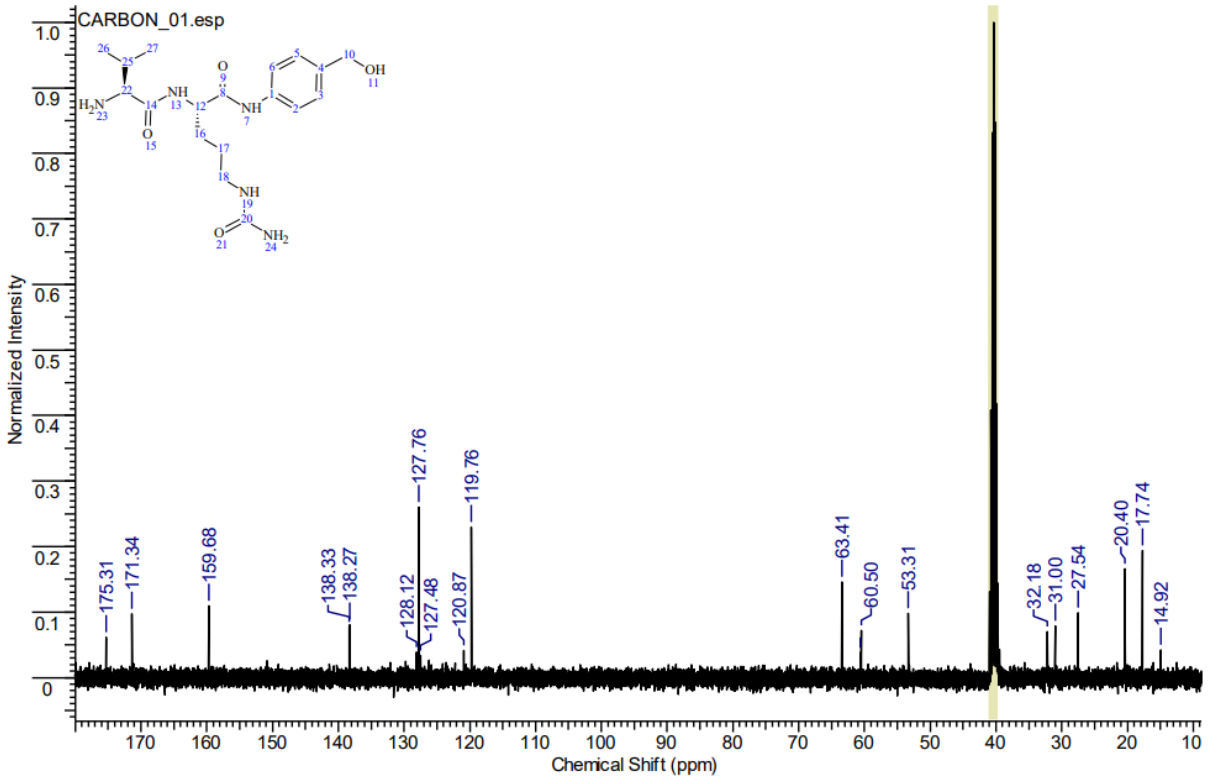

$^1$H NMR spectra for compound **2**



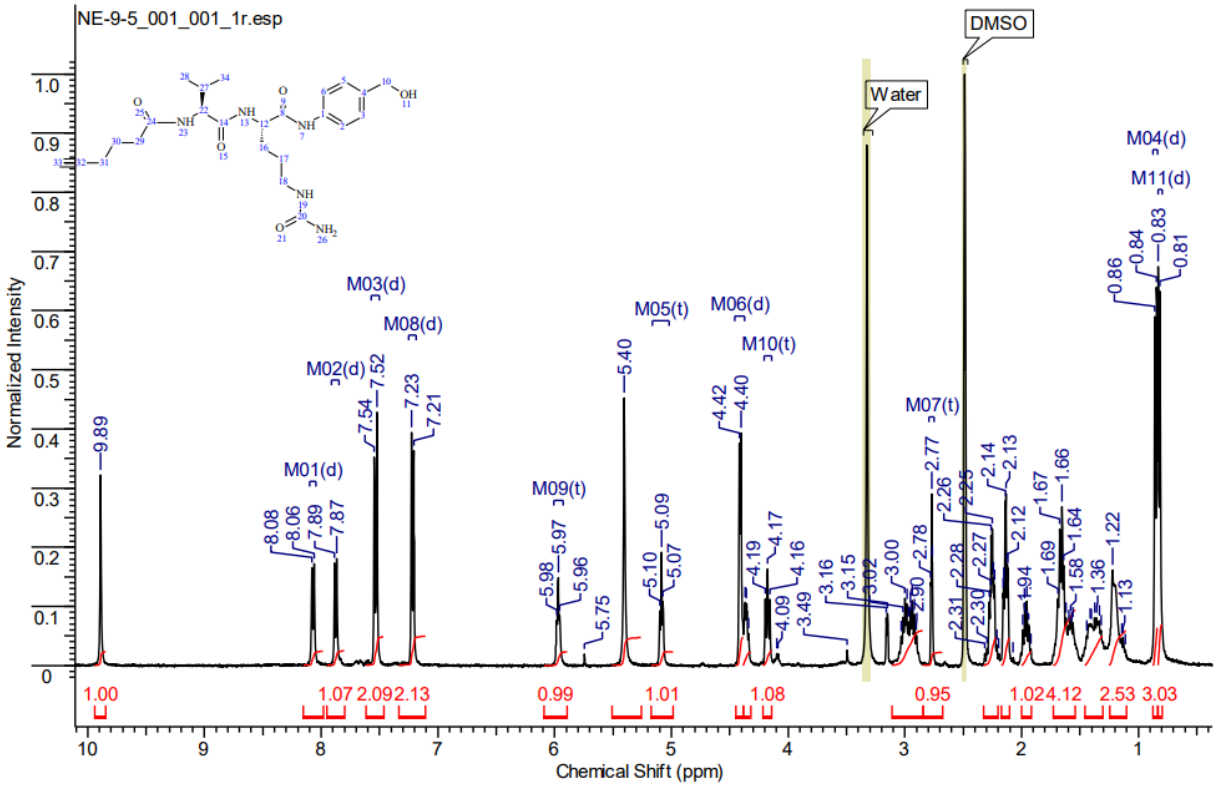
$^{1}$H NMR spectra for compound **7**



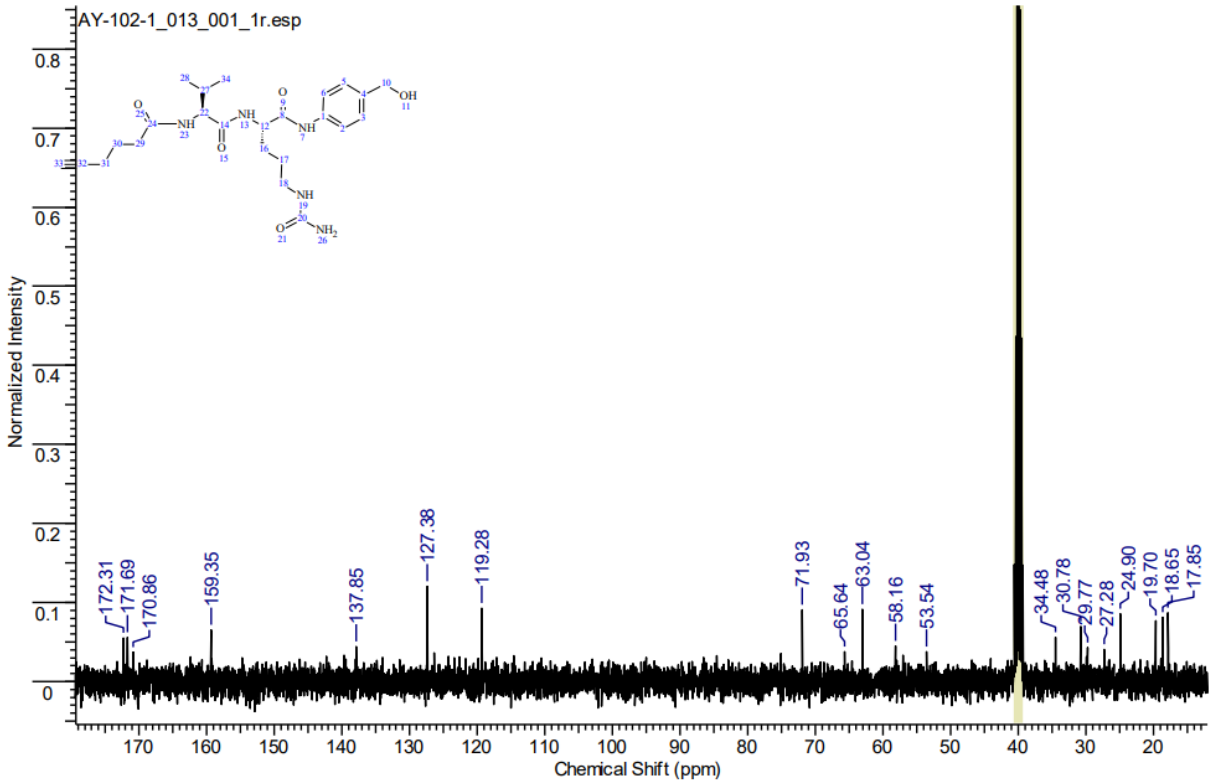

$^{13}$C NMR spectra for compound **7**



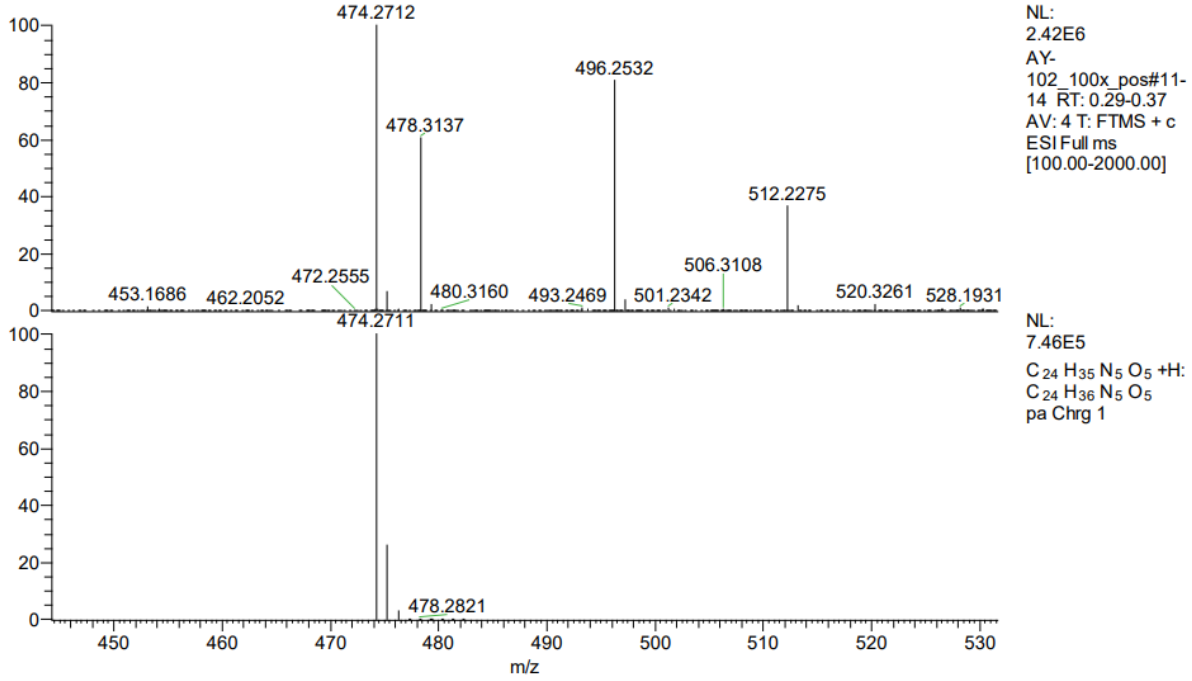
$^{1}$H NMR spectra for compound **8**



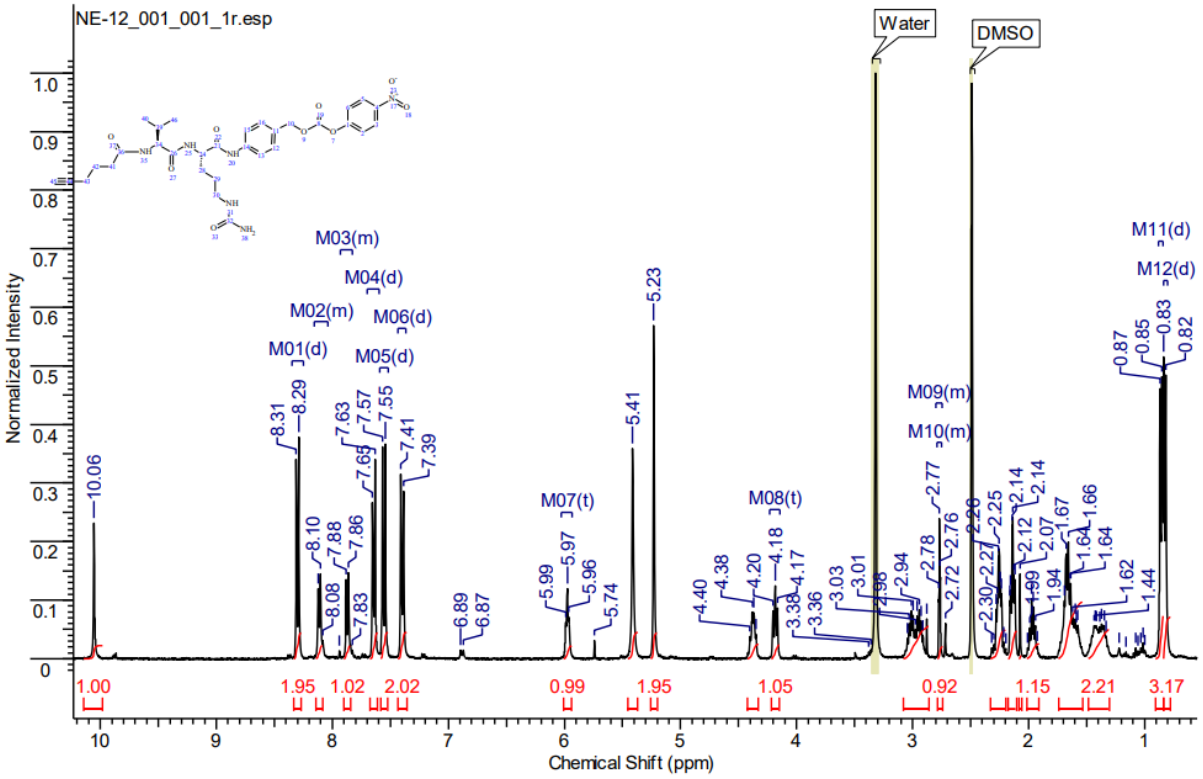

$^{13}$C NMR spectra for compound **8**



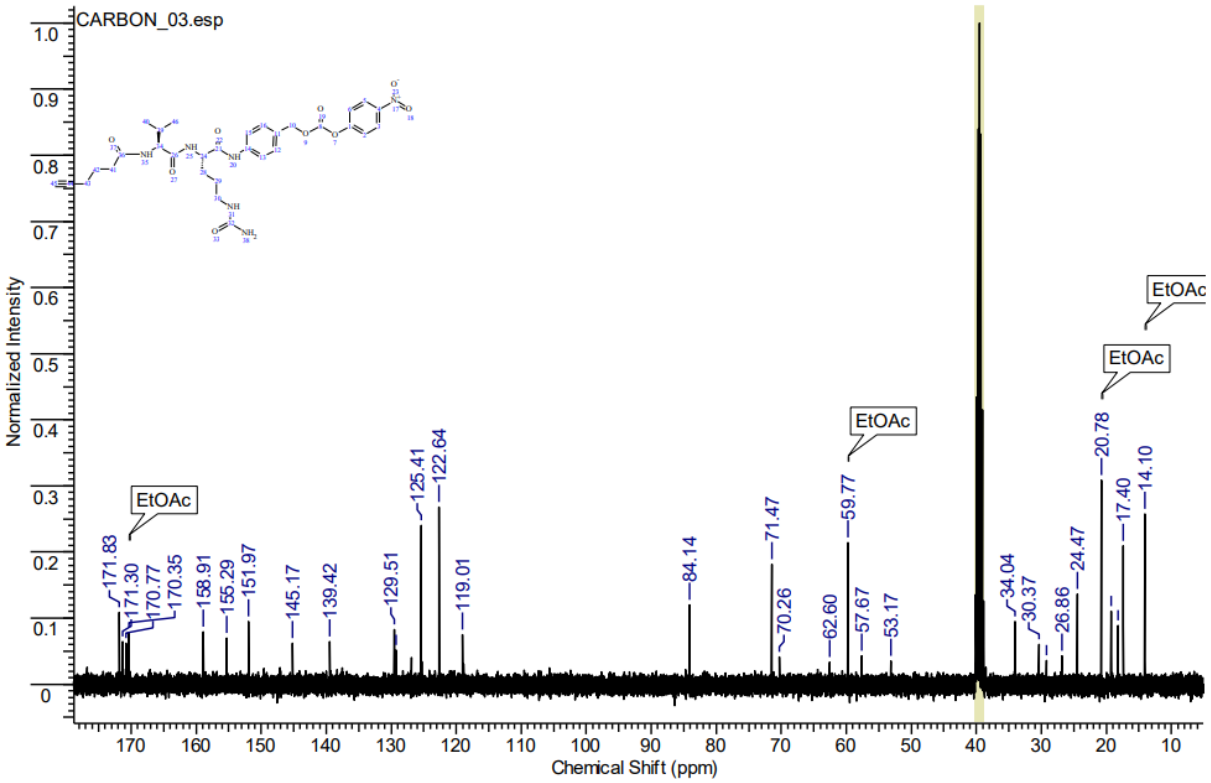
HRMS spectra for compound **8**



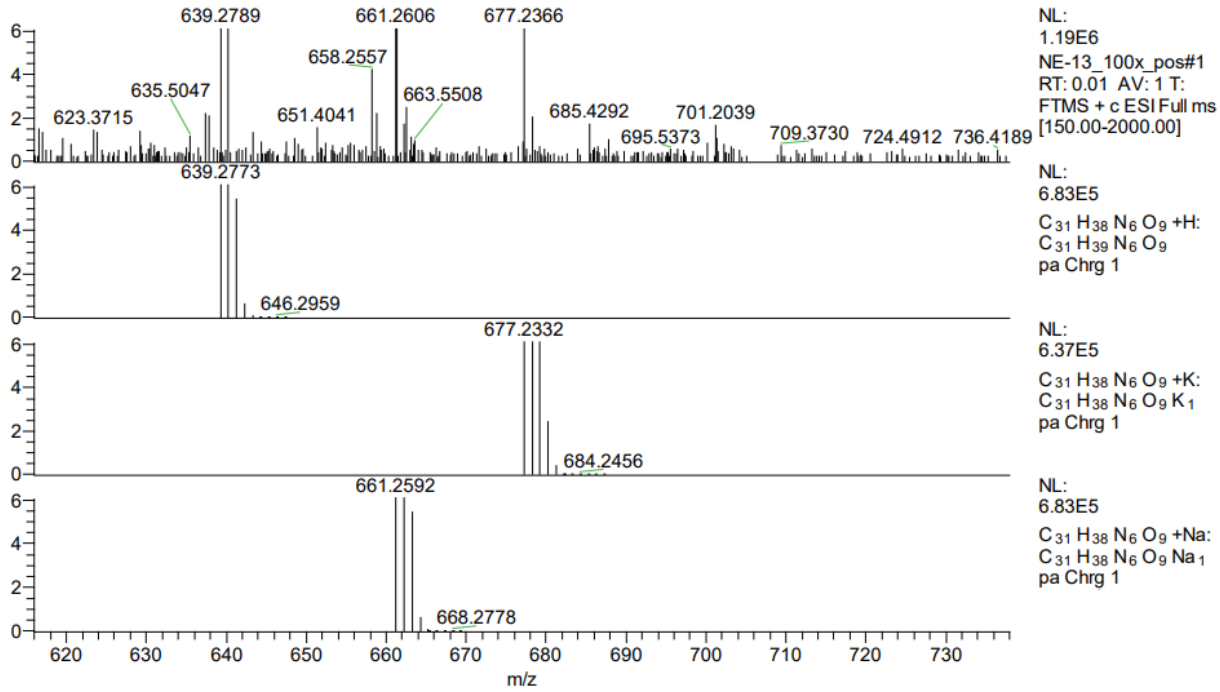

$^{1}$H NMR spectra for compound **9**



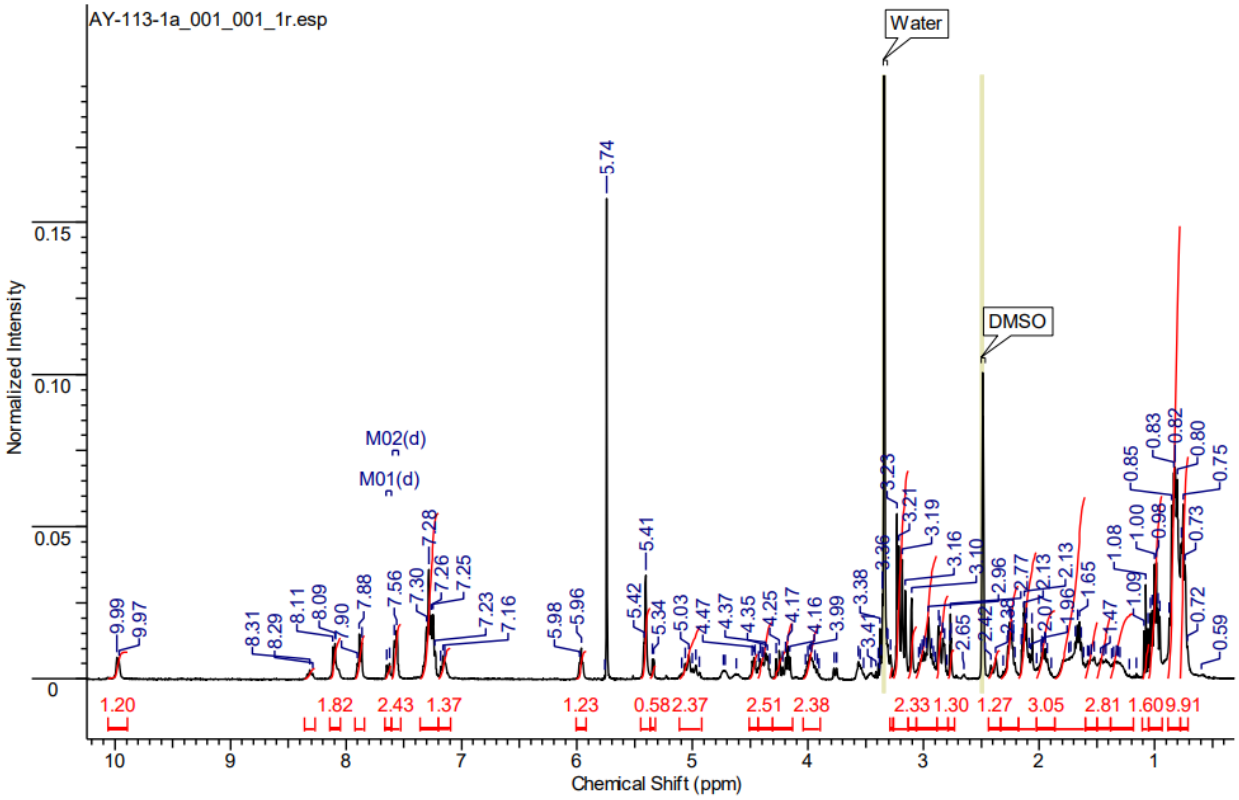
$^{13}$C NMR spectra for compound **9**



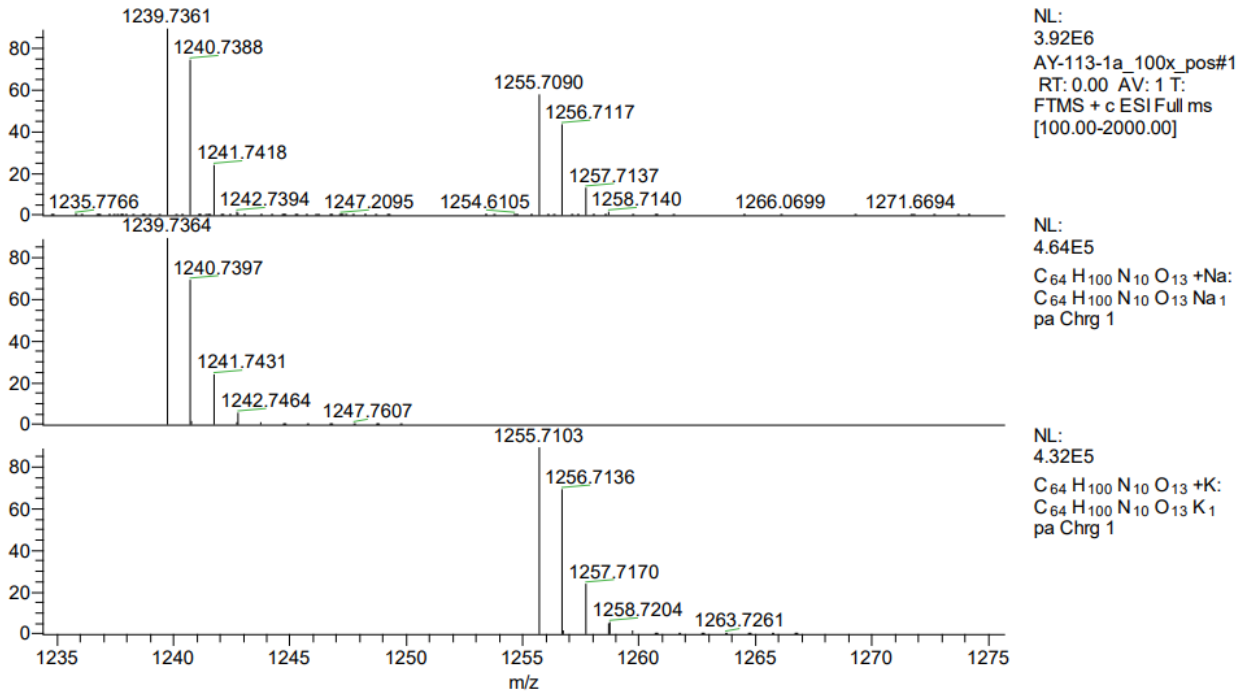

HRMS spectra for compound **9**



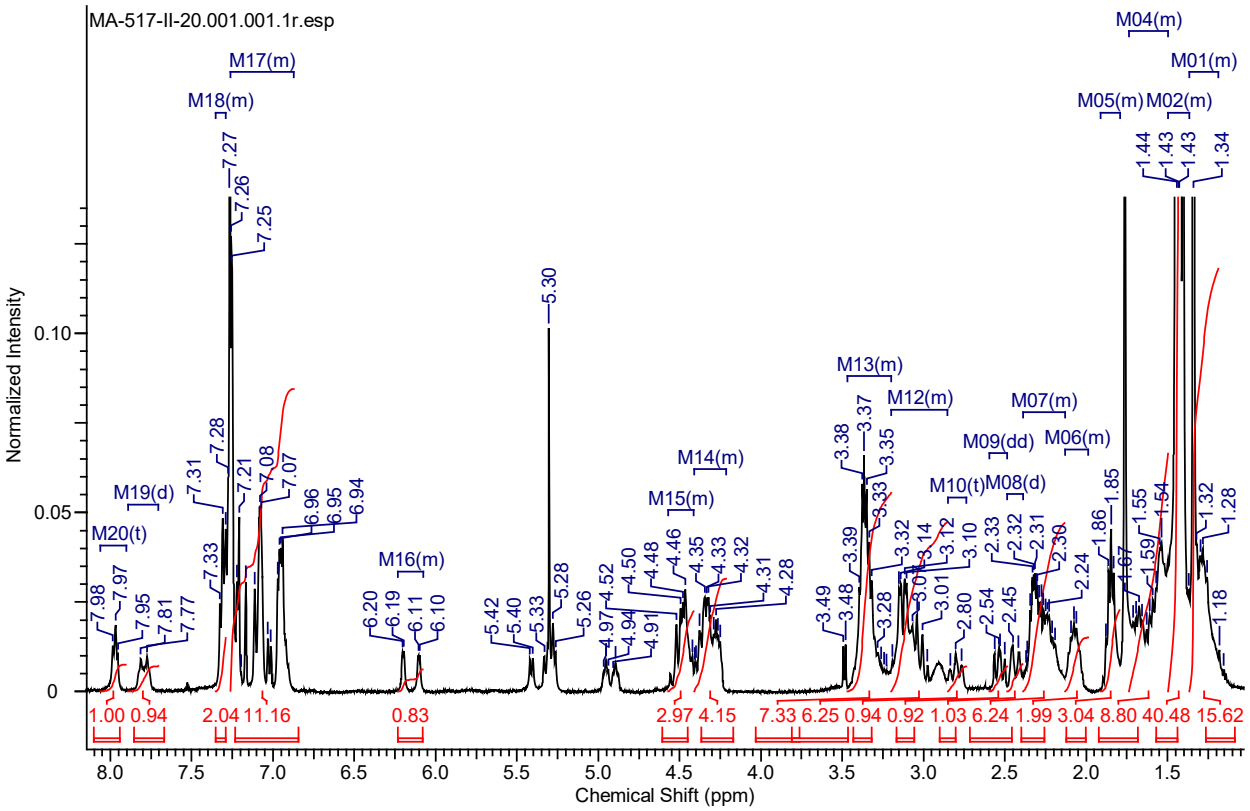
$^{1}$H NMR spectra for compound **3**



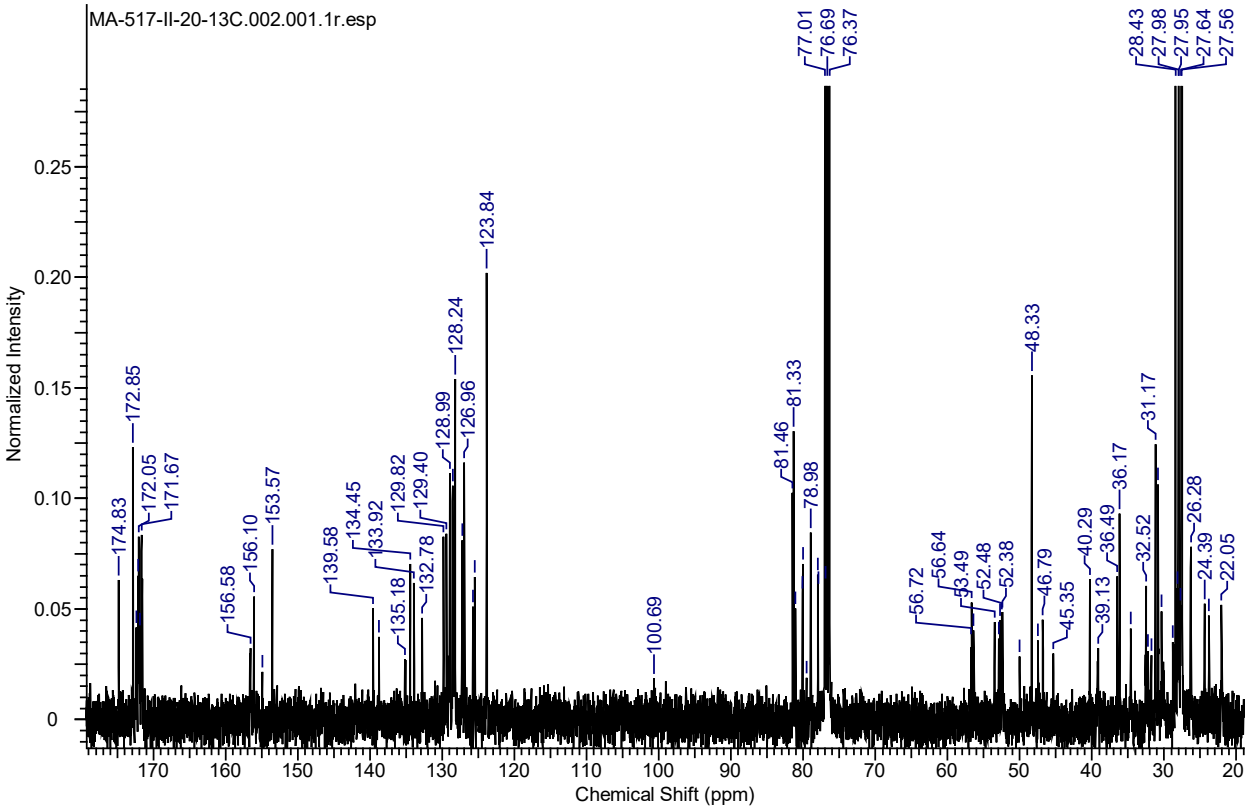

HRMS spectra for compound **3**



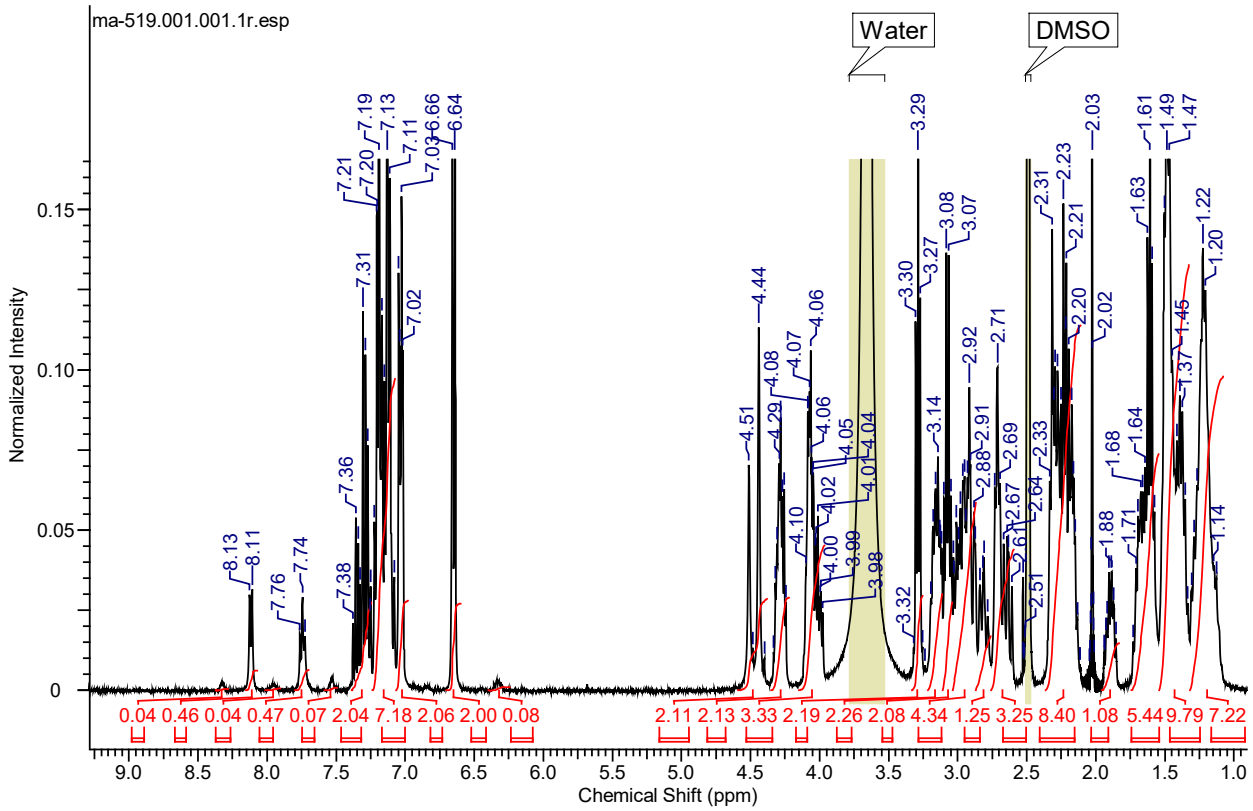
$^{1}$H NMR spectra for compound **11**



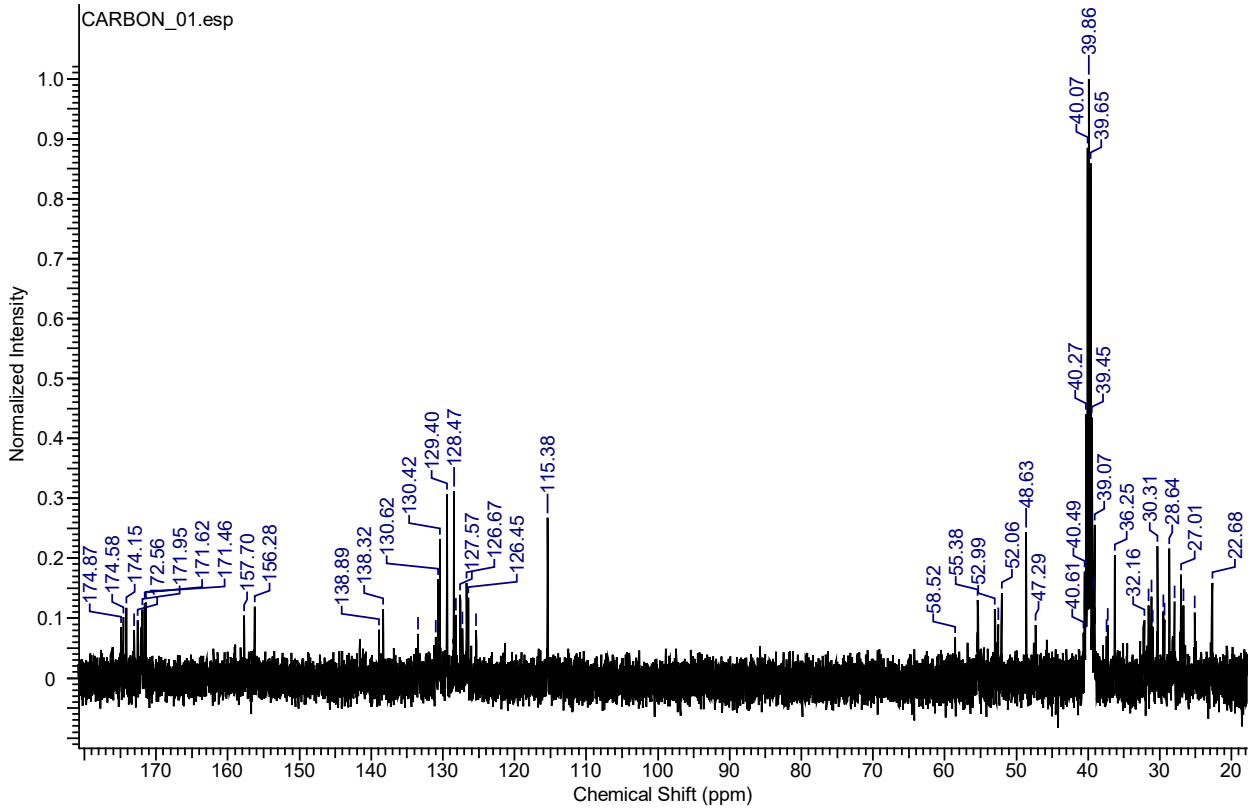

$^{13}$C NMR spectra for compound **11**



$^{1}$H NMR spectra for compound **12**

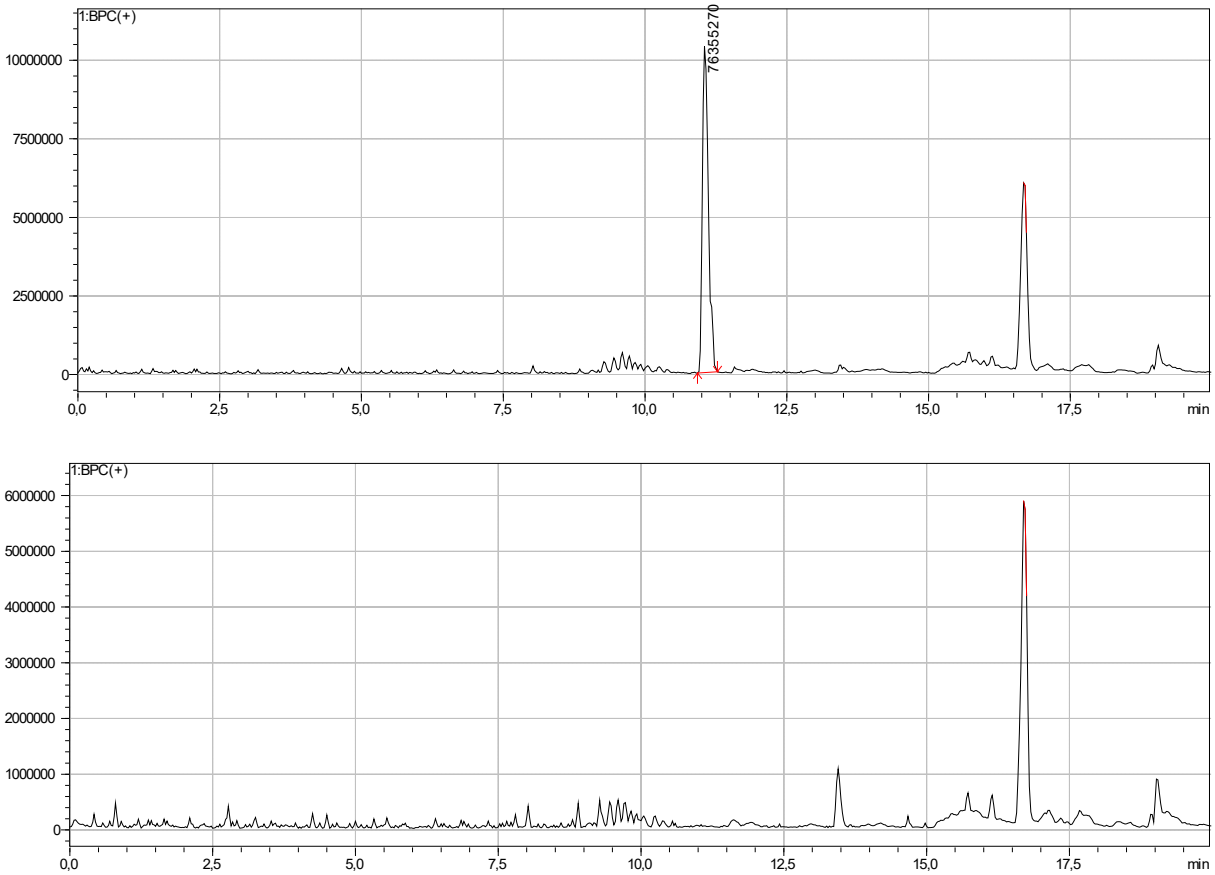



$^{13}$C NMR spectra for compound **12**

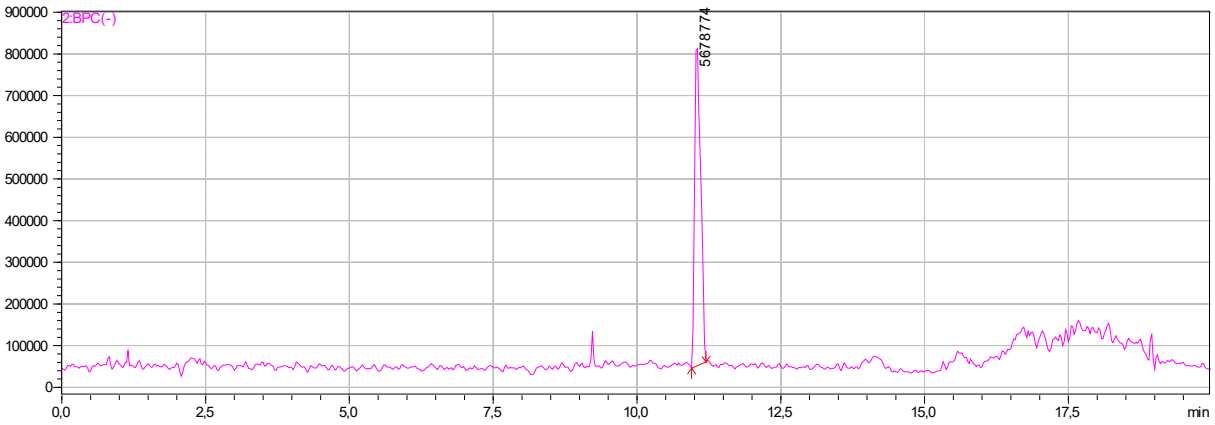


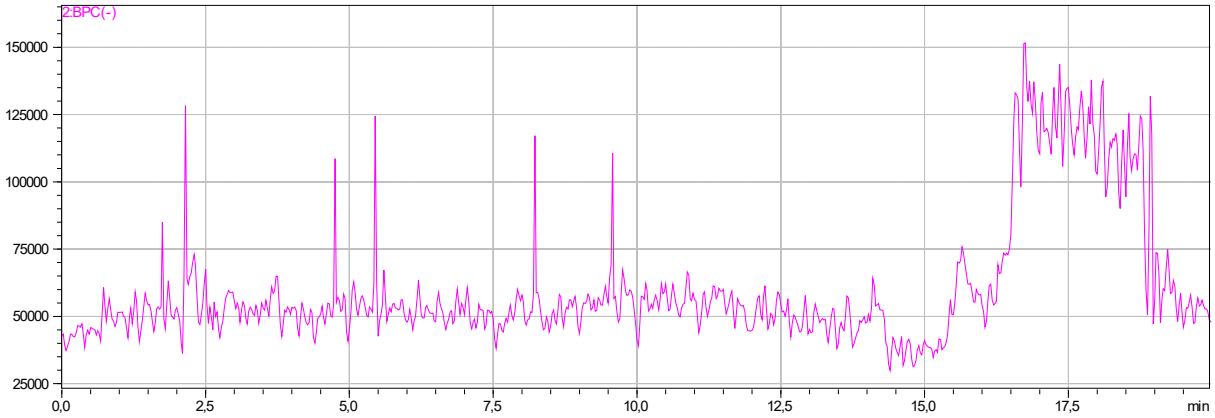
HPLC chromatogram for compound **12**

Positive ions (chromatogram and blank)



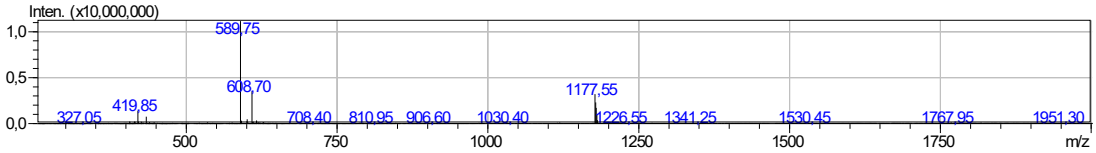

Negative ions (chromatogram and blank)



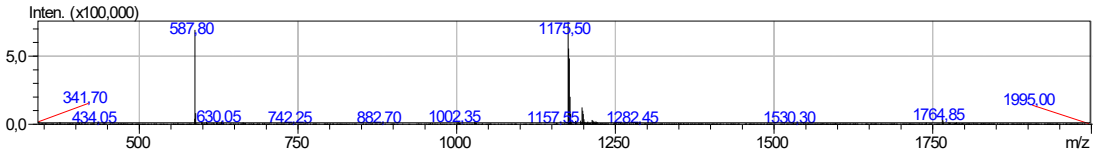



LCMS spectra for compound **12**

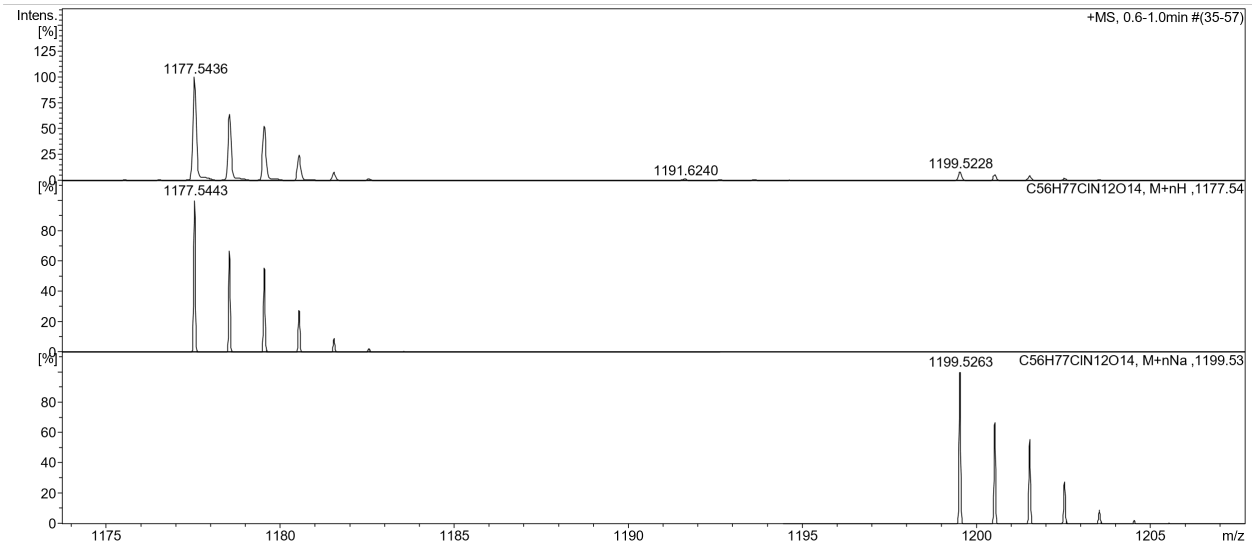
Positive ions spectra for compound **12**



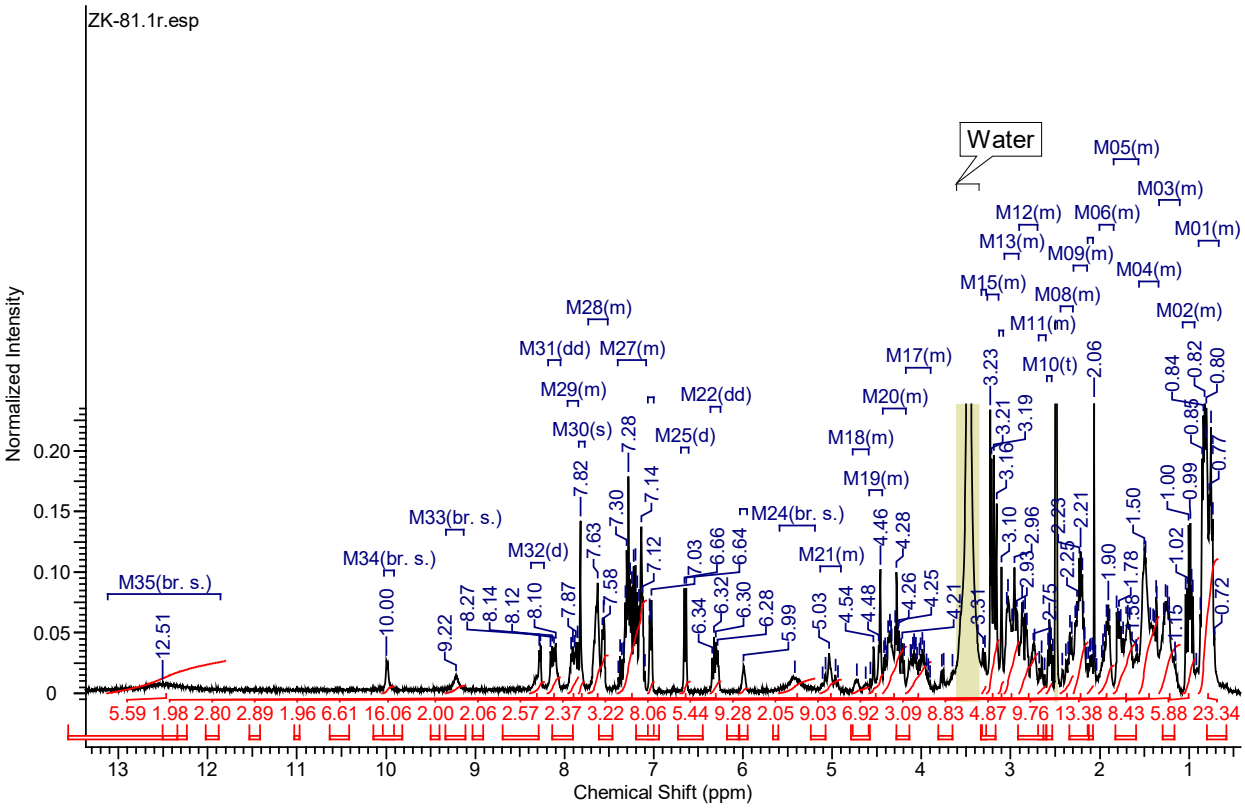

Negative ions spectra for compound **12**



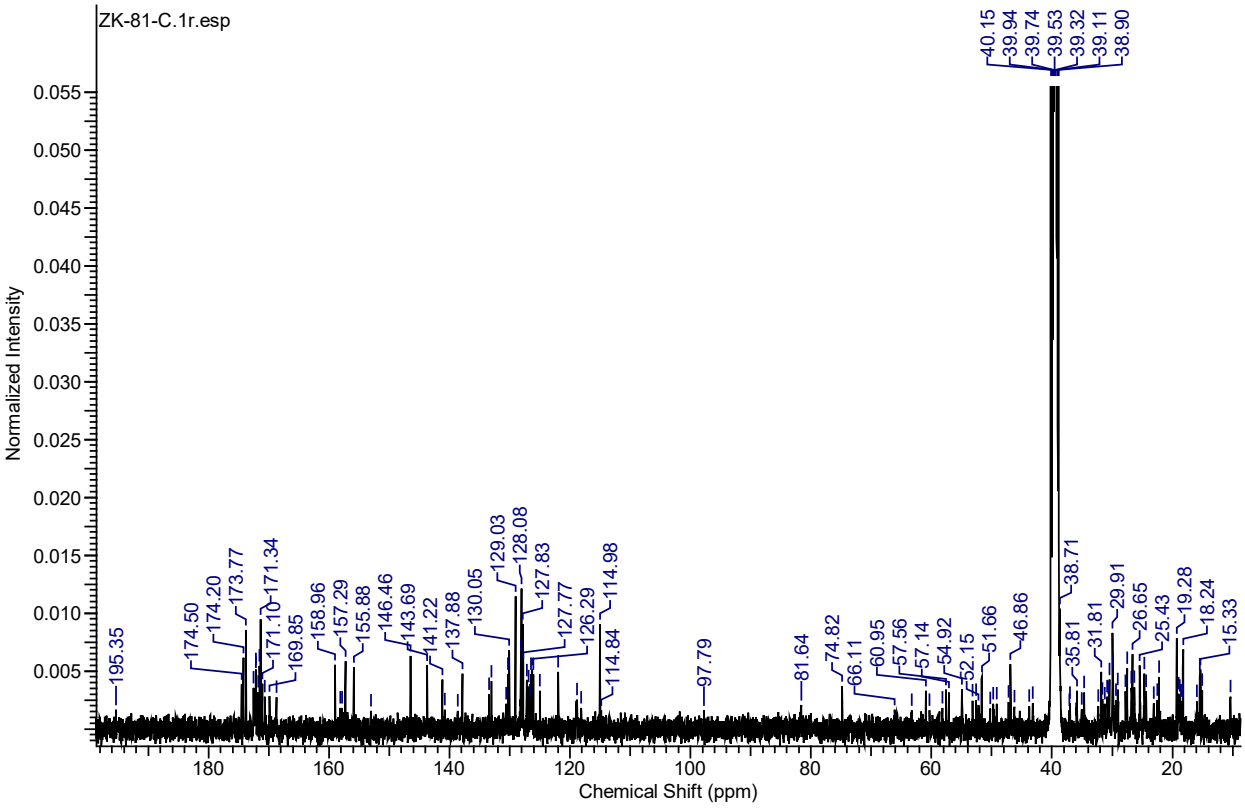

HRMS spectra for compound **12**



$^{1}$H NMR spectra for compound **13**

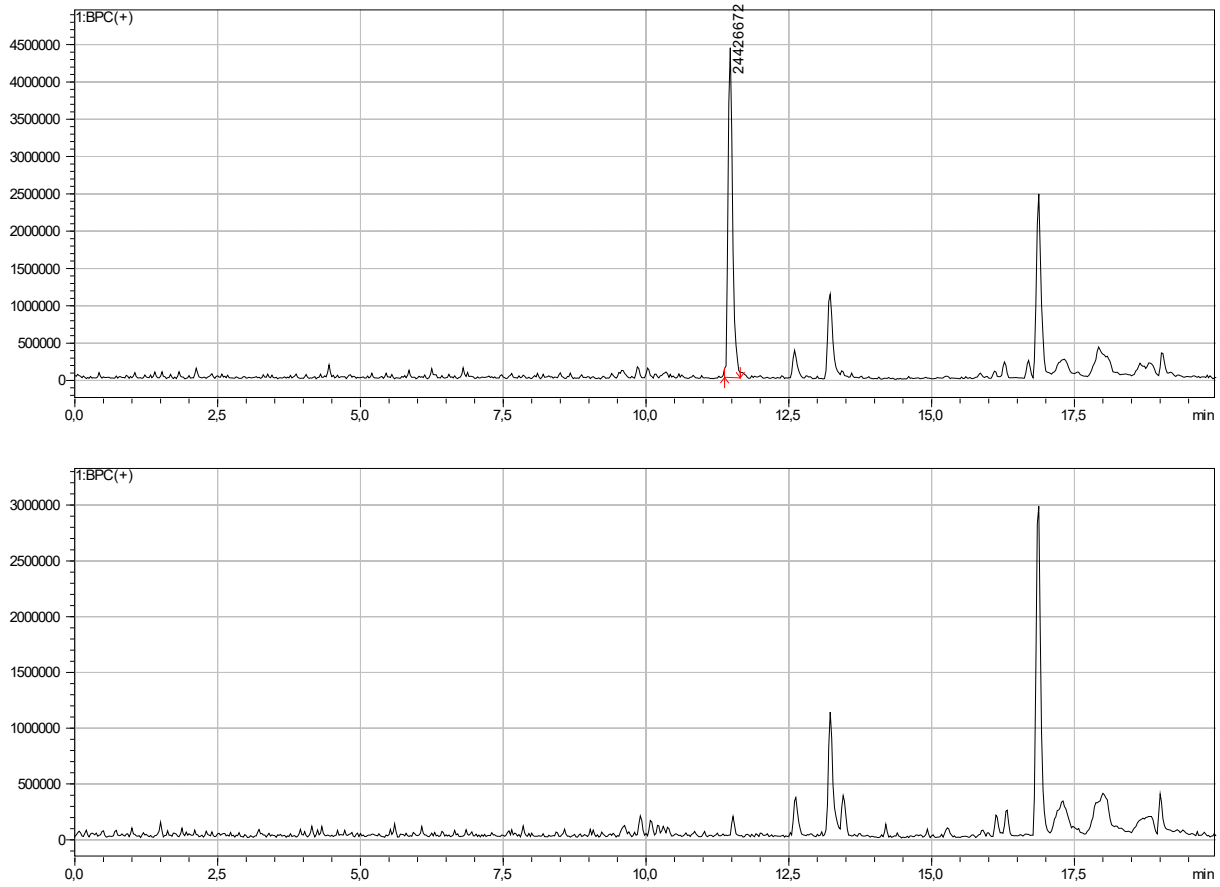



$^{13}$C NMR spectra for compound **13**

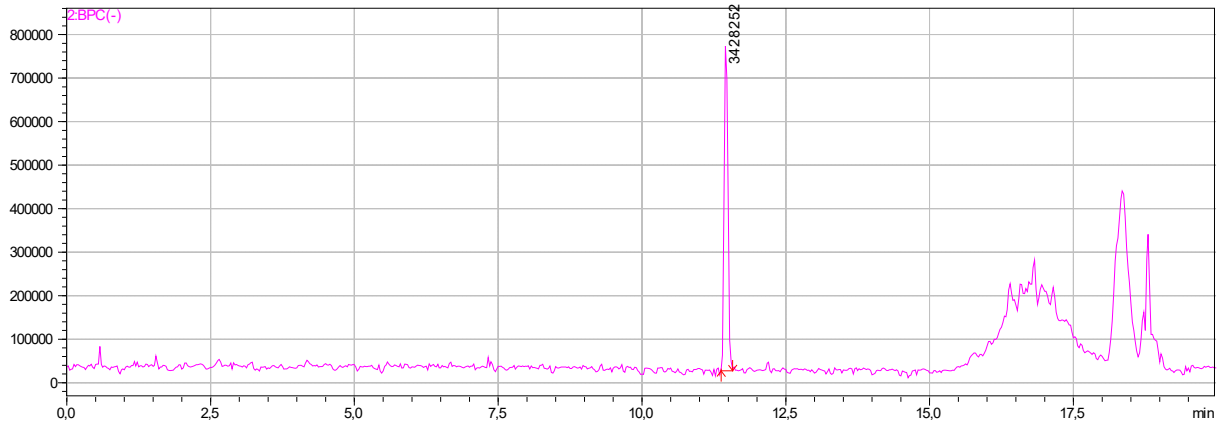


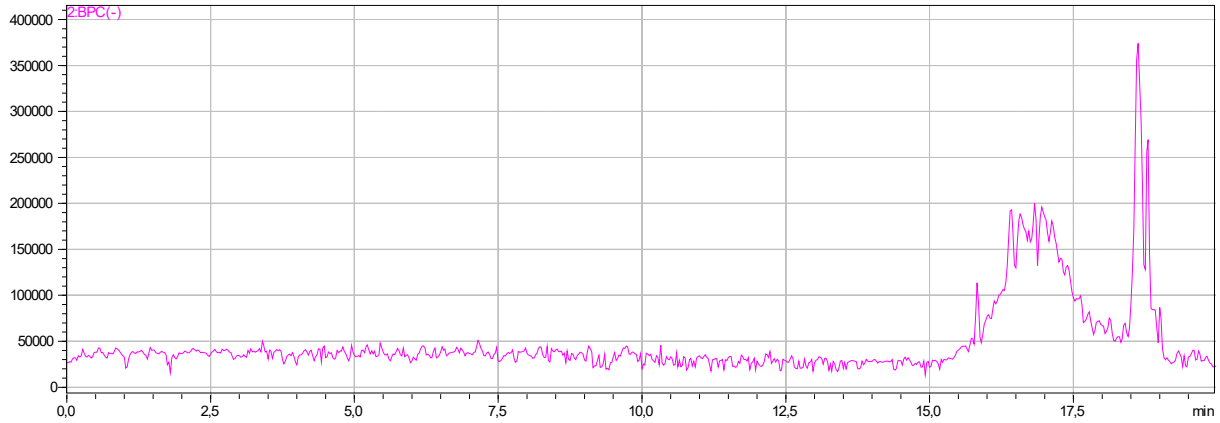
HPLC chromatogram for compound **13**

Positive ions (chromatogram and blank)



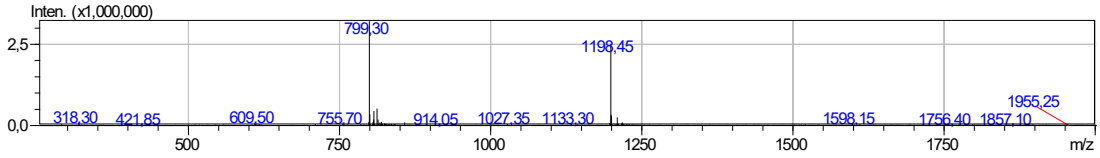

Negative ions (chromatogram and blank)



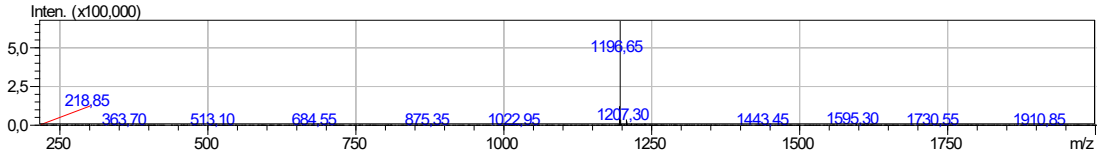



LCMS spectra for compound **13**

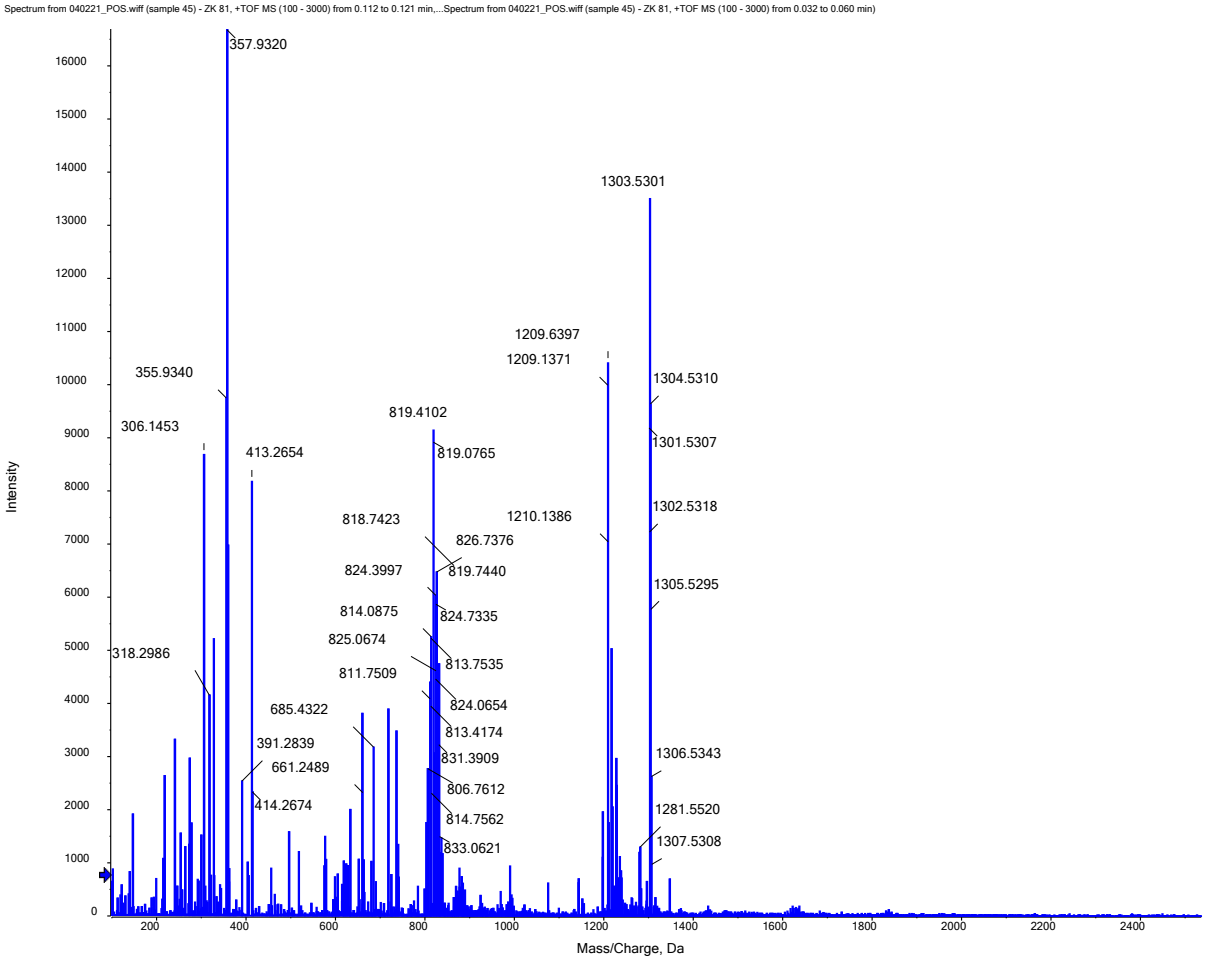
Positive ions spectra for compound **13**



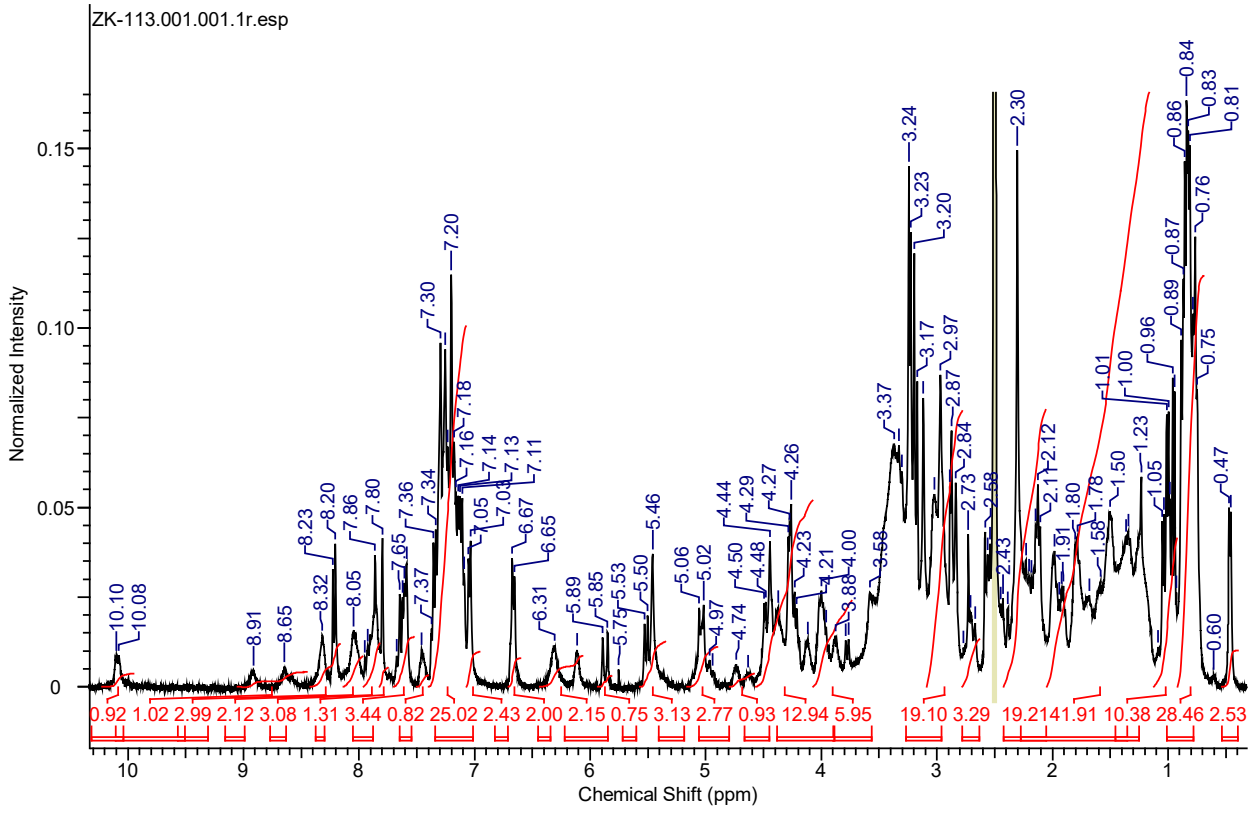

Negative ions spectra for compound **13**



HRMS spectra for compound **13**

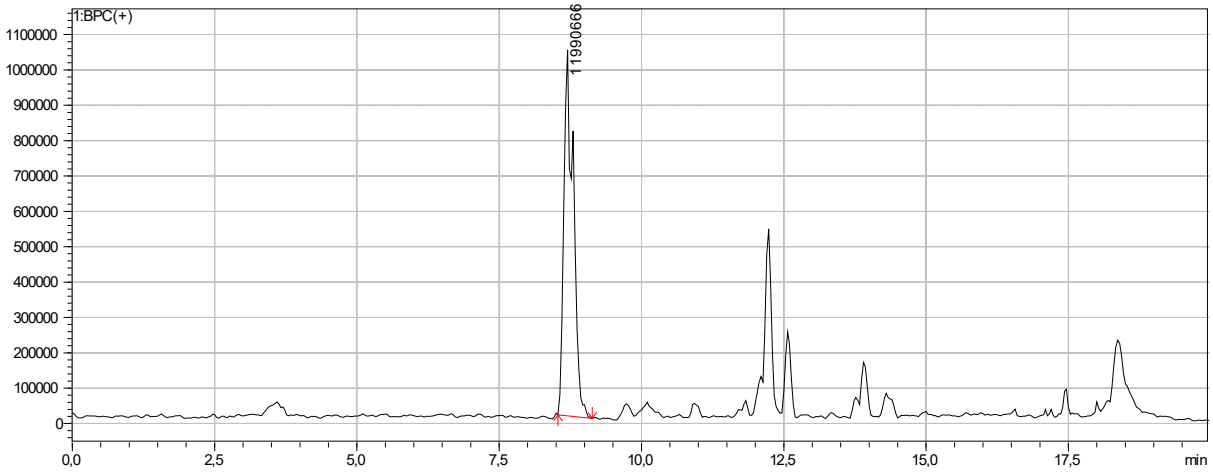


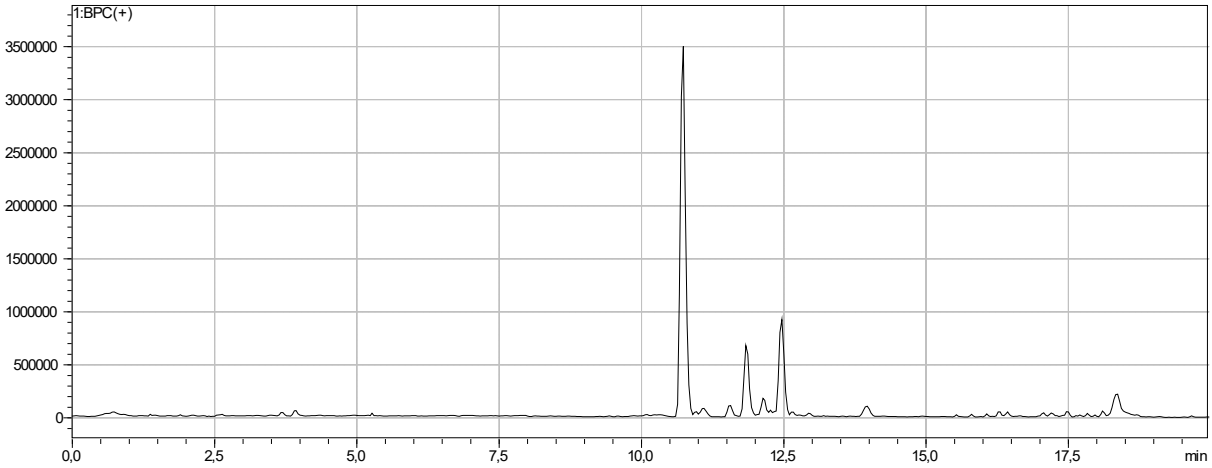

$^{1}$H NMR spectra for compound **4**



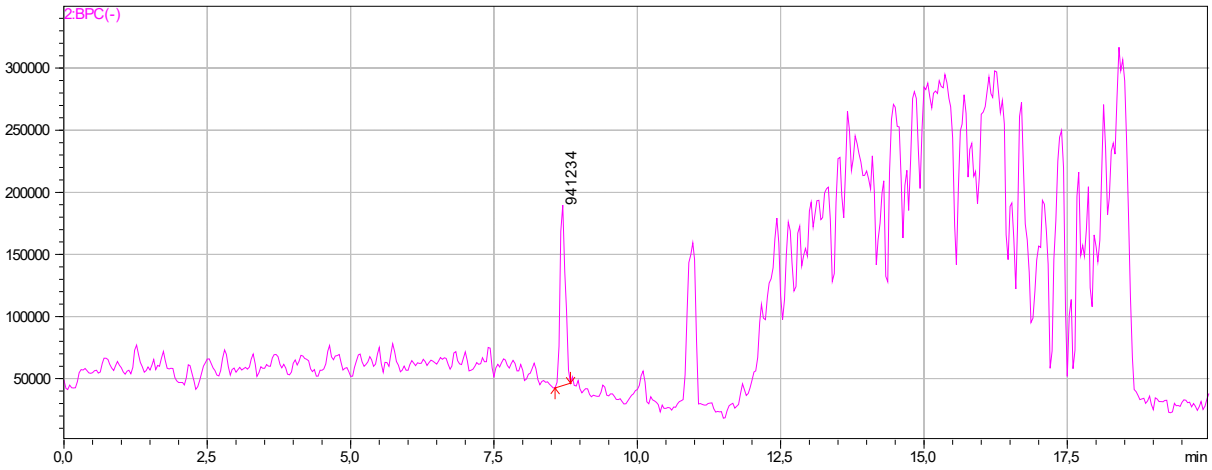
HPLC chromatogram for compound **4**

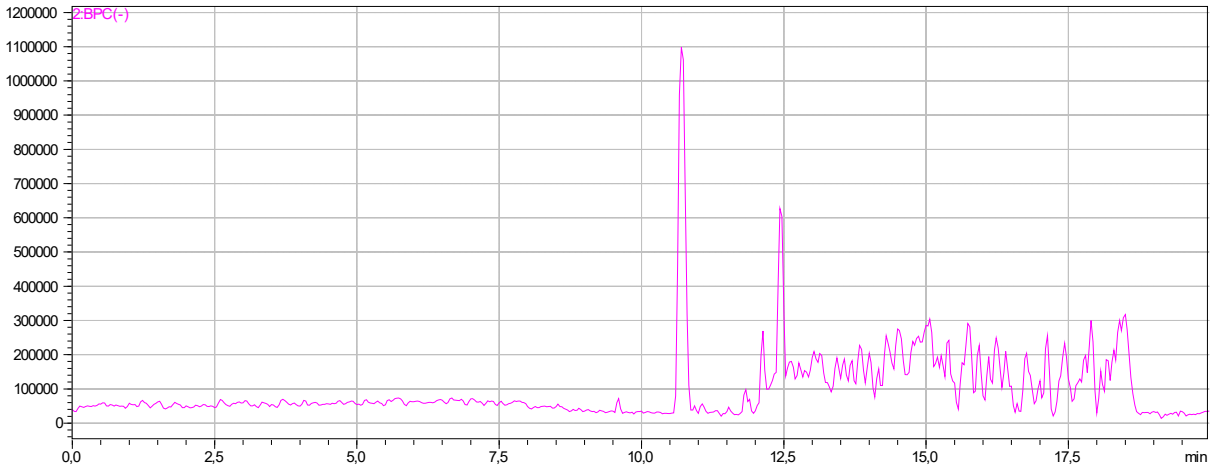
Positive ions (chromatogram and blank)



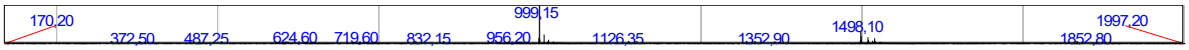

Negative ions (chromatogram and blank)



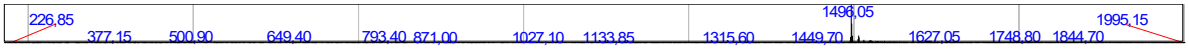



LCMS spectra for compound **4**

Positive ions spectra for compound **4**

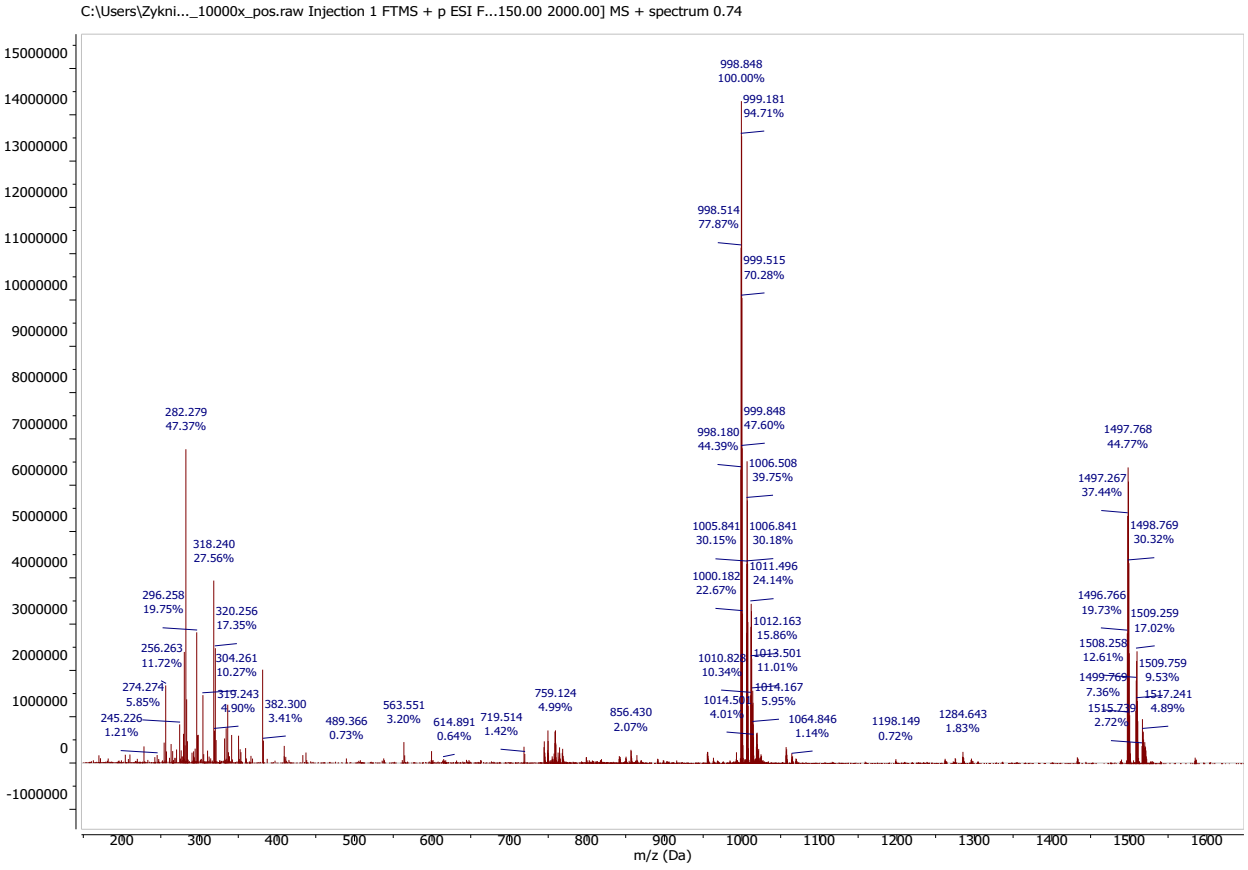



Negative ions spectra for compound **4**

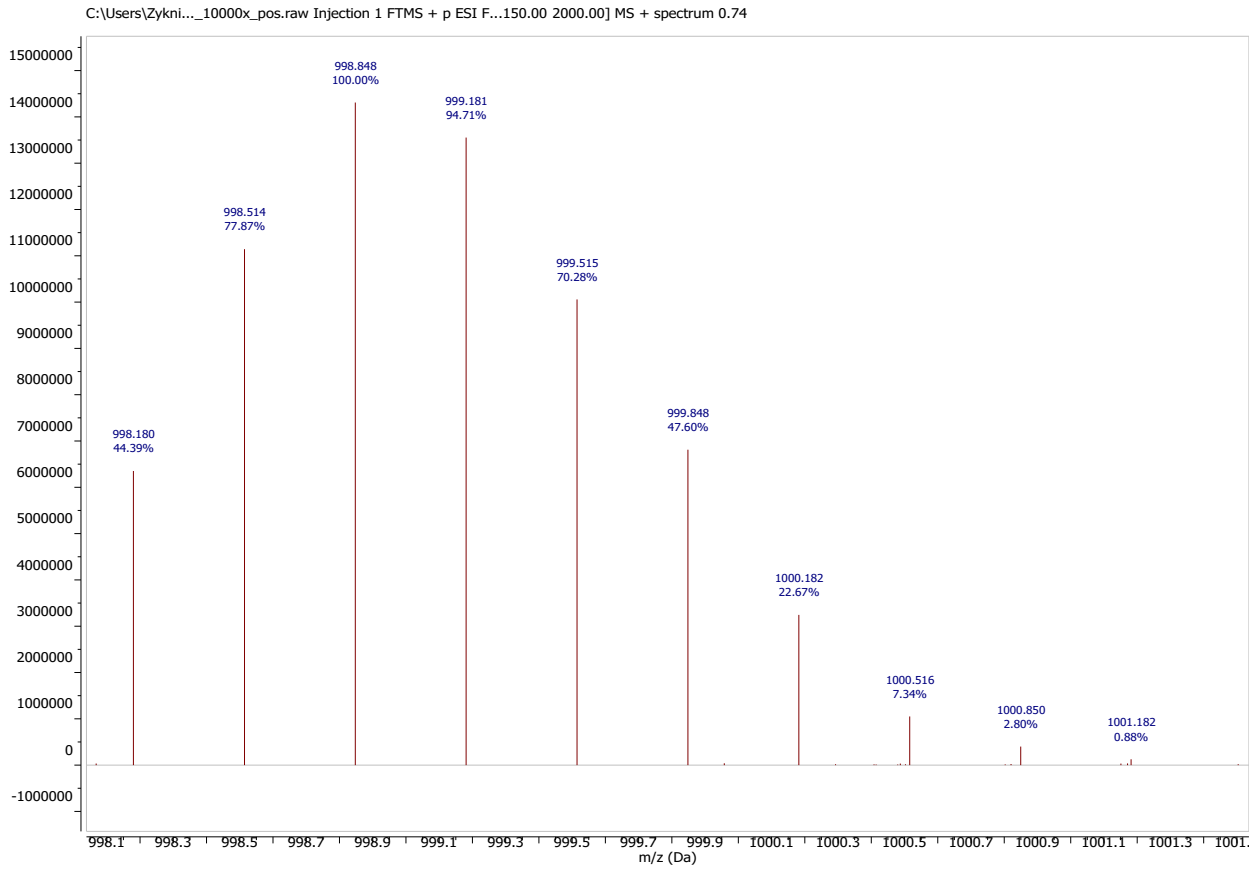



ESI-HRMS spectra for compound **4**

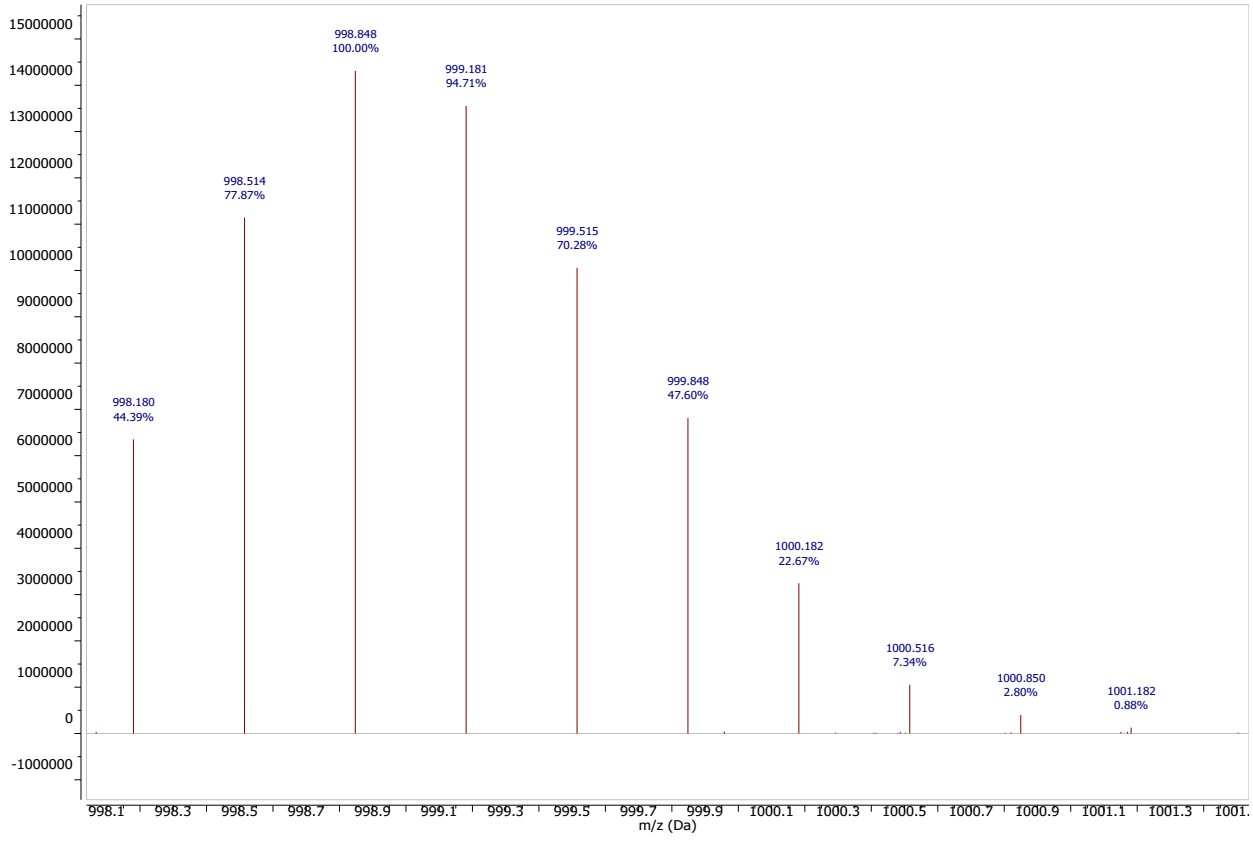
Positive ions:



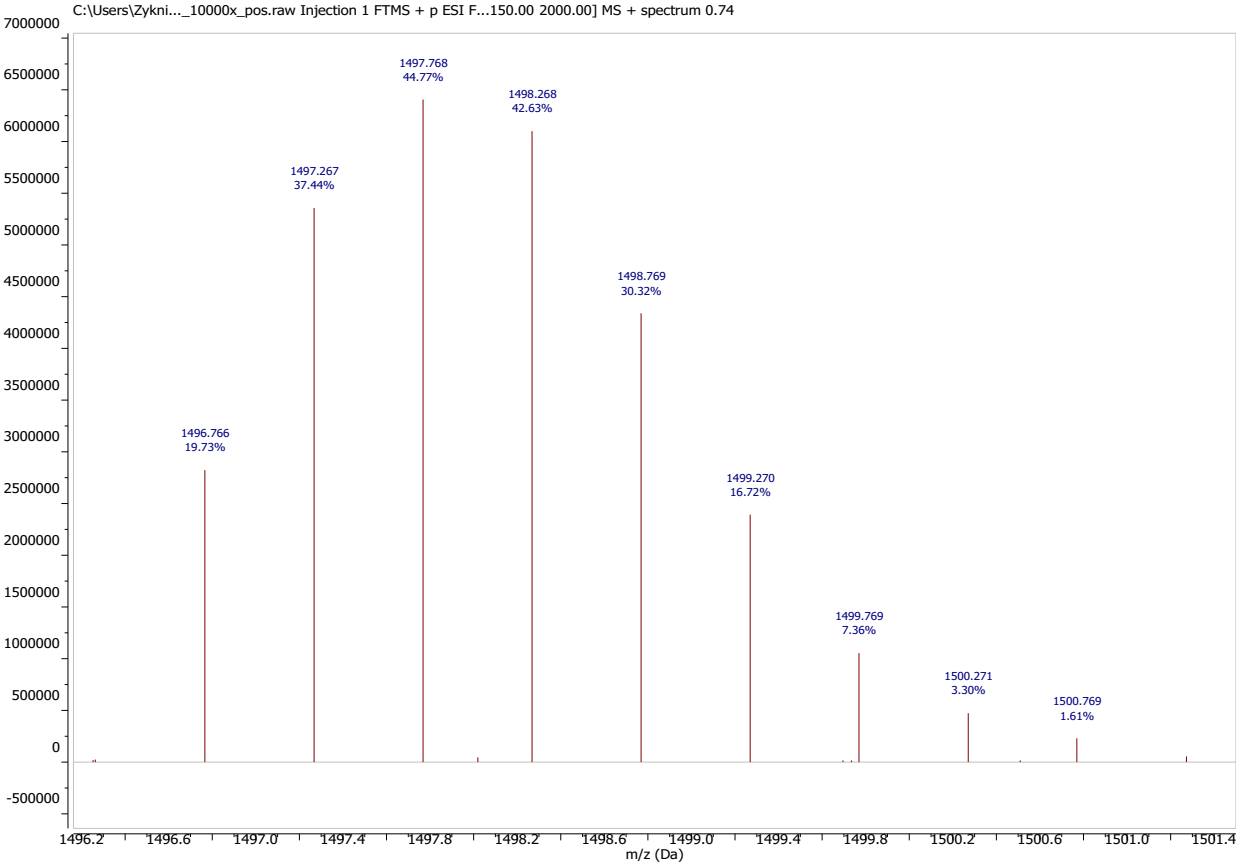
Experimental spectra for m/z $[M+3H]^{3+}$



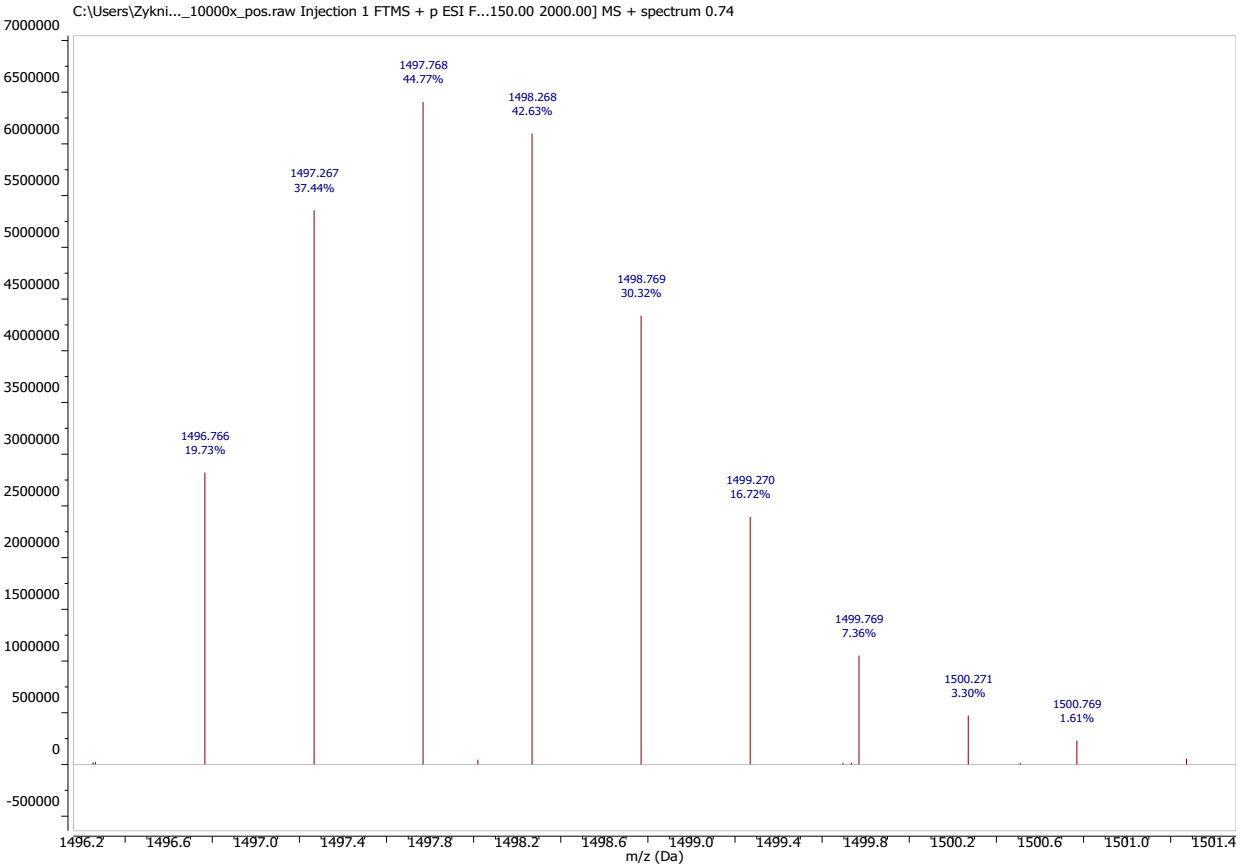

Predicted spectra for m/z $[M+3H]^{3+}$



Experimental spectra for m/z $[M+2H]^{2+}$

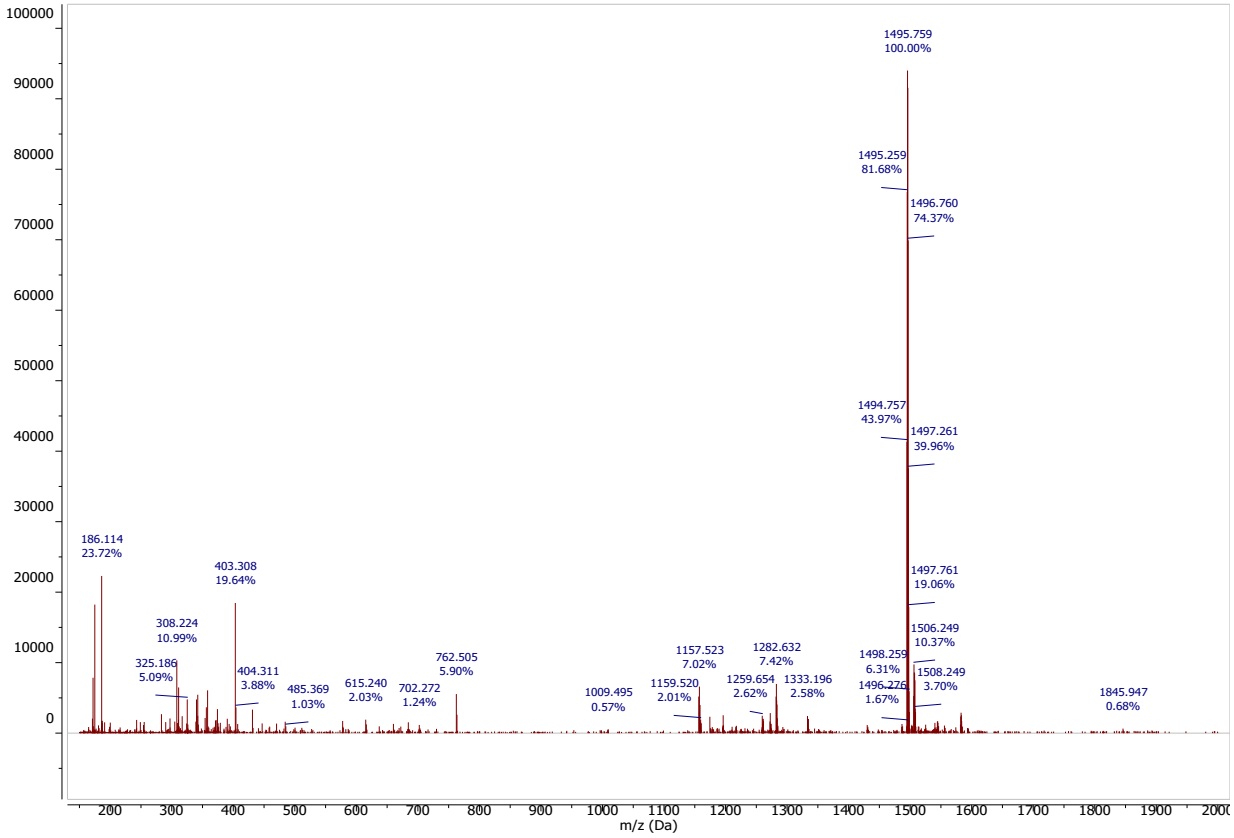



Predicted spectra for m/z $[M+2H]^{2+}$



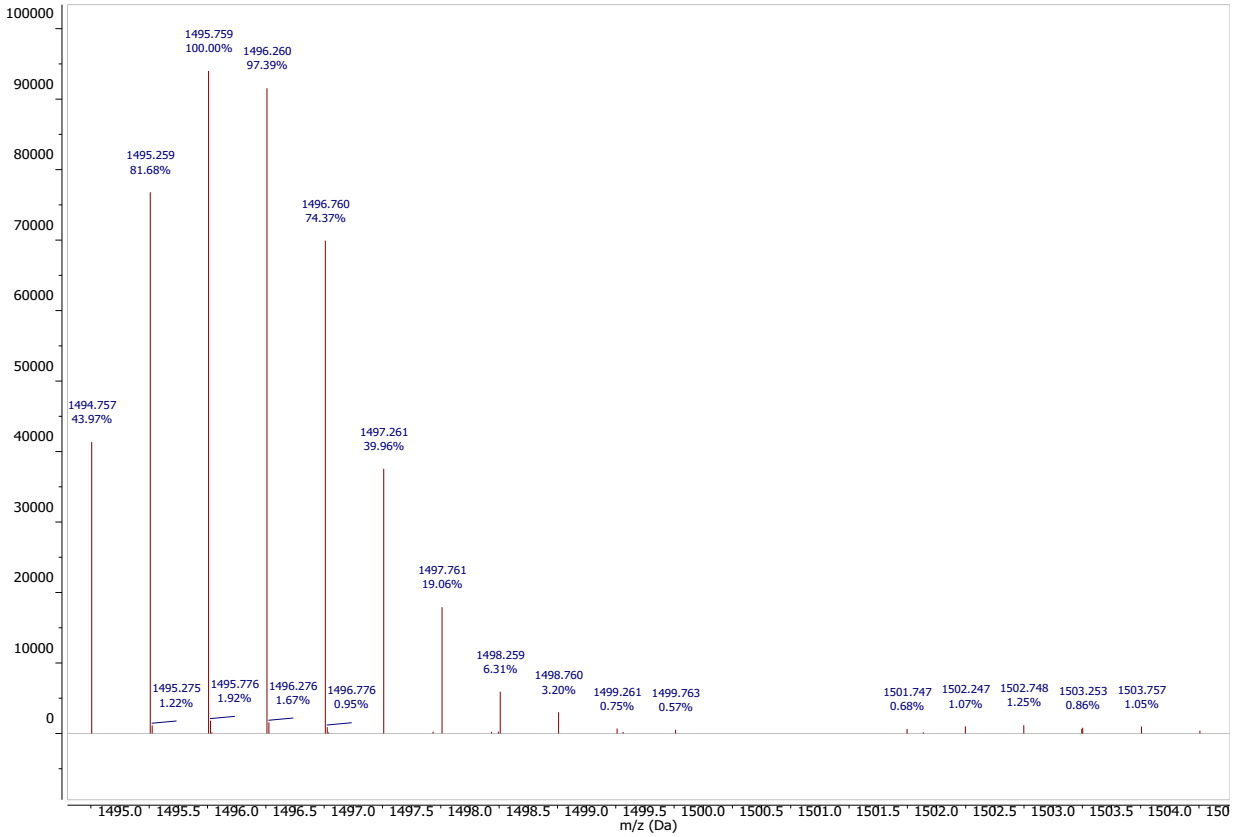
## Negative ions

C:\Users\Zykni..._10000x_neg.raw Injection 1 FTMS p ESI F...150.00 2000.00] MS spectrum 0.65

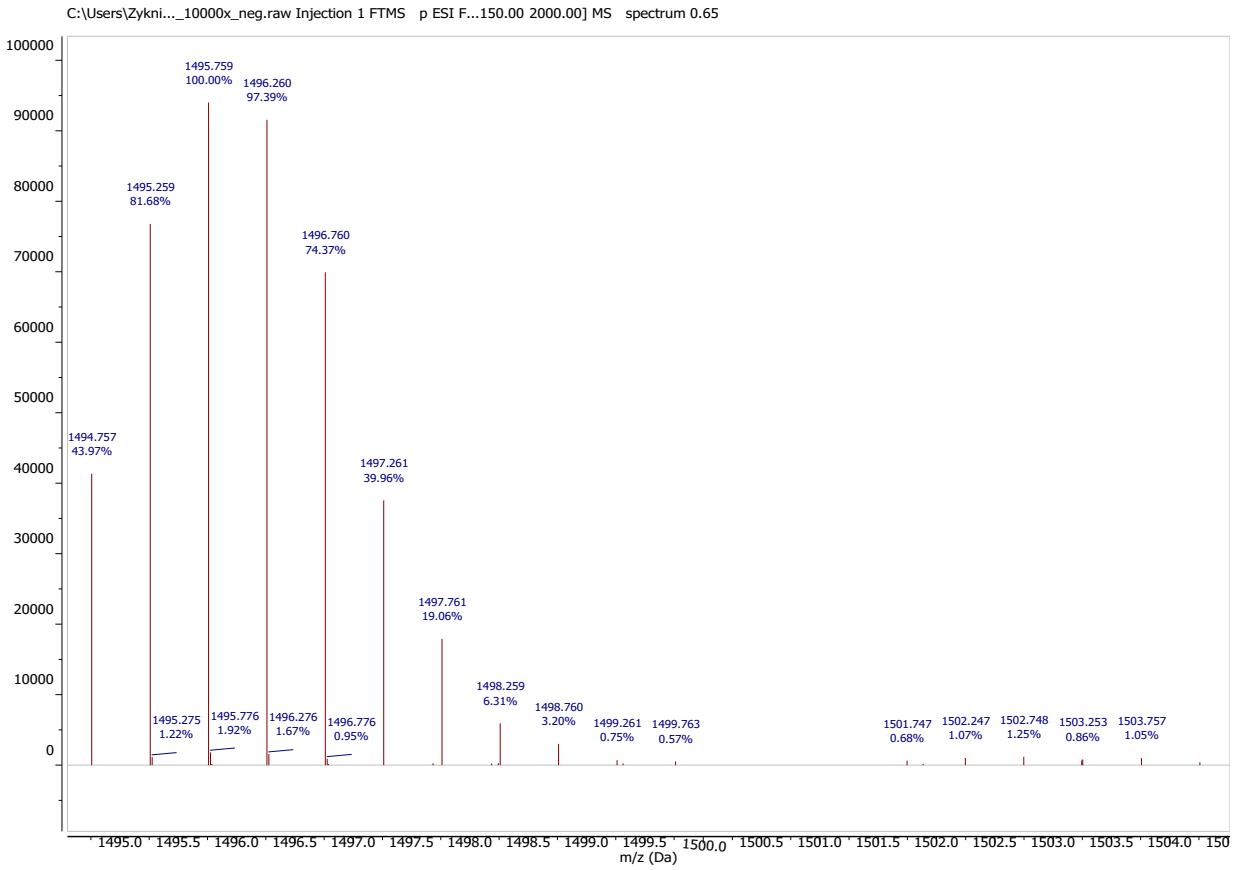



## Experimental spectra for m/z $[M-2H]^{2-}$

C:\Users\Zykni..._10000x_neg.raw Injection 1 FTMS p ESI F...150.00 2000.00] MS spectrum 0.65



Predicted spectra for m/z $[M-2H]^{2-}$



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