"Seasoning" antimalarial drugs' action: chloroquine bile salts as novel triple-stage antiplasmodial hits

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Electronic Supporting Information

1. Chemistry

1.1. Conversion of antimalarial drug phosphate salts 1a and 2a into the respective free bases 1b and 2b. 5% aq. Na_2CO_3 was added to a suspension of **1a** or **2a** in dichloromethane, and the mixture was stirred at RT for 30 min. The mixture was then extracted with DCM (3×), and the organic layers pooled and dried over anhydrous Na_2SO_4. The solid was next removed by filtration and discarded, whereas the filtrate was evaporated to dryness under reduced pressure, on a rotatory evaporator. Data obtained by ^{1}H-NMR analysis agreed with previous reports for **1b** and **2b**.[1,2]

1.2. Synthesis and spectral data of drug-derived bile salts 4a-e and 5a-e. 1 eq. of either **1b** or **2b** was dissolved in MeOH and put under stirring at RT. In parallel, 1 eq. of the relevant bile acid **3** was dissolved also in MeOH and the methanolic solution thus obtained was added dropwise to the drug-containing solution. The final solution was stirred for 30 min, after which MeOH was eliminated by evaporation under reduced pressure on a rotatory evaporator. The solid residue obtained was dried in a vacuum oven at 45 ºC, for 5 h. NMR and mass spectrometry (MS) analyses of the final compounds confirmed their expected structures, according to spectral data given below. Spectral traces and proton correspondence are included further ahead, in section 4.

4a, sugar-like white solid (100%; m.p. 98-105 ºC); **δH (DMSO-d6, 400 MHz)** 8.36 (m, 2H, Q3 and Q8), 7.76 (d, 1H, J= 2.3Hz, Q5), 7.42 (dd, 1H, J= 9.0, 2.3Hz, Q7), 6.91 (d, 1H, J= 8.1Hz, Q2), 6.51 (d, 1H, J= 5.5Hz, -NH-), 4.29 (s, 1H, A3-OH), 4.10 (s, 1H, A7-OH), 4.00 (s, 1H, A12-OH) , 3.78 (s, 1H, A12), 3.73 (q, 1H, J= 6.6Hz, CQ1), 3.61 (s, 1H, A7), 3.19 (m, 1H, A3), 2.45 (q, 4H, J= 7.1Hz, CQ5 and CQ7), 2.36 (t, 2H, J= 6.9Hz, CQ4), 2.29-1.09 (m, 31H), 0.92 (m, 9H, CQ6,CQ8, A21), 0.81(s, 3H, A18), 0.59 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 174.92, 151.77, 149.44, 149.18, 133.23, 127.32,

124.26, 123.67, 117.41, 98.73, 70.90, 70.34, 66.15, 51.91, 47.47, 46.07, 46.00, 45.66, 41.43, 41.27, 35.22, 34.95, 34.78, 34.29, 33.22, 30.82, 30.73, 30.31, 28.44, 27.18, 26.11, 23.13, 22.70, 22.52, 19.75, 16.85, 12.23, 11.35; **ESI-IT MS (+)** ($C_{18}H_{27}ClN_3^+$, 320.19 a.m.u.) m/z: 320.87a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_5^-$, 407.28 a.m.u.) m/z: 407.73 a.m.u. (M^-), 453.33 a.m.u. ($M^- + CH_2O_2$), 815.73 a.m.u. ($2M^-$).

4b, sugar-like white solid (94%; m.p. 90-91 ºC); **δH (DMSO-d6, 400 MHz)** 8.36 (m, 2H, Q3 and Q8), 7.76 (d, 1H, J= 2.3Hz, Q5), 7.42 (dd, 1H, J= 9.0, 2.3Hz, Q7), 6.90 (d, 1H, J= 8.1Hz, Q2), 6.51 (d, 1H, J= 5.6Hz, -NH-), 4.30 (s, 1H, A3-OH), 4.10 (s, 1H, A7-OH), 3.72 (m, 1H, J= 6.6Hz, CQ1), 3.63 (s, 1H, A7), 3.18 (m, 1H, A3), 2.44 (m, 6H, CQ4-5 and CQ7), 2.29 – 0.96 (m, 33H) , 0.91 (m, 9H, CQ6,CQ8, A21), 0.84(s, 3H, A18), 0.60 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 174.80, 151.77, 149.42, 149.19, 133.22, 127.32, 124.24, 123.67, 117.40, 98.73, 70.23, 66.07, 55.45, 51.90, 49.89, 47.46, 46.08, 41.83, 41.32, 35.21, 34.83, 34.72, 34.64, 33.20, 32.17, 30.69, 30.63, 30.46, 27.67, 23.05, 22.61, 20.15, 19.74, 18.07, 11.55, 11.32; **ESI-IT MS (+)** ($C_{18}H_{27}ClN_3^+$, 320.19 a.m.u.) m/z: 320.47 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_4^-$, 391.21 a.m.u.) m/z: 437.47 a.m.u. ($M^- + CH_2O_2$), 783.67 a.m.u. ($2M^-$).

4c, sugar-like white solid (99%; m.p. 90-93 ºC); **δH (DMSO-d6, 400 MHz)** 8.36 (m, 2H, Q3 and Q8), 7.76 (d, 1H, J= 2.3Hz, Q5), 7.42 (dd, 1H, J= 9.0, 2.3Hz, Q7), 6.90 (d, 1H, J= 8.0Hz, Q2), 6.51 (d, 1H, J= 5.5Hz, -NH-), 4.44 (s, 1H, A3-OH), 4.19 (s, 1H, A12-OH), 3.79 (s, 1H, A12), 3.73 (m, 1H, J= 6.6Hz, CQ1), 3.37 (m, 1H, A3), 2.42 (q, 4H, J= 7.2Hz, CQ5 and CQ7), 2.37 (t, 2H, J= 6.9Hz, CQ4), 2.30-0.95 (m, 33H), 0.91 (m, 9H, CQ6,CQ8, A21), 0.84 (s, 3H, A18), 0.59 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 174.87, 151.78, 149.42, 149.21, 133.21, 127.33, 124.25, 123.65, 117.41, 98.73, 70.90, 69.84, 51.98, 47.48, 47.35, 46.08, 45.90, 41.51, 36.21, 35.55, 35.05, 34.87, 33.72, 33.27, 32.82, 30.82, 30.69, 30.14, 28.49, 27.07, 26.88, 25.99, 25.39, 23.40, 23.29, 22.99, 19.75,

16.82, 12.33, 11.51; **ESI-IT MS (+)** ($C_{18}H_{27}ClN_3^+$, 320.19 a.m.u.) m/z: 320.27 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_4^-$, 391.21 a.m.u.) m/z: 391.80 a.m.u. (M^-), 437.00 a.m.u. ($M^- + CH_2O_2$), 783.47 a.m.u. ($2M^-$).

4d, sugar-like white solid (98%; m.p. 75-76ºC); **δH (DMSO-d6, 400 MHz)** 8.36 (m, 2H, Q3 and Q8), 7.76 (d, 1H, J= 2.2Hz, Q5), 7.42 (dd, 1H, J= 9.0, 2.2Hz, Q7), 6.91 (d, 1H, J= 8.0Hz, Q2), 6.50 (d, 1H, J= 5.6Hz, -NH-), 3.72 (m, 1H, CQ1), 3.38 (q, 1H,A3), 2.43 (q, 4H, J= 7.1Hz, CQ5 and CQ7), 2.38 (t, 2H, J= 6.9Hz, CQ4), 2.29-0.96 (m, 33H), 0.89 (m, 12H, CQ6,CQ8, A21, A18), 0.60 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 175.01, 151.82, 149.52, 149.26, 133.30, 127.39, 124.33, 123.73, 117.49, 98.79, 69.85, 56.01, 55.53, 52.01, 47.57, 46.12, 42.23, 41.51, 36.28, 35.35, 35.13, 34.82, 34.18, 33.33, 30.97, 30.76, 30.37, 27.68, 26.87, 26.12, 23.81, 23.29, 23.23, 20.37, 19.81, 18.12, 11.83, 11.51, 11.48; **ESI-IT MS (+)** ($C_{18}H_{27}ClN_3^+$, 320.19 a.m.u.) m/z: 320.13 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_3^-$, 375.29 a.m.u.) m/z: 421.13 a.m.u. ($M^- + CH_2O_2$), 694.27 a.m.u. ($2M^- - CH_2O_2$), 751.20 a.m.u. ($2M^-$).

4e, sugar-like white solid (100%; m.p. 123-125 ºC); **δH (DMSO-d6, 400 MHz)** 8.36 (m, 2H, Q3 and Q8), 7.88 (t, 1H, J= 5.7Hz, -$NH_{(A)}$-), 7.77 (d, 1H, J= 2.2Hz, Q5), 7.43 (dd, 1H, J= 9.0, 2.2Hz, Q7), 6.93 (d, 1H, J= 8.1Hz, Q2), 6.51 (d, 1H, J= 5.5Hz, -$NH_{(CQ)}$-), 4.00 (s, 1H, A12-OH) , 3.78 (s, 1H, A12), 3.73 (m, 1H, J= 6.6Hz, CQ1), 3.62 (m, 3H, A7 and A25), 3.17 (m, 1H, A3), 2.55 (m, 4H, CQ5 and CQ7), 2.30-1.05 (m, 31H), 0.95 (m, 9H, CQ6,CQ8, A21), 0.81(s, 3H, A18), 0.58 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 172.61, 171.56, 151.70, 149.47, 149.10, 133.28, 127.25, 124.27, 123.71, 117.40, 98.74, 70.92, 70.35, 66.16, 51.52, 47.43, 46.09, 45.94, 45.65, 41.44, 41.26, 35.22, 35.06, 34.79, 34.29, 33.04, 32.22, 31.52, 30.32, 28.46, 27.18, 26.11, 22.71, 22.53, 22.46, 19.73, 17.02, 12.26, 10.69; **ESI-IT MS (+)** ($C_{18}H_{27}ClN_3^+$, 320.19 a.m.u.) m/z: 320.47 a.m.u. (M^+),

785.13 a.m.u. ($C_{18}H_{27}ClN_3^{2+}$ + $C_{26}H_{42}O_6^-$); **ESI-IT MS (-)** ($C_{26}H_{42}NO_6^-$, 464.30 a.m.u.) m/z: 464.53 a.m.u. (M^-), 929.47 a.m.u. ($2M^-$).

5a, sugar-like brown solid (65%; m.p. 101-106 ºC); **δH (DMSO-d6, 400 MHz)** 8.53 (dd, 1H, *J*= 4.2, 1.7Hz, Q8), 8.07 (dd, 1H, *J*= 8.3, 1.6Hz, Q6), 7.42 (dd, 1H, *J*= 8.3, 4.2Hz, Q7), 6.47 (d, 1H, *J*= 2.6Hz, Q2), 6.26 (d, 1H, *J*= 2.6Hz, Q4), 6.12 (d, 1H, *J*= 8.6Hz, -NH-), 4.30 (s, 1H, A3-OH), 4.10 (s, 1H, A7-OH), 4.00 (s, 1H, A12-OH), 3.82 (s, 3H, -OCH_3), 3.78 (s, 1H, A12), 3.61 (s, 2H, A7 and PQ1), 3.19 (m, 1H, A3), 2.59 (t, 2H, PQ4), 2.37 (m, 37H), 0.58 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 158.91, 144.55, 144.14, 134.70, 134.43, 129.48, 122.00, 95.97, 91.51, 70.90, 70.34, 66.15, 47.05, 46.10, 45.65, 41.43, 41.25, 41.03, 35.22, 35.06, 34.77, 34.29, 33.34, 31.72, 31.11, 30.31, 28.77, 28.44, 27.19, 26.11, 22.72, 22.52, 20.15, 16.92, 12.26; **ESI-IT MS (+)** ($C_{15}H_{22}N_3O^+$, 260.18 a.m.u.) m/z: 260.40 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_5^-$, 407.28 a.m.u.) m/z: 407.33 a.m.u. (M^-), 815.40 a.m.u. ($2M^-$).

5b, sugar-like brown solid (99%; m.p. 94-99 ºC); **δH (DMSO-d6, 400 MHz)** 8.53 (dd, 1H, *J*= 4.2, 1.7Hz, Q8), 8.07 (dd, 1H, *J* =8.3, 1.7Hz, Q6), 7.42 (m, 1H, Q7), 6.47 (d, 1H, *J*= 2.5Hz, Q2), 6.26 (d, 1H, *J*= 2.5Hz, Q4), 6.13 (d, 1H, *J*= 8.6Hz, -NH-), 4.10 (s, 1H, A7-OH), 3.82 (s, 3H, -OCH_3), 3.63 (s, 1H, A7 and PQ1), 3.18 (m, 1H, A3), 2.62 (t, 2H, *J* =6.7Hz, PQ4), 2.37-0.74 (m, 39H) 0.60 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 175.35, 158.91, 144.55, 144.14, 134.70, 134.44, 129.48, 122.00, 96.00, 91.53, 70.23, 66.07, 55.58, 54.89, 49.89, 47.01, 41.82, 41.33, 40.60, 35.22, 34.96, 34.71, 34.64, 34.00, 33.25, 32.17, 31.72, 31.07, 30.47, 27.70, 23.07, 22.61, 20.14, 18.15, 11.56; **ESI-IT MS (+)** ($C_{15}H_{22}N_3O^+$, 260.18 a.m.u.) m/z: 260.53 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_4^+$, 391.27 a.m.u.) m/z: 437.53 a.m.u. (M^- + CH_2O_2).

5c, sugar-like brown solid (100%; m.p. 97-98 ºC); δH (DMSO-d6, 400 MHz) 8.53 (dd, 1H, *J* = 4.2, 1.7Hz, Q8), 8.07 (dd, 1H, *J* =8.3, 1.7Hz, Q6), 7.42 (m, 1H, Q7), 6.47 (d, 1H,

J =2.5Hz, Q2), 6.26 (d, 1H, *J* =2.5Hz, Q4), 6.13 (d, 1H, *J* =8.7Hz, -NH-), 4.49 (s, 1H, A3-OH), 3.82 (s, 3H, -OCH3), 3.78 (s, 1H, A12), 3.61 (m, 1H, PQ1), 3.36 (m, 1H, A3), 2.32 (t, , *J* =6.8Hz, 2H, PQ4), 2.21-0.78 (m, 39H), 0.59 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 175.61, 158.91, 144.55, 144.14, 134.70, 134.45, 129.49, 122.00, 96.01, 91.54, 70.92, 69.85, 54.89, 47.34, 47.00, 46.21, 45.89, 41.53, 40.42, 36.21, 35.56, 35.06, 35.03, 33.72, 33.24, 32.82, 32.14, 31.24, 30.14, 28.50, 27.72, 27.10, 26.89, 26.00, 23.42, 22.98, 20.13, 16.92, 12.36. **ESI-IT MS (+)** ($C_{15}H_{22}N_3O^+$, 260.18 a.m.u.) m/z: 260.93 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_4^-$, 391.27 a.m.u.) m/z: 437.53 a.m.u. ($M^- + CH_2O_2$), 783.73 a.m.u. ($2M^-$)

5d, sugar-like brown solid (100%; m.p. 82-83 ºC); **δH (DMSO-d6, 400 MHz)** 8.54 (dd, 1H, *J*= 4.2, 1.7Hz, Q8), 8.08 (dd, 1H, *J*= 8.3, 1.7Hz, Q6), 7.43 (dd, 1H, *J*= 8.3, 4.2Hz, Q7), 6.48 (d, 1H, *J*= 2.5Hz, Q2), 6.27 (d, 1H, *J*= 2.5Hz, Q4), 6.13 (d, 1H, *J*= 8.6Hz, -NH-), 4.33 (s, 1H, A3-OH), 3.83 (s, 3H, $-OCH_3$), 3.62 (m, 1H, PQ1), 3.37 (m, 1H, A3), 2.63 (t, 2H, *J*= 6.8Hz, PQ4), 1.22 (m, 43H), 0.61 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 158.99, 144.63, 144.22, 134.78, 134.52, 129.56, 122.08, 96.07, 91.60, 69.84, 56.04, 55.65, 54.97, 47.11, 42.24, 41.52, 36.29, 35.36, 35.13, 34.94, 34.19, 33.36, 30.37, 27.70, 26.87, 26.13, 23.84, 23.25, 20.38, 20.22, 18.21, 11.86. **ESI-IT MS (+)** ($C_{15}H_{22}N_3O^+$, 260.18 a.m.u.) m/z: 260.73 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{24}H_{39}O_3^-$, 375.29 a.m.u.) m/z: 421.13 a.m.u. (M^-+ CH_2O_2), 751.40 a.m.u. ($2M^-$), 797.33 a.m.u. ($2M^- + CH_2O_2$), 1127.60 a.m.u. ($3M^-$).

5e, sugar-like brown solid (100%; m.p. 111-127 ºC); **δH (DMSO-d6, 400 MHz)** 8.54 (dd, 1H, *J*= 4.2, 1.7Hz, Q8), 8.08 (dd, 1H, *J*= 8.3, 1.7Hz, Q6), 7.43 (m, 1H, Q7), 7.33 (m, 1H,-$NH_{(P1g)}$-), 6.48 (d, 1H, *J*= 2.5Hz, Q2), 6.28 (d, 1H, *J*= 2.5Hz, Q4), 6.14 (d, 1H, *J*= 8.8Hz, -NH-), 4.30 (s, 1H, A3-OH), 4.10 (s, 1H, A7-OH), 3.99 (s, 1H, A12-OH), 3.82 (s, 3H, $-OCH_3$), 3.78 (s, 1H, A12), 3.64 (m, 2H, PQ1 and A7), 3.39(d, 2H, A25), 3.19 (m,

1H, A3), 2.73 (t, 2H, *J* =6.9Hz, PQ4), 2.30-0.74 (m, 39H), 0.58 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 171.83, 171.05, 158.90, 144.52, 144.15, 134.72, 134.44, 129.49, 122.02, 96.11, 91.60, 70.91, 70.35, 66.15, 46.83, 46.11, 45.65, 42.89, 41.44, 41.24, 35.22, 35.13, 34.77, 34.29, 32.91, 32.46, 31.60, 30.31, 28.44, 27.19, 26.10, 25.19, 22.53, 20.12, 17.02, 12.26. **ESI-IT MS (+)** ($C_{15}H_{22}N_3O^+$, 260.18 a.m.u.) m/z: 260.40 a.m.u. (M^+); **ESI-IT MS (-)** ($C_{26}H_{42}NO_6^-$, 464.30 a.m.u.) m/z: 464.87 a.m.u. (M^-), 929.80 a.m.u. ($2M^-$).

1.3. Synthesis of chloroquine analogue 1d. This synthesis was earlier reported by our group.[3] Briefly, **1c** (1 eq.) and 1,4-diaminobutane (10 eq.) were stirred at 100 ºC for 3 h. After cooling to RT, the mixture was diluted with DCM (25 mL), and the solution was washed with 5% aqueous Na_2CO_3 (3×). The organic layers were combined and dried over Na_2SO_4, which was then filtered and discarded. The filtrate was evaporated to dryness under reduced pressure on a rotary evaporator. The solid obtained **1d** (0.22 g, 0.88 mmol) was further dried in a vacuum oven at 45 ºC, for 5 h. Spectroscopic data agreed with previous reports.[3]

1.4. Synthesis and spectral data of amide 6a. Compound **1d** (1 eq.) was dissolved in DCM. In parallel, **3a** (1.1 eq.), TBTU (1.1 eq.), DIEA (2 eq.) were dissolved in DMF, and this solution was placed under magnetic stirring for 30 min at 0 ºC. The solution of **1b** was then added dropwise, and stirring was prolonged for more 24 h, at RT in the dark. The mixture was diluted with DCM and washed (1×) with 5% aq. Na_2CO_3. A precipitate formed after this step, which was subsequently filtered and dried in a vacuum oven at 45 ºC, for 5 h. Structural data for this solid, as obtained by ^{1}H-NMR, ^{13}C-RMN and ESI-IT MS, agreed with the expected structure for **6a**, as shown by spectral data given below. Spectral traces and proton correspondence are included further ahead, in section 4.

6a, beige solid (39%); **δH (DMSO-d6, 400 MHz)** 8.36 (d, 1H, *J*= 5.4 Hz Q3), 8.27 (d, 1H, *J*= 9.0 Hz, Q8), 7.78 (t, 1H, *J*= 5.7Hz, $NH_{(amide)}$), 7.75 (d, 1H, *J*= 2.3Hz, Q5), 7.40 (dd, 1H, *J*= 9.0, 2.3Hz, Q7), 7.32 (t, 1H, *J*= 5.4Hz, $NH_{(amine)}$), 6.44 (d, 1H, *J*= 5.5Hz, Q2), 4.33 (s, 1H, A3-OH), 4.06 (s, 1H, A7-OH), 3.99 (s, 1H, A12-OH), 3.72 (s, 1H, A12), 3.56 (s, 1H, A7), 3.25 (m, 2H, CQ1), 2.36 (t, 2H, CQ4), 2.39-0.71 (m, 34H), 0.48 (s, 3H, A19); **δC (DMSO-d6, 100 MHz)** 173.03, 152.30, 150.59, 149.55, 133.82, 127.88, 124.48, 117.95, 99.05, 71.50, 70.93, 66.72, 46.64, 46.15, 42.65, 42.01, 41.80, 38.49, 35.52, 34.82, 33.09, 32.28, 30.87, 28.97, 27.78, 27.37, 26.65, 25.65, 23.20, 23.03, 17.52, 12.68. **ESI-IT MS (+)** ($C_{37}H_{54}ClN_3O_4$, 640.29 a.m.u.) m/z: 641.47 a.m.u. (MH^+).

2. Thermal characterization

The thermal degradation of the compounds under study was evaluated by thermogravimetric analysis (TGA) in a Hitachi 7200RV simultaneous thermal analyser (STA), with the compounds subjected to heating from 25 to 500 ºC, at a rate of 5 ºC/min, under a N_2 gas flow rate of 50 $mL \cdot min^{-1}$. The melting process of the compounds was studied by differential scanning calorimetry (DSC) using a Hitachi DSC7020 heat flux DSC calorimeter, with the compounds subjected to heating in Al crucibles between 25-250 ºC, at a rate of 10 ºC/min, and a N_2 gas flow rate of 50 $mL \cdot min^{-1}$. In parallel, the melting was also studied by light microscopy through an Olympus BX51 microscope equipped with a Linkam TP94 heating stage (a few solid crystals were placed between slide and cover slip and observed until complete disappearance of the crystals and formation of a liquid).

3. *in vitro* assays

3.1. *Plasmodium* hepatic stage. Inhibition of the hepatic stage *Plasmodium* infection by the compounds was assessed by measuring the luminescence intensity of lysates of Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, as previously described.[4] Briefly, Huh-7 cells, a human hepatoma cell line, were cultured in 1640 Roswell Park Memorial Institute (RPMI) medium supplemented with 10% v/v fetal calf serum, 1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2. For infection assays, Huh-7 cells (1.2×10^4 per well) were seeded in 96-well plates the day before drug treatment and infection. The cell culture medium was replaced by medium containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes, reared at the arthropode facility of Instituto de Medicina Molecular João Lobo Antunes. Sporozoite addition to the cells was followed by centrifugation at 1700 g for 5 min. Parasite load was evaluated at 48 h infection by luminescence analysis of cell lysates, following addition of the luciferin substrate. The effect of the compounds on the viability of Huh-7 cells was assessed by the AlamarBlue assay (Invitrogen, UK), using the manufacturer's protocol.

3.2. *Plasmodium* blood stage. Laboratory-adapted *P. falciparum* 3D7 (chloroquine and mefloquine sensitive; 3D7-GFP; MRA-1029, MR4, ATCC® Manassas Virginia) and Dd2 (chloroquine-resistant, mefloquine-resistant) were continuously cultured, and sorbitol synchronized, as previously described by Nogueira *et al.*.[5] Staging and

parasitaemia were determined by light microscopy of Giemsa-stained thin blood smears. All compounds were screened for their *in vitro* antimalarial activity[6] against chloroquine-susceptible 3D7-GFP. Unsynchronized culture with 0,6% hematocrit and 0,5% parasitaemia, was incubated in a 96-well flat bottom plate with 10 μM of each compound for 72 h (37 °C and 5% CO_2). Parasite growth was assessed by flow cytometry[7] (Beckman Coulter, Cytoflex) with a 96-well plate reader, using Fl-1 (green fluorescent protein [GFP]; excitation wavelength, 488 nm). Typically, 20,000 to 40,000 erythrocytes (RBC) were counted for each well. Samples were analyzed using FlowJo software (Tree Star Inc.). Dose-response assay to estimate the correspondent IC_{50} was performed as described elsewhere[7] with modifications. Unsynchronized culture with 1% hematocrit and 0,6% parasitemia, was incubated with the tested compounds in 3-fold serial dilutions ranging from 10 to 0.014 μM. After 72 h at 37 °C and 5% CO_2, parasite growth was assessed by flow cytometry above for the as described for the antimalarial activity screening. Half-maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism 5 (trial version). At least three experiments, each in duplicate, were performed to obtain the mean IC_{50} presented.

3.3. *Plasmodium* gametocyte stage. Laboratory-adapted *P. falciparum* 3D7HT-GFP (MRA-1029, MR4, ATCC Manassas Virginia) were continuously cultured and sorbitol synchronized, as described by Lobo *et al.*.[8] Parasite growth was monitored microscopically using Giemsa-stained smears until ∼10% parasitemia, and sorbitol-synchronized ring stage parasites (>95%) were obtained. Parasites were returned to culture and allowed to mature to schizonts, then culture was loaded on a 20% over 60% Percoll cushion and centrifuged for 5 min at 2000 g. Schizonts were collected, washed once in phosphate buffered saline (PBS), re-suspended in complete medium, hematocrit

adjusted to 3% and returned to culture conditions until stage-III gametocytes were apparent. Gametocytocidal activity assay was performed as previously described,[9,10] with modifications. Upon the appearance of stage III gametocytes, 1 mL-microcultures (1-2% gametocytemia and 2.5% hematocrit) were incubated with either 10 μM of each tested compound, 10 μM of primaquine (PQ, reference drug) or dimethylsulfoxide (DMSO; < 0.3%), during 72 h under standard culture conditions. Afterwards, medium was replaced by compound-free medium and incubated for another 24 h. After 4 days, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting the number of stage IV and stage V gametocytes in a total number of 3000 RBC. Assays were performed in duplicate, and slides were examined by two independent microscopists. Gametocyte growth inhibition rate was calculated as follows: 100 – ((% of stage IV/V gametocytes in treated microcultures/% of IV/V gametocytes in drug free control)×100).

3.4. ***Hemolytic Activity.*** The hemolytic activity of the compounds was evaluated as described by Aguiar *et al*.[11] First, the blood plasma was removed by centrifuging the blood samples (Centurion scientific Ltd centrifuge with a BRK1011) for 5 min at 1020 g and 4 °C. The RBC were then washed (×3) with 0.01 M PBS, pH 7.4, and diluted to a final 6% hematocrit in PBS. Next, the RBC were plated in a 96-well plate, 100 μL of 6% hematocrit in the presence of 100 μL of the compound at 20 μM in PBS with 1.3% DMSO. For positive and negative controls, 1% Triton X-100 and PBS with 0.65% DMSO were used, respectively. The plate was incubated at 37 ºC for 1 h, after which it was centrifuged (Sigma 3-30K centrifuge from Sigma Centriguges GmbH with a swing-out rotor 11222) for 10 min at 859 g and 25 ºC; 80 μL of the supernatants were transferred to another plate, and the supernatant absorbance at 450 nm was measured

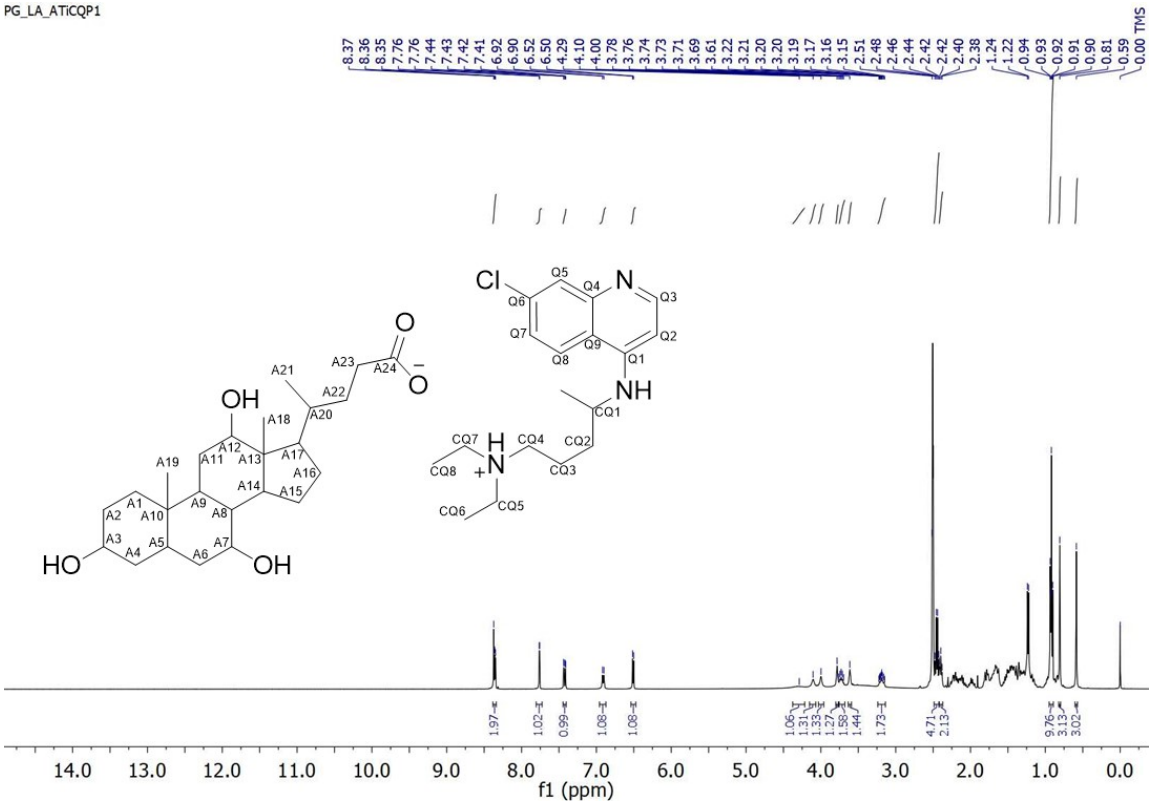
in a multi-mode microplate reader (Multiskan GO- Thermo Scientific). Absorbance values were treated using the following equation:

$$Hemolysis\ \% = \frac{Abs - Abs\ (negative\ control)}{Abs\ (Positive\ control) - Abs\ (negative\ control)} \times 100$$

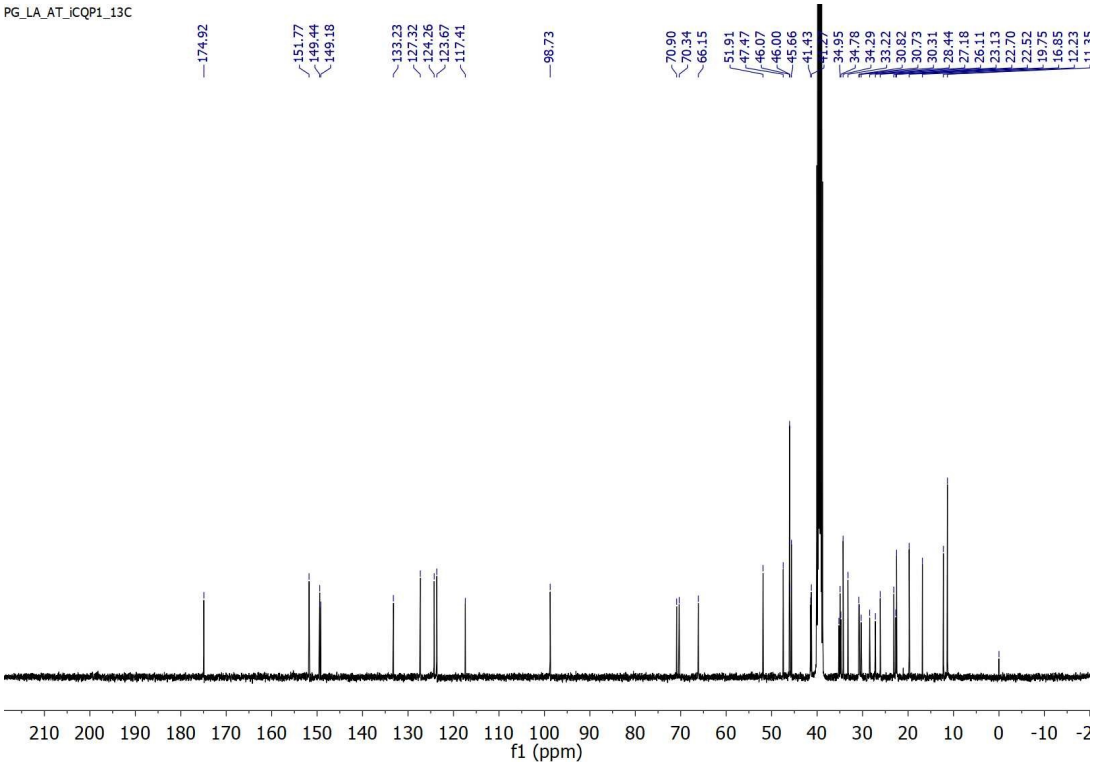
Ethics statement: The human blood used in this work was obtained, free of charge, from the *Serviço de Hematologia Clínica do Centro Hospitalar Universitário do Porto*. Prior to their use, blood units underwent the analytical checks specified in the current legislation. Before being delivered to us, unit data were anonymized and irreversibly dissociated, the only information we had on the information tag that came with the blood sample was the date of birth, blood group, and sex of the donor. The blood samples will not be used for studies other than those made explicit in this research.

4. Spectral traces

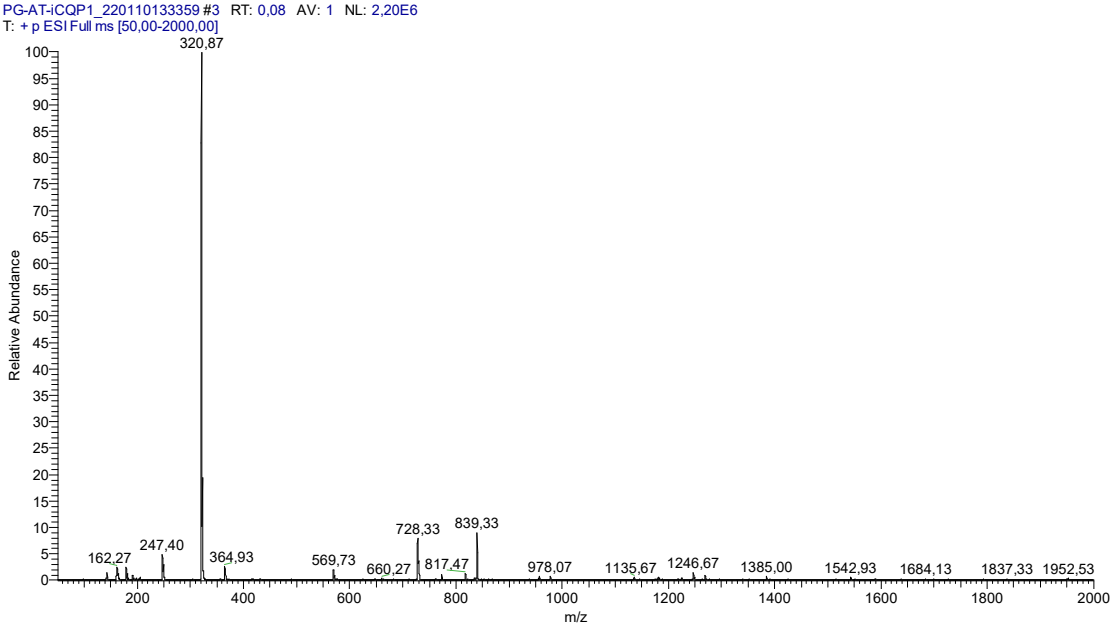
4.1. Bile salts 4 and 5



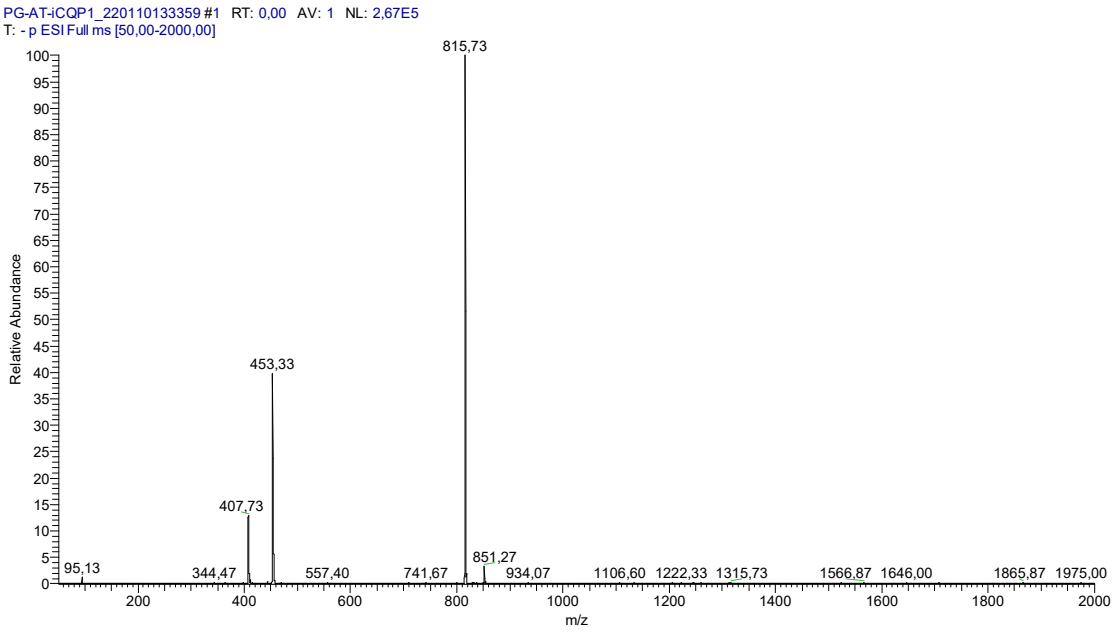
^{1}H-NMR spectrum of **4a** (400 MHz, DMSO-d_6).



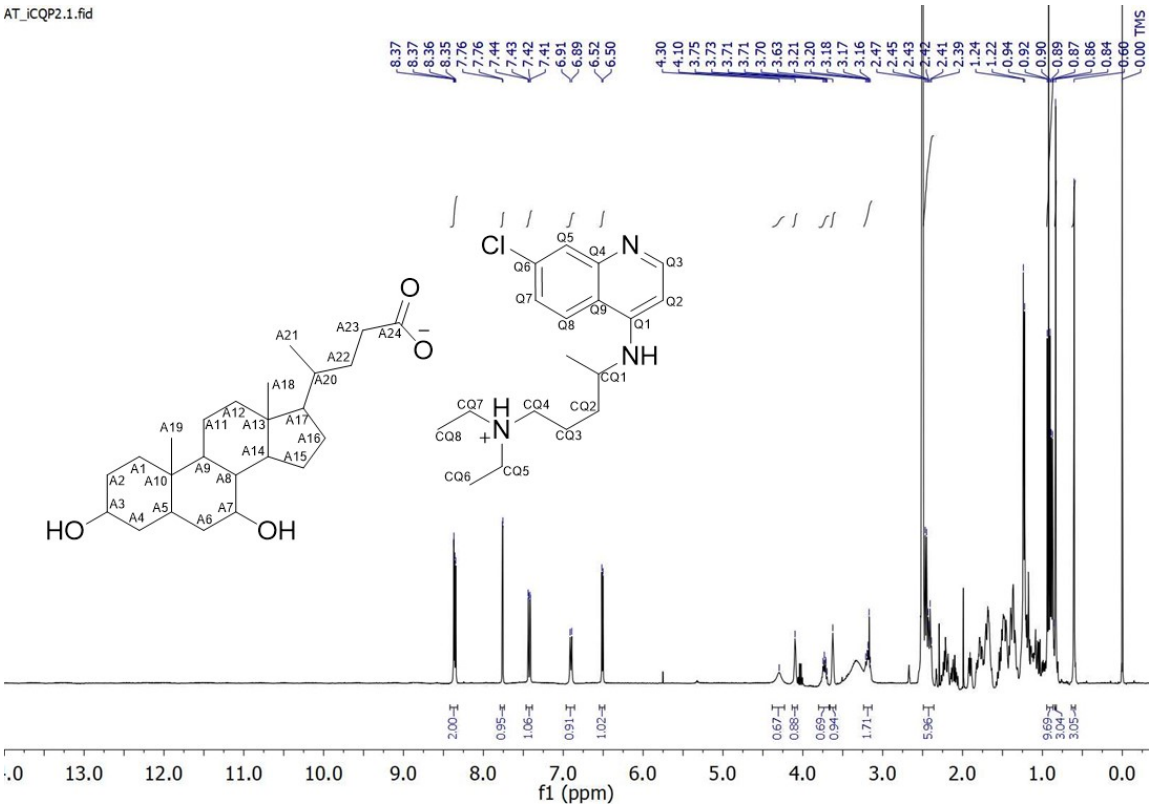
^{13}C-NMR spectrum of **4a** (100 MHz, DMSO-d_6).



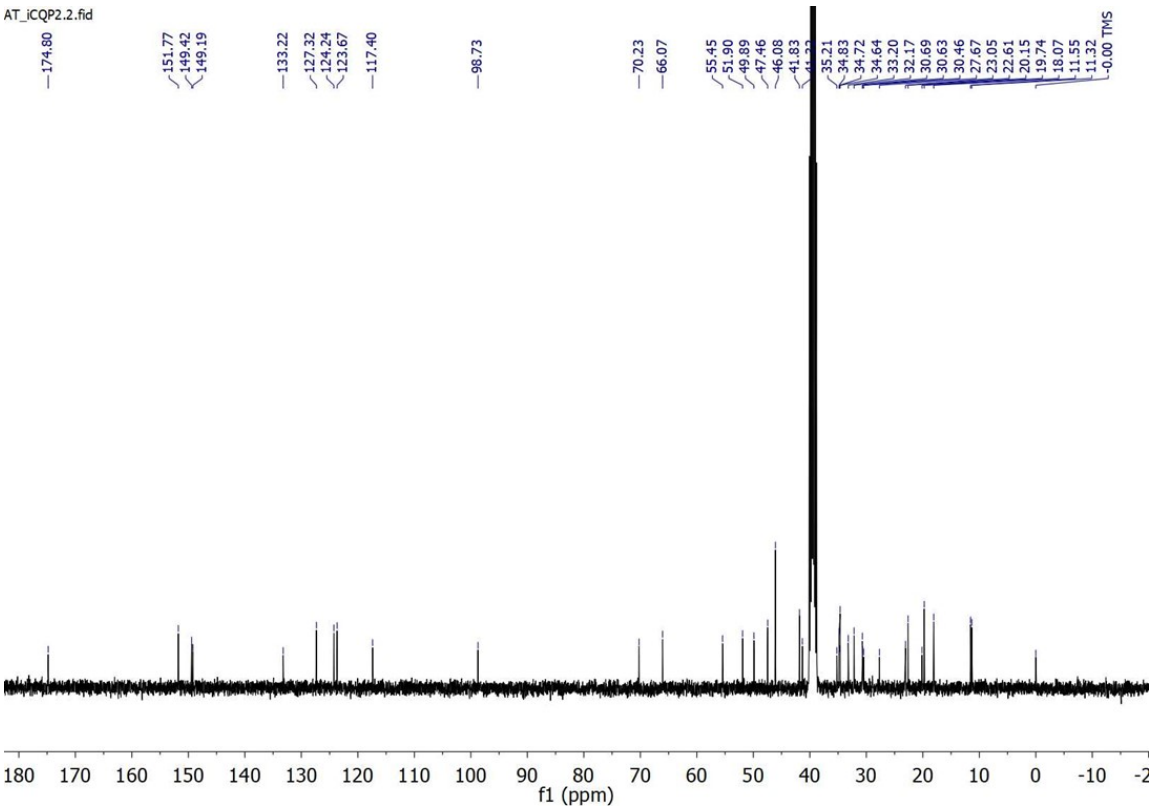
ESI-IT mass spectrum for **4a** (positive mode).



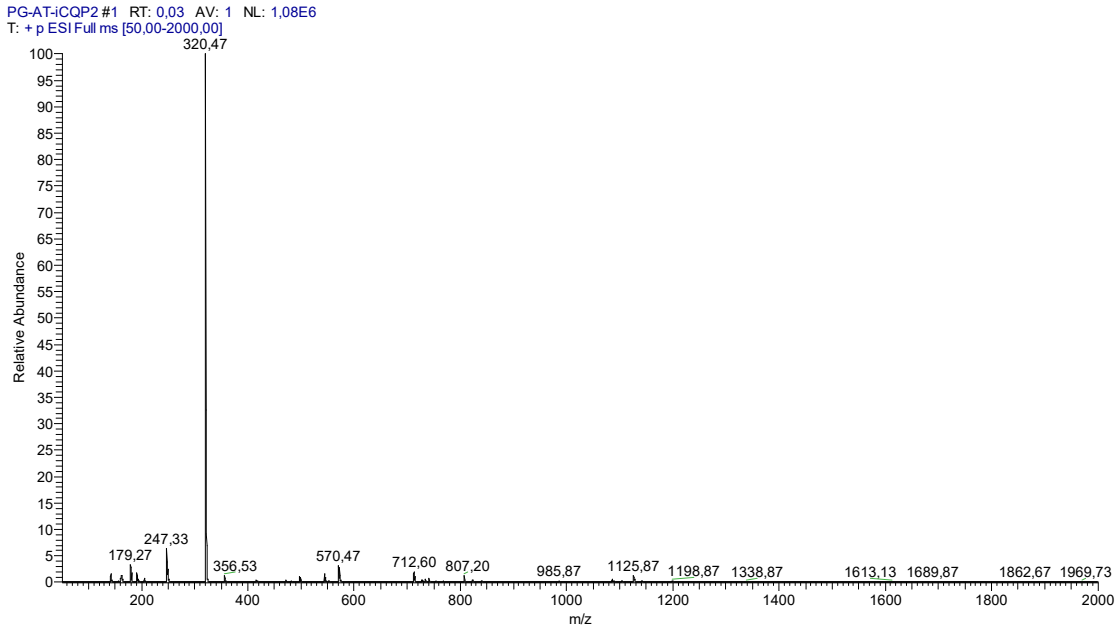
ESI-IT mass spectrum for **4a** (negative mode).



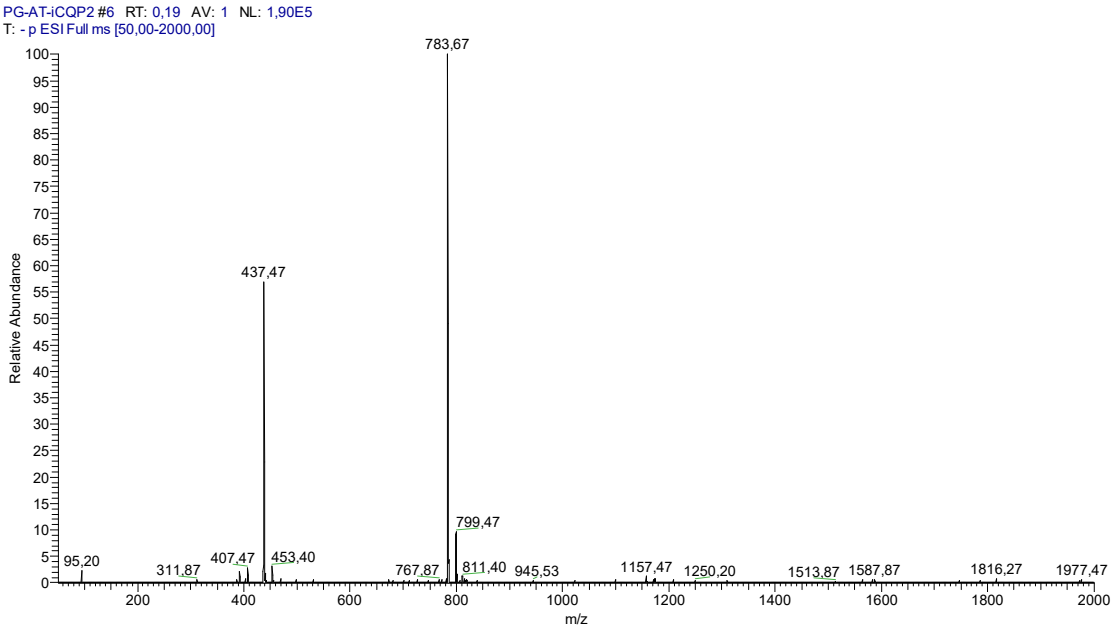
^{1}H-NMR spectrum of **4b** (400 MHz, DMSO-d_6).



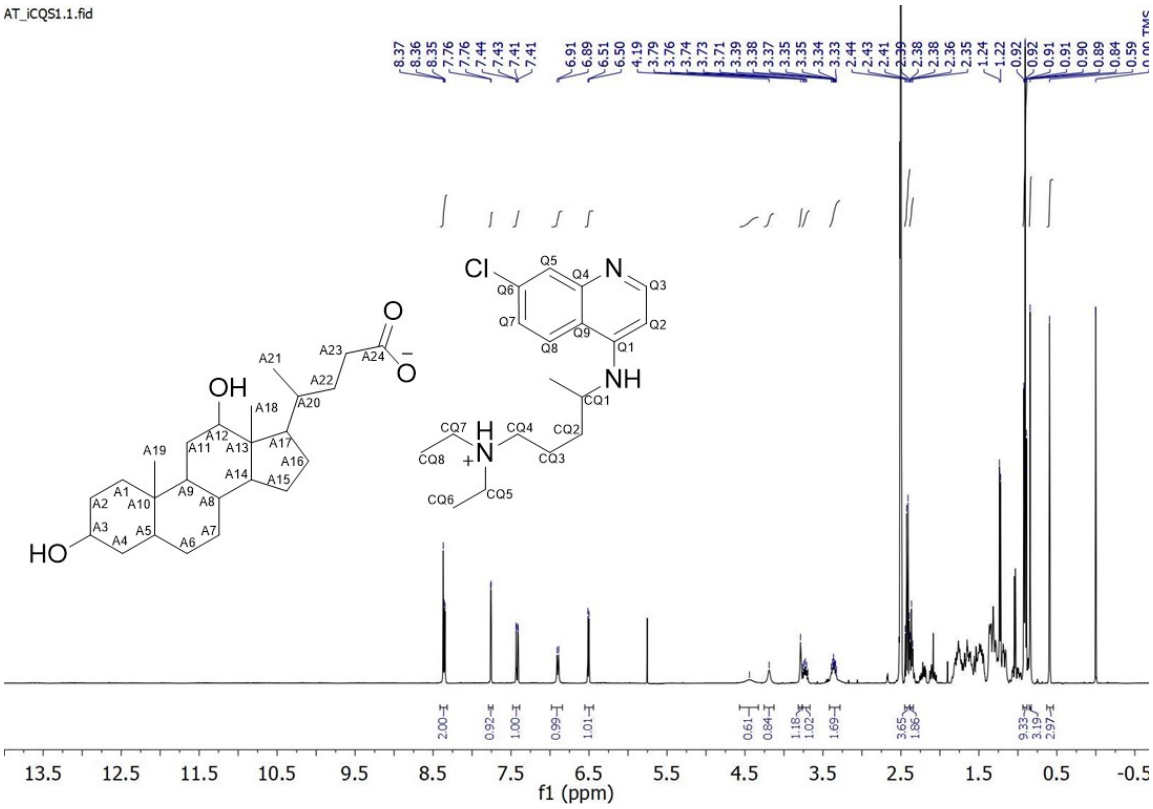
^{13}C-NMR spectrum of **4b** (100 MHz, DMSO-d_6).



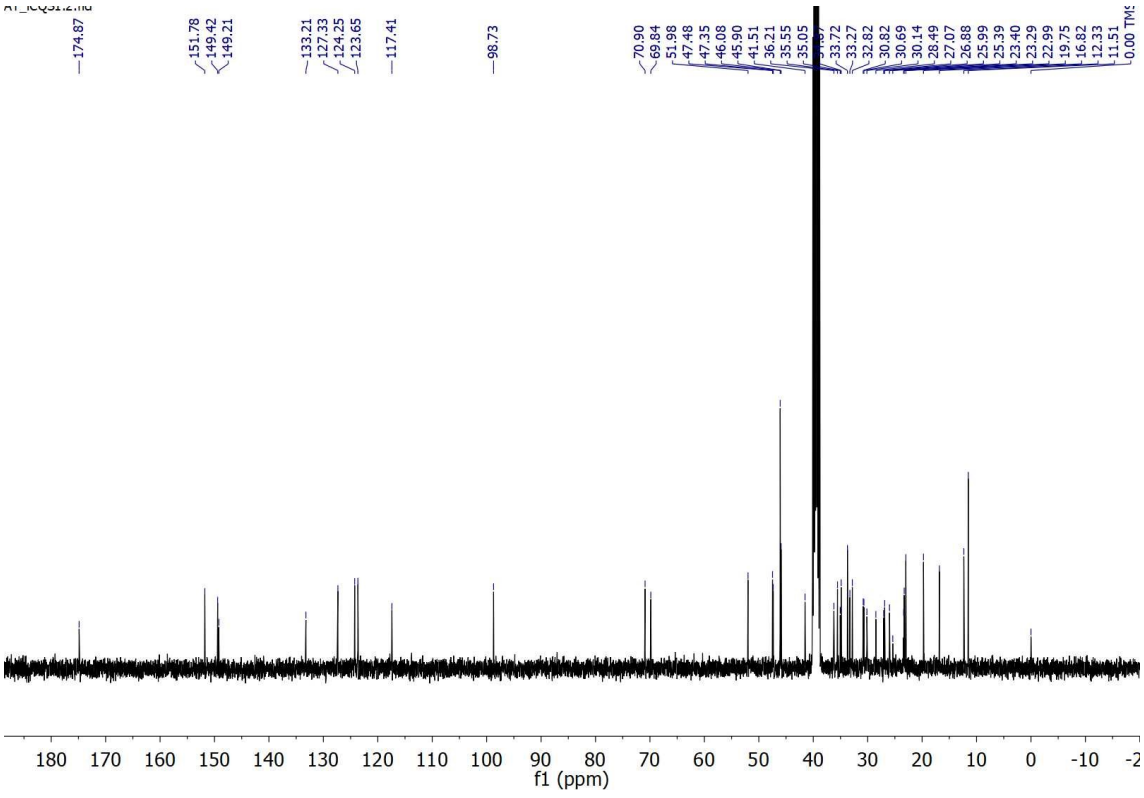
ESI-IT mass spectrum for **4b** (positive mode).



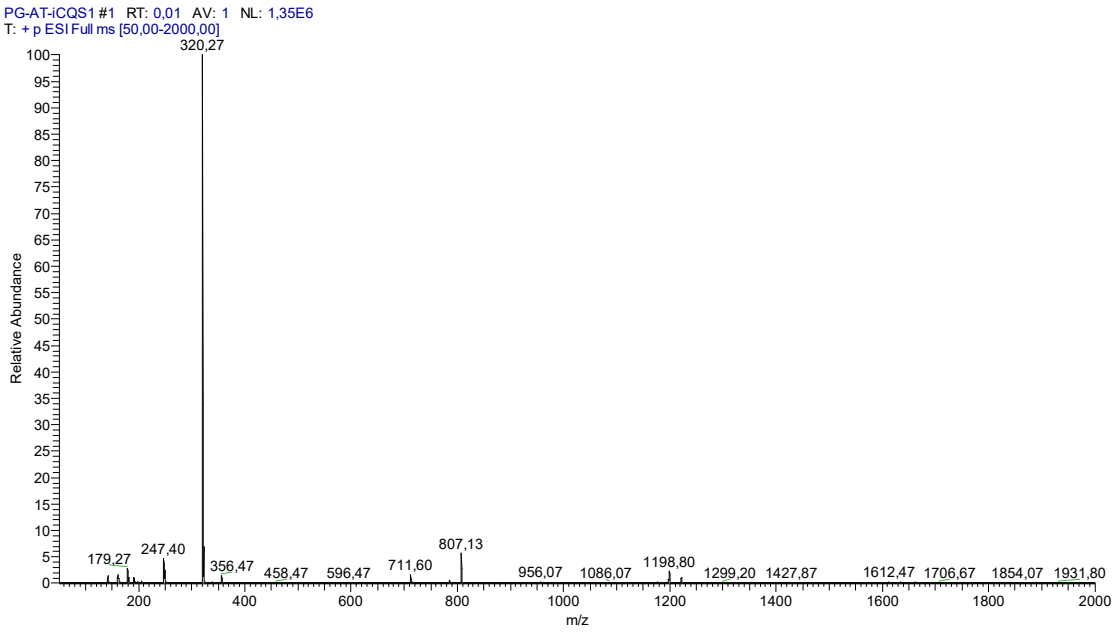
ESI-IT mass spectrum for **3b** (negative mode).



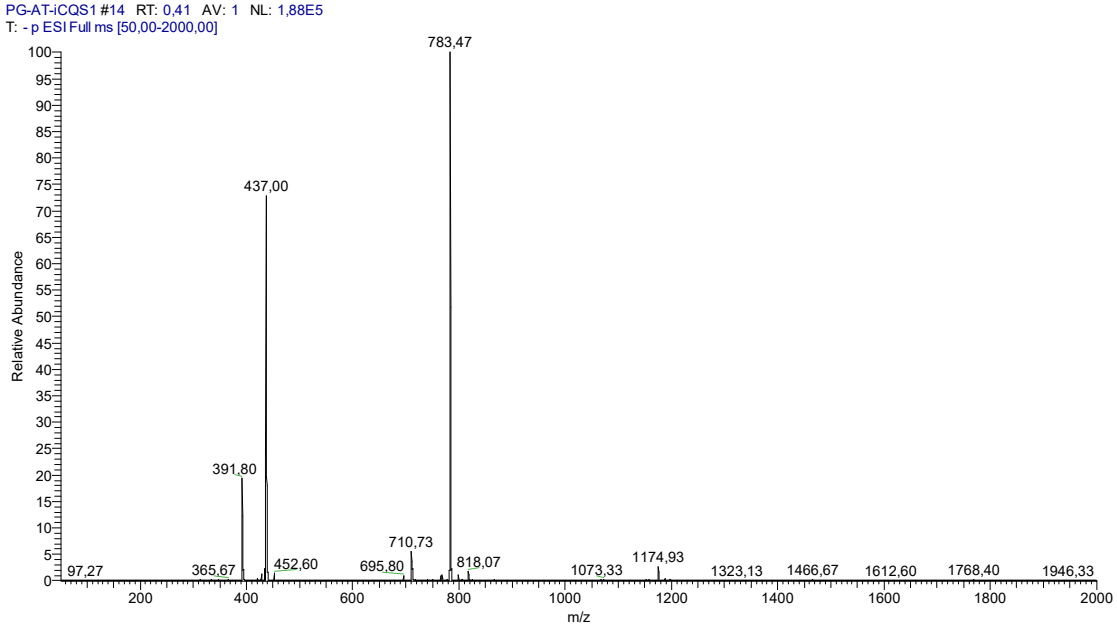
^{1}H-NMR spectrum of **4c** (400 MHz, DMSO-d_6).



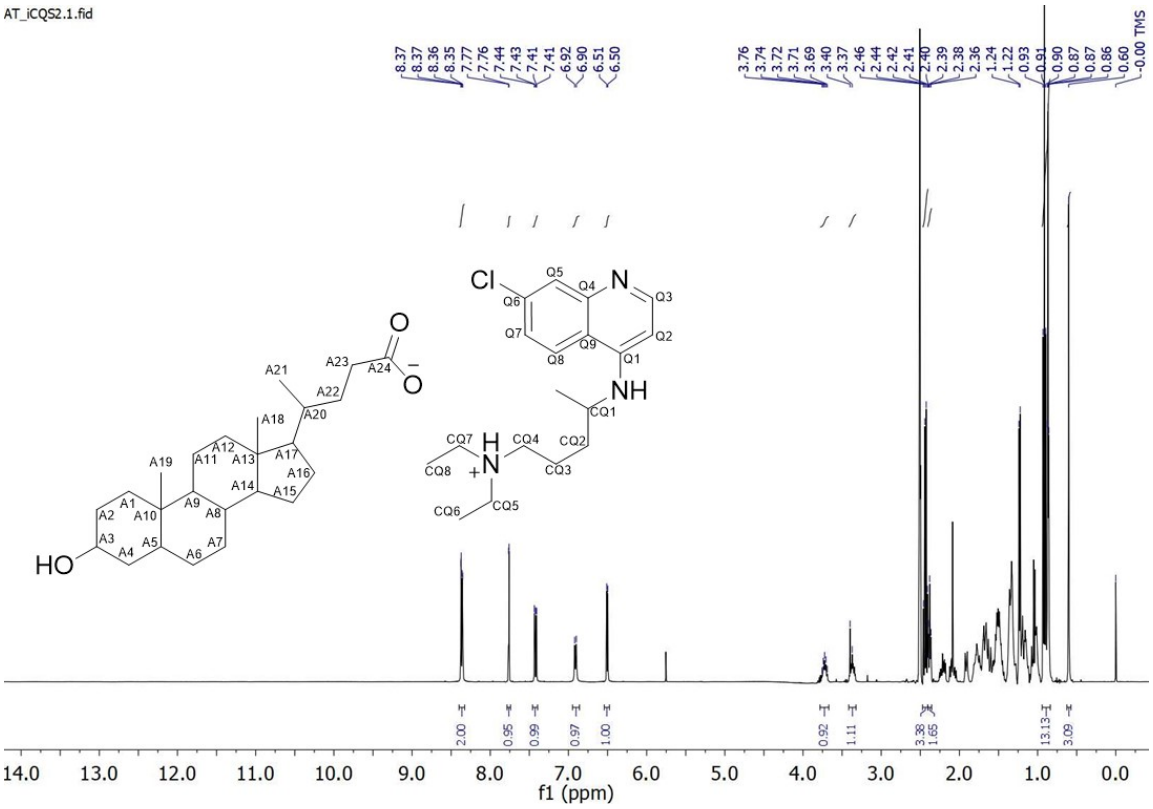
^{13}C-NMR spectrum of **4c** (100 MHz, DMSO-d_6).



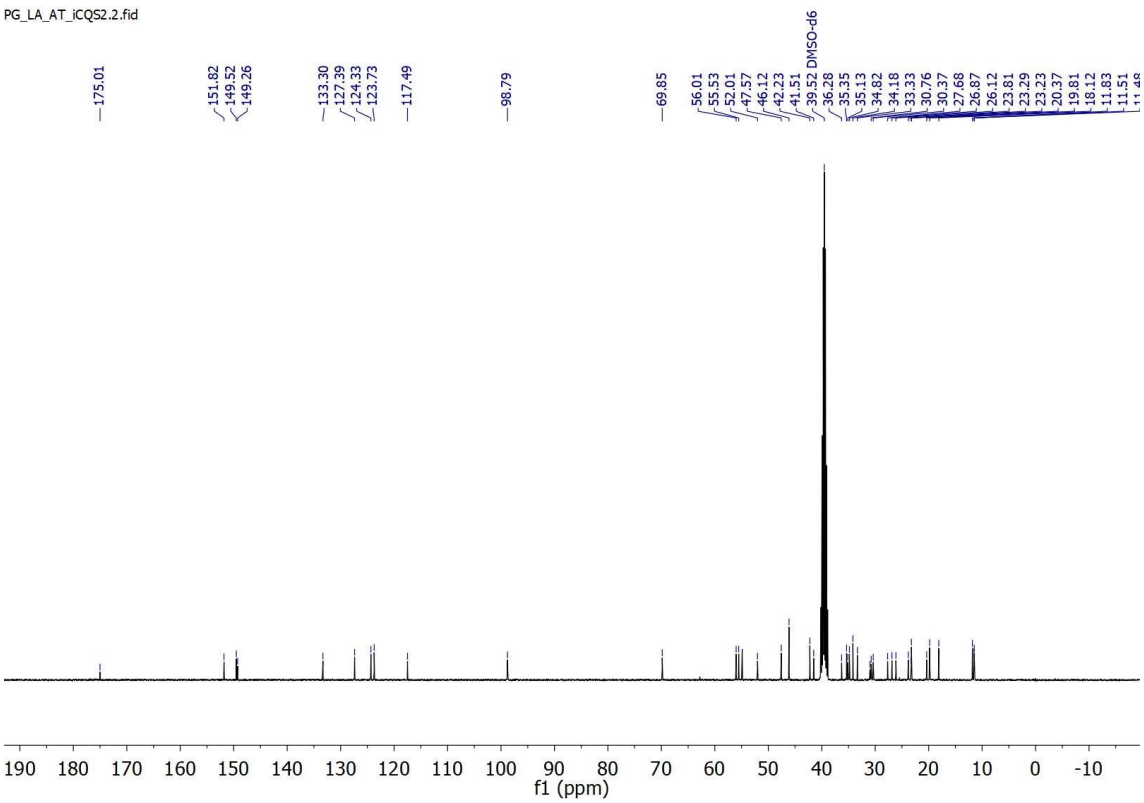
ESI-IT mass spectrum for **4c** (positive mode).



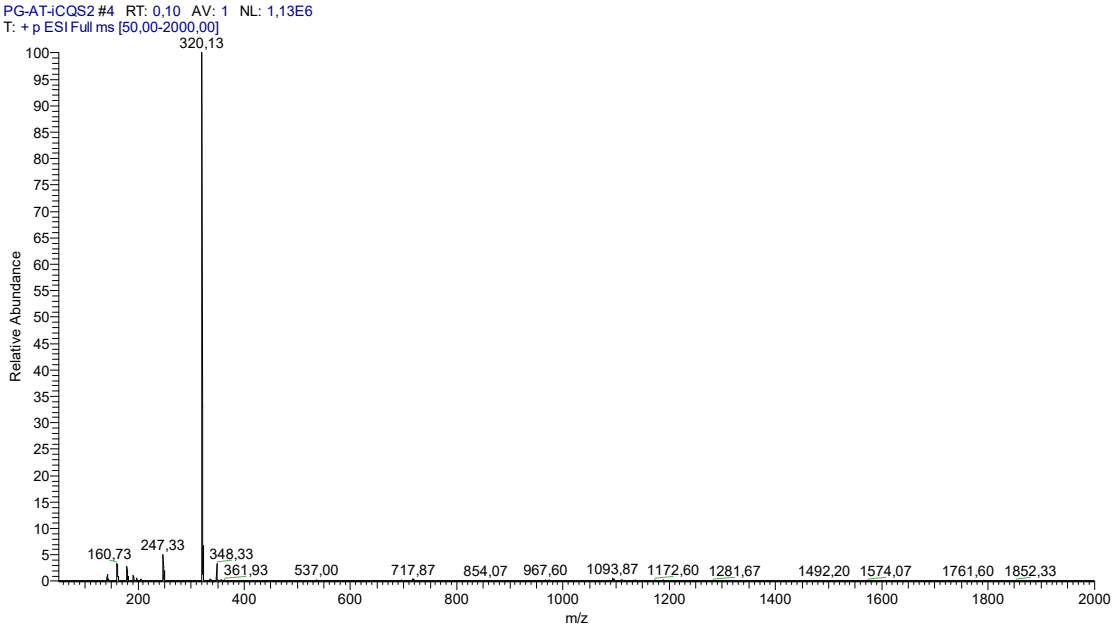
ESI-IT mass spectrum for **4c** (negative mode).



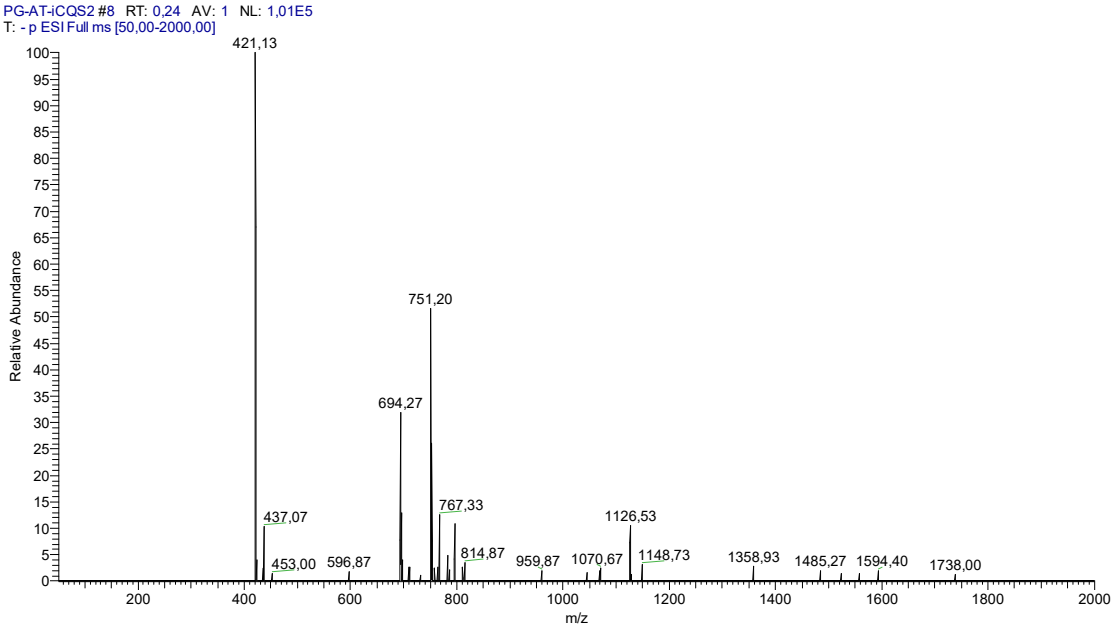
^{1}H-NMR spectrum **4d** (400 MHz, DMSO-d_6).



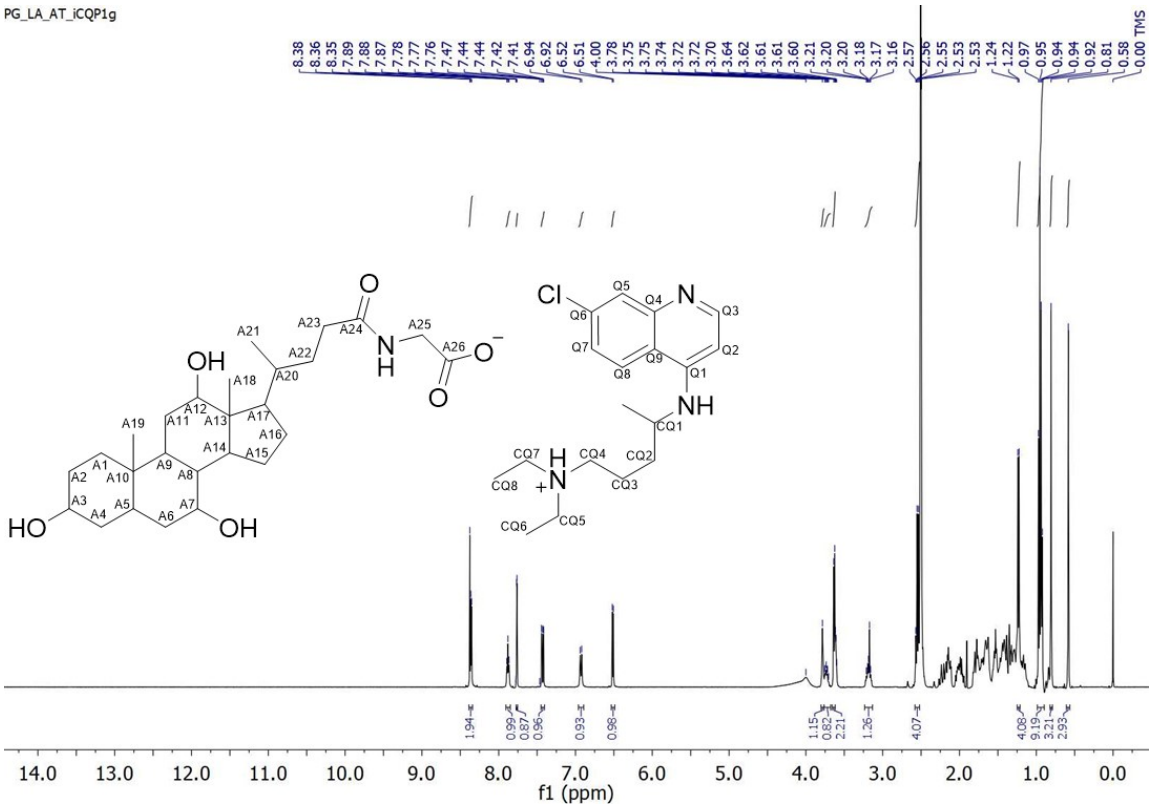
^{13}C-NMR spectrum of **4d** (100 MHz, DMSO-d_6).



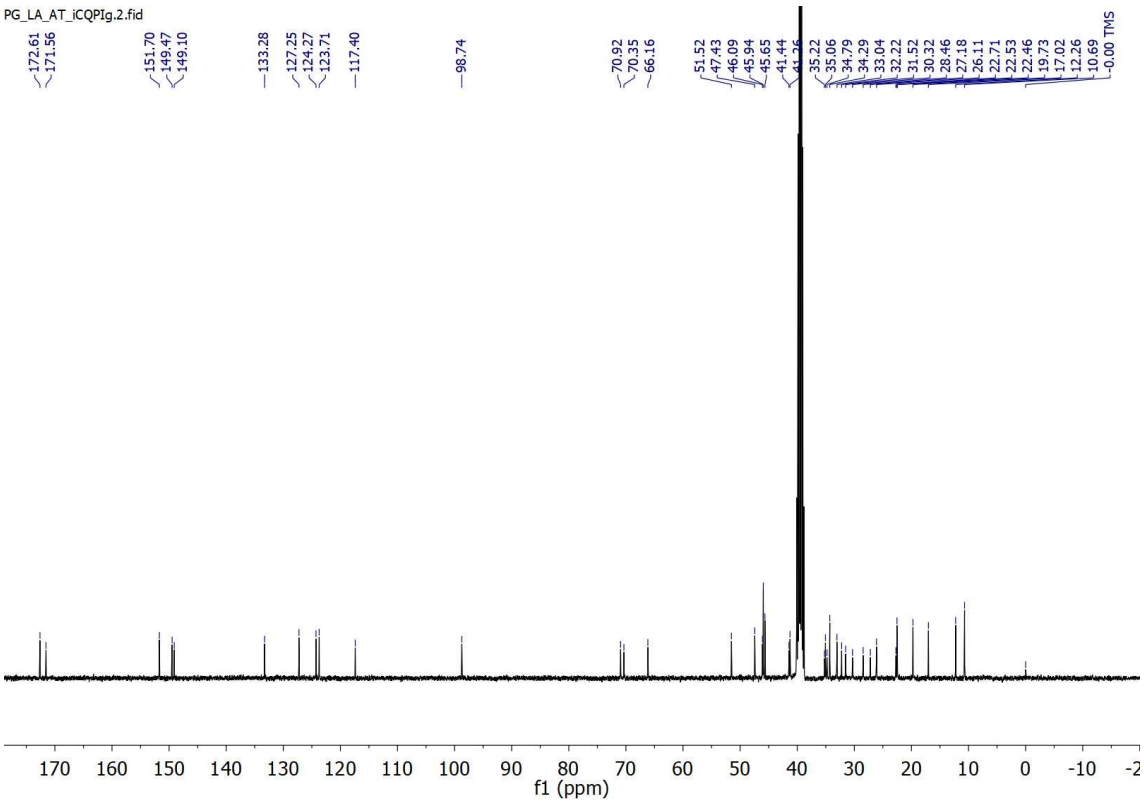
ESI-IT mass spectrum for **4d** (positive mode).



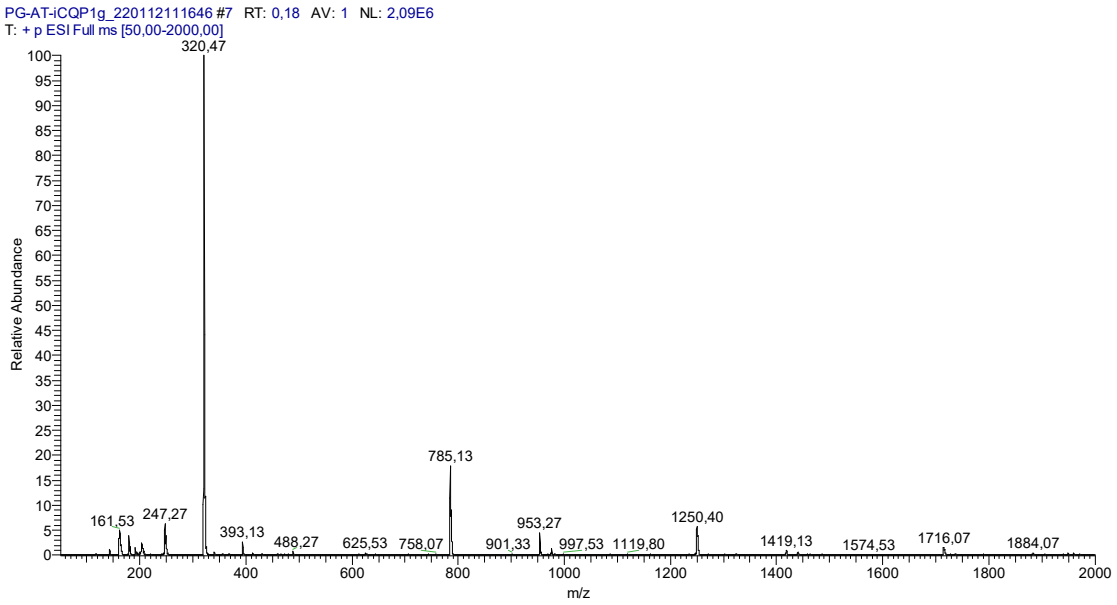
ESI-IT mass spectrum for **4d** (negative mode).



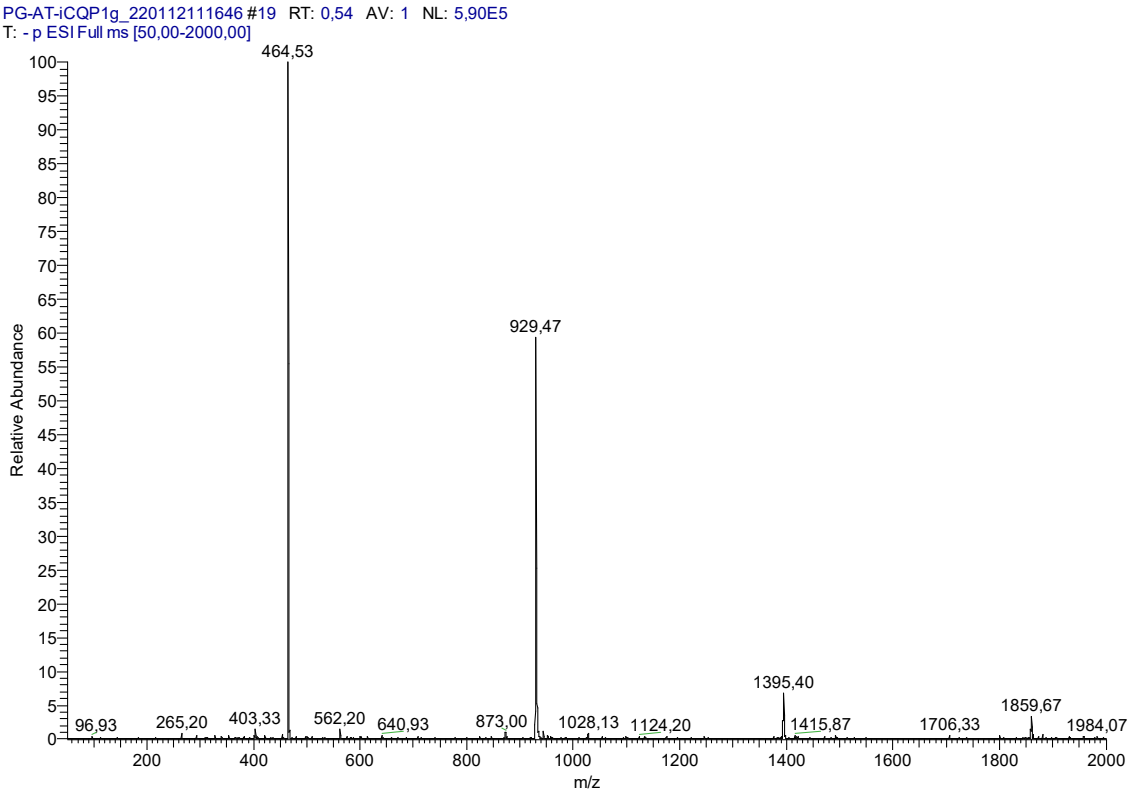
^{1}H-NMR spectrum of **4e** (400 MHz, DMSO-d_6).



^{13}C-NMR spectrum of **4e** (100 MHz, DMSO-d_6).



ESI-IT mass spectrum for **4e** (positive mode).



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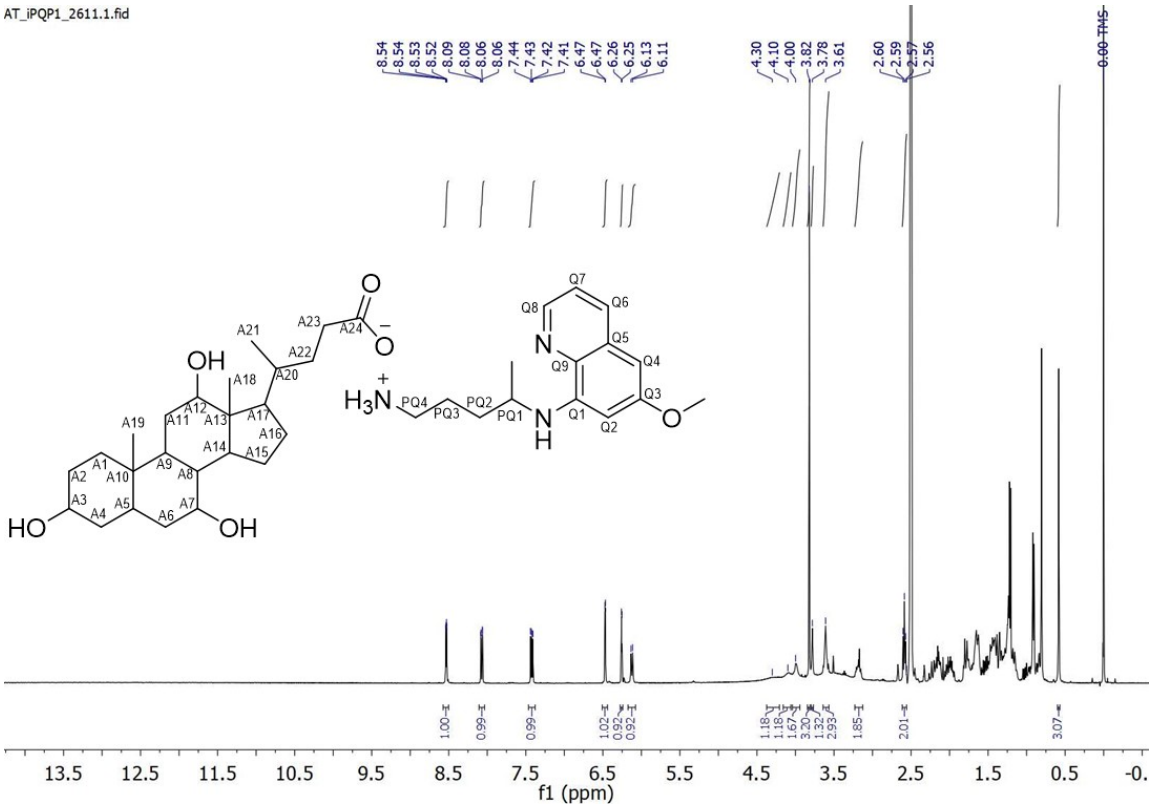
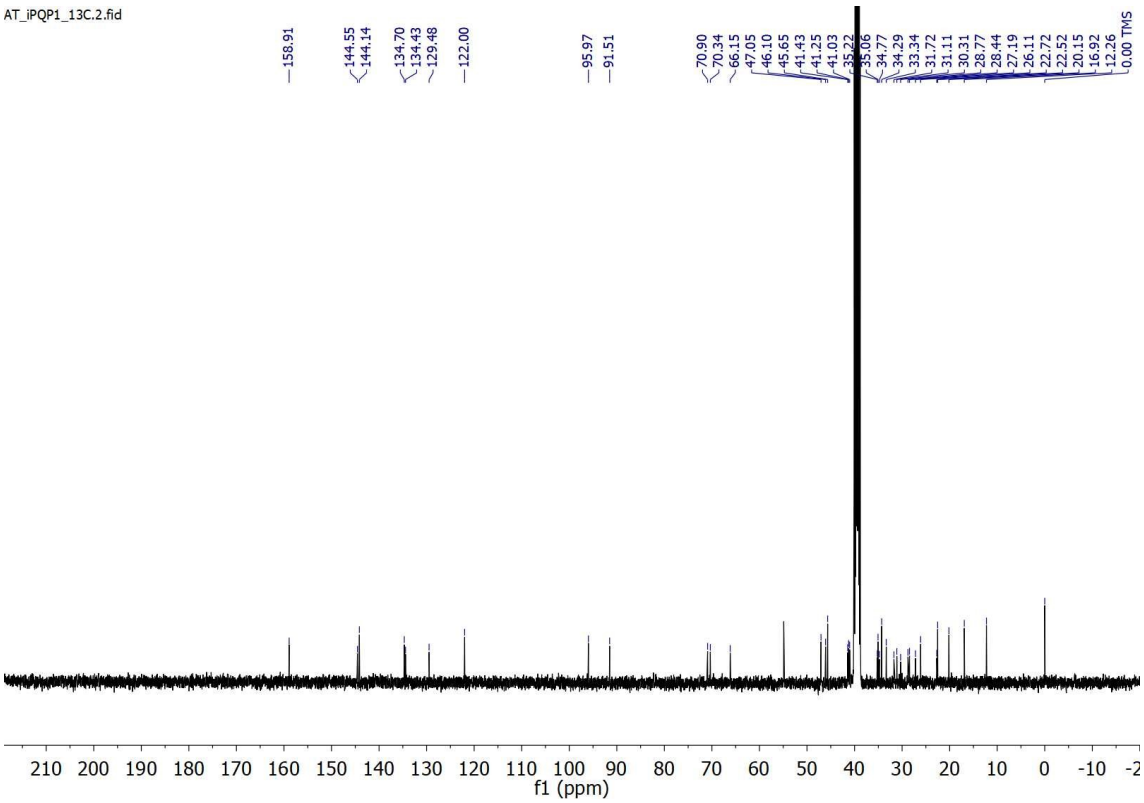
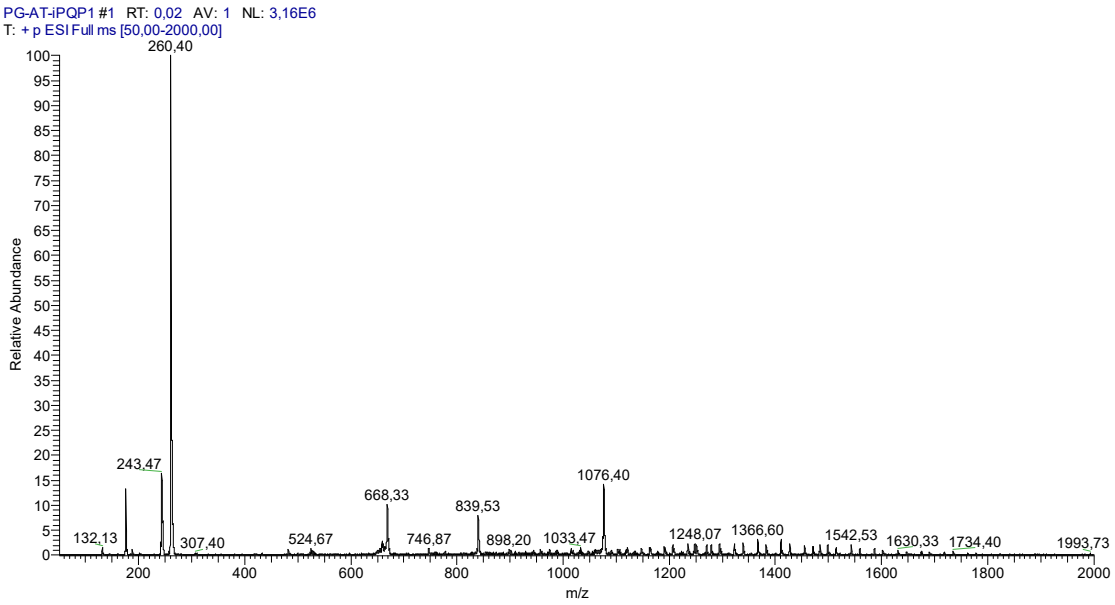


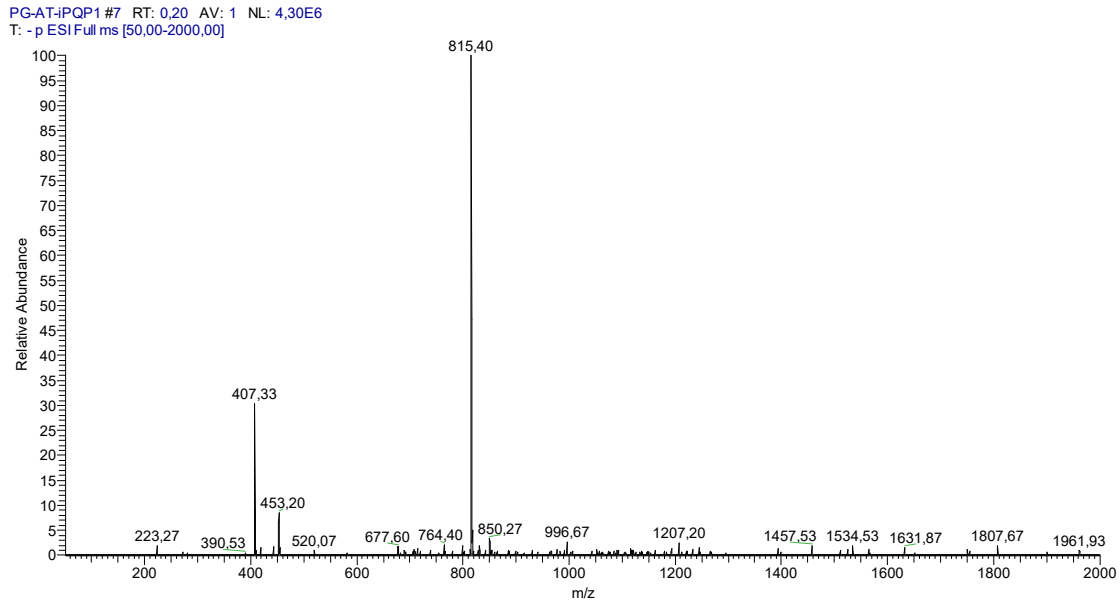
^{1}H-NMR spectrum of **5a** (400 MHz, DMSO-d_6).



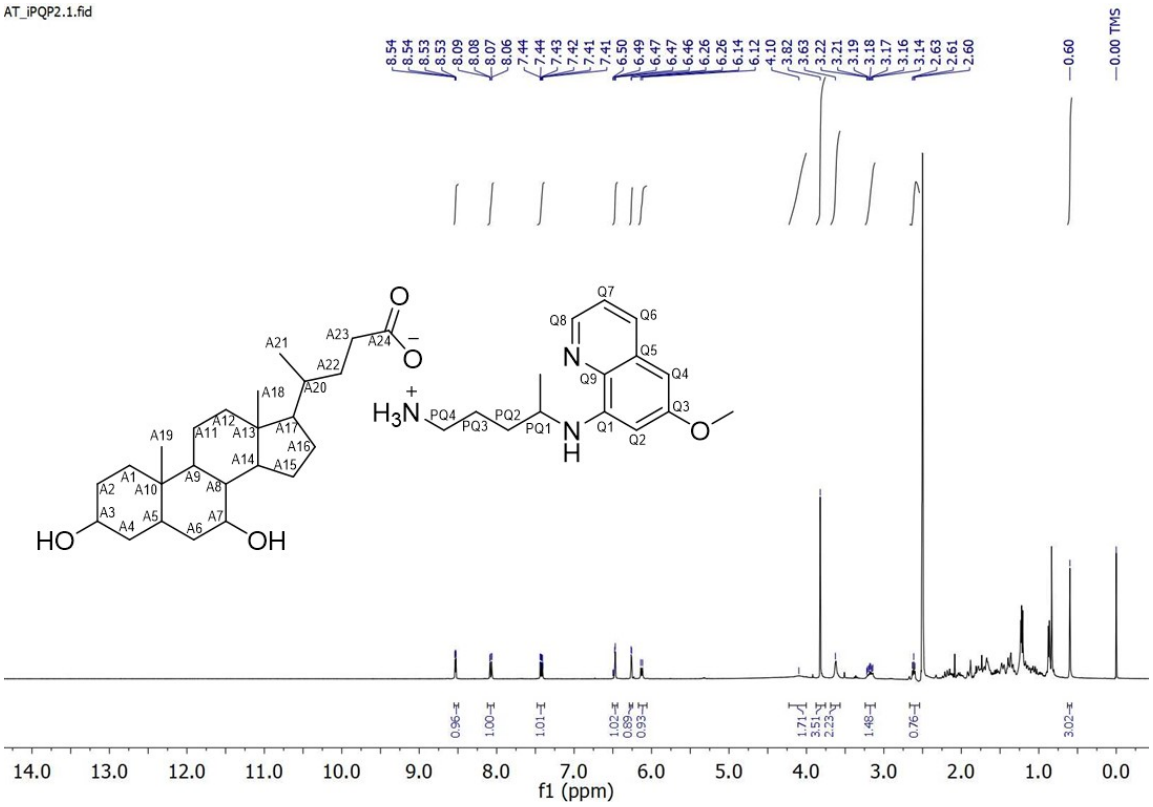
^{13}C-NMR spectrum **5a** (100 MHz, DMSO-d_6).



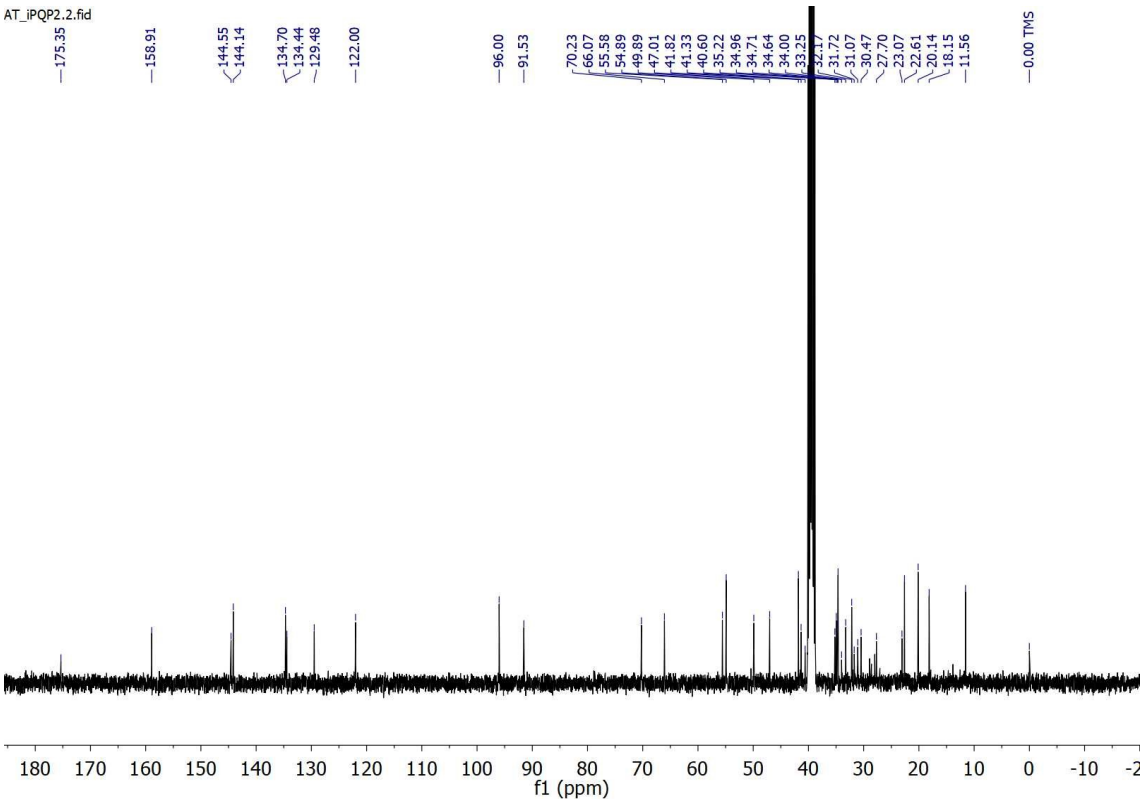
ESI-IT mass spectrum for **5a** (positive mode).



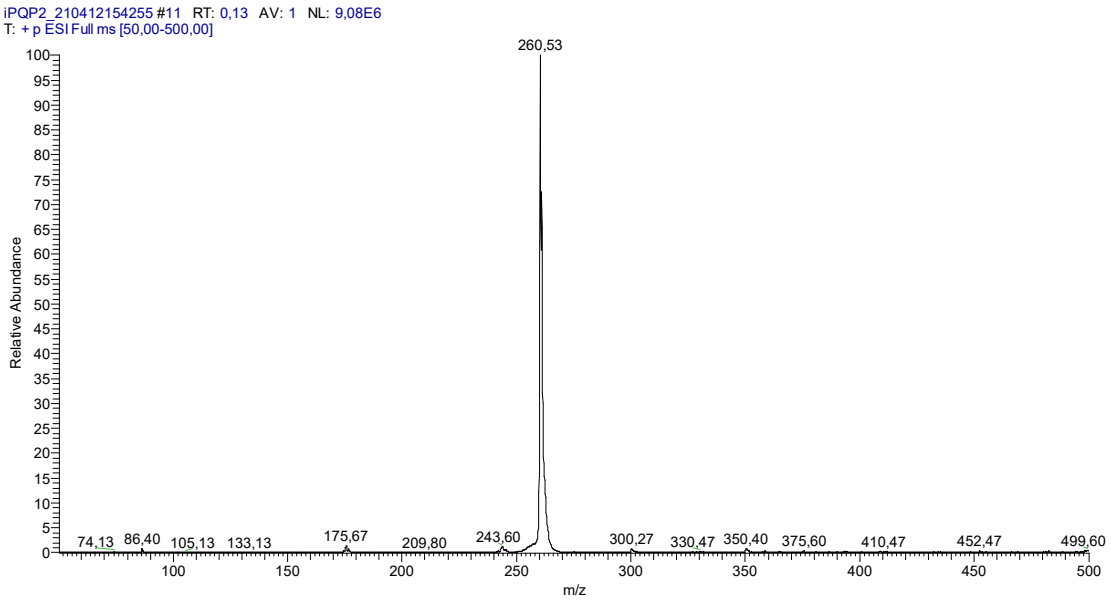
ESI-IT mass spectrum for **5a** (negative mode).



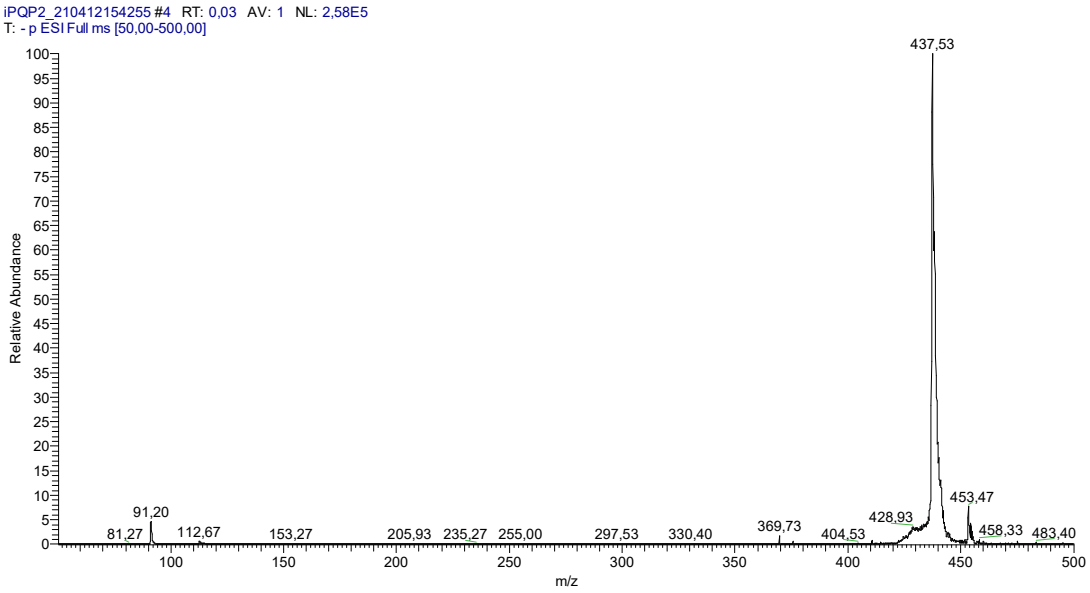
^{1}H-NMR spectrum of **5b** (400 MHz, DMSO-d_6).



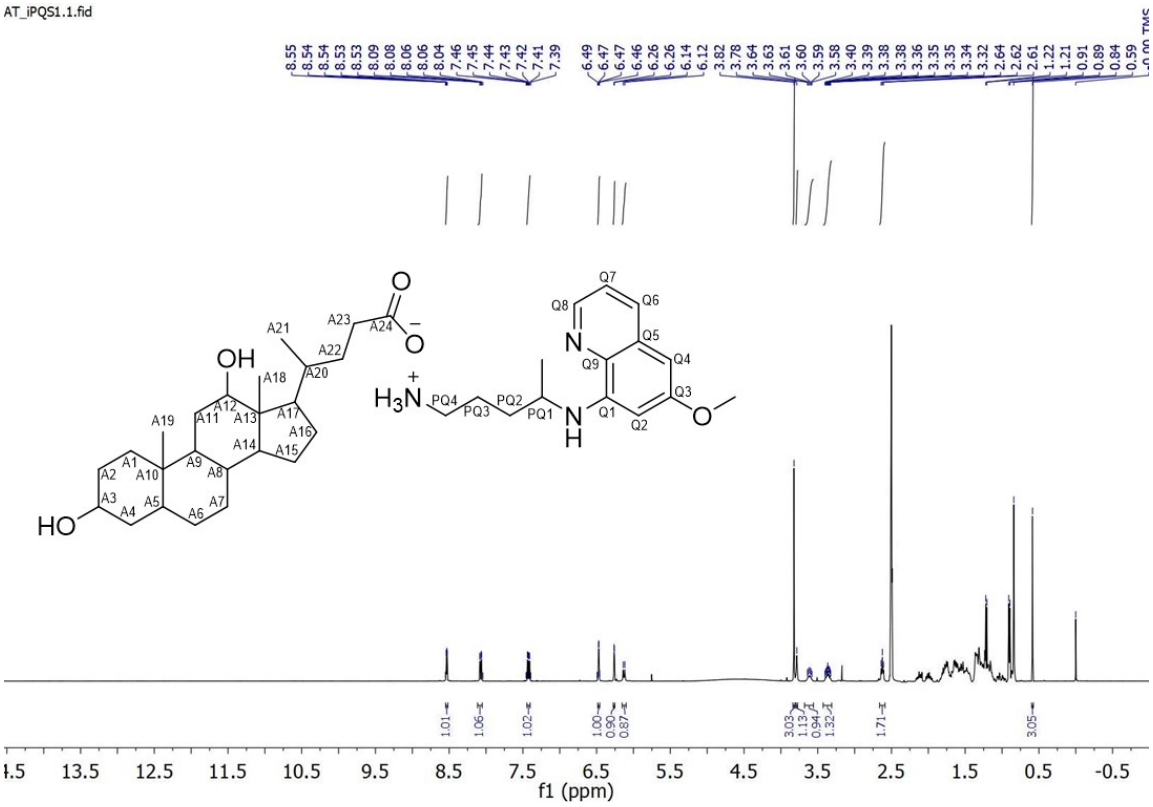
^{13}C-NMR spectrum of **5b** (100 MHz, DMSO-d_6).



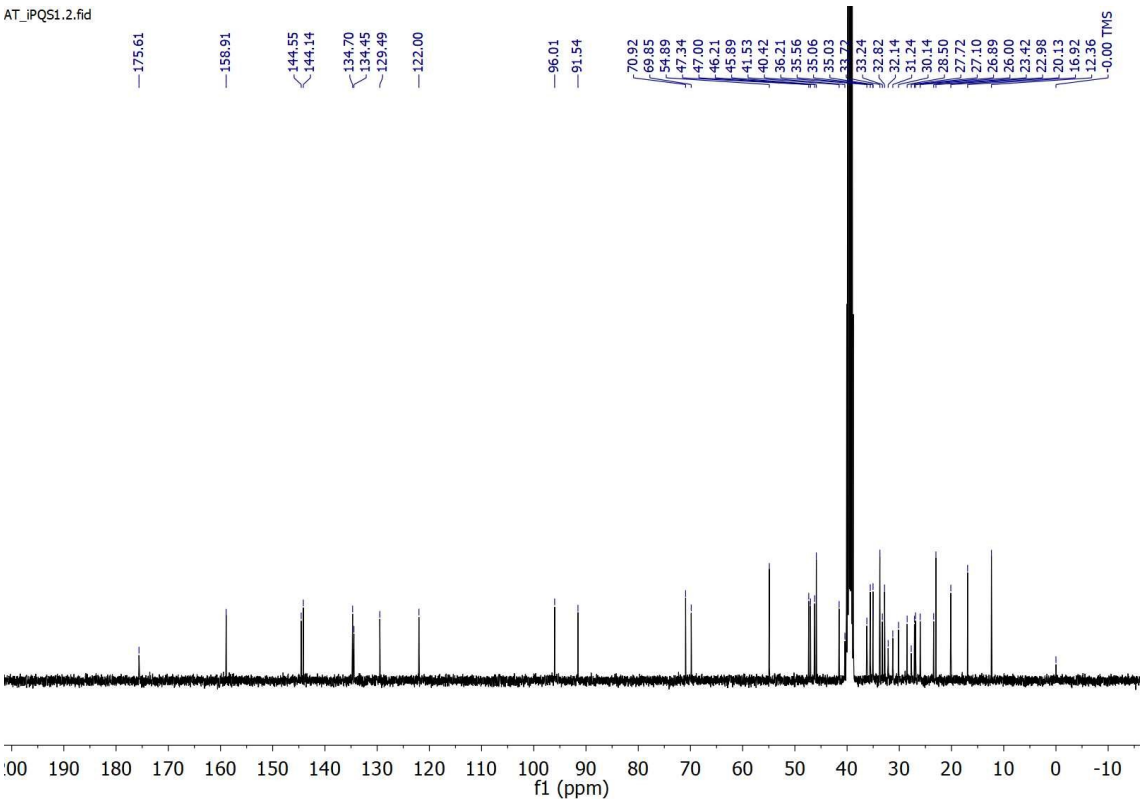
ESI-IT mass spectrum for **5b** (positive mode).



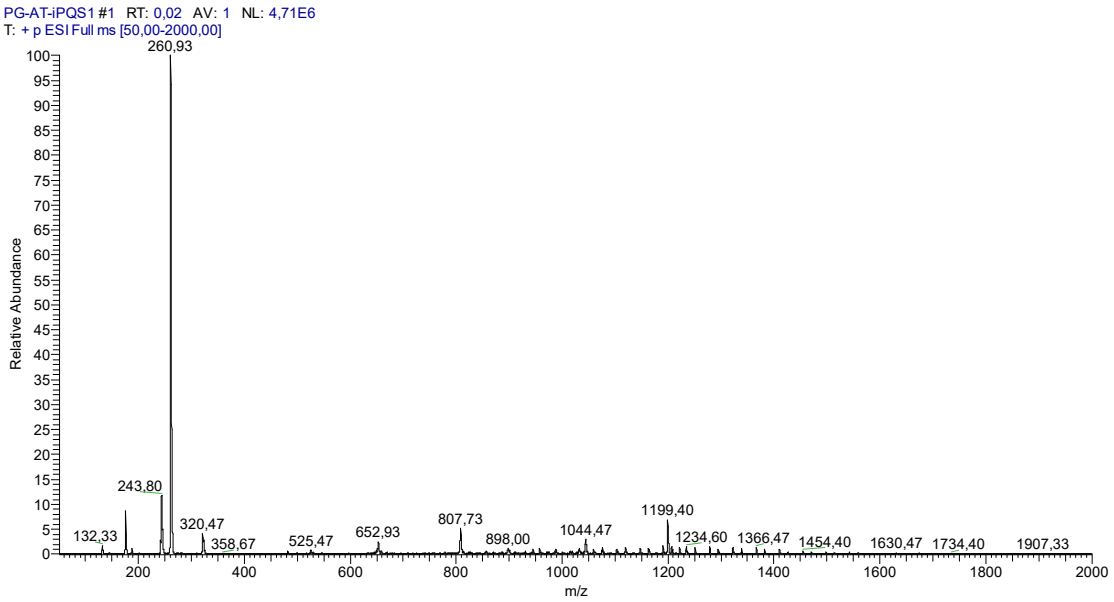
ESI-IT mass spectrum for **5b** (negative mode).



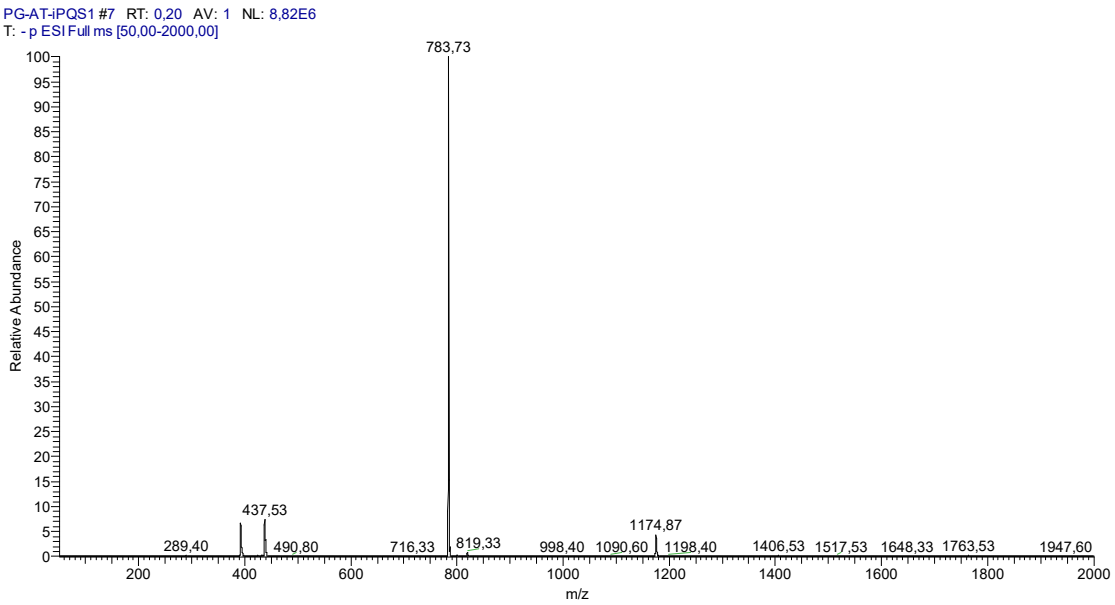
^{1}H-NMR spectrum of **5c** (400 MHz, DMSO-d_6).



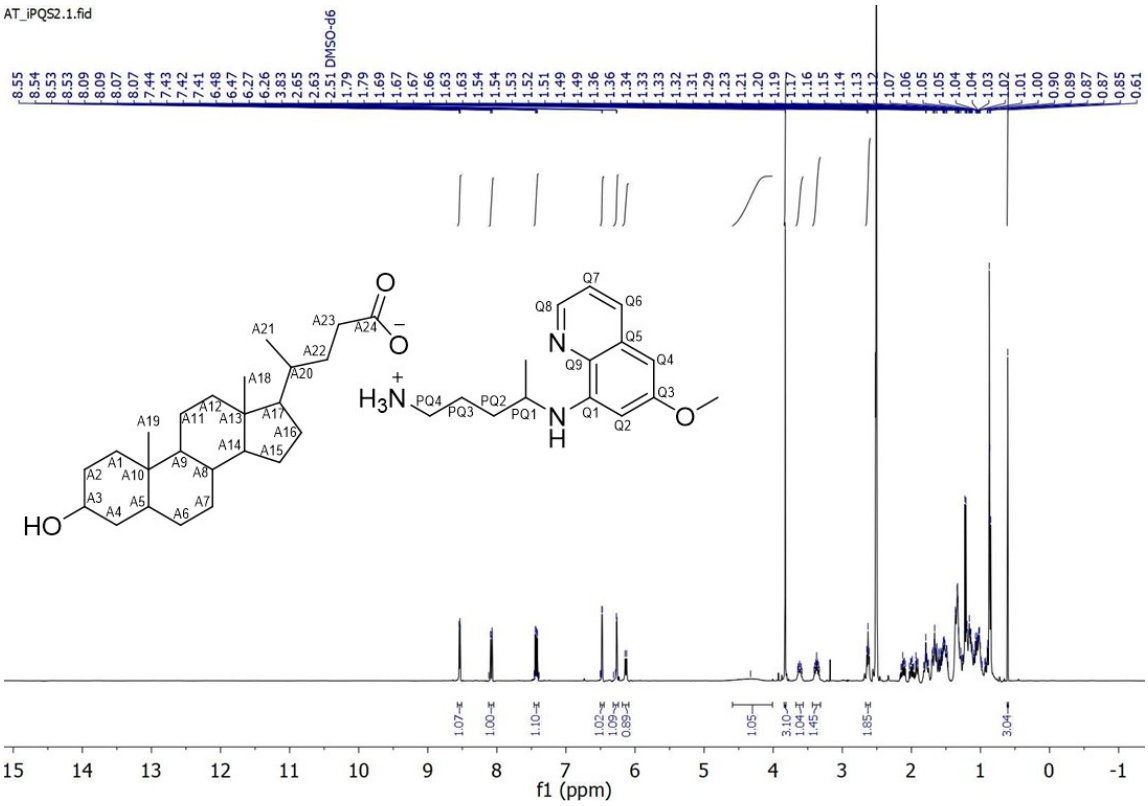
^{13}C-NMR spectrum of **5c** (100 MHz, DMSO-d_6).



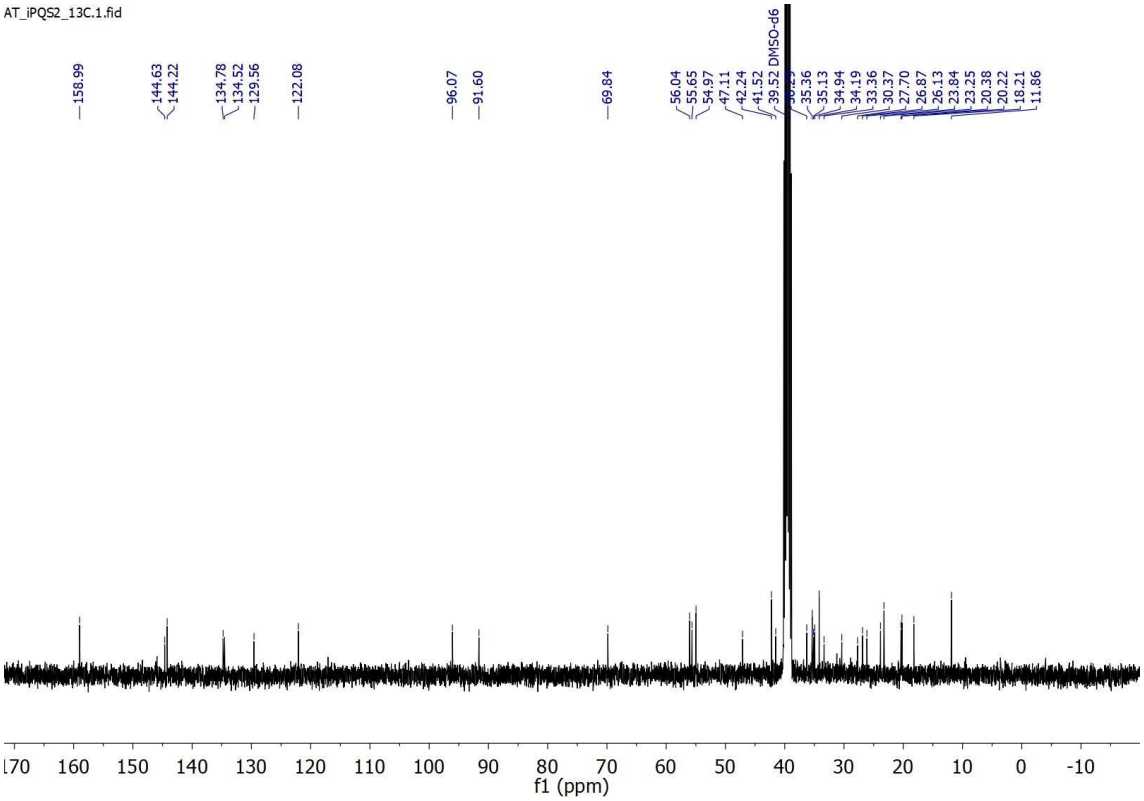
ESI-IT mass spectrum for **5c** (positive mode).



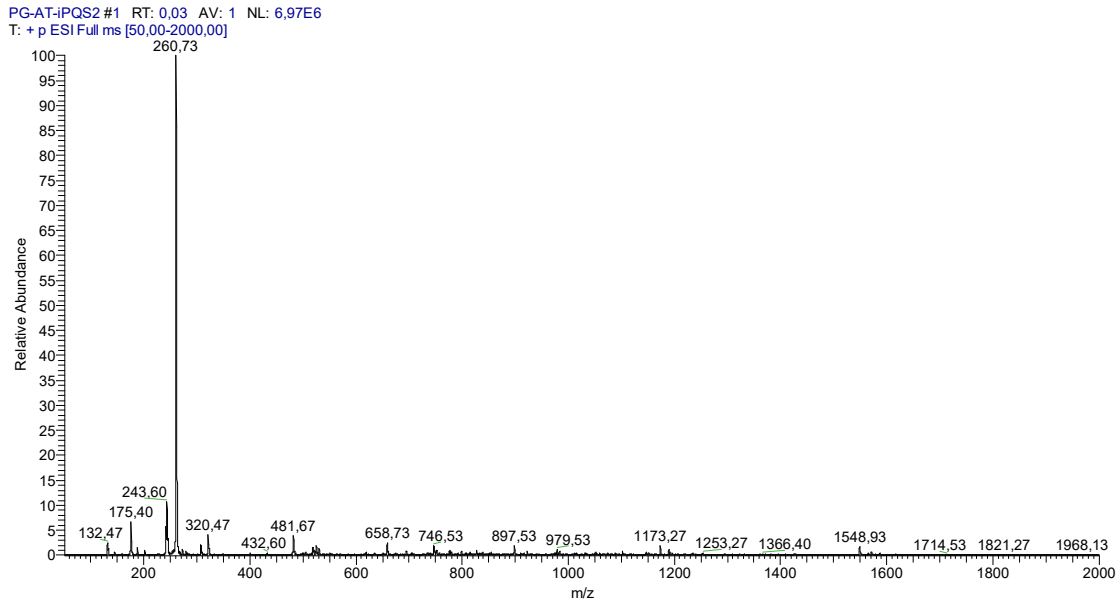
ESI-IT mass spectrum for **5c** (negative mode).



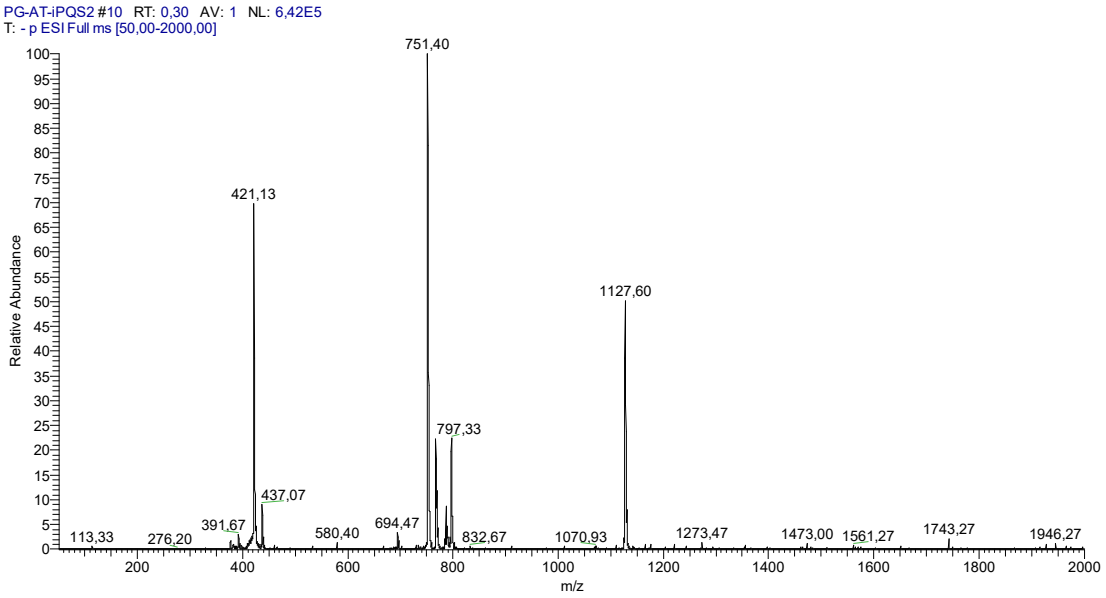
^{1}H-NMR spectrum of **5d** (400 MHz, DMSO-d_6).



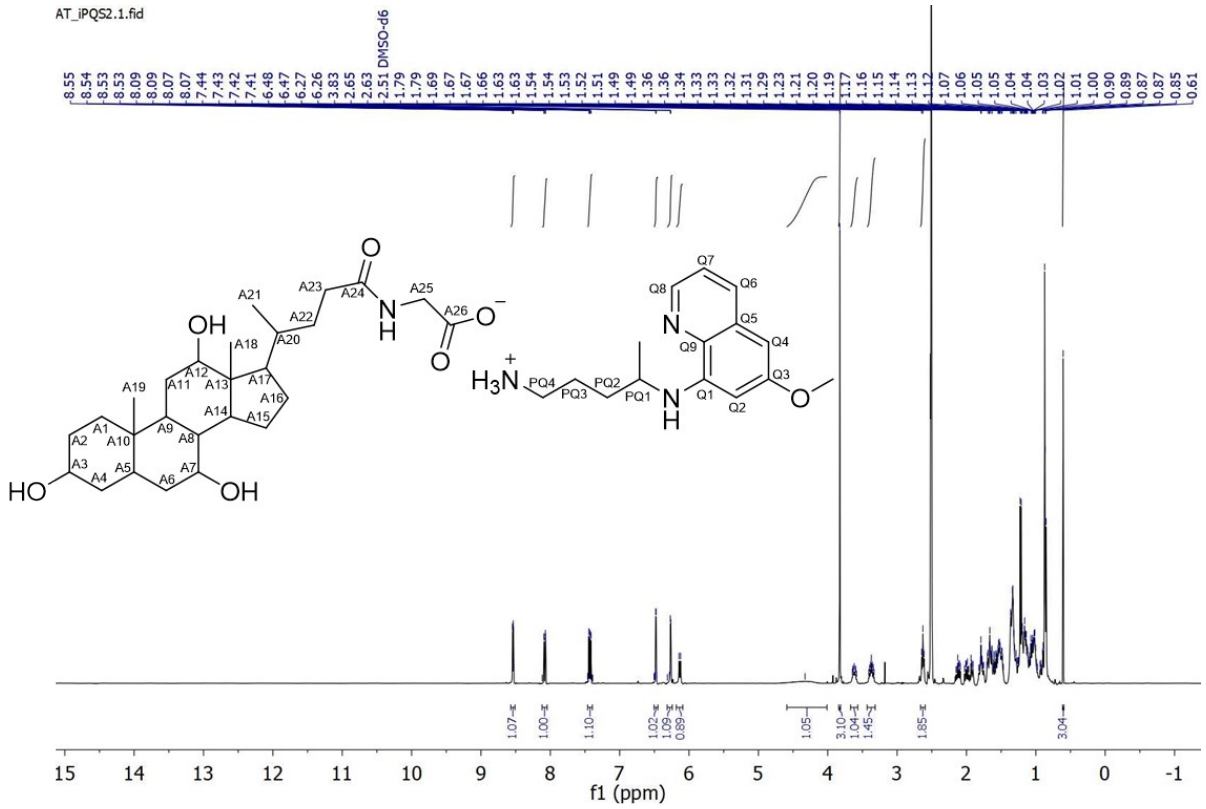
^{13}C-NMR spectrum of **5d** (100 MHz, DMSO-d_6).



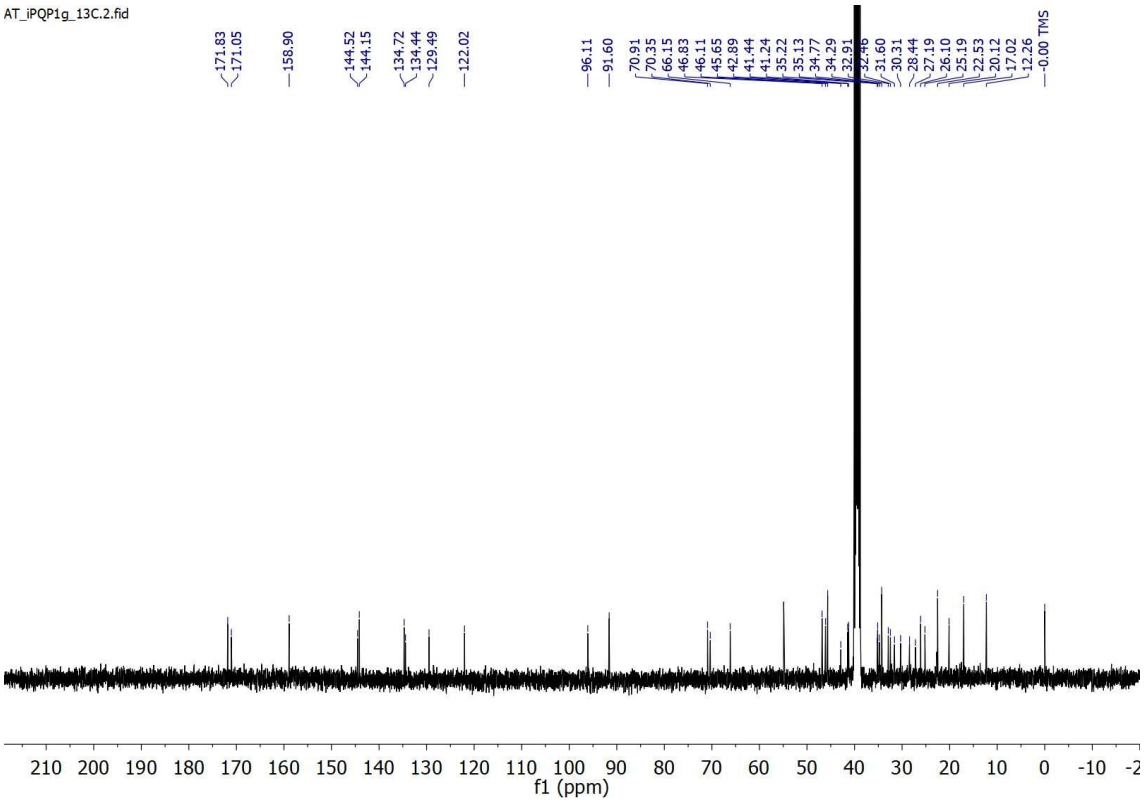
ESI-IT mass spectrum for **5d** (positive mode).



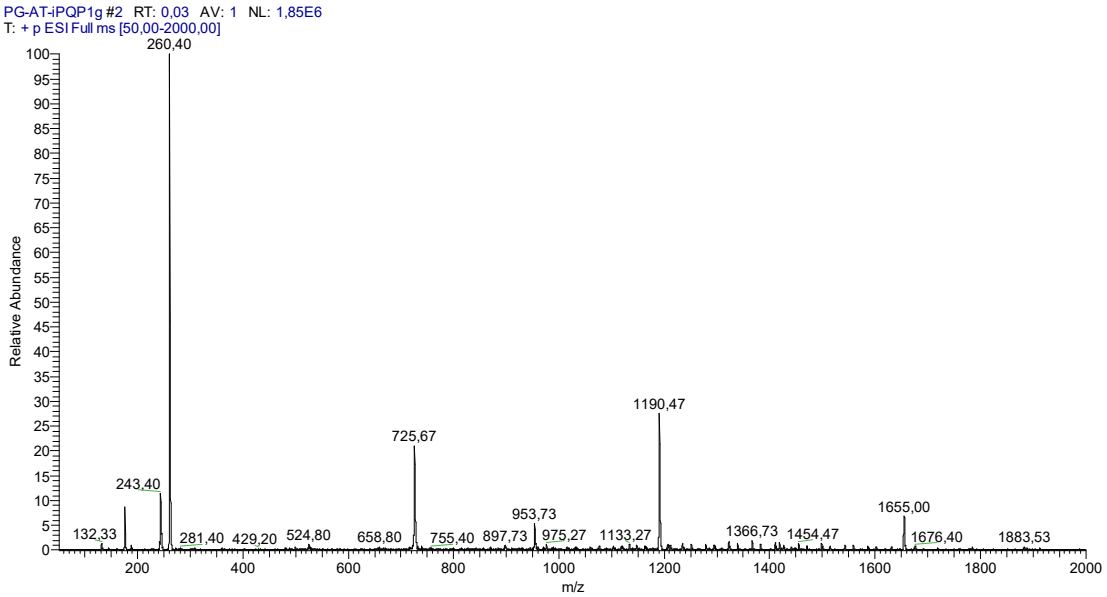
ESI-IT mass spectrum for **5d** (negative mode).



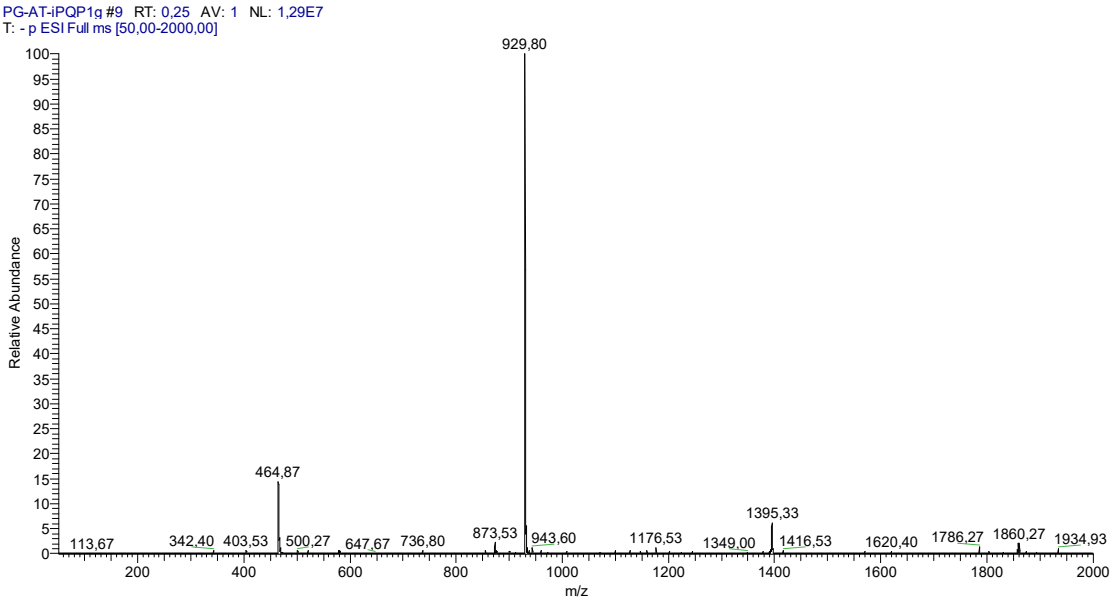
^{1}H-NMR spectrum of **5e** (400 MHz, DMSO-d_6).



^{13}C-NMR spectrum of **5e** (100 MHz, DMSO-d_6).

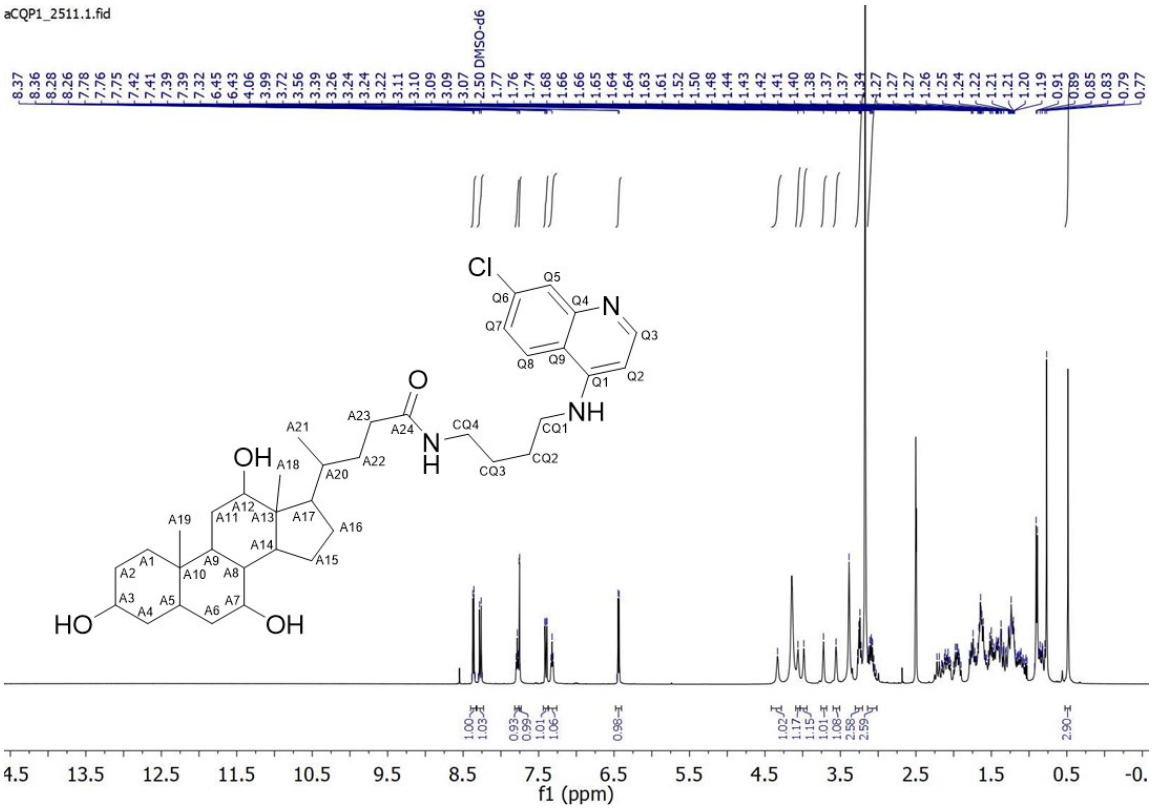


ESI-IT mass spectrum for **5e** (positive mode).

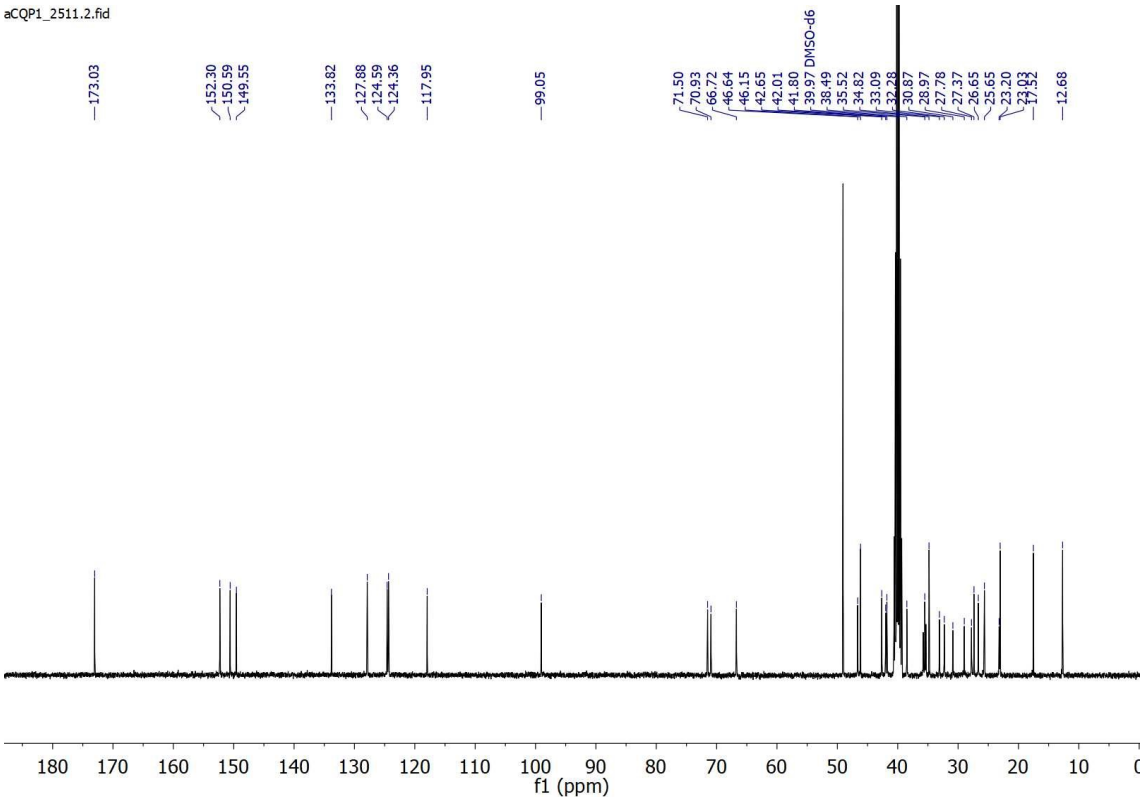


ESI-IT mass spectrum for **5e** (negative mode).

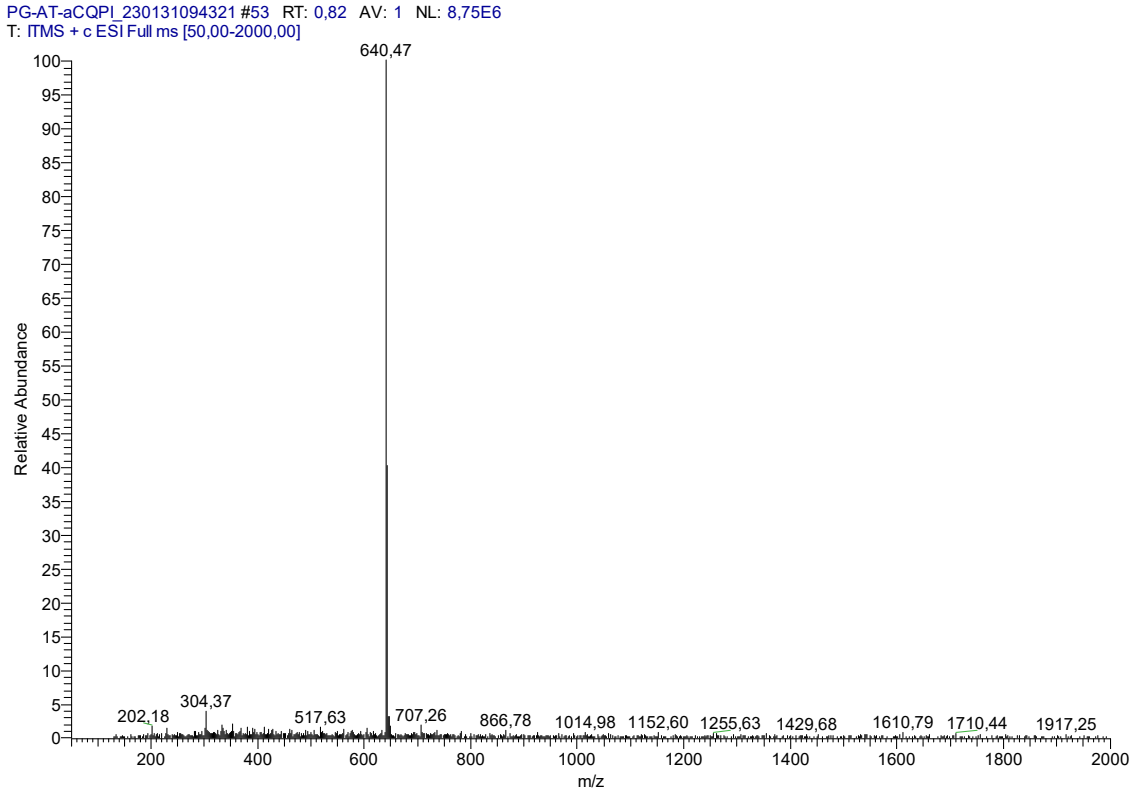
4.2. Amide 6a



^{1}H-NMR spectrum of **6a** (400 MHz, DMSO-d_6).

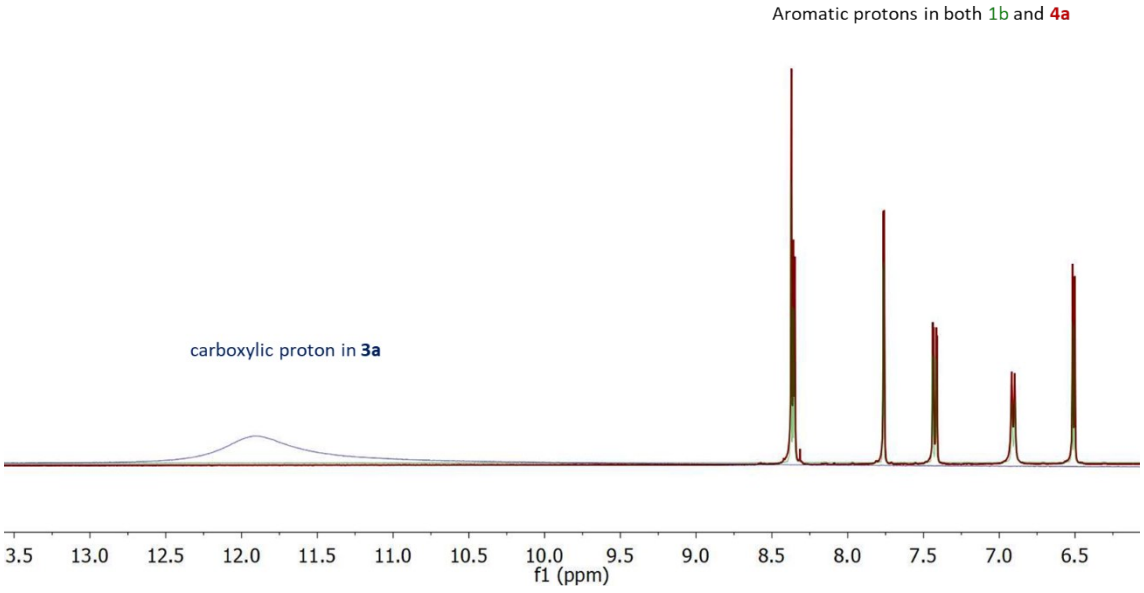


^{13}C-NMR spectrum of **6a** (100 MHz, DMSO-d_6).



ESI-IT mass spectrum for **6a** (positive mode)

4.3. **Figure S1:** Superimposed ^{1}H NMR spectra of cholic acid **3a** (blue), basic chloroquine **1b** (green), and their derived bile salt **4a** (red); the carboxylic proton peak at ca. 12 ppm is exclusively observed in the spectrum of **3a**, while missing in the spectrum of **4a**, thus confirming the complete transfer of the acidic proton to the basic antimalarial drug.



5. References

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