Electronic Supplementary Material (ESI) for Organic Chemistry Frontiers.

Supporting Information

General

^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 400 and 500 MHz spectrometer, and were calibrated using the solvent signals; coupling constants are given in Hertz (Hz). Products were purified by flash chromatography and semi-preparative HPLC. Analytical RP-HPLC was performed on a Beijing ChuangXinTongHeng LC3000 (analytic) instrument with a C18 column (5 μm, 4.6 × 150 mm) at 40 °C. The column was eluted with a linear gradient of 2–90% acetonitrile containing 0.1% TFA for 30 min at a flow rate of 1 mL min^{-1}. Preparative HPLC was performed on a Beijing ChuangXinTongHeng LC3000 (preparative) instrument with a preparative column (Waters, C18, OBD, 5 μm, 19 × 250 mm). The column was eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA at a flow rate of 8 mL min^{-1}. The ESI-HRMS spectra were measured on an Agilent 6230 LC–TOF MS spectrometer. Glycoconjugates and other intermediates were analyzed using a short guard column and eluted with 70% methanol containing 0.1% formic acid. Mass spectra were recorded in the mass range of 200-3000 or 600-2000 under high resolution mass-spec mode (HR-MS, standard 3200 m/z, 4 GHz). Key source parameters: drying nitrogen gas flow of 11 L/min; nebulizer pressure of 40 psi; gas temperature of 350 ^{o}C; fragmenter voltage of 175 V; skimmer voltage of 65 V; and capillary voltage of 4000 V.

The antibody and antibody drug conjugate LC-MS analysis was performed on an Agilent 6230 LC–TOF MS spectrometer. The antibodies were measured with a Thermo Mab column (4 mm, 3.0 x 100 mm) at 70 ^{o}C. The column was eluted isocratic gradient of 20% acetonitrile (Buffer B) and 80% water containing 0.1% formic acid (Buffer A) in first 3 min, a linear gradient of 20-50% acetonitrile in additional 2.5 min and isocratic gradient of 50% acetonitrile in another 2 min, another linear gradient of 50-90% acentonitrile in 0.5 min and an isocratic 90% acetonitrile for 2 min at a flow rate of 0.4 mL/min. The mass spectra of antibodies were performed under a mode of protein analysis with resolution of 1 GHz and were collected among the mass range of 800-

5000. The multiple charged peaks of antibodies were deconvoluted using Agilent Mass Hunter Bioconfirm software (Agilent technology).

Synthesis

Synthesis of ethyl 6-(3-cyano-4-nitrophenoxy)hexanoate 3

To a solution of ethyl 6-hydroxyhexanoate (352 mg, 2.2 mmol) in THF (10 ml), NaH (60% in oil, 88 mg, 2.2 mmol) was added at 0 ^{o}C. The mixture was stirred at 0 ^{o}C for 15 min. To this mixture, 5-fluoro-2-nitrobenzonitrile (332 mg, 2 mmol) was slowly added. The reaction mixture was then allowed to warm to room temperature and stirred for 1 h. Methanol was added to quench the reaction and the solution was concentrated and flash chromatographed [on silica gel; elution with petroleum ether-EtOAc (4:2)] to afford compound **3** (199 mg, yield 65%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (d, *J* = 9.3 Hz, 1H), 7.30 (d, *J* = 2.7 Hz, 1H), 7.18 (dd, *J* = 9.3, 2.8 Hz, 1H), 4.25 – 4.01 (m, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.87 (dt, *J* = 14.2, 6.5 Hz, 2H), 1.71 (dt, *J* = 15.1, 7.4 Hz, 2H), 1.58 – 1.46 (m, 2H) and 1.26 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 163.3, 141.5, 128.0, 121.0, 118.6, 115.2, 110.2, 69.6, 60.5, 34.2, 28.6, 25.5, 24.6 and 14.4.

Synthesis of ethyl 6-(4-amino-3-cyanophenoxy)hexanoate 4

To solution of compound **3** (153 mg, 0.5 mmol) in ethanol (10 ml), iron (300 mg) and con. HCl (0.5 ml) was added. This mixture was diluted with water (10 ml) and was vigorously stirred at 80 ^{o}C for 1 h. The reaction mixture was filtered and neutralized with aq. $NaHCO_3$ then filtered and concentrated in vacuum. The crude product was purified by flash chromatography [on silica gel; elution with petroleum ether-EtOAc (2:1)] to afford compound **4** (55 mg, yield 40%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.96 (dd, *J* = 9.0, 2.9 Hz, 1H), 6.86 (d, *J* = 2.8 Hz, 1H), 6.73 (d, *J* = 9.0 Hz, 1H), 4.36 (br s, 2H), 4.13 (q, *J* = 7.1 Hz, 2H), 3.86 (t, *J* = 6.4 Hz, 2H), 2.33 (t, *J* = 7.5 Hz, 2H), 1.79-1.66 (m, 4H), 1.54 – 1.41 (m, 2H) and 1.25 (t, *J* = 7.1 Hz, 3H); 13C NMR (100 MHz, $CDCl_3$) δ 174.0, 151.5, 143.4, 123.2, 117.6, 117.6, 115.8, 96.9, 68.6, 60.5, 34.4, 29.0, 25.7, 24.8 and 14.2.

Synthesis of 6-(4-amino-3-cyanophenoxy)hexanoic acid 5

To solution of compound **4** (55 mg, 0.2 mmol) in methanol-water mixture (1:1) 5 ml, LiOH (10 mg, 0.4 mmol) was added and stirred at room temperature for 4 h. After completion of reaction, the reaction mixture was evaporated at reduced pressure and subjected to flash chromatography [petroleum ether- EtOAc (1:1)] to afford compound **5** (44.6 mg, yield 90%). HRMS, calculated for $C_{13}H_{17}N_2O_3$ $[M+H]^+$ 249.1239, found 249.1246. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.96 (dd, *J* = 9.0, 2.9 Hz, 1H), 6.86 (d, J = 2.8 Hz, 1H), 6.69 (d, *J* = 9.0 Hz, 1H), 5.99 (br s, 2H), 3.87 (t, *J* = 6.4 Hz, 7H), 2.40 (t, *J* = 7.4 Hz, 2H), 1.80-1.68 (m, 4H) and 1.54-1.48 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.3, 151.2, 144.2, 123.4, 117.7, 117.2, 115.9, 96.5, 68.6, 33.9, 29.0, 25.7 and 24.5.

Compounds **8-10** were synthesis by reported method with little modification.[1]

Synthesis of ethyl 3-oxo-5-(tritylthio)pentanoate 8

A solution of 3-(tritylthio)propranoic acid (287 mg, 0.824 mmol), Meldrum's acid (118.7 mg, 0.824 mmol), and 4-dimethylaminopyridine (100.7 mg, 0.824 mmol) in 5 mL anhydrous DCM was cooled to 0 °C. To this solution was added *N,N'*-dicyclohexylcarbodiimide (168.7 mg, 0.819 mmol). The reaction was stirred at 0 °C for 30 min, then allowed to warm to room temperature and stirred for 6 h. The reaction was then diluted with 25 mL DCM filtered and washed with 3x25 mL 1M HCl (aq), 3x25mL brine. The organic phase was collected then dried over MgSO4, filtered and evaporated under reduced pressure. The resultant oil was then dissolved in 15 mL anhydrous ethanol and refluxed for 4 h. The crude reaction mixture was evaporated under reduced pressure and flash chromatographed [on silica gel; elution with hexane–Et_2O (10:1)] to afford compound **3** (280 mg, yield 81%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.39 (m, 6H), 7.29 (t, *J* = 7.5 Hz, 6H), 7.21 (t, *J* = 7.2 Hz, 3H), 4.15 (q, *J* = 7.1 Hz, 2H), 3.29 (s, 2H), 2.43 (dd, *J* = 9.2, 5.2 Hz, 4H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.0, 166.9, 144.7, 129.7, 128.1, 126.8, 67.0, 61.5, 49.3, 42.1, 25.6, 14.2.

Synthesis of ethyl 2-(5-oxo-3-(2-(tritylthio)ethyl)-4,5-dihydro-1H-pyrazol-1-yl)acetate 9

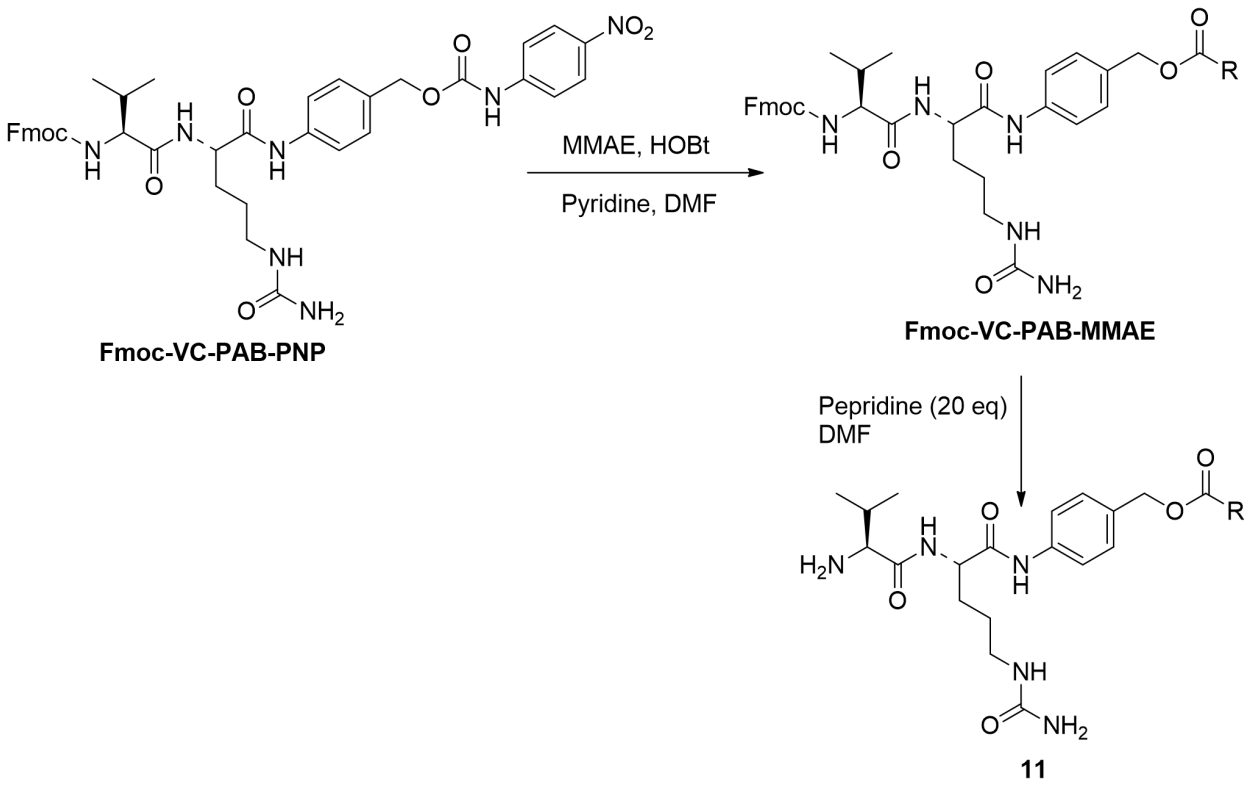
A solution of ethyl 3-oxo-5-(tritylthio)pentanoate **8** (499 mg, 1.24 mmol), ethyl 2-hydrazinylacetate (191 mg, 1.23 mmol), triethylamine (17 mL, 0.124 mmol) in 10 mL

ethanol was warmed to 50 °C and maintained for 2 h. The crude reaction was concentrated in vacuo and flash chromatographed [on silica gel; elution with hexane–Et_2O (1:1)] to afford the desired compound **9** (402 mg, yield 71%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.38 (m, 6H), 7.29 (t, *J* = 7.5 Hz, 6H), 7.22 (dd, *J* = 8.3, 6.1 Hz, 3H), 4.38 (s, 2H), 4.19 (q, *J* = 7.1 Hz, 2H), 3.01 (s, 2H), 2.46 – 2.31 (m, 4H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 168.0, 157.7, 144.6, 129.7, 128.1, 126.9, 67.2, 61.7, 45.6, 39.8, 30.5, 28.7, 14.2.

Synthesis of 2-(5-hydroxy-3-(2-(tritylthio)ethyl)-1H-pyrazol-1-yl)acetic acid 10

To a solution of compound **9** (236 mg, 0.5 mmol) in 10 mL of 2:3:1 THF:MeOH:water was added 25.0 mg of lithium hydroxide. The reaction was stirred for 4 h then partitioned between 40 mL of water and 40 mL of diethyl ether. The aqueous layer was acidified to pH 2 and washed 3x60mL with DCM. The organic phases were combined, dried over $MgSO_4$, filtered, concentrated at reduced pressure and purified by preparative thin layer chromatography [DCM–MeOH (4:1)] (160 mg, yield, 72%) ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 – 7.29 (m, 12H), 7.27 – 7.22 (m, 3H), 5.14 (s, 1H), 4.54 (s, 2H), 2.41 (t, *J* = 7.2 Hz, 2H), 2.31 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.2, 154.1, 148.6, 144.5, 129.1, 128.1, 126.7, 85.9, 66.2, 47.1, 30.7, 27.3.

Conjugation of cytotoxic drug (MMAE) to a cleavable Linker (VC)



The cleavable activated ester linker (**Fmoc-VC-PAB-PNP**)[2] was synthesized by reported method.

Synthesis of Fmoc-VC-PAB-MMAE [3]

The cleavable activated ester linker (**Fmoc-VC-PAB-PNP**) (64 mg, 84 µmol, 1.1 eq.), MMAE (56 mg, 76 µmol, 1.0 eq.), and HOBt (10 mg, 1.0 equivalents) were dissolved in anhydrous DMF (2 mL) and dry pyridine (0.5 mL) and stirred at room temperature. The progress of reaction was monitored with high performance liquid chromatography (HPLC). After completion of reaction, the crude product was purified by semi preparative-HPLC. Lyophilization resulted in the desired product as pale yellow solid **Fmoc-VC-PAB-MMAE** (65 mg, yield 78%). HRMS, calculated for $C_{73}H_{104}N_{10}O_{14}$ [M + H]$^+$ 1345.7811, found 1345.7798. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.88 (d, *J* = 7.4 Hz,

2H), 7.77 – 7.70 (m, 2H), 7.58 (d, *J* = 6.7 Hz, 2H), 7.41 (td, *J* = 7.5, 4.2 Hz, 2H), 7.36 – 7.23 (m, 7H), 7.17 (m, 2H), 5.14 – 4.96 (m, 3H), 4.74 (s, 1H), 4.62 (s, 1H), 4.49 (d, *J* = 5.9 Hz, 1H), 4.45 – 4.38 (m, 2H), 4.35 – 4.19 (m, 5H), 4.06 – 3.94 (m, 3H), 3.92 (d, *J* = 6.9 Hz, 1H), 3.77 (d, *J* = 9.1 Hz, 1H), 3.32 (d, *J* = 9.9 Hz, 1H), 3.24 (d, *J* = 8.5 Hz, 3H), 3.19 (d, *J* = 13.2 Hz, 3H), 3.12 (s, 2H), 3.08-2.92 (m, 5H), 2.86 (d, *J* = 18.2 Hz, 3H), 2.41 (d, *J* = 16.2 Hz, 1H), 2.28 (dd, *J* = 13.3, 10.8 Hz, 2H), 2.30-2.25 (m, 2H), 2.04 – 1.92 (m, 3H), 1.88 – 1.64 (m, 6H), 1.65 – 1.50 (m, 3H), 1.49-144 (m, 2H), 1.41 – 1.26 (m, 3H), 1.09 – 0.96 (m, 6H), 0.91 – 0.71 (m, 20H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.5, 171.3, 170.5, 169.8, 168.9, 159.0, 158.4, 156.2, 144.0, 143.8, 143.7, 143.6, 140.8, 128.3, 127.9, 127.8, 127.2, 126.6, 126.5, 125.4, 120.2, 119.1, 85.5, 81.7, 77.0, 74.8, 65.8, 61.0, 60.4, 60.1, 58.8, 57.3, 57.2, 53.1, 49.7, 49.1, 46.8, 46.4, 43.8, 43.3, 38.6, 30.4, 30.0, 29.5, 26.8, 25.4, 24.5, 23.2, 19.3, 19.0, 18.9, 18.3, 15.5, 15.4, 15.0, 10.4, 10.4.

Synthesis of NH_2-Val-Cit-PAB-MMAE 11

To a 5 mL round bottom flask containing a stir bar was added **Fmoc-VC-PAB-MMAE** (45 mg, 33.4 μmol, 1 eq.). Pepridine (66 μL, 668μmol, 20 eq.) in DMF (0.5 mL) was added. The solution was stirred at room temperature for 20 min. After completion of reaction, the crude product was purified by reverse-phase preparative-HPLC. Lyophilization resulted in the desired product as white solid **11** (26.5 mg, yield 70.7%). HRMS, calculated for $C_{58}H_{94}N_{10}O_{12}$ $[M + H]^+$ 1123.7031, found 1123.7079. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.19 (s, 1H), 8.68 (d, *J* = 6.9 Hz, 1H), 8.21– 8.12 (m, 1H), 7.92 (d, *J* = 8.6 Hz, 1H), 7.72 (d, *J* = 8.6 Hz, 1H), 7.56–7.54 (m, 2H), 7.33–7.22 (m, 6H), 7.19 – 7.07 (m, 1H), 5.10 – 4.98 (m, 2H), 4.71 (s, 1H), 4.60 (s, 1H), 4.49 – 4.40 (m, 2H), 4.41 (d, *J* = 6.6 Hz, 5H), 4.20 (q, *J* = 10.6 Hz, 2H), 3.73 (d, *J* = 9.7 Hz, 1H), 3.64 (s, 1H), 3.56-3.49 (m, 2H), 3.29 (d, *J* = 10.2 Hz, 1H), 3.26 – 3.17 (m, 5H), 3.15 (s, 2H), 3.09 (s, 2H), 3.07 – 2.92 (m, 4H), 2.84 (d, *J* = 18.8 Hz, 3H), 2.37 (d, *J* = 15.6 Hz, 1H), 2.32 – 2.17 (m, 1H), 2.17 – 2.01 (m, 3H), 1.98-190 (m, 1H), 1.85 – 1.57 (m, 5H), 1.58 – 1.34 (m, 5H), 1.28 (s, 1H), 1.02 (dd, *J* = 6.3, 4.1 Hz, 3H), 0.99 (d, *J* = 6.6 Hz, 3H), 0.93 (d, *J* = 6.6 Hz, 6H), 0.86 (d, *J* = 6.4 Hz, 2H), 0.82 (dd, *J* = 9.5, 6.7 Hz, 6H), 0.79 – 0.69 (m, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.0, 170.5, 170.2, 169.5, 168.2, 159.8, 156.8,

143.8, 143.7, 138.7, 132.4, 128.6, 128.2, 127.2, 126.9, 119.5, 85.7, 82.1, 78.1, 77.3, 75.3, 66.5, 64.0, 61.3, 60.7, 59.1, 58.7, 57.6, 57.6, 55.7, 54.5, 53.6, 50.0, 49.5, 47.73, 44.15, 43.64, 37.47, 35.52, 32.24, 32.00, 30.41, 30.24, 30.03, 29.47, 26.93, 25.67, 24.68, 23.5, 19.2, 19.0, 18.9, 18.7, 18.6, 18.0, 16.4, 16.0, 15.9, 15.7, 15.2, 10.7.

Synthesis of benzonitrile substituted Val-Cit-PAB-MMAE 12

To a dried 5 mL round bottom flask containing a stir bar was added **11** (25.2 mg, 0.0225 mmol), **5** (5.6 mg, 0.0225 mmol), DIEA (7.83 µL, 0.045 mmol) and 1 mL anhydrous DMF. To this solution was added HATU (8.5 mg, 0.0225 mmol) and stirred for 1 h. After confirmation of completion of reaction by analytical RP HPLC, the reaction mixture was then purified by reverse-phase preparative-HPLC to get the desired product as white solid **12** after lyophilization (24 mg, yield 80%). HRMS, calculated for $C_{71}H_{109}N_{12}O_{14}$ $[M+H]^+$ 1353.8184, found 1353.8181, $[M+2H]^{2+}$ calculated 677.4054, found 677.4121. ^{1}H NMR (500 MHz, CD_3OD) δ 7.58-7.57 (m, 2H), 7.39 – 7.29 (m, 6H), 7.22-7.19 (m, 1H), 7.06-6.92 (m, 3H), 5.19-5.13 (m, 2H), 5.06 (dd, *J* =13.0, 3.4 Hz, 1H), 4.68-4.52 (m, 4H), 4.24-4.18 (m, 3H), 4.07 (br s, 1H), 3.89 (t, *J* = 6.3 Hz, 2H), 3.86 (dd, *J* = 9.1, 2.0 Hz, 1H), 3.74-3.66 (m, 2H), 3.56-3.52 (m, 1), 3.43-3.37 (m, 2H), 3.35-3.34 (m, 4H), 3.29-3.26 (m, 3H), 3.22-3.15 (m, 2H), 3.13-3.06 (m, 2H), 2.99-2.88 (m, 3H), 2.53-2.46 (m, 2H), 2.32-2.29 (m, 2H), 2.25-2.19 (m, 2H), 2.14-2.11 (m, 1H), 2.10-2.03 (m, 2H), 1.99-1.83 (m, 4H), 1.80-1.71 (m, 4H), 1.69-1.66 (m, 2H), 1.62-1.54 (m, 3H), 1.51-1.45 (m, 2H), 143-140 (m, 2H), 1.31-1.23 (m, 1H), 1.77 (dd, *J* = 6.6, 4.4 Hz, 2H), 1.13 (dd, *J* = 10.2, 6.8 Hz, 2H), 1.08-1.04 (m, 1H), 0.99 (d, *J* = 6.5 Hz, 2H), 0.97-0.91 (m, 8H) and 0.90-0.68 (m, 11H); ^{13}C NMR (125 MHz, CD_3OD) δ 176.4, 175.7, 175.4, 175.1, 174.0, 172.2, 171.7, 162.3, 158.8, 144.0, 143.8, 139.5, 133.9, 130.1, 129.6, 129.5, 129.2, 128.6, 128.4, 128.0, 127.9, 124.1, 121.1, 120.1, 118.1, 117.3, 115.6, 86.7, 83.5, 77.5, 77.3, 69.8, 68.3, 62.0, 61.5, 60.8, 60.6, 60.5, 58.6, 58.4, 56.0, 54.9, 51.4, 50.9, 48.1, 45.9, 45.5, 40.4, 36.6, 31.7, 30.4, 30.0, 27.8, 27.0, 26.7, 26.7, 26.6, 25.8, 25.7, 24.5, 19.8, 19.7, 18.9, 15.9, 15.8, 15.0 and 11.0.

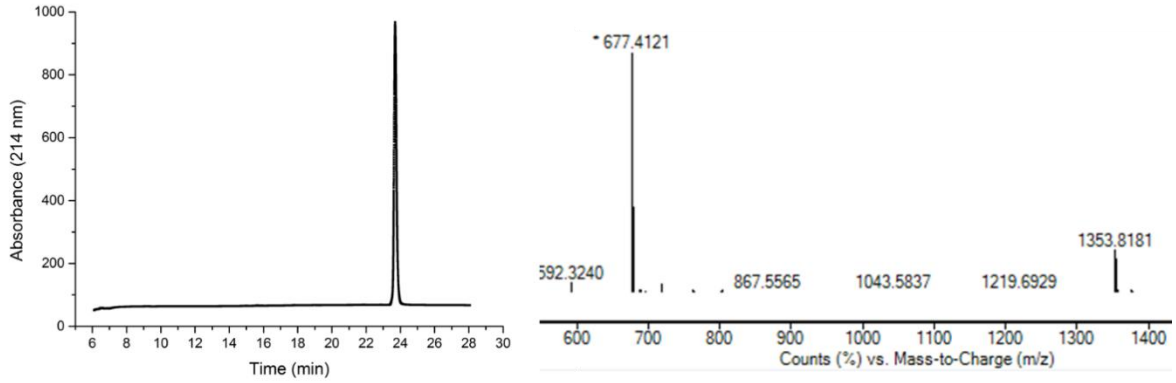


Figure S1: HPLC chromatogram and LC-MS spectrum of Compound **12**

Synthesis of 2-amino benzamidoxime substituted Val-Cit-PAB-MMAE 13

To solution of **12** (13.2 mg, 0.01 mmol) in 1ml of methanol was added the mixture of 5 eq. of hydroxylamine hydrochloride and sodium bicarbonate (1:1) in 0.5 ml water. Then the reaction mixture was kept under stirring at 65 ^{o}C for 24 h. The progress of reaction was monitored by analytical RP HPLC. After completion of reaction, the reaction mixture was then purified by reverse-phase preparative-HPLC to get the desired product as white solid **13** after lyophilization (13 mg, yield 94%). HRMS, calculated for $C_{71}H_{112}N_{13}O_{15}$ $[M+H]^+$ 1386.8401, found 1386.8385, $[M+2H]^{2+}$ calculated 693.9262, found 693.9264. ^{1}H NMR (500 MHz, CD_3OD) δ 7.58-7.57 (m, 2H), 7.39-7.28 (m, 6H), 7.22-7.19 (m, 1H), 7.02-6.90 (m, 3H), 5.19-5.05 (m, 2H), 4.68-4.49 (m, 4H), 4.26-4.18 (m, 4H), 4.06 (t, *J* = 6.2 Hz, 1H), 3.93 (t, *J* = 6.2 Hz, 2H), 3.86 (dd, *J* = 9.1, 1.9 Hz, 1H), 3.72-3.66 (m,1H), 3.57-3.52 (m, 1H), 3.43-3.37 (m, 1H), 3.35-3.34 (m, 4H), 3.28 (d, J = 14.1 Hz, 3H), 3.23-3.15 (m, 2H), 3.10-3.06 (m, 2H), 2.96-2.92 (m, 3H), 2.54-2.46 (m, 2H), 2.34-2.30 (m, 2H), 2.24-2.19 (m, 2H), 2.15-2.03 (m, 2H), 2.00-1.86 (m, 4H), 1.84-1.66 (m, 7H), 1.62-1.42 (m, 5H), 1.40-1.37 (m, 2H), 1.33-1.29 (m, 1H), 1.18 (t, *J* = 6.1 Hz, 3H), 1.13 (dd, *J* = 11.5, 6.8 Hz, 3H), 1.00-0.96 (m, 10H), 0.94-0.92 (m, 3H), 0.90-0.83 (m, 9H) and 0.80-0.76 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 176.4, 175.7, 175.4, 175.1, 174.0, 172.2, 171.7, 162.3, 144.1, 143.8, 139.5, 134.0, 129.6, 129.5, 129.2, 128.6, 128.4, 128.0, 127.9, 121.7, 121.5, 121.1, 116.1, 115.4, 83.5, 77.5, 77.3, 69.7, 68.3, 66.0, 62.0, 61.5, 60.8, 60.5, 58.6, 58.4, 56.1, 56.0, 54.9, 51.4, 50.8, 49.5, 48.1,

45.9, 45.5, 36.6, 31.9, 31.7, 30.5, 30.3, 30.0, 29.9, 27.8, 27.0, 26.7, 26.7, 26.6, 25.8, 25.6, 24.5, 19.8, 19.8, 18.9, 16.0, 15.8, 15.0, 10.9.

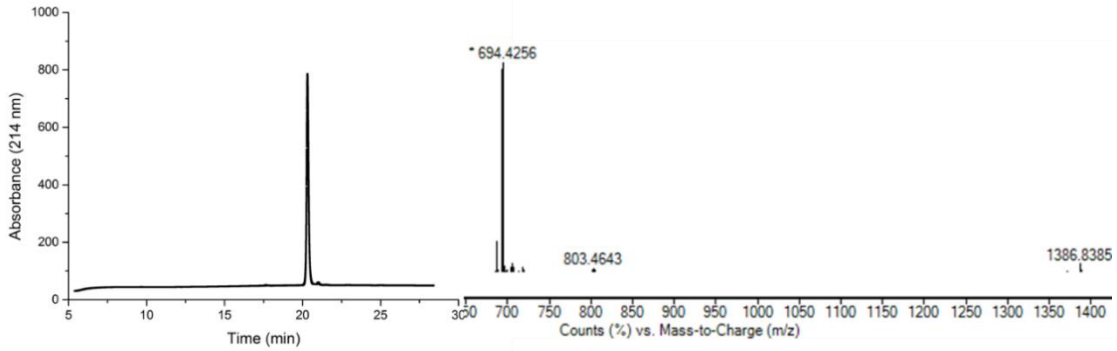


Figure S2: HPLC chromatogram and LC-MS spectrum of Compound **13.**

Synthesis of trt-*S*-thioPz- NH-Val-Cit-PAB-MMAE 14

To a dried 5mL round bottom flask containing a stir bar was added **11** (25.2 mg, 0.0225 mmol), **10** (10 mg, 0.0225 mmol), DIEA (7.83 µL, 0.045 mmol) and 1mL anhydrous DMF. To this solution was added HATU (8.5 mg, 0.0225 mmol) and stirred for 1 h. After confirmation of completion of reaction by analytical RP HPLC, the reaction mixture was then purified by reverse-phase preparative-HPLC to get the desired product as white solid **14** after lyophilization (20 mg, yield 57.6%). HRMS, calculated for $C_{84}H_{116}N_{12}O_{14}S$ $[M\text{ -Trt}]^+$ 1307.7437, found 1307.7329.

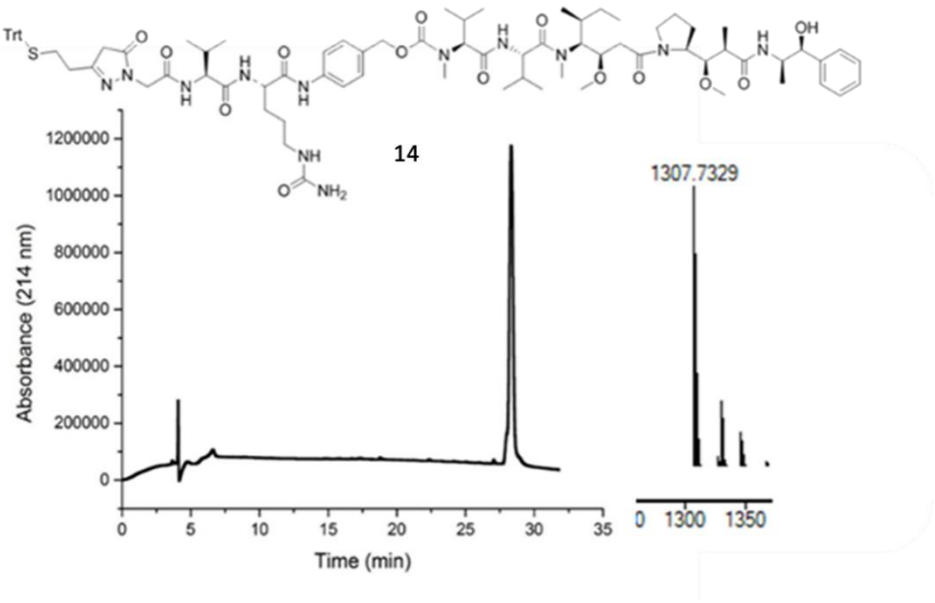


Figure S3: HPLC chromatogram and LC-MS spectrum of Compound **14**

Synthesis of thioPz- NH-Val-Cit-PAB-MMAE 15

To a 1 mL glass vial containing a stir bar was added **14** (15.4 mg, 0.01 mmol), DCM (130 µL) and cooled to 0 °C. To this solution was added water (10 µL), triisopropylsilane (10 µL), and trifluoroacetic acid (80 µL). The reaction was warmed to room temperature and stirred for 30 min. The crude product was purified by reverse-phase preparative-HPLC. Lyophilization resulted in compound **15** (8 mg, yield 61%). HRMS, calculated for $C_{65}H_{102}N_{12}O_{14}S$ $[M + H]^+$ 1307.7437, found 1307.7329.

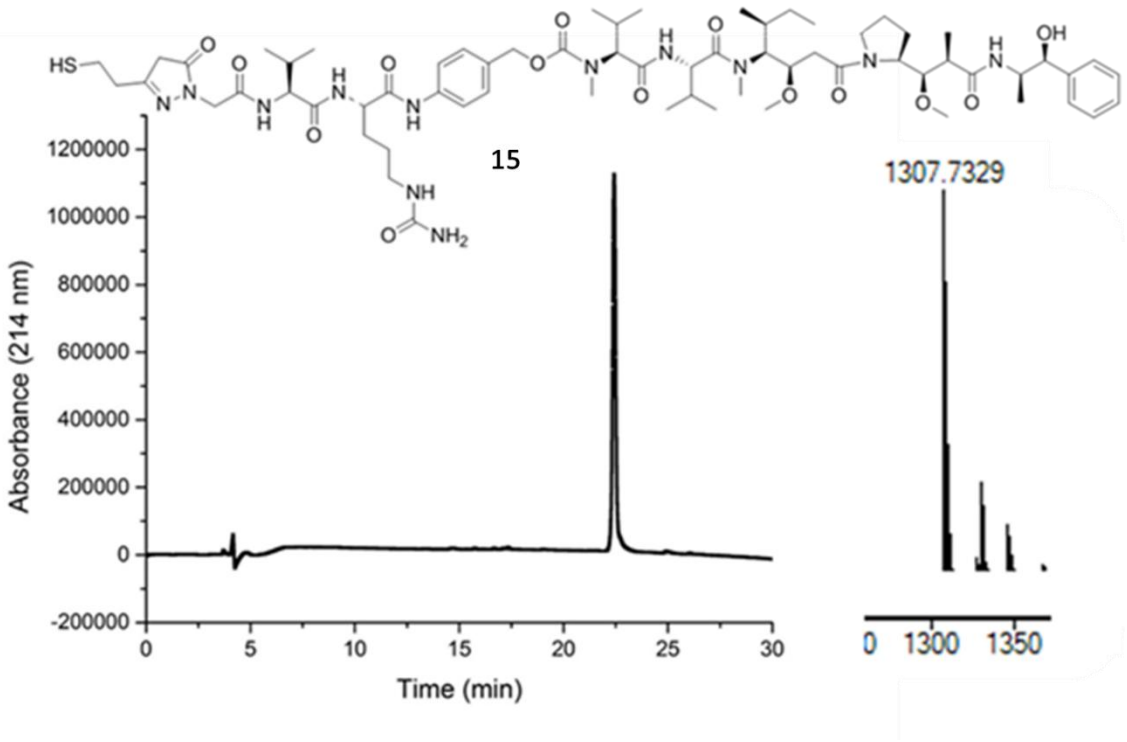


Figure S4: HPLC chromatogram and LC-MS spectrum of Compound **15**

Synthesis of glycoconjugate 17

A general method for milligram scale synthesis is reported here only.

To the solution of CHO-SGP **16** (2.7 mg, 1.0 µmol, 1eq.) in 200 µL phosphate buffer (pH = 6.0, 100 mM), compound **13** (5.5 mg, 4.0 µmol, 4eq.) in acetonitrile-water 100 µL (1:1) was added and stirred at room temperature for 5 h. The progress of reaction was monitored by analytical RP HPLC. After completion of reaction, the reaction mixture was purified by reverse-phase preparative-HPLC and lyophilized to get the desired product as light green solid **17.** HRMS, calculated for $C_{250}H_{353}N_{41}O_{94}$ $[M + 4H]^{4+}$ 1369.9347, found 1370.1658, $[M + 5H]^{5}+$ 1096.14778, found 1096.3357.

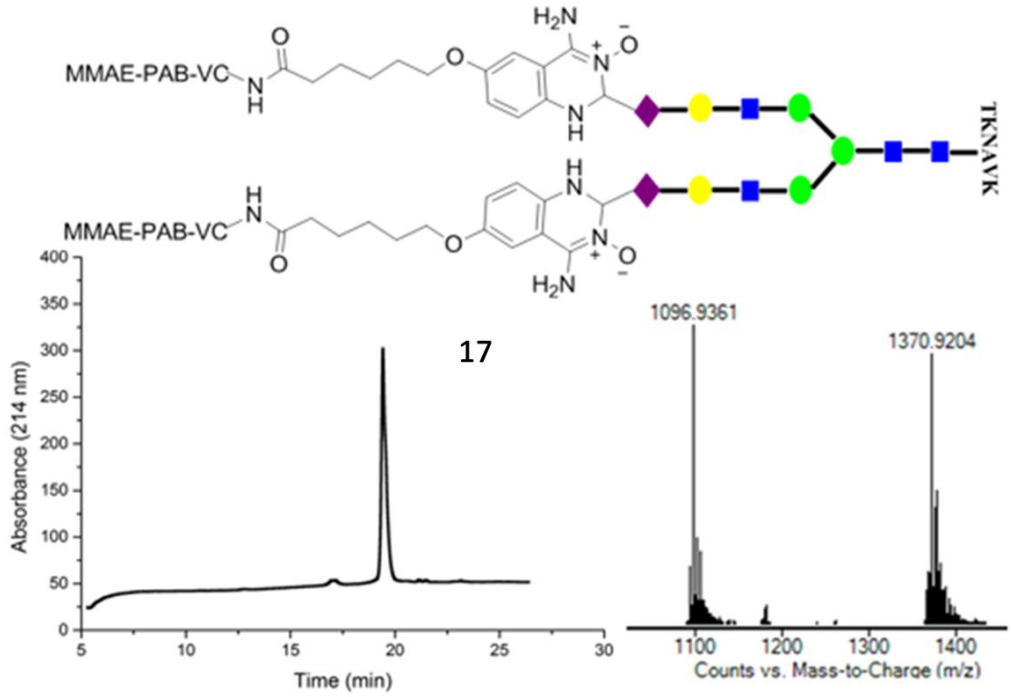


Figure S5: HPLC chromatogram and LC-MS spectrum of Compound **17**

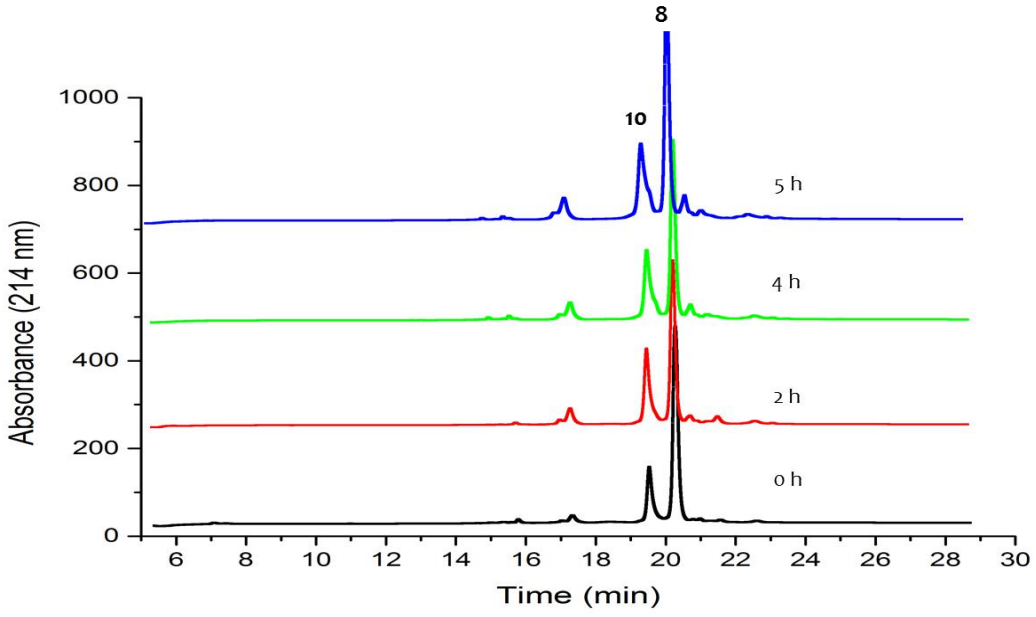
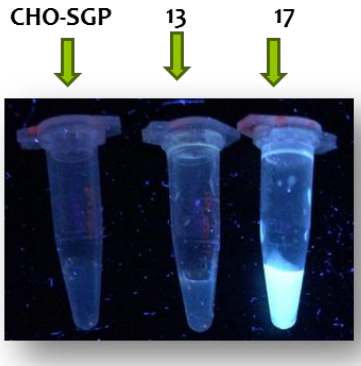
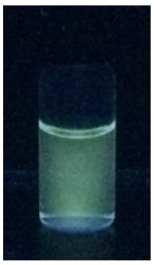


Figure S6: Reaction progress of conjugation of drug-linker **13** with CHO-SGP **16** monitored by HPLC



Pictogram of CHO-SGP , drug-linker **13** and crude glycoconjugate **17** upon irradiation with a 365 nm UV-lamp.



Pictogram of solution of **17** (after purification) upon irradiation with a 365 nm UV-lamp.

Figure S7: Pictograms of reactants, reaction mixture and pure product upon irradiation with a 365 nm UV-lamp

Synthesis of SGP-thioPz- NH-Val-Cit-PAB-MMAE 18

To the solution of CHO-SGP **16** (2.3 mg, 0.839 µmol, 1eq.) in PBS solution (0.8 ml, pH = 7.4, 10 mM), compound **15** (4.5 mg, 3.445 µmol, 4eq.) was added. Acetonitrile was added until to get a clear solution. The reaction mixture was stirred at room temperature for 24 h. In the analytical HPLC chromatogram, two new peaks were identified with exactly same molar mass in the complete accordance with the target conjugate **18**. The reaction mixture was then subjected to semi-preparative HPLC purification and lyophilization to give the compound **18** as white solid in the pure form as isomeric mixture (3.2 mg, 71%). HRMS, calculated for $C_{238}H_{377}N_{39}O_{92}S_2$ $[M + 4H]^{4+}$ 1330.3865, found 1330.3746; $[M + 3H]^{3+}$ 1773.5154, found 1773.5008

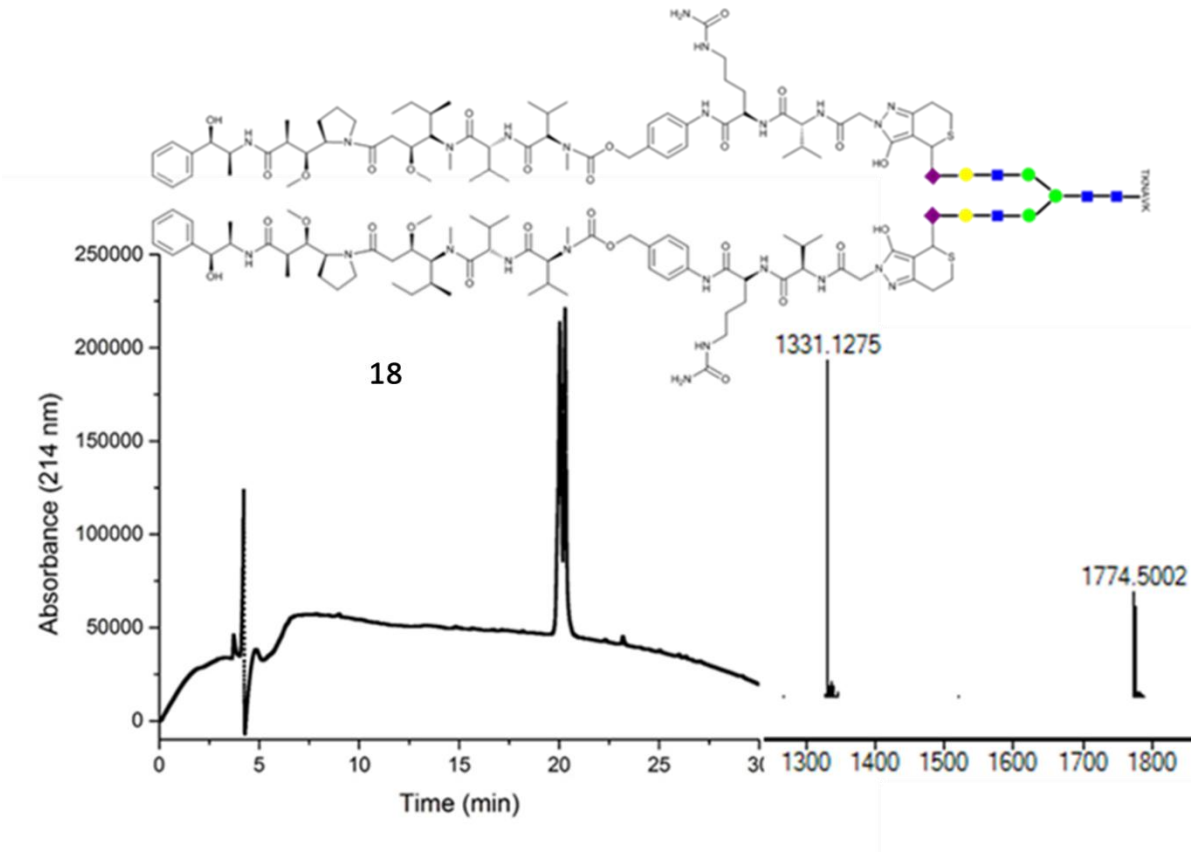


Figure S8: HPLC chromatogram and LC-MS spectrum of Compound **18**

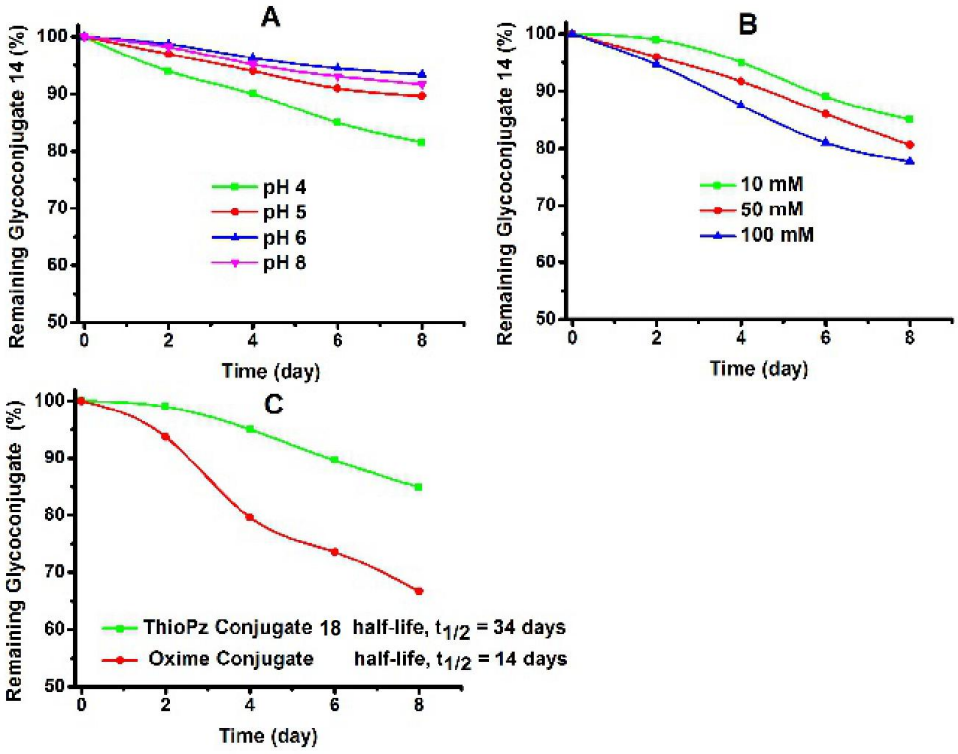


Figure S9: Stability assay of drug-glycan conjugates via ThioPz ligation (**18**) and Oxime ligation. Panel A: the hydrolysis rate of ThioPz-glycoconjugate **18** at various pH (4, 5, 6, and 8) under room temperature; Panel B: the hydrolysis rate of glycoconjugate **18** under different buffer strength (PB, pH 7.4, 10 mM, 50 mM, and 100 mM) at 37 °C; Panel C: Comparison of the hydrolysis rate of thioPz-linked glycoconjugate **18** and oxime-linked glycoconjugate at 37 °C in PB solution (pH = 7.4, 10 mM).

Preparation of Herceptin containing aldehyde group 22

Firstly, wild-type Herceptin was deglycosylated **19** and transglycosylated with sialo-complex-type oxazoline (SCT-ox) **20** to give SCT-Herceptin **21** as previously reported.[4] Then, SCT-Herceptin **21** (1.0 mg·mL-1) in 50 mM pH 6.0 phosphate buffer was oxidized by sodium periodate ($NaIO_4$, 2.0 mM) in an ice bath for 20 mins with following de-salting, buffer-exchanging and concentration with reaction buffer (50 mM sodium citrate, 50 mM NaCl, pH 5.5) in a 30 KD centrifuge tube. The obtained aldehyde group tagged Herceptin **22** (CHO-SCT-Her, 10 mg) and SCT-Herceptin (10 mg) was incubated with

50 mM DTT for 10 mins and was subjected to LC-MS for mass spectra analysis separately.

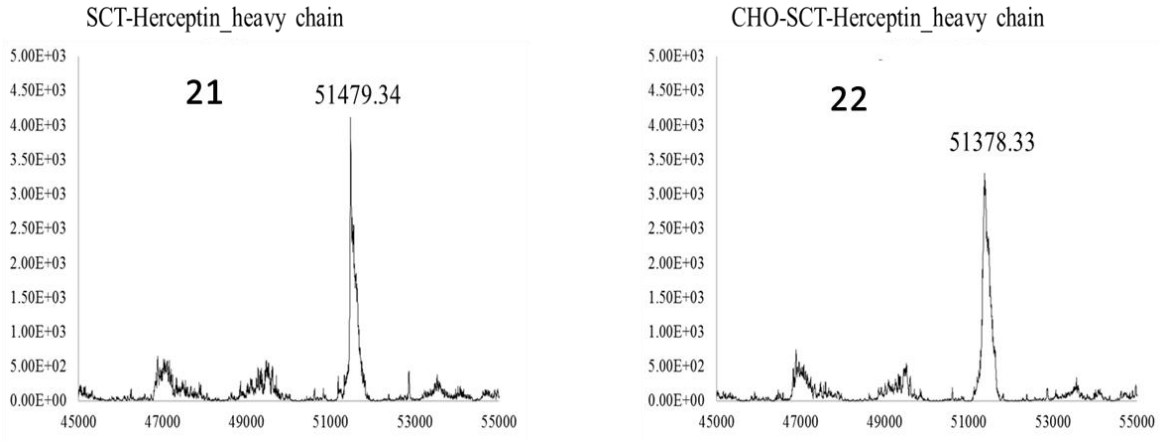


Figure S10: LC-MS spectra of **21** and **22**

Synthesis of gsADC 23a

CHO-SCT-Herceptin **22** (1.0 mg) was incubated with ABAO-VC-PAB-MMAE **13** (0.7 mg, 0.5 μmol, 18.7 equiv. per aldehyde) in 10% DMF containing phosphate buffer (500 μL, 50 mM, pH 5.5) at 37 ^{o}C for 24 hours and at 4 ^{o}C for another 24 hours to give gsADC **23a**. Then the reaction mixture was subjected to a Milipore ultra centrifuge tube (10 KDa cutting) and repeated 5 times buffer-exchange with 10% DMF containing phosphate buffer (300 μL, 50 mM, pH 7.0) and 5 times buffer-exchange with another phosphate buffer (300 μL, 50 mM, pH 7.0). The concentration was determined with Nanodrop (Thermo Fisher).

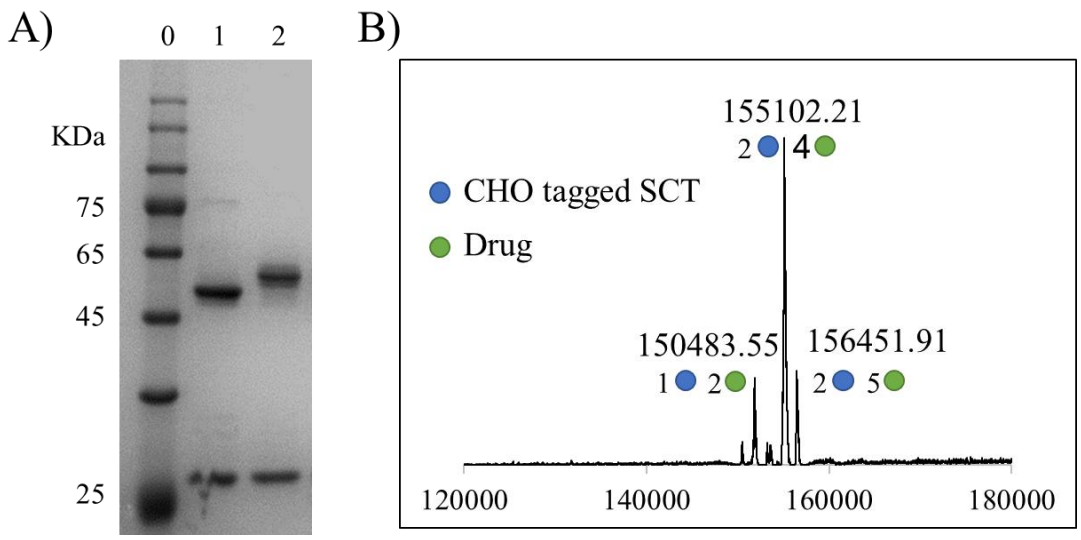


Figure S11: SDS-PAGE and LCMS profiles of glycol-specific ADC armed with ABAO-VC-PAB-MMAE **23a**. A) SDS-PAGE of gsADC **23a**. Lane0: Protein marker; Lane1: CHO-SCT-Herceptin; Lane2: CHO-SCT-Herceptin after incubation with ABAO-MMAE. B) Mass spectra of gsADC **23a**. Calculated for 4 drugs (2 glycan) is 155103.33, found 155102.21; Calculated for 5 drugs (2 glycan) is 156489.16, found 156451.91. Calculated for 2 drugs (1 glycan) is 150454.67, found 150483.55.

Synthesis of gsADC 23b

CHO-Herceptin **22** (10 mg·mL^{-1}) in sodium citrate buffer (pH 5.5, 50 mM) and NaCl (50 mM) was incubated with compound **15** (20 eq, 1.3 mM, 5 eq. per aldehyde), 10% (v/v) DMF and 0.1% (v/v) Triton X-100 together with EDTA (1.0 mM). The reaction mixture was incubated at 37 °C for 20 hours and samples were taken out for SDS-PAGE and LC-MS analysis at certain time point.

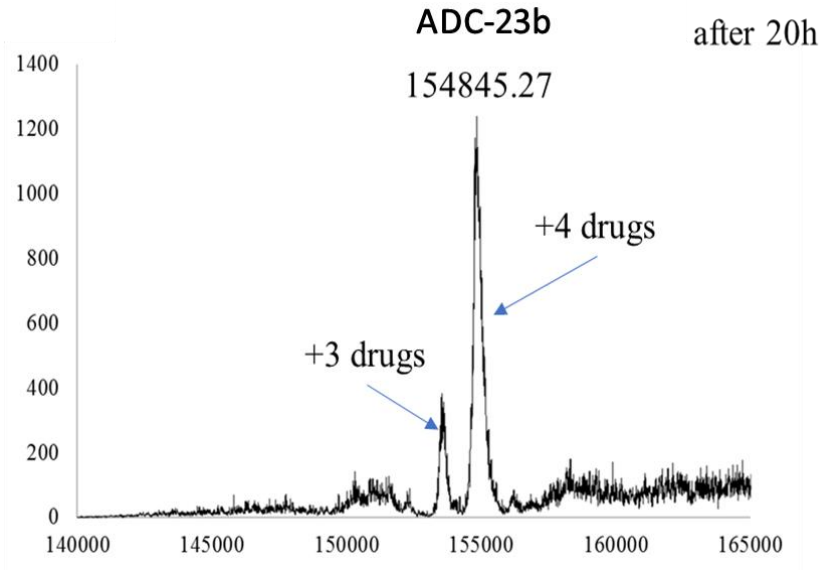


Figure S12: LC-MS spectrum of **ADC-23b.**

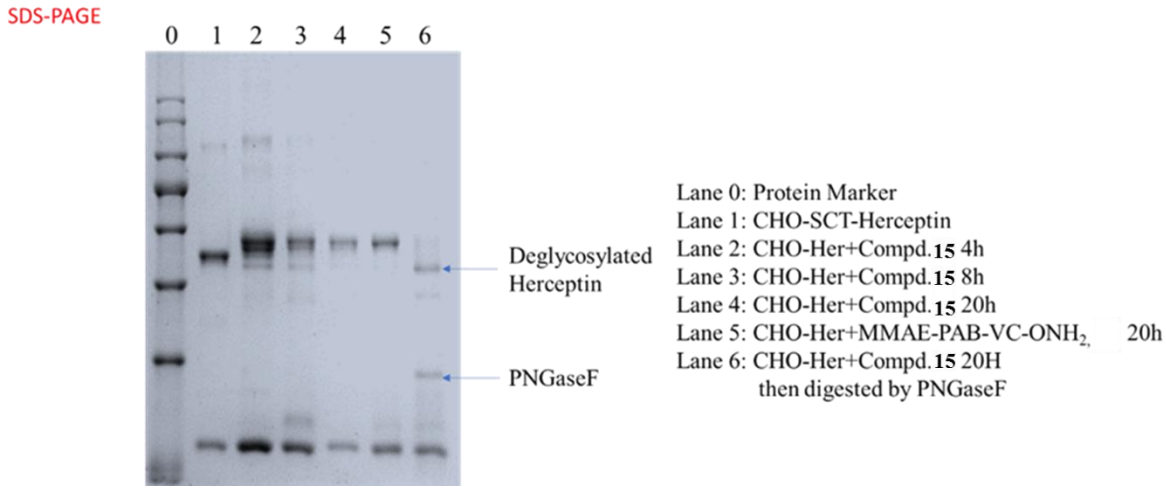
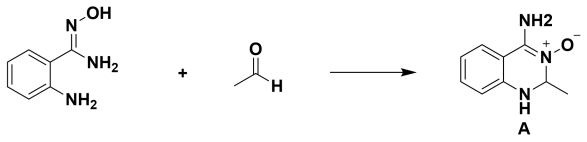


Figure S13: SDS-PAGE of **ADC-23b**

Synthesis of compound A as the fluorescent standard



^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.62 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.52 – 7.45 (m, 1H), 6.94 (ddd, *J* = 8.2, 7.2, 1.1 Hz, 1H), 6.87 (dd, *J* = 8.3, 1.1 Hz, 1H), 5.17 (q, *J* = 5.9 Hz, 1H), 1.50 (d, *J* = 5.9 Hz, 3H).HRMS, calculated for $C_9H_{11}N_3O$ $[M+H]^+$ 178.0980, found 178.0985.

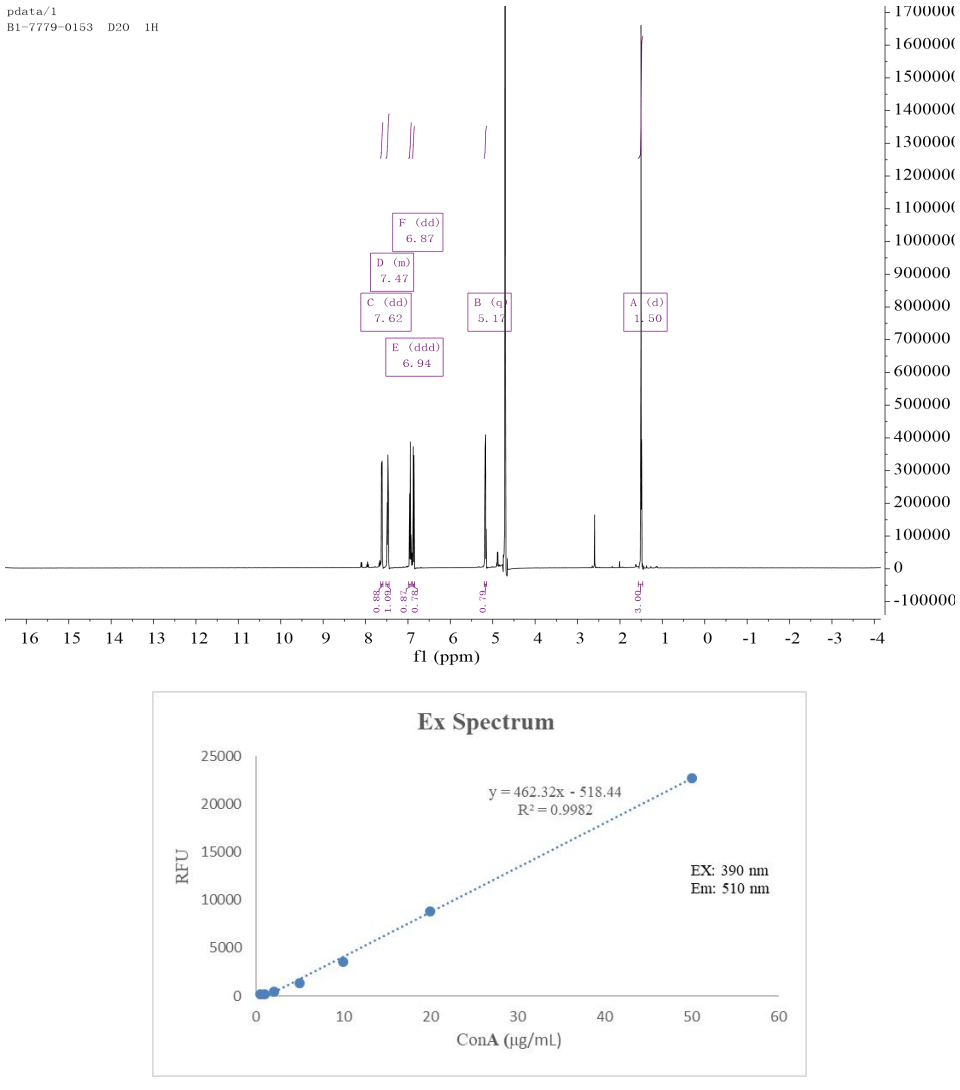


Figure S14: Standard curve of compound A in fluorescent detection

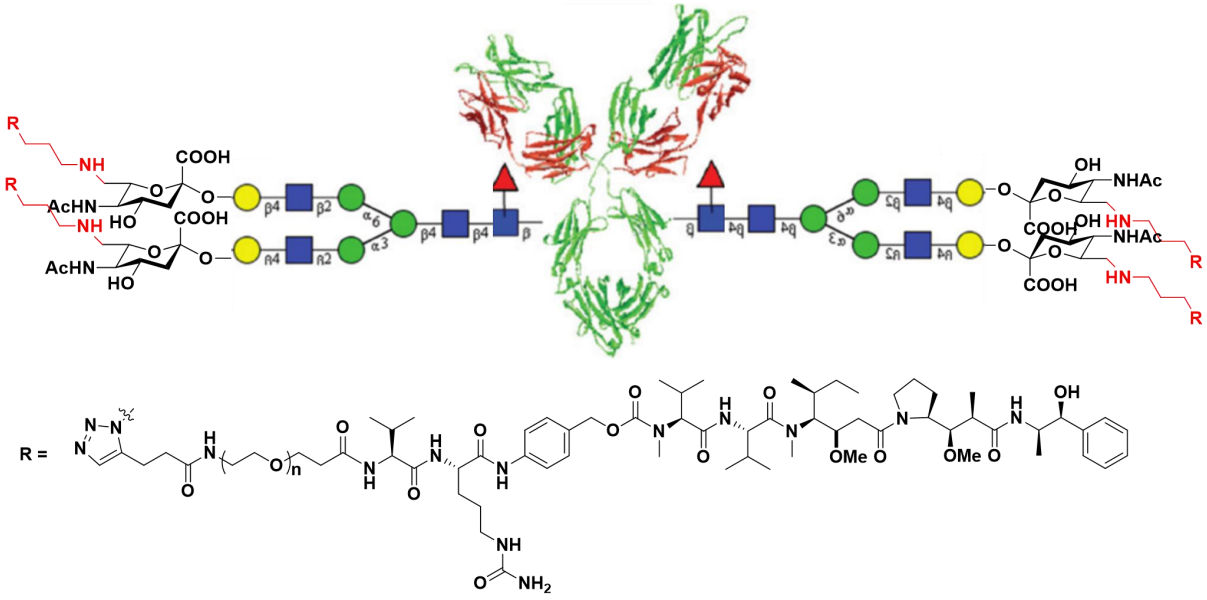


Figure S15: Structure of ADC-1 prepared following the literature (*Organic & Biomolecular Chemistry* **2016**, *14*, 9501-9518**)**

Table S1. Thermostability of gsADCs.

gsADCs	Tm (°C)
ADC-23a	62.5
ADC-23b	62.8
ADC-1	59.5

*The melting point (Tm) values were measured by DSF.

Cell Cytotoxicity against cancer cells

Two different gsADCs, **14** and **15**, were valuated against SK-Br-3 (Her2+), NCI-N87 (Her2+), and MDA-MB-231 (Her2-) cell lines respectively. Those cells were separately plated and cultured in RPMI 1640 (Hyclone, 90μL/well) supplemented with 10% fetal bovine serum (FBS) in 96-well plates (Corning, 6000 cells/well) overnight at 37 oC and 5% CO_2. Two gsADCs were diluted into three-fold dilution series, ranging from 10

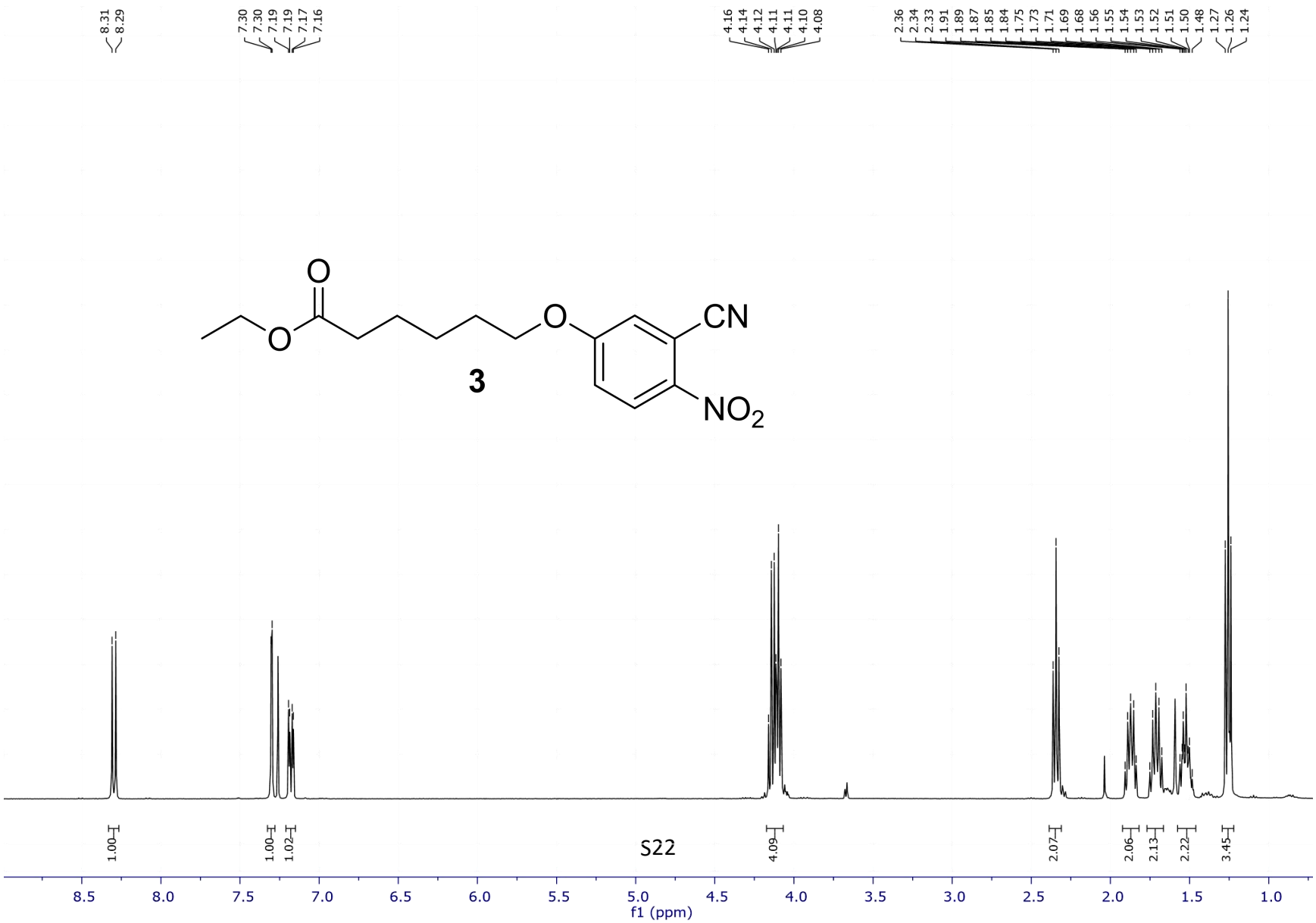
μg/mL to 1.5 ng/mL. 10 μL of the diluted samples were added to triple wells and incubated at 37 °C and 5% CO_2 for three days before the addition of MTT solution (10 μL, 5 mg/mL in Hyclone PBS). To each well was added 90 μL of 10% SDS solution after 4 hours incubation with MTT solution at 37 °C and 5% CO_2. Optical density (OD) of each well was measured at 570 nm using an Epoch (BioTek) after overnight incubation (or at least 7 hours incubation) at 37 °C and 5% CO_2, and EC_{50} values and cell viability curve were calculated and plotted by GraphPad software.

[1] R. Kudirka, Robyn M. Barfield, J. McFarland, Aaron E. Albers, Gregory W. de Hart, Penelope M. Drake, Patrick G. Holder, S. Banas, Lesley C. Jones, Albert W. Garofalo, D. Rabuka, *Chemistry & Biology* **2015**, *22*, 293-298.

[2] G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch, P. A. Trail, *Bioconjugate Chemistry* **2002**, *13*, 855-869.

[3] J. A. Francisco, C. G. Cerveny, D. L. Meyer, B. J. Mixan, K. Klussman, D. F. Chace, S. X. Rejniak, K. A. Gordon, R. DeBlanc, B. E. Toki, C.-L. Law, S. O. Doronina, C. B. Siegall, P. D. Senter, A. F. Wahl, *Blood* **2003**, *102*, 1458-1465.

[4] F. Tang, Y. Yang, Y. Tang, S. Tang, L. Yang, B. Sun, B. Jiang, J. Dong, H. Liu, M. Huang, M.-Y. Geng, W. Huang, *Organic & Biomolecular Chemistry* **2016**, *14*, 9501-9518.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4.10
4.08
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2.34
2.33
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1.89
1.87
1.85
1.84
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1.69
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1.51
1.50
1.48
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1.26
1.24
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O
O
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1.00
1.02
4.09
2.07
2.06
2.13
2.22
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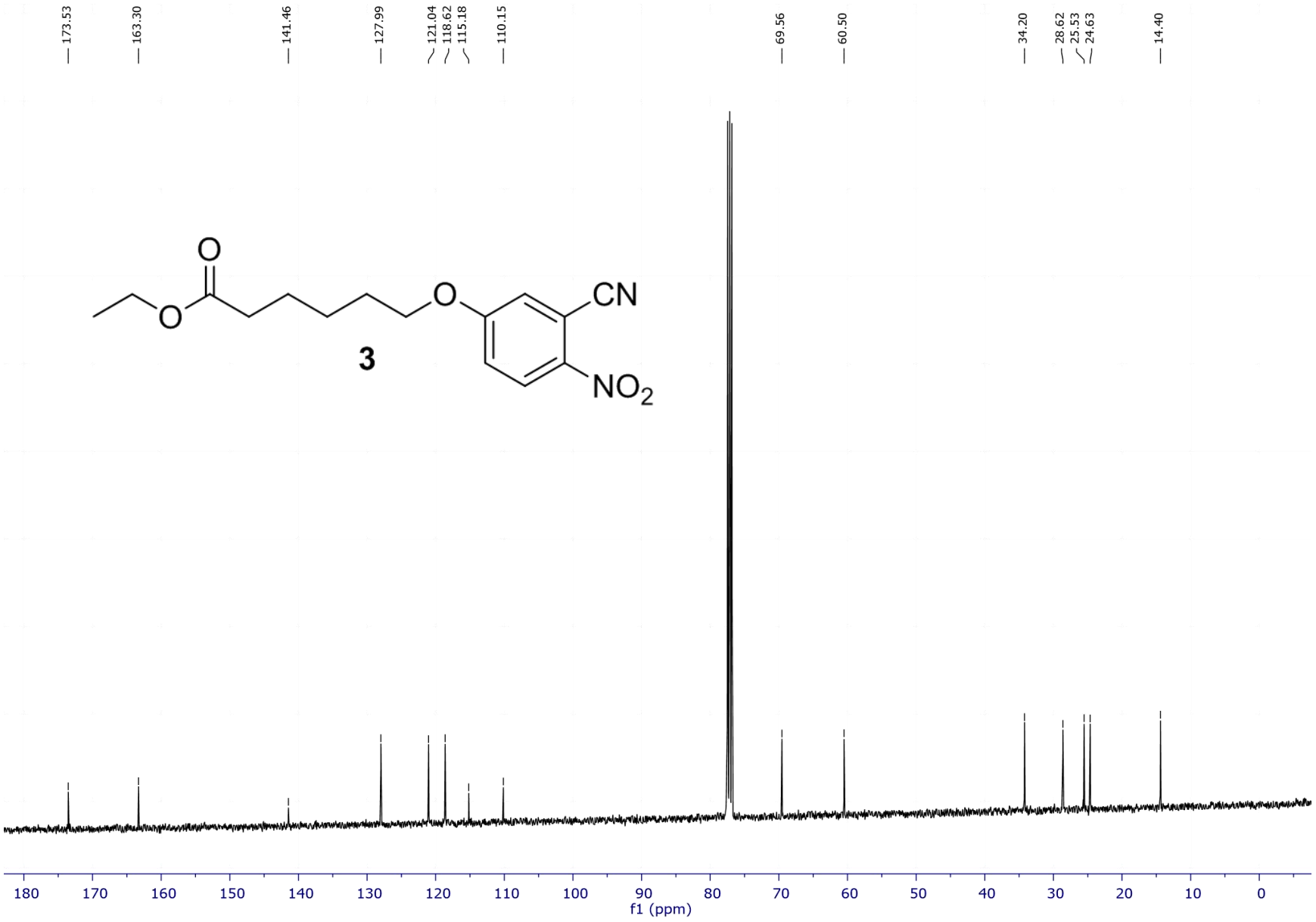
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O
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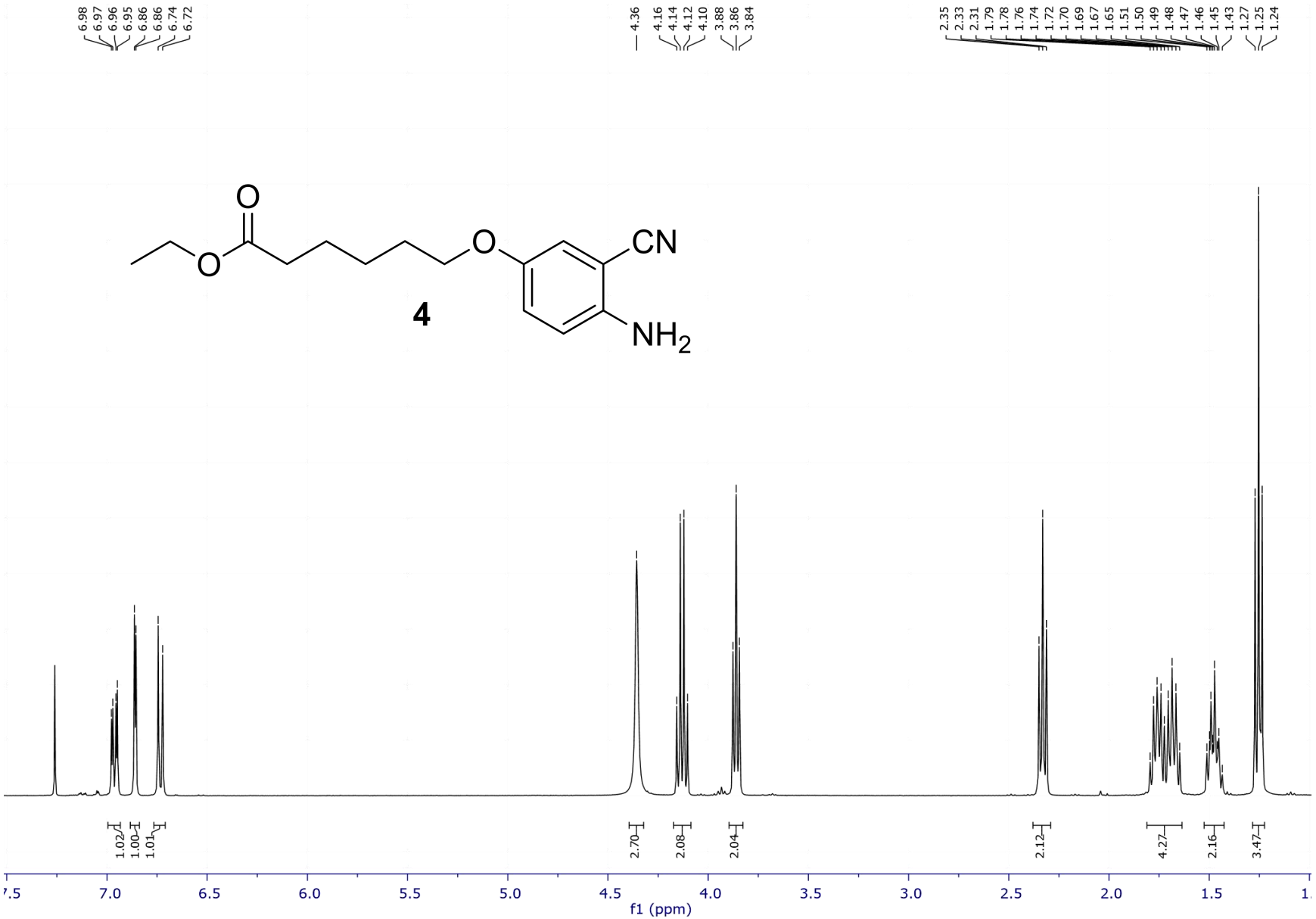
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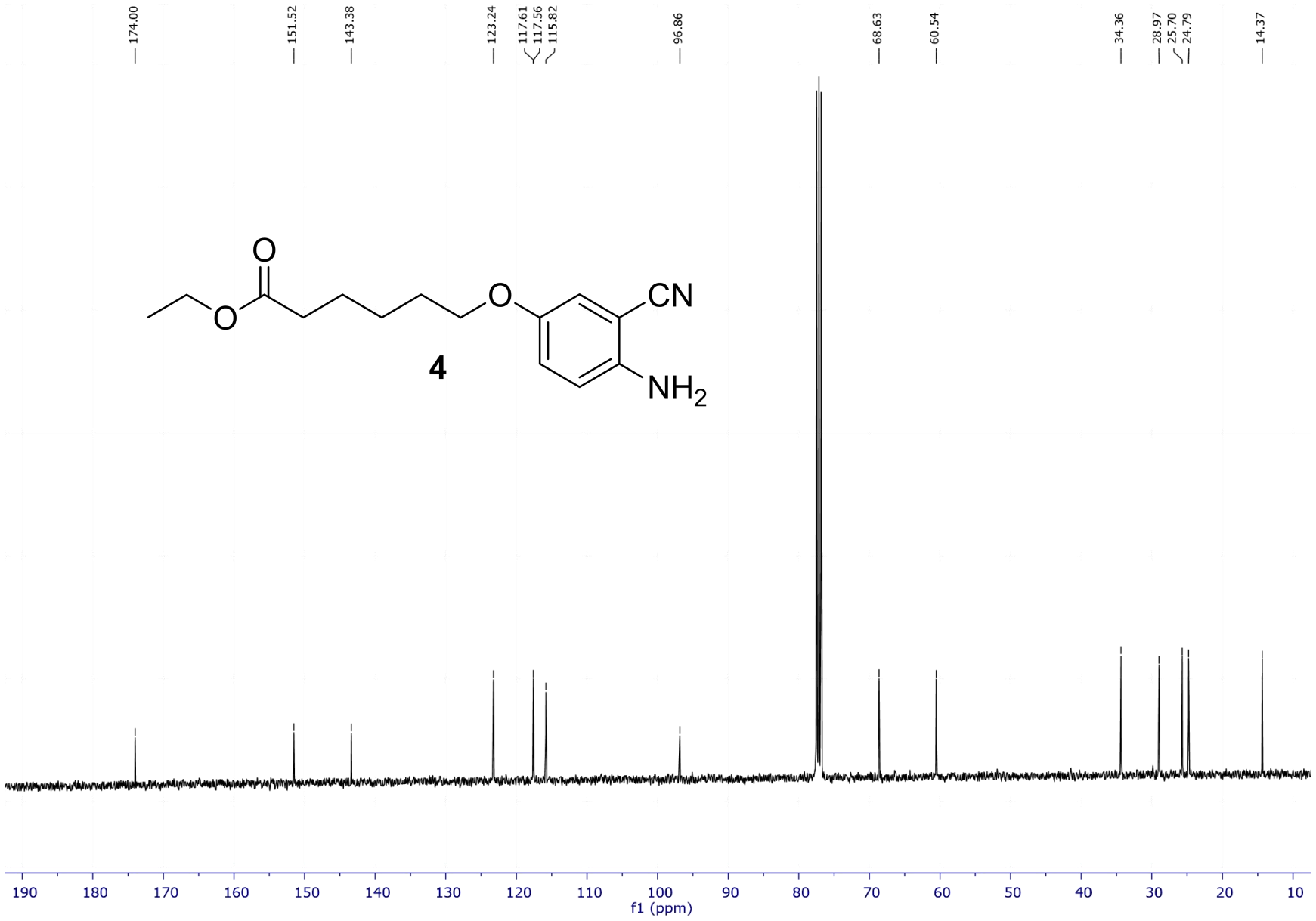
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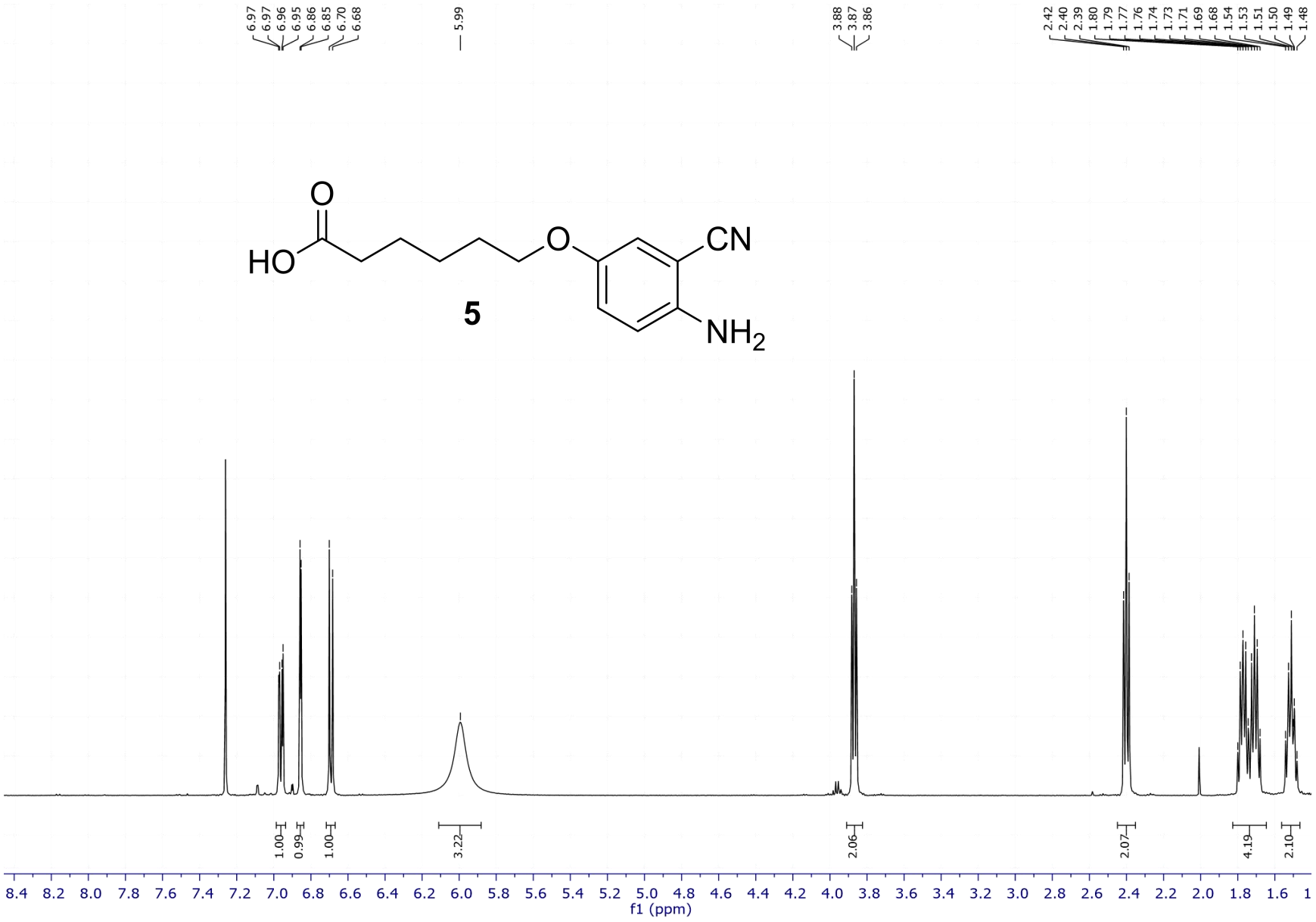
f1 (ppm)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CN
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2.07
4.19
2.10
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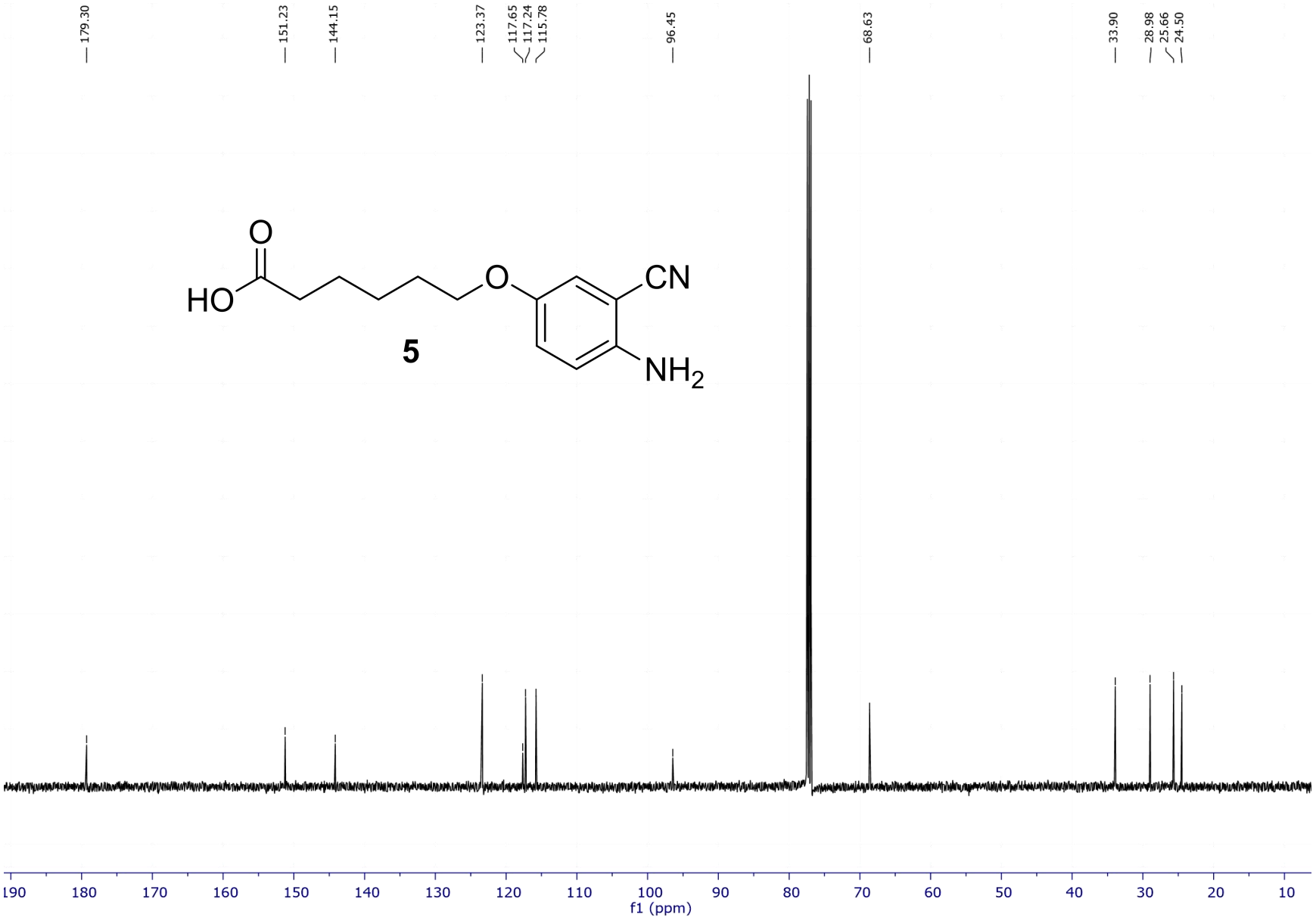
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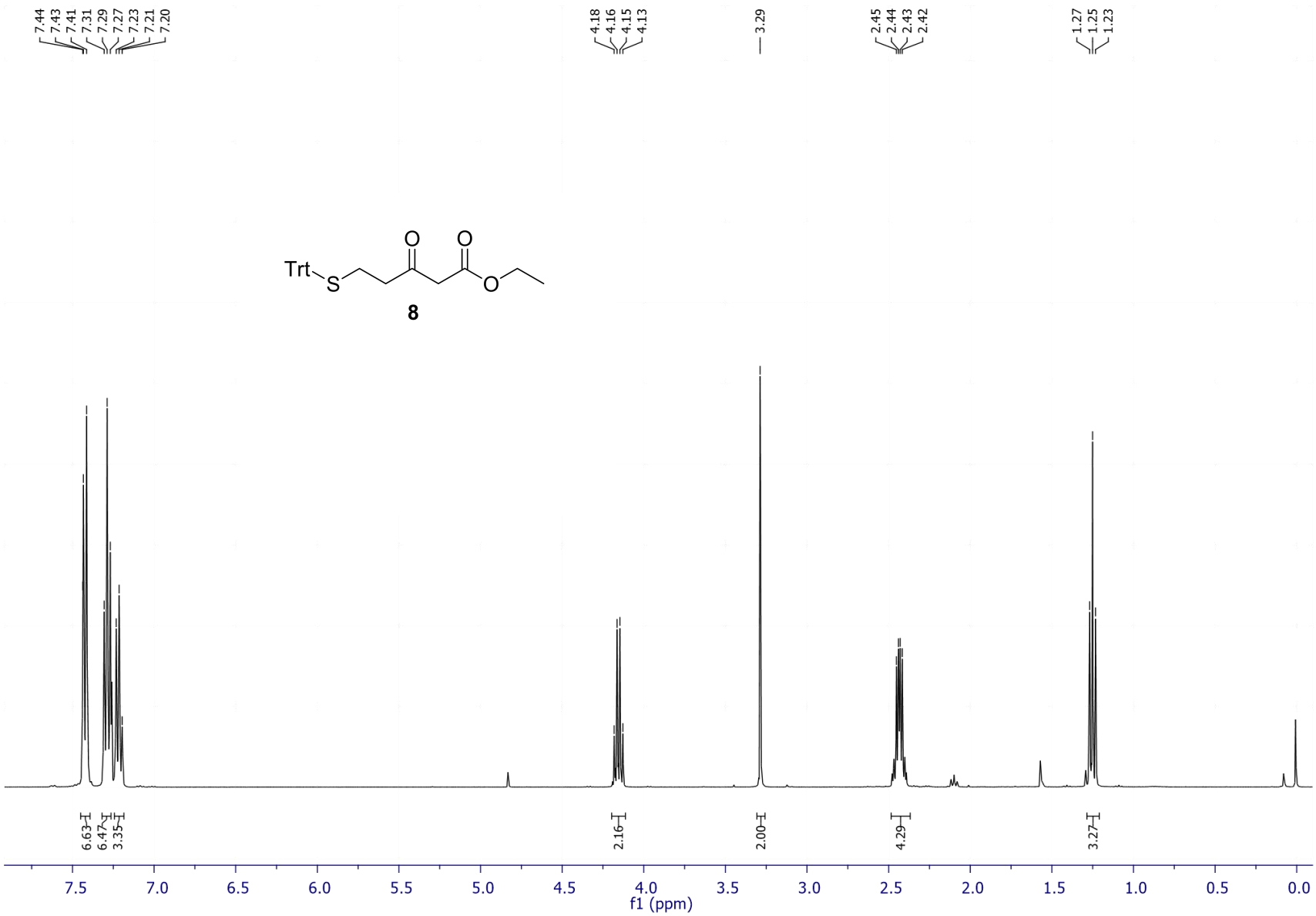
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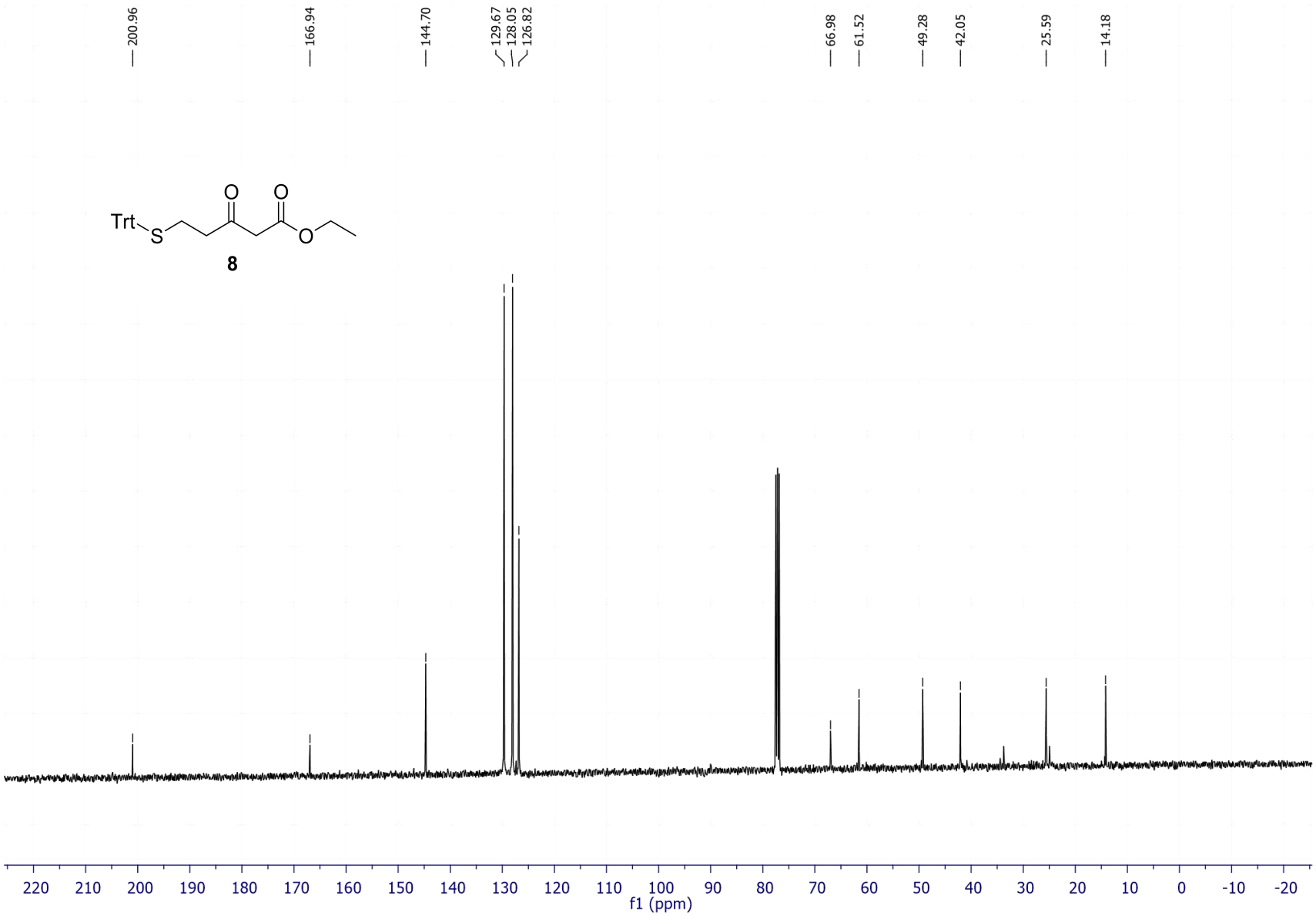
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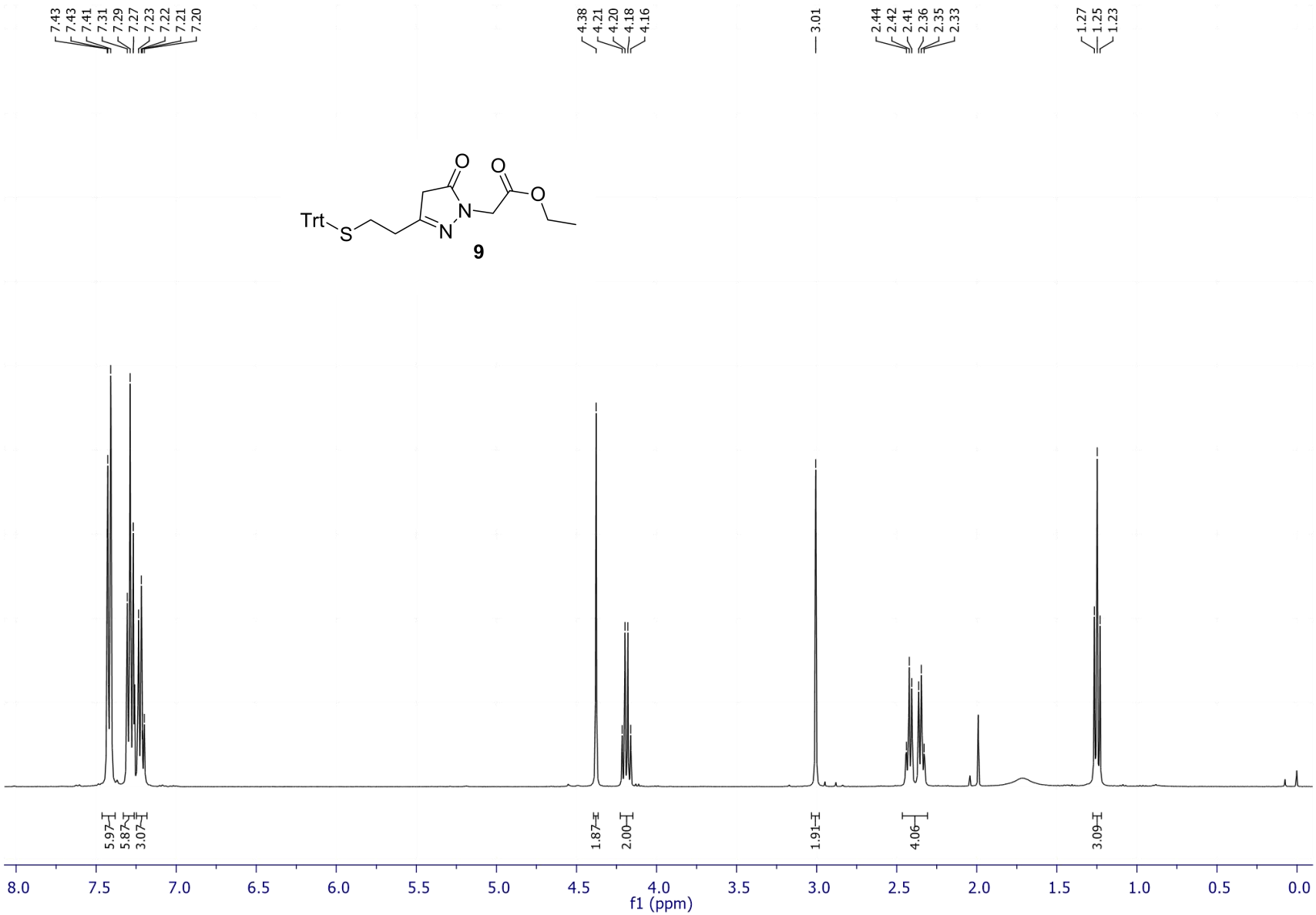
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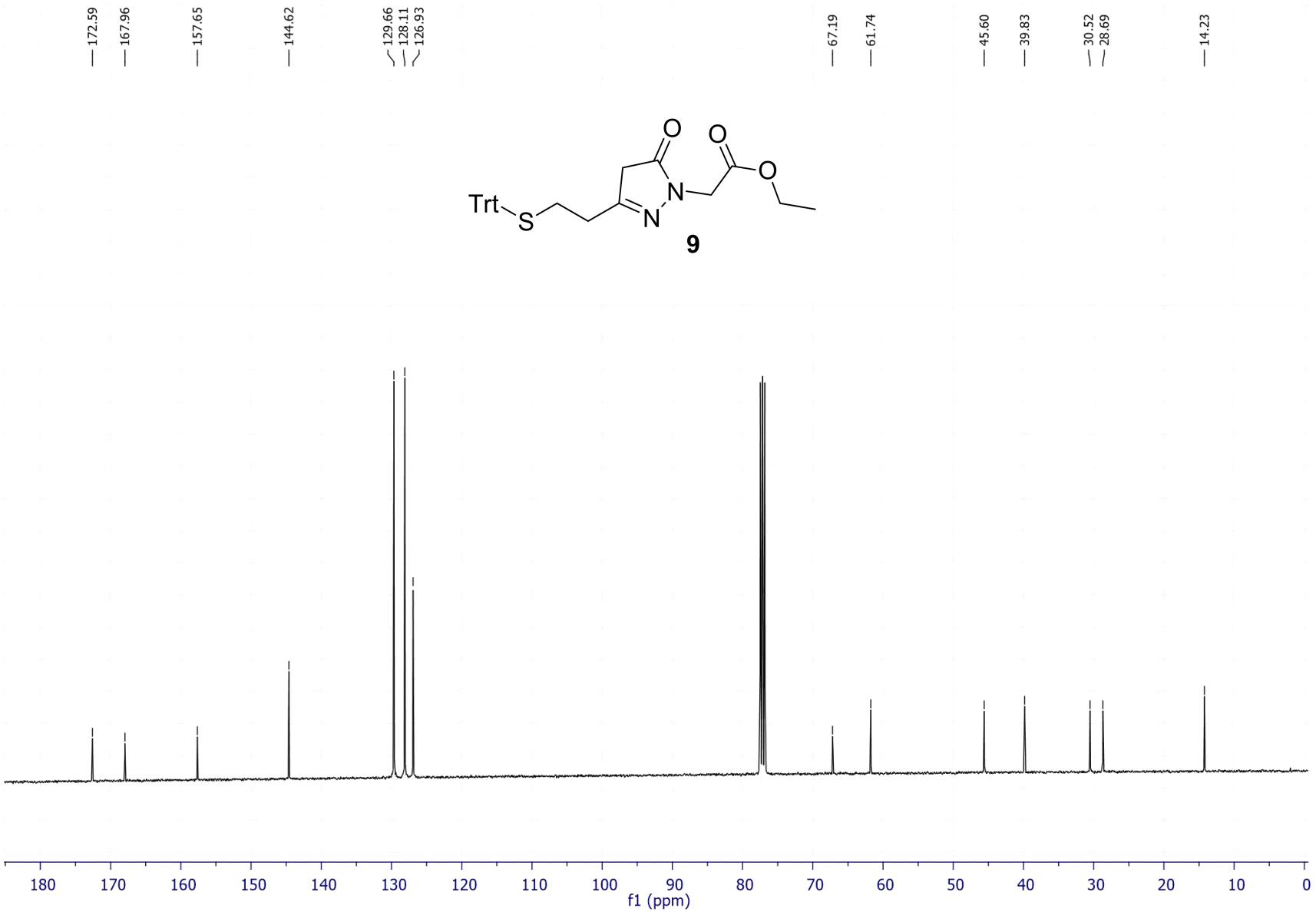
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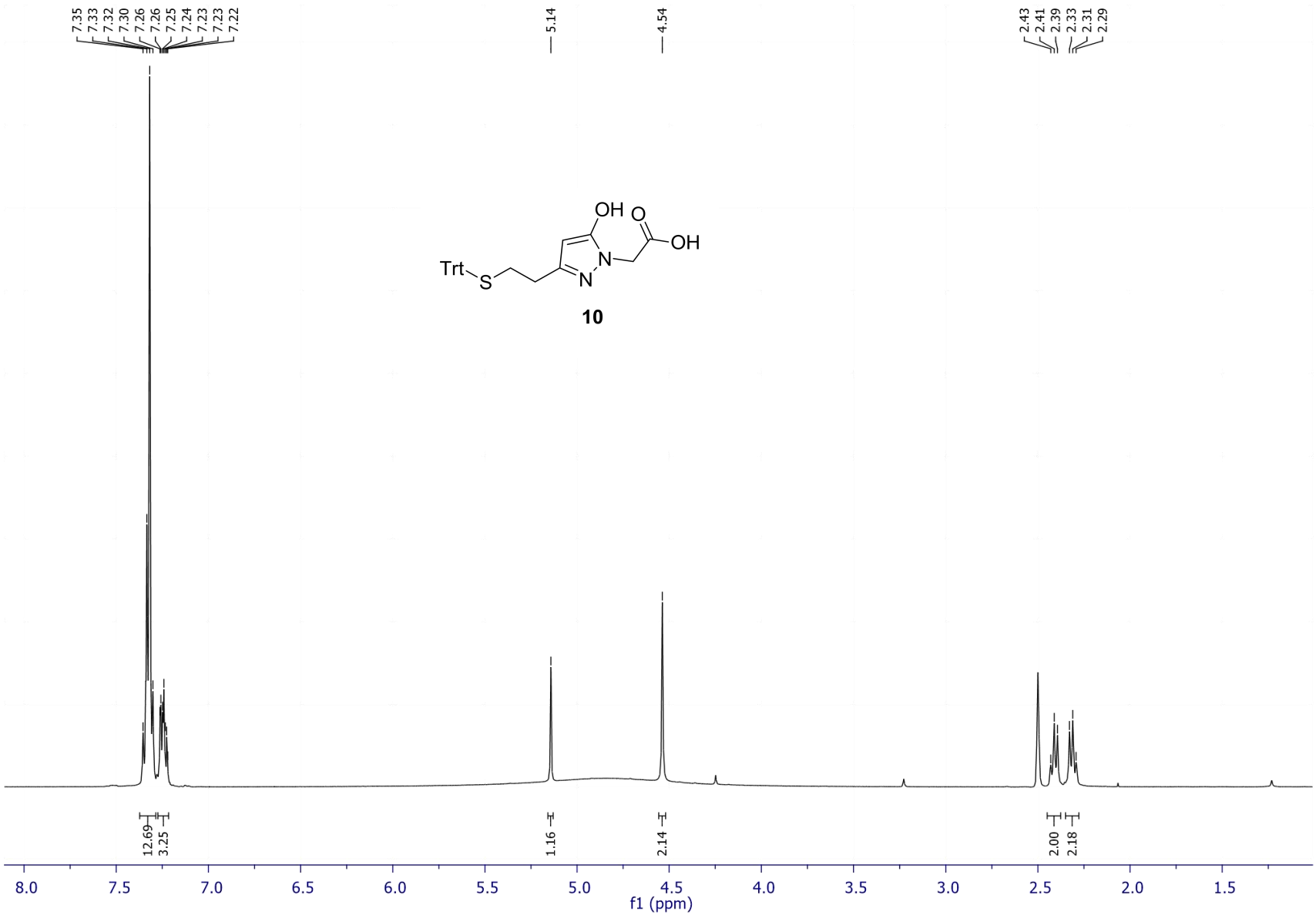
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2.33
2.31
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O
OH
N
N
Trt
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7.0
6.5
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4.0
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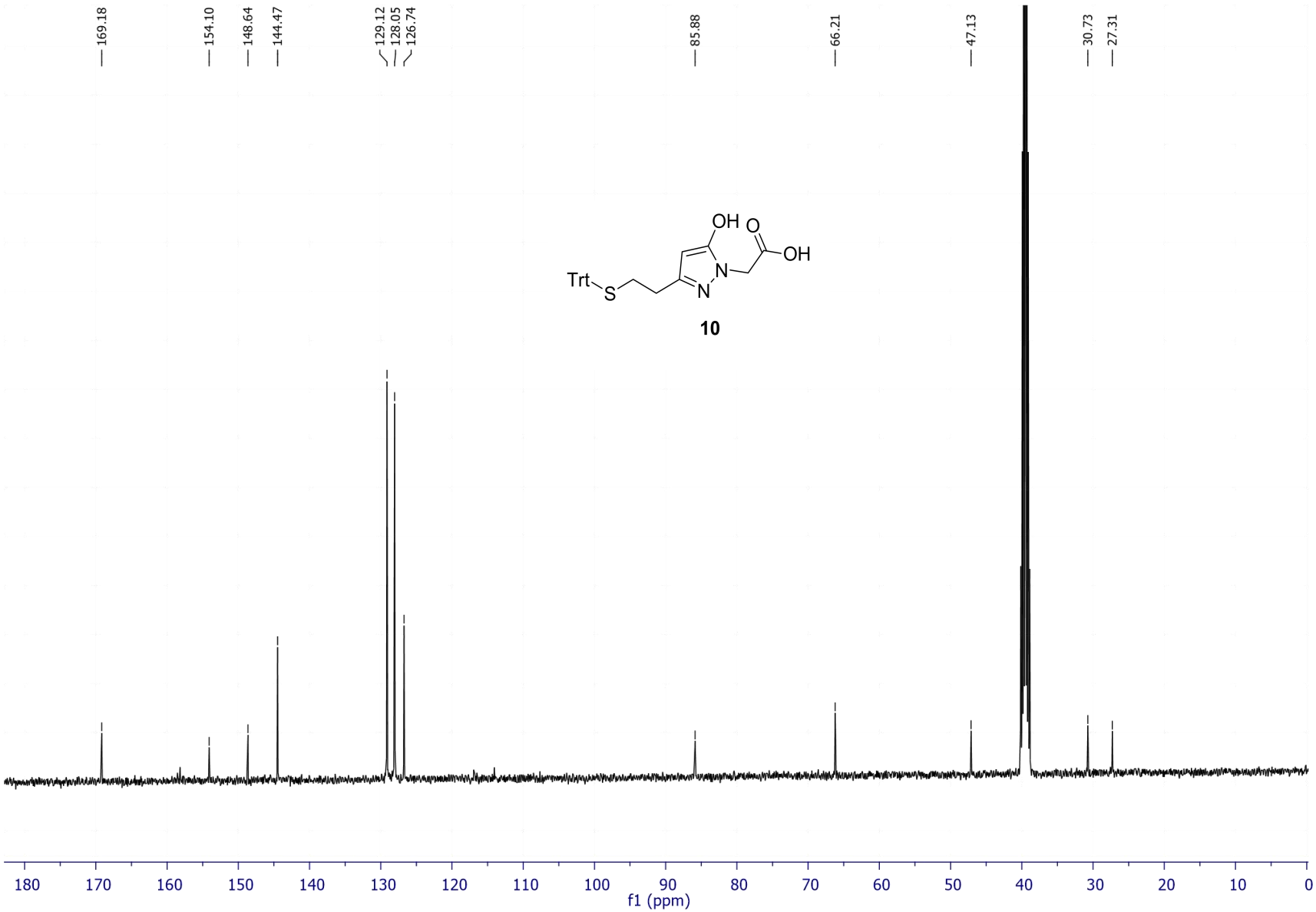
2.0
1.5
f1 (ppm)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154.10
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144.47
129.12
128.05
126.74
85.88
66.21
47.13
30.73
27.31
OH
O
OH
N
N
Trt
S
10
180
170
160
150
140
130
120
110
100
90
80
70
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50
40
30
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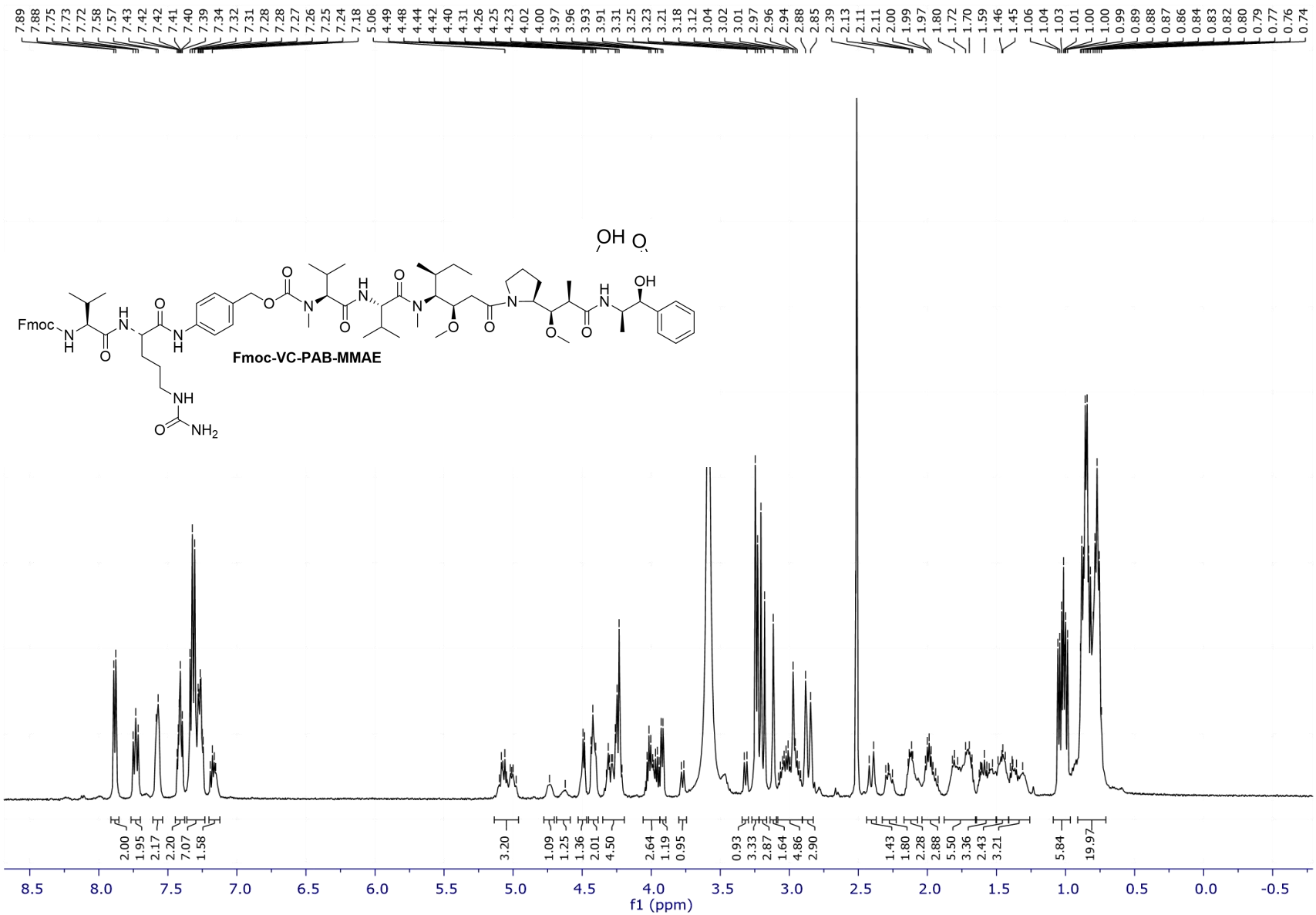
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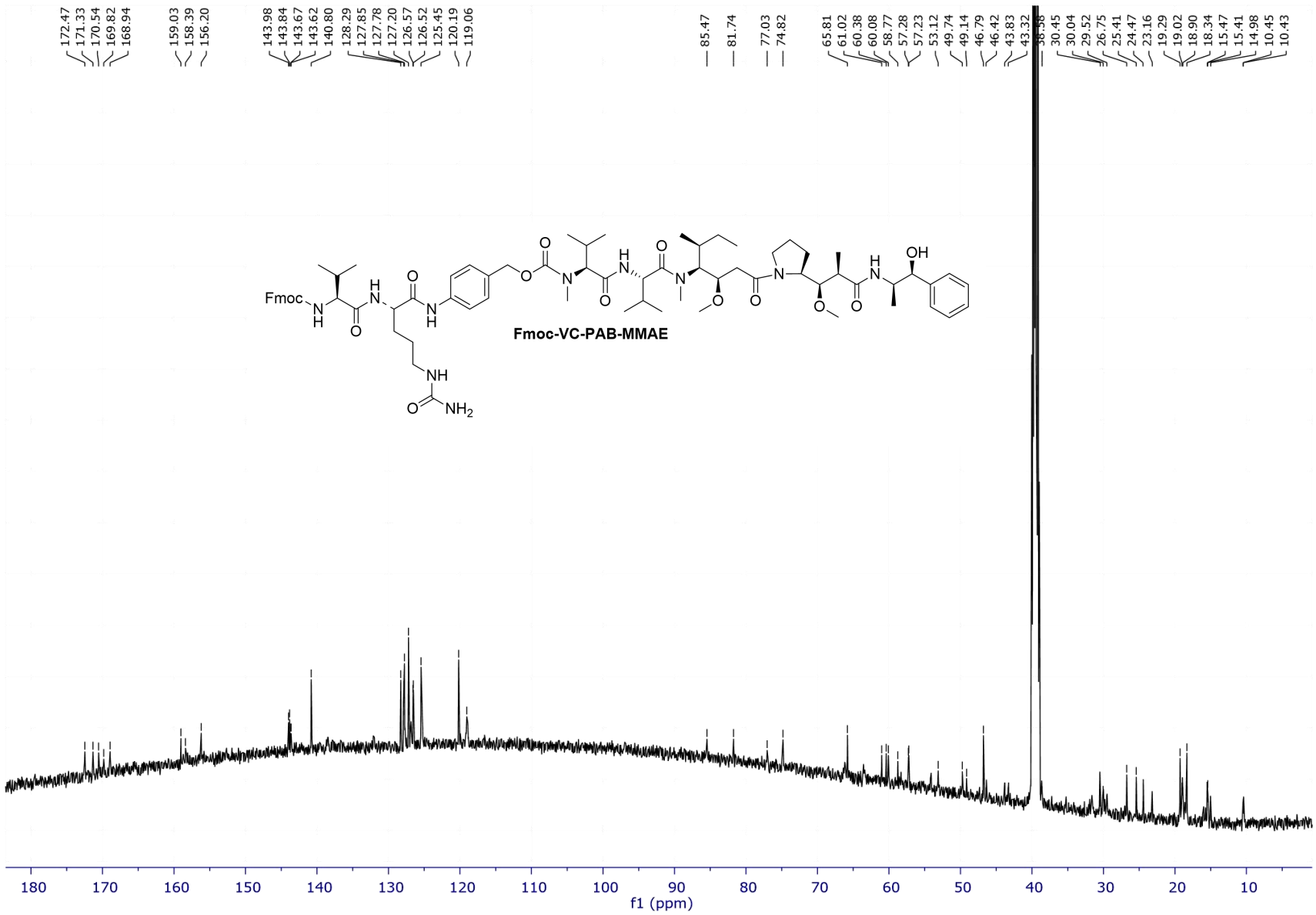
f1 (ppm)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171.33
170.54
169.82
168.94
159.03
158.39
156.20
143.98
143.84
143.67
143.62
140.80
128.29
127.85
127.78
127.20
126.57
126.52
125.45
120.19
119.06
85.47
81.74
77.03
74.82
65.81
61.02
60.38
60.08
58.77
57.28
57.23
53.12
49.74
49.14
46.79
46.42
43.83
43.32
38.58
30.45
30.04
29.52
26.75
25.41
24.47
23.16
19.29
19.02
18.90
18.34
15.47
15.41
14.98
10.45
10.43
Fmoc
OH
NH
NH2
Fmoc-VC-PAB-MMAE
180
170
160
150
140
130
120
110
100
90
80
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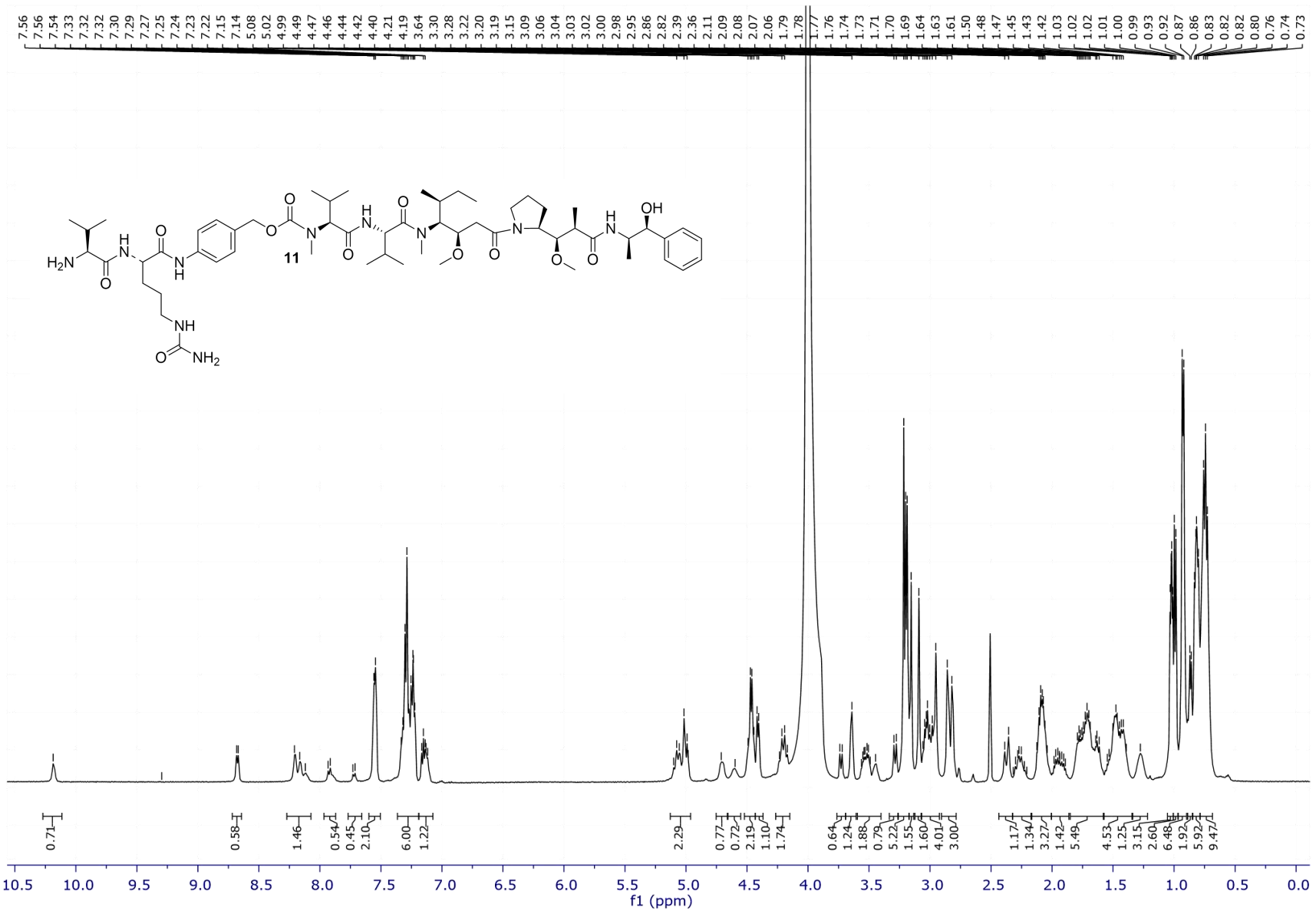
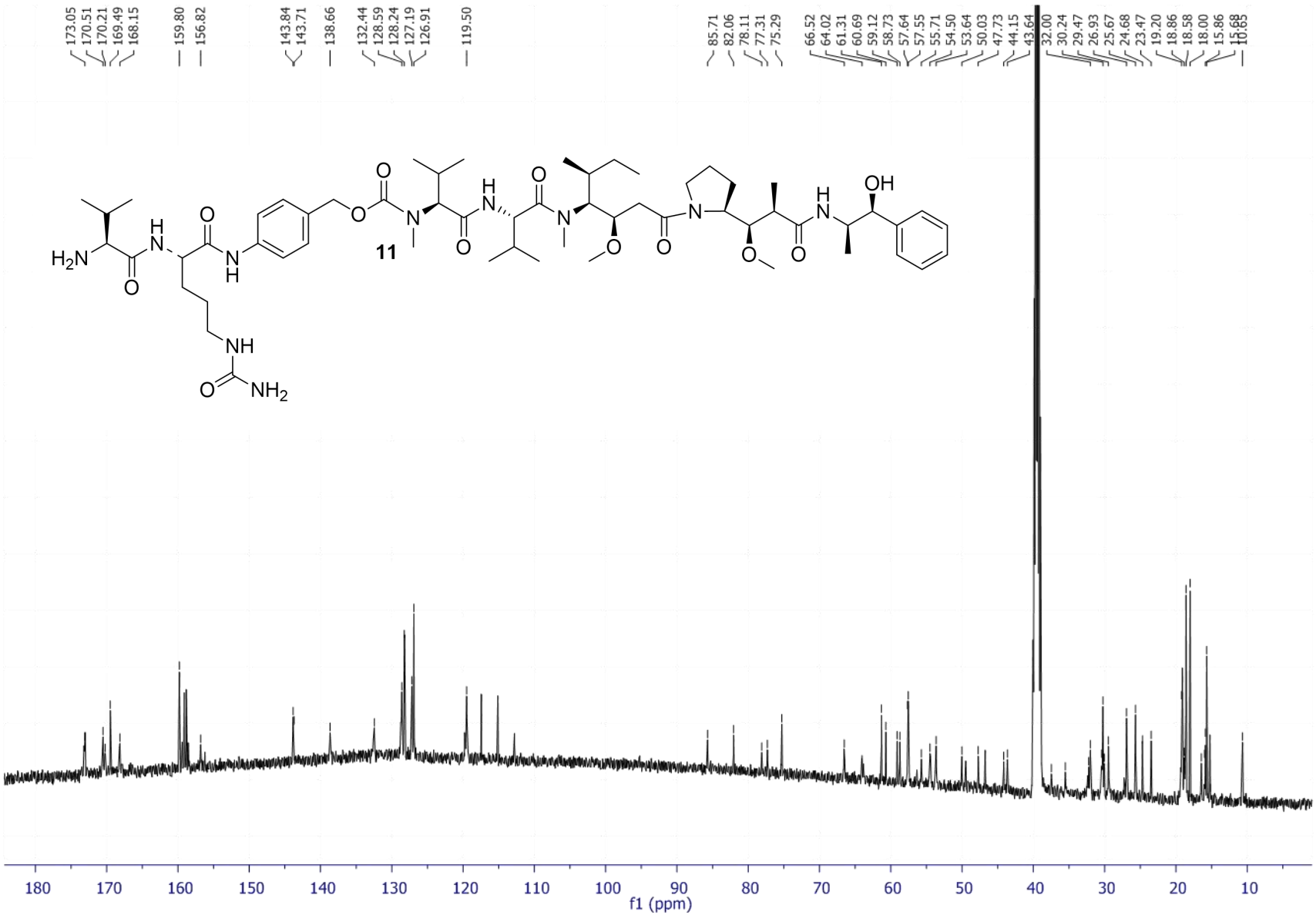
60
50
40
30
20
10
f1 (ppm)


11
f1 (ppm)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143.84
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128.24
127.19
126.91
119.50
85.71
82.06
78.11
77.31
75.29
66.52
64.02
61.31
60.69
59.12
58.73
57.64
57.55
55.71
54.50
53.64
50.03
47.73
44.15
43.64
32.00
30.24
29.47
26.93
25.67
24.68
23.47
19.20
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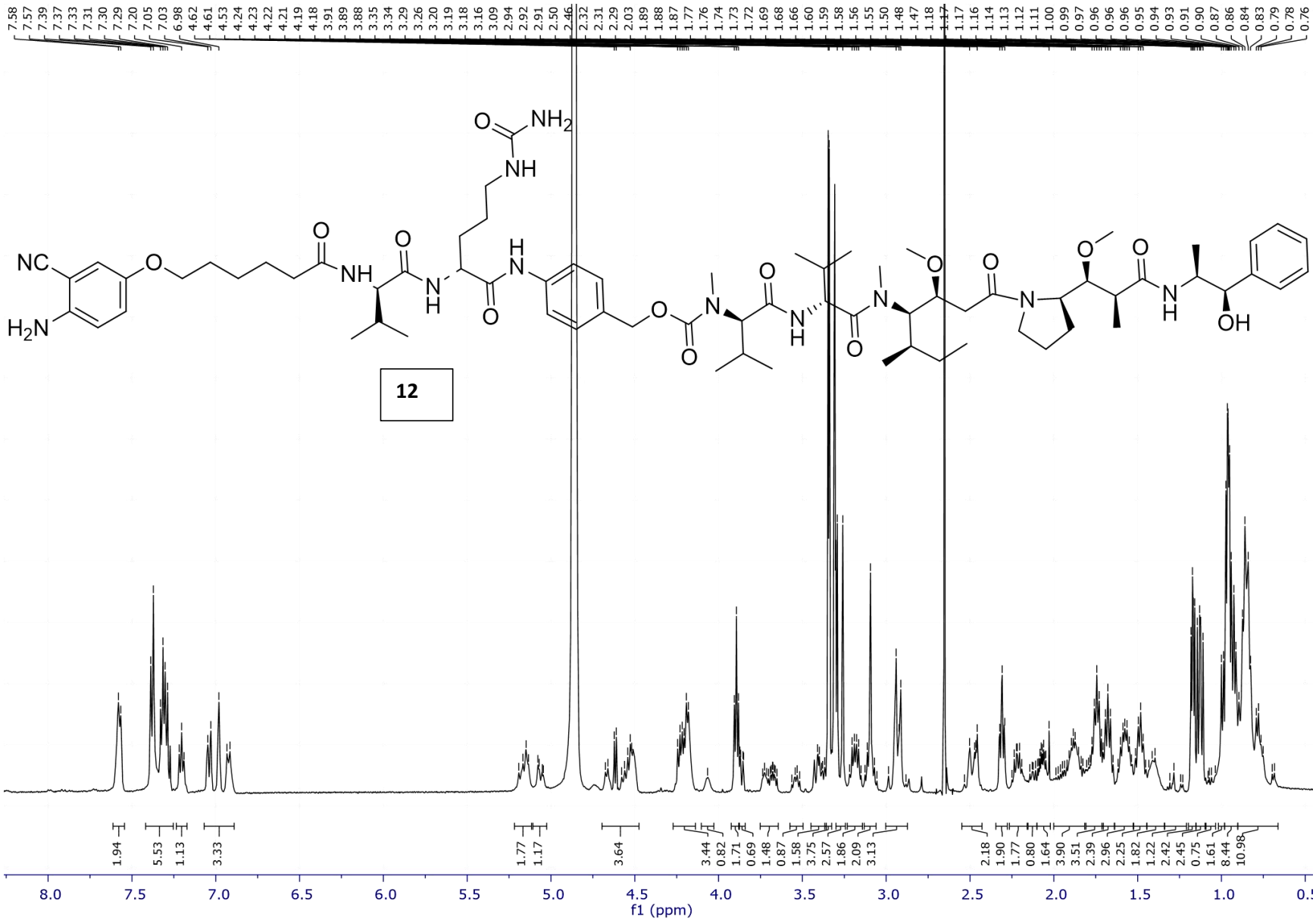
18.58
18.00
15.86
15.68
10.65
11
f1 (ppm)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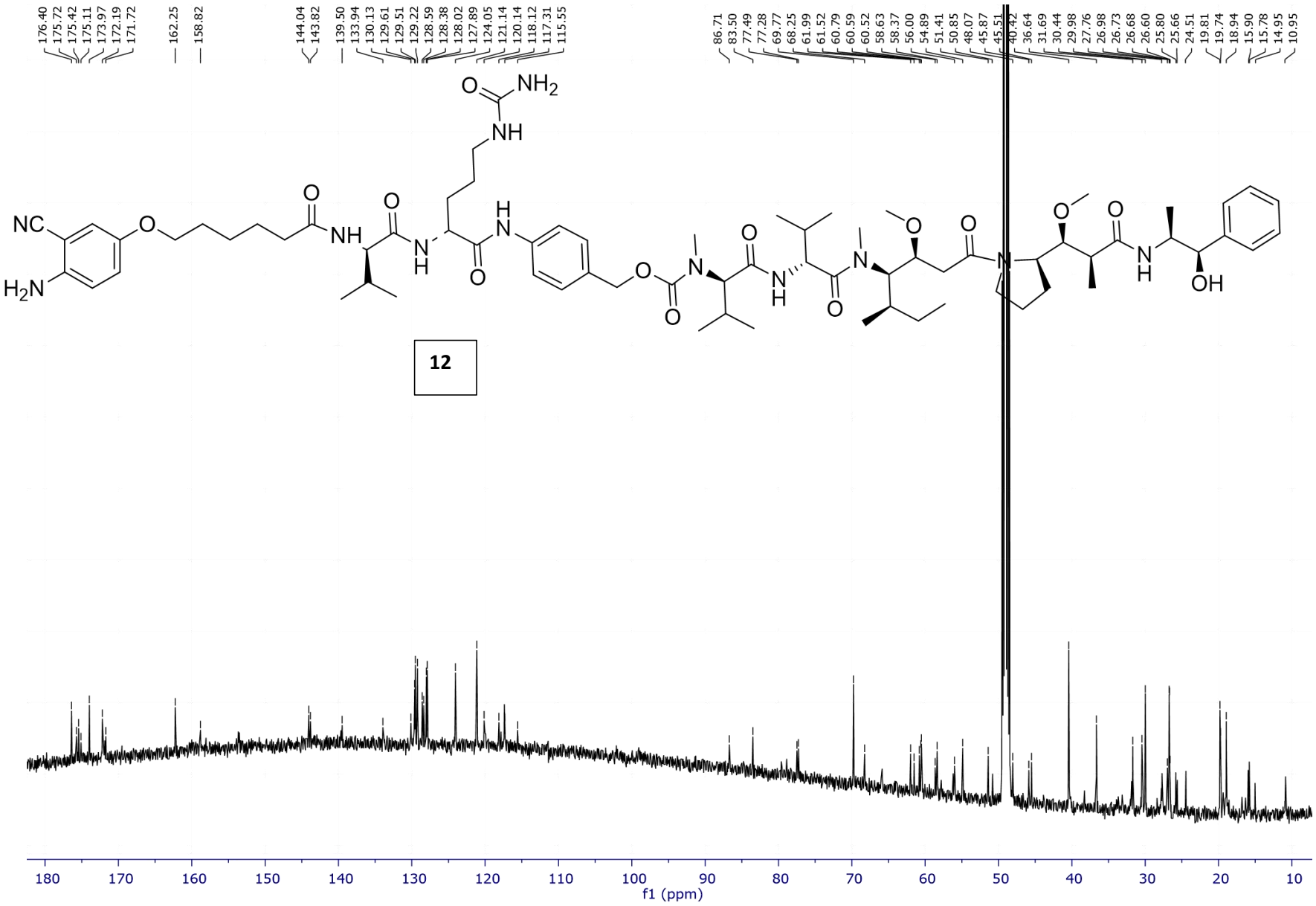
f1 (ppm)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172.19
171.72
162.25
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144.04
143.82
139.50
133.94
130.13
129.61
129.51
129.22
128.59
128.38
128.02
127.89
124.05
121.14
120.14
118.12
117.31
115.55
86.71
83.50
77.49
77.28
69.77
68.25
61.99
61.52
60.79
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60.52
58.63
58.37
56.00
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51.41
50.85
48.07
45.87
45.51
40.42
36.64
31.69
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29.98
27.76
26.98
26.73
26.68
26.60
25.80
25.66
24.51
19.81
19.74
18.94
15.90
15.78
14.95
10.95
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NH2
OH
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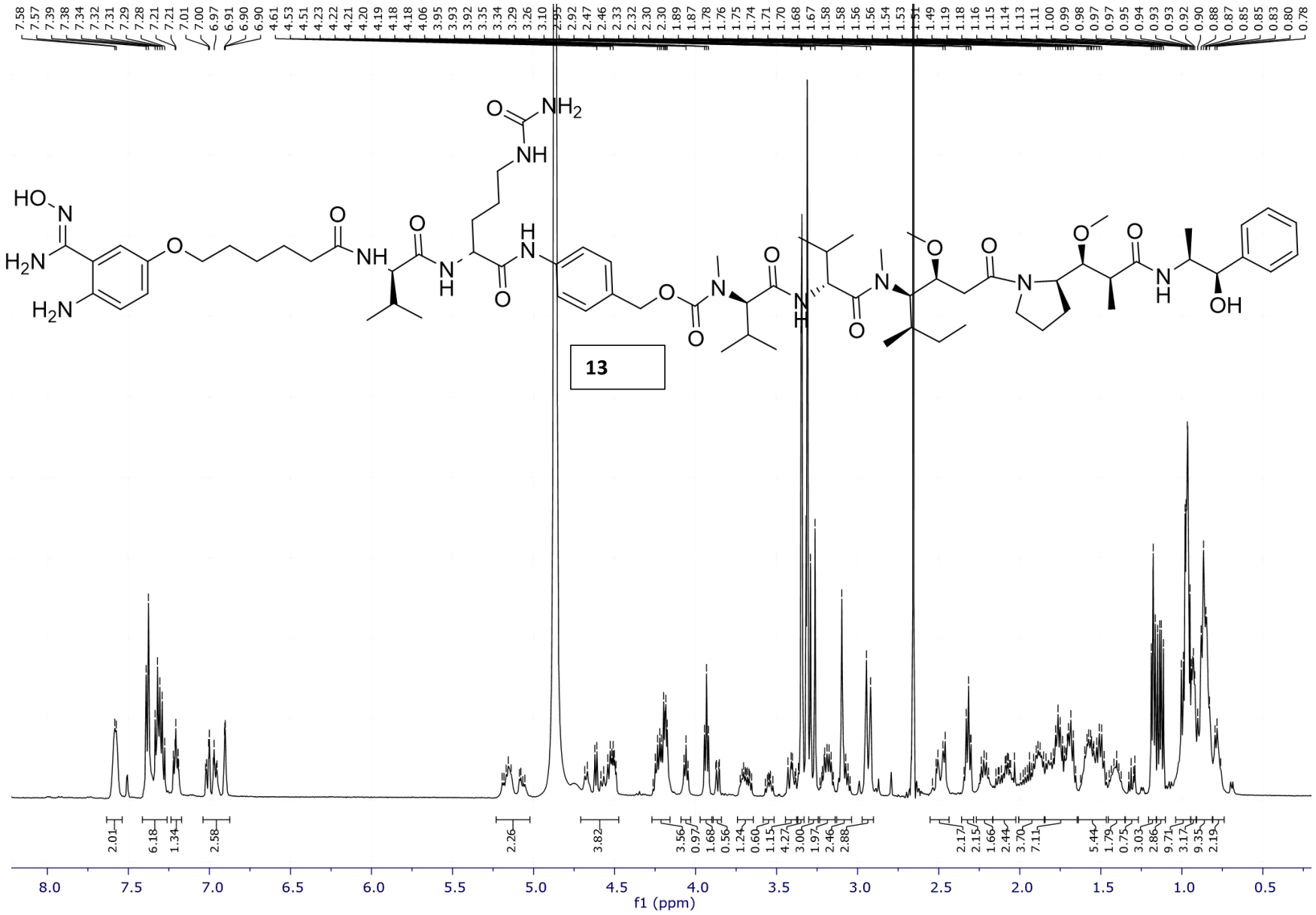
f1 (ppm)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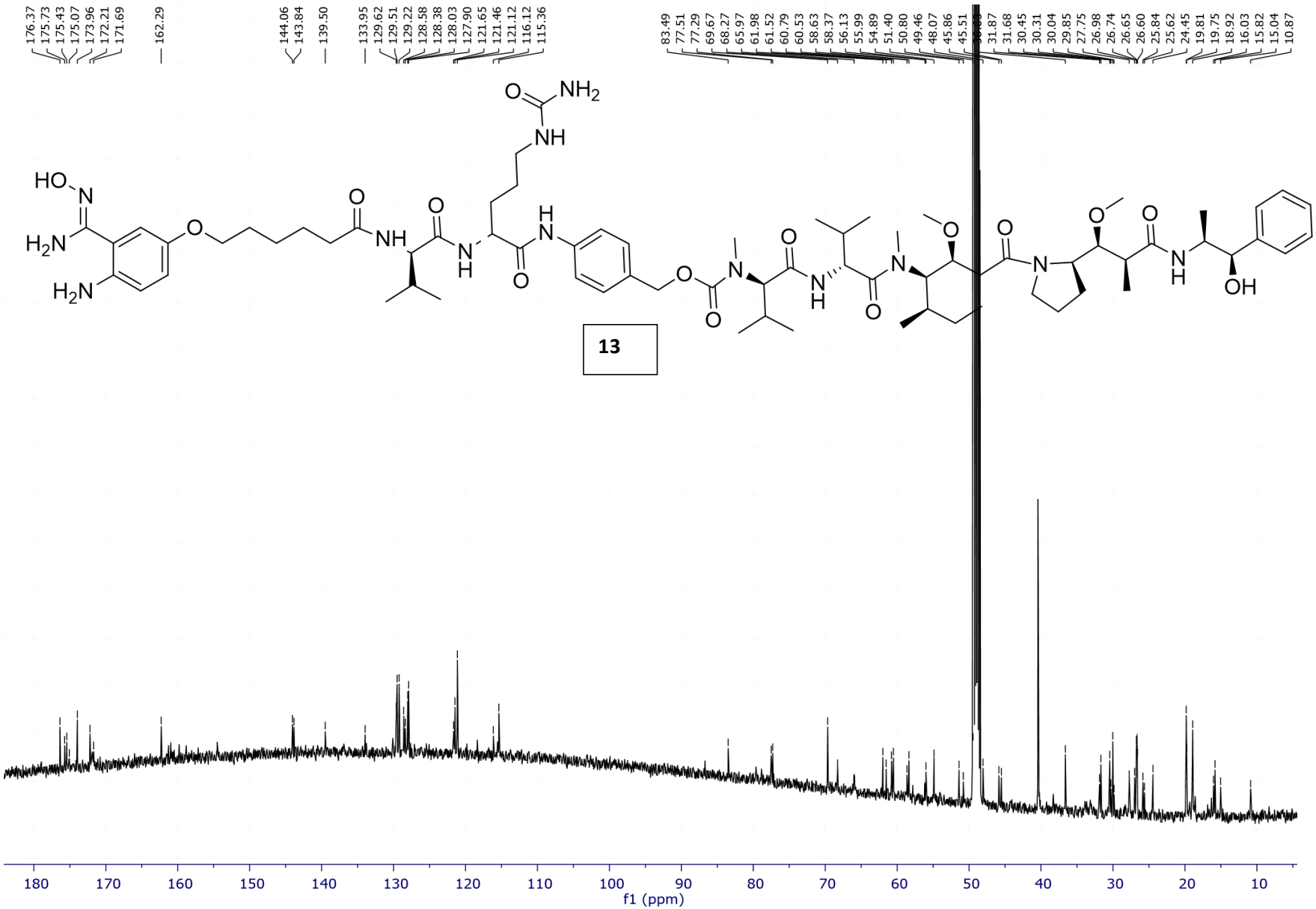
f1 (ppm)


176.37
175.73
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175.07
173.96
172.21
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162.29
144.06
143.84
139.50
133.95
129.62
129.51
129.22
128.58
128.38
128.03
127.90
121.65
121.46
121.12
116.12
115.36
83.49
77.51
77.29
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f1 (ppm)