Supporting Information for

Development and Characterization of Fluorescent Probes for the G Protein-Coupled Receptor 35

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A. Experimental Section

General.

All experimental reagents and solvents were obtained from various providers and used without any additional purification or drying except for tetrahydrofuran, which was distilled over calcium. The purity of all the target compounds was ≥ 95%. The reactions were monitored by thin layer chromatography (TLC). If necessary, the products were purified with column chromatography. NMR data were collected on a Bruker Ascend 600 MHz NMR spectrometer at 600 MHz (^{1}H) or 151 MHz (^{13}C). The chemical shifts were reported in parts per million (ppm) relative to the deuterated solvent DMSO-d_6; that is: δ ^{1}H, 2.49 ppm; ^{13}C, 39.7 ppm. High-resolution mass spectra (HRMS) were performed on an Agilent 1290 Infinity LC instrument (Agilent, USA) coupled to an Agilent 6540 series QTOF-MS (Agilent, USA) equipped with an ESI source, a diode-array detector (DAD), an automatic sample injector, a degasser and a column thermostat.

The purity of all final compounds analyzed by high-pressure liquid chromatography (HPLC) was ＞ 95%. The determination of purity was conducted on a Waters ACQUITY UPLCTM system (Waters Corp., Milford, MA, USA) with an ACQUITY UPLC® HSS T3 column (2.1×100 mm, 1.8 μm). Elution was performed with a gradient of water/acetonitrile (containing 0.1% formic acid) from 95/5 to 5/95 for 10 min and maintained at 5/95 for another 10 min. The flow rate was 200 μL/min. The peaks were detected at 254 nm.

The synthesis and structural characterization data of the compounds and their intermediates were described below. No unexpected or unusually high safety hazards were encountered.

General Procedure of method A for the Synthesis of Compounds 5-7. A mixture of the **1** (1.0 equiv) and iodoalkynes (1.2 equiv) in degassed 20 mL anhydrous DMF was evacuated and flushed with nitrogen. Cs_2CO_3 (2.0 equiv) was added, and the reaction mixture was stirred at 50^{o}C under nitrogen atmosphere overnight. Hydrochloric acid solution (2 M) was added to adjust pH to 4~5, and a large amount of cold water was added to the solution to precipitate a yellow solid. Then the solid precipitate was filtered, washed with water, diluted with CH_2Cl_2 and dried over anhydrous Na_2SO_4, concentrated under reduce pressure. The crude product was purified by column chromatography (1:1 DCM/EtOAc).

*Dimethyl 4-hydroxy-10-methyl-6-(pent-4-yn-1-yloxy)pyrido[3,2-g]quinoline-2,8- dicarboxylate (**5**).* Compound **5** was obtained from **1** and 5-iodopent-1-yne as described for method A; 34% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.99 (s, 1H), 8.84 (s, 1H), 7.44 (s, 1H), 6.60 (s, 1H), 4.44 (t, J = 6.1 Hz, 2H), 4.01 (s, 3H), 3.98 (s, 3H), 3.01 (s, 3H), 2.90 (t, J = 2.5 Hz, 1H), 2.48 – 2.46 (m, 2H), 2.17 – 2.11 (m, 2H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 179.2, 165.9, 163.9, 163.12, 151.1, 147.0, 140.3, 137.9, 125.5, 125.2, 118.5, 118.0, 108.5, 99.4, 84.0, 72.4, 68.4, 54.3, 53.4, 27.8, 15.2, 11.6. HRMS m/z: 409.1391 $[M + H]^+$, calcd for $C_{22}H_{21}N_2O_6^+$, 409.1394.

*Dimethyl 4-(hex-5-yn-1-yloxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8- dicarboxylate (**6**).* Compound **6** was obtained from **1** and 6-iodopent-1-yne as described for method A; 41% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.66 (s, 1H), 8.57 (s, 1H), 7.23 (s, 1H), 6.49 (s, 1H),

4.29 (t, *J* = 6.2 Hz, 2H), 4.01 (s, 3H), 3.97 (s, 3H), 2.86 (s, 3H), 2.85 (t, *J* = 2.4 Hz, 1H), 2.37 – 2.34 (m, 2H), 2.05 – 1.99 (m, 2H), 1.78 – 1.73 (m, 2H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 179.0, 165.8, 163.7, 163.0, 150.7, 146.6, 139.6, 137.3, 132.1, 129.2, 125.0, 124.7, 118.2, 117.6, 108.3, 99.1, 84.7, 72.0, 69.0, 54.3, 53.3, 28.0, 25.1, 17.9, 11.3. HRMS m/z: 423.1556 $[M + H]^+$, calcd for $C_{23}H_{23}N_2O_6^+$, 423.1551.

*Dimethyl 4-(hept-6-yn-1-yloxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8- dicarboxylate (**7**).* Compound **7** was obtained from **1** and 6-iodopent-1-yne as described for method A; 46% yield as a yellow solid. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.75 (s, 1H), 8.64 (s, 1H), 7.29 (s, 1H), 6.51 (s, 1H), 4.29 (s, 2H), 4.01 (s, 3H), 3.98 (s, 3H), 2.90 (s, 3H), 2.81 (s, 1H), 2.26 (td, *J* = 6.5, 2.5 Hz, 2H), 1.98 – 1.91 (m, 2H), 1.68 – 1.58 (m, 4H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 179.1, 165.9, 163.8, 163.1, 150.8, 146.7, 139.8, 137.5, 125.1, 124.8, 118.3, 117.7, 108.4, 99.2, 84.8, 71.9, 69.4, 54.3, 53.3, 28.2, 28.0, 25.0, 18.1, 11.4. HRMS m/z: 437.1704 $[M + H]^+$, calcd for $C_{24}H_{25}N_2O_6^+$, 437.1707.

General Procedure of method B for the Synthesis of Compounds 10-13. A 100 mL round bottom flask was charged with alkynyl derivatives (**5-7**) (1.0 equiv), **9** (1.1 equiv), $CuSO_4 \cdot H_2O$ (0.05 equiv) and sodium ascorbate (0.1 equiv) in THF/H_2O. The resulting mixture was stirred at 50°C overnight, and then tested on TLC to confirm completion of the reaction. The mixture was diluted with water and ethyl acetate. The organic layer was separated, dried over anhydrous Na_2SO_4, concentrated under reduce pressure, and the residue was purified by column chromatography with DCM/MeOH from 100:1 to 40:1.

*BF_2 Chelate of dimethyl (Z)-4-(3-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)propoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylate (**10**).* Compound **10** was obtained from **5** and **9** as described for method B; 76% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.99 (s, 1H), 8.84 (s, 1H), 8.07 (s, 1H), 7.43 (s, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.6 Hz, 2H), 6.59 (s, 1H), 6.10 (s, 2H), 4.76 (t, *J* = 4.8 Hz, 2H), 4.49 – 4.38 (m, 4H), 4.00 (s, 3H), 3.96 (s, 3H), 2.98 (s, 3H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.42 (s, 6H), 2.34 – 2.26 (m, 2H), 1.30 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 178.1, 166.8, 164.9, 162.9, 162.1, 157.8, 154.0, 150.1, 146.0, 145.3, 142.0, 141.3, 139.2, 136.8, 131.1, 130.8, 130.4, 128.5, 128.1, 125.8, 124.39, 124.1, 122.3, 120.6, 117.5, 116.8, 114.6, 107.4, 98.4, 67.7, 65.7, 53.2, 52.3, 52.0, 48.3, 27.4, 20.9, 13.6, 13.5, 10.5. HRMS m/z: 818.3272 $[M + H]^+$, calcd for $C_{43}H_{43}BF_2N_7O_7^+$, 818.3280.

*BF_2 Chelate of dimethyl (Z)-4-(4-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)butoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylate (**11**).* Compound **11** was obtained from **6** and **9** as described for method B; 81% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.73 (s, 1H), 8.73 (s, 1H), 8.02 (s, 1H), 7.35 (s, 1H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.05 (d, *J* = 8.7 Hz, 2H), 6.49 (s, 1H), 5.99 (s, 2H), 4.76 (t, *J* = 4.9 Hz, 2H), 4.45 (t, *J* = 5.0 Hz, 2H), 4.34 (s, 2H), 4.00 (s, 3H), 3.98 (s, 3H), 2.92 (s, 3H), 2.80 (t, *J* = 7.3 Hz, 2H), 2.38 (s, 6H), 2.00 – 1.94 (m, 2H), 1.92 – 1.83 (m, 2H), 1.25 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 178.0, 166.8, 164.9, 162.7, 162.0, 157.9, 153.9, 149.8, 146.0, 145.6, 141.9, 141.1, 138.6, 136.4, 131.1, 130.8, 130.3, 128.5, 128.1, 125.8, 122.2, 120.45,

117.2, 116.6, 114.6, 107.2, 98.1, 96.8, 96.6, 68.3, 65.9, 59.2, 52.2, 52.0, 48.3, 27.2, 24.9, 24.0, 13.5, 13.5. HRMS m/z: 832.3431 $[M + H]^+$, calcd for $C_{44}H_{45}BF_2N_7O_7^+$, 832.3436.

*BF_2 Chelate of dimethyl (Z)-4-((5-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)pentyl)oxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylate (**12**)*. Compound **12** was obtained from **7** and **9** as described for method B; 79% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.89 (s, 1H), 8.76 (s, 1H), 8.00 (s, 1H), 7.37 (s, 1H), 7.20 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 8.6 Hz, 2H), 6.57 (s, 1H), 6.10 (s, 2H), 4.75 (t, J = 4.9 Hz, 2H), 4.43 (t, J = 5.0 Hz, 2H), 4.33 (s, 2H), 4.00 (s, 3H), 3.96 (s, 3H), 2.96 (s, 3H), 2.71 (t, J = 7.4 Hz, 2H), 2.42 (s, 6H), 2.00 – 1.94 (m, 2H), 1.80 – 1.73 (m, 2H), 1.62 – 1.55 (m, 2H), 1.31 (s, 6H).^{13}C NMR (151 MHz, DMSO-*d6*) δ 178.1, 164.8, 162.8, 162.0, 157.9, 154.0, 150.0, 146.2, 145.8, 142.0, 141.3, 139.00, 136.6, 131.1, 130.4, 128.5, 128.1, 125.8, 124.2, 123.9, 122.0, 120.6, 117.4, 116.7, 114.6, 107.3, 98.2, 68.5, 65.7, 52.2, 52.0, 48.2, 28.0, 27.4, 24.4, 24.3, 13.6, 13.5. HRMS m/z: 846.3597 $[M + H]^+$, calcd for $C_{45}H_{47}BF_2N_7O_7^+$, 846.3593.

*BF_2 Chelate of dimethyl (Z)-4-(2-((1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)ethoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylate (**13**)*. Compound **13** was obtained from **8** and **9** as described for method B; 73% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.82 (s, 1H), 8.73 (s, 1H), 8.28 (s, 1H), 7.38 (d, J = 8.5 Hz, 1H), 7.17 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.52 (s, 1H), 6.05 (s, 2H), 4.84 – 4.79 (m, 2H), 4.73 (s, 2H), 4.49 (s, 2H), 4.47 – 4.45 (m, 2H), 4.02 (s, 2H), 3.99 (d, J = 6.4 Hz, 3H), 3.97 (s, 3H), 2.94 (s, 3H), 2.40 (s, 6H), 1.24 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 179.2, 167.8, 165.9, 163.8, 163.0, 158.9, 155.0, 151.0, 146.9, 144.4, 143.1, 142.3, 139.9, 137.6, 132.1, 131.9, 131.4, 129.6, 129.2, 126.9, 125.3, 125.1, 124.9, 121.6, 118.3, 117.9, 115.6, 108.4, 99.4, 69.0, 68.1, 66.7, 64.1, 55.4, 54.3, 53.3, 53.1, 49.5, 14.6, 14.6, 11.5. HRMS m/z: 834.3222 $[M + H]^+$, calcd for $C_{43}H_{43}BF_2N_7O_8^+$, 834.3229.

General Procedure of method C for the Synthesis of Compounds 14-17. The ethyl carboxylate derivatives **10-13** (1.0 equiv) were added into a solution of LiOH (2.5 equiv) in water:MeOH (1:1) (10 mL). The reaction mixture was stirred at rt for 1 h until a clear solution was obtained. The mixture was acidified with hydrochloric acid solution (2 M) until pH 4-5 was reached. The obtained precipitate was filtered and washed with a modicum of MeOH (3 × 2 mL), and dried under vacuum at 50°C.

*BF_2 Chelate of (Z)-4-(3-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)propoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylic acid (**14**)*. Compound **14** was obtained from **10** as described for method C; 78% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.06 (s, 1H), 7.45 (s, 1H), 7.18 (d, J = 8.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 6.66 (s, 1H), 6.10 (s, 2H), 4.76 (t, J = 5.1 Hz, 2H), 4.46 – 4.42 (m, 4H), 3.03 (s, 3H), 2.94 (t, J = 7.4 Hz, 2H), 2.42 (s, 6H), 2.32 – 2.28 (m, 2H), 1.30 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 166.9, 164.2, 164.0, 158.9, 155.1, 152.0, 146.8, 146.4, 143.1, 142.4, 131.5, 129.6, 126.9, 124.9, 123.3, 121.7, 118.6, 117.6, 115.7, 99.14, 68.7, 66.8, 49.3, 28.5, 22.0, 14.7, 14.6. HRMS m/z: 790.2961 $[M + H]^+$, calcd for

$C_{41}H_{39}BF_2N_7O_7^+$, 790.2967.

*BF_2 Chelate of (Z)-4-(4-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)butoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylic acid (**15**).* Compound **15** was obtained from **11** as described for method C; 81% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.03 (s, 1H), 7.43 (s, 1H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.05 (t, *J* = 8.2 Hz, 2H), 6.56 (s, 1H), 6.01 (s, 2H), 4.76 (t, *J* = 5.0 Hz, 2H), 4.45 (t, *J* = 5.0 Hz, 2H), 4.38 (t, *J* = 6.5 Hz, 2H), 3.01 (s, 3H), 2.79 (t, *J* = 7.4 Hz, 2H), 2.39 (s, 6H), 2.01 – 1.95 (m, 2H), 1.92 – 1.83 (m, 2H), 1.26 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 166.9, 164.0, 163.8, 159.0, 155.0, 151.8, 147.1, 146.7, 143.0, 142.2, 131.4, 129.6, 126.9, 124.8, 123.2, 121.6, 118.4, 117.5, 115.7, 107.2, 98.9, 69.3, 66.9, 49.4, 28.3, 26.0, 25.1, 14.6, 14.6, 11.3. HRMS m/z: 804.3127 $[M + H]^+$, calcd for $C_{42}H_{41}BF_2N_7O_7^+$, 804.3123.

*BF_2 Chelate of (Z)-4-((5-(1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl)(3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)pentyl)oxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylic acid (**16**).* Compound **16** was obtained from **12** as described for method C; 65% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.88 (s, 1H), 7.99 (s, 1H), 7.45 (s, 1H), 7.20 (d, *J* = 8.7 Hz, 2H), 7.06 (d, *J* = 8.7 Hz, 2H), 6.63 (s, 1H), 6.11 (s, 2H), 4.74 (t, *J* = 5.1 Hz, 2H), 4.44 (t, *J* = 5.1 Hz, 2H), 4.38 (t, *J* = 6.4 Hz, 2H), 3.03 (s, 3H), 2.71 (t, *J* = 7.5 Hz, 2H), 2.42 (s, 6H), 2.01 – 1.96 (m, 2H), 1.76 (dt, *J* = 15.2, 7.6 Hz, 2H), 1.59 (dt, *J* = 15.1, 7.6 Hz, 2H), 1.32 (s, 6H).. ^{13}C NMR (151 MHz, DMSO-*d6*) δ 165.8, 162.9, 162.7, 157.9, 154.0, 150.8, 146.2, 145.7, 142.0, 141.3, 130.4, 128.5, 125.8, 123.8, 121.9, 120.6, 117.4, 116.5, 114.6, 106.2, 97.9, 68.5, 65.7, 48.2, 28.0, 27.4, 24.5, 24.3, 13.6, 13.5, 10.3. HRMS m/z: 818.3286 $[M + H]^+$, calcd for $C_{43}H_{43}BF_2N_7O_7^+$, 818.3280.

*BF_2 Chelate of (Z)-4-(2-((1-(2-(4-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)phenoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)ethoxy)-6-hydroxy-10-methylpyrido[3,2-g]quinoline-2,8-dicarboxylic acid (**17**).* Compound **17** was obtained from **13** as described for method C; 72% yield as an orange solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.88 (s, 1H), 8.28 (s, 1H), 7.47 (s, 1H), 7.18 (d, *J* = 7.1 Hz, 2H), 7.04 (d, *J* = 8.0 Hz, 2H), 6.59 (s, 1H), 6.07 (s, 2H), 4.81 (t, *J* = 4.9 Hz, 2H), 4.72 (s, 2H), 4.55 – 4.51 (m, 2H), 4.47 (t, *J* = 4.7 Hz, 2H), 4.05 – 3.99 (m, 2H), 3.02 (s, 3H), 2.41 (s, 6H), 1.28 (s, 6H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 166.9, 164.0, 163.6, 158.9, 155.1, 151.8, 146.7, 144.4, 143.1, 142.3, 138.0, 131.4, 129.6, 126.9, 125.1, 124.8, 121.7, 118.3, 117.8, 115.7, 107.0, 99.1, 69.0, 68.2, 66.7, 64.1, 49.5, 14.6, 14.6, 11.6. HRMS m/z: 806.2911 $[M + H]^+$, calcd for $C_{41}H_{39}BF_2N_7O_8^+$, 806.2916.

*Preparation of dimethyl 6-(2-(but-3-yn-1-yloxy)ethoxy)-10-methyl-4-oxo- 1,4-dihydropyrido[3,2-g]quinoline-2,8-dicarboxylate **(8).*** Under the nitrogen atmosphere and protection from light, compound 1 (1.0 equiv), triphenylphosphine (2.5 equiv) and 2-(prop-2-yn-1-yloxy)ethan-1-ol (1.2 equiv) were dissolved in anhydrous THF (50 mL) and cooled at 0 ºC. Diisopropylazadicarboxylate (2.5 equiv) was added dropwise at 0 ºC. The reaction mixture was stirred for another 1 h and then overnight at room temperature. The solvent was dissolved in EtOAc and washed with saturated brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated under reduce pressure, and

the residue was purified by column chromatography with DCM/EtOAc from 9:1 to 1:4. 35% yield as a golden yellow solid. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) 1H NMR (600 MHz, DMSO) δ 9.87 (s, 1H), 8.76 (s, 1H), 7.39 (s, 1H), 6.56 (s, 1H), 4.50 (d, *J* = 11.7 Hz, 2H), 4.33 (d, *J* = 2.1 Hz, 2H), 4.02 – 3.98 (m, 8H), 3.22 (s, 1H), 2.95 (s, 3H). ^{13}C NMR (151 MHz, DMSO-*d6*) δ 179.2, 165.8, 163.8, 163.1, 151.0, 146.9, 140.0, 137.7, 125.3, 125.0, 118.3, 118.0, 108.4, 99.4, 80.7, 78.0, 68.9, 67.6, 58.1, 54.3, 53.4, 11.5. HRMS m/z: 425.1339 $[M + H]^+$, calcd for $C_{22}H_{21}N_2O_7^+$, 425.1343.

Optical Property Evaluation of Fluorescent Probes for GPR35.

Probes are dissolved in either n-octanol or HBSS to measure absorbance curves and extinction coefficients by a FLUOstar Omega Microplate Reader (BMG LAB-TECH).

ChronosFD time-resolved spectrofluorometer coupled to PMT detectors were used to measure emission curves. All compounds were excited at a fixed excitation wavelength of 402 nm, and coumarin 153 (C153) in EtOH was used as an internal standard for determination of relative quantum yield (Φ C153 = 0.546, λ_{ex} = 402 nm). All experiments were performed at 25°C.

The solution of **15** in *n*-octanol (2 μM) was irradiated at room temperature with a mercury lamp 250 W through a Carl Zeiss JENA filter at 365nm. The area of the light flux was 2.02 cm^2 at a specific power W_{365} = 1.47 mW/m^2 of UV lamp.

Materials and Cell Culture

Zaprinast, PQQ, cromolyn, ellagic acid and pamoic acid were obtained from Sigma-Aldrich. ML-145 and ML-194 were obtained from Tocris. Epic® 384-well biosensor microplates were obtained from Corning Incorporated (Corning, NY). HT-29 and CHO-K1 cells were cultured in McCoy's 5A and Ham's F12K, respectively. Either medium was supplemented with 10% FBS and 1% penicillin/streptomycin, and the cells were maintained in a 5% CO_2 incubator at 37ºC.

Transfection of hGPR35 cell line

CHO-K1 cells were transfected with 8 μg of pcDNA3.1-hGPR35 plasmid mixed with 24 μL of lipofectamine 2000 reagent (Invitrogen). After 24 hours post-transfection, the medium was replaced by fresh complete medium containing 400 μg/mL zeocin (TransGen Biotech Co., Ltd, Beijing, China). Stable clones were selected after 3~4 weeks of zeocin treatment and transfected CHO-K1-hGPR35 cell lines were obtained. After 3~4 months, a stably transfected CHO-K1-hGPR35 cell line was obtained. The function expression of hGPR35 was detected every two weeks using zaprinast as the probe in DMR assays.

DMR Assays Using Epic BT System

All DMR assays were performed using an Epic BT system (Corning Incorporated). Epic is a swept wavelength interrogation reader system tailored for resonant waveguide grating biosensors in microtiter plates. Cells were directly seeded in Epic plates and cultured overnight to form a confluent monolayer in the cell culture medium. After being washed, the cells were maintained in Hank's Balanced Salt Solution and further incubated inside the system for 1 h. For agonist profiling, a 2 min baseline was established. After a compound was added, cellular responses were recorded immediately. For desensitization assays, cells were initially treated with compounds for 1 h, followed by stimulation with zaprinast at 1μM for HT-29 or 400 nM for CHO-K1-hGPR35. The

cellular responses were recorded throughout the assays. All EC_{50} or IC_{50} described in the main text were calculated based on the amplitudes of DMR signals at 8 min post-stimulation. All GPR35 agonists led to a sustained positive-DMR signal. The data represented mean ± sd from two independent measurements, each with four replicates (n=8).

BRET-based GPR35 Binding Assays.

NLuc-GPR35 plasmid DNA was purchased from Sandon Biotech. The sequence of the DNA construct was showed in the supporting information. For BRET-based binding assays, CHO-K1 cells were seeded into 96-well plates at a density of 40,000 cells per well (in F12K supplemented with 10% FBS (PAN)) at 37°C prior to the experiment. After 24 h, cells were transfected with NLuc-GPR35 using Lipofectamine 3000 (Invitrogen) at a 3:1 lipid/DNA ratio, with 100 ng of DNA per well. Twenty-four h post-transfection, the medium was removed from each well and replaced with HBSS containing the required concentration of the fluorescent ligand.

For BRET-based equilibrium binding assay, serially diluted fluorescent probe (for total binding) and zaprinast (10 μM, final concentration, for nonspecific binding) were prepared in HBSS and added to the cells. After 30 min of incubation at room temperature, substrate furimazine (10 μM, final concentration) was added, and BRET signals were recorded immediately.

For competition binding assay, **15** at a fixed concentration and a competitor compound at various dilutions were added to the cells separately for 30 min incubation at room temperature. After addition of the substrate, BRET signals were recorded.

For kinetic assay, substrate furimazine (10 μM, final concentration) was added to the cells prior to the experiment and incubated at room temperature in the plate reader for 5 min to balance the signal before the measurement. Then the injector module started the association with 5 nM **15** and measurement for 10 min. Then 10 μM ML145 was added to start the dissociation procedure. The measurement continued for 15 min.

All measurements were performed on a BioTek Cytation5 plate reader at room temperature using 485±20 nm and 528±20 nm filters with an integration time of 1 s per data point for both channels. For all experiments, specific binding was calculated by subtraction of nonspecific binding from total binding.

Data Analysis.

All data were analyzed using GraphPad Prism 6. Total and nonspecific saturation binding curves were created using the following equation of BRET ratio:

$$BRET\ ratio = \frac{B_{max} \times [B]}{[B] + K_d} + ((M \times [B]) + C)$$

where B_{max} is the maximal response, $[B]$ is the concentration of the fluorescent ligand in nM, K_D is the equilibrium dissociation constant in nM, M is the slope of the nonspecific binding component, and C is the intercept with the y axis. The Cheng−Prusoff equation was used to calculate the K_i of the unlabeled ligands fitted in the competition binding curves:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

where $[L]$ is the concentration of the fluorescent ligand in nM and K_d is the K_d of fluorescent ligand in nM. The calculated K_d values used were as calculated from the saturation binding experiments.

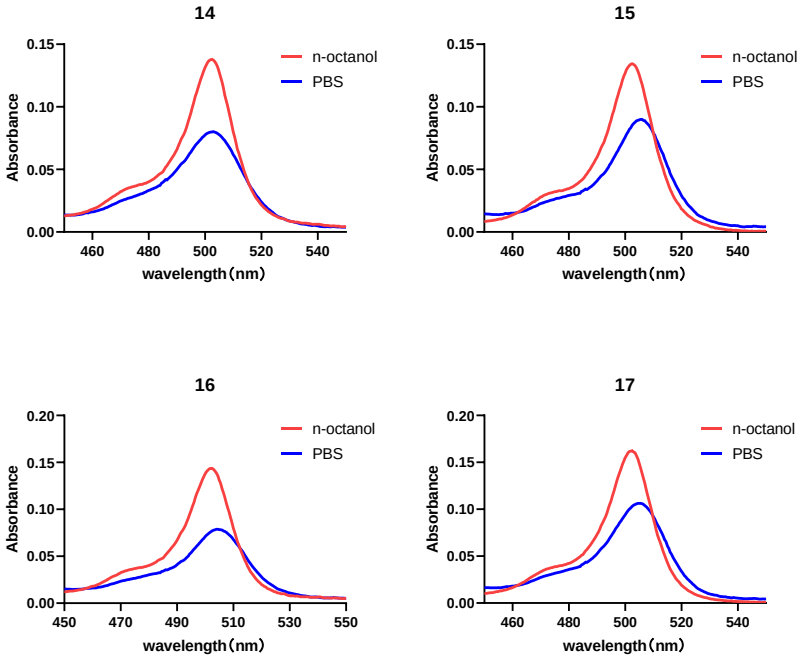

Fig. S1 Absorption spectra of probes in n-octanol and PBS (2μM).

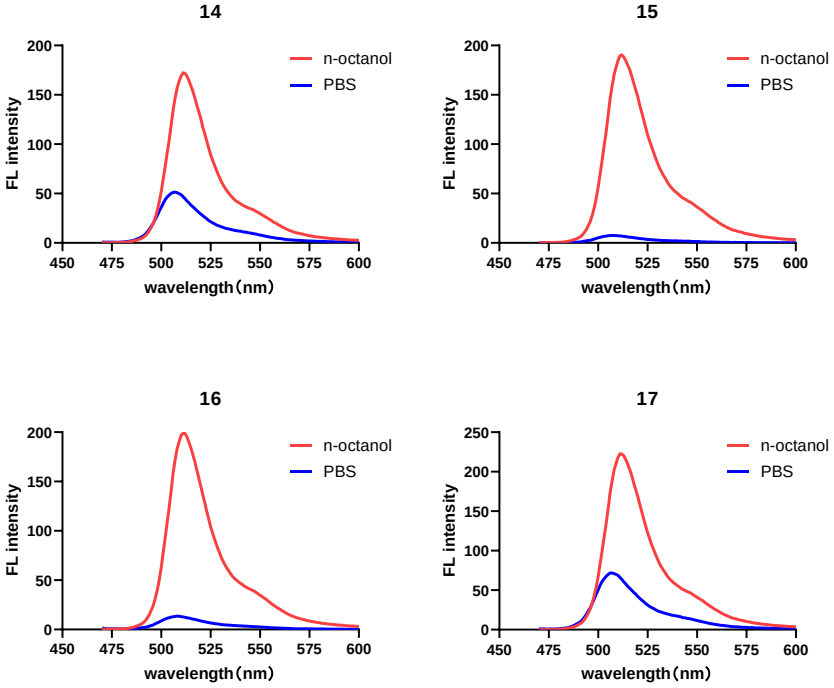

Fig. S2 Emission spectra of probes at different concentration in n-octanol and PBS (2μM).

Table S1. Fluorescence characterization of probes 14–17.

Compound	*n*-octanol				PBS	
	λ_{abs} (nm)	λ_{em} (nm)	SS[a] (nm)	Φ[b]	λ_{abs} (nm)	λ_{em} (nm)
14	502	512	10	0.49	503	507
15	502	512	10	0.77	505	508
16	502	512	10	0.58	504	508
17	502	512	10	0.72	505	507

[a]SS: Stokes shift. [b]Φ: relative quantum yield.

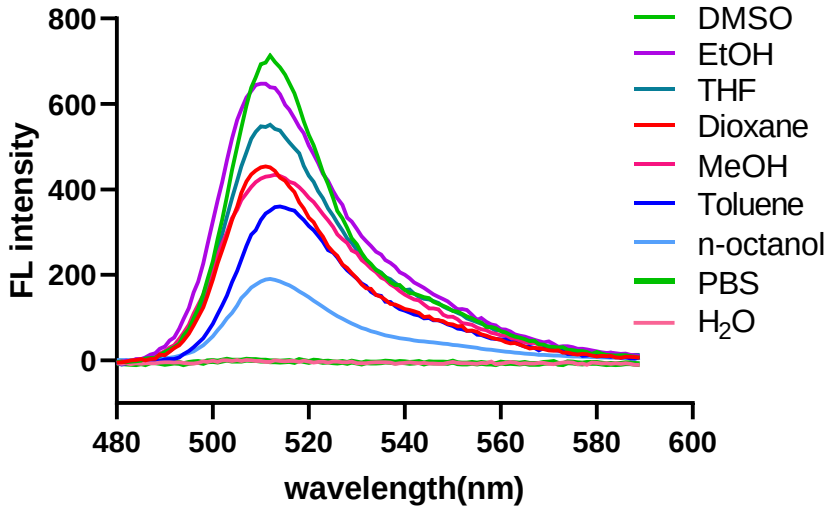

Fig. S3 Emission spectra of 15 obtained in different polarity solvents

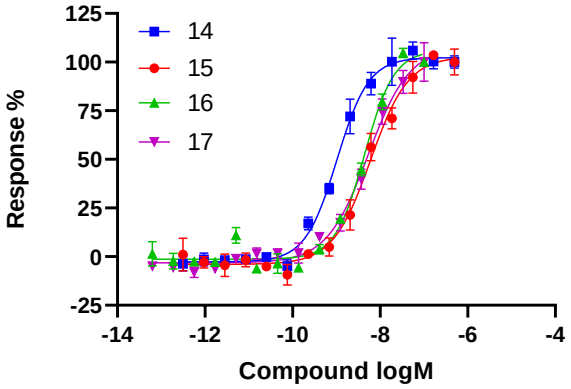

Fig. S4. Amplitudes of the DMR induced by fluorescent probes 14-17 as a function of concentrations. The data respresents mean ± sd from two independent measurements, each with four replicates (n = 8)

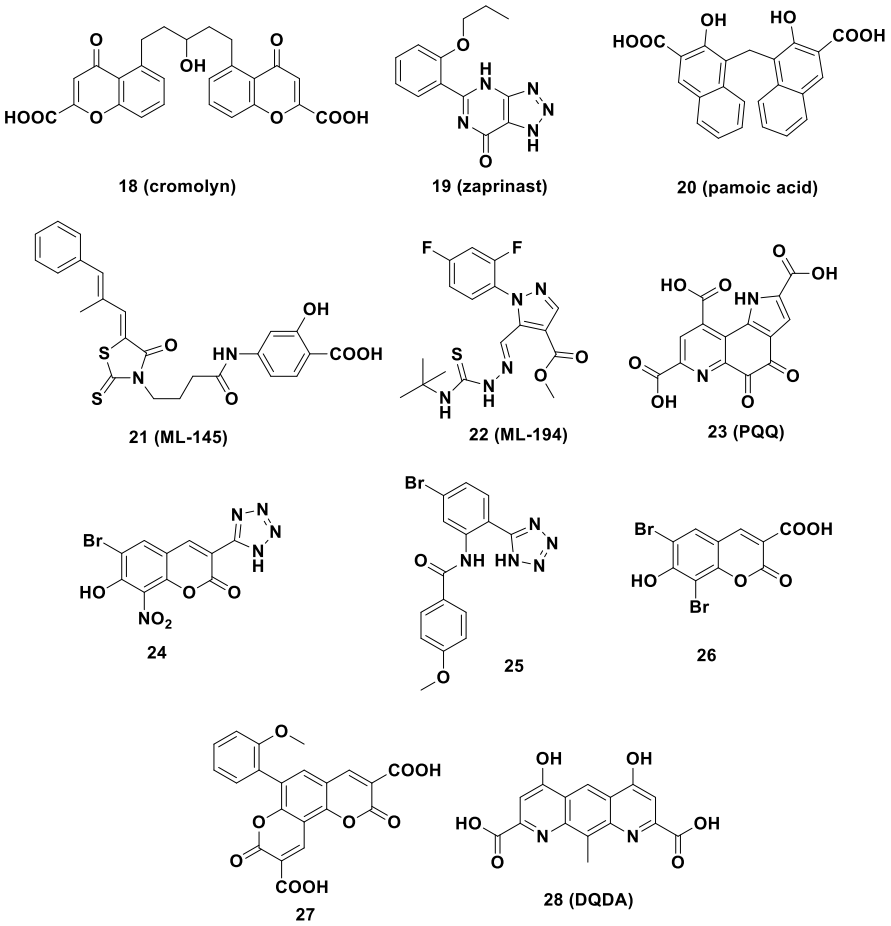

Fig. S5. Structures of the selected GPR35 ligands.

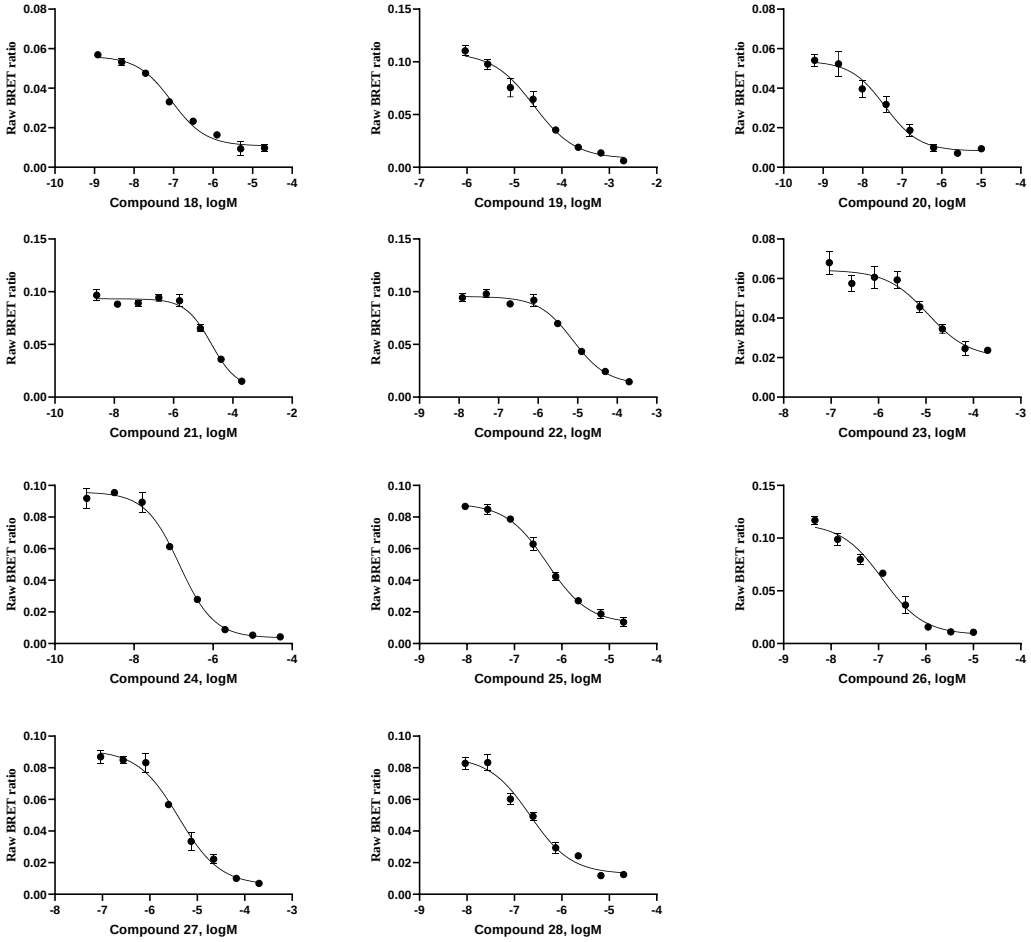

Fig. S6 Competitive binding of selected compounds 18-28 with probe 15 (25nM) on GPR35. The data respresents mean ± sd from three independent measurements, each with three replicates (n = 9).

GPR35a-Nluc

GPR35a

TCCGGAatggtccttctgttgatcctgtcagtcttacttttgaaagaagatgtccgtgggGTCTTCACACTCG
AAGATTTCGTTGGGGACTGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTTG
AACAGGGAGGTGTGTCCAGTTTGTTTCAGAATCTCGGGGTGTCCGTAACTCCGATCC
AAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCC
CGTATGAAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGTGG
TGTACCCTGTGGATGATCATCACTTTAAGGTGATCCTGCACTATGGCACACTGGTAA
TCGACGGGGTTACGCCGAACATGATCGACTATTTCGGACGGCCGTATGAAGGCATCG
CCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACGGCAACAAAA
TTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAGTAACCATCAA
CGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGgatcatcaggaaatggcaccta
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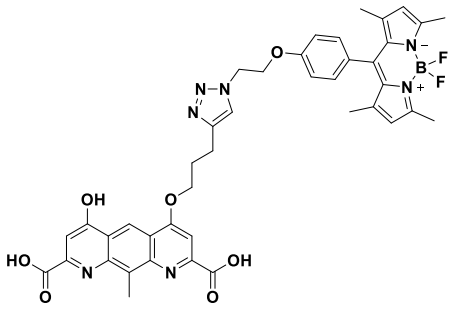
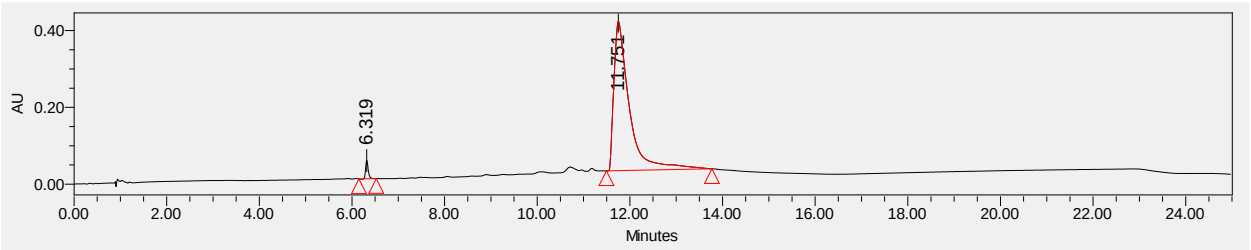
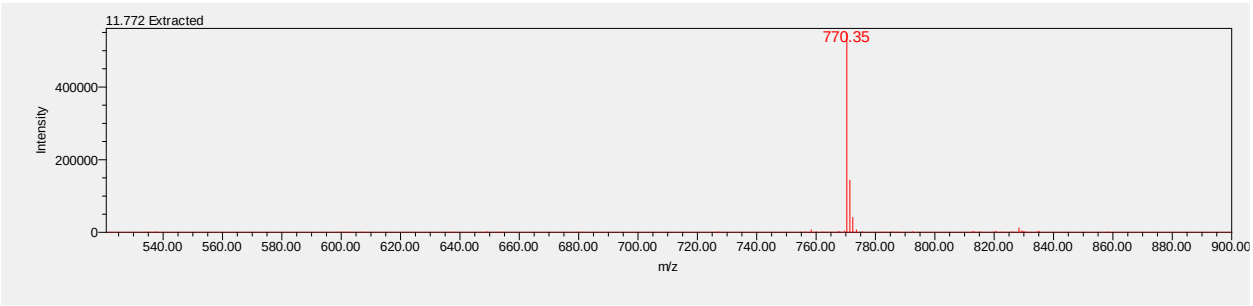


14





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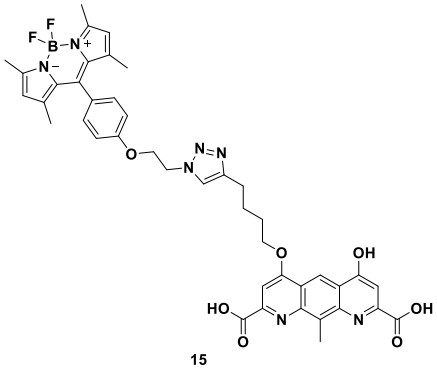

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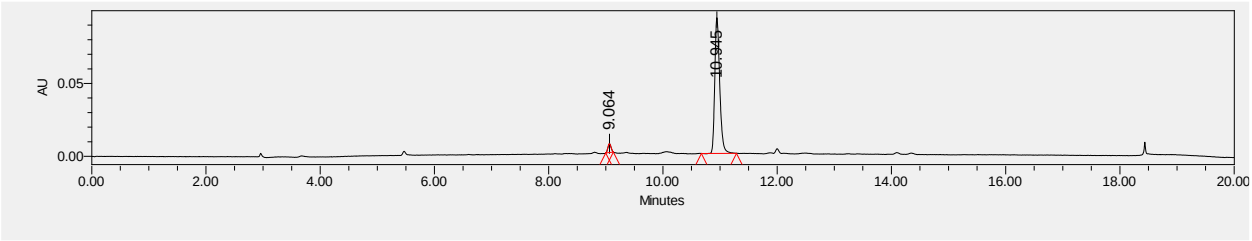

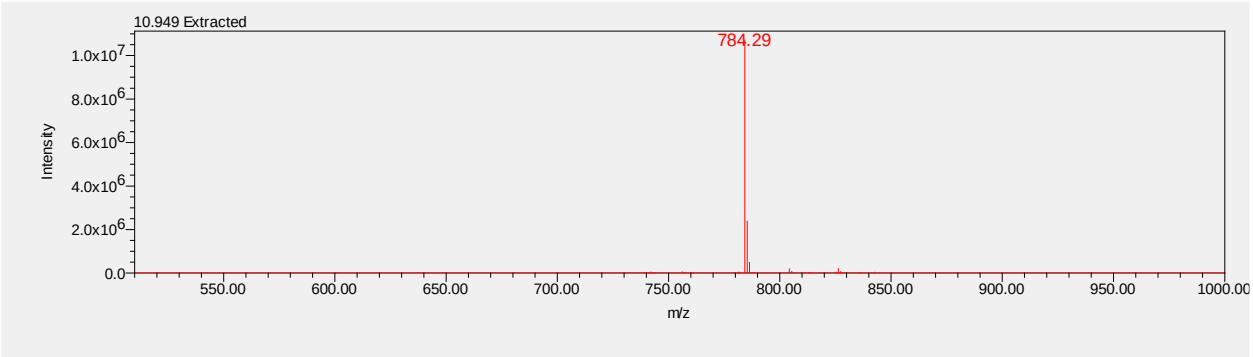

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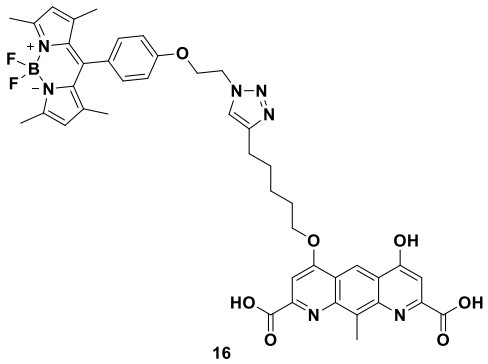
2	10.945	728349	96.39	120804	bb



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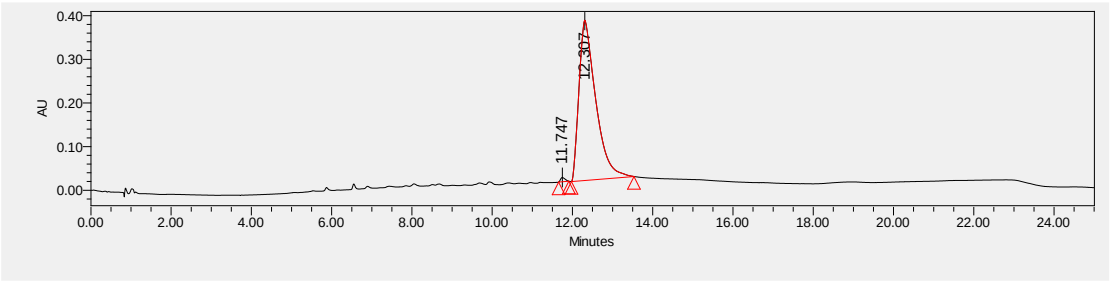

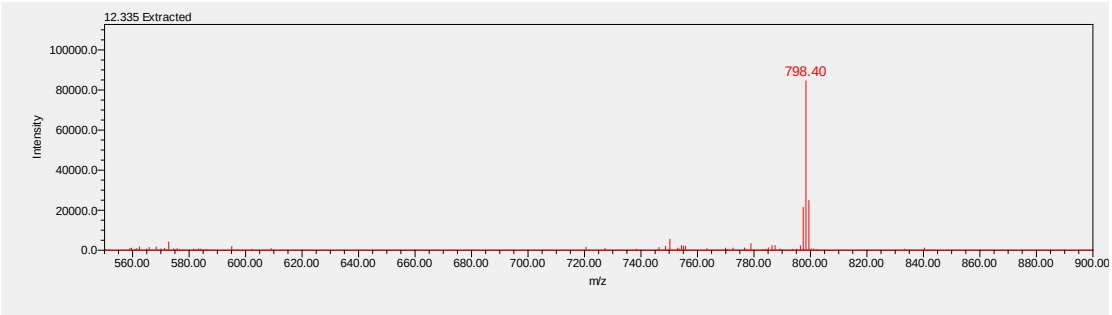

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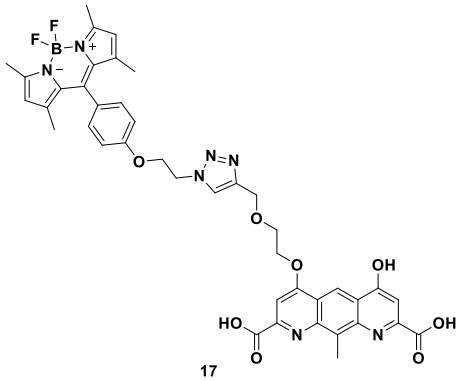

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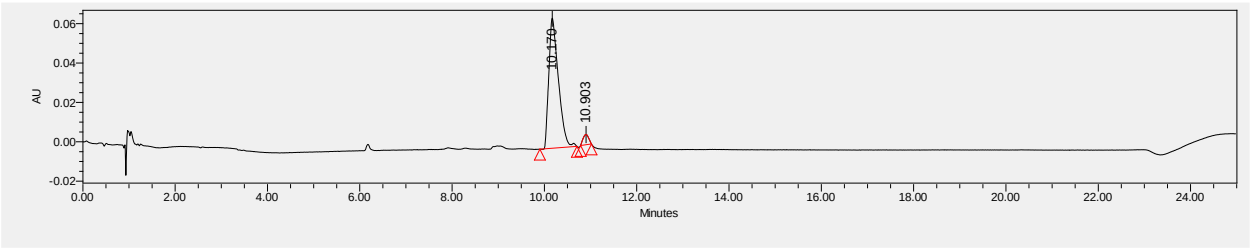
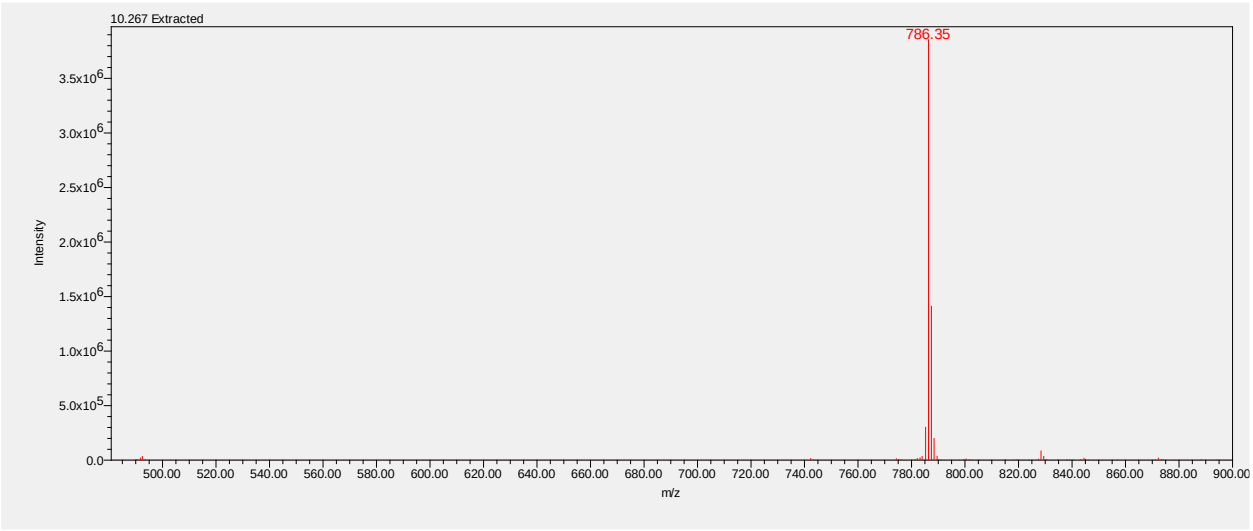
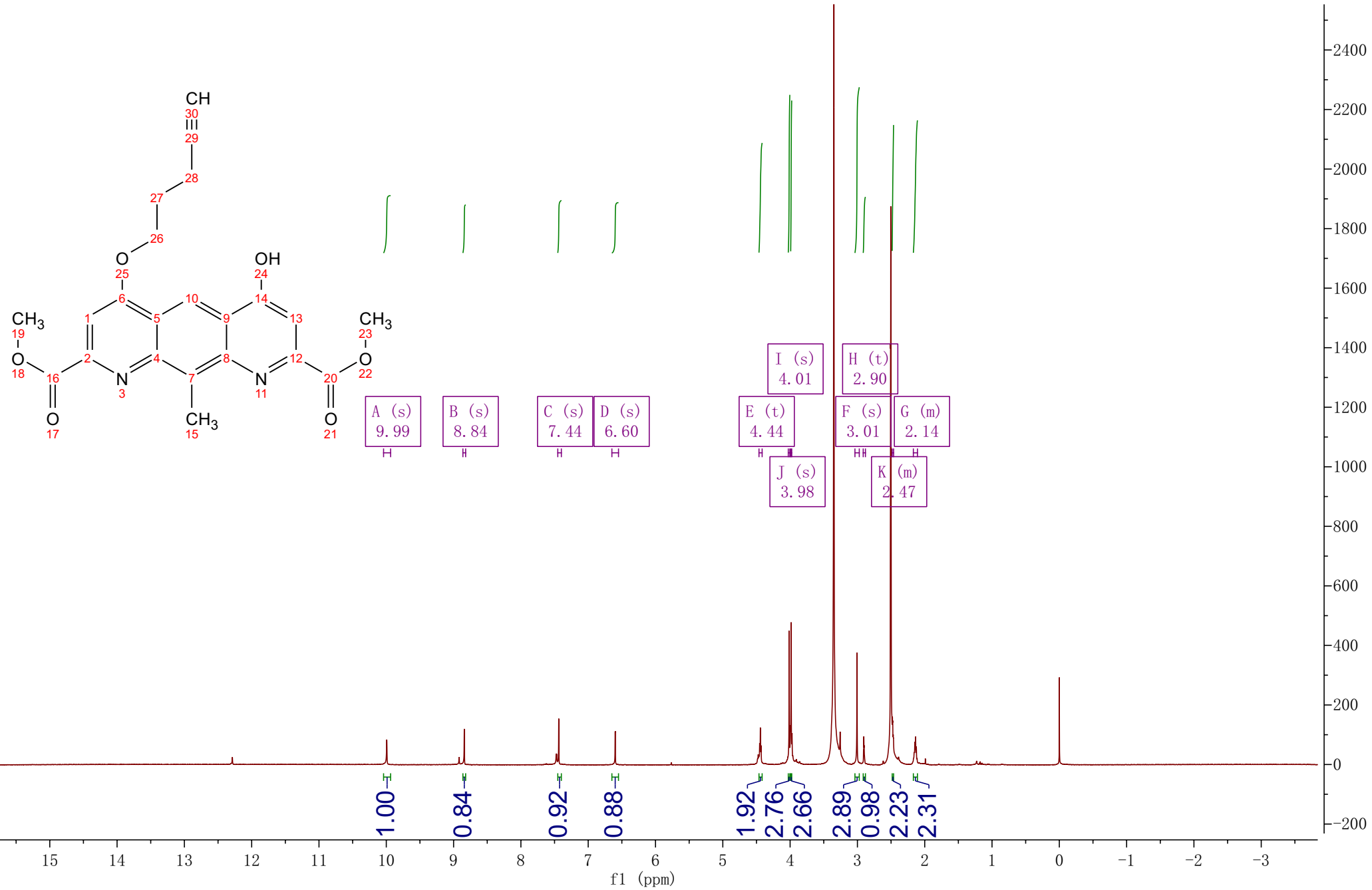


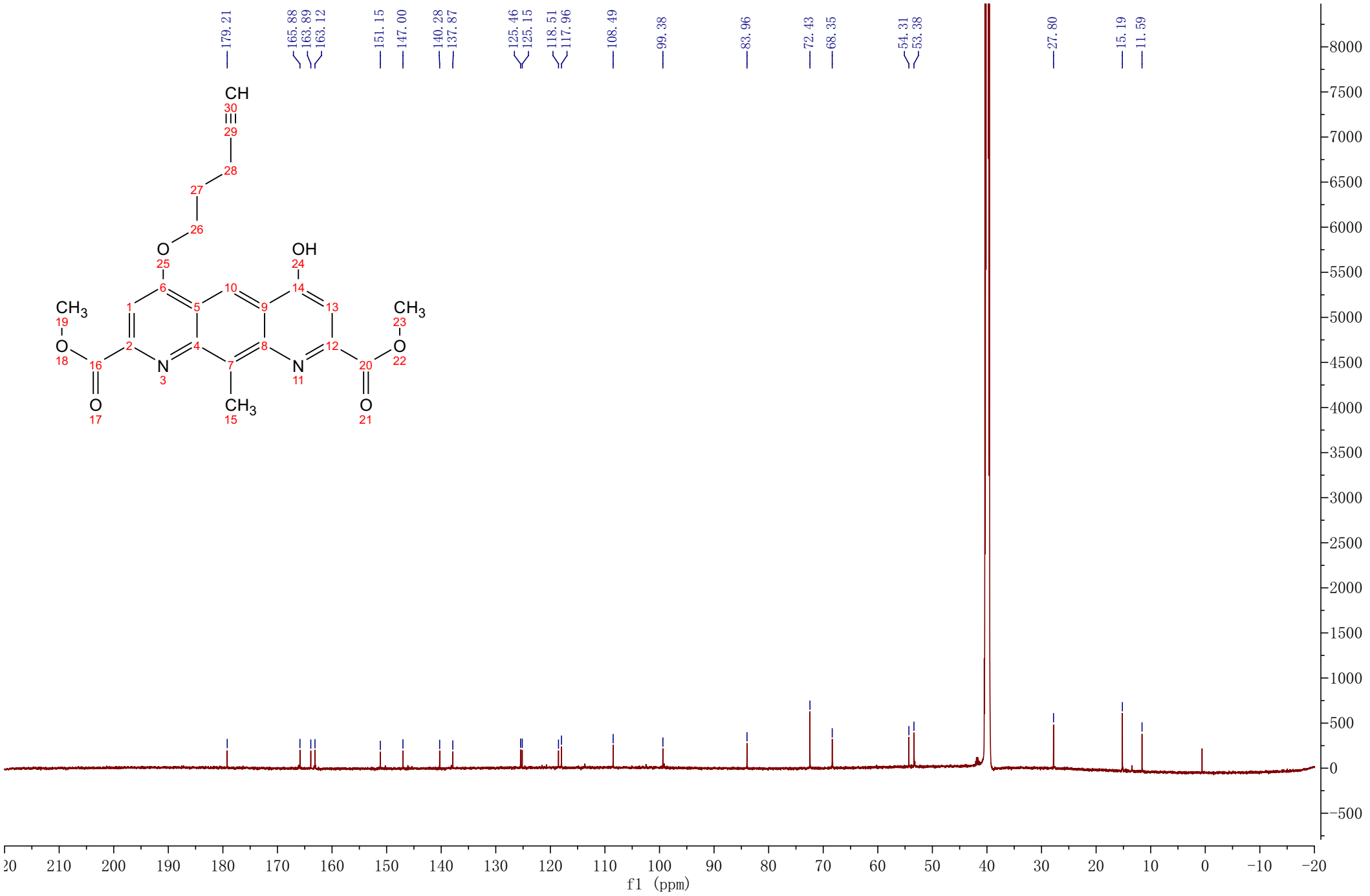


	Retention Time	Area	% Area	Height	Int Type
1	10.170	953475	95.60	66024	bb
2	10.903	43856	4.40	5203	bb

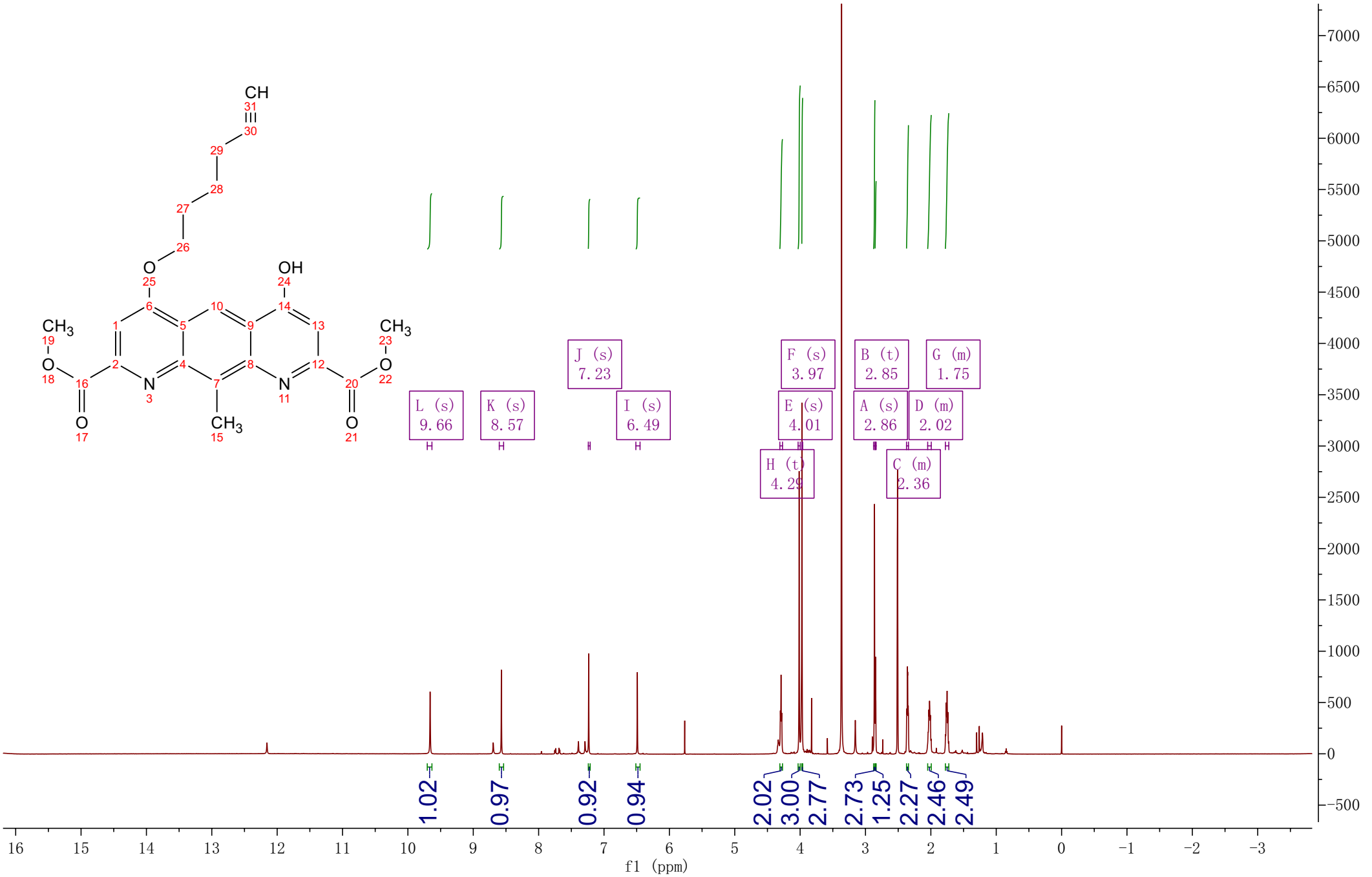
^{1}H NMR (600 MHz, DMSO) δ 9.99 (s, 1H), 8.84 (s, 1H), 7.44 (s, 1H), 6.60 (s, 1H), 4.44 (t, J = 6.1 Hz, 2H), 4.01 (s, 3H), 3.98 (s, 3H), 3.01 (s, 3H), 2.90 (t, J = 2.5 Hz, 1H), 2.48 – 2.46 (m, 2H), 2.17 – 2.11 (m, 2H).



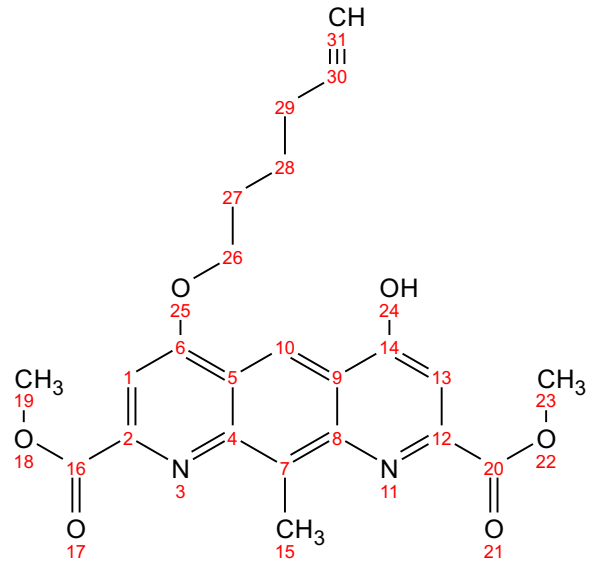
^{13}C NMR (151 MHz, DMSO) δ 179.21, 165.88, 163.89, 163.12, 151.15, 147.00, 140.28, 137.87, 125.46, 125.15, 118.51, 117.96, 108.49, 99.38, 83.96, 72.43, 68.35, 54.31, 53.38, 27.80, 15.19, 11.59.



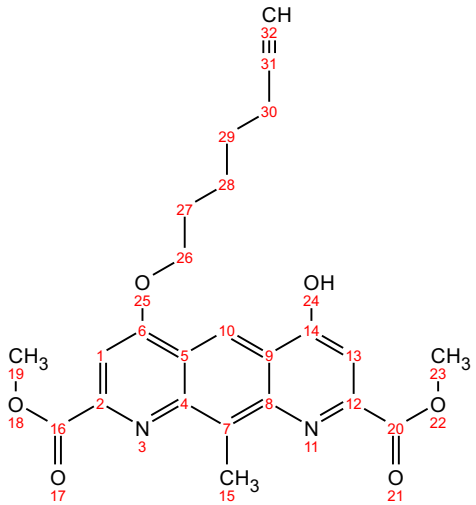
^{1}H NMR (600 MHz, DMSO) δ 9.66 (s, 1H), 8.57 (s, 1H), 7.23 (s, 1H), 6.49 (s, 1H), 4.29 (t, J = 6.2 Hz, 2H), 4.01 (s, 3H), 3.97 (s, 3H), 2.86 (s, 3H), 2.85 (t, J = 2.4 Hz, 1H), 2.37 – 2.34 (m, 2H), 2.05 – 1.99 (m, 2H), 1.78 – 1.73 (m, 2H).



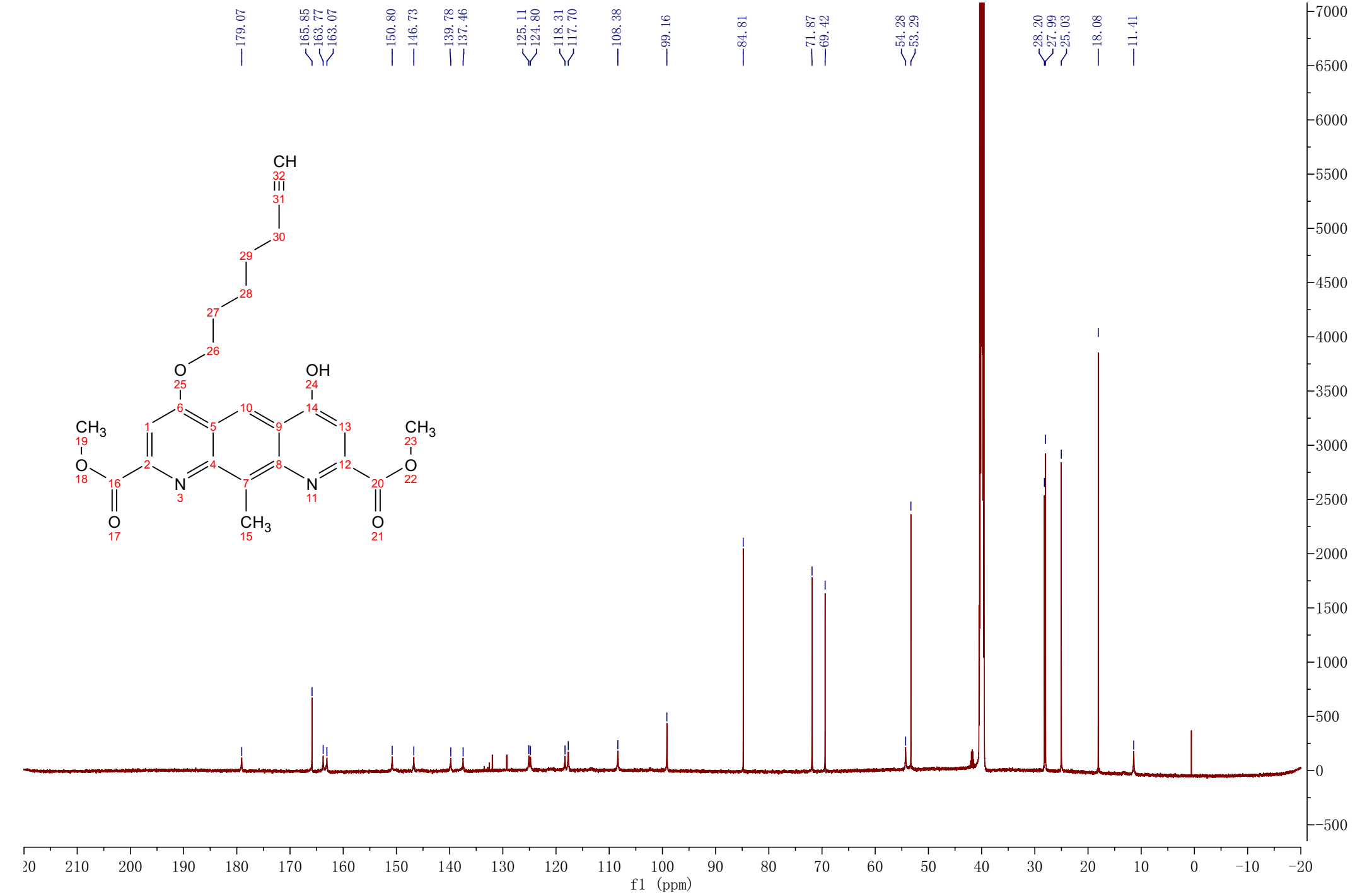
^{13}C NMR (151 MHz, DMSO) δ 179.00, 165.78, 163.66, 163.02, 150.66, 146.63, 139.61, 137.34, 132.13, 129.16, 125.01, 124.69, 118.16, 117.57, 108.34, 99.11, 84.73, 72.04, 68.99, 54.29, 53.29, 27.95, 25.09, 17.94, 11.33.



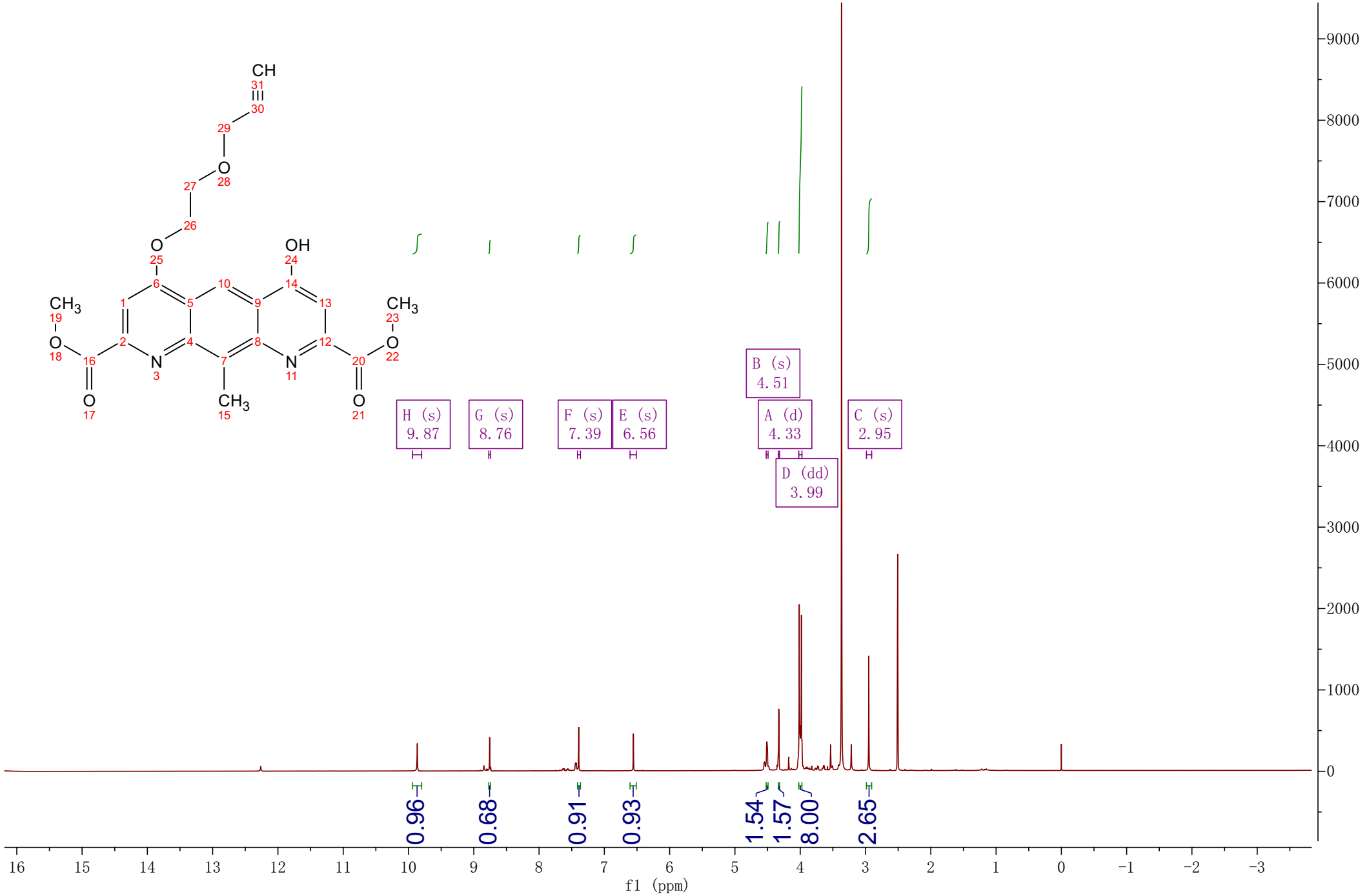
^{1}H NMR (600 MHz, DMSO) δ 9.75 (s, 1H), 8.64 (s, 1H), 7.29 (s, 1H), 6.51 (s, 1H), 4.29 (s, 2H), 4.01 (s, 3H), 3.98 (s, 3H), 2.90 (s, 3H), 2.81 (s, 1H), 2.26 (td, *J* = 6.5, 2.5 Hz, 2H), 1.98 – 1.91 (m, 2H), 1.68 – 1.58 (m, 4H).



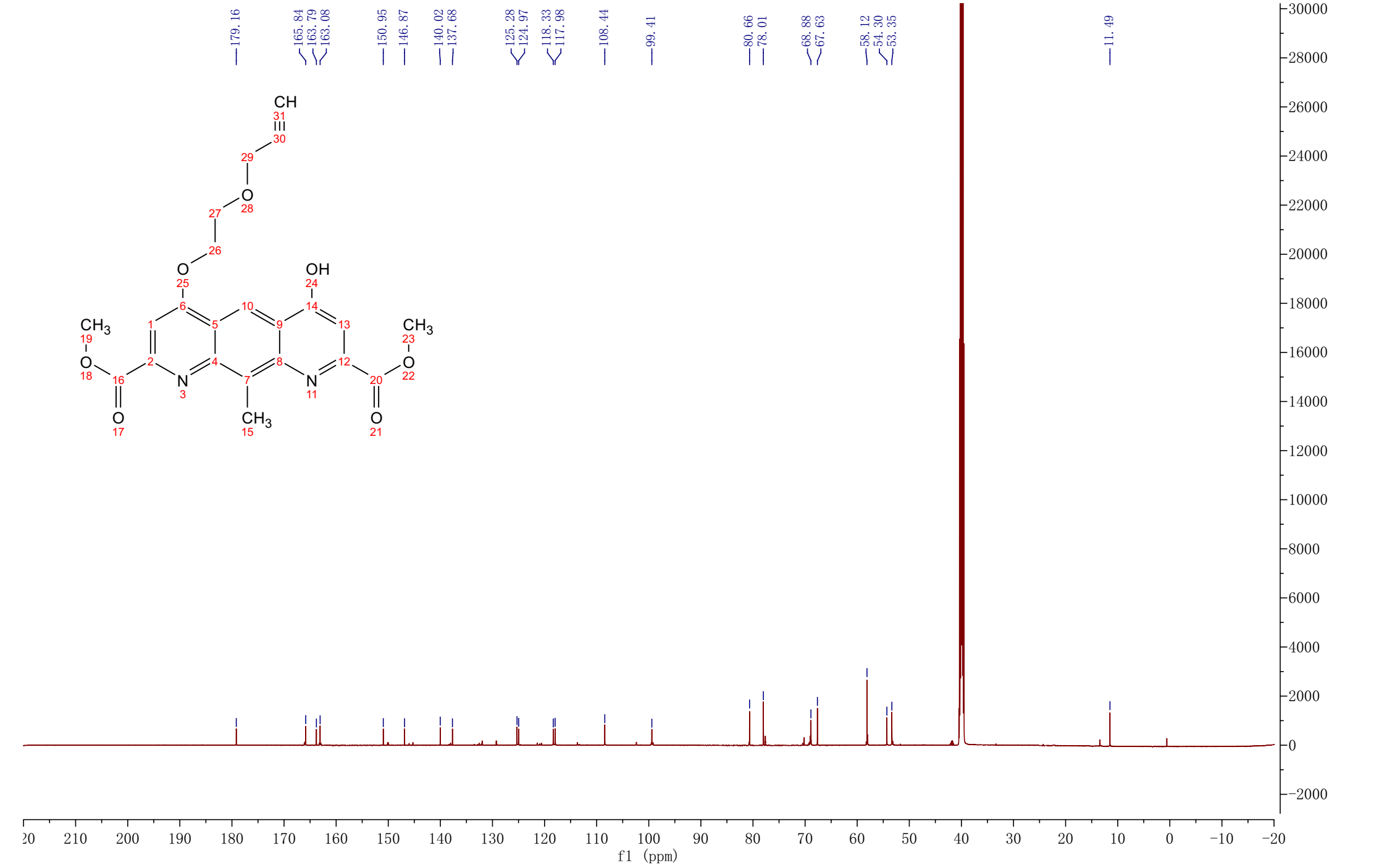
^{13}C NMR (151 MHz, DMSO) δ 179.07, 165.85, 163.77, 163.07, 150.80, 146.73, 139.78, 137.46, 125.11, 124.80, 118.31, 117.70, 108.38, 99.16, 84.81, 71.87, 69.42, 54.28, 53.29, 28.20, 27.99, 25.03, 18.08, 11.41.



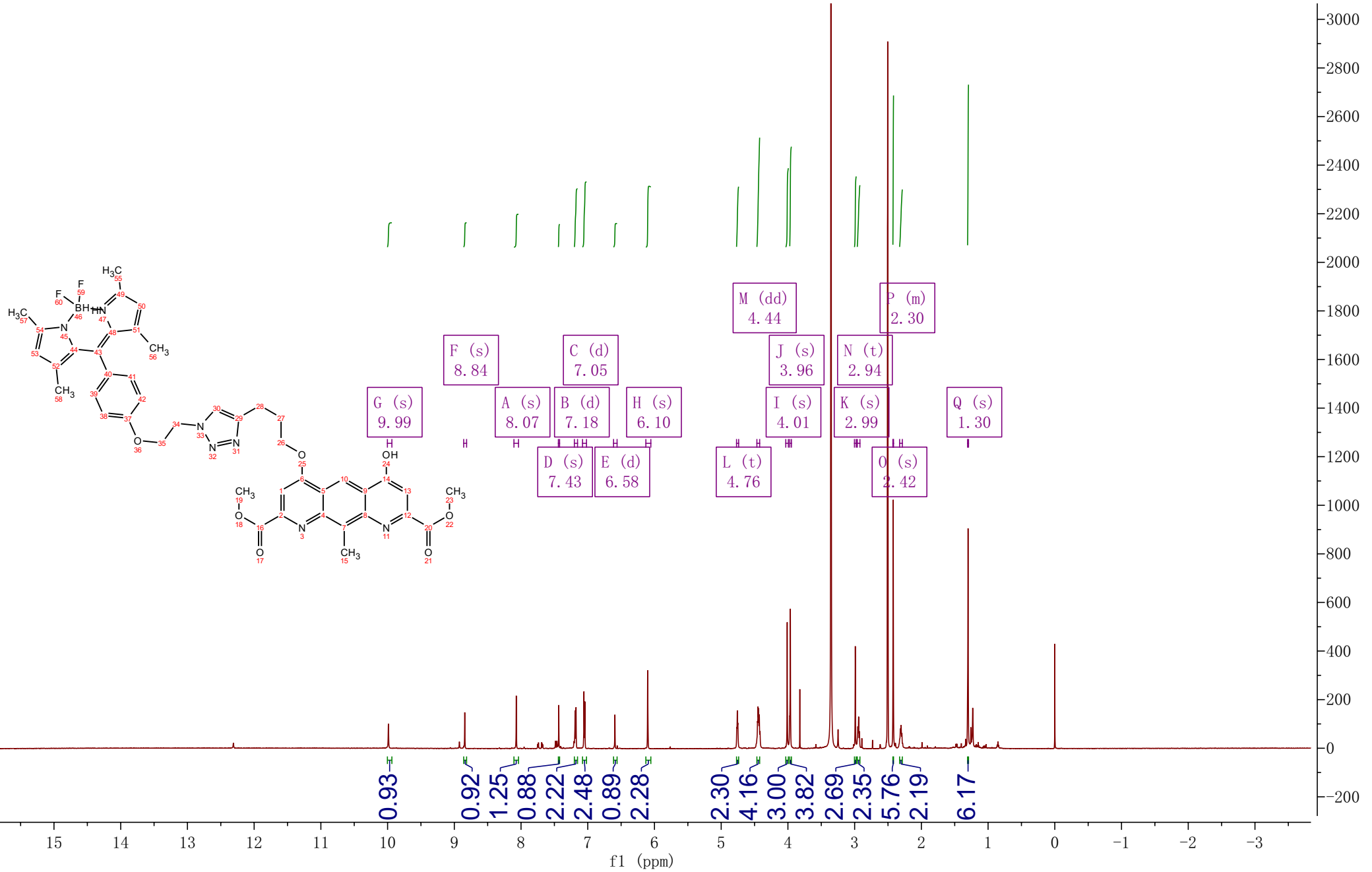
^{1}H NMR (600 MHz, DMSO) δ 9.87 (s, 1H), 8.76 (s, 1H), 7.39 (s, 1H), 6.56 (s, 1H), 4.51 (s, 2H), 4.33 (d, J = 2.1 Hz, 2H), 3.99 (dd, J = 17.2, 7.1 Hz, 8H), 2.95 (s, 3H).



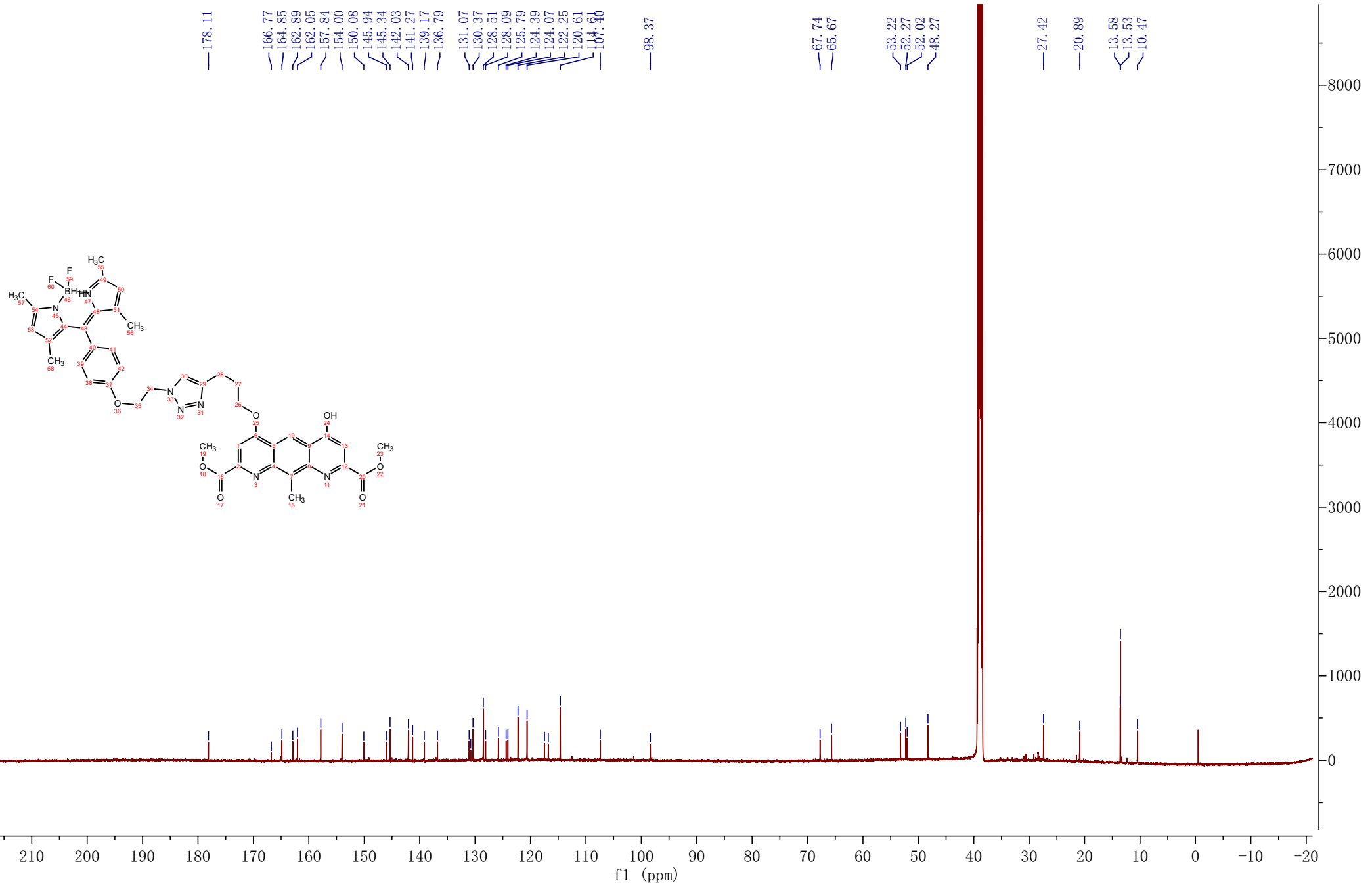
^{13}C NMR (151 MHz, DMSO) δ 179.16, 165.84, 163.79, 163.08, 150.95, 146.87, 140.02, 137.68, 125.28, 124.97, 118.33, 117.98, 108.44, 99.41, 80.66, 78.01, 68.88, 67.63, 58.12, 54.30, 53.35, 11.49.



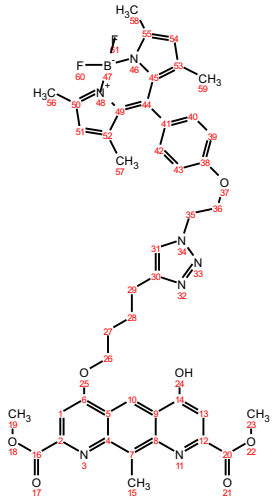
^{1}H NMR (600 MHz, DMSO) δ 9.99 (s, 1H), 8.84 (s, 1H), 8.07 (s, 1H), 7.43 (s, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.6 Hz, 2H), 6.58 (d, *J* = 19.1 Hz, 1H), 6.10 (s, 2H), 4.76 (t, *J* = 4.8 Hz, 2H), 4.44 (dd, *J* = 10.5, 5.6 Hz, 4H), 4.01 (s, 3H), 3.96 (s, 4H), 2.99 (s, 3H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.42 (s, 6H), 2.33 – 2.29 (m, 2H), 1.30 (s, 6H).



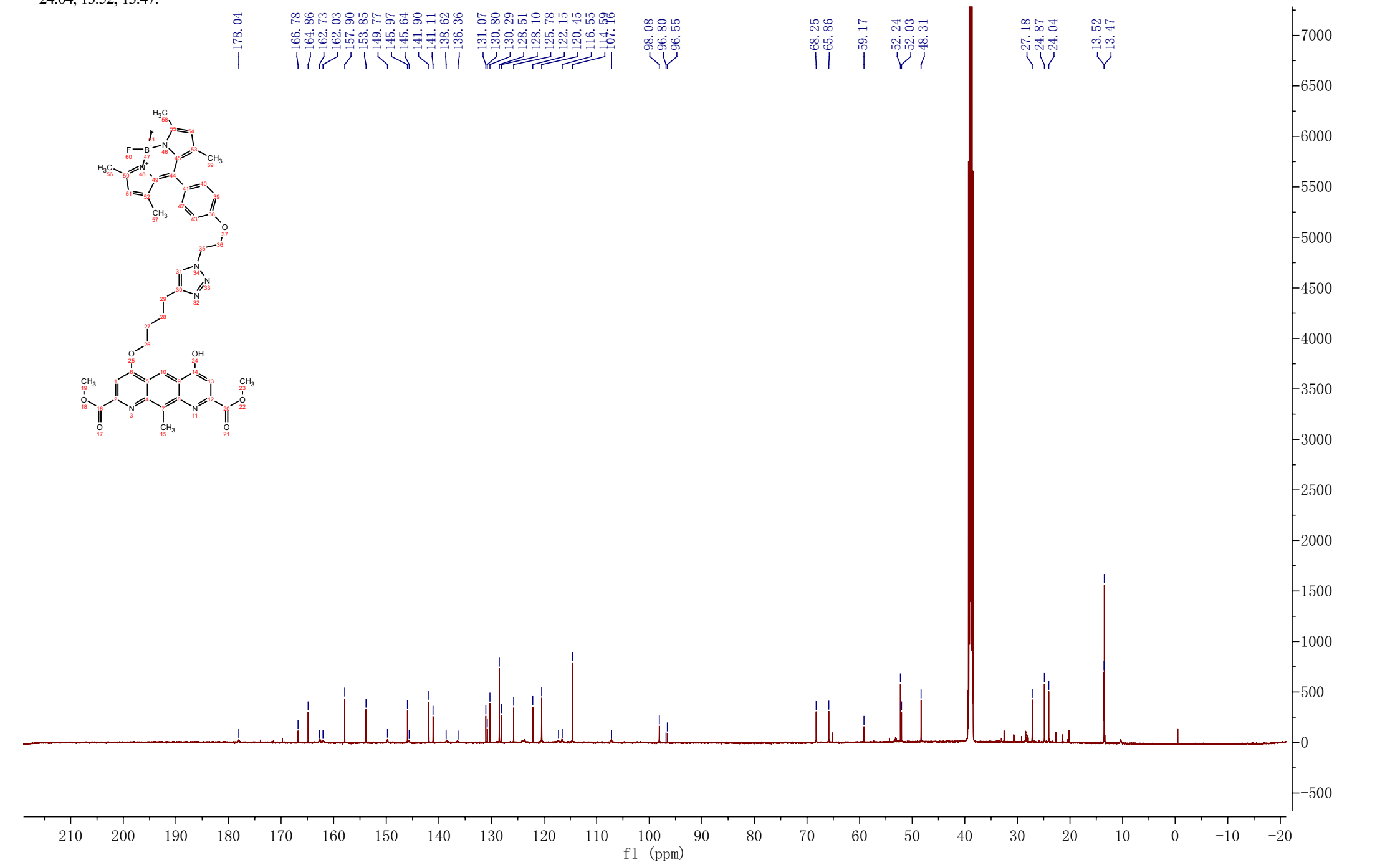
^{13}C NMR (151 MHz, DMSO) δ 178.11, 166.77, 164.85, 162.89, 162.05, 157.84, 154.00, 150.08, 145.94, 145.34, 142.03, 141.27, 139.17, 136.79, 131.07, 130.79, 130.37, 128.51, 128.09, 125.79, 124.39, 124.07, 122.25, 120.61, 117.47, 116.78, 114.61, 107.40, 98.37, 67.74, 65.67, 53.22, 52.27, 52.02, 48.27, 27.42, 20.89, 13.58, 13.53, 10.47.



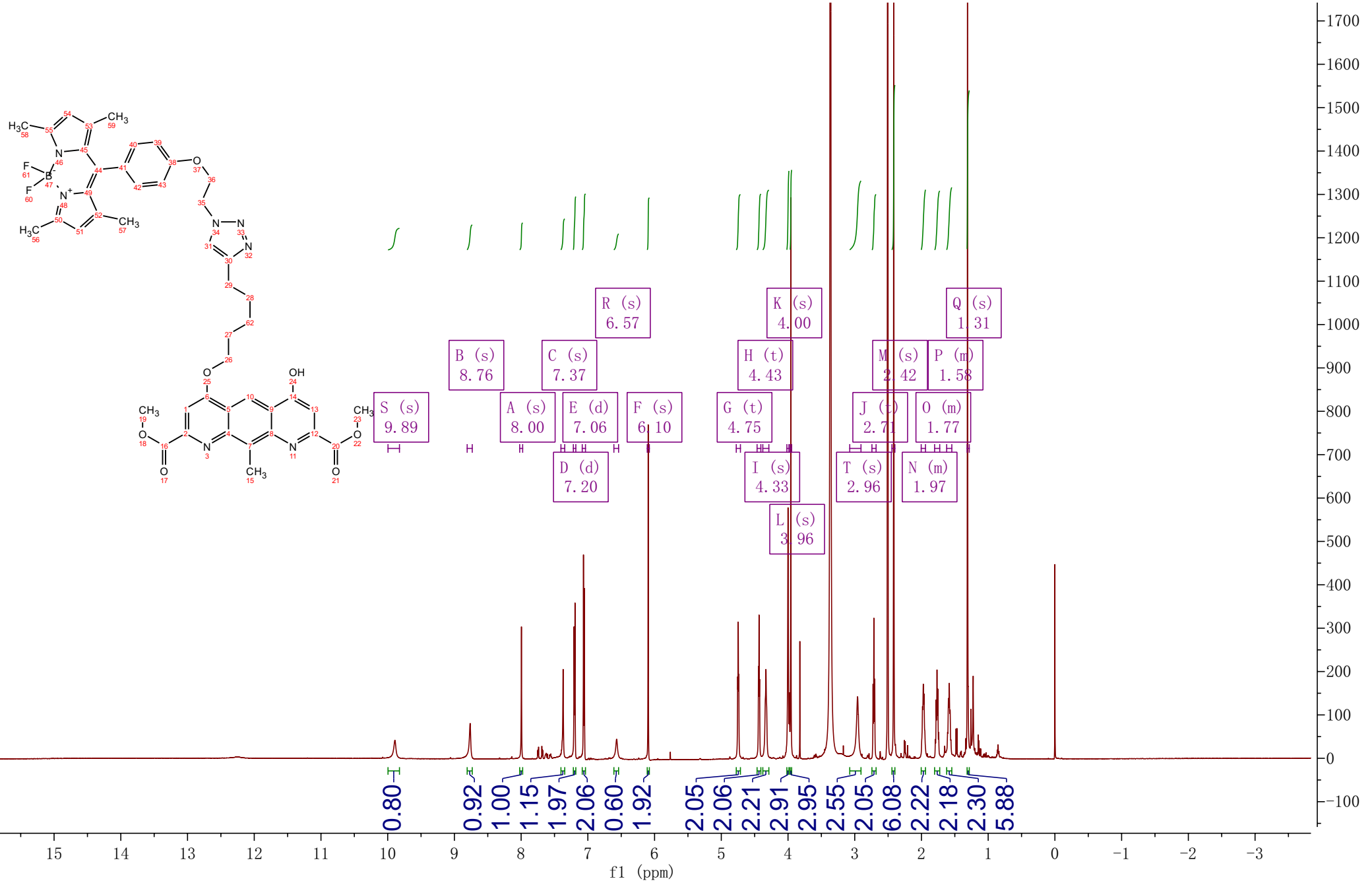
^{1}H NMR (400 MHz, DMSO) δ 8.73 (s, 1H), 8.02 (s, 1H), 7.35 (s, 1H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.05 (d, *J* = 8.7 Hz, 2H), 6.49 (s, 1H), 5.99 (s, 2H), 4.76 (t, *J* = 4.9 Hz, 2H), 4.45 (t, *J* = 5.0 Hz, 2H), 4.32 (s, 2H), 4.00 (s, 3H), 3.98 (s, 3H), 2.92 (s, 3H), 2.80 (t, *J* = 7.3 Hz, 2H), 2.38 (s, 6H), 2.00 – 1.94 (m, 2H), 1.92 – 1.83 (m, 2H), 1.25 (s, 6H).



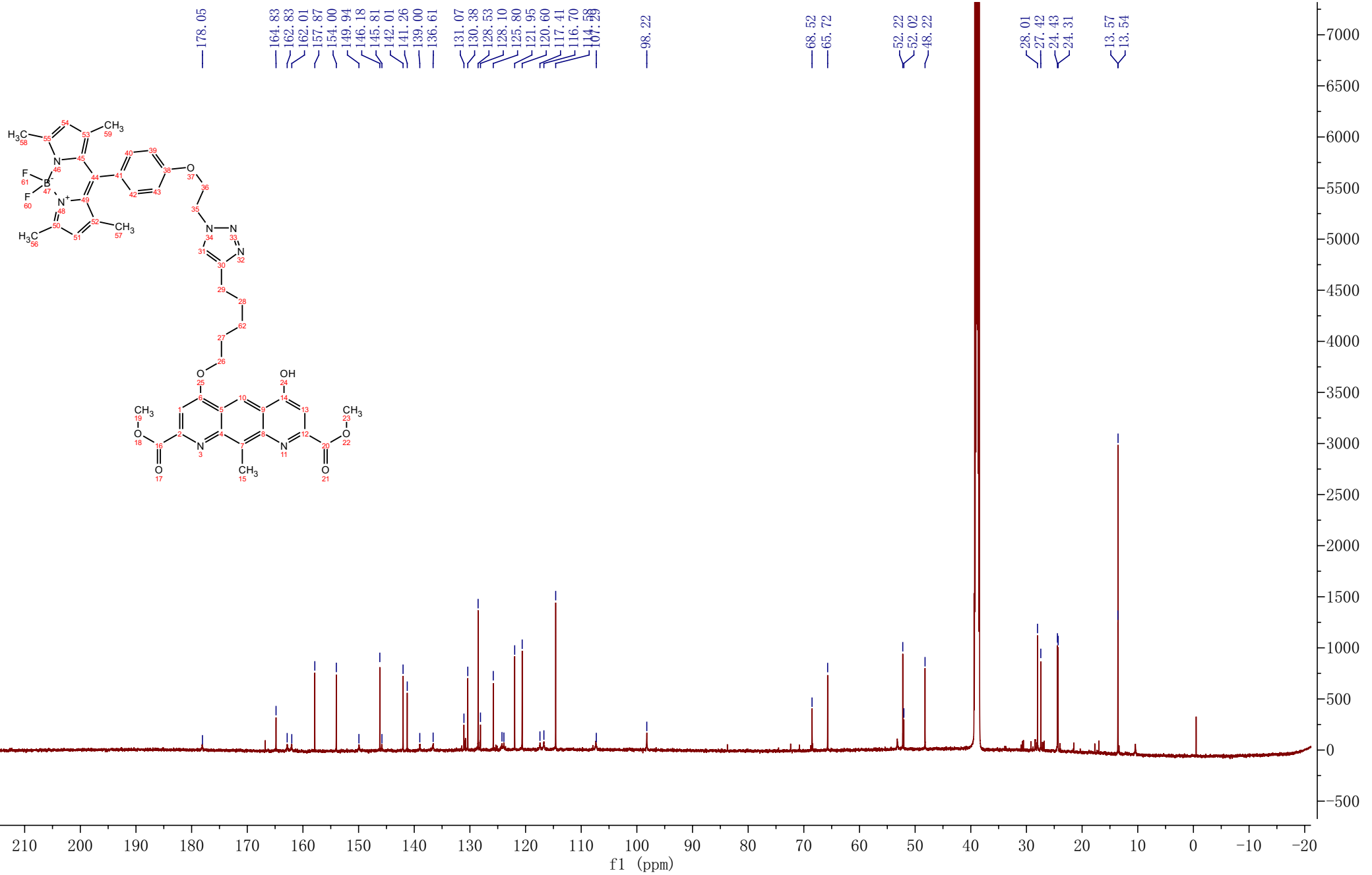
^{13}C NMR (151 MHz, DMSO) δ 178.04, 166.78, 164.86, 162.73, 162.03, 157.90, 153.85, 149.77, 145.97, 145.64, 141.90, 141.11, 138.62, 136.36, 131.07, 130.80, 130.29, 128.51, 128.10, 125.78, 122.15, 120.45, 117.25, 116.55, 114.59, 107.16, 98.08, 96.80, 96.55, 68.25, 65.86, 59.17, 52.24, 52.03, 48.31, 27.18, 24.87, 24.04, 13.52, 13.47.



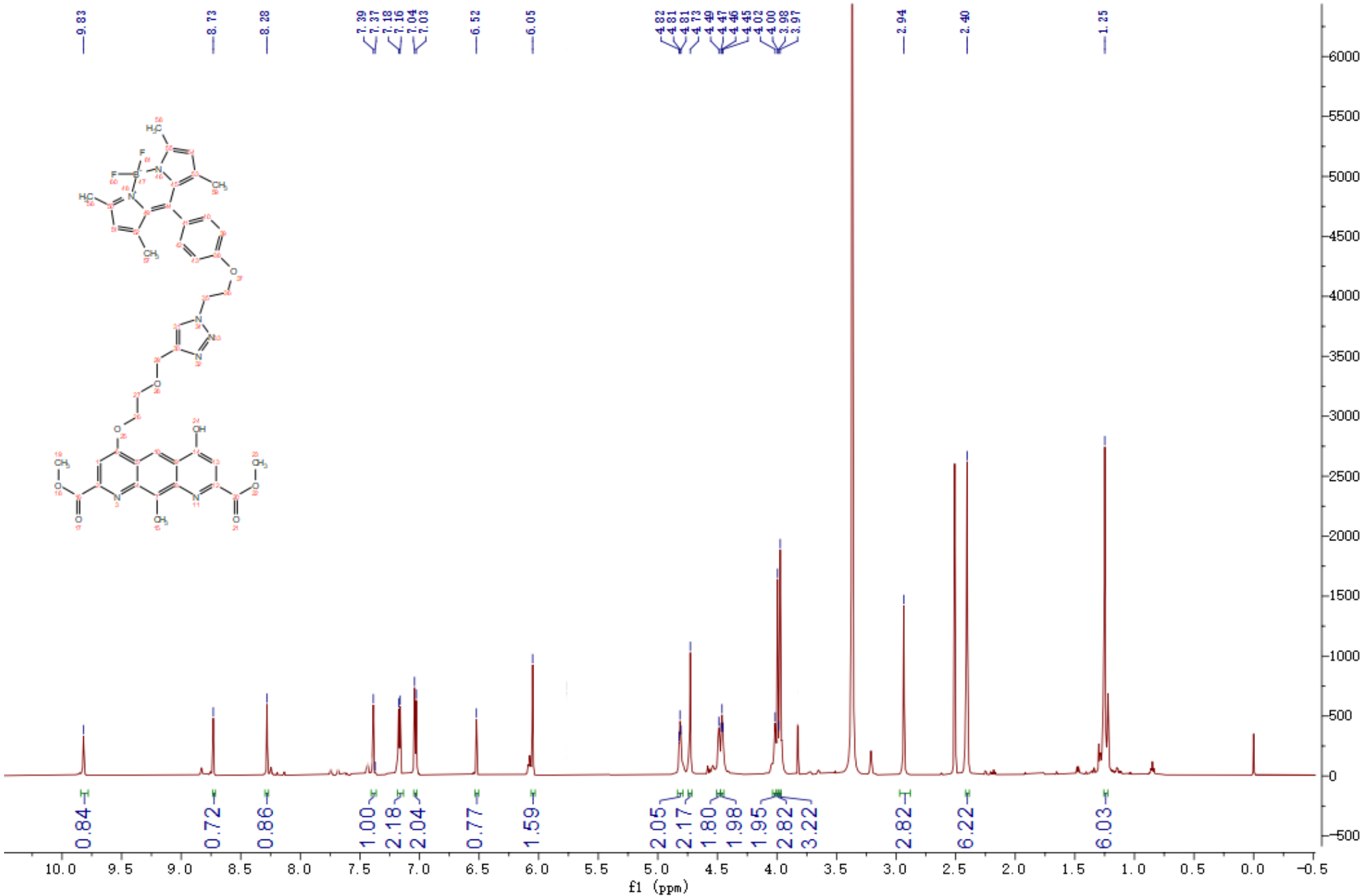
^{1}H NMR (600 MHz, DMSO) δ 9.89 (s, 1H), 8.76 (s, 1H), 8.00 (s, 1H), 7.37 (s, 1H), 7.20 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 8.6 Hz, 2H), 6.57 (s, 1H), 6.10 (s, 2H), 4.75 (t, J = 4.9 Hz, 2H), 4.43 (t, J = 5.0 Hz, 2H), 4.33 (s, 2H), 4.00 (s, 3H), 3.96 (s, 3H), 2.96 (s, 3H), 2.71 (t, J = 7.4 Hz, 2H), 2.42 (s, 6H), 2.00 – 1.94 (m, 2H), 1.80 – 1.73 (m, 2H), 1.62 – 1.55 (m, 2H), 1.31 (s, 6H).



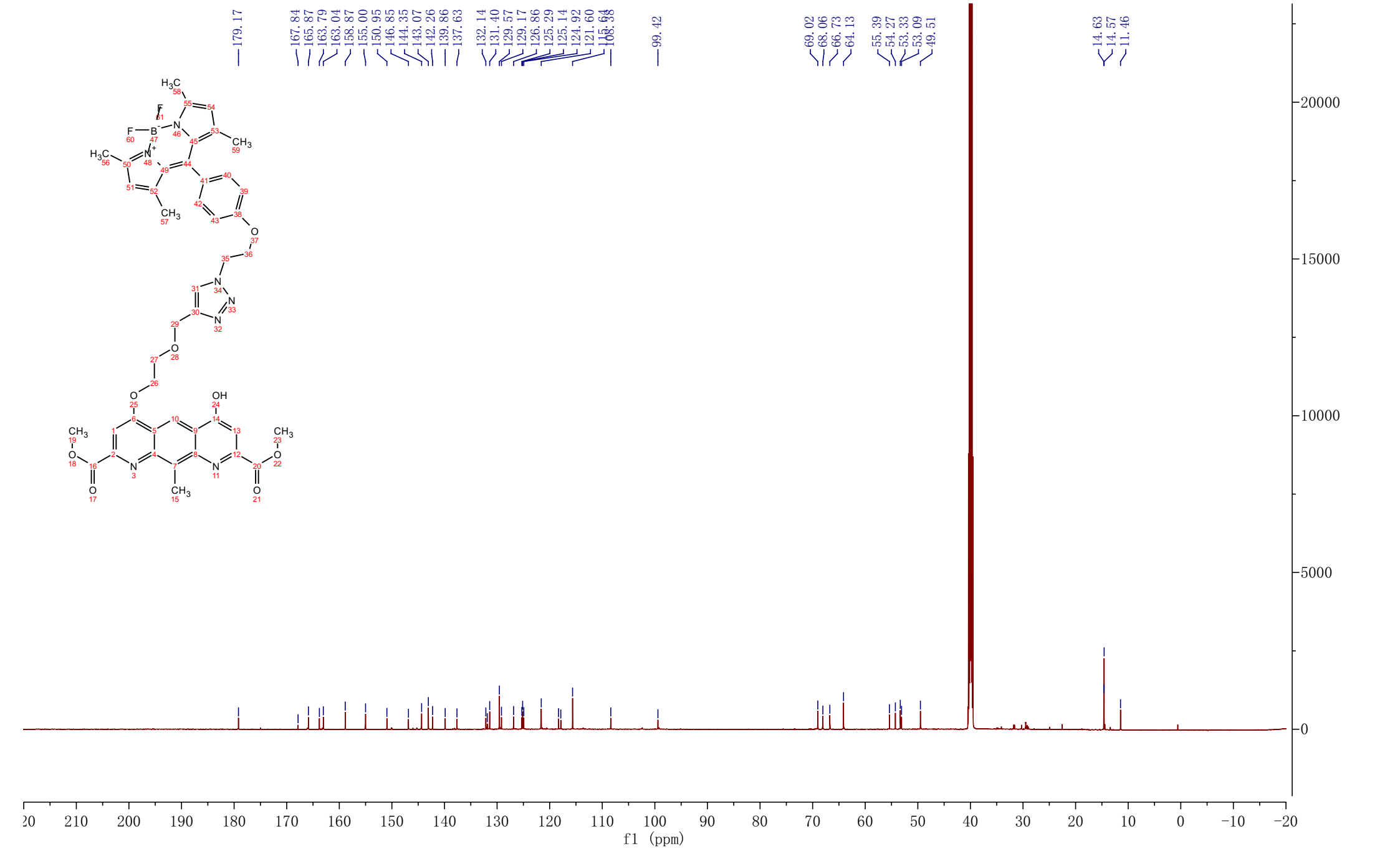
^{13}C NMR (151 MHz, DMSO) δ 178.05, 164.83, 162.83, 162.01, 157.87, 154.00, 149.94, 146.18, 145.81, 142.01, 141.26, 139.00, 136.61, 131.07, 130.38, 128.53, 128.10, 125.80, 124.23, 123.89, 121.95, 120.60, 117.41, 116.70, 114.58, 107.29, 98.22, 68.52, 65.72, 52.22, 52.02, 48.22, 28.01, 27.42, 24.43, 24.31, 13.57, 13.54.



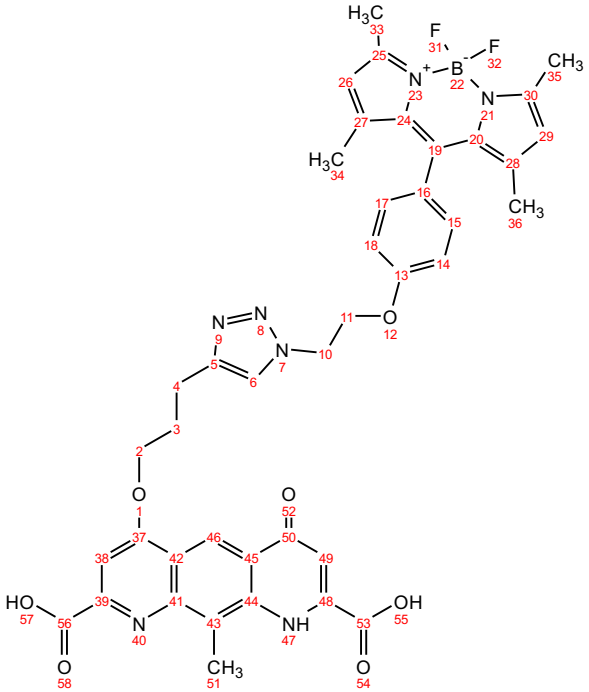
^{1}H NMR (600 MHz, DMSO) δ 9.83 (s, 1H), 8.73 (s, 1H), 8.28 (s, 1H), 7.38 (d, J = 8.5 Hz, 1H), 7.17 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.52 (s, 1H), 6.05 (s, 2H), 4.84 – 4.79 (m, 2H), 4.73 (s, 2H), 4.49 (s, 2H), 4.47 – 4.45 (m, 2H), 4.02 (s, 2H), 3.99 (d, J = 6.4 Hz, 3H), 3.97 (s, 3H), 2.94 (s, 3H), 2.40 (s, 6H), 1.25 (s, 6H).



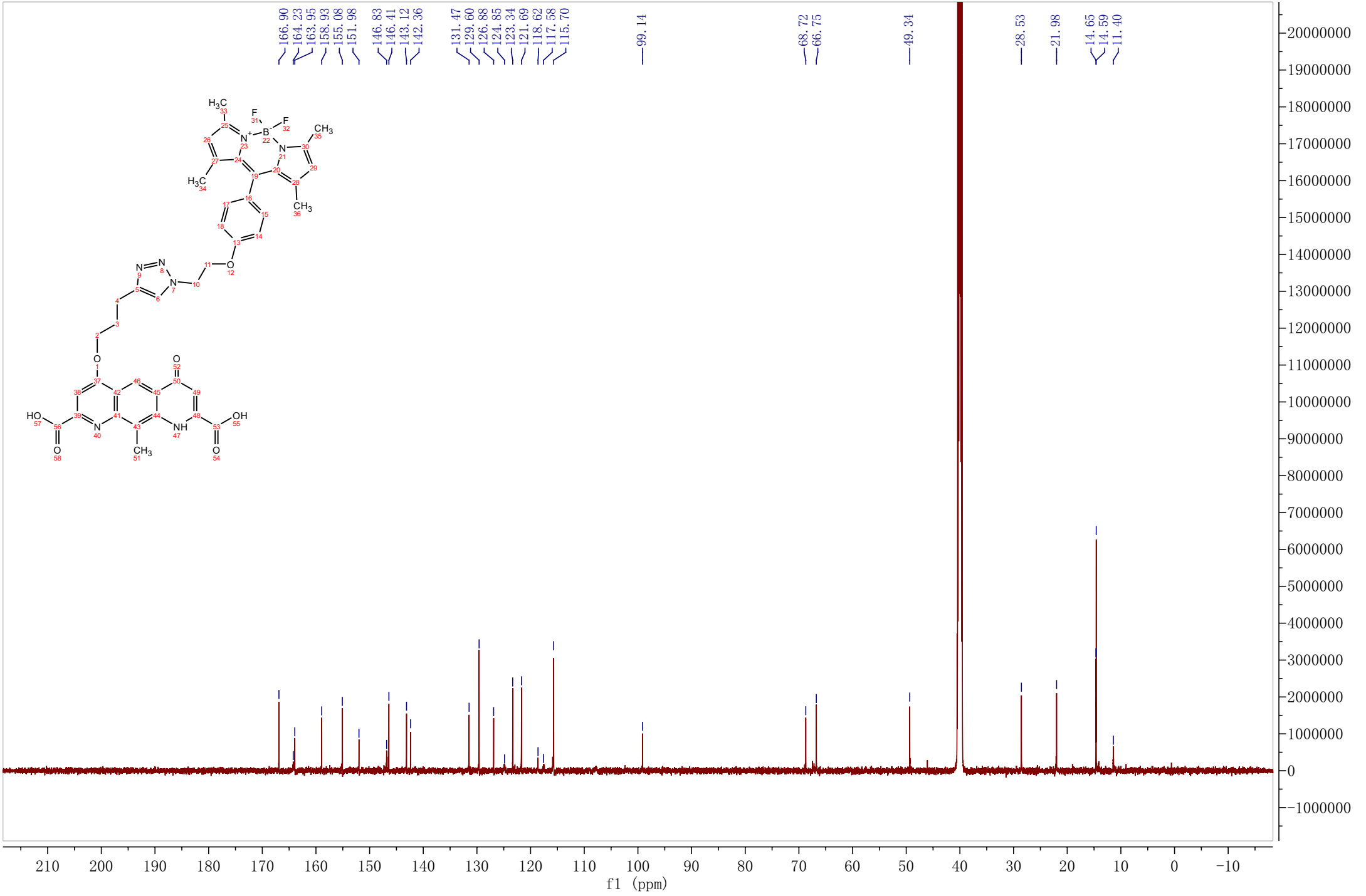
^{13}C NMR (151 MHz, DMSO) δ 179.17, 167.84, 165.87, 163.79, 163.04, 158.87, 155.00, 150.95, 146.85, 144.35, 143.07, 142.26, 139.86, 137.63, 132.14, 131.87, 131.40, 129.57, 129.17, 126.86, 125.29, 125.14, 124.92, 121.60, 118.32, 117.88, 115.64, 108.38, 99.42, 69.02, 68.06, 66.73, 64.13, 55.39, 54.27, 53.33, 53.09, 49.51, 14.63, 14.57, 11.46.



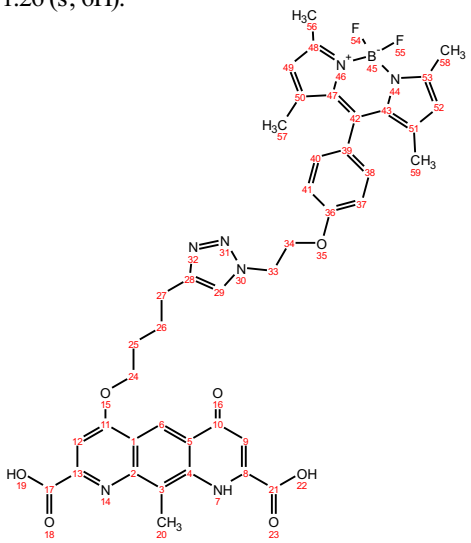
^{1}H NMR (600 MHz, DMSO) δ 8.87 (s, 1H), 8.06 (s, 1H), 7.45 (s, 1H), 7.18 (d, *J* = 8.7 Hz, 2H), 7.05 (d, *J* = 8.7 Hz, 2H), 6.66 (s, 1H), 6.10 (s, 2H), 4.76 (t, *J* = 5.1 Hz, 2H), 4.46 – 4.42 (m, 4H), 3.03 (s, 3H), 2.94 (t, *J* = 7.4 Hz, 2H), 2.42 (s, 6H), 2.32 – 2.28 (m, 2H), 1.30 (s, 6H).



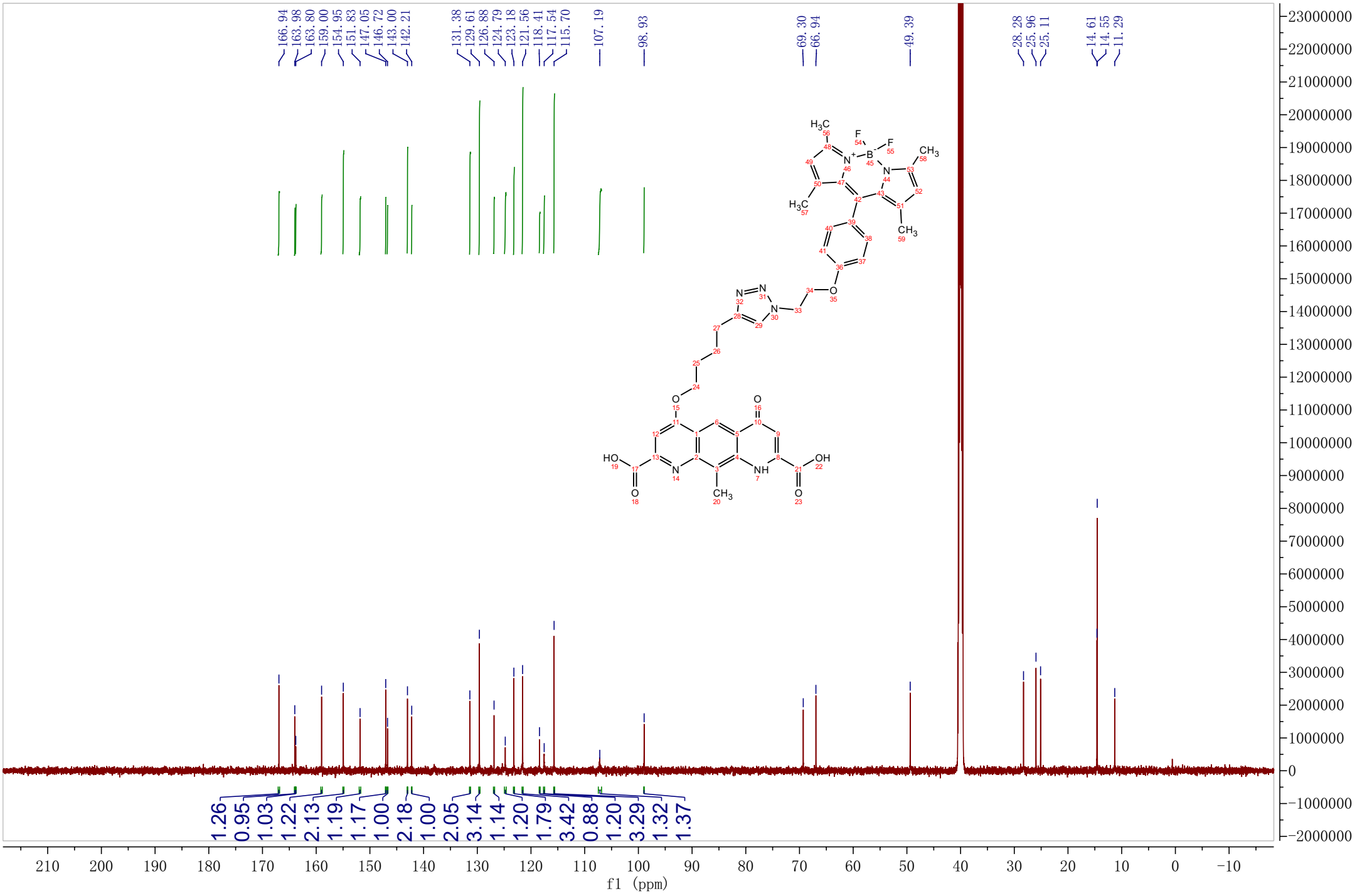
^{13}C NMR (151 MHz, DMSO) δ 166.90, 164.23, 163.95, 158.93, 155.08, 151.98, 146.83, 146.41, 143.12, 142.36, 131.47, 129.60, 126.88, 124.85, 123.34, 121.69, 118.62, 117.58, 115.70, 99.14, 68.72, 66.75, 49.34, 28.53, 21.98, 14.65, 14.59.



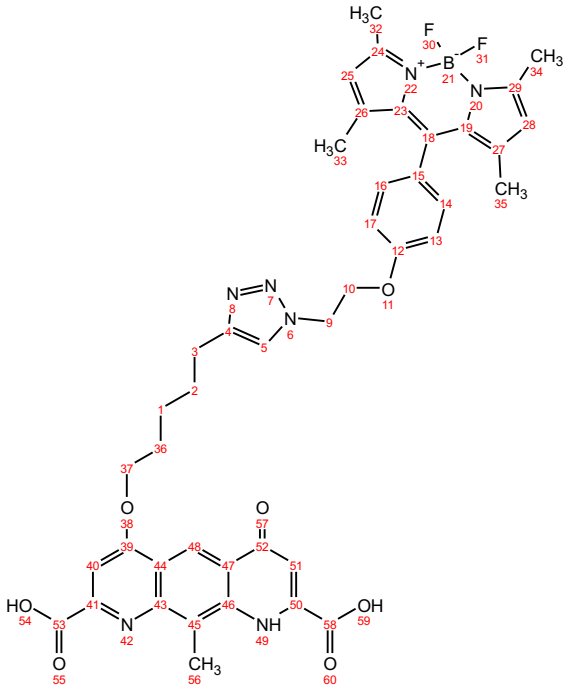
^{1}H NMR (600 MHz, DMSO) δ 8.84 (s, 1H), 8.03 (s, 1H), 7.43 (s, 1H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.05 (t, *J* = 8.2 Hz, 2H), 6.56 (s, 1H), 6.01 (s, 2H), 4.76 (t, *J* = 5.0 Hz, 2H), 4.45 (t, *J* = 5.0 Hz, 2H), 4.38 (t, *J* = 6.5 Hz, 2H), 3.01 (s, 3H), 2.79 (t, *J* = 7.4 Hz, 2H), 2.39 (s, 6H), 2.01 – 1.95 (m, 2H), 1.92 – 1.83 (m, 2H), 1.26 (s, 6H).



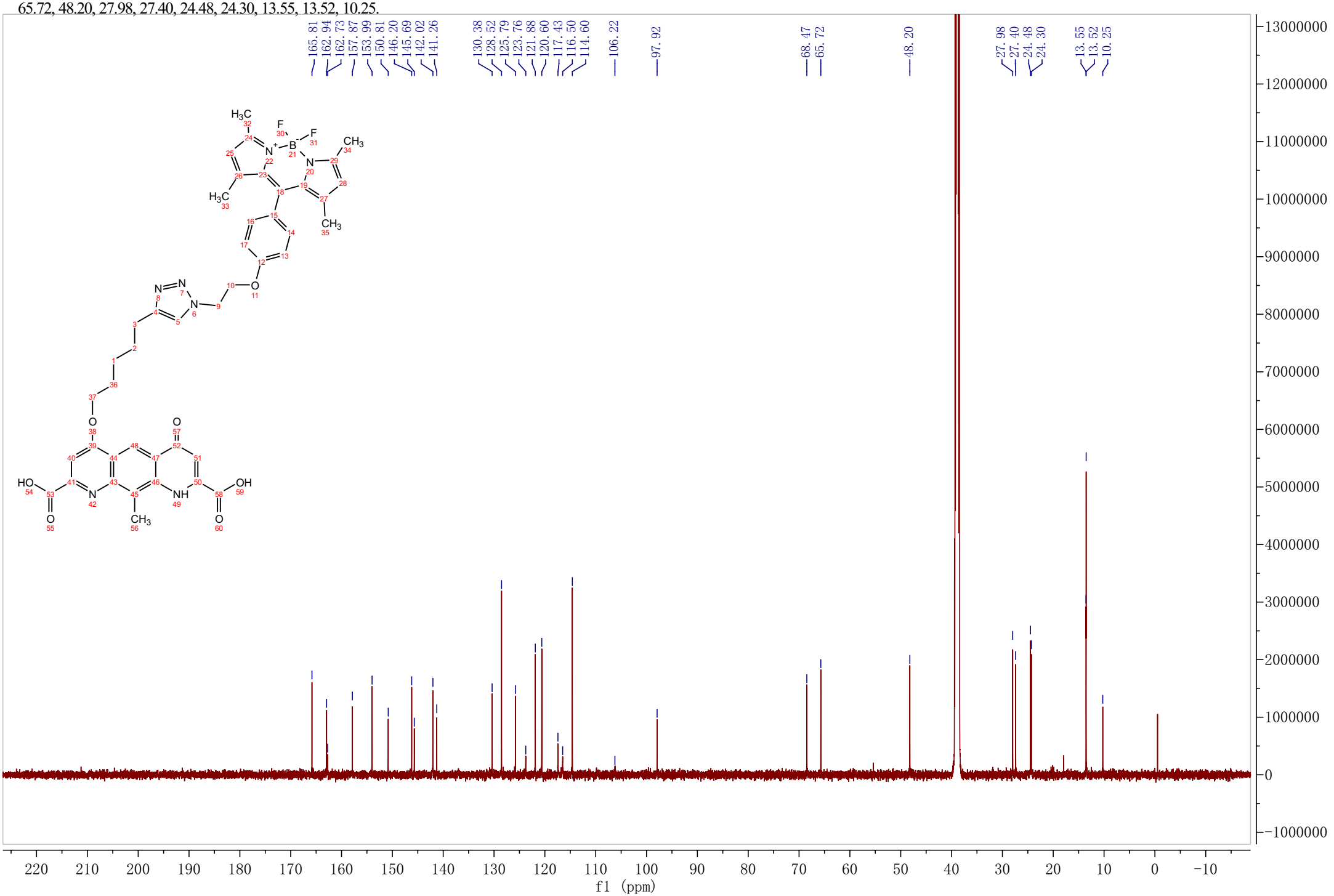
^{13}C NMR (151 MHz, DMSO) δ 166.94, 163.98, 163.80, 159.00, 154.95, 151.83, 147.05, 146.72, 143.00, 142.21, 131.38, 129.61, 126.88, 124.79, 123.18, 121.56, 118.41, 117.54, 115.70, 107.19, 98.93, 69.30, 66.94, 49.39, 28.28, 25.96, 25.11, 14.61, 14.55, 11.29.



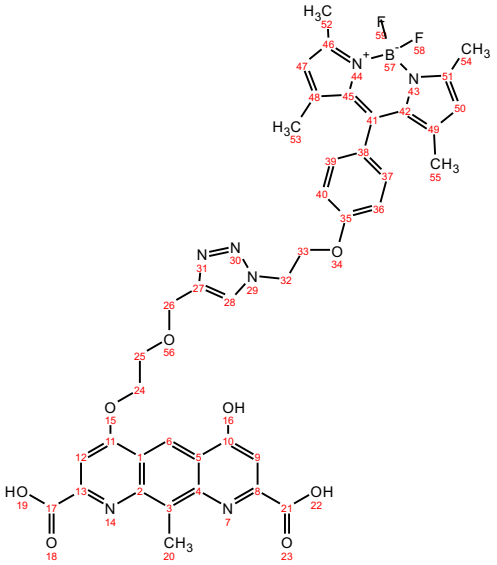
^{1}H NMR (600 MHz, DMSO) δ 8.88 (s, 1H), 7.99 (s, 1H), 7.45 (s, 1H), 7.20 (d, J = 8.7 Hz, 2H), 7.06 (d, J = 8.7 Hz, 2H), 6.63 (s, 1H), 6.11 (s, 2H), 4.74 (t, J = 5.1 Hz, 2H), 4.44 (t, J = 5.1 Hz, 2H), 4.38 (t, J = 6.4 Hz, 2H), 3.03 (s, 3H), 2.71 (t, J = 7.5 Hz, 2H), 2.42 (s, 6H), 2.01 – 1.96 (m, 2H), 1.76 (dt, J = 15.2, 7.6 Hz, 2H), 1.59 (dt, J = 15.1, 7.6 Hz, 2H), 1.32 (s, 6H).



^{13}C NMR (151 MHz, DMSO) δ 165.81, 162.94, 162.73, 157.87, 153.99, 150.81, 146.20, 145.69, 142.02, 141.26, 130.38, 128.52, 125.79, 123.76, 121.88, 120.60, 117.43, 116.50, 114.60, 106.22, 97.92, 68.47, 65.72, 48.20, 27.98, 27.40, 24.48, 24.30, 13.55, 13.52, 10.25.



^{1}H NMR (600 MHz, DMSO) δ 8.88 (s, 1H), 8.28 (s, 1H), 7.47 (s, 1H), 7.18 (d, *J* = 7.1 Hz, 2H), 7.04 (d, *J* = 8.0 Hz, 2H), 6.59 (s, 1H), 6.07 (s, 2H), 4.81 (t, *J* = 4.9 Hz, 2H), 4.72 (s, 2H), 4.55 – 4.51 (m, 2H), 4.47 (t, *J* = 4.7 Hz, 2H), 4.05 – 3.99 (m, 2H), 3.02 (s, 3H), 2.41 (s, 6H), 1.28 (s, 6H).



^{13}C NMR (151 MHz, DMSO) δ 166.92, 163.95, 163.59, 158.88, 155.07, 151.83, 146.71, 144.41, 143.11, 142.26, 137.95, 131.40, 129.58, 126.87, 125.10, 124.83, 121.66, 118.32, 117.77, 115.67, 106.99, 99.05, 68.98, 68.15, 66.74, 64.14, 49.54, 14.62, 14.55, 11.25.