Supporting Information

A Dual-Channel Hill-Type Small-Molecule pH Probe

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CONTENTS

Experimental section

Materials and General Methods

All chemicals were purchased from TCI and J&K without further purification. Silica gel P60 (Qingdao Haiyang) was used for column chromatography. All the organic solvents were of analytical grade. Water was purified by a Milli-Q system.

Experimental

The stability test of PHHF

The probe was placed in an aqueous solution of pH 4.77 and 7.10, and its fluorescence emission spectra were measured every 1 minute with the excitation wavelength at 462 nm. The ratio of fluorescence intensity (I_{580}/I_{550}) is plotted over time to observe the stability of the molecule.

Cell culture

Human Osteosarcoma cell-lines (U2OS) were from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). U2OS cells were cultured in McCoy's 5A medium (GIBCO) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37°C (v/v) in a 5% CO_2 / 95% air incubator. Cell culture dish was a 60 mm diameter petri dish (NEST). Before imaging, cells were cultured in a 15 mm diameter glass substrate (NEST).

Cell viability assay

Toxicity toward U2OS cells was determined by cell counting kit-8 (CCK-8). About 1×10^4 cells per well were seeded in 96-well plates and cultured overnight. The medium was replaced with fresh medium, **PHHF** (0, 1.0, 5.0, 10.0, 15.0, 20.0 μM) was added to achieve final volume of 100 mL. Five replicates for each concentration. 24 hours later, 10 μL CCK-8 was added to each well for additional 2 h incubation. The absorbance was measured in an ELISA plate reader (model 550, BioRad) at a wavelength of 450 nm.

Real-time confocal imaging.

Real-time imaging of **PHHF** was carried out in U2OS cells. U2OS cells were incubated with **PHHF** (5 μM) for 30 mins and then immediately imaged with a Leica SP5 confocal microscope.

The synthetic procedure of PHHF

Synthesis of 6-(isopropylamino)-4,4-dimethylchroman-2-one (2).

Add compound **1** (5.66 g, 29.59 mmol, 1.0 equiv.), 2 - iodoethane (6.50 mL, 65.09 mmol, 2.2 equiv.), K_2CO_3 (4.09 g, 29.59 mmol, 1.0 equiv.) and KI (0.98 g, 5.92 mmol, 0.2 equiv.) into a 250 mL round-bottomed flask, 80 mL DMF was added to dissolve them. The mixture was heated to 90°C for 6 hours to get the crude product, which was purified by silica column chromatography (eluent: PE:EA = 100:1). The pure compound was a light pink solid (4.50 g) with a yield of 65%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.88 (d,

J = 8.6 Hz, 1 H), 6.50 (d, *J* = 2.6 Hz, 1 H), 6.46 (dd, J_1 = 8.6 Hz, J_2 = 2.5 Hz, 1 H), 3.58 (d, *J* = 6.3 Hz, 1 H), 2.57 (s, 2 H), 1.31 (s, 6 H), 1.21 (d, *J* = 6.3 Hz, 6 H).

Synthesis of tert-butly 4-(((4,4-dimethyl-2-oxochroman-6-yl)(isopropyl)amino)methyl)benzoate (3)

Add compound **2** (10.00 g, 42.86 mmol, 1.0 equiv.), tert-butyl 4-(bromomethyl) benzoate (12.78 g, 47.15 mmol, 1.1 equiv.) and K_2CO_3 (5.92 g, 42.86 mmol, 1.0 equiv.) in a 250 mL round-bottomed flask, add 120 mL DMF to dissolve it, the mixture was heated to 140°C refluxing overnight to get the crude product, DMF was removed with diaphragm pump, the mixture was recrystallized with ethanol to obtain pure compound as white solid (9.60 g), with a yield of 53%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.1 Hz, 2 H), 7.34 (d, *J* = 7.9 Hz, 2 H), 6.86 (d, *J* = 8.5 Hz, 1 H), 6.56 (s, 2 H), 4.40 (s, 2 H), 4.24 – 4.14 (m, 1 H), 2.54 (s, 2 H), 1.58 (s, 10 H), 1.22 (s, 12 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.82, 165.67, 146.26, 145.47, 142.22, 132.10, 130.56, 129.69, 126.16, 117.50, 113.10, 109.13, 80.91, 49.23, 48.65, 43.75, 33.40, 28.19, 27.92, 27.79, 27.59, 19.83. ESI-HRMS (m/z): $[M]^+$ calcd. for $C_{26}H_{33}NO$, 423.24; found 424.2487.

Synthesis of tert-butyl 4-(((3',6'-bis(diethylamino)-4,4-dimethylspiro[chromane-2,9'-xanthen]-6-yl)(isopropyl)amino)methyl)benzoate (4).

Add compound 3, 3'-Oxybis[4-bromo-N,N-diethylbenzenamine] (1.67 g, 3.54 mmol, 1.5 equiv.) in a dry flask, replace argon for three times, add 15 mL dry THF to dissolve it under argon protection, the mixture was stirred at - 78°C for 30 mins, and then add 2.5 M n - BuLi (2.83 mL, 7.08 mmol, 3.0 equiv.) by syringe and continue to react for 30 mins, in the process of which the color of the mixture gradually became yellow from purple, Compound **3** (1.00 g, 2.36 mmol, 1.0 equiv.) was dissolved in 5 mL dry THF and then added into the flask, then the mixture was moved to room temperature for overnight reaction to get the crude product. Saturated NH_4Cl solution was added into the flask to quench the excess lithium reagent, then the mixture was extracted with DCM repeatedly. collect the organic layer, dried with $MgSO_4$ and filtered. DCM was removed under reduced pressure to get the crude product, which was purified by silica gel column chromatography (eluent: PE:EA:TEA = 200:1:0.5). the pure compound was white solid (697 mg) with a yield of 41%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 8.2 Hz, 2 H), 7.42 (d, *J* = 8.2 Hz, 2 H), 7.20 (d, *J* = 8.7 Hz, 2 H), 6.87 (d, *J* = 8.8 Hz, 1 H), 6.64 (d, *J* = 2.8 Hz, 1 H), 6.61 (dd, J_1 = 8.8 Hz, J_2 = 2.9 Hz, 1 H), 6.41 (d, *J* = 2.4 Hz, 2 H), 6.36 (dd, J_1 = 8.7 Hz, J_2 = 2.5 Hz, 2 H), 4.38 (s, 2 H), 4.09 (dt, J_1 = 13.2 Hz, J_2 = 6.5 Hz, 1 H), 3.32 (dt, J_1 = 11.3 Hz, J_2 = 7.3 Hz, 8 H), 2.04 (s, 2 H), 1.58 (s, 9 H), 1.20 (d, *J* = 6.5 Hz, 6 H), 1.14 (t, *J* = 7.0 Hz, 12 H), 1.05 (s, 6 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.87, 152.54, 147.95, 147.28, 146.75, 143.28, 131.31, 130.28, 129.54, 126.56, 117.37, 116.67, 115.04, 113.23, 107.31, 98.83, 80.75, 72.12, 52.13, 50.11, 50.05, 49.63, 44.45, 31.78, 31.40, 28.23, 19.88, 12.59. ESI-HRMS (m/z): $[M]^+$ calcd. for $C_{46}H_{59}N_3O_4$, 717.45; found 718.4583.

Synthesis of 4-(((3',6'-bis(diethylamino)-4,4-dimethylspiro[chromane-2,9'-xanthen]-6-yl)(isopropyl)amino)methyl)benzoic acid (5).

Add compound **4** (1.00 g, 1.39 mmol, 1.0 equiv.) into the round-bottomed flask. then add TFA (2.67 mL, 41.78 mmol, 30.0 equiv.) to it under 0 °C. the crude product was yield in 2 h, then neutralize the mixture with 1 M NaOH aqueous solution, the

resulting mixture was extracted repeatedly with DCM. collect the organic layer, The crude product was dried as purple solid and then purified by silica gel column chromatography (eluent: DCM : CH_3OH : CH_3COOH = 100 : 1 : 0.25) to obtain pure **5** (876 mg) in a 95% yield. ^{1}H NMR (600 MHz, CD_3OD) δ 7.77 (d, *J* = 8.0 Hz, 2 H), 7.73 (d, *J* = 9.6 Hz, 2 H), 7.09 (d, *J* = 7.9 Hz, 2 H), 6.84 – 6.78 (m, 2 H), 6.74 (dd, J_1 = 10.6 Hz, J_2 = 6.4 Hz, 4 H), 6.06 (s, 1 H), 4.18 (s, 2 H), 3.88 (dt, J_1 = 13.0 Hz, J_2 = 6.5 Hz, 1 H), 3.73 (s, 2 H), 3.64 (dd, J_1 = 13.6 Hz, J_2 = 6.6 Hz, 8 H), 3.31 (s, 2 H), 1.99 (s, 2 H), 1.30 (t, *J* = 7.1 Hz, 12 H), 1.28 (s, 6 H), 1.06 (d, *J* = 6.5 Hz, 6 H). ^{13}C NMR (151 MHz, CD_3OD) δ 159.41, 157.00, 155.12, 130.81, 129.28, 127.31, 116.88, 114.49, 112.57, 95.39, 45.24, 41.01, 35.81, 28.14, 18.24, 11.50. ESI-HRMS (m/z): $[M]^+$ calcd. for $C_{42}H_{51}N_3O_4$, 661.39; found 662.3959.

Synthesis of 6-bromo-2-(2-morpholinoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (7).

Add compound **6** (5.00 g, 18.05 mmol, 1.0 equiv.) in the round-bottomed flask and dissolve it with DMF, the mixture was stirred at room temperature for 5 mins, the color of the solution was light yellow, then add 4-morpholineethanamine (2.35 g, 18.05 mmol, 1.0 equiv.) slowly to it and the color gradually became dark brown, the crude product was yielded as the mixture continue stirring at room temperature for 4 h, the pure product (4.42 g) was purified by silica gel column chromatography (eluent: PE : EA = 20 : 1), and the yield was 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (dd, J_1 = 7.3 Hz, J_2 = 0.9 Hz, 1 H), 8.56 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1 H), 8.40 (d, *J* = 7.9 Hz, 1 H), 8.04 (d, *J* = 7.9 Hz, 1 H), 7.84 (dd, J_1 = 8.4 Hz, J_2 = 7.4 Hz, 1 H), 4.35 (t, *J* = 6.8 Hz, 2 H), 3.73 – 3.66 (m, 4 H), 2.74 (t, *J* = 6.7 Hz, 2 H), 2.63 (s, 4 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.66, 133.37, 132.08, 131.19, 130.65, 130.39, 129.05, 128.10, 123.02, 122.15, 66.81, 56.03, 53.72, 37.12. ESI-HRMS (m/z): $[M]^+$ calcd. for $C_{18}H_{17}BrN_2O_3$, 388.04; found 389.0500.

Synthesis of 6-((2-aminoethyl)amino)-2-(2-morpholinoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (8).

Add compound **7** (3.33 g, 8.55 mmol) in the 100 mL round-bottomed flask, add about 20 mL ethanediamine to dissolve it and the crude product was yielded after reflux for 2 h. remove the ethylenediamine under reduced pressure. the product was purified as yellow solid (1.73 g) by silica gel column chromatography (eluent: DCM : CH_3OH = 50 : 1), and the yield was 55%. ^{1}H NMR (400 MHz, DMSO) δ 8.84 (d, *J* = 8.1 Hz, 1 H), 8.44 (d, *J* = 6.8 Hz, 1 H), 8.28 (d, *J* = 8.5 Hz, 1 H), 8.12 (s, 2 H), 7.98 (s, 1 H), 7.70 (dd, J_1 = 8.2 Hz, J_2 = 7.6 Hz, 1 H), 6.86 (d, *J* = 8.6 Hz, 1 H), 4.14 (t, *J* = 7.0 Hz, 2 H), 3.66 (d, *J* = 5.2 Hz, 2 H), 3.57 – 3.48 (m, 4 H), 3.16 (t, *J* = 6.1 Hz, 2 H), 2.53 (t, *J* = 5.1 Hz, 2 H), 2.45 (s, 4 H). ^{13}C NMR (101 MHz, DMSO) δ 164.20, 163.39, 150.71, 134.58, 131.28, 129.73, 124.91, 122.24, 120.87, 108.96, 104.46, 66.69, 56.23, 53.90, 41.04, 37.83, 36.90. ESI-HRMS (m/z): $[M]^+$ calcd. for $C_{20}H_{24}N_4O_3$, 368.18; found 369.1926.

Synthesis of 4-(((3',6'-bis(diethylamino)-4,4-dimethylspiro[chromane-2,9'-xanthen]-6-yl)(isopropyl)amino)methyl)-N-(2-((2-(2-morpholinoethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)amino)ethyl)benzamide (PHHF).

Add compound **5** (226.00 mg, 0.34 mmol, 1.0 equiv.) and TSTU (113.14 mg, 0.41 mmol, 1.2 equiv.) into a 50 mL round-bottomed flask and 30 mL of DMF was added to dissolve them. after DIPEA (0.28 mL, 1.71 mmol, 5.0 equiv.) was added and then

stirred for 1 h at r.t., compound **8** (125.80 mg, 0.34 mmol, 1.0 equiv.) was added and continue to stir, the crude product was yielded in 4 h. by silica gel column chromatography separation (eluent: DCM : H_2O : TEA = 100 : 1 : 0.25), A purplish solid (107 mg) was obtained with a yield of 31%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 7.3 Hz, 1 H), 8.34 (dd, J_1 = 8.2 Hz, J_2 = 4.3 Hz, 2 H), 7.79 (d, *J* = 8.2 Hz, 2 H), 7.59 (t, *J* = 7.9 Hz, 1 H), 7.47 – 7.41 (m, 3 H), 7.17 (d, *J* = 8.7 Hz, 2 H), 6.97 (s, 1 H), 6.83 (d, *J* = 8.8 Hz, 1 H), 6.63 (d, *J* = 2.6 Hz, 1 H), 6.57 (dd, J_1 = 8.9 Hz, J_2 = 2.6 Hz, 1 H), 6.48 (d, *J* = 8.5 Hz, 1 H), 6.40 (d, *J* = 2.4 Hz, 2 H), 6.34 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 2 H), 4.36 (s, 2 H), 4.34 – 4.29 (m, 2 H), 4.07 (dt, J_1 = 12.8 Hz, J_2 = 6.3 Hz, 1 H), 3.90 (s, 2 H), 3.71 – 3.66 (m, 4 H), 3.46 (s, 2 H), 3.30 (dt, J_1 = 10.7 Hz, J_2 = 7.2 Hz, 8 H), 2.72 – 2.66 (m, 2 H), 2.61 (s, 4 H), 2.03 (s, 2 H), 1.19 (d, *J* = 6.5 Hz, 6 H), 1.12 (t, *J* = 7.0 Hz, 12 H), 1.03 (s, 6 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.27, 164.82, 164.16, 152.53, 150.24, 147.97, 147.38, 146.55, 143.16, 134.50, 131.61, 131.11, 129.77, 128.08, 127.71, 127.71, 126.95, 126.53, 124.81, 120.34, 117.35, 116.56, 115.20, 113.36, 109.49, 107.27, 103.15, 98.83, 72.16, 67.06, 56.39, 53.85, 52.09, 49.63, 44.43, 36.89, 31.77, 31.38, 19.86, 12.58, 0.01. ESI-HRMS (m/z): $[M+H]^+$ calcd. for $C_{67}H_{73}N_7O_6$, 1012.5695; found 1012.5700.

Synthesis of PHFb

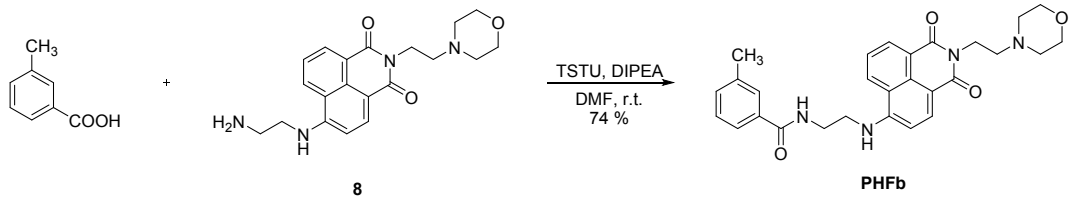

Figure S1 The synthesis of **PHFb**

Add m-methylbenzoic acid (58.02 mg, 0.43 mmol, 1.0 equiv.) and TSTU (153.95 mg, 0.51 mmol, 1.2 equiv.) into a 50 mL round-bottomed flask, add 25 mL DMF to dissolve it, DIPEA (275.37 mg, 2.13 mmol, 5.0 equiv.) was added during the stirring process. After the reaction system was stirred at room temperature for 1 h, add compound **8** (157 mg, 0.43 mmol, 1.0 equiv.) into the flask stirring for another 4 h. The DMF was removed by the diaphragm pump and extracted with DCM/H_2O for 3 times. The organic layer was collected, dried over anhydrous sodium sulfate, and separated by silica gel column chromatography (DCM:H_2O = 100:1), pure compound as yellow solid (153 mg) was obtained with a yield of 87%. ^{1}H NMR (600 MHz, DMSO) δ 8.70 (t, *J* = 5.2 Hz, 1 H), 8.63 – 8.57 (m, 1 H), 8.43 – 8.37 (m, 1 H), 8.23 (d, *J* = 8.5 Hz, 1 H), 7.91 (d, *J* = 5.1 Hz, 1 H), 7.66 (q, *J* = 7.3 Hz, 3 H), 7.34 (d, *J* = 7.2 Hz, 2 H), 6.89 (d, *J* = 8.6 Hz, 1 H), 4.13 (t, *J* = 7.0 Hz, 2 H), 3.63 – 3.55 (m, 4 H), 3.53 (t, *J* = 4.3 Hz, 4 H), 2.52 (dd, J_1 = 11.7 Hz, J_2 = 4.6 Hz, 2 H), 2.45 (s, 4 H), 2.34 (s, 3 H). ^{13}C NMR (151 MHz, DMSO) δ 166.46, 163.11, 162.24, 150.01, 136.95, 133.71, 133.54, 131.24, 130.07, 128.79, 127.79, 127.62, 127.16, 123.74, 121.22, 119.51, 107.19, 103.13, 65.63, 55.19, 52.83, 41.95, 37.38, 35.80, 20.35. ESI-HRMS (m/z): $[M+H]^+$ calcd. for $C_{28}H_{30}N_4O_4$, 487.2340; found 487.2344.

pH titration of rhodamine moiety (PHFa)

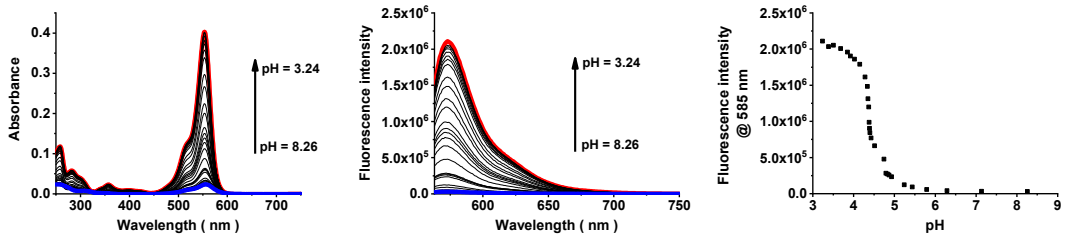

Figure S2 The UV-Vis absorption spectra and fluorescence emission spectra of **PHFa** (5 μM in H_2O with 0.1% DMSO) at different pH values (3.24 - 8.26), λ_{ex} = 550 nm.

pH titration of naphthimide moiety (PHFb)

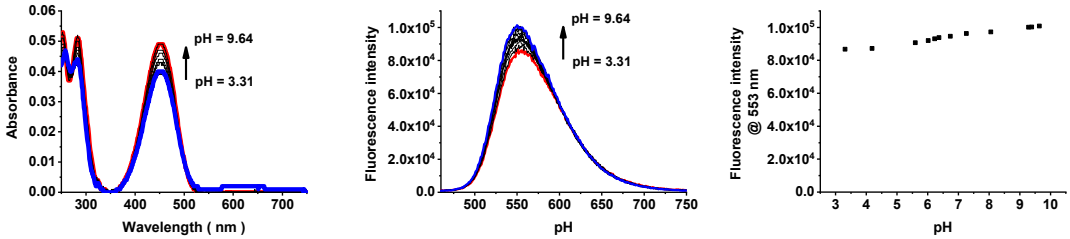

Figure S3 The UV-Vis absorption spectra and fluorescence emission spectra of **PHFb** (5 μM in H_2O with 0.1% DMSO) at different pH (3.31 - 9.64), λ_{ex} = 450 nm .

Stability experiment

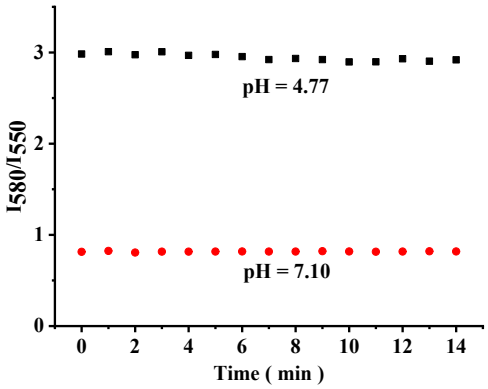

Figure S4 Time courses of the fluorescence intensity ratio I_{580}/I_{550} of **PHHF** (5 μM in H_2O with 0.1% DMSO) at pH 4.77 and pH 7.10. λ_{ex} = 462 nm.

Supplementary figures

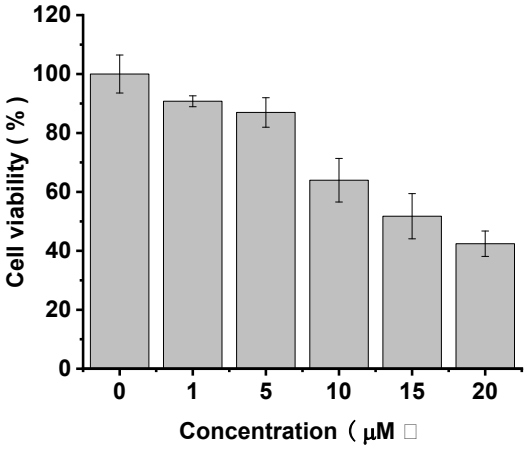

Figure S5 Cell viability of U2OS cells toward **PHHF** by CCK-8 assay for 24 h.

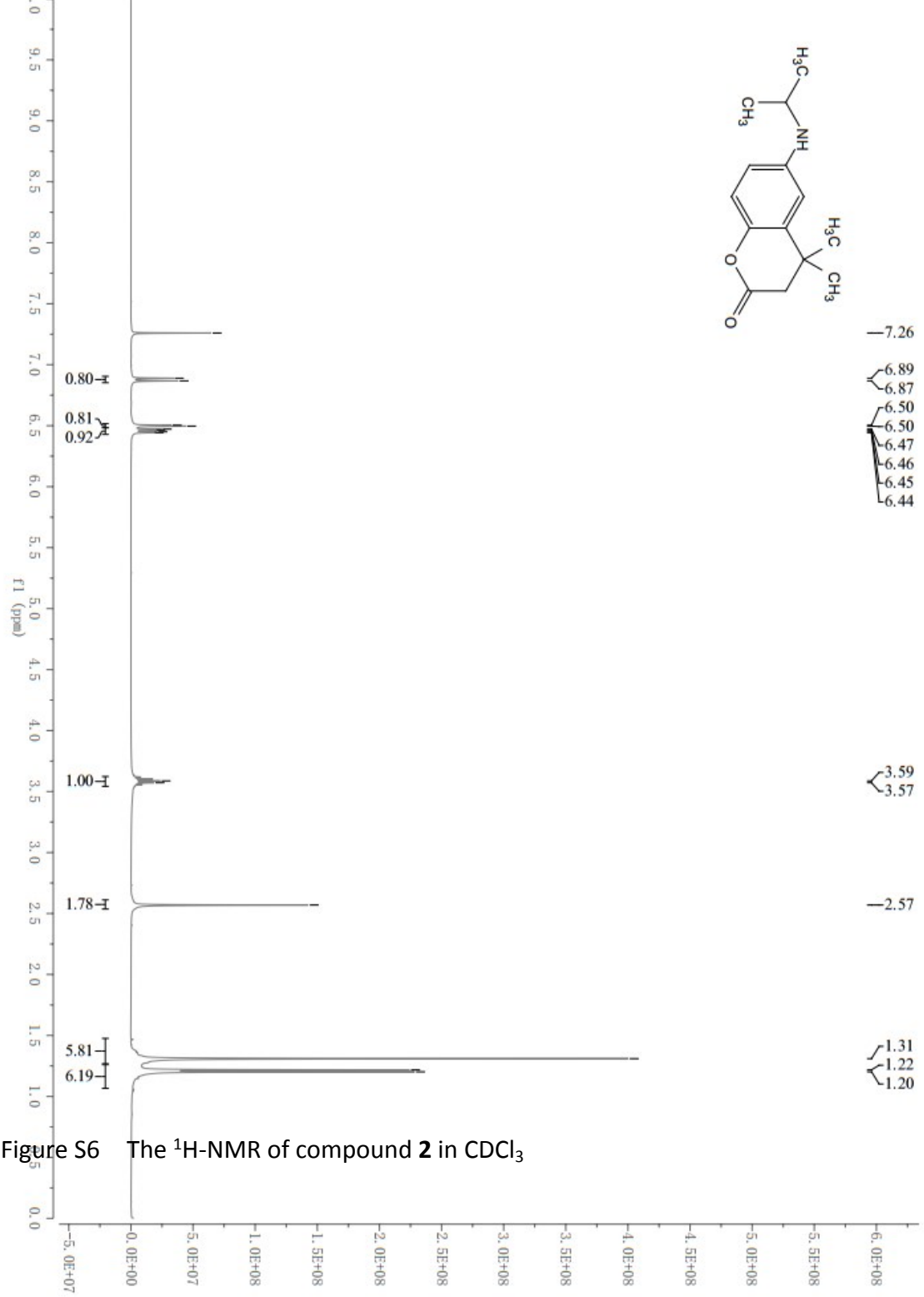

Figure S6 The ^{1}H-NMR of compound **2** in $CDCl_3$

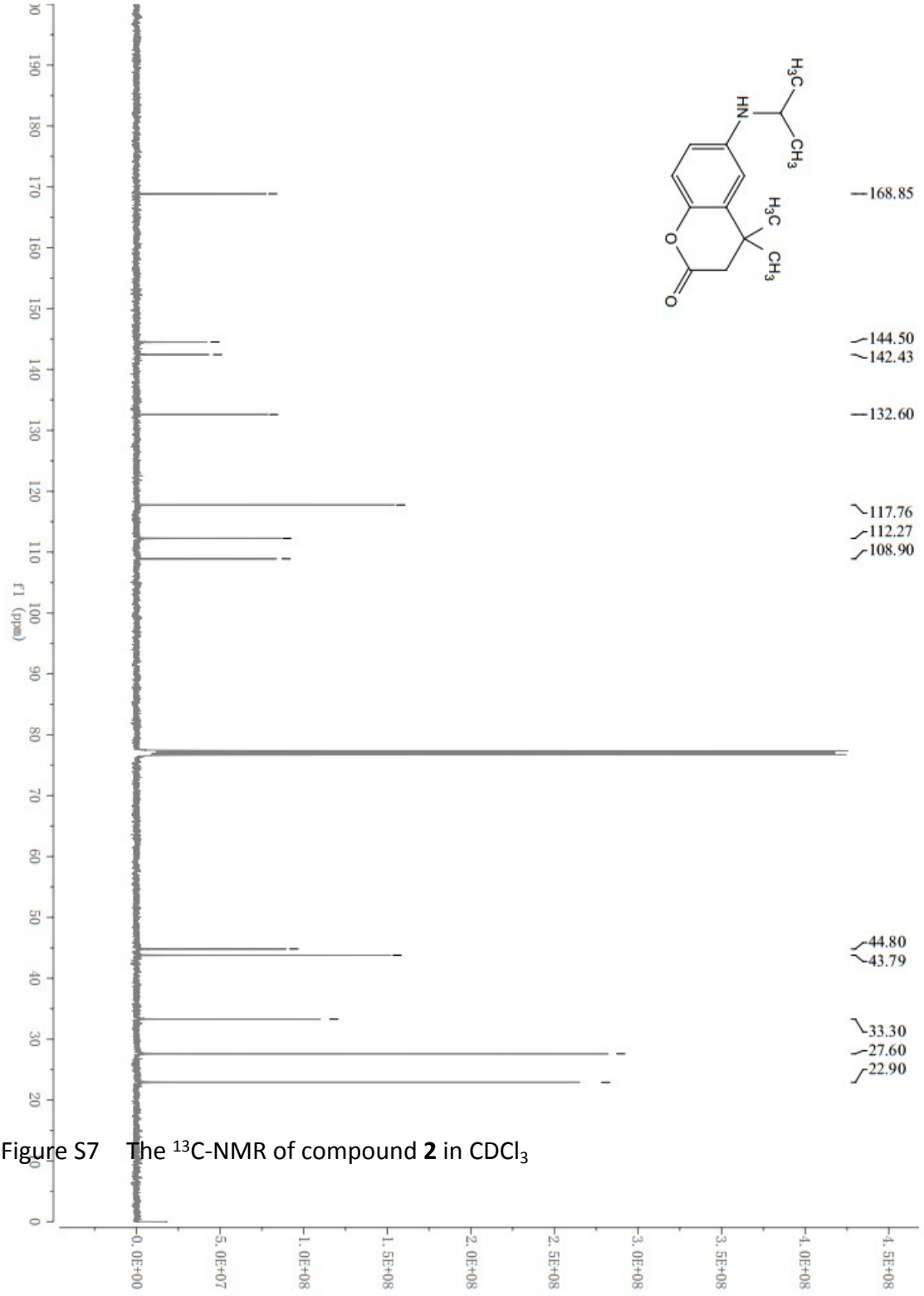

Figure S7 The ^{13}C-NMR of compound **2** in $CDCl_3$

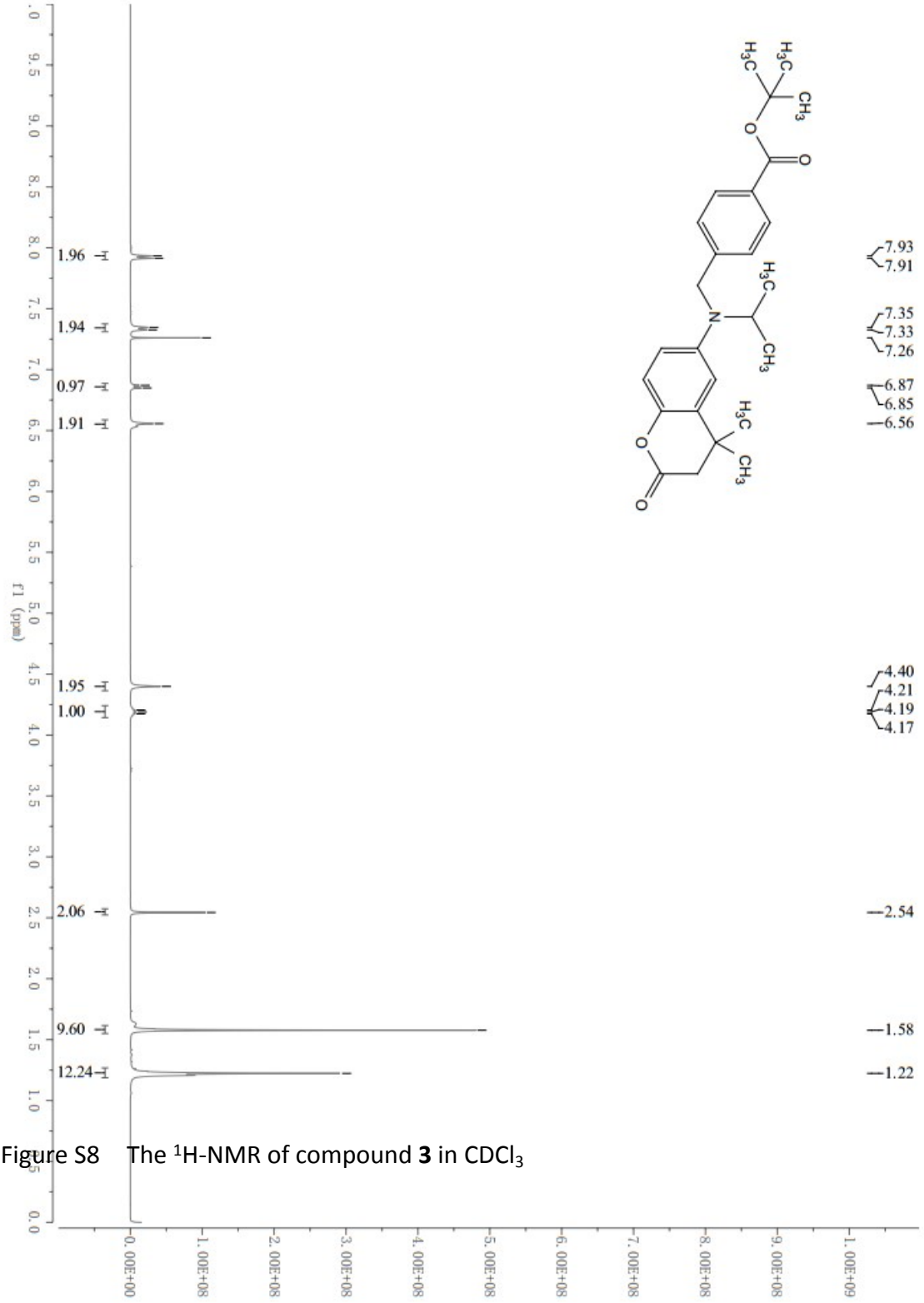

Figure S8 The ^{1}H-NMR of compound **3** in $CDCl_3$

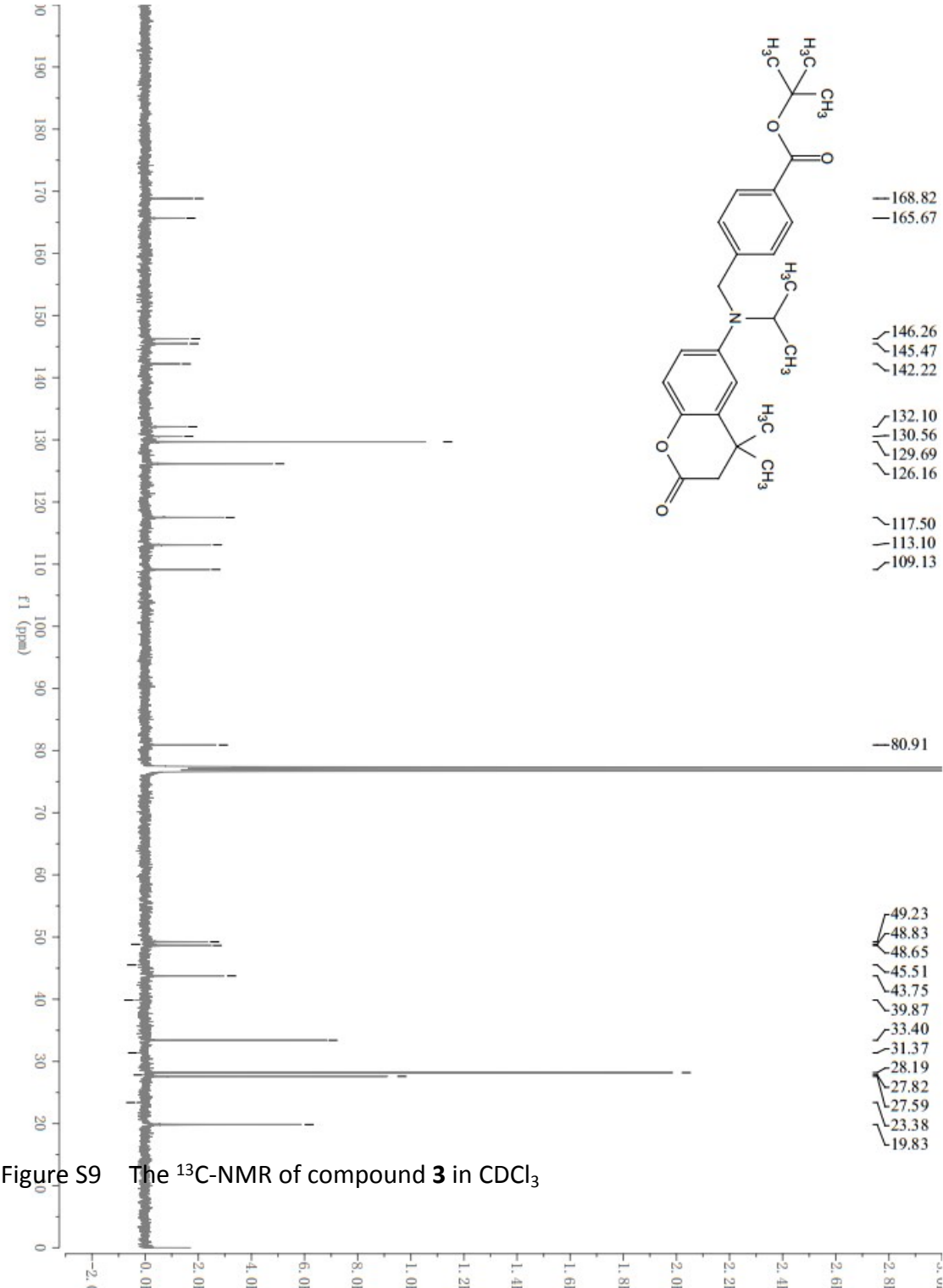

Figure S9 The ^{13}C-NMR of compound **3** in $CDCl_3$

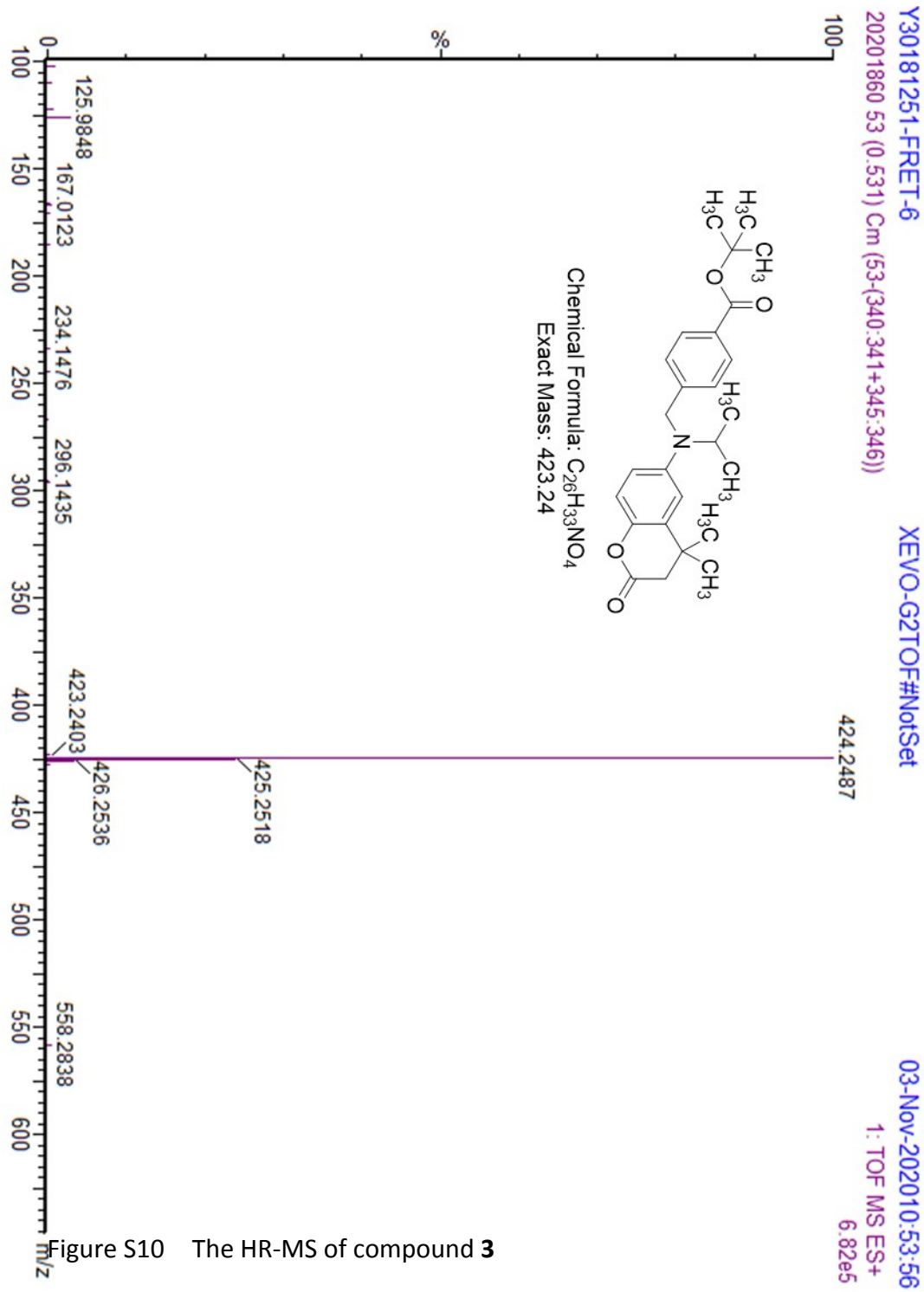

Figure S10 The HR-MS of compound **3**

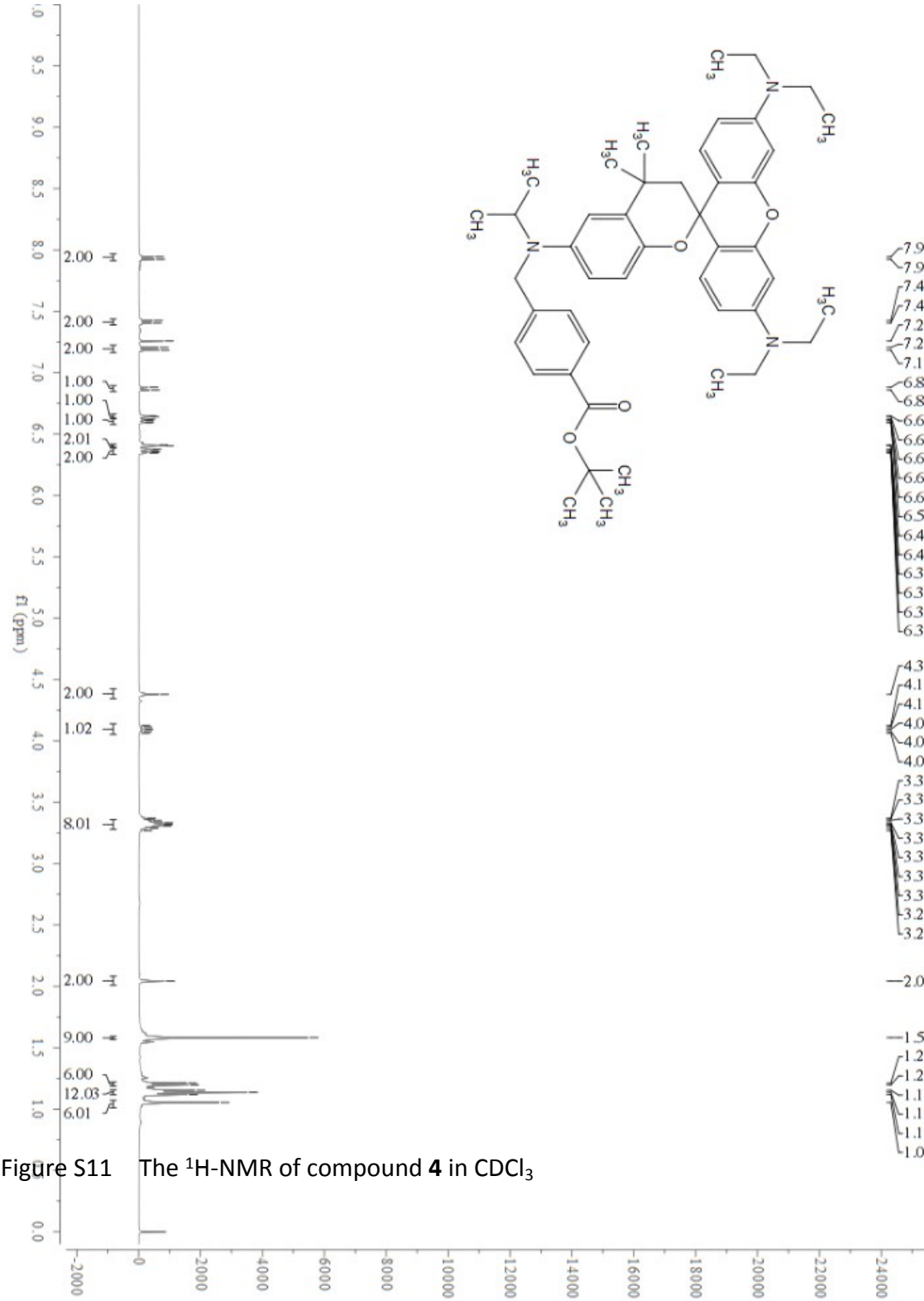

Figure S11 The ^{1}H-NMR of compound **4** in $CDCl_3$

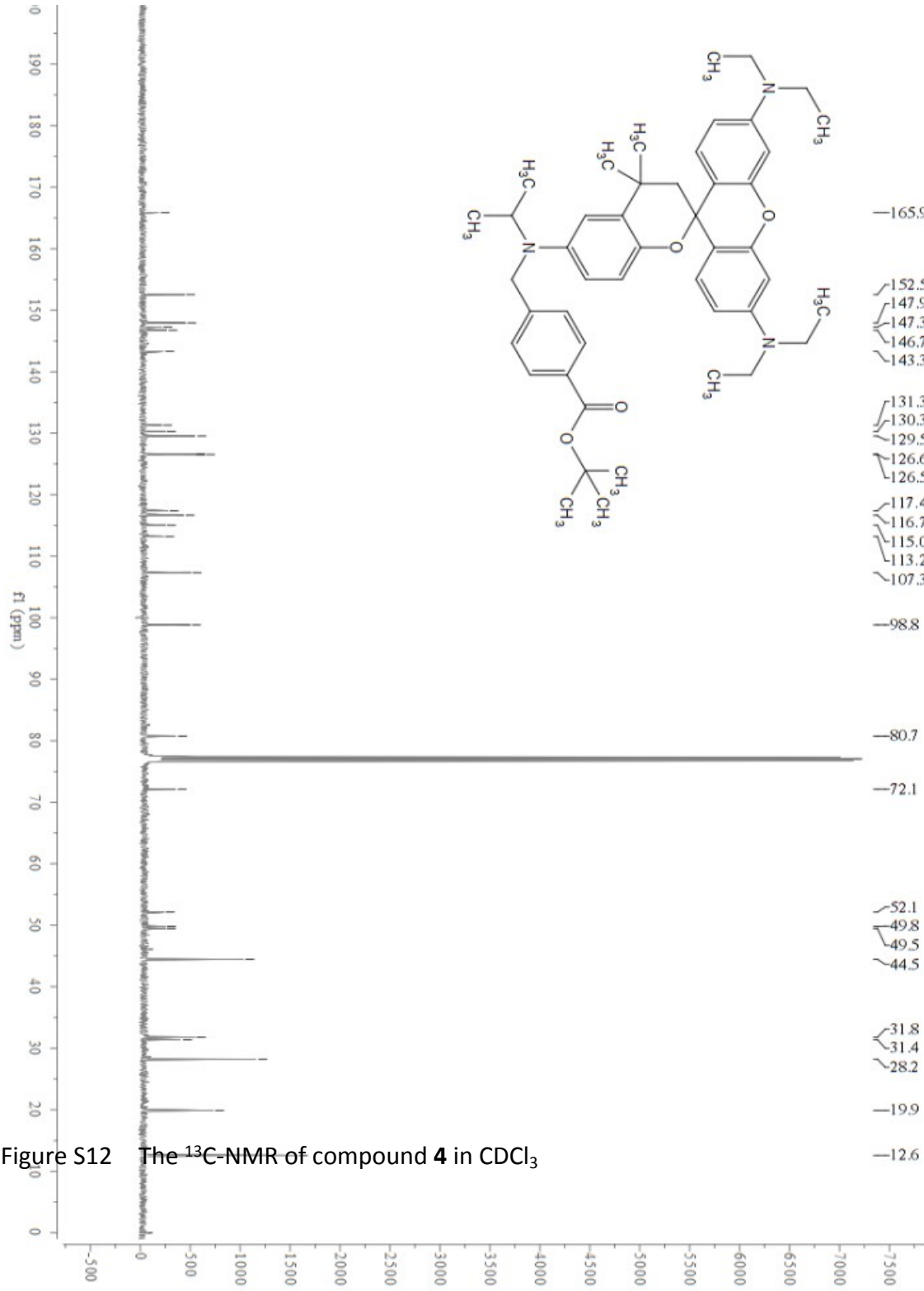

Figure S12 The ^{13}C-NMR of compound **4** in $CDCl_3$

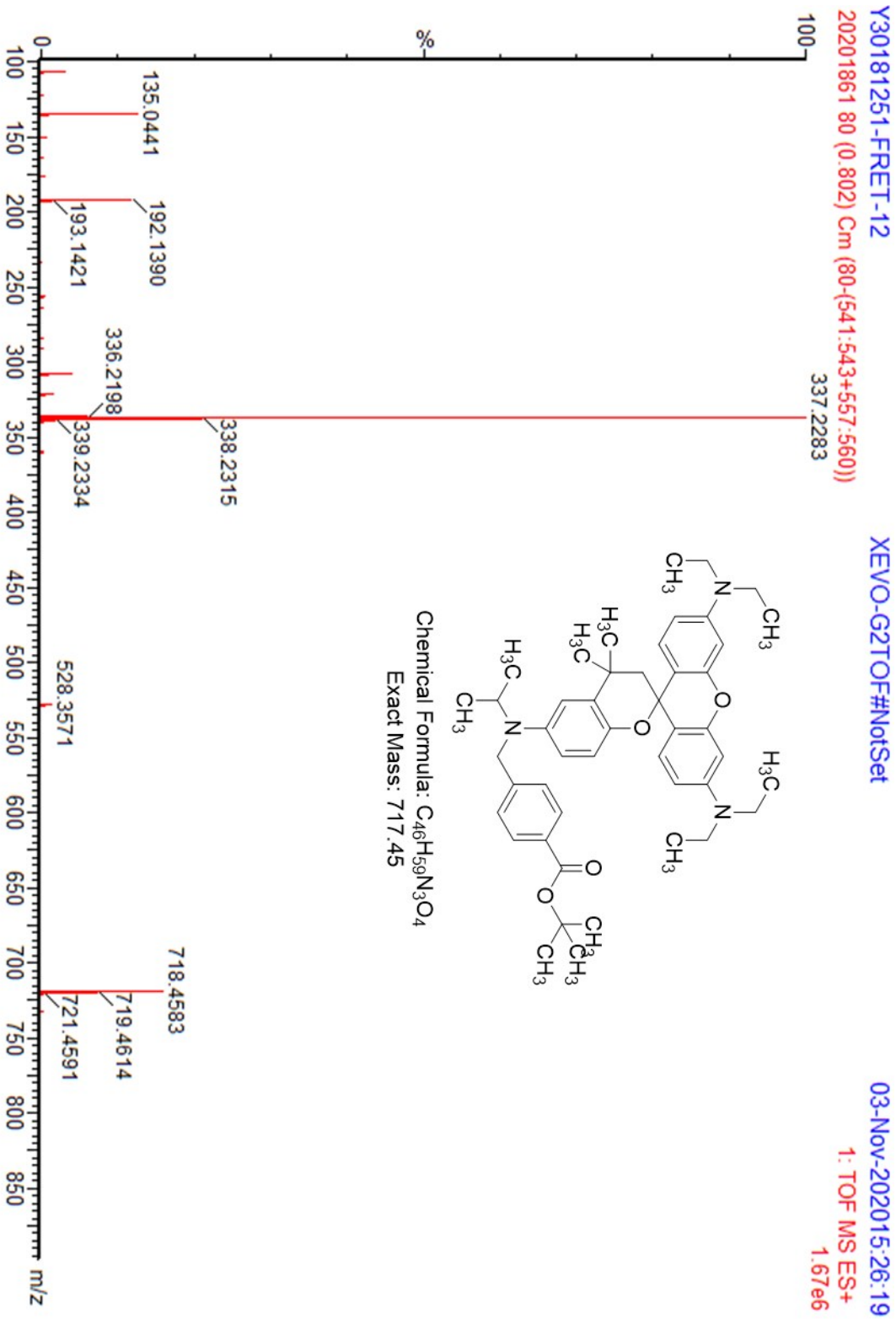

Figure S13 The HR-MS of compound **4**

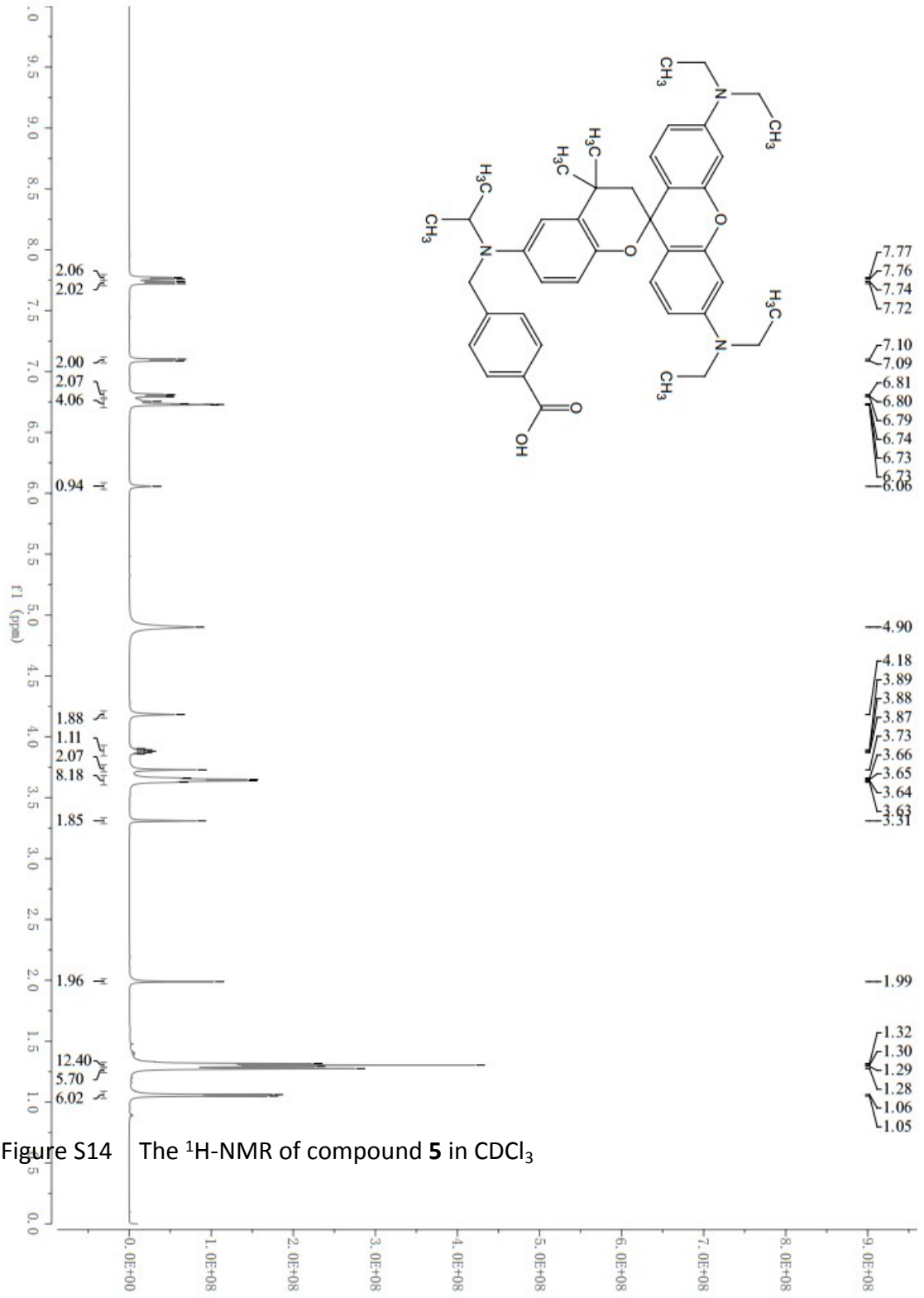

Figure S14 The ^{1}H-NMR of compound **5** in $CDCl_3$

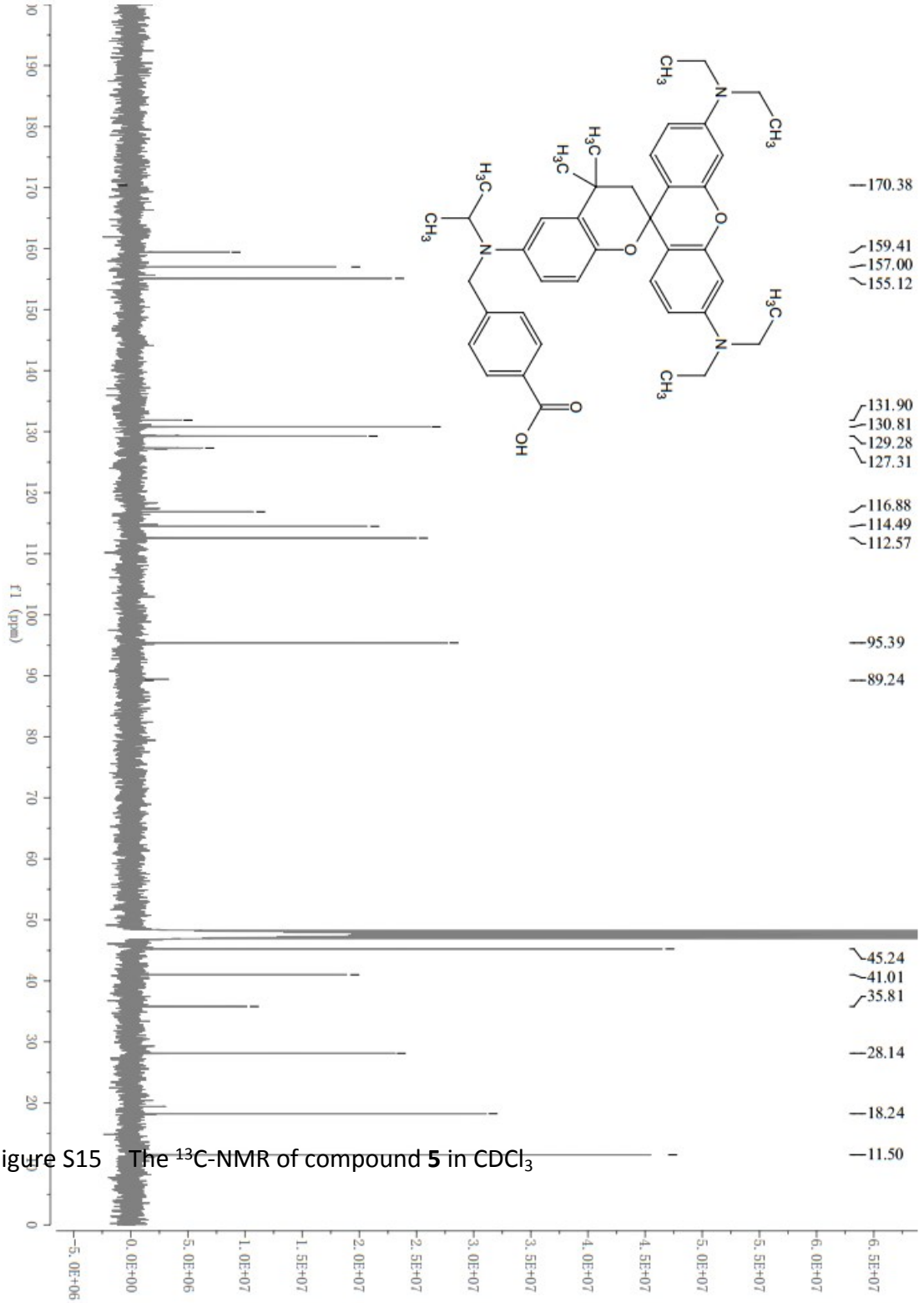

Figure S15 The ^{13}C-NMR of compound **5** in $CDCl_3$

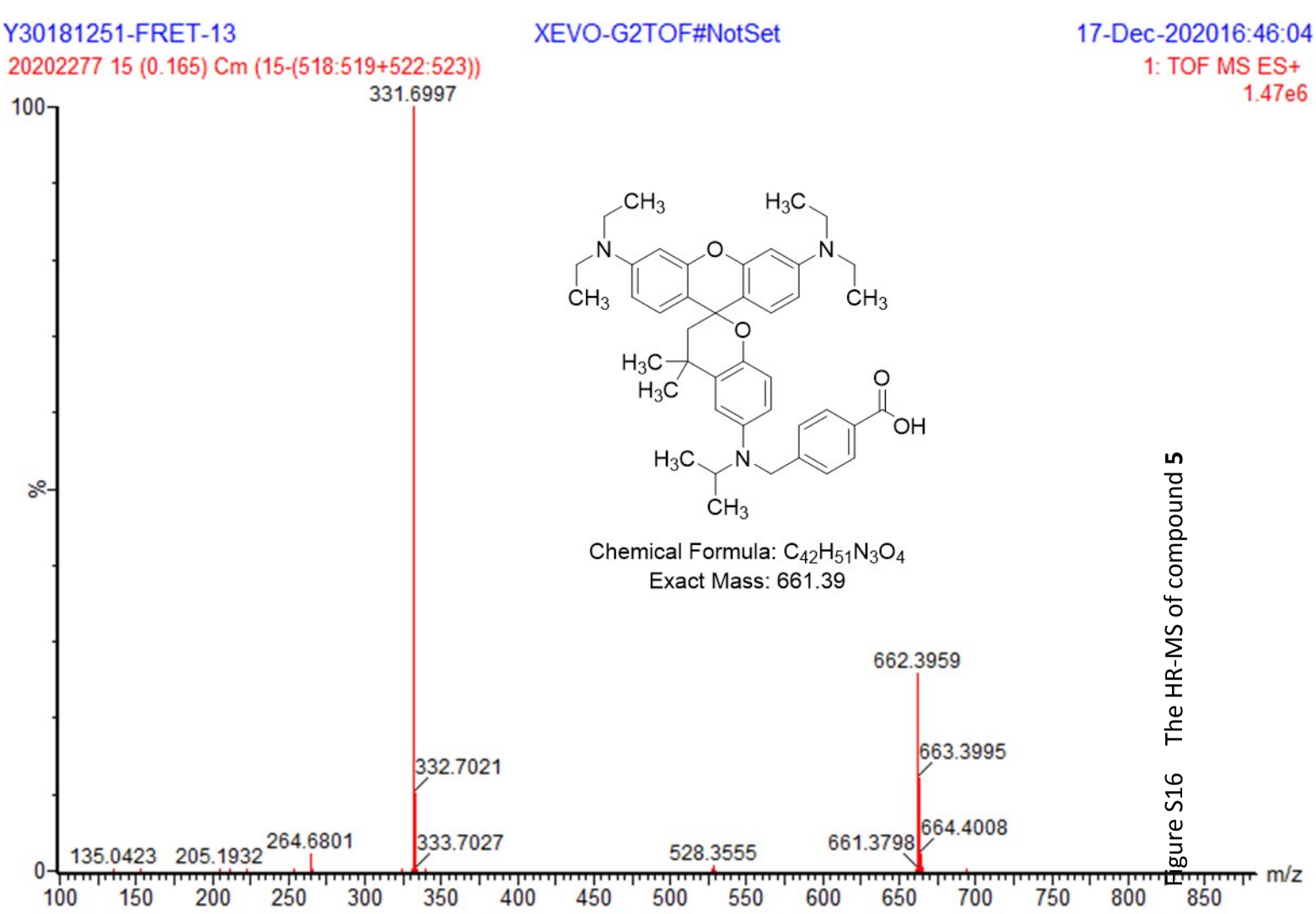

Figure S16 The HR-MS of compound **5**

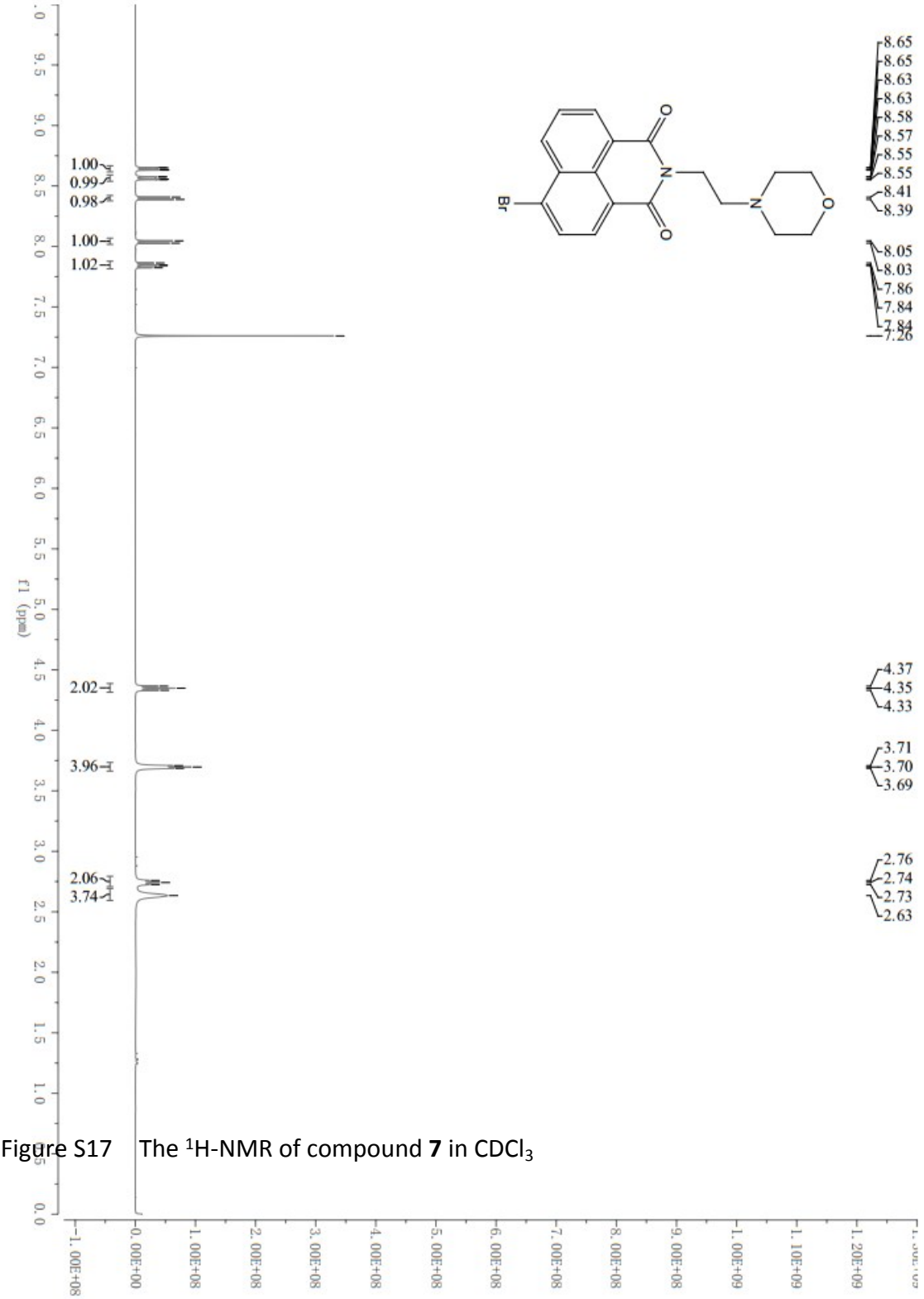

Figure S17 The ^{1}H-NMR of compound **7** in $CDCl_3$

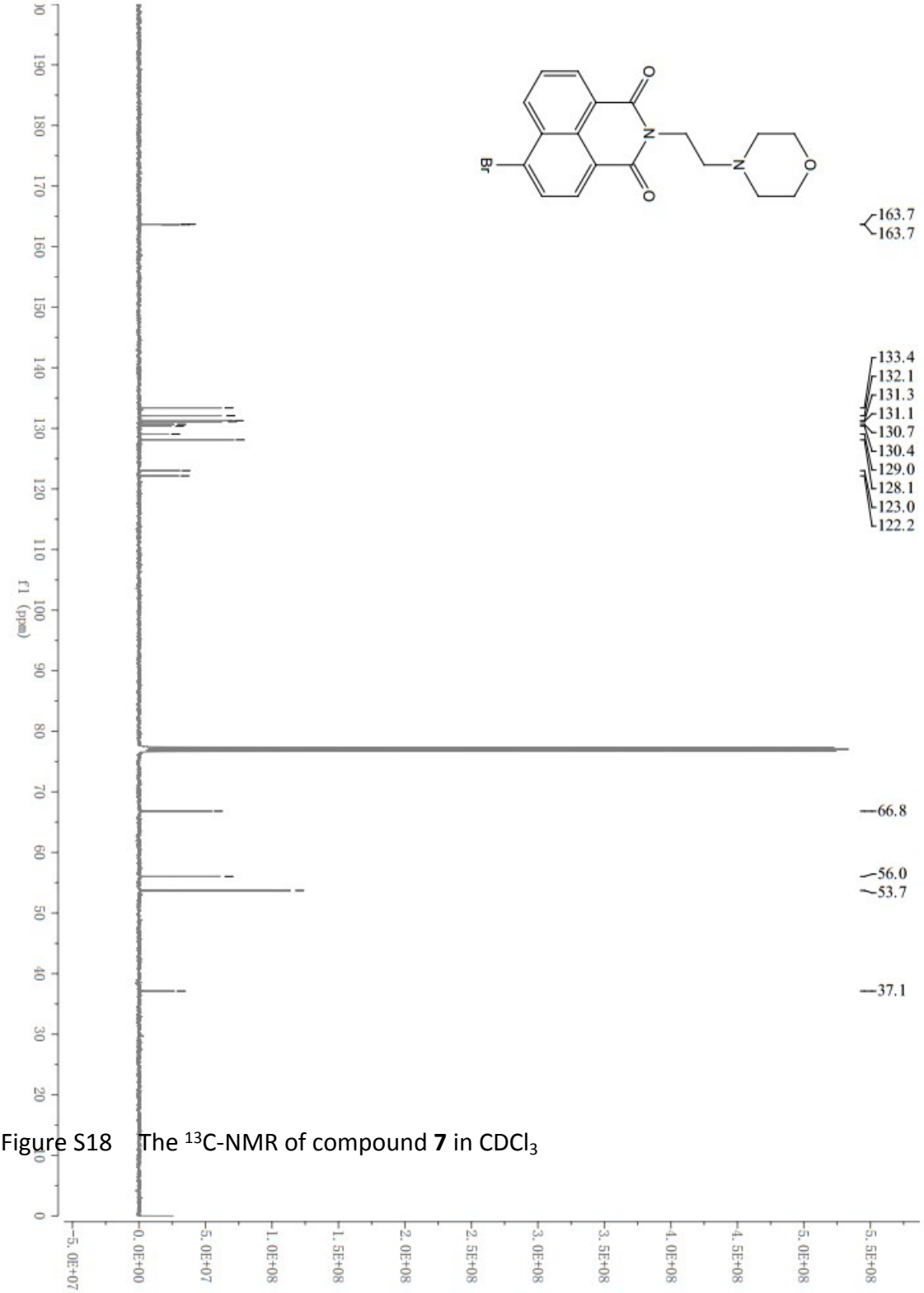

Figure S18 The ^{13}C-NMR of compound **7** in $CDCl_3$

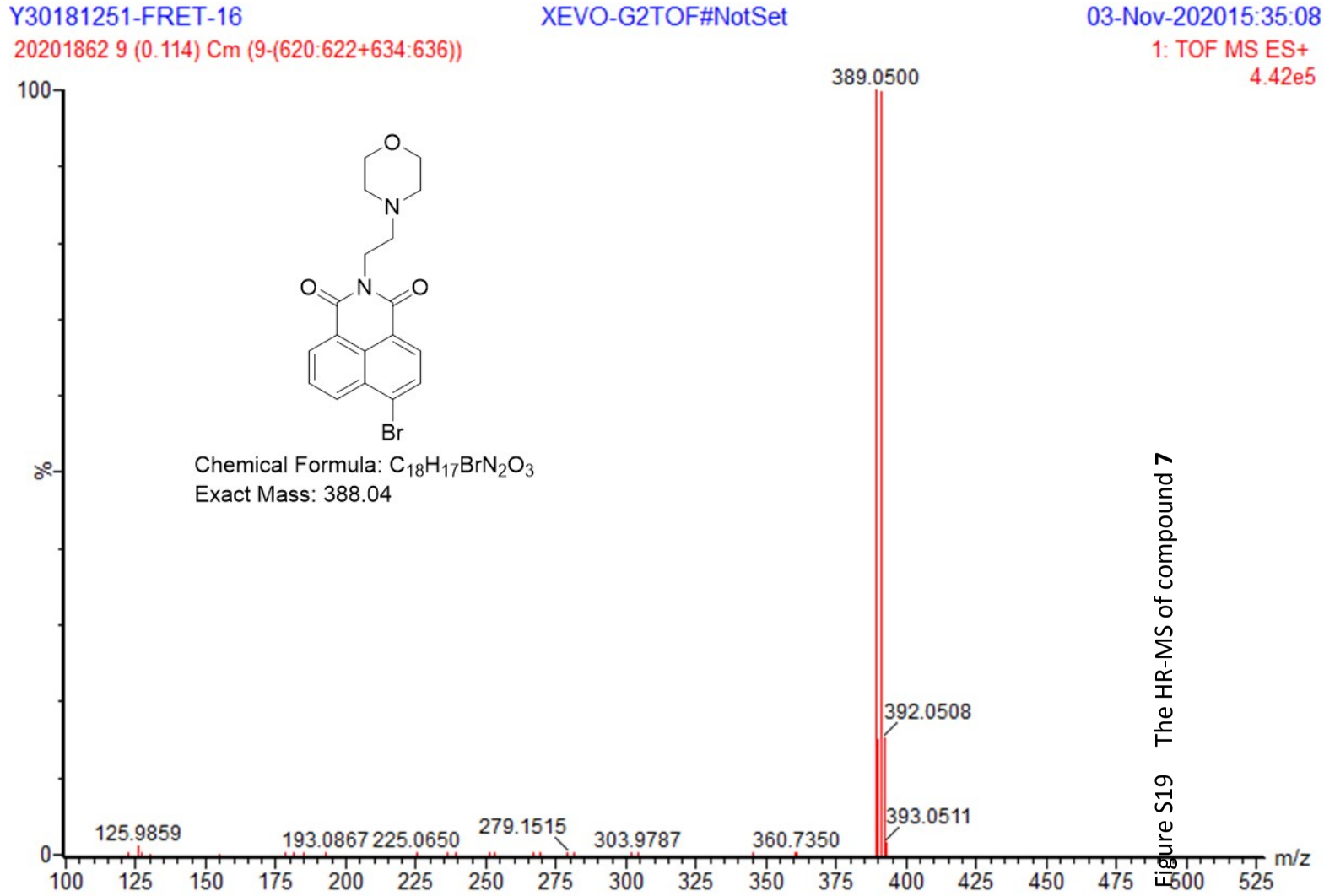

Figure S19 The HR-MS of compound **7**

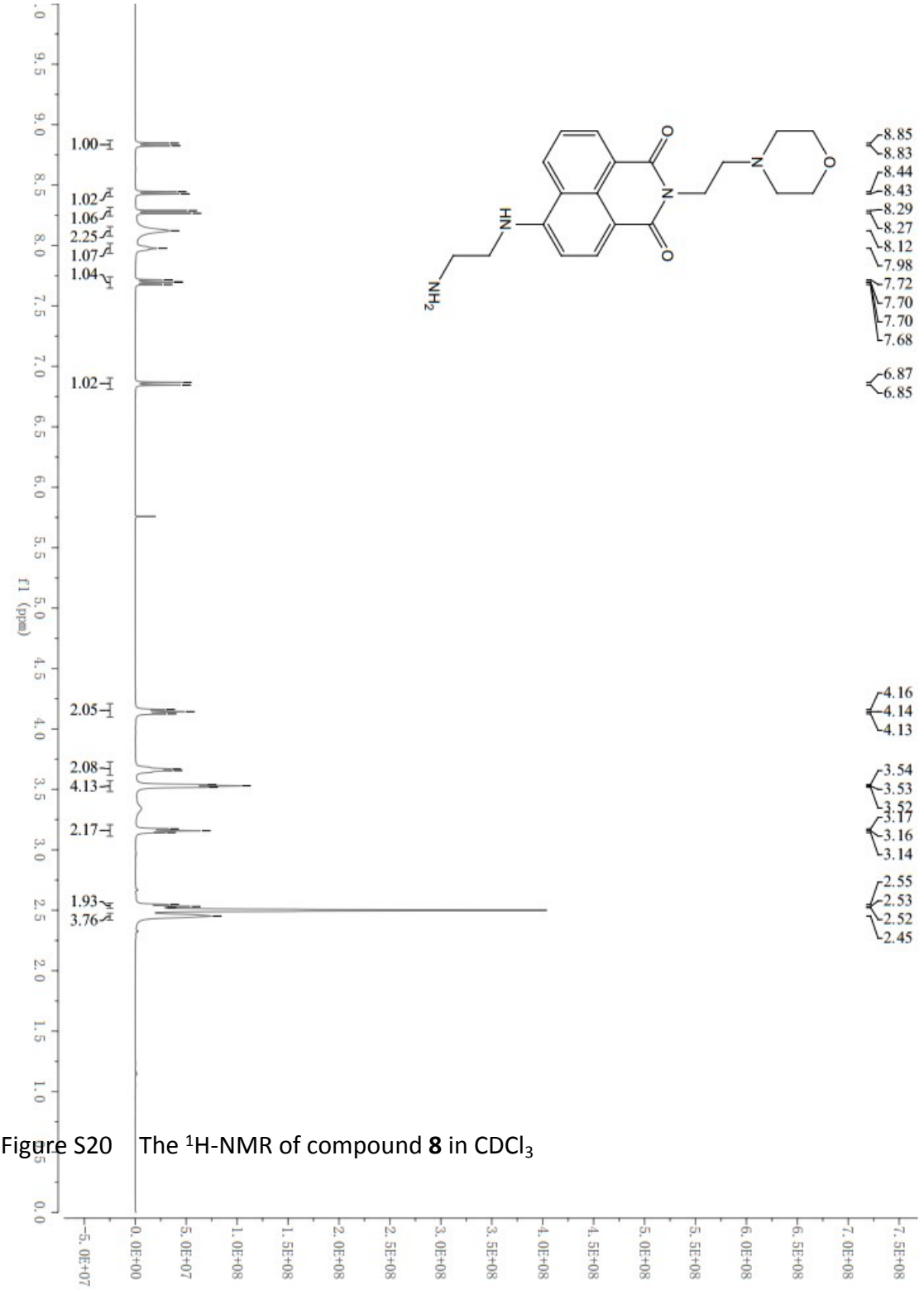

Figure S20 The ^{1}H-NMR of compound **8** in $CDCl_3$

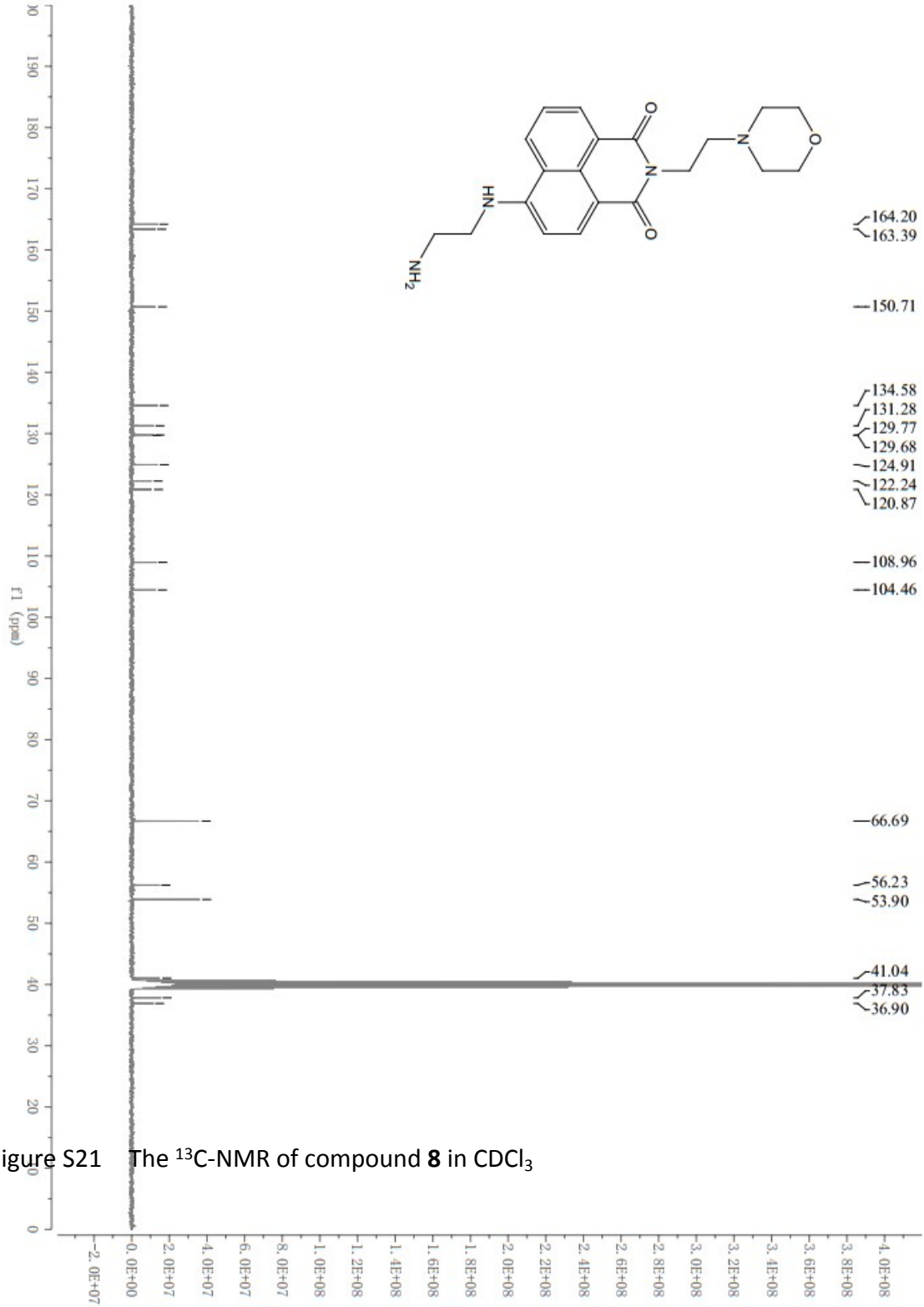

Figure S21 The ^{13}C-NMR of compound **8** in $CDCl_3$

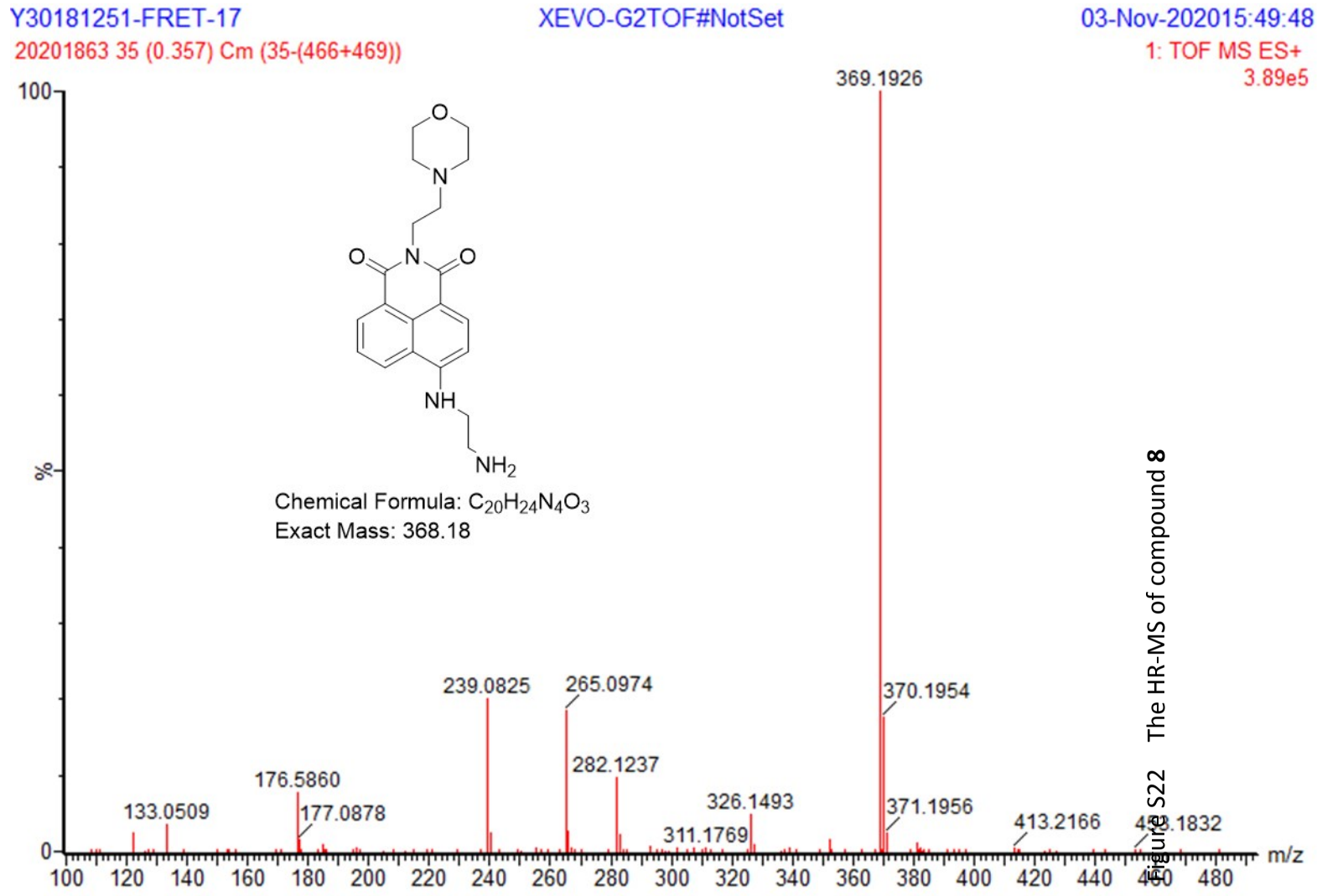

Figure S22 The HR-MS of compound **8**

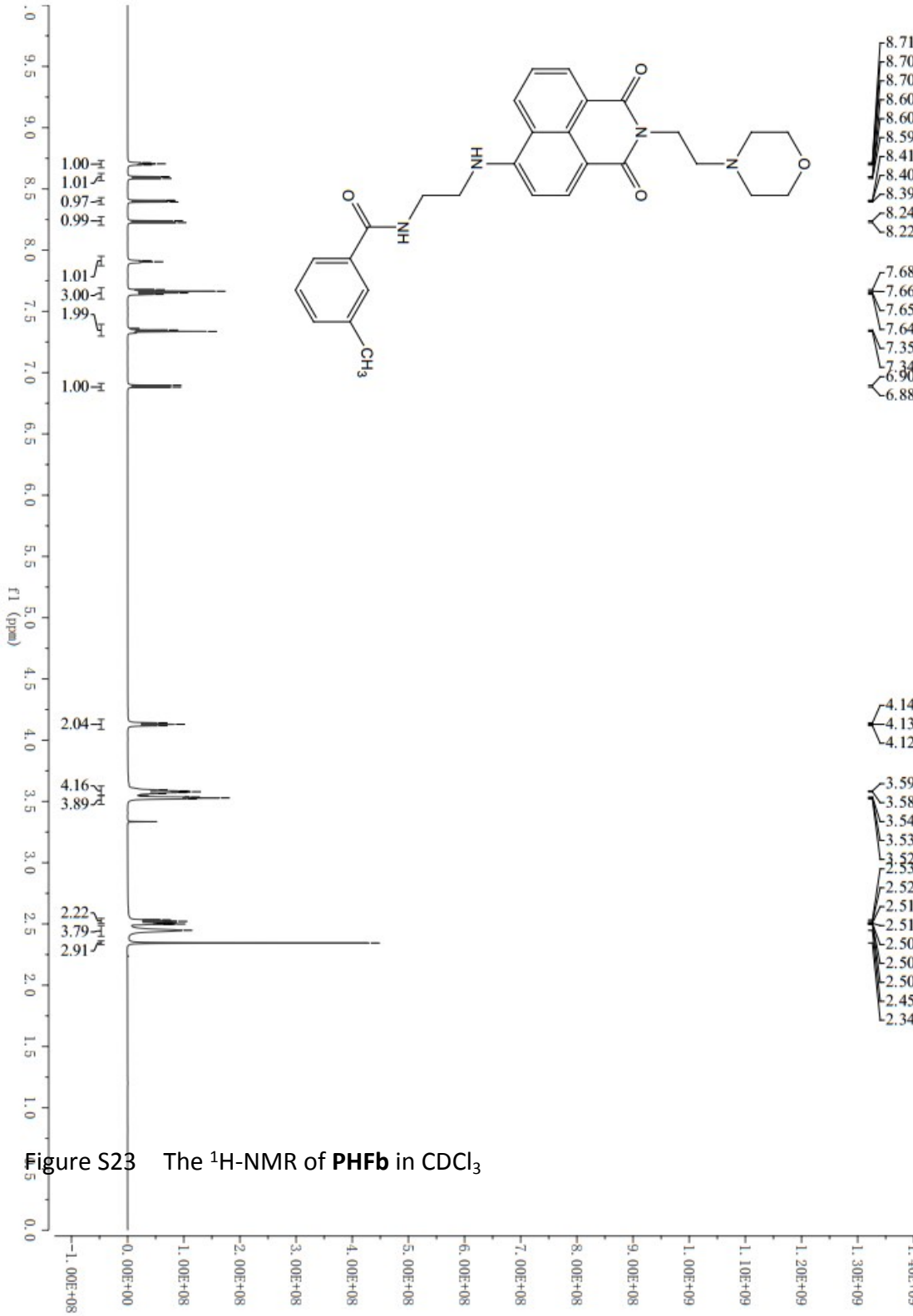

Figure S23 The ^{1}H-NMR of **PHFb** in $CDCl_3$

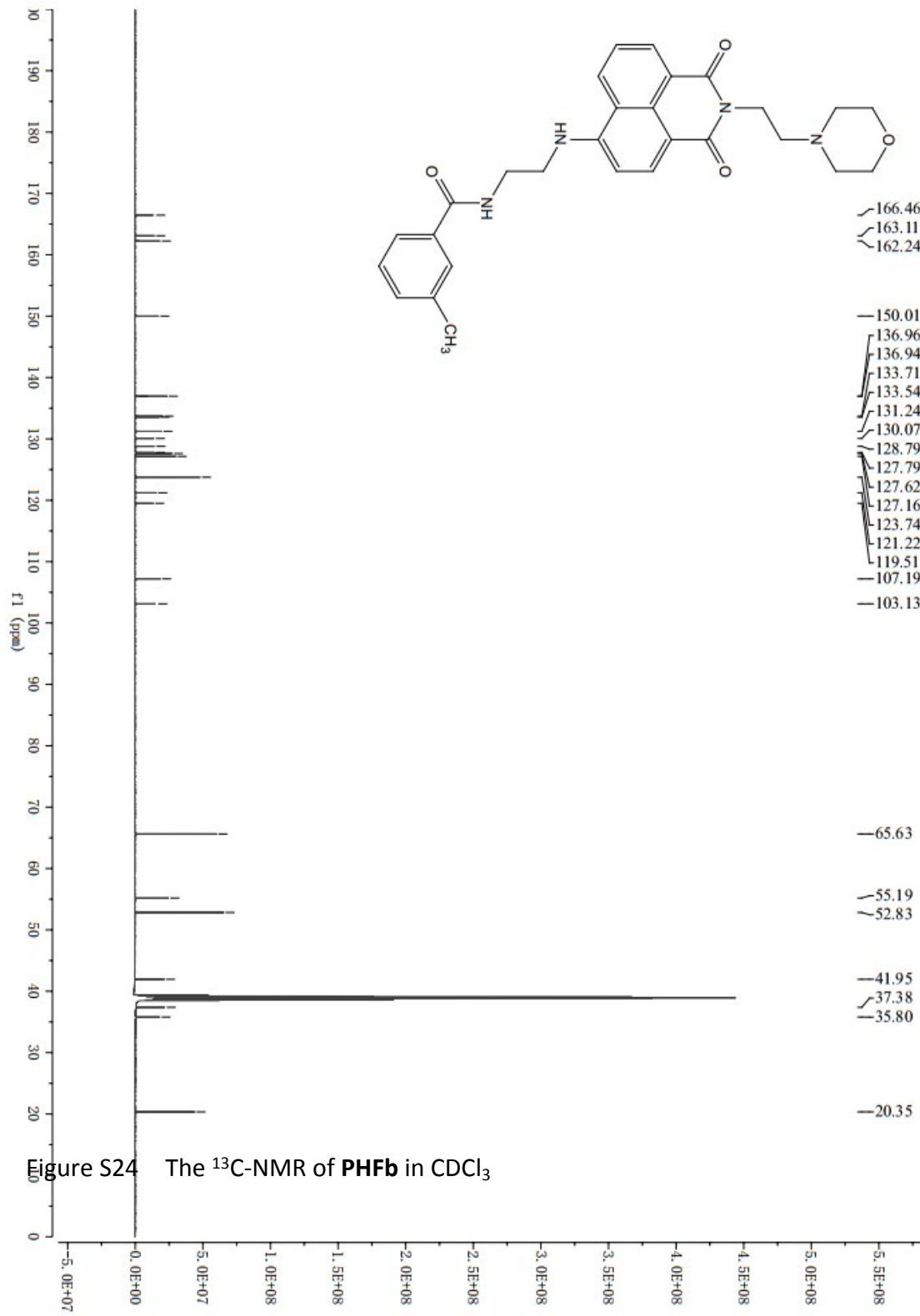

Figure S24 The ^{13}C-NMR of **PHFb** in $CDCl_3$

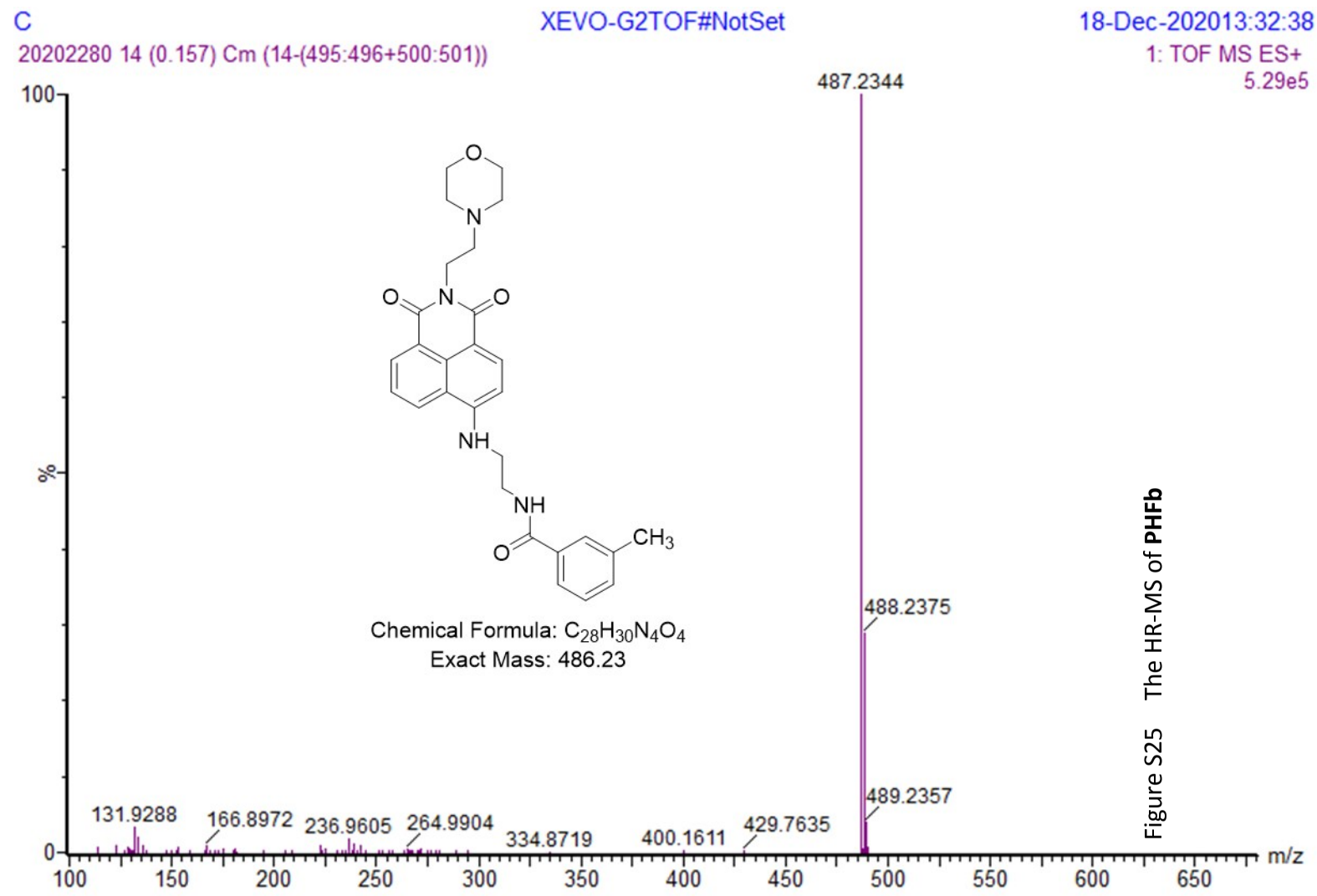

Figure S25 The HR-MS of **PHFb**

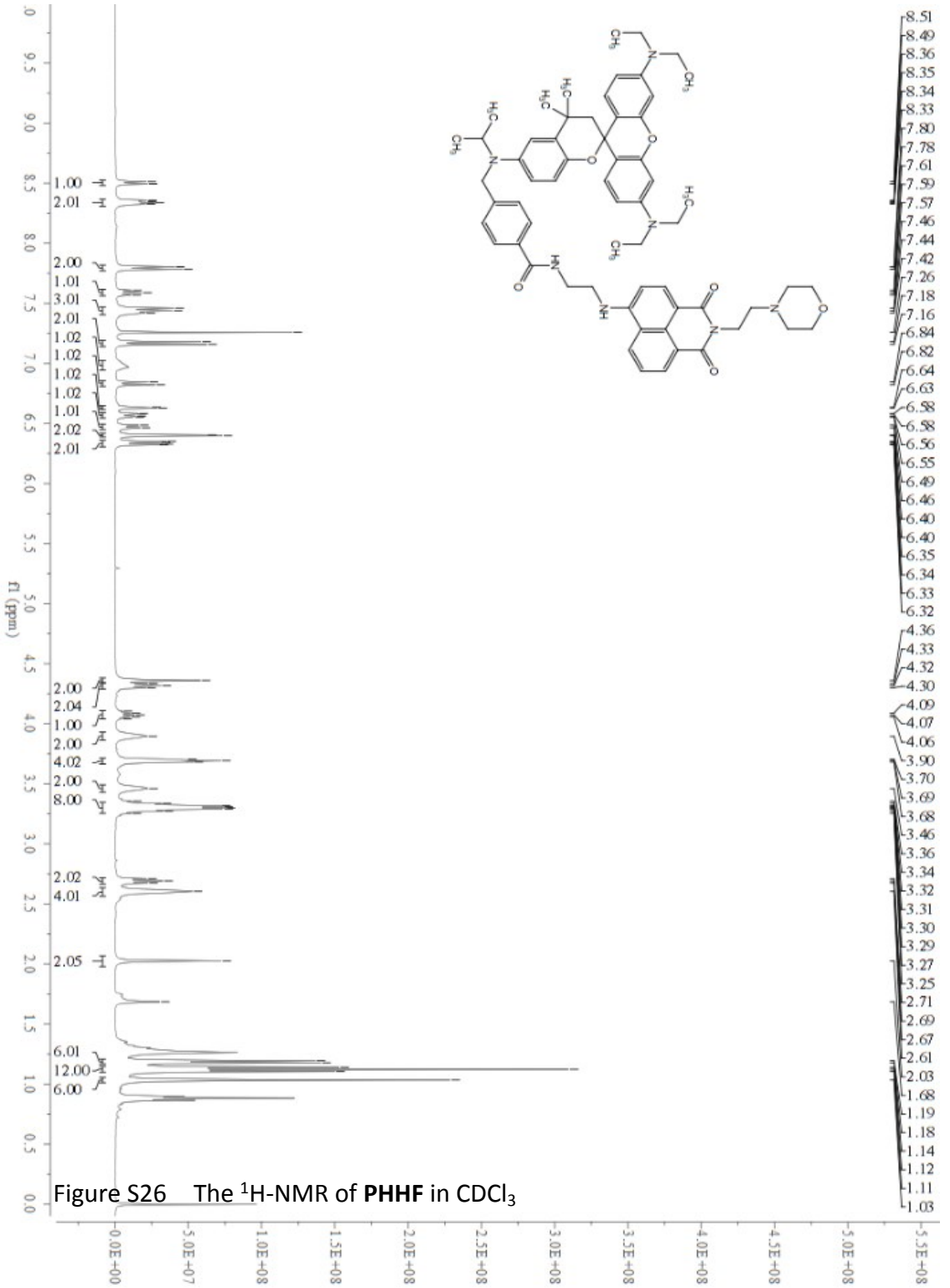

Figure S26 The ^{1}H-NMR of **PHHF** in $CDCl_3$

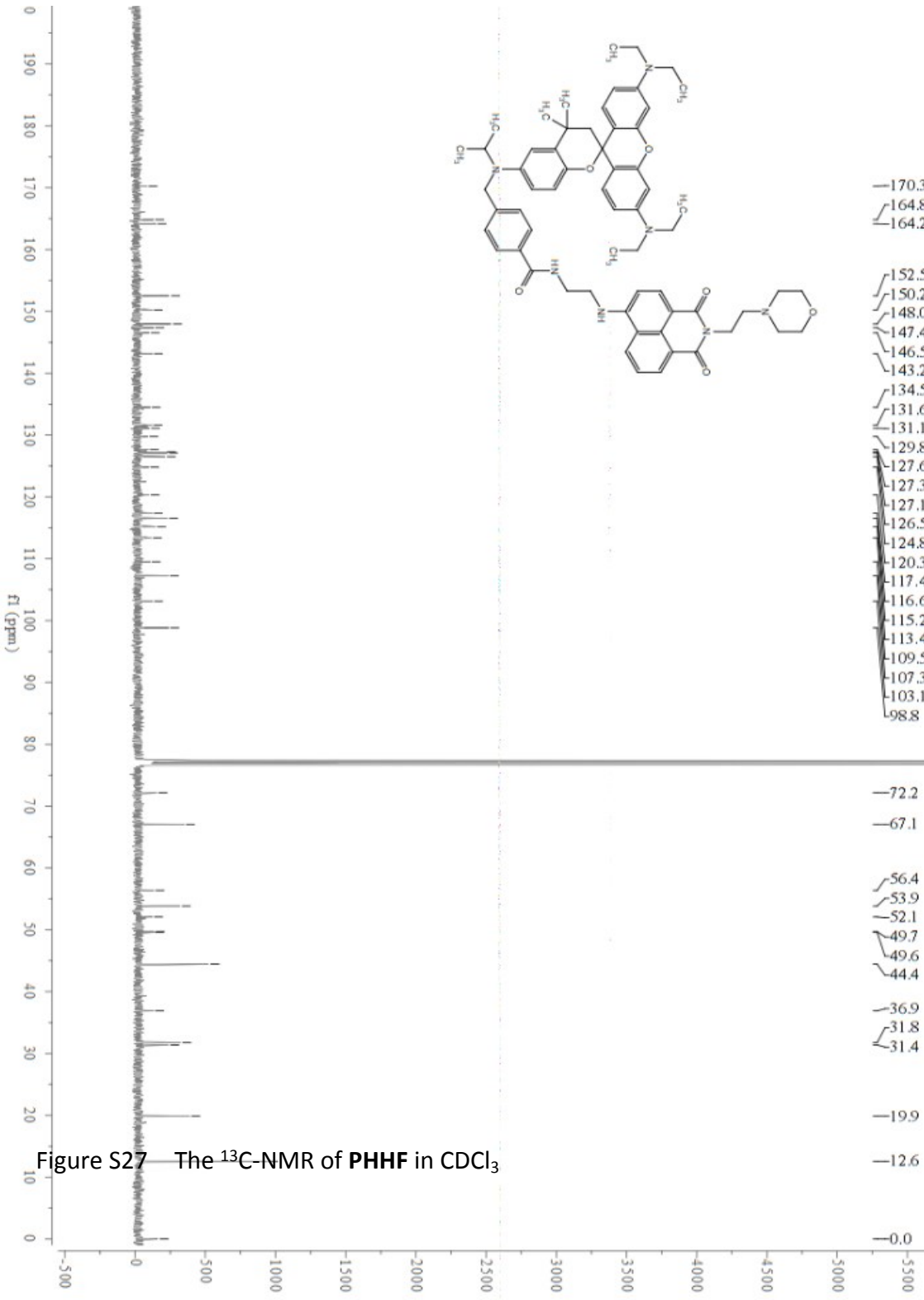

Figure S27 The ^{13}C-NMR of **PHHF** in $CDCl_3$

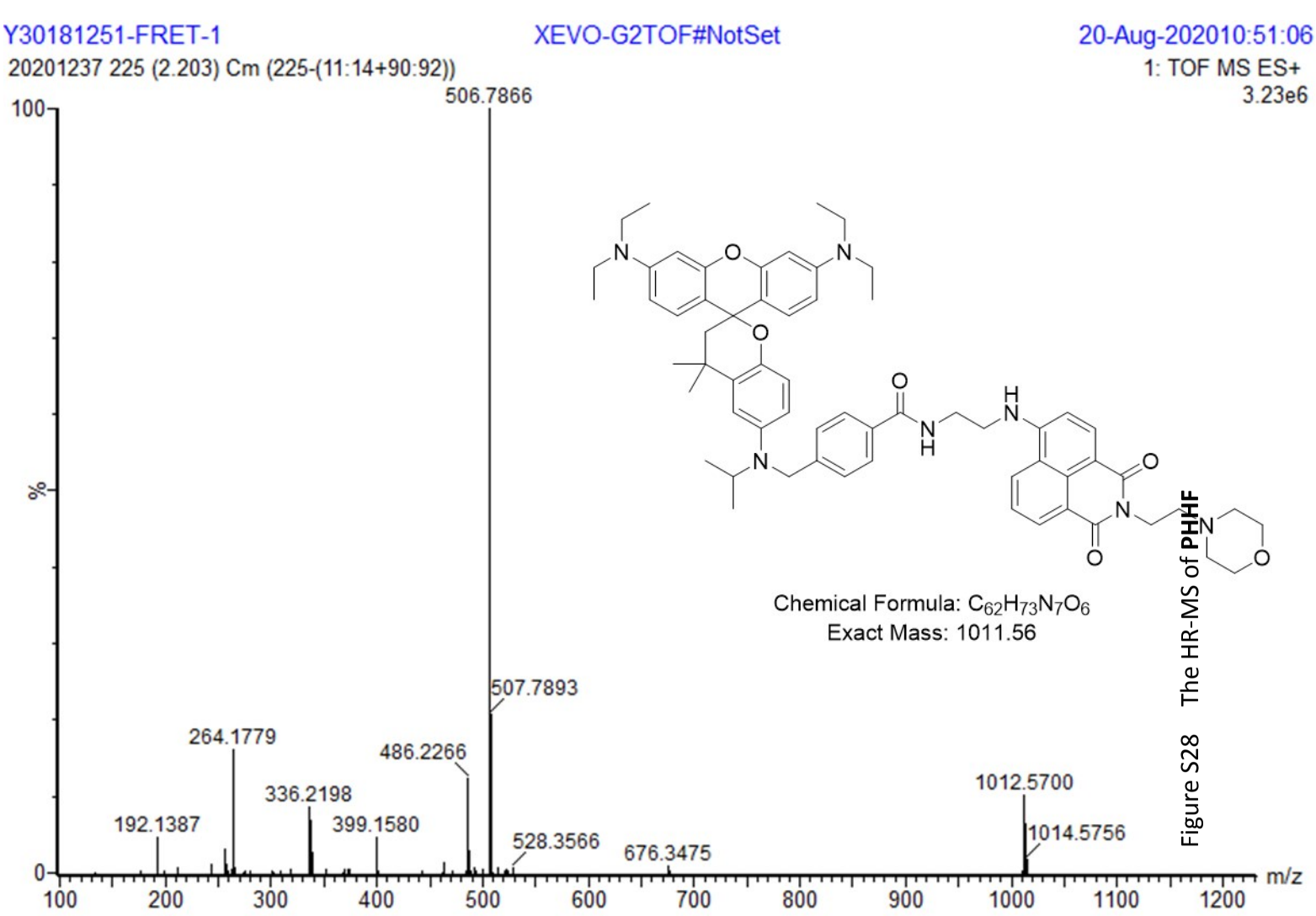

Figure S28 The HR-MS of **PHF**