Table of Contents

1. General Experimental·····S3
2. Chemical Synthesis·····S3
3. Primer and Gene Sequences·····S8
4. Construction of Plasmids·····S9
5. Protein Expression and Purification ·····S10
6. Protein Labeling Procedures·····S11
7. MS Spectrometry Analysis·····S12
8. FRET Assay·····S12
9. References·····S12
10. Supplementary Figures·····S13
11. NMR Spectra of Synthesized Compounds·····S18

1. General Experimental

All reactions involving moisture sensitive reagents were conducted in oven-dried glassware under an argon atmosphere. Anhydrous solvents were obtained through standard laboratory protocols. Analytical thin-layer chromatography (TLC) was performed on Whatman SiO_2 60 F-254 plates. Visualization was accomplished by UV irradiation at 254 nm or by staining with ninhydrin (0.3% w/v in glacial acetic acid/n-butyl alcohol 3:97). Flash column chromatography was performed with flash silica gel (particle size 32-63 μm) from Dynamic Adsorbents Inc (Atlanta, GA).

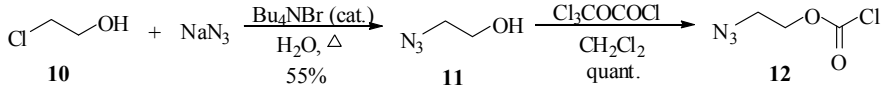
Specific rotations of chiral compounds were obtained at the designated concentration and temperature on a Rudolph Research Analytical Autopol II polarimeter using a 0.5 dm cell. Proton and carbon NMR spectra were obtained on Varian 300 and 500 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) as referenced to the residual solvents: chloroform (7.27 ppm for ^1H and 77.23 ppm for ^{13}C), methanol (3.31 ppm for ^1H and 49.15 ppm for ^{13}C), DMSO (2.50 ppm for ^1H and 39.51 ppm for ^{13}C), or water (4.80 ppm for ^1H). A minimal amount of 1,4-dioxane was added as the reference standard (67.19 ppm for ^{13}C) for carbon NMR spectra in deuterium oxide, and a minimal amount of sodium hydroxide pellet or concentrated hydrochloric acid was added to the NMR sample to aid in the solvation of amino acids which have low solubility in deuterium oxide under neutral conditions. ^1H NMR spectra are tabulated as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), number of protons, and coupling constant(s). Mass spectra were obtained at the Laboratory for Biological Mass Spectrometry at the Department of Chemistry, Texas A&M University.

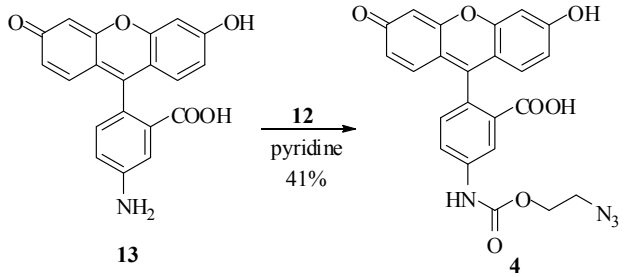
Compounds **1**,[2] **2** and **3**,[3] and **8** and **9**[2] were prepared according to literature procedures as previously described. Compound **6** was purchased from Click Chemistry Tools (Scottsdale, AZ). All other reagents were obtained from commercial suppliers and used as received.

2. Chemical Synthesis

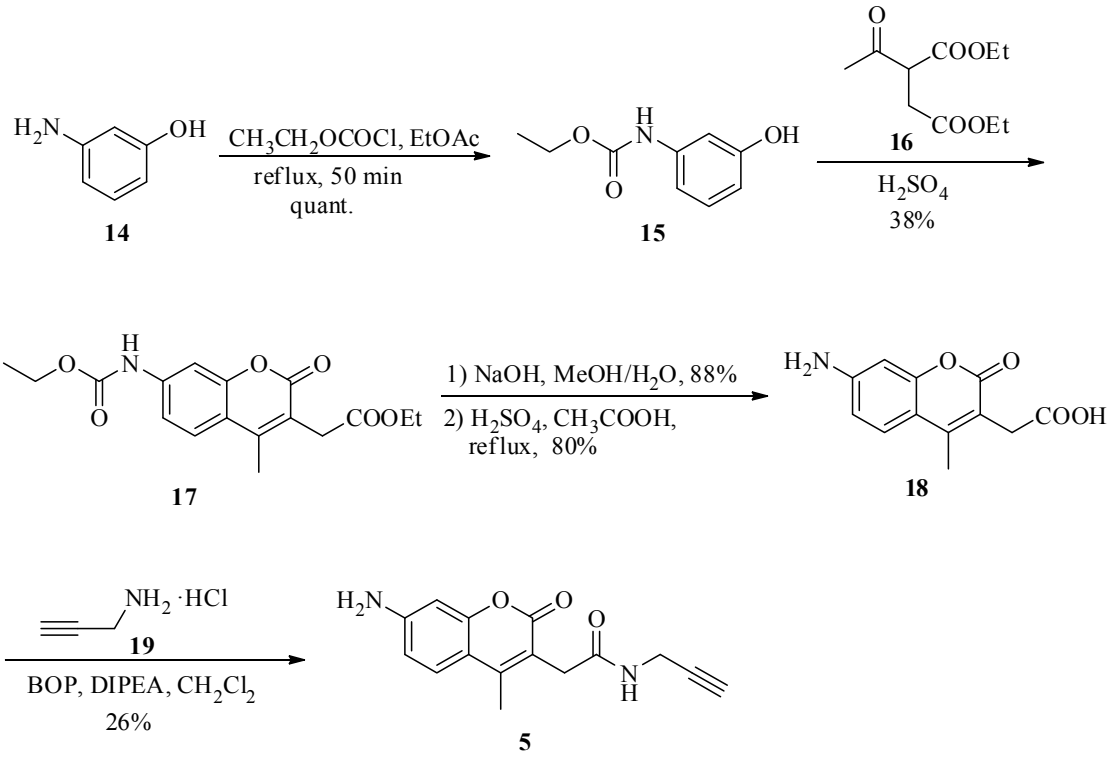
Compounds **4** was synthesized from fluorescein amine **13** through carbamate formation (***Scheme 1***). Compounds **5** was synthesized by amide coupling between and **18** and **19** (***Scheme 2***). Compound **7** was similarly obtained by coupling between **26** and **22** followed by deprotection (***Scheme 3***).

Scheme 1. Synthesis of **4**.



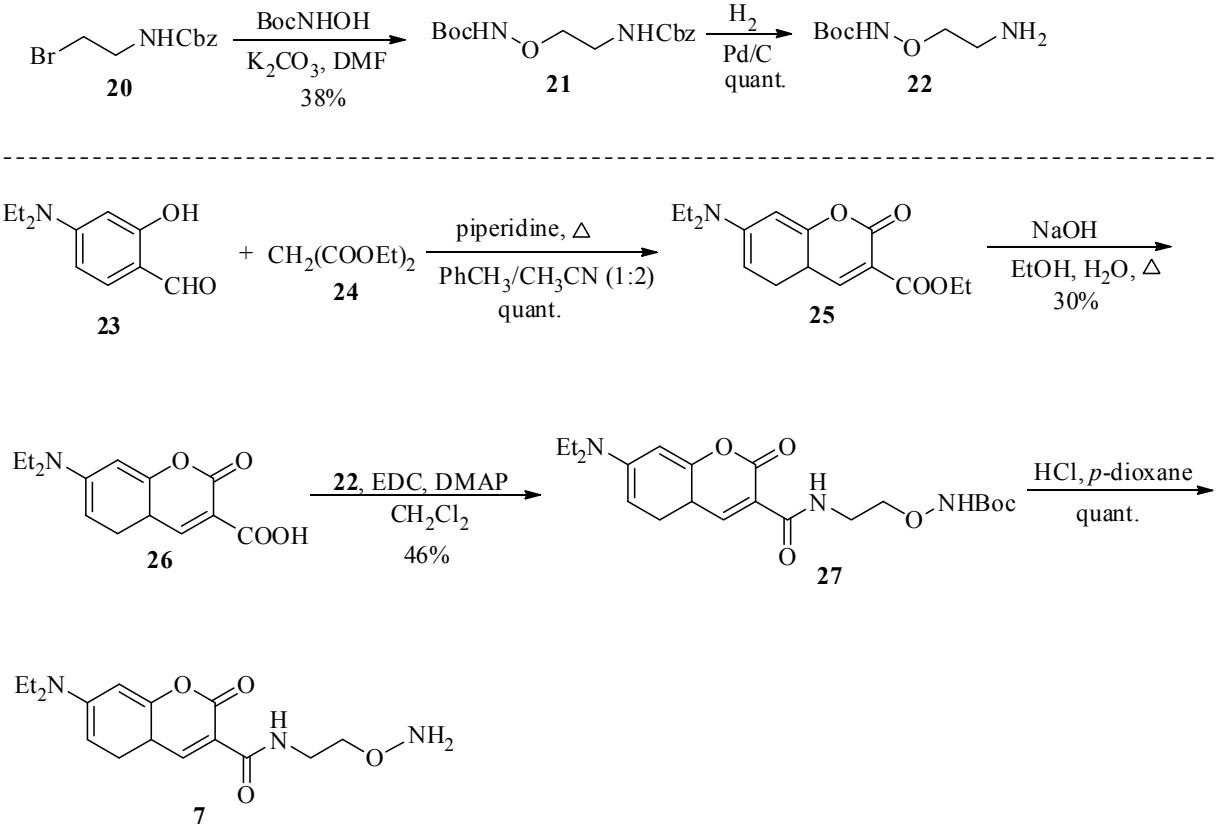


Scheme 2. Synthesis of **5**.



14 15 17 18 5

Scheme 3. Synthesis of **7**.



20 21 22 23 24 25 26 27 7

2-Azidoethanol (11).[4] To a solution of 2-chloroethanol (**10**, 25.2 g, 0.31 mol) in water (80 mL)

was added sodium azide (26.3 g, 0.40 mol) and tetrabutylammonium bromide (2.0 g, 6.2 mmol), and the mixture was stirred at room temperature for 2 h before being heated at 120 °C for 2 h. Sodium chloride was added to the cooled yellow solution until saturation, and the mixture was extracted with ethyl acetate (60 mL x 3). The combined organics were dried ($MgSO_4$), filtered, and evaporated to give a crude yellow oil (31 g). Distillation under an oil pump-generated vacuum (~0.1 mmHg, bp 87-90 °C) afforded **11** (14.9 g, 55%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.80-3.78 (m, 2 H), 3.46 (t, 2 H, *J* = 5.0 Hz), 1.88 (bs, 1 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 61.7, 53.7.

2-Azidoethyl chloroformate (12). To a solution of **11** (82 mg, 0.94 mmol) in anhydrous dichloromethane (0.5 mL) was added trichloromethyl chloroformate (0.13 mmol, 1.1 mmol), and the mixture was stirred at room temperature for 12 h. The volatiles were evaporated to leave crude **12** (0.14 g, quant.) as a yellow oil which was used without further purification. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.43 (t, 2 H, *J* = 5.2 Hz), 3.63 (t, 2 H, *J* = 5.0 Hz).

5-((2'-Azidoethyl)oxycarbonylamino)fluorescein (4). To a solution of fluorescein amine isomer I (Aldrich, 0.20 g, 0.58 mmol) in pyridine (2.0 mL, 24.7 mmol) cooled in an ice/water bath was added **12** (0.14 g, 0.94 mmol) dropwise over 5 min with the aid of a small amount of anhydrous dichloromethane (0.1 mL). The mixture was then stirred at room temperature for 48 h, and water (5 mL) was added. The mixture was diluted in ethyl acetate (50 mL), washed with water (10 mL), hydrochloric acid (0.1 *N*, 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated, chromatographed (EtOAc/hexanes, 1:1), and crystallized in ethyl acetate/hexanes to give **4** (0.11 g, 41%) as an orange solid. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.32 (s, 1 H), 10.12 (s, 2 H), 8.12 (s, 1 H), 7.78 (dd, 1 H, *J* = 8.5, 1.5 Hz), 7.20 (d, 1 H, *J* = 8.5 Hz), 6.66 (d, 2 H, *J* = 2.0 Hz), 6.59 (d, 2 H, *J* = 8.5 Hz), 6.54 (dd, 2 H, *J* = 8.7, 2.2 Hz), 4.31 (t, 2 H, *J* = 4.7 Hz), 3.65 (t, 2 H, *J* = 5.2 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 168.6, 159.5, 153.3, 151.9, 146.2, 140.7, 129.1, 127.1, 125.7, 124.6, 112.6, 112.4, 109.7, 102.2, 83.3, 63.1, 49.7; HRMS (ESI) calcd for $C_{23}H_{17}N_4O_7$ ($[M+H]^+$) 461.1097, found 461.1094.

3-*N*-(Carbethoxy)aminophenol (15).[5] To a 500 mL round-bottomed flask fitted with a condenser, an addition funnel with pressure-equilibrating side arm, and a magnetic stir bar was charged 3-aminophenol (**14**, 50.0 g, 0.45 mol) and ethyl acetate (170 mL), which was dried over $MgSO_4$ and filtered prior to use. The mixture was heated at reflux to give a grey solution, and ethyl chloroformate (22.2 mL, 0.22 mol) was added dropwise over 50 min. The mixture was cooled to room temperature, filtered, washed with ethyl acetate (100 mL x 3) and hexanes (100 mL x 3), and dried in vacuo to give the recovered hydrochloride salt of 3-aminophenol (32.9 g, quant.) as an off-white powder. The combined filtrate was evaporated to remove most of the solvents, and hexanes (50 mL) was added. The mixture was frozen at -20 °C for 1 h, crushed, filtered, washed with hexanes (100 mL), and dried in vacuo to give pure **15** (37.0 g) as an off-white solid. The filtrate was again concentrated, mixed with hexanes (50 mL) and frozen, and filtered and treated as above to give more **15** (4.5 g, quant.) as a brown solid (slightly impure). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.42 (s, 1 H), 7.14 (t, 1 H, *J* = 8.0 Hz), 6.96 (bs, 1 H), 6.78 (s, 1 H), 6.63 (d, 1 H, *J* = 8.0 Hz), 6.59 (dd, 1 H, *J* = 9.0, 2.5 Hz), 4.24 (q, 2 H, *J* = 7.0 Hz), 1.32 (t, 3 H, *J* = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 157.2, 154.3, 139.0, 130.1, 110.9, 110.5, 106.1, 61.9, 14.6.

Ethyl 7-carbethoxyamido-4-methylcoumarin-3-acetate (17).[6] To a brown solution of **15**

(4.56 g, 25.2 mmol) in 71% sulfuric acid (18 mL) was added diethyl acetylsuccinate (**16**, 5.7 mL, 27.6 mmol) dropwise through an addition funnel over 30 min. After 4 h stirring at room temperature, the mixture was poured into a mixture of ice and water (~100 g). Upon stirring and trituration the initial white gel solidified, which was filtered and dried to give an off-white solid (6.6 g). The material was suspended in sodium hydroxide (1.0 *N*, 100 mL) and extracted with dichloromethane (120 mL x 3). The combined organics were washed with sodium hydroxide (0.5 *N*, 50 mL x 2) and brine (50 mL), dried ($MgSO_4$), evaporated, suspended in ether (50 mL), filtered, and dried in vacuo to give **17** (3.2 g, 38%) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.52 (d, 1 H, *J* = 9.0 Hz), 7.41 (d, 1 H, *J* = 2.0 Hz), 7.34 (d, 1 H, *J* = 8.5 Hz), 7.04 (s, 1 H), 4.26 (q, 2 H, *J* = 7.2 Hz), 4.20 (q, 2 H, *J* = 7.2 Hz), 3.72 (s, 2 H), 2.38 (s, 3 H), 1.33 (t, 3 H, *J* = 7.2 Hz), 1.28 (t, 3 H, *J* = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.7, 161.9, 153.4, 153.2, 149.0, 141.3, 125.7, 117.8, 115.8, 114.6, 105.8, 61.9, 61.5, 33.2, 15.5, 14.7, 14.4; HRMS (ESI) calcd for $C_{17}H_{19}NO_6Li$ ($[M+Li]^+$) 340.1372, found 340.1373.

7-Carbethoxyamido-4-methylcoumarin-3-acetic acid (18).[6] To a suspension of **17** (1.37 g, 4.1 mmol) in a mixed solvent of methanol (20 mL) and water (20 mL) was added sodium hydroxide (1.0 g, 25.0 mmol), and the mixture was stirred at room temperature for 36 h to give a milky solution. Most of the methanol was evaporated, and the remaining solution was diluted in water (10 mL) and extracted with ethyl acetate (30 mL). The separated aqueous phase was adjusted to pH 1 with concentrated hydrochloric acid, and the precipitate was filtered, washed with water (50 mL) and dichloromethane (50 mL), and dried to give a 7:1 mixture of the corresponding acid of **17** with **18** (1.11 g, 88%) as a pink solid. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.17 (s, 1 H, minor product), 10.13 (s, 1 H), 7.72 (d, 1 H, *J* = 8.0 Hz), 7.54 (s, 1 H), 7.39 (d, 1 H, *J* = 8.5 Hz), 7.44 (d, 1 H, *J* = 8.5 Hz, minor product), 6.57 (d, 1 H, *J* = 7.5 Hz, minor product), 6.41 (s, 1 H, minor product), 6.05 (s, 2 H, minor product), 4.16 (q, 2 H, *J* = 6.8 Hz), 3.70 (s, 2 H, minor product), 3.56 (s, 2 H), 2.34 (s, 3 H), 2.25 (s, 3 H, minor product), 1.25 (t, 3 H, *J* = 7.0 Hz); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 171.7, 161.0, 153.5, 152.6, 149.0, 142.4, 126.3, 117.4, 114.6, 114.5, 104.3, 60.8, 32.8, 15.0, 14.5.

The above crude mixture of products (1.11 g, 3.6 mmol) was dissolved in conc. sulfuric acid (1.5 mL) and acetic acid (1.5 mL) in a 100 mL flask and heated at reflux for 3 h to give a dark solution. Upon cooling down to room temperature ice/water (15 g) was added followed by another 25 mL of water. Charcoal (0.5 g) and Celite 545 (0.5 g) were then added, and the mixture was heated at reflux for 10 min. The hot mixture was carefully filtered to give a red filtrate, which was evaporated to a volume of ~15 mL. The solution was allowed to stand at room temperature overnight, and further cooling at -20 ^{o}C did not yield more precipitate. The mixture was filtered, washed with cold water (4 mL) and cold ethanol (10 mL), and dried to give **18** (0.71 g, 80%) as a brown solid. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.50 (d, 1 H, *J* = 9.0 Hz), 6.65 (dd, 1 H, *J* = 8.7, 1.8 Hz), 6.51 (d, 1 H, *J* = 2.0 Hz), 3.50 (s, 2 H), 2.27 (s, 3 H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.0, 161.5, 153.9, 150.8, 149.7, 126.6, 113.6, 112.3, 110.2, 99.8, 32.6, 14.9.

2-(7-Amino-4-methyl-2-oxo-2*H*-chromen-3-yl)-*N*-(prop-2-yn-1-yl)acetamide (5). To a solution of **18** (10 mg, 43 μmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 26 mg, 62 μmol), *N*,*N*-diisopropylethylamine (20 μL, 0.12 mmol) in anhydrous DMF (0.2 mL) was added propargylamine hydrochloride (**19**, 7.5 mg, 78 μmol), and the mixture was stirred at room temperature for 14 h. The mixture was diluted in ethyl acetate (40 mL), washed with sodium hydroxide (0.5 *N*, 10 mL), hydrochloric acid (0.5 *N*, 10 mL) and

brine (10 mL), dried (Na_2SO_4), evaporated, and chromatographed (EtOAc/hexanes, 2:1 followed by 5% MeOH in CH_2Cl_2) to give **5** (3.0 mg, 26%) as a yellow solid. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.31 (t, 1 H, *J* = 5.7 Hz), 7.45 (d, 1 H, *J* = 8.5 Hz), 6.57 (dd, 1 H, *J* = 8.7, 2.2 Hz), 6.40 (d, 1 H, *J* = 2.0 Hz), 6.04 (s, 2 H), 3.83 (dd, 2 H, *J* = 5.5, 2.5 Hz), 3.40 (s, 2 H), 3.09 (s, 2 H), 2.24 (s, 3 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 169.1, 161.6, 154.1, 152.4, 150.0, 126.3, 113.1, 111.3, 109.4, 99.4, 81.3, 72.8, 33.5, 28.0, 14.9; HRMS (ESI) calcd for $C_{15}H_{14}N_2O_3Na$ ([M+Na]$^+$) 293.0902, found 293.0914.

***N*-Boc-*O*-(2-(benzyloxycarbonylamino)ethyl)hydroxylamine (21)**. To a solution of **20**[7] (0.30 g, 1.2 mmol) and *N*-Boc-hydroxylamine (0.16 g, 1.2 mmol) in anhydrous DMF (1.5 mL) was added potassium carbonate (0.44 g, 3.2 mmol), and mixture was stirred at room temperature for 24 h. Water (10 mL) was then added, and the mixture was extracted with ether (50 mL). The organic phase was washed with sodium hydroxide (0.5 *N*, 10 mL), hydrochloric acid (0.5 *N*, 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated, and chromatographed (EtOAc/hexanes, 1:5) to give **21** (0.14 g, 38%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.47 (bs, 1 H), 7.38-7.29 (m, 5 H), 5.80 (bs, 1 H), 5.12 (s, 2 H), 3.86 (t, 2 H, *J* = 4.5 Hz), 3.44-3.43 (m, 2 H), 1.47 (s, 9 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 158.0, 157.4, 137.2, 129.1, 128.9, 128.6, 82.7, 76.2, 67.3, 39.7, 28.8; HRMS (ESI) calcd for $C_{15}H_{22}N_2O_5Na$ ([M+Na]$^+$) 333.1426, found 333.1418.

***tert*-Butyl 2-aminoethoxycarbamate (22)**. To a solution of **21** (0.33 g, 1.1 mmol) in methanol (20 mL) was added palladium on activated carbon (10% Pd, 0.11 g, 0.1 mmol), and the mixture was hydrogenated under a H_2 balloon at room temperature for 5 h. The mixture was filtered over Celite and evaporated to give **22** (0.20 g, quant.) as a grey oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.93 (t, 2 H, *J* = 5.0 Hz), 2.99 (t, 2 H, *J* = 5.2 Hz), 1.48 (s, 9 H), 1.46 (s, 2 H). The material was used without further purification.

Ethyl 7-(diethylamino)coumarin-3-carboxylate (25).[8] To a solution of 4-(diethylamino)salicylaldehyde (**23**, 5.93 g, 30.1 mmol) and diethyl malonate (7.6 g, 47.0 mmol) in a mixed solvent of toluene and acetonitrile (1:2, 210 mL) was added piperidine (8.9 mL, 90.1 mmol), and the red solution was heated at reflux for 10 h. The solvent was evaporated under reduced pressure, and the residue was directly chromatographed (EtOAc/hexanes, 1:3) to give **25** (9.2 g, quant.) as a yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.34 (s, 1 H), 7.29 (d, 1 H, *J* = 8.5 Hz), 6.54 (dd, 1 H, *J* = 8.7, 2.2 Hz), 6.35 (d, 1 H, *J* = 2.5 Hz), 4.29 (q, 2 H, *J* = 7.2 Hz), 3.37 (q, 2 H, *J* = 7.2 Hz), 1.32 (t, 3 H, *J* = 7.2 Hz), 1.16 (t, 6 H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.2, 158.4, 152.9, 149.2, 131.1, 109.6, 108.8, 107.6, 96.6, 61.1, 45.1, 14.4, 12.5.

7-(Diethylamino)coumarin-3-carboxylic acid (26).[8] To a solution of **25** (3.34 g, 11.5 mmol) in ethanol (30 mL) was added sodium hydroxide (1.0 *N*, 20.0 mL, 20.0 mmol), and a yellow precipitate quickly formed. The mixture was heated at reflux for 3 h to give a clear red solution, which was cooled to room temperature and filtered. The filtrate was adjusted to pH 3 with hydrochloric acid (2 *N*, ~18 mL), filtered, washed with water (30 mL), ethanol (10 mL) and ether (30 mL), and dried to give **26** (0.90 g, 30%) as an orange solid. ^{1}H NMR (CD_3OD, 500 MHz) δ 8.62 (s, 1 H), 7.58 (d, 1 H, *J* = 9.0 Hz), 6.86 (dd, 1 H, *J* = 9.0, 2.5 Hz), 6.62 (d, 1 H, *J* = 2.5 Hz), 3.56 (q, 2 H, *J* = 7.0 Hz), 1.25 (t, 6 H, *J* = 7.2 Hz); HRMS (ESI) calcd for $C_{14}H_{16}NO_4$ ([M+H]$^+$) 262.1079, found 262.1073.

***tert*-Butyl 2-(7-(diethylamino)-2-oxo-4a,5-dihydro-2*H*-chromene-3-carboxamido)ethoxy-**

carbamate (27). To a solution of **26** (0.146 g, 0.56 mmol), 4-(dimethylamino)pyridine (44 mg, 0.36 mmol) and **22** (74 mg, 0.42 mmol) in anhydrous dichloromethane (2 mL) was added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC hydrochloride, 0.136 g, 0.71 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was diluted in ethyl acetate (50 mL), washed with sodium hydroxide (0.5 *N*, 10 mL), hydrochloric acid (0.5 *N*, 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated, and chromatographed (EtOAc/hexanes, 1:3 to 1:1) to give **27** (81 mg, 46%) as a yellow oil which solidified upon standing. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.06 (t, 1 H, *J* = 6.0 Hz), 8.67 (s, 1 H), 8.07 (s, 1 H), 7.40 (d, 1 H, *J* = 9.0 Hz), 6.63 (dd, 1 H, *J* = 8.8, 2.5 Hz), 6.48 (d, 1 H, *J* = 2.4 Hz), 3.96 (t, 2 H, *J* = 5.1 Hz), 3.70 (dt, 2 H, *J* = 5.4, 5.4 Hz), 3.44 (q, 4 H, *J* = 7.2 Hz), 1.47 (s, 9 H), 1.22 (t, 6 H, *J* = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.2, 162.8, 157.8, 156.9, 152.6, 148.4, 131.3, 110.2, 108.6, 96.9, 81.5, 74.9, 45.3, 37.8, 28.4, 12.5; HRMS (ESI) calcd for $C_{21}H_{30}N_3O_6$ ($[M+H]^+$) 420.2135, found 420.2129; calcd for $C_{21}H_{29}N_3O_6Li$ ($[M+Li]^+$) 426.2216, found 426.2214; calcd for $C_{21}H_{29}N_3O_6Na$ ($[M+Na]^+$) 442.1954, found 442.2010.

***N*-(2-(Aminooxy)ethyl)-7-(diethylamino)-2-oxo-4a,5-dihydro-2*H*-chromene-3-carboxamide (7)**. To a solution of **27** (62 mg, 0.15 mmol) in 1,4-dioxane (1.0 mL) was added hydrogen chloride in dioxane (4.0 M, 0.3 mL, 1.2 mmol), and the mixture was stirred at room temperature for 4 h. Water (10 mL) was added followed by saturated sodium bicarbonate (20 mL), and the mixture was extracted with chloroform (50 mL). The separated organic phase was washed with brine (10 mL), dried (Na_2SO_4), and evaporated to afford **7** (48 mg, quant.) as a yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.03 (s, 1 H), 8.69 (s, 1 H), 7.42 (d, 1 H, *J* = 8.5 Hz), 6.63 (dd, 1 H, *J* = 9.2, 1.7 Hz), 6.48 (d, 1 H, *J* = 2.0 Hz), 3.88 (bs, 2 H), 3.69 (bs, 2 H), 3.44 (q, 4 H, *J* = 7.2 Hz), 1.23 (t, 6 H, *J* = 7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 163.9, 162.9, 157.8, 152.7, 148.4, 131.3, 110.2, 110.1, 108.5, 96.7, 45.2, 38.5, 12.6; HRMS (ESI) calcd for $C_{16}H_{22}N_3O_6$ ($[M+H]^+$) 320.1610, found 320.1616.

3. Primer and Gene Sequences

3.1 Primer Sequences

Forward primer NP2: agcgcggccgcgtcgacggtaccctcgagtctggtaaag
Reverse primer NP2: attgcggccgcccatggtatatctccttcttatacttaac
F1-QBP3TAG: tatacatatggcctaggattaaaaattagttgtcgc
R1-QBP141TAA: cagttccatataggcttaatcgatgttcgggaa
F2-QBP141TAA: ttcccgaagaatcgattaagcctatatggaactg
R2-QBP: gagggtacctcagtgatggtgatggtgatgtttcggttcagtacc
PylRS->AcKRS: acctgcgataccggtttccacccaag
PylRS->AcKRS R: cttaagttacaggttggtagaaatccc

3.2 Genes Sequences

QBP2m:
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4. Construction of Plasmids

All the plasmid structures were confirmed by DNA sequencing. All oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. All the PCR reactions were performed with Phusion® High-Fidelity DNA Polymerase from New England Biolabs Inc. (Ipswich, MA). All the restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase (T4 PNK) were purchased from New England Biolabs Inc. (Ipswich, MA).

4.1 Construction of pETtrio-PylT-PylRS-MCS

The plasmid pPylRS-PylT-MCS was derived from pPylRS-pylT-GFP1TAG149TAA.[2] Forward

primer NP2 and reverse primer NP2 were used to clone the whole plasmid without the gene GFP1TAG149TAA. Meanwhile, four restriction sites, *Nco I, Not I, Sal I and Kpn I* were introduced to the positions where GFP1TAG149TAA was located in the original plasmid.

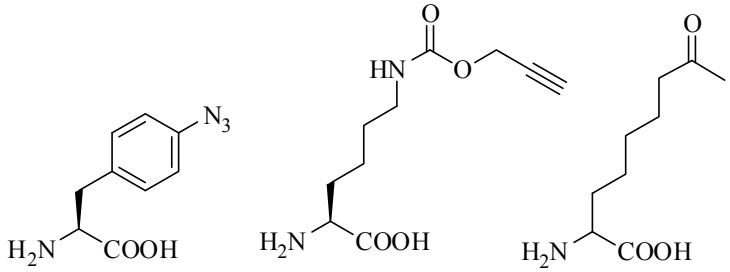
4.2 Construction of pETtrio-pylT-PylRS-QBP3TAG141TAA

F1-QBP3TAG and R1-QBP141TAA were used to clone the first part of site-mutated glutamine binding protein (QBP) from *E. coli* TOP 10 cell. The second part of QBP3TAG141TAA gene was cloned out in the same manner by using F2-QBP141TAA and R2-QBP as the two primers. Overlap PCR was performed with F1-QBP3TAG and R2-QBP as the two primers and the two fragments obtained from the PCR reactions mentioned above to afford QBP3TAG141TAA, which was inserted to pETtrio-PylT-PylRS-MCS with *Nco I* at 5' end and *Kpn I* at 3'end.

4.3 Construction of pETtrio-pylT-MmAcKRS-pylT-QBP3TAG141TAA

pKTS-MmAcKRS, the plasmid contains MmAcKRS that takes **4** efficiently evolved from MmPylRS was constructed by Prof. Dieter Söll's group (Yale University, New Haven, CT) and given to us as a gift.[9] To construct pETtrio-pylT-MmAcKRS-pylT-QBP3TAG141TAA, pETtrio-pylT-PylRS-QBP3TAG141TAA was used. Since MmAcKRS was derived from PylRS and all the mutations are beyond the 600 base pairs in the gene, PylRS->AcKRS F, a forward primer basing on the sequence of the PylRS at 450 bp was designed. Together with PylRS->AcKRS R, PylRS was converted to MmAcKRS with *Age I* at 5' end and *AflII* at 3' end.

5. Protein Expression and Purification



1 **2** **3**

5.1 Expression of QBP(1+2)

E. coli BL21 cells co-transformed with pETtrio-pylT-MmAcKRS-pylT-QBP3TAG141TAA and pEVOL-AzFRS[10] were grown in 2TY medium (150 mL) with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol overnight. The culture was inoculated into 2YT medium (450 mL) with the same concentration of antibiotics. IPTG (500 mM), arabinose (0.2% w/v), together with **1** and **2** (both 1 mM) were added into the cell culture after the OD_{600} reached 1.2~1.4. The cell culture was incubated at 37 ^{o}C for 8 h, and the cells were harvested by centrifugation at 4000 r.p.m. for 20 min at 4 ^{o}C and re-suspended in 50 mL of lysis buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.8). The re-suspended cells were sonicated in an ice/water bath four times (4 min each, 10 min interval to cool the suspension below 10 ^{o}C before the next run) and the lysate was clarified by centrifugation at 10000 r.p.m. for 40 min at 4 °C. The supernatant was then incubated with 3 mL of Ni Sepharose^{TM} 6 Fast Flow from GE Healthcare (Little Chalfont, United Kingdom) for 1 h, and then washed with 100 mL of lysis buffer. QBP(**1**+**2**) was then eluted out with 12 mL of elution buffer (50 mM HEPES, 500 mM NaCl, 250 mM imidazole, pH 7.8) and concentrated by Amicon Ultra-15 Centrifugal Filter Units – 10,000 NMWL from Millipore (Billerica, MA) to 3 mL. The buffer was then changed to ammonium

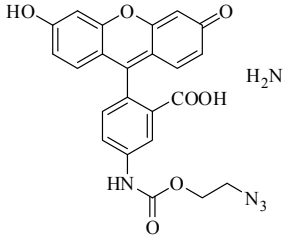
bicarbonate (ABC, 20 mM, pH 8.1) by dialysis. The concentration was determined by BCA protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL). According to the concentration, QBP(**1**+**2**) expression yield was 12 mg/L from the 2YT medium.

5.2 Expression of QBP(1+3)

E. coli BL21 cells were co-transformed with pETtrio-pylT-MmAcKRS-pylT-QBP3TAG141TAA and pEVOL-AzFRS[2] for the expression of QBP(**1**+**3**), which followed the same procedure of the expression of QBP(**1**+**2**) except **3** (2 mM) was supplemented with **1** (1 mM) instead of **2**. Purified QBP(**1**+**3**) was dialyzed against phosphate buffered saline (pH 6.4) for the following labeling reactions. The expression yield for QBP(**1**+**3**) was 11 mg/L from the 2YT medium.

6. Protein Labeling Procedures

6.1 Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)



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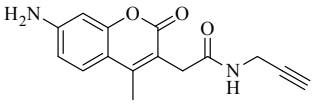

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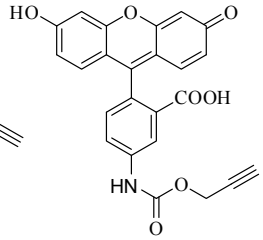

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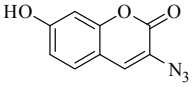
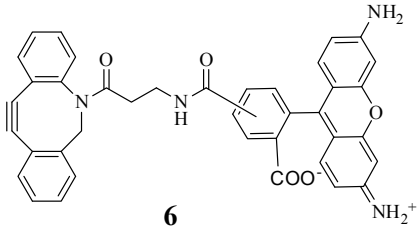


9

To QBP(**1**+**2**) (concentration varied from 0.017 mM to 0.072 mM in 20 mM ABC buffer, 270 μL, pH 8.1) was added $CuSO_4$ (100 μM), $NiCl_2$ (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, stock solution in DMSO, 500 μM) and one of the dyes (**4, 5, 8** and **9**, stock solutions in DMSO, 50 equiv. to the protein) sequentially, followed by sodium ascorbate (5 mM). The reaction was performed at room temperature for 3 h. Then ethylenediaminetetraacetic acid (EDTA, pH 8.0, 5 μL, 0.5 M final concentration) was added to the reaction mixture to chelate the two metals. The reaction product was transferred into lysis buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.8, 10 mL) with Ni Sepharose^{TM} 6 Fast Flow (1 mL) and incubated at 4 ^{o}C for 1 h. The resin was loaded onto an empty column and the catalysts were washed away by lysis buffer (100 mL). The labeled QBP(**1**+**2**) was eluted out by elution buffer (50 mM HEPES, 500 mM NaCl, 250 mM imidazole, pH 7.8, 6 mL), concentrated, dialyzed against ABC buffer (20 mM, pH 8.1) and then analyzed by mass spectrometry. The second CuAAC labeling was performed in the same manner with the appropriate dye to afford doubly labeled QBP(**1**+**2**).

6.2 Catalyst-free labeling



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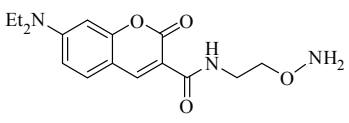

7

To QBP(**1**+**3**) (0.024 mM to 0.035 mM) was added **6** (50 equiv.) and **7** (10 equiv.). The reaction

was performed at room temperature overnight. The reaction product was transferred into lysis buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.8, 10 mL) with Ni Sepharose™ 6 Fast Flow (1 mL) and incubated at 4 °C for 1 h. The resin was loaded onto an empty column and the dye was washed away by lysis buffer (400 mL). The labeled QBP(**1**+**3**) was eluted out by elution buffer (50 mM HEPES, 500 mM NaCl, 250 mM imidazole, pH 7.8, 6 mL), concentrated, dialyzed against ABC buffer (20 mM, pH 8.1) and then analyzed by mass spectrometry. It is noteworthy that excessive lysis buffer must be used to completely remove **6**, which has very poor solubility in water.

7. Mass Spectrometry Analysis

Nanoelectrospray ionization in positive mode was performed using an Applied Biosystems QSTAR Pulsar (Concord, ON, Canada) equipped with a nanoelectrospray ion source. Solution was flowed at 700 nL/min through a 50 μm ID fused-silica capillary that was tapered at the tip. Electrospray needle voltage was held at 2100 V.

8. FRET Assay

QBP(**1**+**2**)-**5**-**4** and QBP(**1**+**3**)-**6**-**7** were diluted with various concentrations of guanidine hydrochloride (GndCl, 0 M, 1 M, 2 M, 3 M, 4 M, 5 M and 6 M, respectively) in PBS buffer (pH 7.8). The fluorescent emission of those solutions was tested by QuantaMaster™ 40 Intensity Based Spectrofluorometer from Photon Technology International Inc. (Birmingham, NJ) with excitation at 350 nm (QBP(**1**+**2**)) or 430 nm (QBP(**1**+**3**)). Emission change based on the concentration of GndCl was plotted. All measurements were taken on freshly made samples and the data was collected every 0.2 second with 0.5 nm intervals.

9. References

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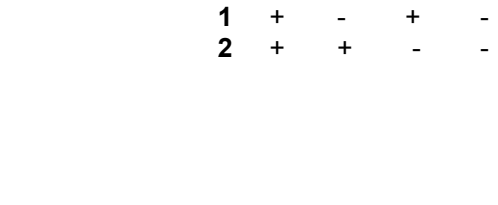
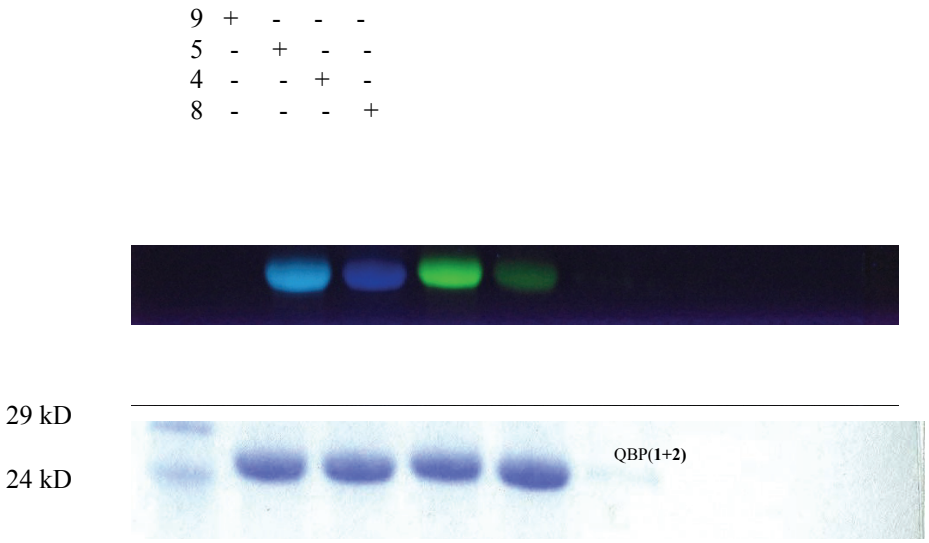
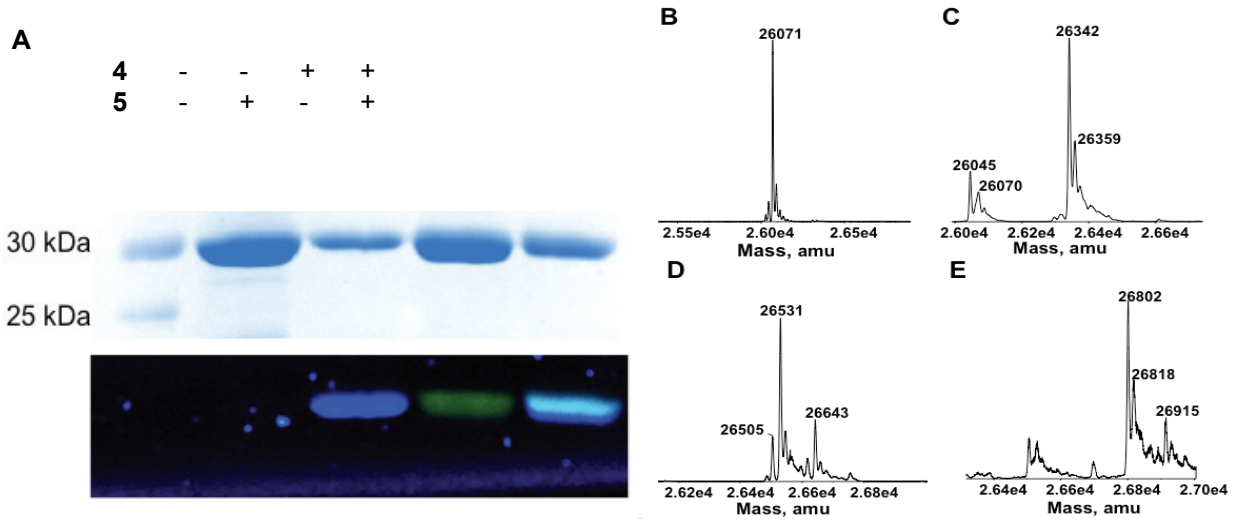
10. Supplementary Figures



Supplementary Figure 1.The expression of QBP(**1**+**2**) supplemented with different NAAs. Visualization was effected by Coomassie blue staining.

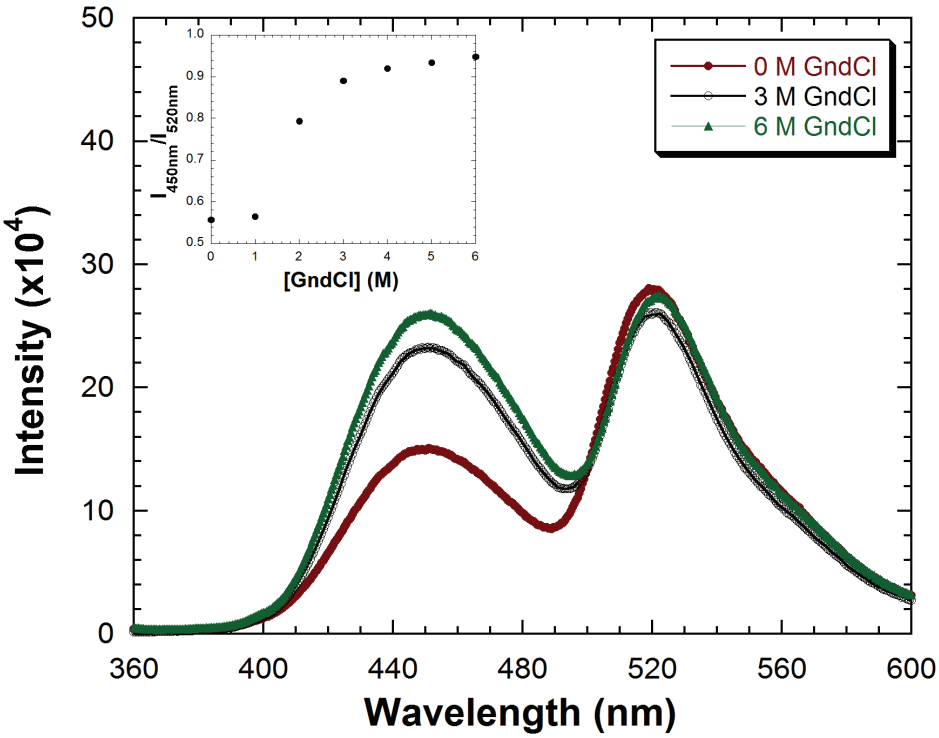


Supplementary Figure 2. QBP(**1**+**2**) labeled with **4, 5, 8, 9,** respectively. Visualization was upon UV excitation at 365 nm.

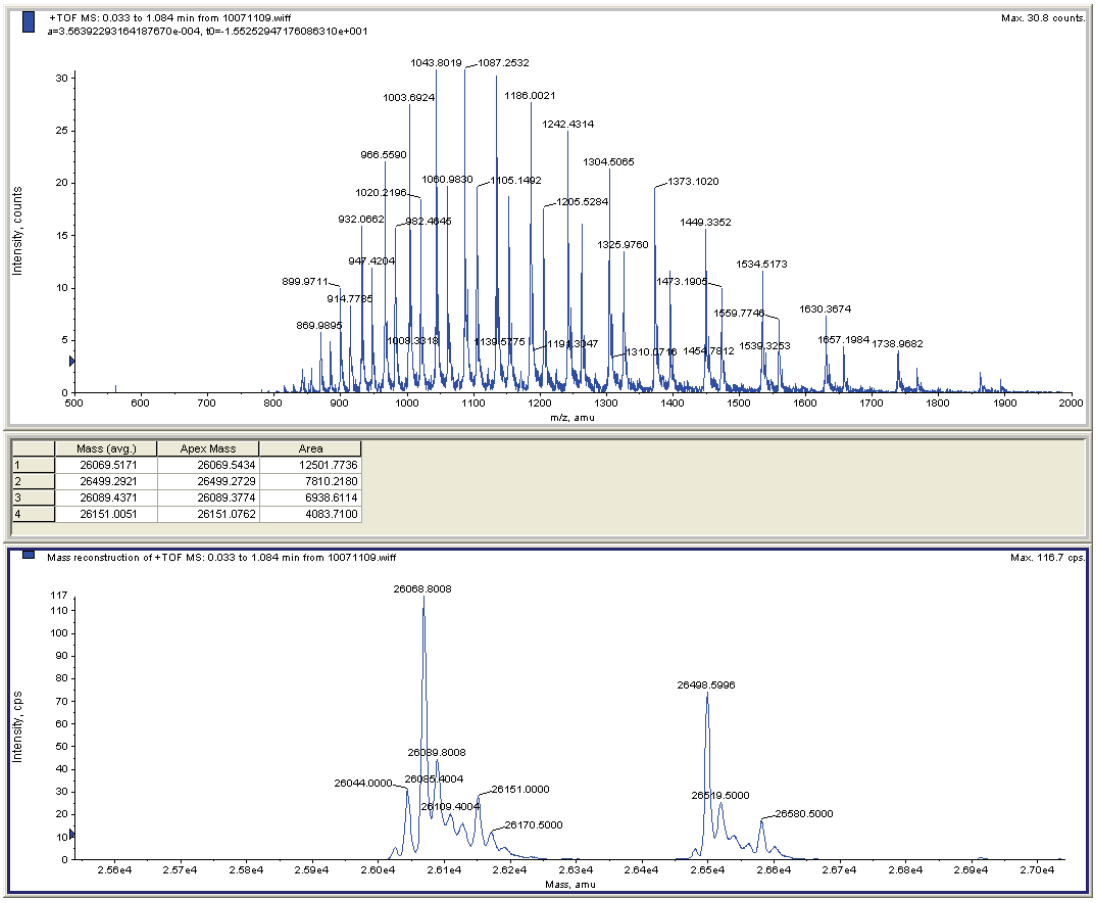


Supplementary Figure 3. **(A)** QBP(**1**+**2**) labeled with **4, 5, 4+5,** respectively. Visualization was effected by UV excitation at 365 nm. (**B**) QBP (**1**+**2**), (**C**) QBP (**1**+**2**)-**5**, (**D**) QBP (**1**+**3**)-**4**, (**E**) QBP (**1**+**2**)-**5**-**4.** The theoretic molecular weights of QBP(**1**+**2**), QBP(**1**+**2**)-**5**, QBP(**1**+**2**)-**4**,and QBP(**1**+**2**)-**5**-**4** are 26,069, 26,339, 26,529, and 26,799 Da, respectively.

During labeling QBP(**1**+**2**) with **4**, we noticed that a significant amount of protein aggregated. This aggregation process was time dependent. While 47% of QBP(**1**+**2**) was recovered after labeling with **4** for 1 h, only 12% was recovered after a 5 h reaction. Since the two labeling reactions have to be performed sequentially for 3 h each and the protein has to be purified by Ni-NTA resins after each round to remove residual dyes, the protein recovery rate is low. The finally obtained product QBP(**1**+**2**)-**5**-**4** only accounted for 9% of the original QBP(**1**+**2**). Besides the protein aggregation issue, there is another problem associated with the CuAAC reaction: protein oxidation. It has been demonstrated in the previous literature that Cu(I) can promote the formation of reactive oxygen species, leading to protein oxidation. As confirmed by the ESI-MS analysis, our finally obtained QBP(**1**+**2**)-**5**-**4** did show a large unexpected peak at 26,818 Da which matches the molecular weight of the desired product with one additional oxygen atom (***Supplementary Figure 3 E***). The final product also has a quite messy ESI-MS spectrum that might result from undesired modifications of the protein during the labeling process.

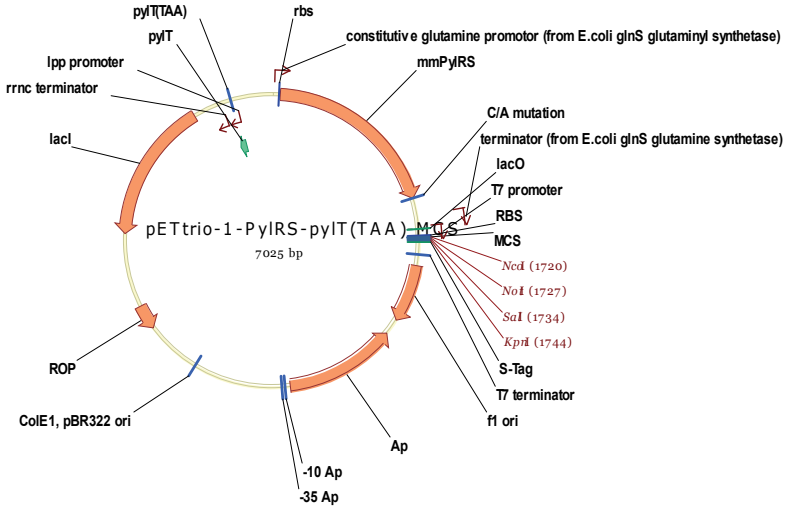


Supplementary Figure 4. Fluorescent emission spectra of QBP(**1**+**2**)-**5**-**4** at different concentrations of GndHCl. Only spectra from three GndHCl concentrations are shown for clarity. The excitation wavelength was at 350 nm. The inset shows the dependence of I_{450nm}/I_{520nm} on the concentration of GndHCl. Comparing with QBP(**1**+**3**)-**6**-**7**, QBP(**1**+**2**)-**5**-**4** denatures with lower GndHCl concentration. We conclude that because QBP(**1**+**2**)-**5**-**4** suffers from oxidation issue, the protein is less stable compared with QBP(**1**+**3**)-**6**-**7**.



Supplementary Figure 3. Labeling of QBP(**1**+**2**) with **8** for 6 h. Calculated protein mass without labeling: 26069; calculated protein mass with labeling: 26499. The labeling was not efficient because aromatic azides are much less reactive than aliphatic ones. Therefore, **8** and **9** were not chosen to be the dyes for double labeling.

a)



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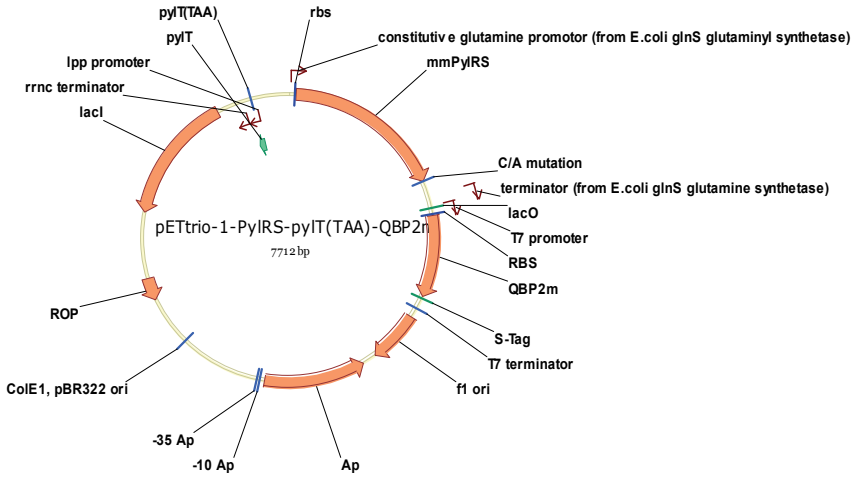

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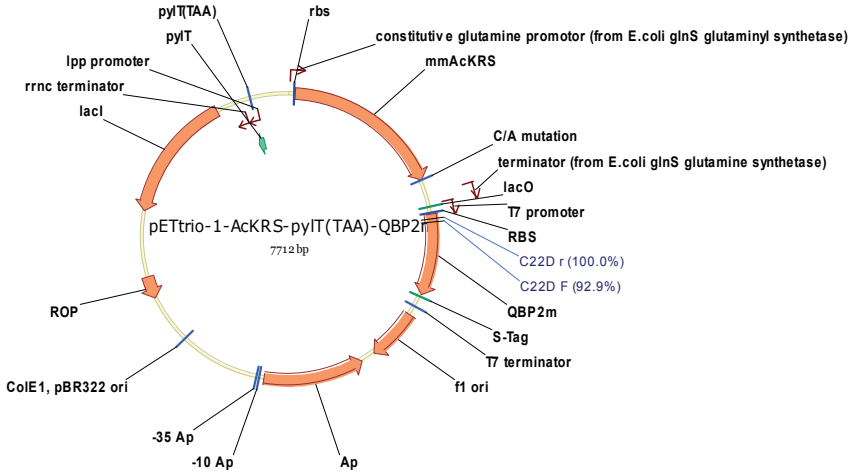
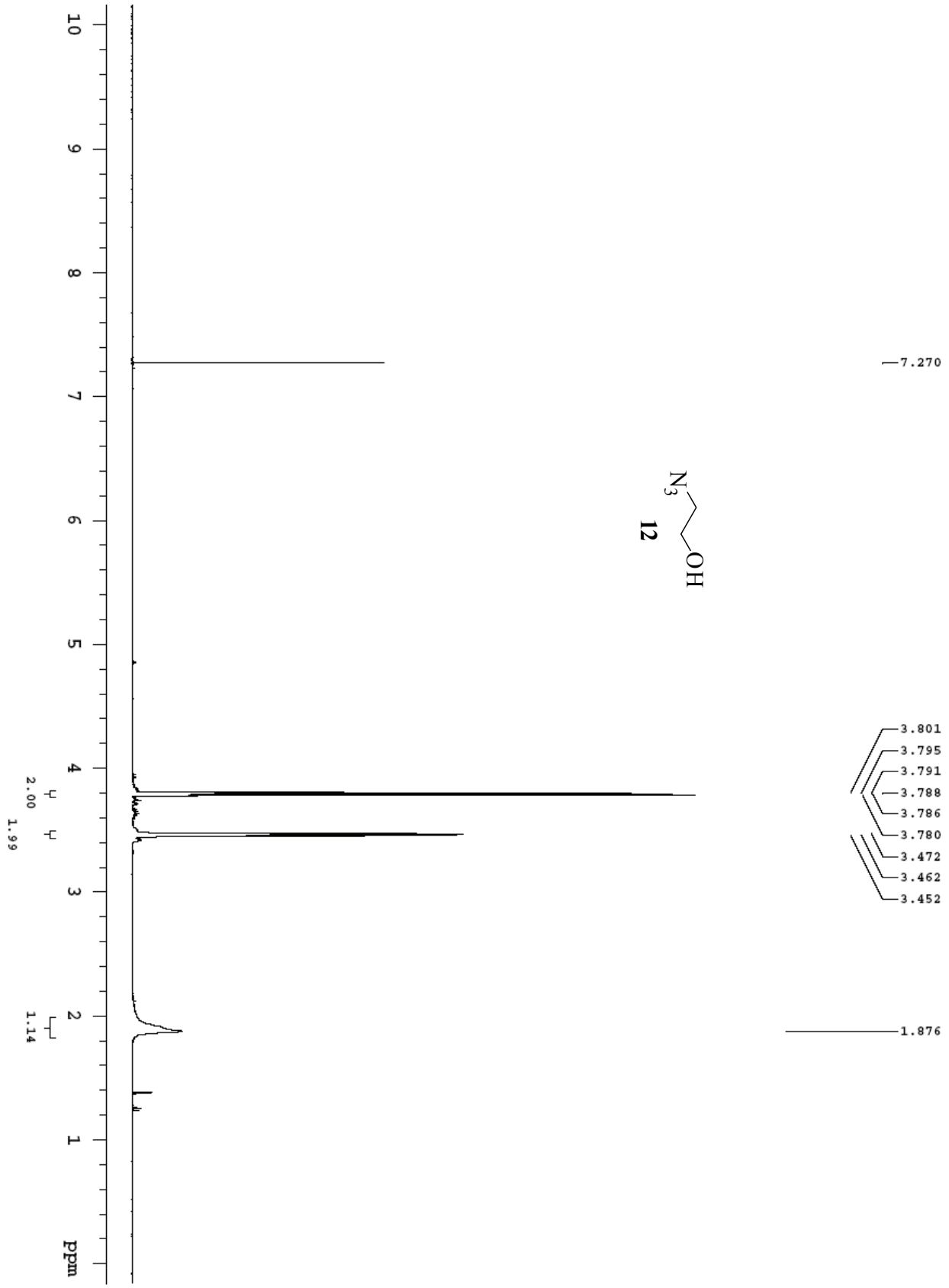
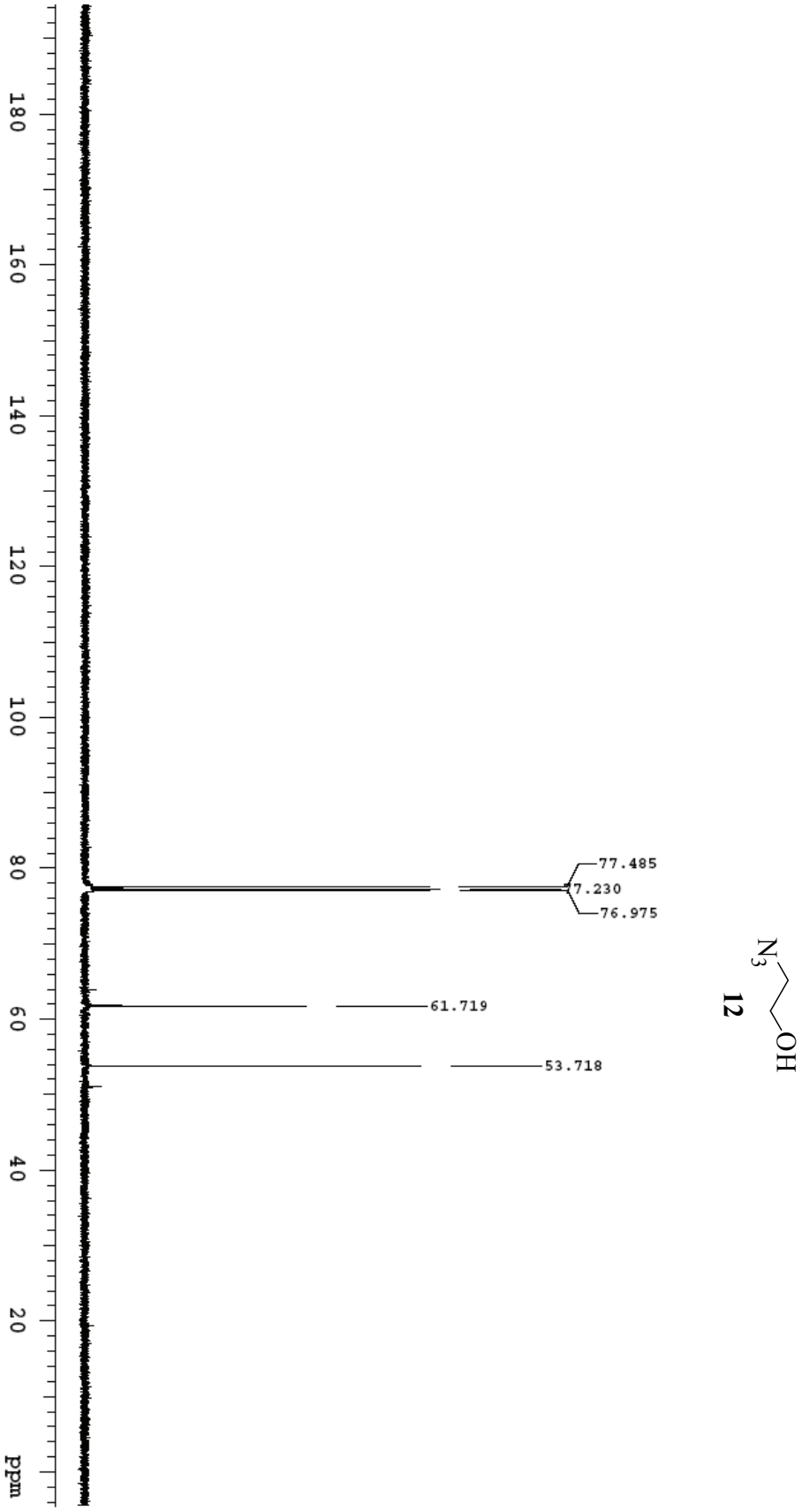


Supplementary Figure 5. Plasmid maps of a) pETTrio-PylT-PylRS-MCS. b) pETtrio-pylT-PylRS-QBP3TAG141TAA. c) pETTrio-PylT-MmAcKRS -QBP3TAG141TAA.

11. NMR spectra of synthesized compounds.

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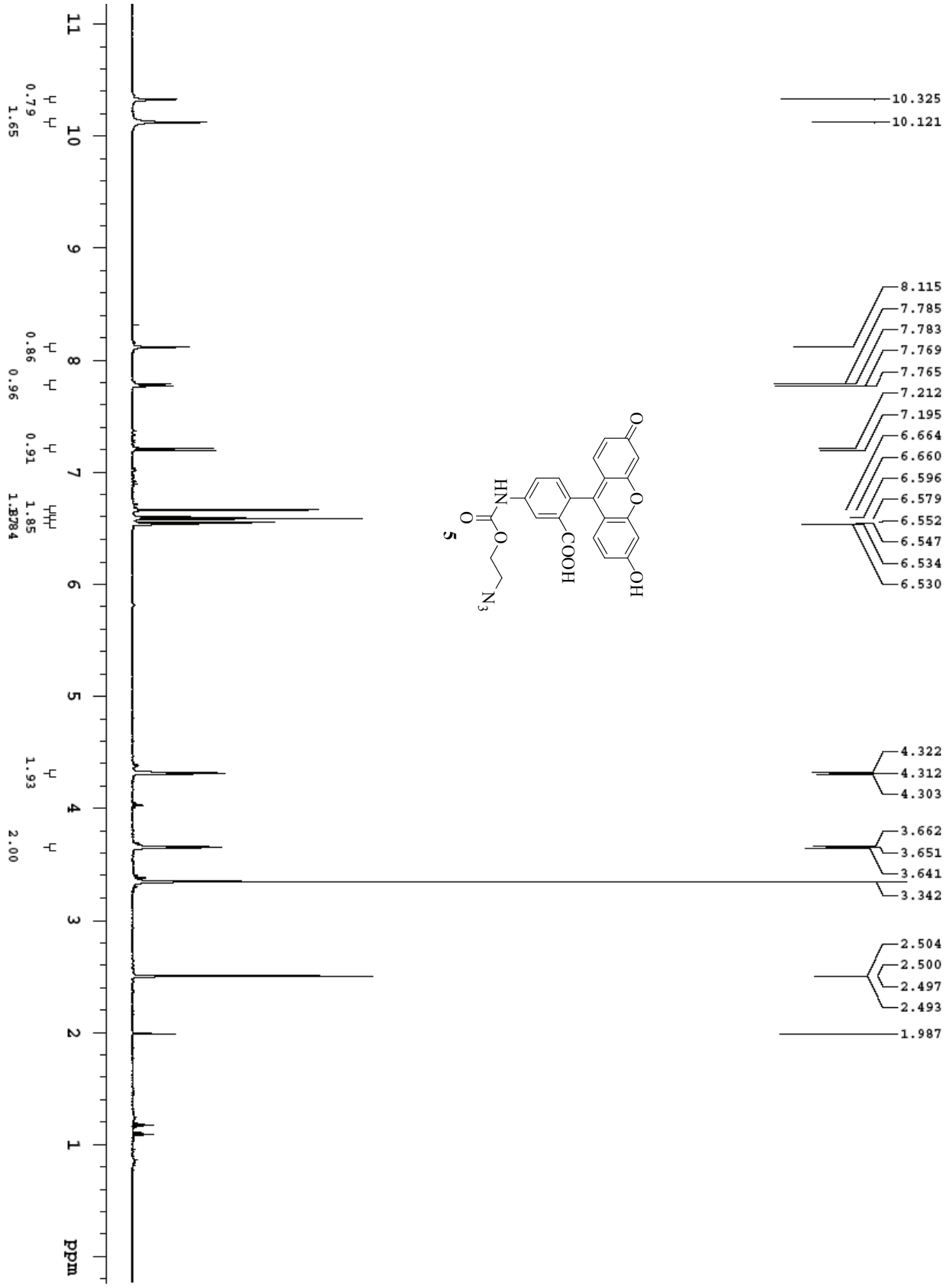
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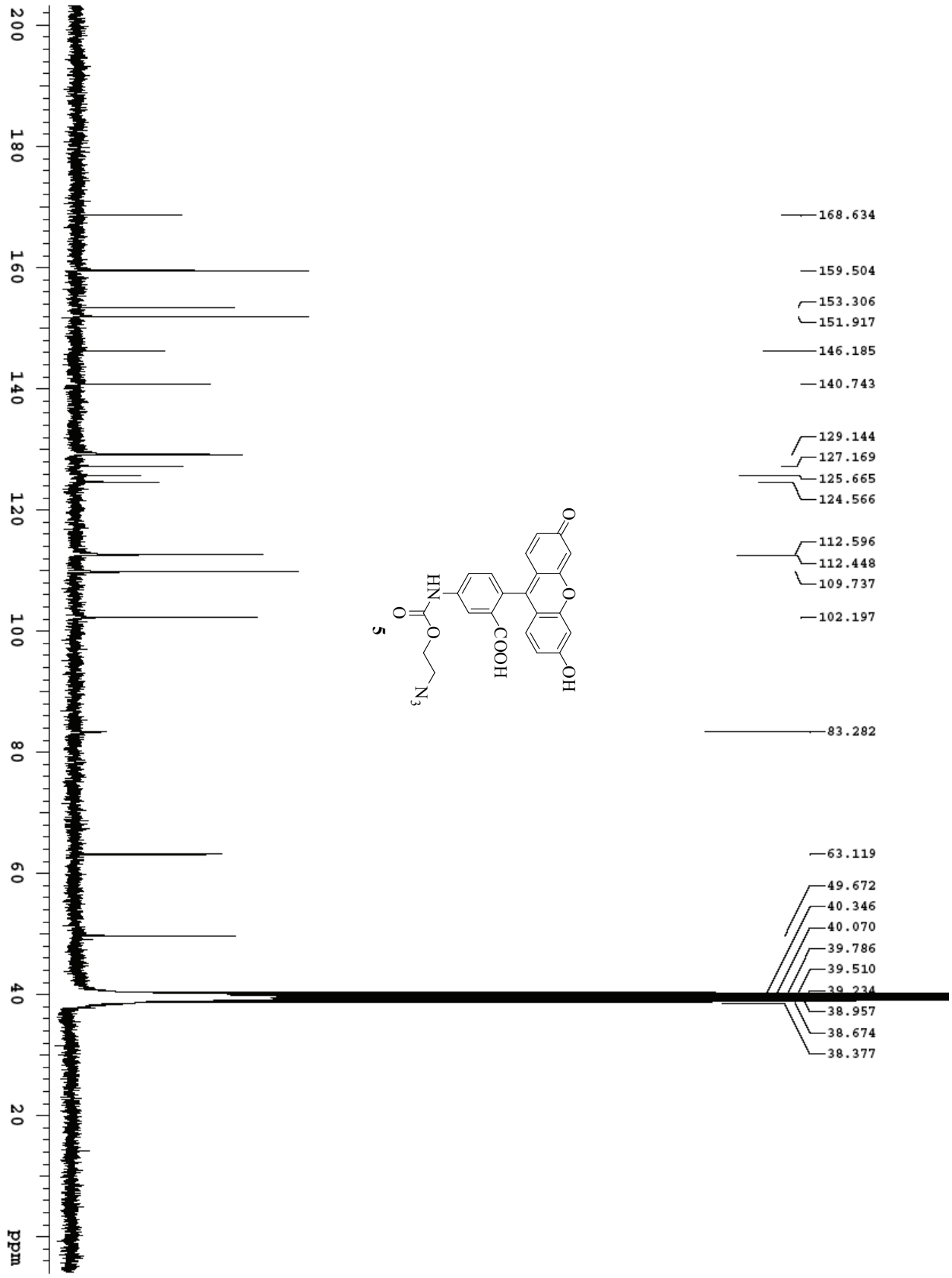
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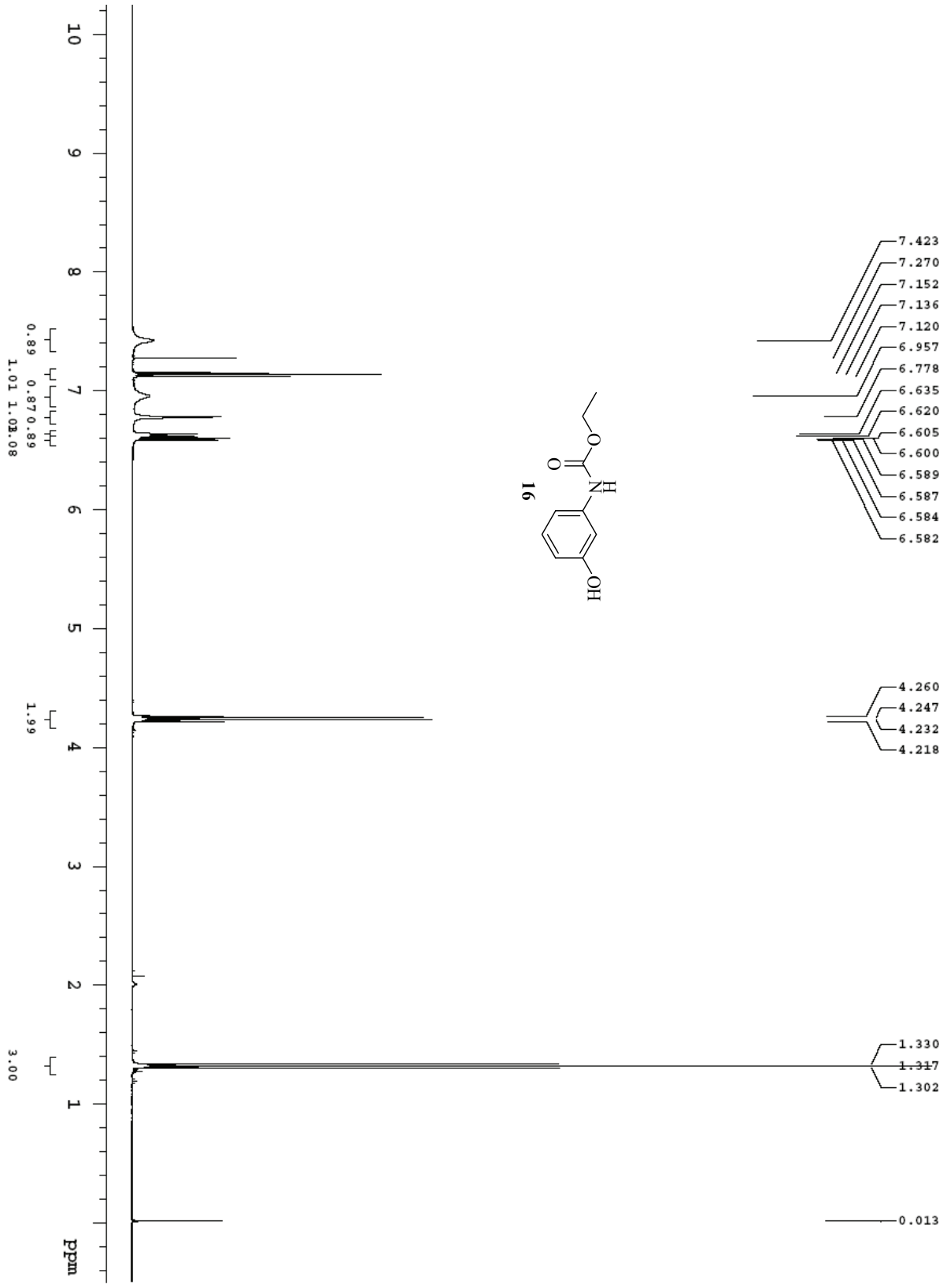
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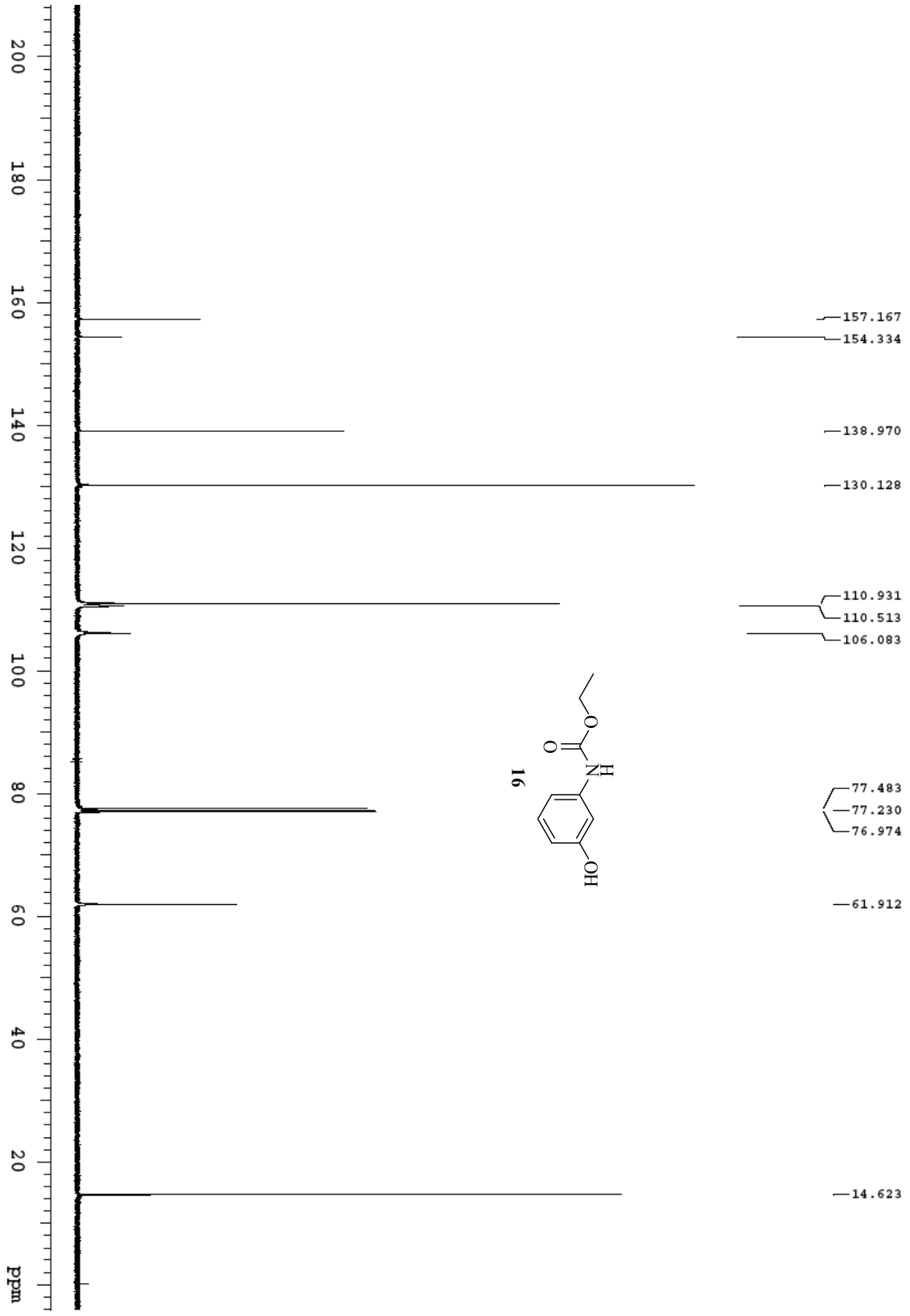
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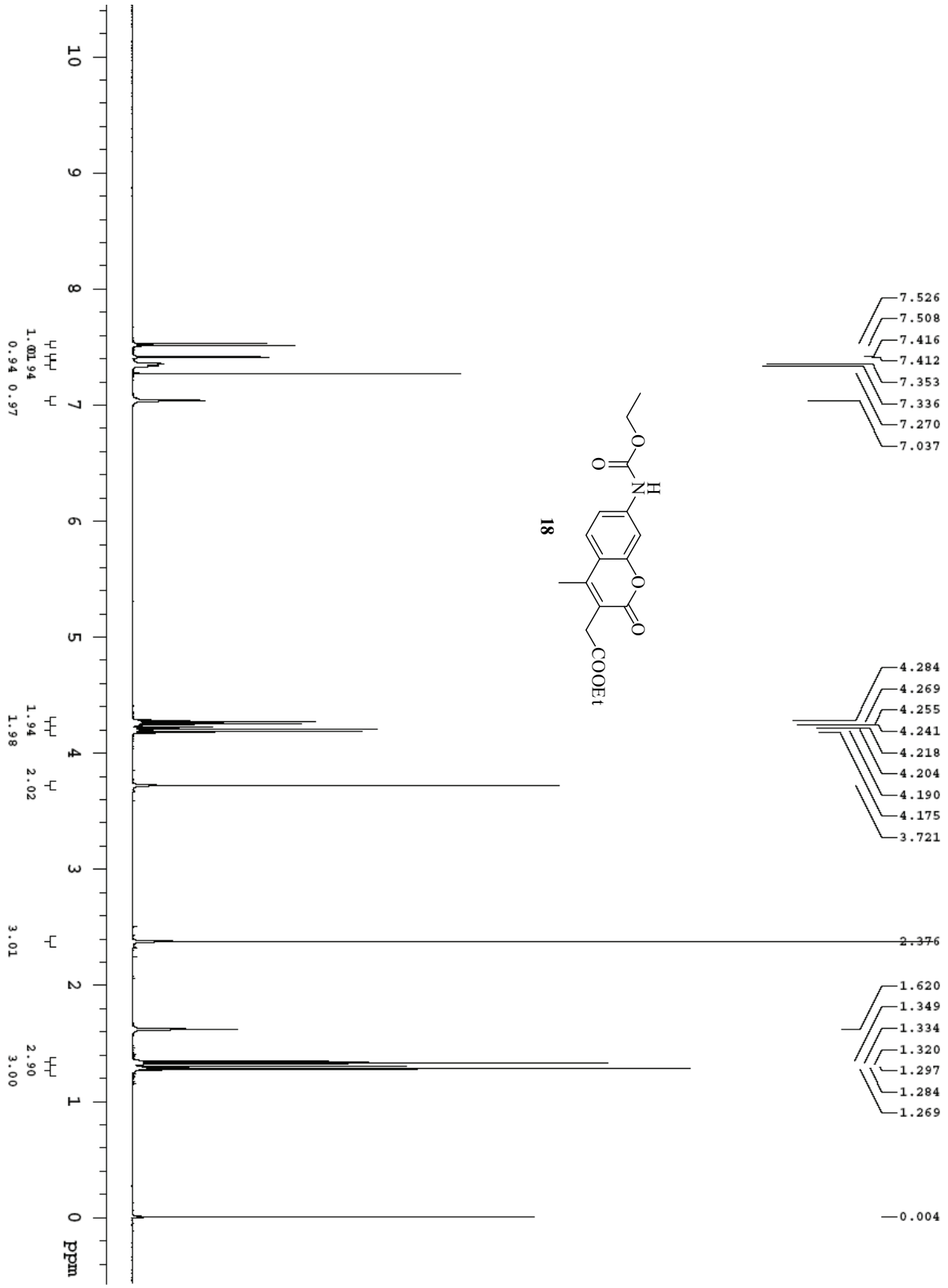
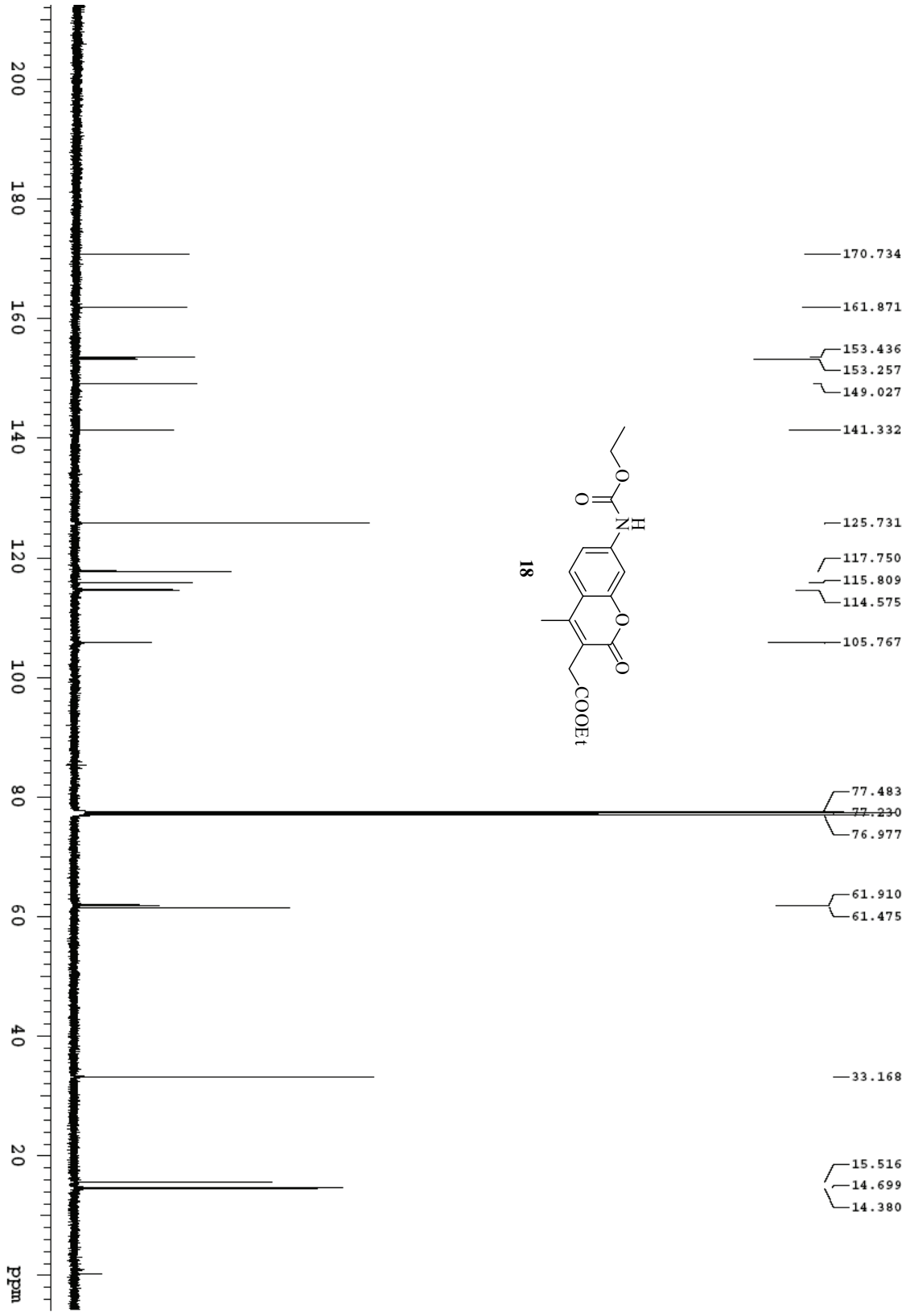
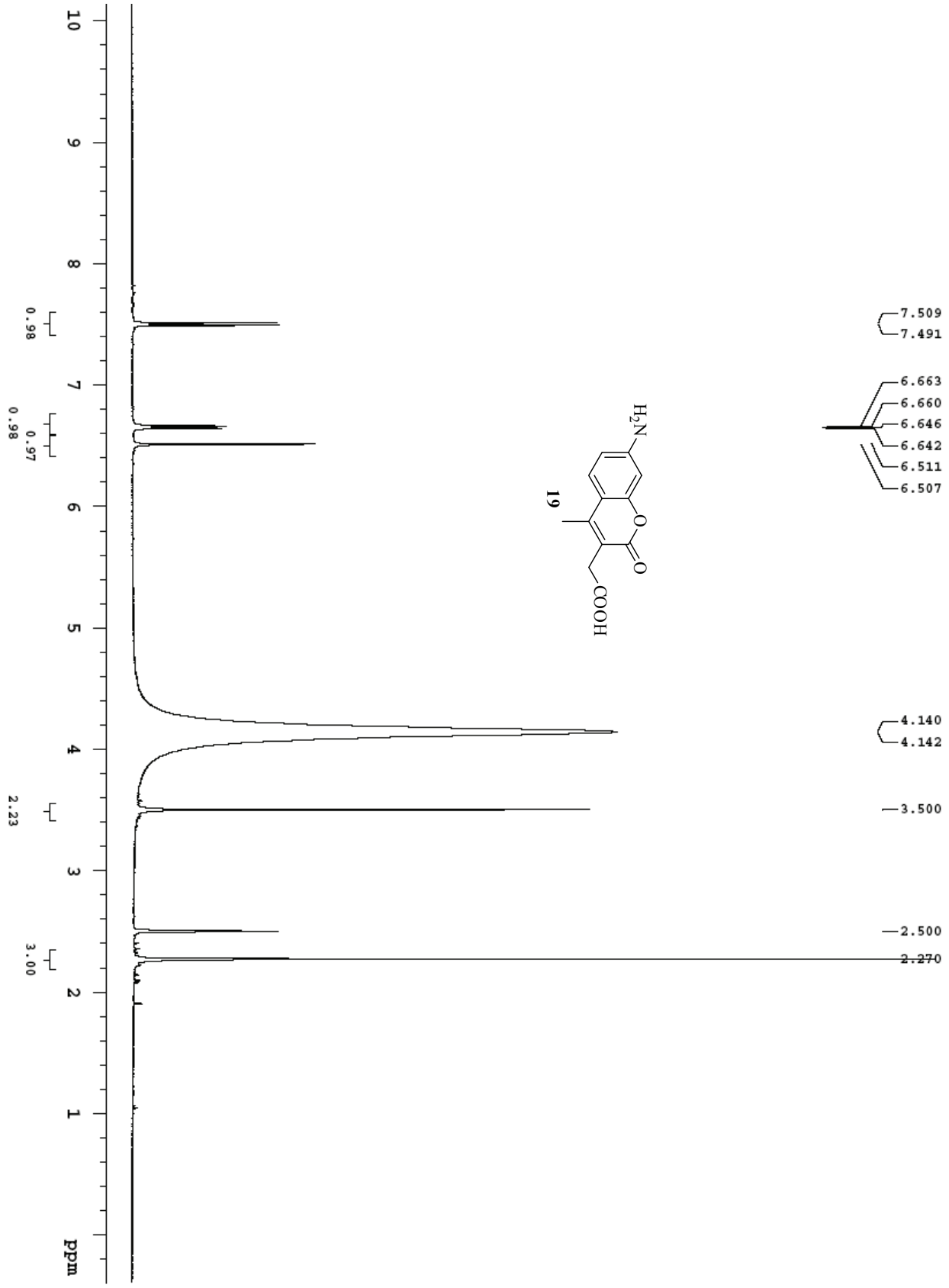
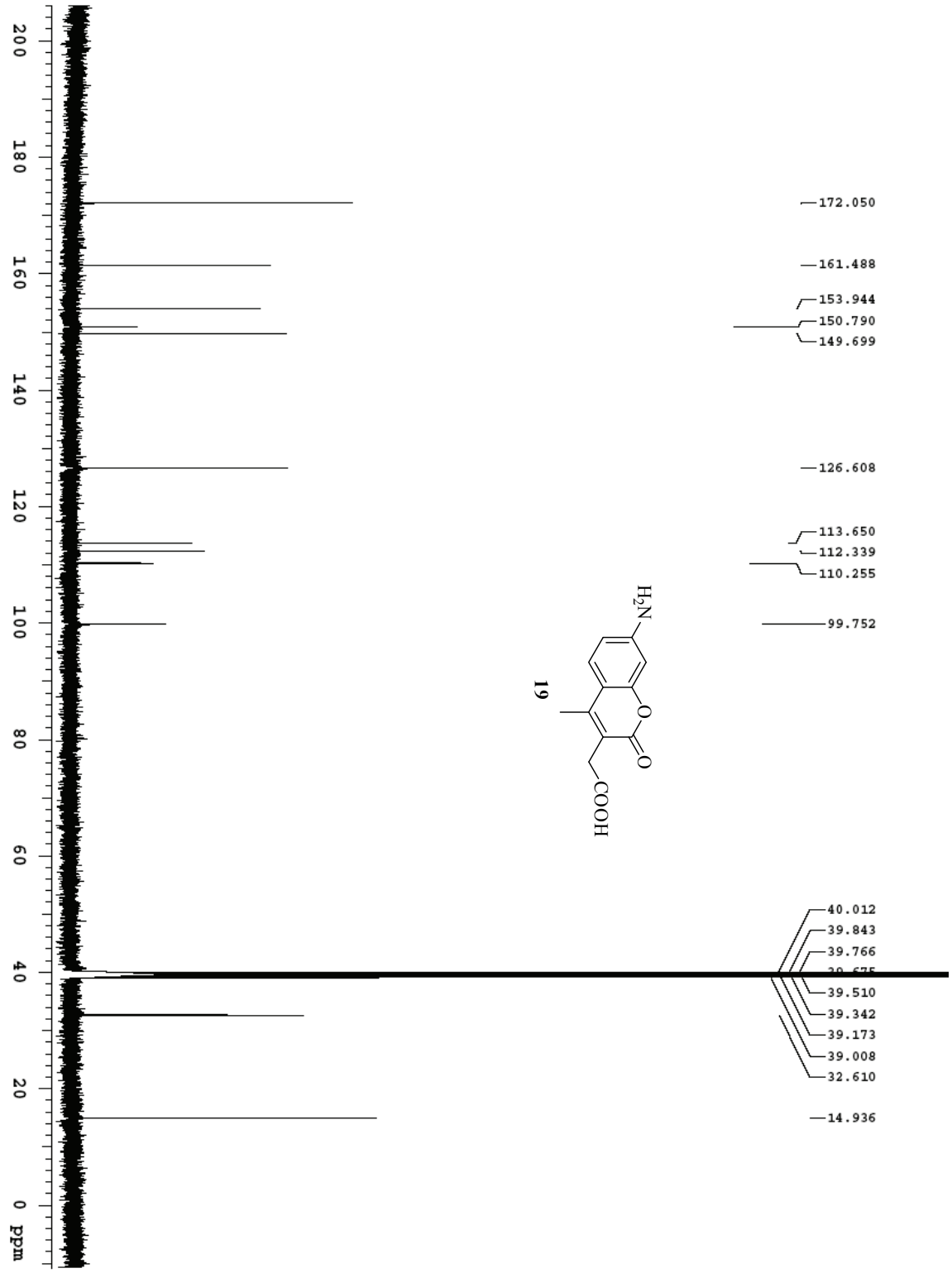
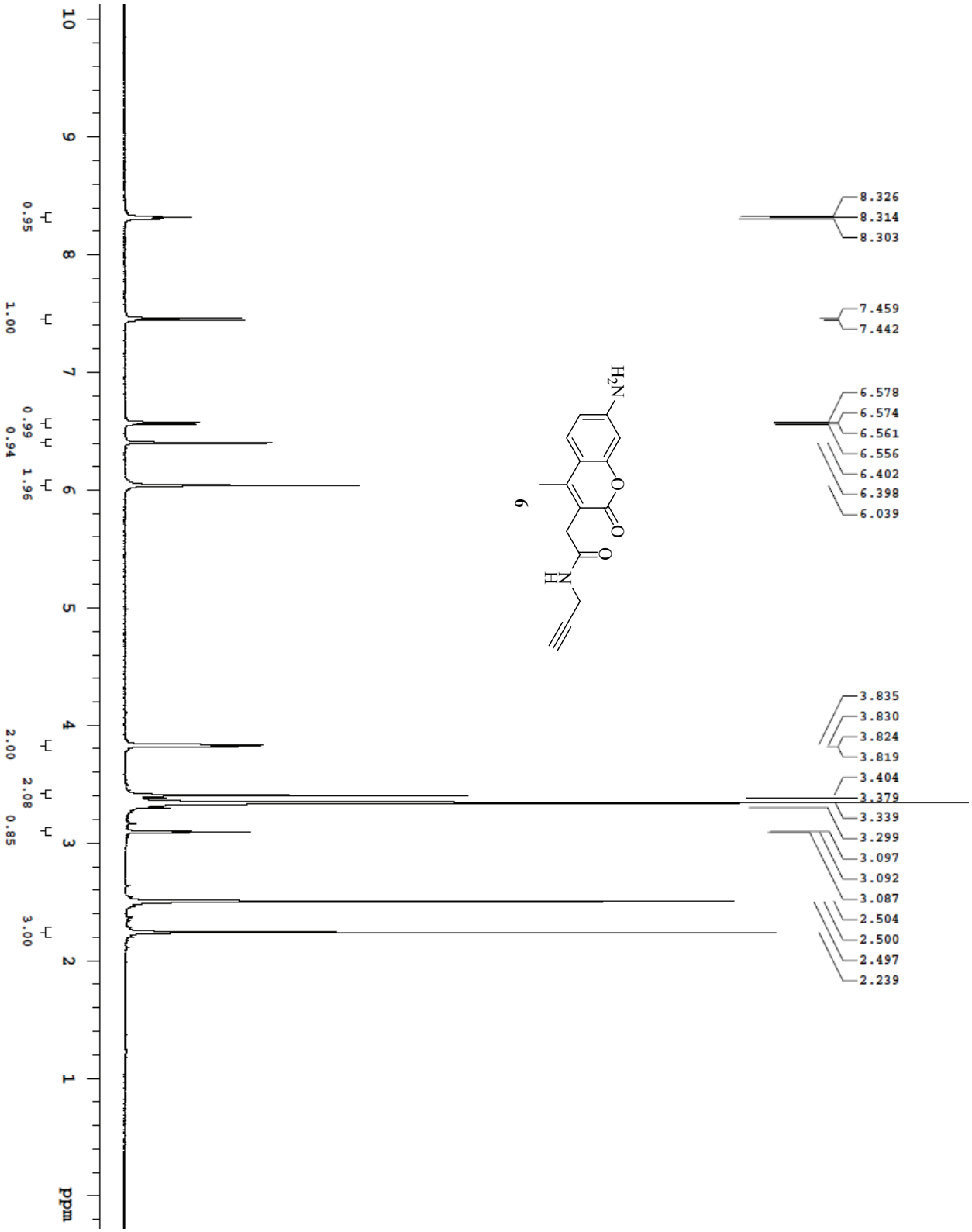
20
ppm
157.167
154.334
138.970
130.128
110.931
110.513
106.083
77.483
77.230
76.974
61.912
14.623
16
O
O
N
H
OH

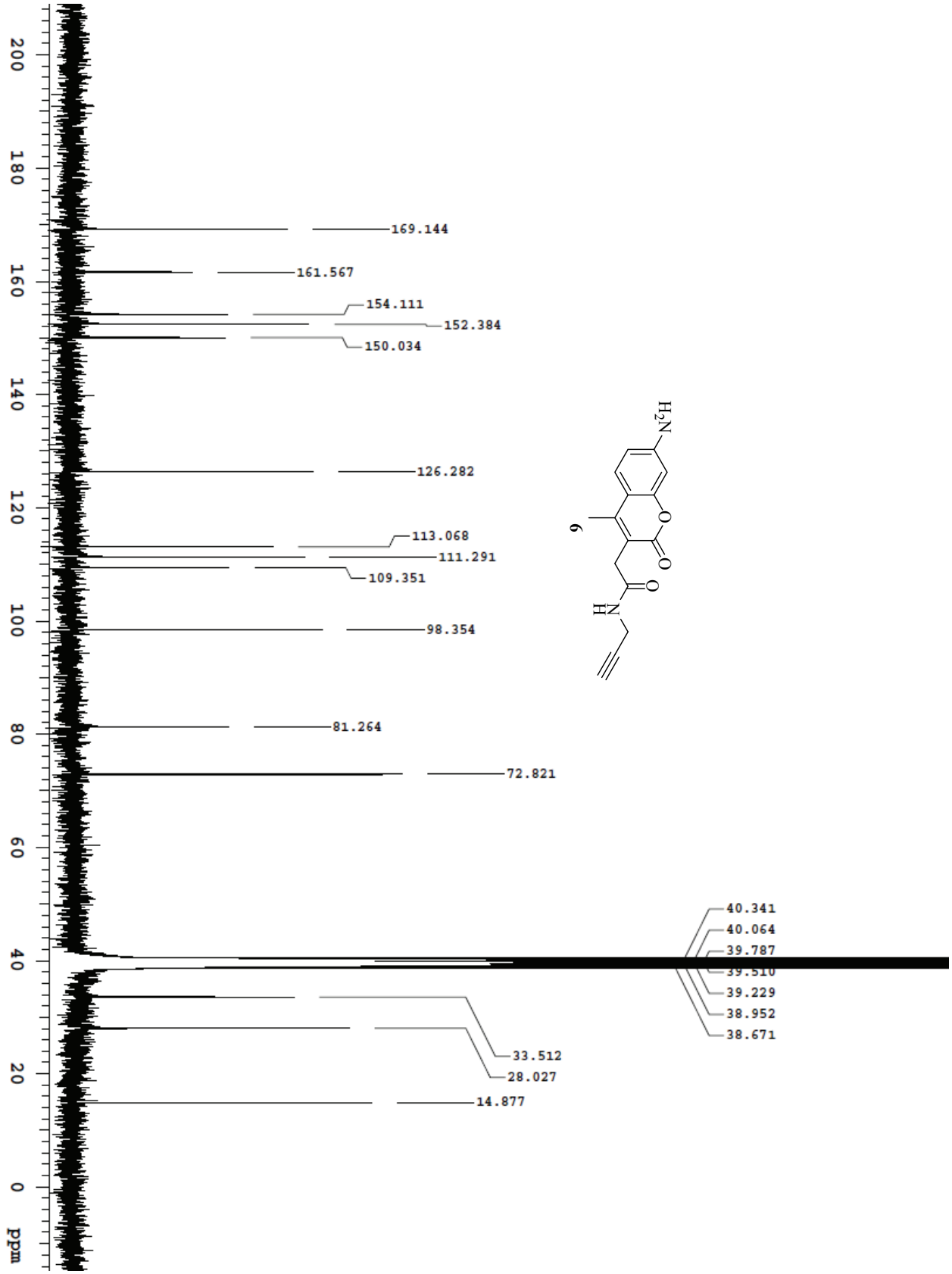

7.526
7.508
7.416
7.412
7.353
7.336
7.270
7.037
4.284
4.269
4.255
4.241
4.218
4.204
4.190
4.175
3.721
2.376
1.620
1.349
1.334
1.320
1.297
1.284
1.269
0.004
1.00 1.01 1.94 0.94 0.97
1.94 2.02 1.98
3.01
2.90 3.00
ppm
18

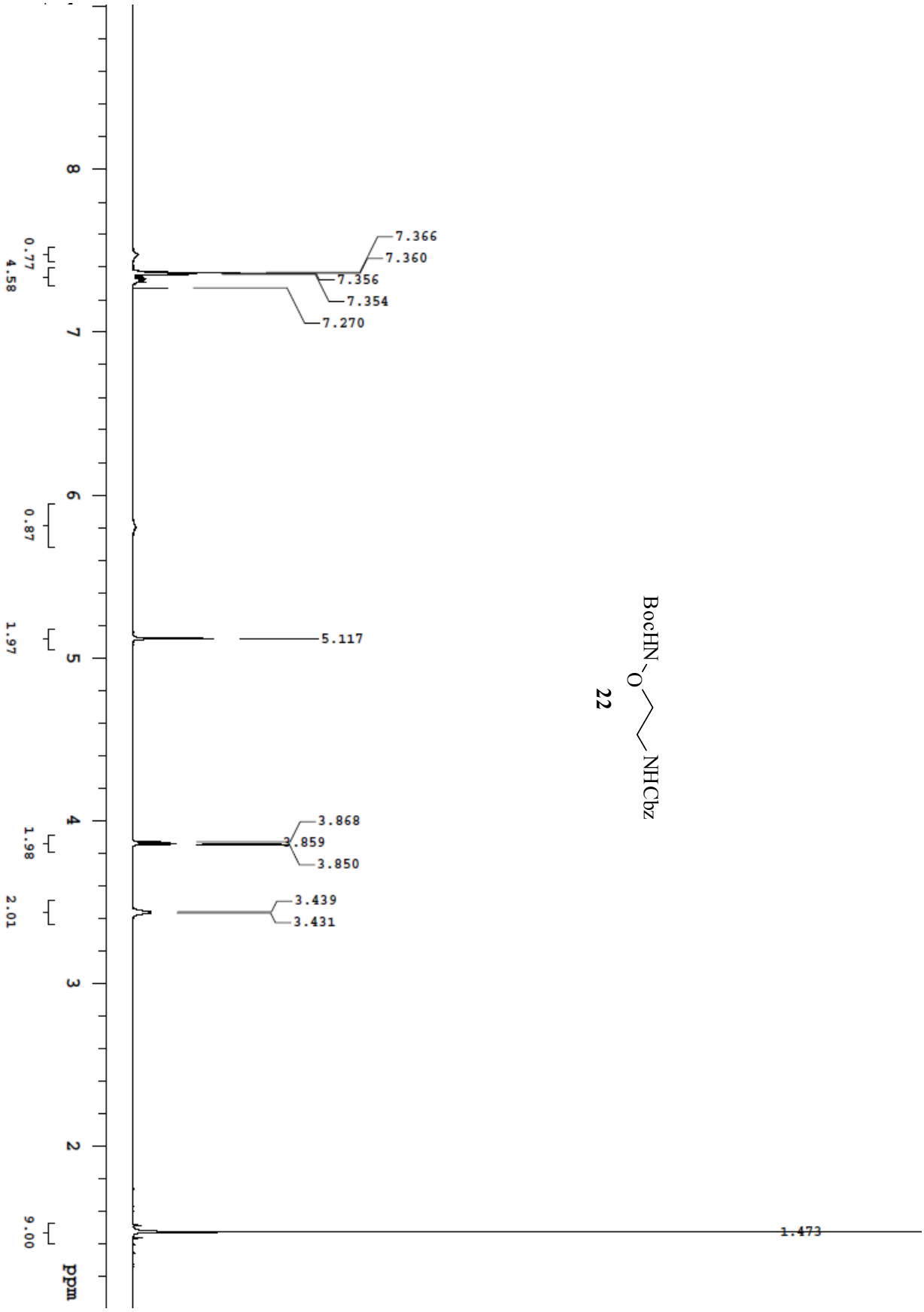

170.734
161.871
153.436
153.257
149.027
141.332
125.731
117.750
115.809
114.575
105.767
77.483
77.230
76.977
61.910
61.475
33.168
15.516
14.699
14.380
200
180
160
140
120
100
80
60
40
20
ppm
18
COOEt

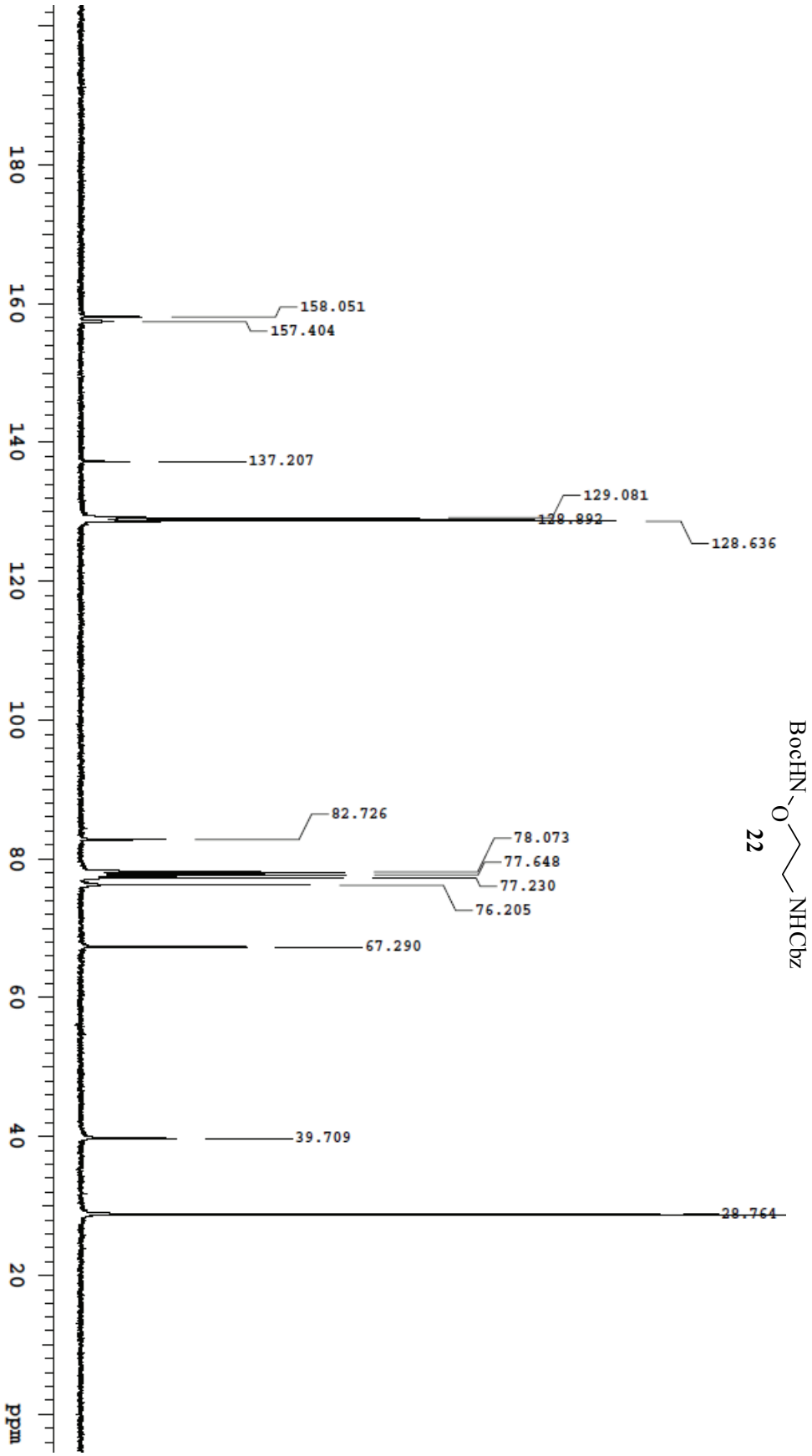

7.509
7.491
6.663
6.660
6.646
6.642
6.511
6.507
4.140
4.142
3.500
2.500
2.270
0.98
0.98
0.97
2.23
3.00
ppm
19
H2N
O
O
COOH

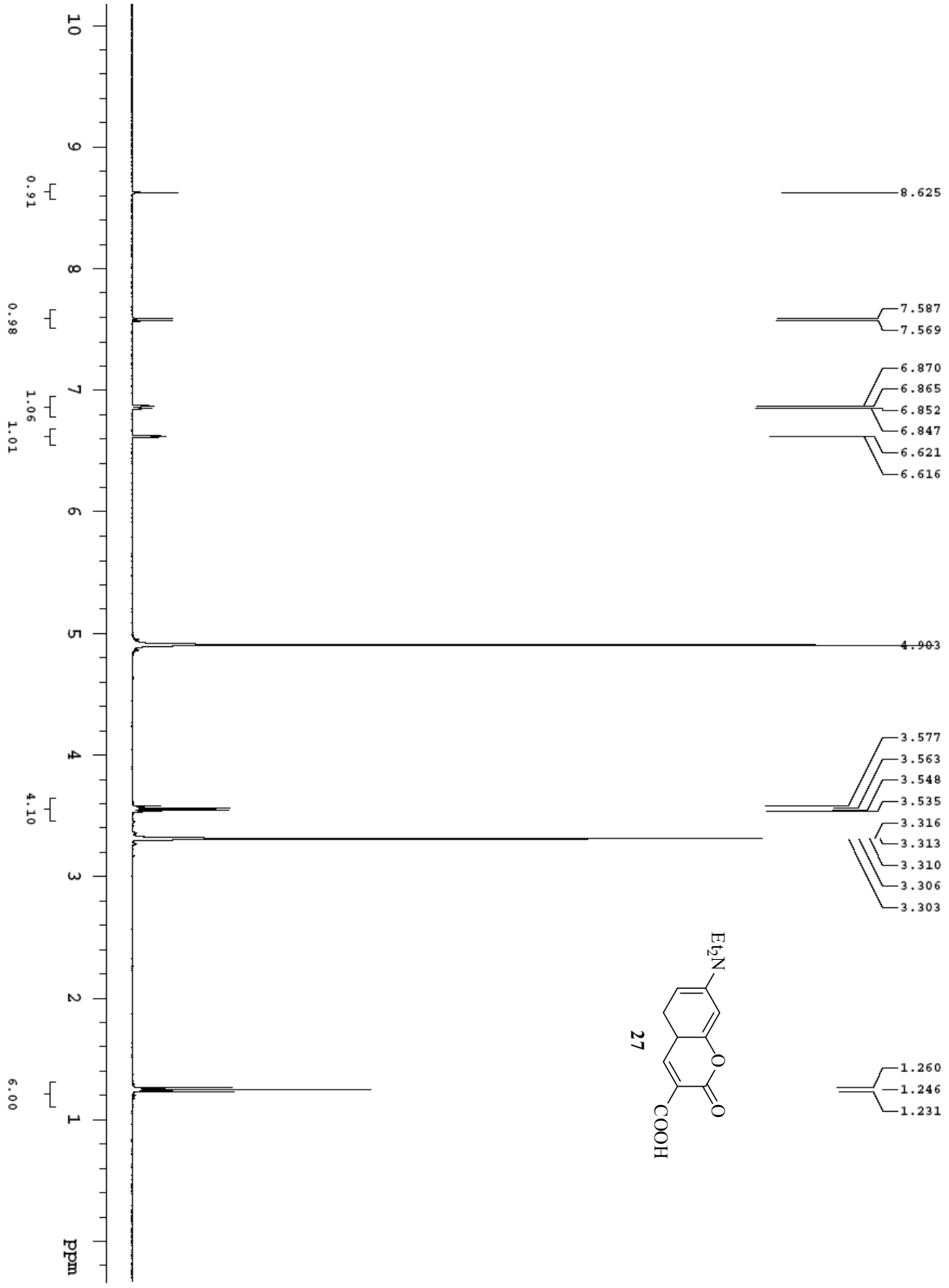

200
180
160
140
120
100
80
60
40
20
0 ppm
172.050
161.488
153.944
150.790
149.699
126.608
113.650
112.339
110.255
99.752
40.012
39.843
39.766
39.510
39.342
39.173
39.008
32.610
14.936
19
H2N
O
O
COOH

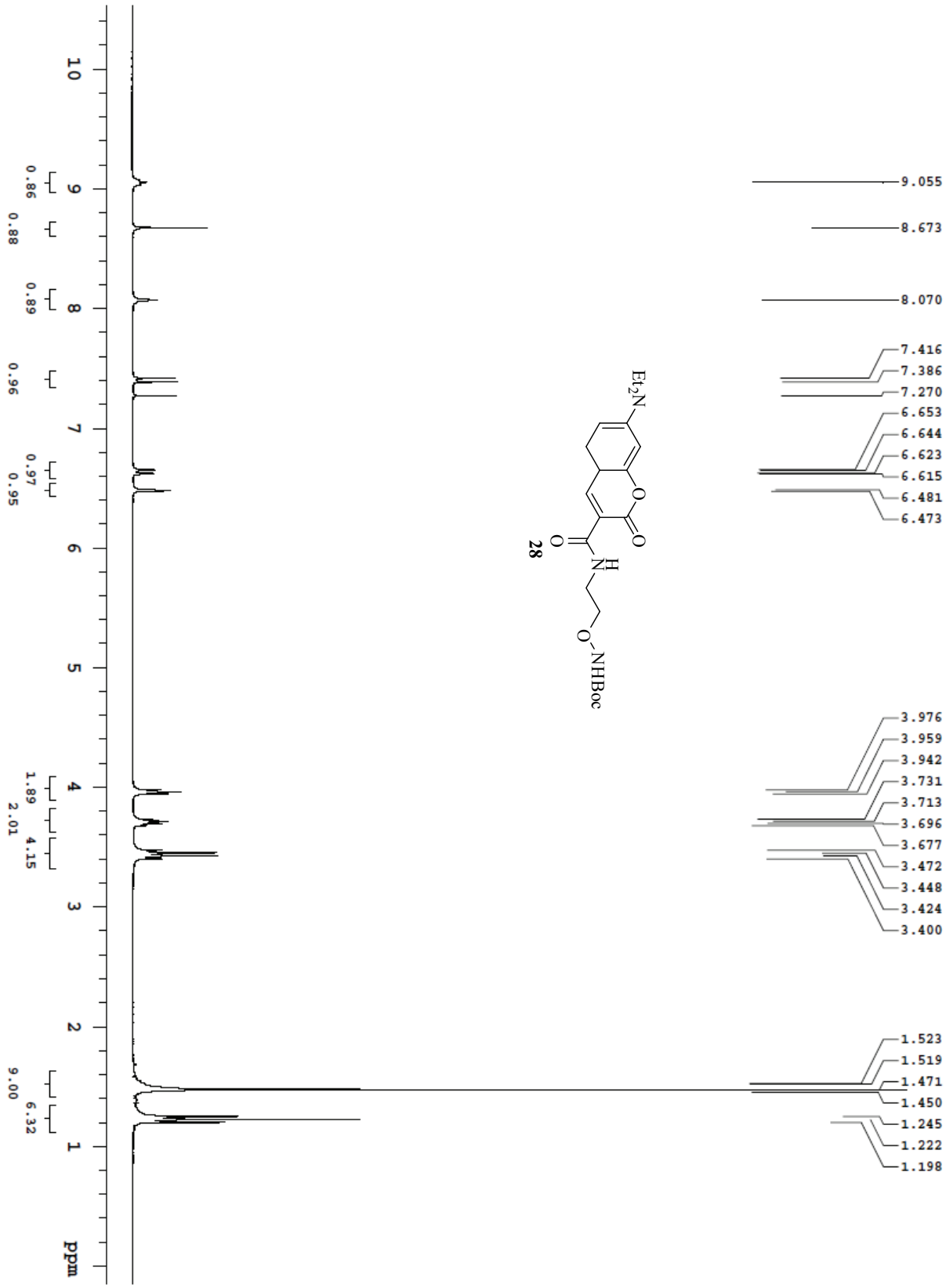

8.326
8.314
8.303
7.459
7.442
6.578
6.574
6.561
6.556
6.402
6.398
6.039
3.835
3.830
3.824
3.819
3.404
3.379
3.339
3.299
3.097
3.092
3.087
2.504
2.500
2.497
2.239
9
ppm

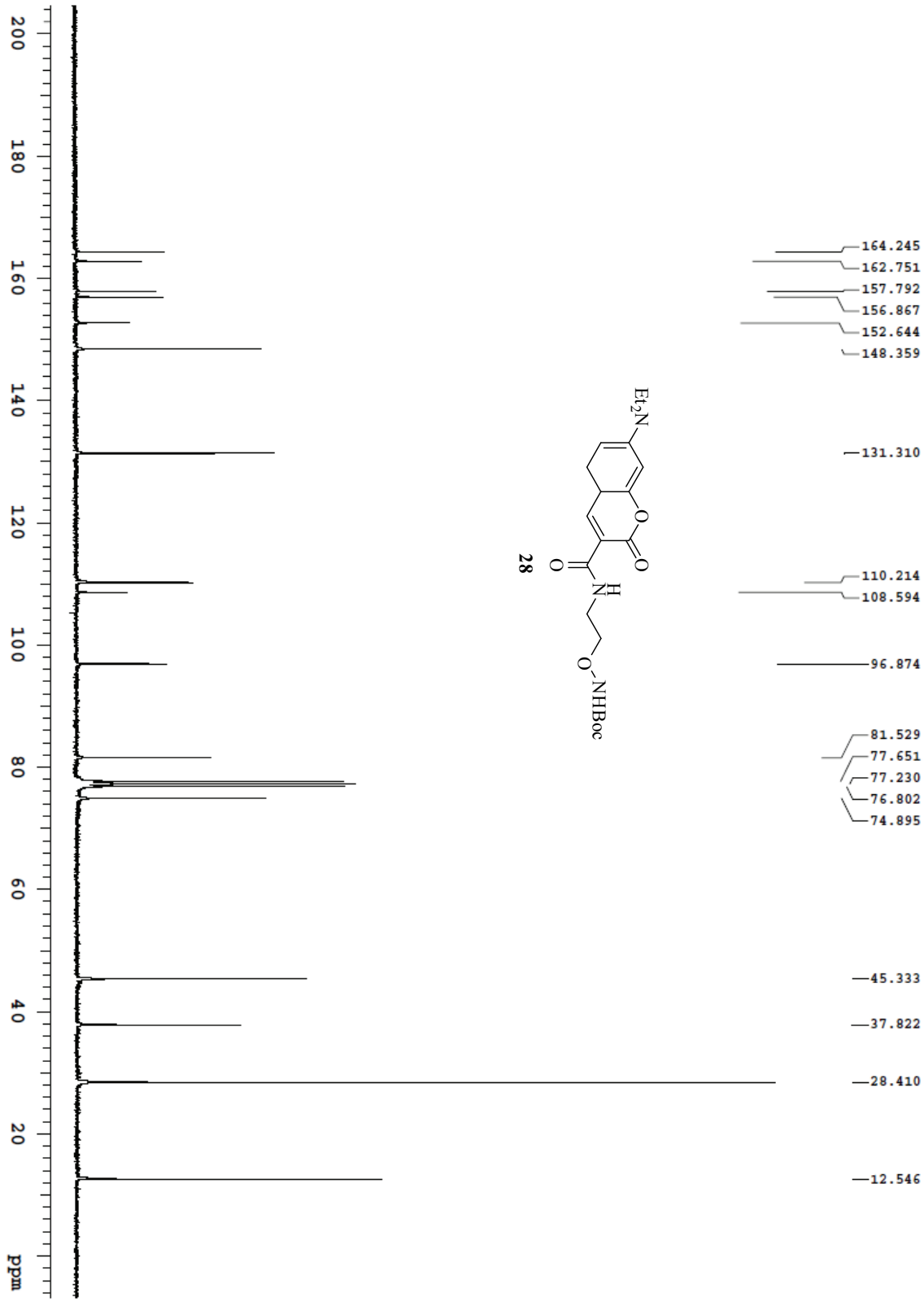

169.144
161.567
154.111
152.384
150.034
126.282
113.068
111.291
109.351
98.354
81.264
72.821
40.341
40.064
39.787
39.510
39.229
38.952
38.671
33.512
28.027
14.877
200
180
160
140
120
100
80
60
40
20
0
ppm
9
O
O
O
H
N
H2N

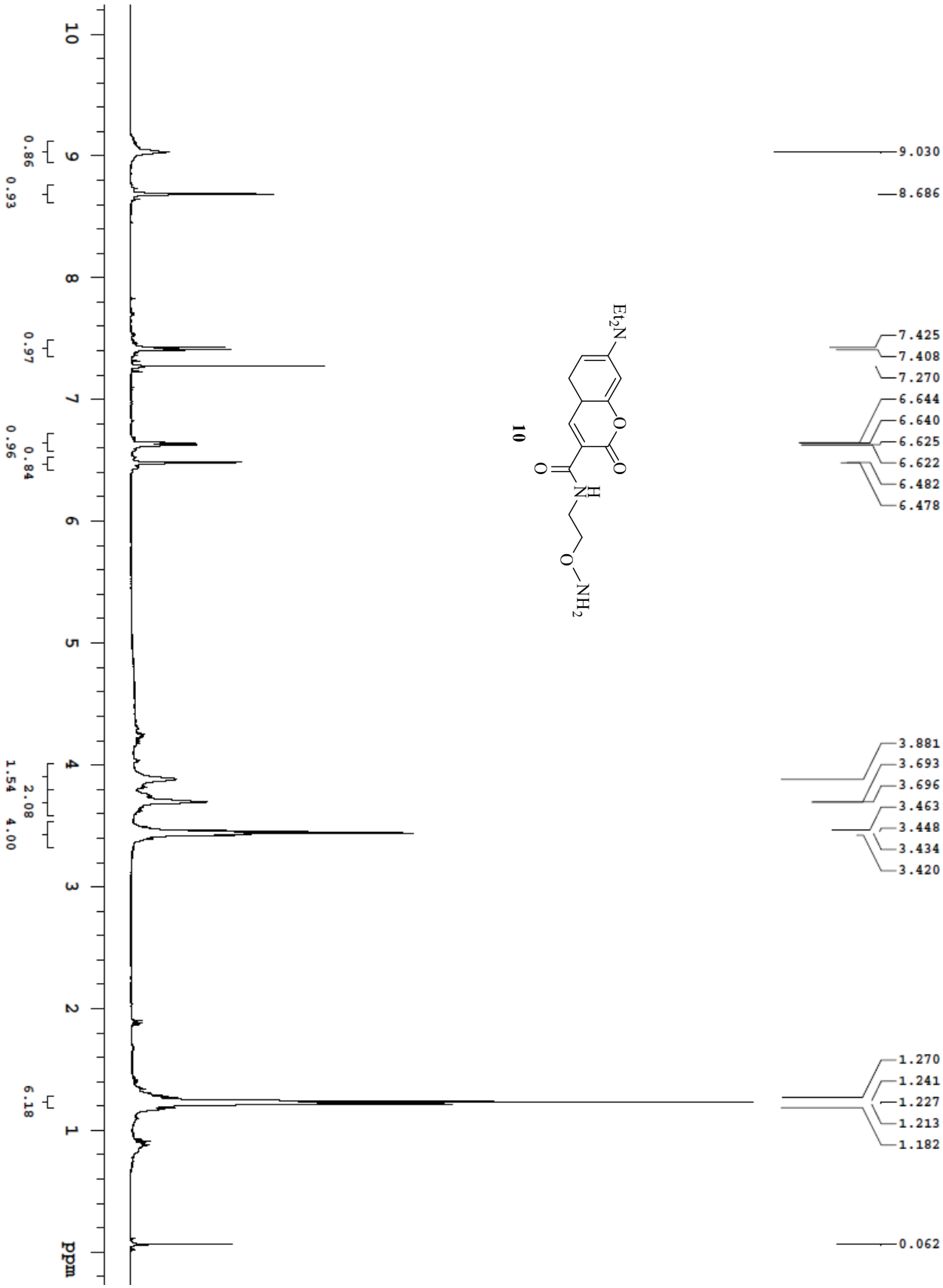

7.366
7.360
7.356
7.354
7.270
5.117
3.868
3.859
3.850
3.439
3.431
1.473
0.77
4.58
0.87
1.97
1.98
2.01
9.00
ppm
BocHN
O
NHCbz
22

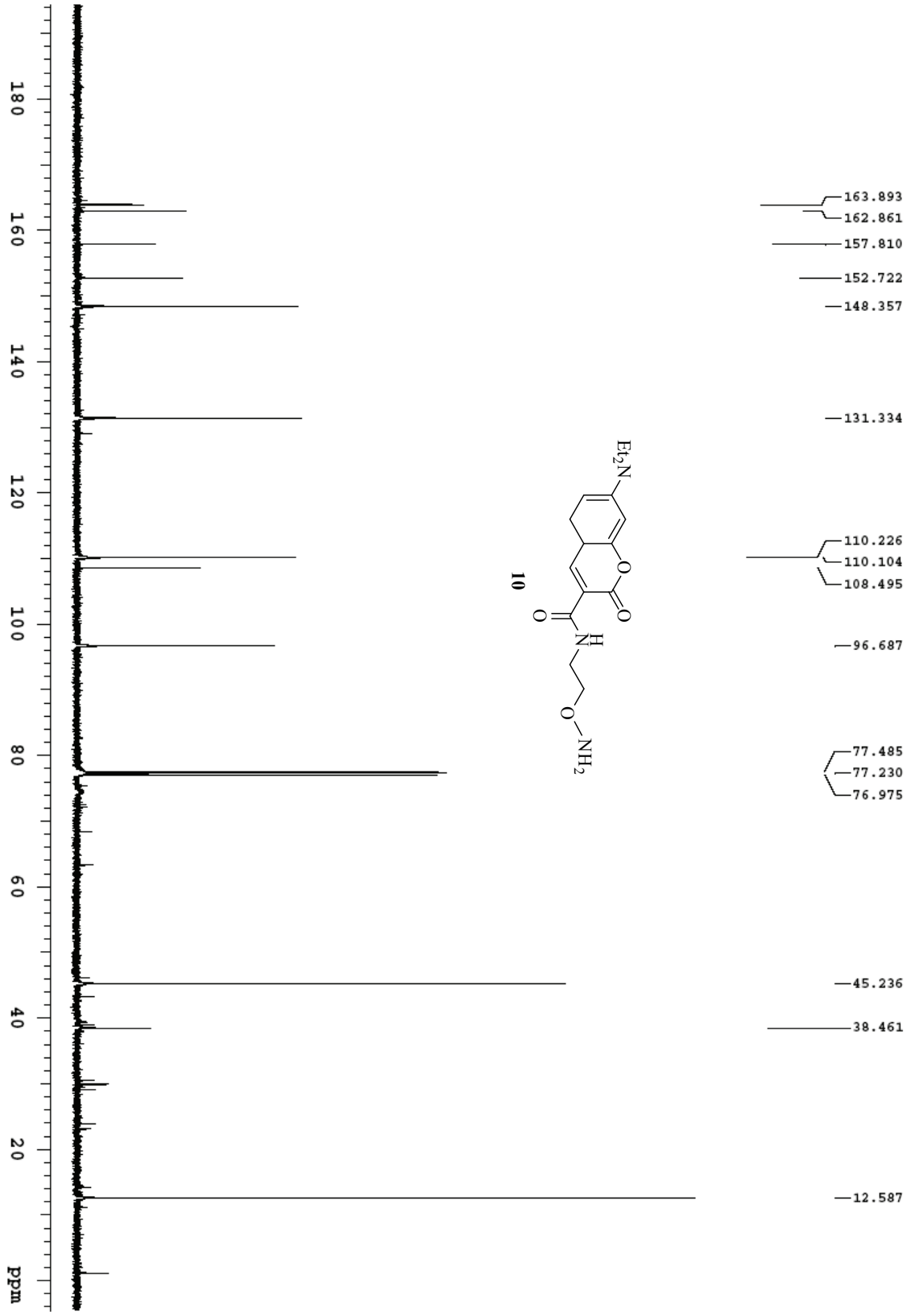

158.051
157.404
137.207
129.081
128.892
128.636
82.726
78.073
77.648
77.230
76.205
67.290
39.709
28.764
ppm
180
160
140
120
100
80
60
40
20
BocHN
O
NHCbz
22


8.625
7.587
7.569
6.870
6.865
6.852
6.847
6.621
6.616
4.903
3.577
3.563
3.548
3.535
3.316
3.313
3.310
3.306
3.303
1.260
1.246
1.231
0.91
0.98
1.06
1.01
4.10
6.00
ppm
Et2N
O
O
COOH
27


9.055
8.673
8.070
7.416
7.386
7.270
6.653
6.644
6.623
6.615
6.481
6.473
3.976
3.959
3.942
3.731
3.713
3.696
3.677
3.472
3.448
3.424
3.400
1.523
1.519
1.471
1.450
1.245
1.222
1.198
0.86
0.88
0.89
0.96
0.97
0.95
1.89
2.01
4.15
9.00
6.32
ppm
Et2N
O
O
O
H
N
O
NHBoc
28


164.245
162.751
157.792
156.867
152.644
148.359
131.310
110.214
108.594
96.874
81.529
77.651
77.230
76.802
74.895
45.333
37.822
28.410
12.546
200
180
160
140
120
100
80
60
40
20
ppm
Et2N
O
O
O
H
N
O
NHBoc
28


9.030
8.686
7.425
7.408
7.270
6.644
6.640
6.625
6.622
6.482
6.478
3.881
3.693
3.696
3.463
3.448
3.434
3.420
1.270
1.241
1.227
1.213
1.182
0.062
0.86
0.93
0.97
0.96
0.84
1.54
2.08
4.00
6.18
ppm
10


163.893
162.861
157.810
152.722
148.357
131.334
110.226
110.104
108.495
96.687
77.485
77.230
76.975
45.236
38.461
12.587
180
160
140
120
100
80
60
40
20
ppm
Et2N
O
O
O
H
N
O
NH2
10