Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

A pyrene-inhibitor fluorescent probe with large Stokes shift for the staining of $A\beta_{1-42}$, α-synuclein and amylin amyloid fibrils as well as amyloid-containing *Staphylococcus aureus* biofilms

Alejandro Mahía, María Conde-Giménez, Sandra Salillas, Irantzu Pallares, Juan J. Galano, Íñigo Lasa, Salvador Ventura, María D. Díaz-de-Villegas, José A. Gálvez, Javier Sancho

Content

I. Synthesis of organic fluorescent compounds.

A. Synthetic methods.

1. General information.

All reactions were performed in round-bottomed or Schlenk flasks. When necessary, an argon atmosphere and dried solvents were used. Chromatographic separations were conducted by column chromatography employing 60 ACC (70 – 200 μm) silica gel as stationary phase. Thin layer chromatography plates (fluorescent indicator F254) were visualized by exposure to ultraviolet light and stain with potassium permanganate basic solution.

2. Experimental procedures.

4-((3-(Ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophen-2-yl)amino)-4-oxobutanoic acid (5). To a stirred suspension of succinic anhydride (60.0 mg, 0.60 mmol) in toluene (3.5 mL) were sequentially added ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate **2** (67.6 mg, 0.30 mmol) and triethylamine (3.0 mg, 0.03 mmol) and the reaction mixture was refluxed for 4 hours. The crude reaction was cooled at room temperature and extracted with saturated $NaHCO_3$ aqueous solution (2 x 3.5 mL). The aqueous phase was acidified with concentrated HCl (pH = 2) and the resulting precipitate was extracted with dichloromethane (2 x 10 mL). The organic phase was dried with anhydrous $MgSO_4$, filtered and solvent was removed under reduced pressure to afford compound 5 as a white solid. Yield 75 %. m.p. (ºC): 157-158. IR (KBr, cm^{-1}): 1659, 1687, 3381. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 11.36 (br.s, 1H), 4.32 (q, 2H, J = 7.2), 2.84 - 2.71 (m, 6H), 2.68 – 2.59 (m, 2H), 1.84 – 1.71 (m, 4H), 1.38 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 177.5, 168.2, 166.5, 147.2, 130.7, 126.7, 111.5, 60.5, 30.9, 28.8, 26.3, 24.3, 22.9, 22.8, 14.2. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{15}H_{19}NNaO_5S$ 348.0876, found 348.0883.

5-((3-(Ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophen-2-yl)amino)-5-oxopentanoic acid (6). To a solution of glutaric anhydride (68.5 mg, 0.60 mmol) and ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate **2** (67.6 mg, 0.30 mmol) in toluene (3.5 mL) was added triethylamine (3.0 mg, 0.03 mmol) and the reaction mixture was stirred and refluxed for 2 hours. The crude reaction was cooled at room temperature and extracted with saturated $NaHCO_3$ aqueous solution (2 x 3.5 mL). The aqueous phase was acidified with concentrated HCl (pH = 2) and the resulting precipitate was extracted with dichloromethane (2 x 10 mL). The organic phase was dried with anhydrous $MgSO_4$, filtered and solvent was removed under reduced pressure to afford compound 6 as a white solid. Yield 89 %. m.p. (ºC): 103-104. IR (nujol, cm^{-1}): 1653, 1683, 1700. ^{1}H-NMR (300 MHz, $CDCl_3$, δ (ppm), J (Hz)): 11.28 (br.s, 1H), 10.83 (br.s, 1H), 4.29 (q, 2H, J = 7.2), 2.78 – 2.66 (m, 2H), 2.65 – 2.56 (m, 2H), 2.56 (t, 2H, J = 7.4), 2.47 (t, 2H, J = 7.4), 2.06 (quint, 2H, J = 7.4), 1.83 – 1.66 (m, 4H), 1.35 (t, 3H, J = 7.2). ^{13}C-NMR (75 MHz, $CDCl_3$, δ (ppm)): 178.4, 169.1, 166.5, 147.2, 130.6, 126.6, 111.4, 60.4, 35.4, 32.8, 26.2, 24.2, 22.9, 22.7, 20.0, 14.2. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{16}H_{21}NNaO_5S$ 362.1033, found 362.1040.

2-(3-Carboxypropanamido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (7). A suspension of compound **5** (195 mg, 0.60 mmol) in 1.8 M NaOH ethanolic

solution (8.0 mL) was stirred at 36 ºC for 48 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and the formed precipitate was filtered, washed with cold water and dried under vacuum to afford **7** as a pale brown solid. Yield 67 %. m.p. (ºC): 204-205. IR (KBr, cm^{-1}): 1653, 1685. ^{1}H-NMR (400 MHz, DMSO-d_6, δ (ppm), J (Hz)): 11.23 (br.s, 1H), 2.75 - 2.63 (m, 4H), 2.62 – 2.52 (m, 4H), 1.77 – 1.63 (m, 4H). ^{13}C-NMR (100 MHz, DMSO-d_6, δ (ppm)): 173.5, 168.9, 167.0, 146.2, 130.9, 125.4, 111.7, 30.8, 28.7, 25.9, 23.7, 22.6, 22.4. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{13}H_{15}NNaO_5S$ 320.0563, found 320.0555.

2-(4-Carboxybutanamido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (8). A suspension of **6** (187 mg, 0.55 mmol) in 1.8 M NaOH ethanolic solution (8.0 mL) was stirred at 36 ºC for 48 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and the formed precipitate was filtered, washed with cold water and dried under vacuum to afford **8** as a pale brown solid. Yield 76 %. m.p. (ºC): 186 – 188. IR (nujol, cm^{-1}): 1667, 1673, 1687. ^{1}H-NMR (400 MHz, CD_3OD, δ (ppm), J (Hz)): 2.85 – 2.71 (m, 2H), 2.70 – 2.59 (m, 2H), 2.56 (t, 2H, J = 7.2), 2.40 (t, 2H, J = 7.2), 1.99 (quint, 2H, J = 7.2), 1.88 -1.68 (m, 4H). ^{13}C-NMR (100 MHz, CD_3OD, δ (ppm)): 176.6, 171.2, 168.9, 148.0, 132.5, 127.6, 113.3, 36.4, 33.9, 27.3, 25.2, 24.1, 23.9, 21.6. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{14}H_{17}NNaO_5S$ 334.0720, found 334.0727.

3-(4-Oxo-5,6,7,8-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*d*][1,3]oxazin-2-yl)-*N*-(pyren-1-ylmethyl)propanamide (1A). A suspension of **7** (98.1 mg, 0.33 mmol) in a mixture of Ac_2O/AcOH 3/1 (3.5 mL) was stirred for 5 hours at room temperature. Solvent was removed under reduced pressure and the resulting solid was washed with ethanol and dried under vacuum to give intermediate **9**, which was used in the next step without purification. To a solution of crude product **9** and 1-pyrenemethylamine hydrochloride (107 mg, 0.40 mmol) in DMF/CH_2Cl_2 1/1 (16 mL) were sequentially added *N*-methylmorpholine (40.4 mg, 0.40 mmol), DCC (68.1 mg, 0.33 mmol) and HOBt (44.6 mg, 0.33 mmol) and the reaction mixture was stirred at room temperature for 18 hours. Saturated $NaHCO_3$ aqueous solution (10 mL) and CH_2Cl_2 (10 mL) were added to the reaction mixture and the aqueous phase was extracted with 10 mL of CH_2Cl_2. The combined organic phases were dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography (eluent 1: Et_2O, eluent 2: AcOEt/Hexane 7:3) and the resulting solid was washed with methanol to afford compound **1A** as a white solid. Yield 21 %. m.p. (ºC): 226 – 227. IR (nujol, cm^{-1}): 3291, 1636, 1757. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 8.19 – 7.87 (m, 9H), 6.45 (t, 1H, J = 5.2), 5.10 (d, 2H, J = 5.2), 3.09 (t, 2H, J = 6.4), 2.67 (t, 2H, J = 6.4), 2.31 – 2.21 (m, 4H), 1.66 – 1.58 (m, 2H), 1.58 – 1.50 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 170.7, 162.7, 159.7, 154.6, 133.5, 131.3, 131.1, 130.9, 130.5, 128.9, 127.8, 127.6, 127.4, 127.1, 126.0, 125.5, 125.2, 124.9, 124.6, 124.6, 122.6, 115.5, 42.4, 31.5, 30.1, 24.4, 22.4, 21.5. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{30}H_{24}N_2NaO_3S$ 515.1400, found 515.1378

4-(4-Oxo-5,6,7,8-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*d*][1,3]oxazin-2-yl)-*N*-(pyren-1-ylmethyl)butanamide (1B). A suspension of **8** (93.4 mg, 0.30 mmol) in a mixture of Ac_2O/AcOH 3/1 (3.5 mL) was stirred for 5 hours at room temperature. Solvent was removed under reduced pressure and the resulting solid was washed with ethanol and dried under vacuum to give intermediate **10**, which was used in the next step without purification. To a solution of crude product **10** and 1-pyrenemethylamine hydrochloride (96.4 mg, 0.36 mmol) in DMF/CH_2Cl_2 1/1 (16 mL) were sequentially added *N*-methylmorpholine (36.4 mg, 0.36 mmol), DCC (61.9 mg, 0.30 mmol) and HOBt (46.1 mg, 0.30 mmol) and the reaction mixture was stirred at room temperature for 18 hours. Saturated $NaHCO_3$ aqueous solution (10 mL) and CH_2Cl_2 (10 mL) were added to the reaction mixture and the aqueous phase was extracted with 10 mL of CH_2Cl_2. The combined organic phases were dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography (eluent : Et_2O) and the resulting solid was washed with methanol to afford compound **1B** as a pale yellow solid. Yield 20 %. m.p. (ºC): 175 – 176. IR (nujol, cm^{-1}): 3326, 1668, 1726. ^{1}H-NMR (300 MHz, $CDCl_3$, 328 K, δ (ppm), J (Hz)): 8.29 -7.91 (m, 9H), 5.95 (br.s, 1H), 5.14 (d, 2H, J = 5.1), 2.85 – 2.76 (m, 2H), 2. 73 (t, 2H, J = 7.2), 2.69 – 2.61 (m, 2H), 2.34 (t, 2H, J = 7.2), 2.17 (quint, 2H, J = 7.2), 1.90 – 1.71 (m, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$, 328 K, δ (ppm)): 171.4, 164.0, 161.3, 155.3, 134.1, 131.9, 131.4, 131.3, 130.9, 129.1, 128.3, 127.6, 127.4, 127.3, 126.1, 125.4, 125.2, 124.8, 122.8, 116.4, 42.1, 35.3, 33.6, 25.1, 24.9, 22.9, 22.0, 21.9. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{31}H_{26}N_2NaO_3S$ 529.1556, found 529.1556

Diethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3,6-dicarboxylate (13). To a solution of 4-(ethoxycarbonyl)cyclohexanone **11** (765 mg, 4.50 mmol) and ethyl cyanoacetate (509 mg, 4.50 mmol) in ethanol (5.0 mL) were sequentially added powdered sulfur (158 mg, 4.95 mmol) and diethylamine (329 mg, 4.5 mmol) and the reaction mixture was stirred for 22 hours at room temperature. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (eluent: hexane/Et_2O 2:1) to afford compound **13** as a white solid. Yield 87 %. m.p. (ºC): 112 -114. IR (nujol, cm^{-1}): 3423, 3306, 1708, 1667. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 5.98 (br.s, 2H), 4.25 (q, 2H, J = 7.2), 4.16 (q, 2H, J = 7.2), 2.98 – 2.87 (m, 1H), 2.79 – 2.57 (m, 4H), 2.20 – 2.11 (m, 1H), 1.79 (dddd, 1H, J = 13.2, J = 10.8, J = 10.4, J = 5.6), 1.32 (t, 3H, J = 7.2), 1.26 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 174.9, 165.9, 162.0, 131.8, 115.4, 105.3, 60.5, 59.4, 40.0, 26.7, 26.1, 25.6, 14.4, 14.2. HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{14}H_{20}NO_4S$ 298.1108, found 298.1116.

Ethyl 2-amino-6-(2-ethoxy-2-oxoethyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (14). To a solution of ethyl 2-(4-oxocyclohexyl)acetate **12** (221 mg, 1.20 mmol) and ethyl cyanoacetate (136 mg, 1.20 mmol) in ethanol (5.0 mL) were sequentially added powdered sulfur (41.6 mg, 1.30 mmol) and diethylamine (87.8 mg, 1.2 mmol) and the reaction mixture was stirred for 22 hours at room temperature. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (eluent: hexane/Et_2O 2:1) to afford compound **13** as a yellow oil. Yield 54 %. IR (nujol, cm^{-1}): 3438, 3330, 1735, 1664. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 5.96 (br.s, 2H), 4.24 (q, 2H, J = 7.2), 4.14 (q, 2H, J = 7.2), 2.92 – 2.80

(m, 1H), 2.71 – 2.56 (m, 2H), 2.41 – 2.18 (m, 4H), 1.93 – 1.83 (m, 1H), 1.51 – 1.37 (m, 1H), 1.32 (t, 3H, J = 7.2), 1.26 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 172.7, 166.0, 161.9, 132.0, 116.1, 105.4, 60.3, 59.4, 40.4, 31.4, 30.3, 28.8, 26.2, 14.4, 14.2. HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{15}H_{22}NO_4S$ 312.1264, found 312.1273.

6-Carboxy-3-(ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophen-2-aminium chloride (15). To a solution of **13** (89.2 mg, 0.30 mmol) in a mixture of THF/EtOH 1/1 (2.0 mL) was added 1M NaOH aqueous solution (0.9 mL, 0.90 mmol) and the reaction mixture was stirred at room temperature for 20 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and the resulting precipitate was filtered and dried under vacuum to afford compound **15** as a white solid. Yield 74 %. m.p. (ºC): 220 – 221 (decomposed). IR (nujol, cm^{-1}): 3426, 3312, 1720, 1631. ^{1}H-NMR (400 MHz, CD_3OD, δ (ppm), J (Hz)): 4.28 – 4.20 (m, 2H), 2.96 – 2.85 (m, 1H), 2.76 – 2.56 (m, 4H), 2.19 – 2.08 (m, 1H), 1.84 – 1.72 (m, 1H), 1.32 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, DMSO-d_6, δ (ppm)): 175.9, 165.0, 163.2, 130.9, 114.0, 102.4, 58.7, 39.1, 26.3, 25.7, 25.2, 14.4. HRMS (ESI^+): m/z $[M]^+$ calcd for $C_{12}H_{16}NO_4S$ 270.0795, found 270.0800.

6-(Carboxymethyl)-3-(ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophen-2-aminium chloride (16). To a solution of **14** (77.8 mg, 0.25 mmol) in a mixture of THF/EtOH 1/1 (1.7 mL) was added 1M NaOH aqueous solution (0.75 mL, 0.75 mmol) and the reaction mixture was stirred at room temperature for 4 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and extracted with dichloromethane (2 x 10 mL). The organic phase was dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure to afford compound **16** as a white solid. Yield 100 %. m.p. (ºC): 166 – 167. IR (nujol, cm^{-1}): 3437, 3332, 1704, 1644. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 4.25 (q, 2H, J = 7.2), 2.92 – 2.81 (m, 1H), 2.74 – 2.59 (m, 2H), 2.48 – 2.36 (m, 2H), 2.35 – 2.21 (m, 2H), 1.97 – 1.88 (m, 1H), 1.54 -1.41 (m, 1H), 1.33 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 178.7, 166.1, 162.0, 132.0, 115.9, 105.4, 59.5, 40.0, 31.2, 30.3, 28.7, 26.1, 14.4. HRMS (ESI^+): m/z $[M]^+$ calcd for $C_{13}H_{18}NO_4S$ 284.0951, found 284.0960.

2-Acetamido-3-(ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-6-carboxylic acid (17). To a stirred suspension of compound **15** (67.3 mg, 0.22 mmol) in toluene (3.0 mL) were added acetic anhydride (44.9 mg, 0.44 mmol) and triethylamine (24.3 mg, 0.24 mmol) and the reaction mixture was refluxed for 4 hours. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (eluent: Et_2O/Hexane 4:1, 5 % AcOH) to afford compound **17** as a white solid. Yield 91 %. m.p. (ºC): 219 – 220. IR (nujol, cm^{-1}): 1639, 1688, 1717. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 11.26 (br.s, 1H), 4.33 (q, 2H, J = 7.2), 3.07 – 2.97 (m, 1H), 2.96 – 2.88 (m, 2H), 2.85 – 2.69 (m, 2H), 2. 26 (s, 3H), 2.28 – 2.19 (m, 1H), 1.89 (dddd, 1H, J = 13.2, J = 10.8, J = 10.4, J = 5.6), 1.38 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 179.7, 167.1, 166.4, 148.2, 130.0, 124.3, 110.9, 60.6, 39.4, 26.3, 25.4, 25.4, 23.7, 14.3. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{14}H_{17}NNaO_5S$ 334.0720, found 334.0728.

2-(2-Acetamido-3-(ethoxycarbonyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophen-6-yl)acetic acid (18). To a stirred suspension of compound **16** (70.4 mg, 0.22 mmol) in toluene (3.0 mL) were added acetic anhydride (44.9 mg, 0.44 mmol) and triethylamine (24.3 mg, 0.24 mmol) and the reaction mixture was refluxed for 4 hours. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (eluent: Et_2O/Hexane 2:1, 5 % AcOH) to afford compound **18** as a white solid. Yield 74 %. m.p. (ºC): 162 – 163. IR (nujol, cm^{-1}): 1660, 1689, 1704. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 11.24 (br.s, 1H), 9.52 (br.s, 1H), 4.30 (q, 2H, J = 7.2), 2.92 (ddd, 1H, J 17.6, J = 4.4, J = 4.4), 2.82 (dd, 1H, J = 15.6, J = 4.0), 2.76 – 2. 64 (m, 1H), 2.45 – 2.33 (m, 3H), 2.33 – 2.24 (m, 1H), 2.24 (s, 3H), 2.02 – 1.90 (m, 1H), 1.55 – 1.42 (m, 1H), 1.36 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 178.4, 167.1, 166.5, 147.8, 130.2, 125.1, 111.0, 60.5, 39.9, 30.9, 30.1, 28.6, 25.6, 23.6, 14.2. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{15}H_{19}NNaO_5S$ 348.0876, found 348.0868.

Ethyl 2-acetamido-6-((pyren-1-ylmethyl)carbamoyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (19). To a solution of **17** (62.3 mg, 0.20 mmol) and 1-pyrenemethylamine hydrochloride (64.3 mg, 0.24 mmol) in a mixture of DMF/CH_2Cl_2 1/1 (12 mL) were sequentially added *N*-methylmorpholine (24.2 mg, 0.24 mmol), EDC (31.0 mg, 0.20 mmol) and HOBt (27.0 mg, 0.20 mmol) and the reaction mixture was stirred at room temperature for 18 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in CH_2Cl_2 (15 mL) and washed with saturated $NaHCO_3$ aqueous solution (15 mL). The organic phase was washed again with 0.5 M HCl aqueous solution (15 mL), dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The resulting residue was purified by column chromatography (eluent 1: Et_2O, eluent 2: AcOEt) to afford compound **19** as a white solid. Yield 90 %. m.p. (ºC): 131 – 132. IR (nujol, cm^{-1}): 3273, 1653. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 10.92 (br.s, 1H), 8.07 – 7.76 (m, 9H), 6.54 (t, 1H, J = 5.2), 4.93 (d, 2H, J = 5.2), 4.14 – 4.00 (m, 2H), 2.76 – 2.54 (m, 3H), 2.45 – 2.24 (m, 2H), 2.11 (s, 3H), 1.96 – 1.85 (m, 1H), 1.68 – 1.54 (m, 1H), 1.21 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 174.5, 166.7, 165.9, 147.5, 131.0, 131.0, 130.5, 129.7, 128.8, 127.9, 127.2, 127.1, 127.1, 125.8, 125.1, 125.1, 124.7, 124.5, 124.4, 124.2, 122.6, 110.6, 60.3, 41.8, 41.4, 27.2, 26.0, 25.4, 23.5, 14.1. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{31}H_{28}N_2NaO_4S$ 547.1662, found 547.1653.

Ethyl 2-acetamido-6-(2-oxo-2-((pyren-1-ylmethyl)amino)ethyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (20). To a solution of **18** (39.0 mg, 0.12 mmol) and 1-pyrenemethylamine hydrochloride (40.2 mg, 0.15 mmol) in a mixture of DMF/CH_2Cl_2 1/1 (7.0 mL) were sequentially added *N*-methylmorpholine (15.2 mg, 0.15 mmol), EDC (18.6 mg, 0.12 mmol) and HOBt (16.2 mg, 0.12 mmol) and the reaction mixture was stirred at room temperature for 18 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in CH_2Cl_2 (20 mL) and washed with saturated $NaHCO_3$ aqueous solution (10 mL). The organic phase was washed again with 0.5 M HCl aqueous solution (10 mL), dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The resulting residue was purified by column chromatography (eluent 1: Et_2O, eluent 2: AcOEt) to afford compound **20** as a white solid. Yield 74 %. m.p. (ºC): 243 – 244. IR (nujol, cm^{-1}): 3272, 1689, 1662,

1634. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 11.03 (br.s, 1H), 8.22 – 7.87 (m, 9H), 6.33 (dd, 1H, J = 5.6, J = 5.0), 5. 15 (dd, 1H, J = 14.4, J = 5.6), 5.06 (dd, 1H, J = 14.4, J = 5.0), 4.22 (q, 2H, J = 7.2), 2.78 – 2.62 (m, 2H), 2.59 – 2.45 (m, 1H), 2.39 – 2.15 (m, 4H), 2.13 (s, 3H), 1.94 – 1.83 (m, 1H), 1.37 – 1.25 (m, 1H), 1.31 (t, 3H, J = 7.2). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 171.4, 166.8, 166.3, 147.4, 131.1, 131.1, 131.1, 130.6, 130.0, 129.0, 128.0, 127.4, 127.3, 127.2, 126.0, 125.3, 125.3, 125.1, 124.9, 124.7, 124.6, 122.8, 110.8, 60.4, 42.7, 41.9, 31.7, 30.2, 28.8, 25.6, 23.6, 14.2. HRMS (ESI$^+$): m/z $[M + Na]^+$ calcd for $C_{32}H_{30}N_2NaO_4S$ 561.1819, found 561.1830.

2-Acetamido-6-((pyren-1-ylmethyl)carbamoyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (21). To a suspension of **19** (83.9 mg, 0.16 mmol) in a mixture of THF/EtOH 1/1 (2.0 mL) was added 1M LiOH aqueous solution (0.48 mL, 0.48 mmol) and the reaction mixture was stirred at 40 ºC for 5 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and the resulting precipitate was filtered, washed with dichloromethane and dried under reduced pressure to afford compound **21** as a pale brown solid. Yield 39 %. m.p. (ºC): 264 – 265. IR (nujol, cm^{-1}): 3284, 1683, 1633. ^{1}H-NMR (400 MHz, DMSO-d_6, δ (ppm), J (Hz)): 11.14 (br.s, 1H), 8.65 (t, 1H, J = 5.6), 8.40 – 8.00 (m, 9H), 5.08 (dd, 1H, J = 14.8, J = 5.6), 5.01 (dd, 1H, J = 14.8, J = 5.6), 3.02 – 2.89 (m, 1H), 2.85 – 2.71 (m, 2H), 2.69 – 2.49 (m, 3H), 2.20 (s, 3H), 2.05 – 1.95 (m, 1H), 1.80 – 1.65 (m, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6, δ (ppm)): 174.1, 167.2, 166.8, 146.5, 133.0, 130.9, 130.4, 130.3, 130.2, 128.2, 127.6, 127.5, 127.1, 126.7, 126.3, 125.3, 125.3, 124.8, 124.3, 124.1, 124.0, 123.3, 111.4, 40.4, 39.9, 26.7, 26.3, 25.5, 23.4. HRMS (ESI$^+$): m/z $[M + Na]^+$ calcd for $C_{29}H_{24}N_2NaO_4S$ 519.1349, found 519.1338.

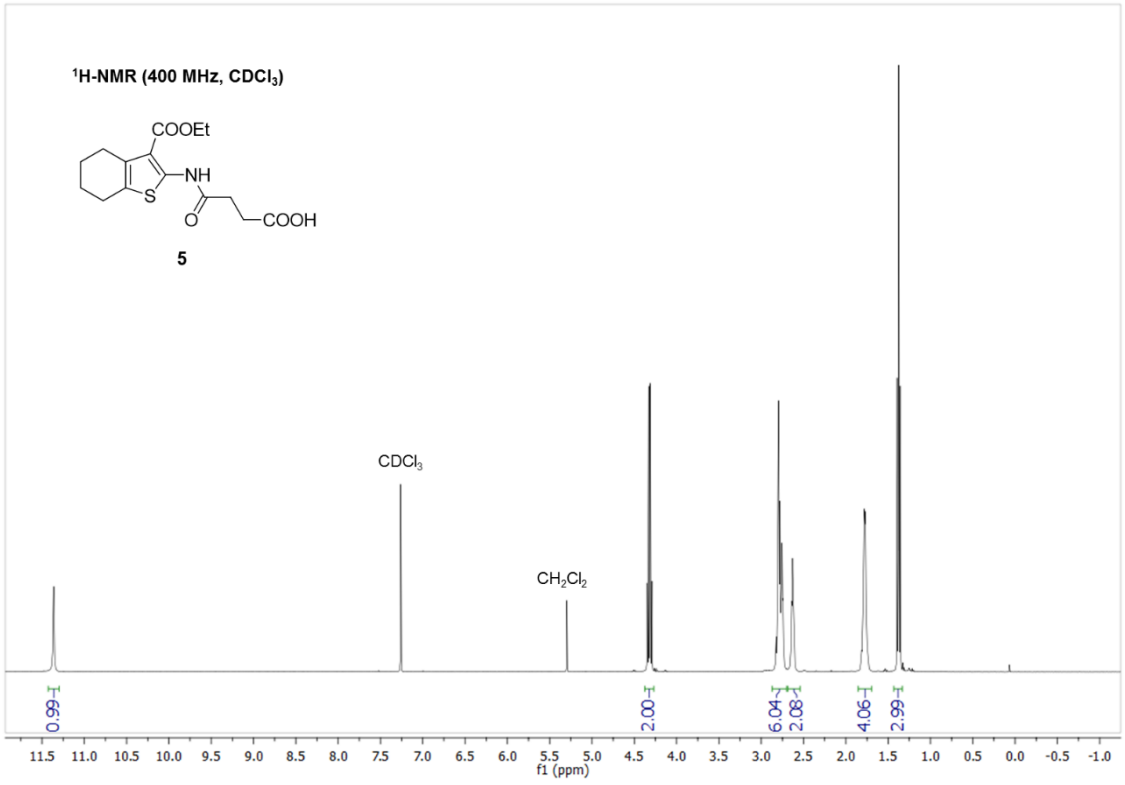
2-Acetamido-6-(2-oxo-2-((pyren-1-ylmethyl)amino)ethyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (22). To a suspension of **20** (75.4 mg, 0.14 mmol) in a mixture of THF/EtOH 1/1 (2.0 mL) was added 1M LiOH aqueous solution (0.42 mL, 0.42 mmol) and the reaction mixture was stirred at 70 ºC for 2 hours. Solvent was removed under reduced pressure and the obtained solid was dissolved in water (10 mL) and washed with dichloromethane (10 mL). The aqueous phase was acidified (pH = 2) with concentrated HCl and the resulting precipitate was filtered, washed with dichloromethane and dried under reduced pressure to afford compound **22** as a pale pink solid. Yield 52 %. m.p. (ºC): 246 – 247. IR (nujol, cm^{-1}): 3288, 1664, 1642. ^{1}H-NMR (400 MHz, DMSO-d_6, δ (ppm), J (Hz)): 11.15 (br.s, 1H), 8.60 (t, 1H, J = 5.8), 8.41- 8.01 (m, 9H), 5.03 (d, 2H, J = 5.8), 5.92 – 5.80 (m, 1H), 2.77 – 2.67 (m, 1H), 2.64 – 2.51 (m, 1H), 2.35 – 2.18 (m, 4H), 2.20 (s, 3H), 1.90 – 1.79 (m, 1H), 1.44 – 1.29 (m, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6, δ (ppm)): 171.0, 167.1, 166.9, 146.4, 133.1, 130.8, 130.4, 130.4, 130.2, 128.2, 127.6, 127.4, 127.1, 126.8, 126.3, 125.3, 125.2, 124.8, 124.6, 124.1, 124.0, 123.4, 111.5, 41.3, 40.5, 31.4, 29.6, 28.2, 25.3, 23.4. HRMS (ESI$^+$): m/z $[M + Na]^+$ calcd for $C_{30}H_{26}N_2NaO_4S$ 533.1506, found 533.1518.

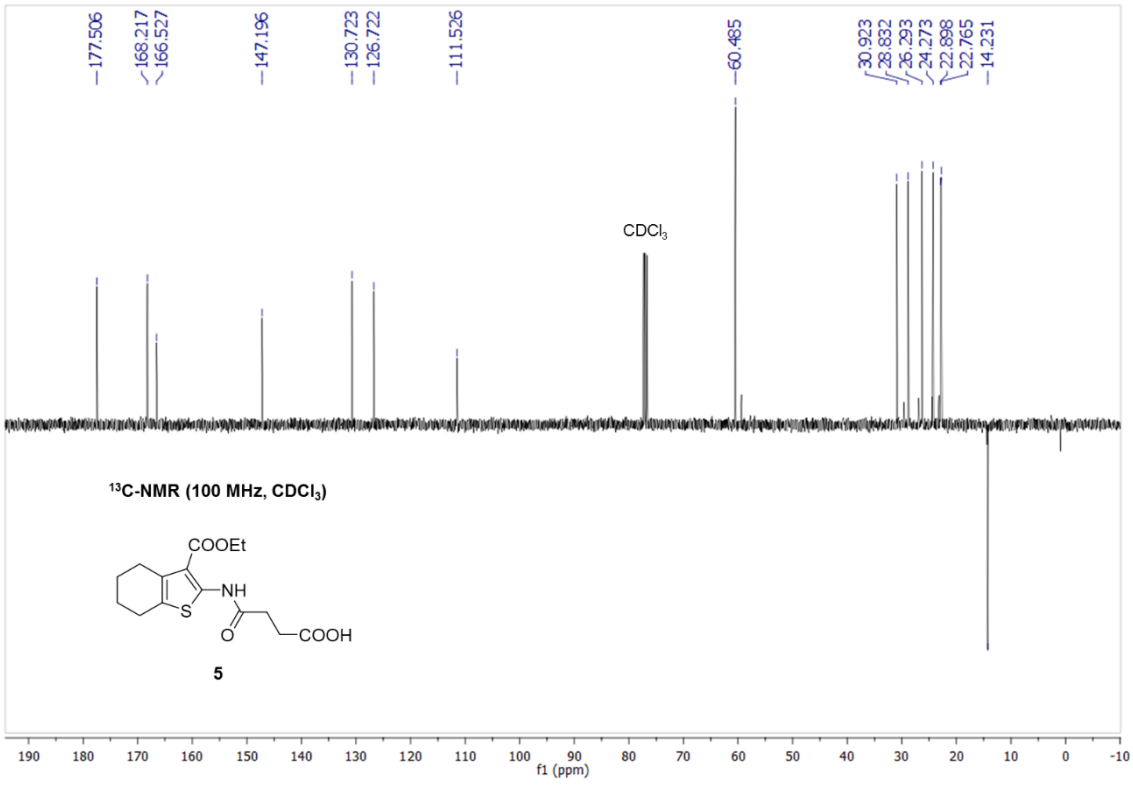
2-Methyl-4-oxo-*N*-(pyren-1-ylmethyl)-5,6,7,8-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*d*][1,3]oxazine-7-carboxamide (1C). A suspension of compound **21** (24.8 mg, 0.05 mmol) in Ac_2O (1.5 mL) was stirred at 130 ºC for 3 hours under argon. Solvent was

removed under reduced pressure to afford compound **1C** as a pale brown solid. Yield 88%. m.p. (ºC): 257 – 258 (decomposed). IR (nujol, cm^{-1}): 3277, 1748, 1635. ^{1}H-NMR (300 MHz, DMSO-d_6, δ (ppm), J (Hz)): 8.68 (dd, 1H, J = 5.7, J = 5.4), 8.40 – 8.00 (m, 9H), 5.09 (dd, 1H, J = 15.0, J = 5.7), 5.01 (dd, 1H, J = 15.0, J = 5.4), 3.05 – 2.84 (m, 3H), 2.81 – 2.57 (m, 2H), 2.39 (s, 3H), 2.09 – 2.00 (m, 1H), 1.90 – 1.73 (m, 1H). ^{13}C-NMR (75 MHz, DMSO-d_6, δ (ppm)): 173.6, 162.6, 161.4, 154.9, 132.9, 132.4, 130.8, 130.4, 130.4, 130.2, 128.2, 127.6, 127.4, 127.1, 126.7, 126.3, 125.3, 125.2, 124.8, 124.1, 124.0, 123.2, 40.3, 40.0, 27.2, 25.5, 24.2, 20.9. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{29}H_{22}N_2NaO_3S$ 501.1243, found 501.1259

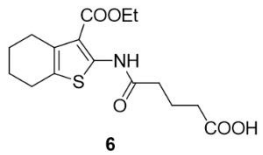
***N*-Acetyl-2-(2-methyl-4-oxo-5,6,7,8-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*d*][1,3]oxazin-7-yl)-*N*-(pyren-1-ylmethyl)acetamide (1D).** A suspension of compound **22** (35.7 mg, 0.07 mmol) in Ac_2O (2.5 mL) was stirred at 130 ºC for 48 hours under argon. Solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (eluent: Et_2O/Hexane 4:1) to afford compound **1D** as a white solid. Yield 97 %. m.p. (ºC): 116 – 117. IR (nujol, cm^{-1}): 1749, 1696, 1685. ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm), J (Hz)): 8.24 – 8.11 (m, 5H), 8.08 – 8.00 (m, 3H), 7.62 – 7.57 (m, 1H), 5.76 (d, 1H, J = 17.4), 5.67 (d, 1H, J = 17.4), 2.93 – 2.81 (m, 3H), 2.81 – 2.65 (m, 2H), 2.58 – 2.48 (m, 1H), 2.52 (s, 3H), 2.40 (s, 3H), 2.32 – 2.22 (m, 1H), 1.93 – 1.83 (m, 1H), 1.53 – 1.41 (m, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$, δ (ppm)): 175.0, 173.8, 161.7, 161.5, 155.2, 132.5, 131.3, 130.9, 130.6, 130.5, 129.4, 128.3, 127.5, 127.4, 127.4, 126.2, 125.6, 125.4, 125.2, 124.8, 124.5, 121.9, 121.1, 115.6, 45.0, 43.0, 30.4, 30.2, 27.6, 26.7, 23.9, 21.2. HRMS (ESI^+): m/z $[M + Na]^+$ calcd for $C_{32}H_{26}N_2NaO_4S$ 557.1505, found 557.1503

B. ^{1}H-NMR and APT (^{13}C-NMR) spectra

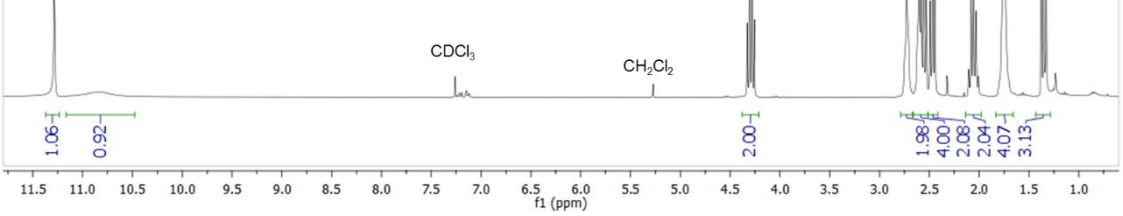


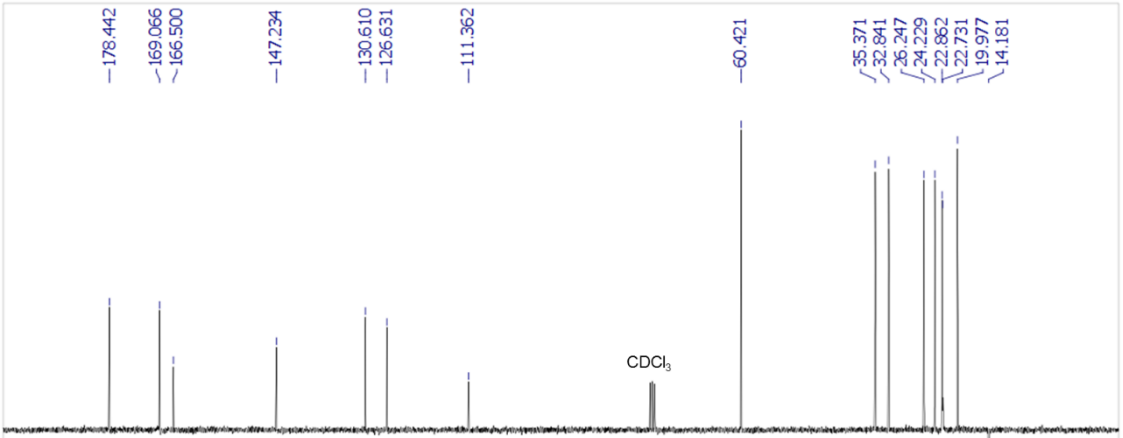


^{1}H-NMR (300 MHz, $CDCl_3$)

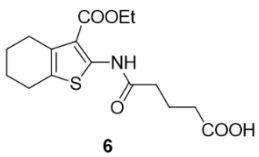


6

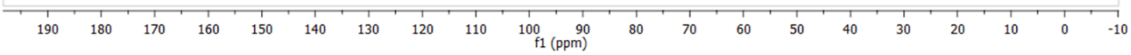


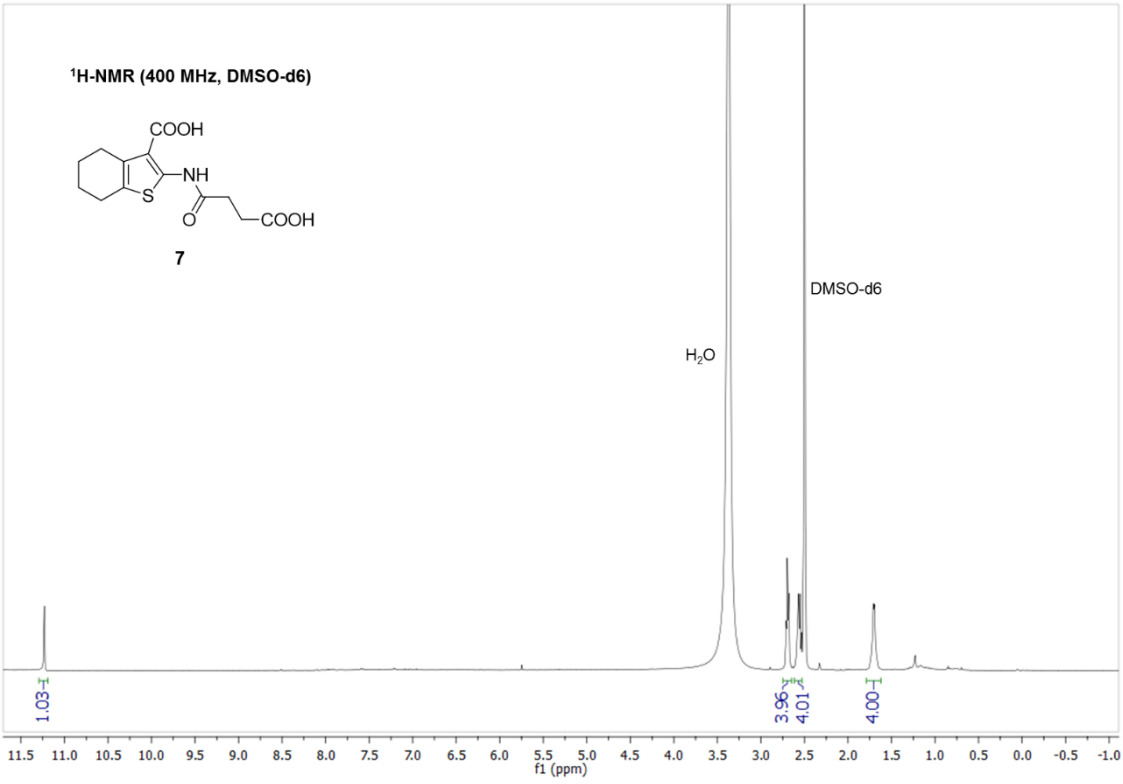


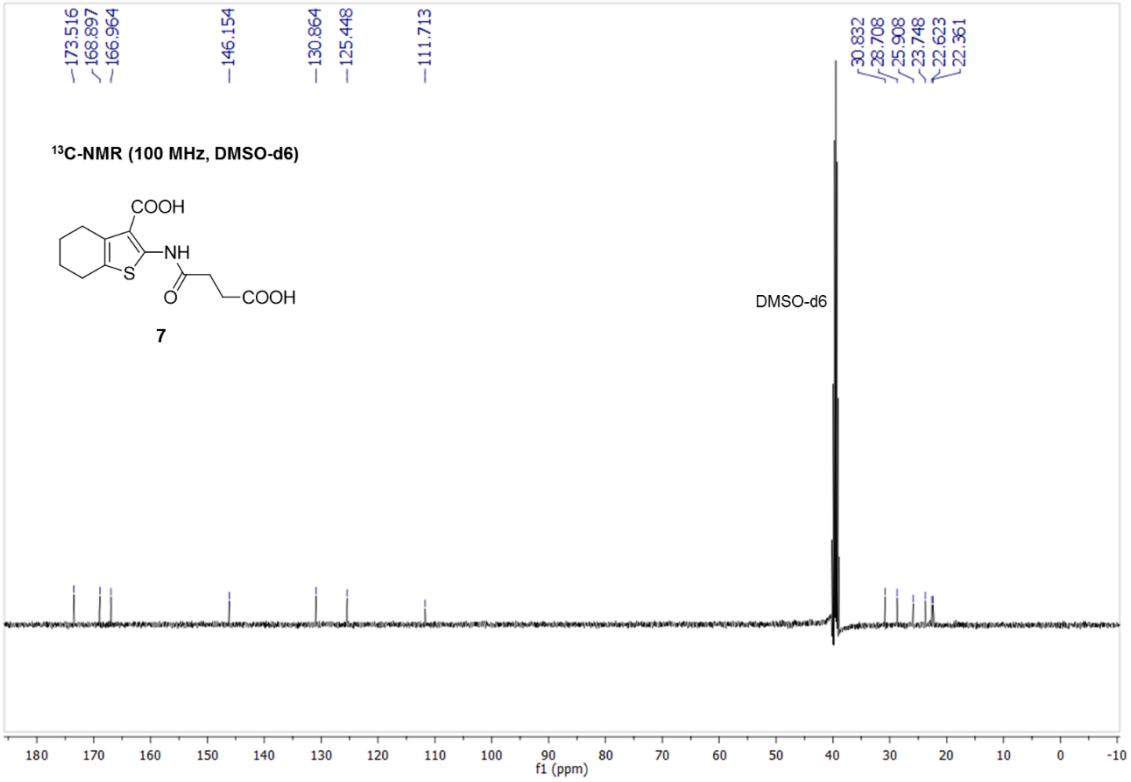
^{13}C-NMR (75 MHz, $CDCl_3$)

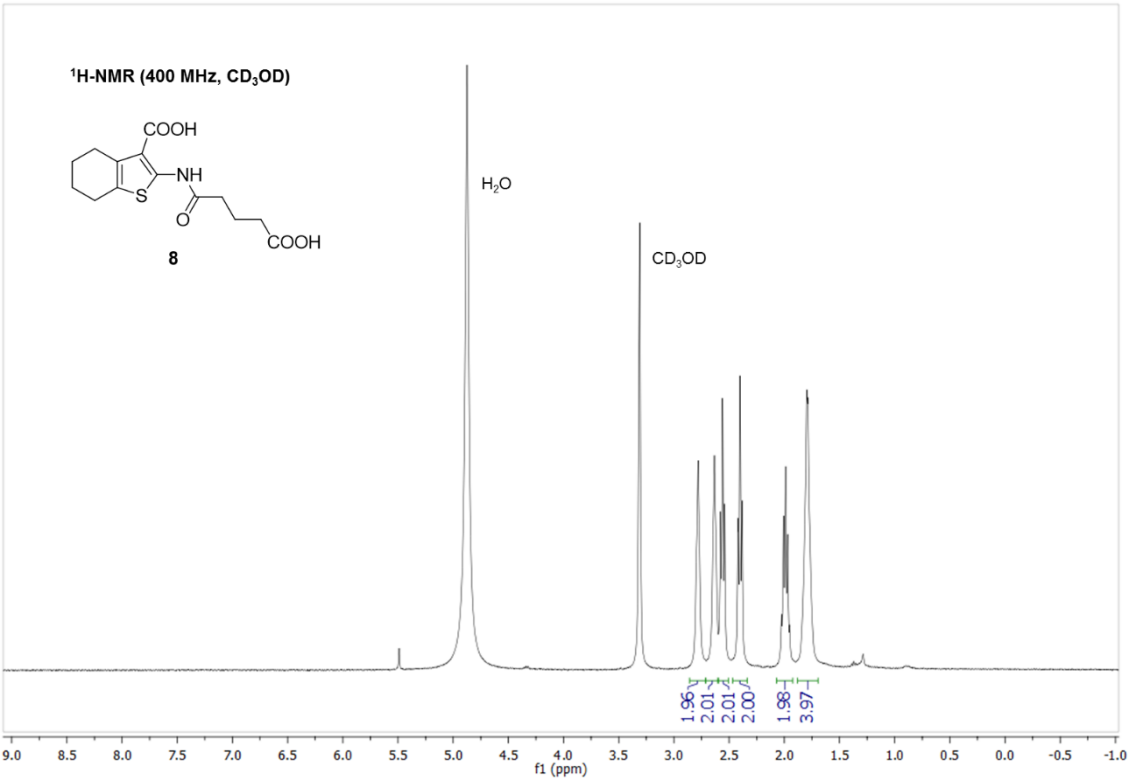


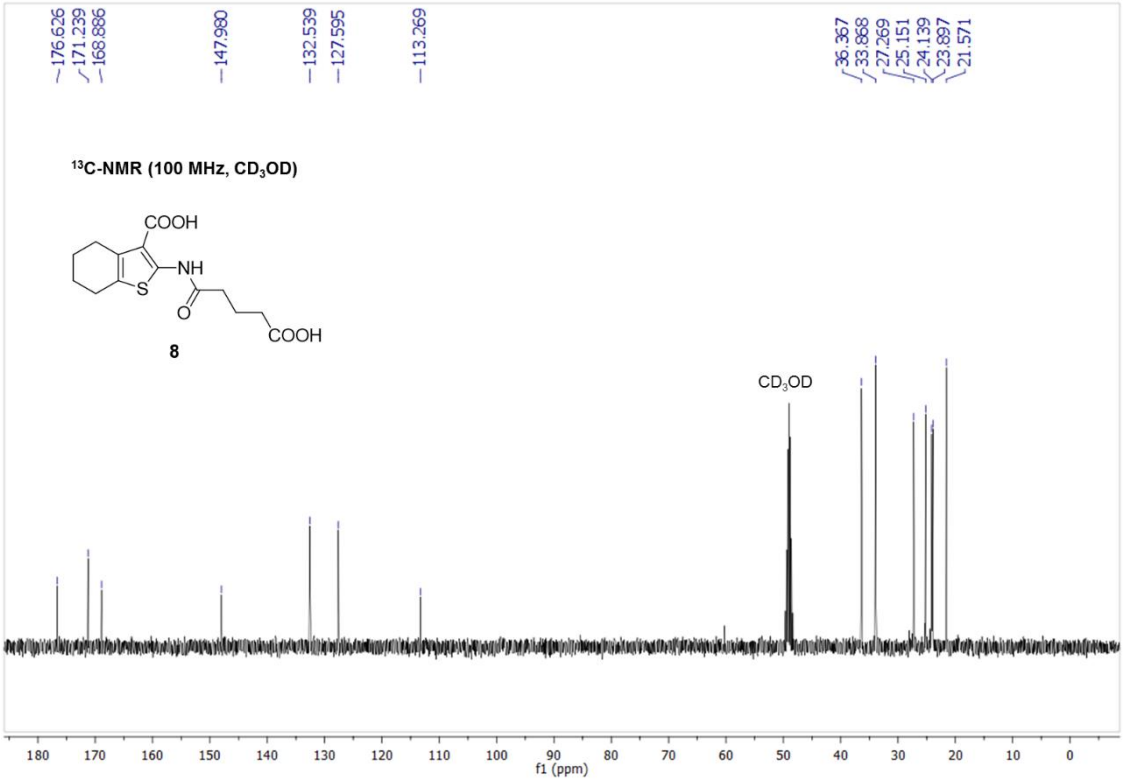
6

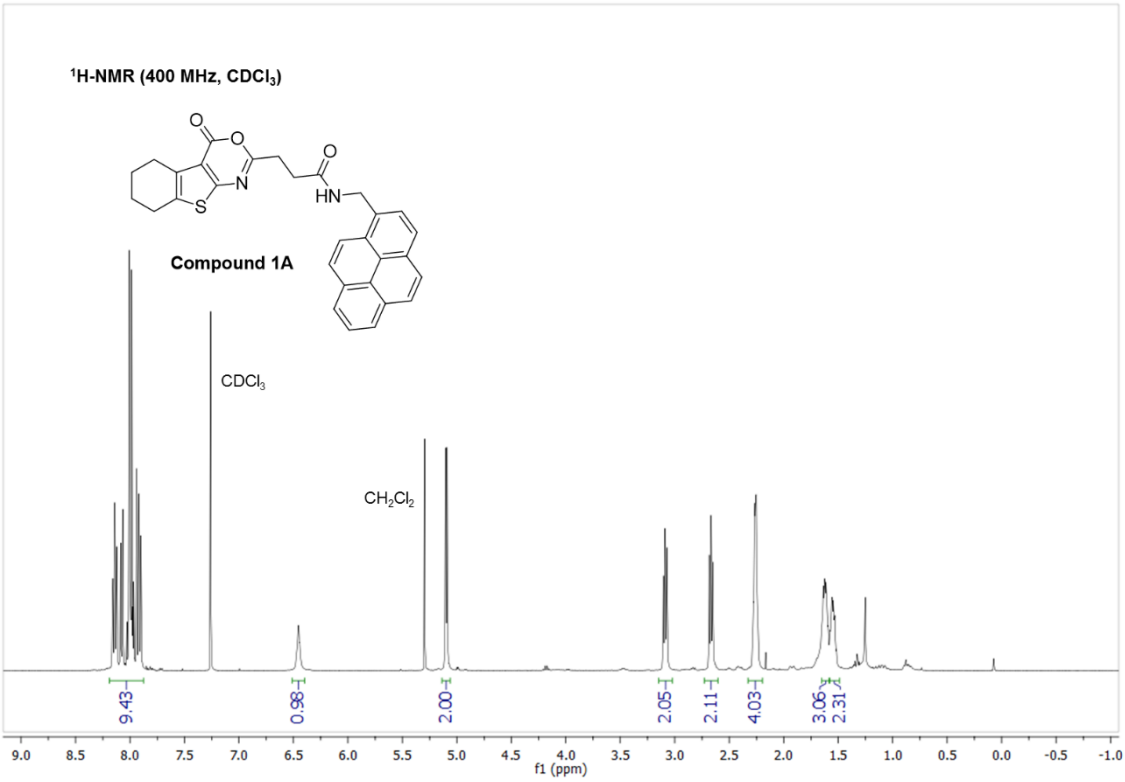


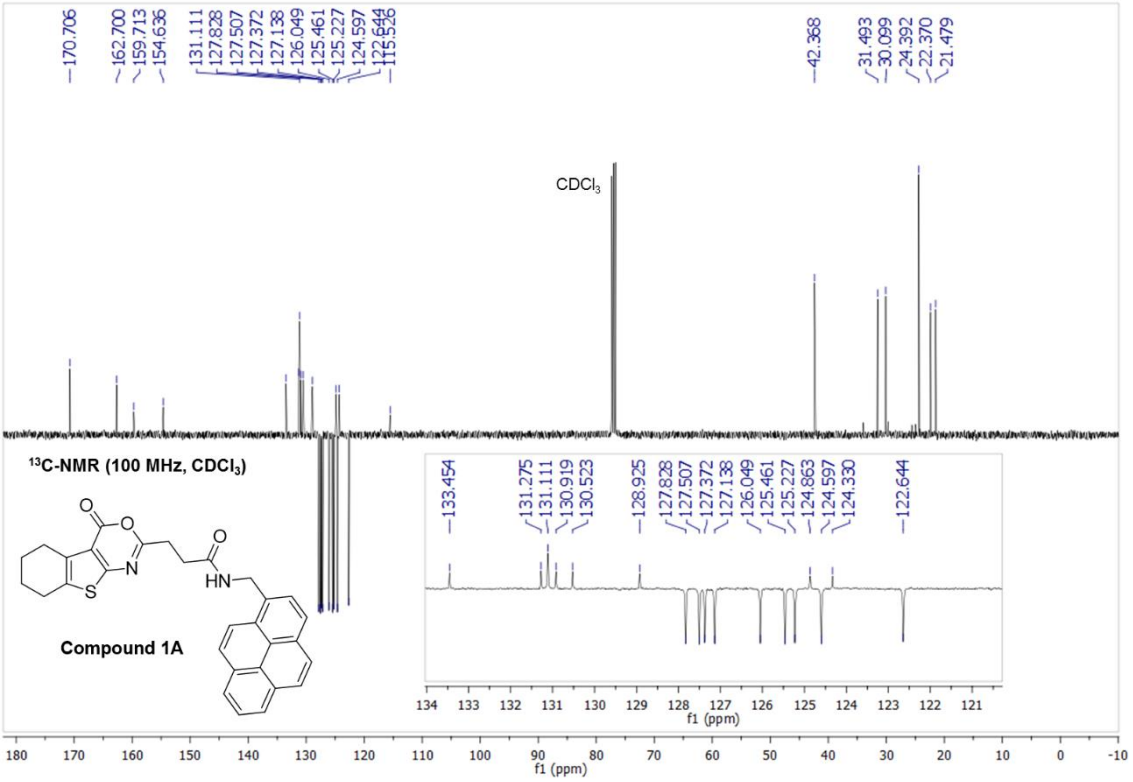

1H-NMR (400 MHz, DMSO-d6)
COOH
NH
S
O
COOH
7
DMSO-d6
H2O
1.03
3.96
4.01
4.00
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


173.516
168.897
166.964
146.154
130.864
125.448
111.713
30.832
28.708
25.908
23.748
22.623
22.361
13C-NMR (100 MHz, DMSO-d6)
COOH
NH
S
O
COOH
7
DMSO-d6
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

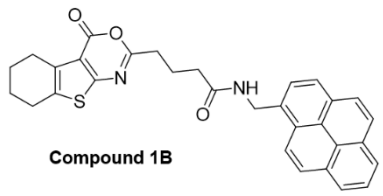

1H-NMR (400 MHz, CD3OD)
COOH
NH
S
O
COOH
8
H2O
CD3OD
1.96
2.01
2.01
2.00
1.98
3.97
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


176.626
171.239
168.886
147.980
132.539
127.595
113.269
36.367
33.868
27.269
25.151
24.139
23.897
21.571
13C-NMR (100 MHz, CD3OD)
COOH
NH
S
O
COOH
8
CD3OD
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

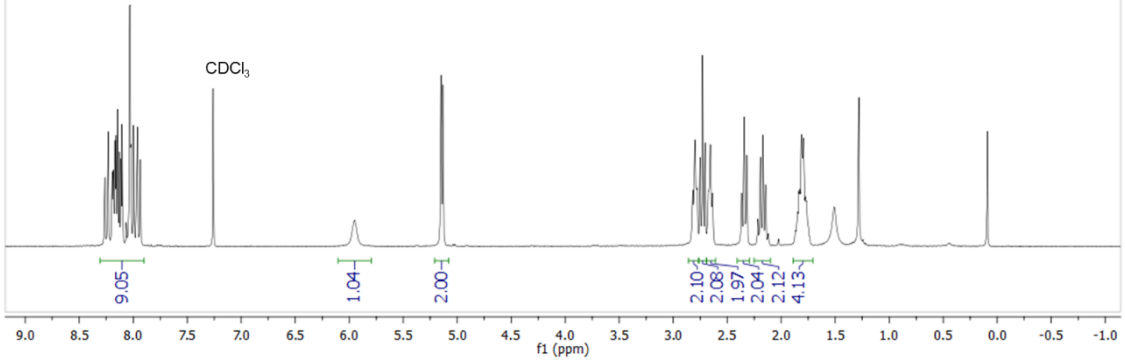

^{1}H-NMR (400 MHz, $CDCl_3$)
Compound 1A
$CDCl_3$
CH_2Cl_2
9.43
0.98
2.00
2.05
2.11
4.03
3.06
2.31
f1 (ppm)

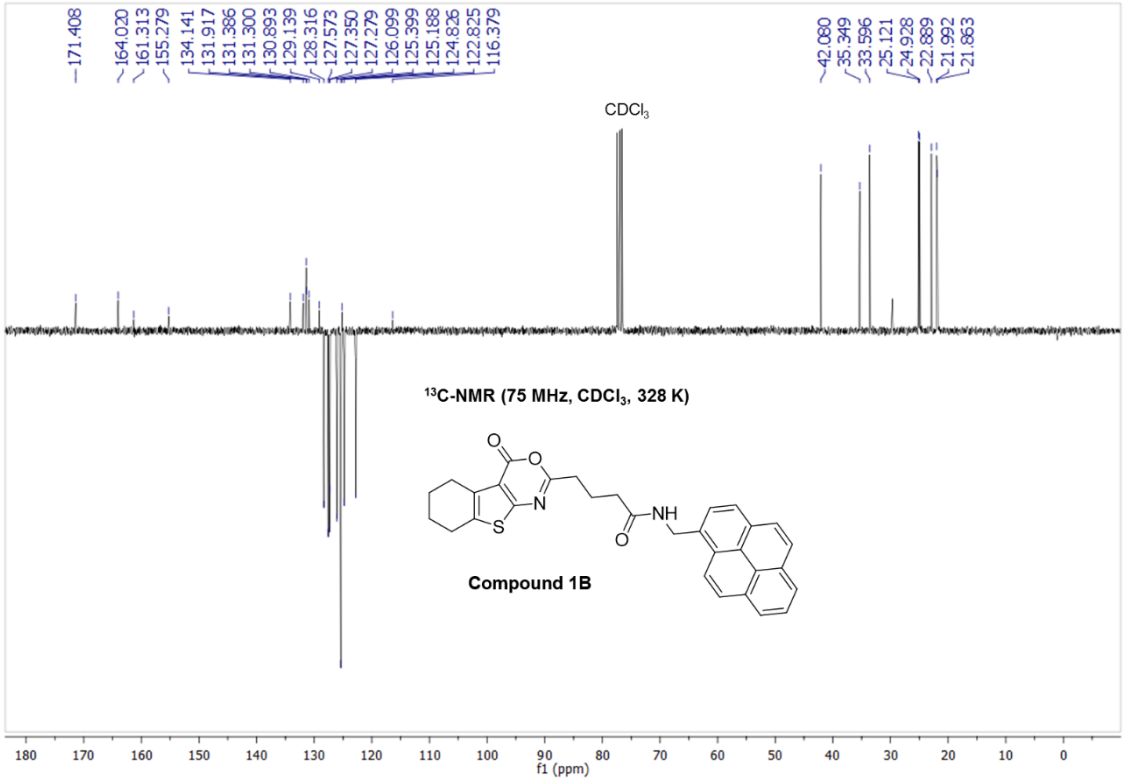

170.706
162.700
159.713
154.636
131.111
127.828
127.507
127.372
127.138
126.049
125.461
125.227
124.597
122.644
115.526
42.368
31.493
30.099
24.392
22.370
21.479
$CDCl_3$
^{13}C-NMR (100 MHz, $CDCl_3$)
Compound 1A
133.454
131.275
131.111
130.919
130.523
128.925
127.828
127.507
127.372
127.138
126.049
125.461
125.227
124.863
124.597
124.330
122.644
f1 (ppm)

^{1}H-NMR (300 MHz, $CDCl_3$, 328 K)



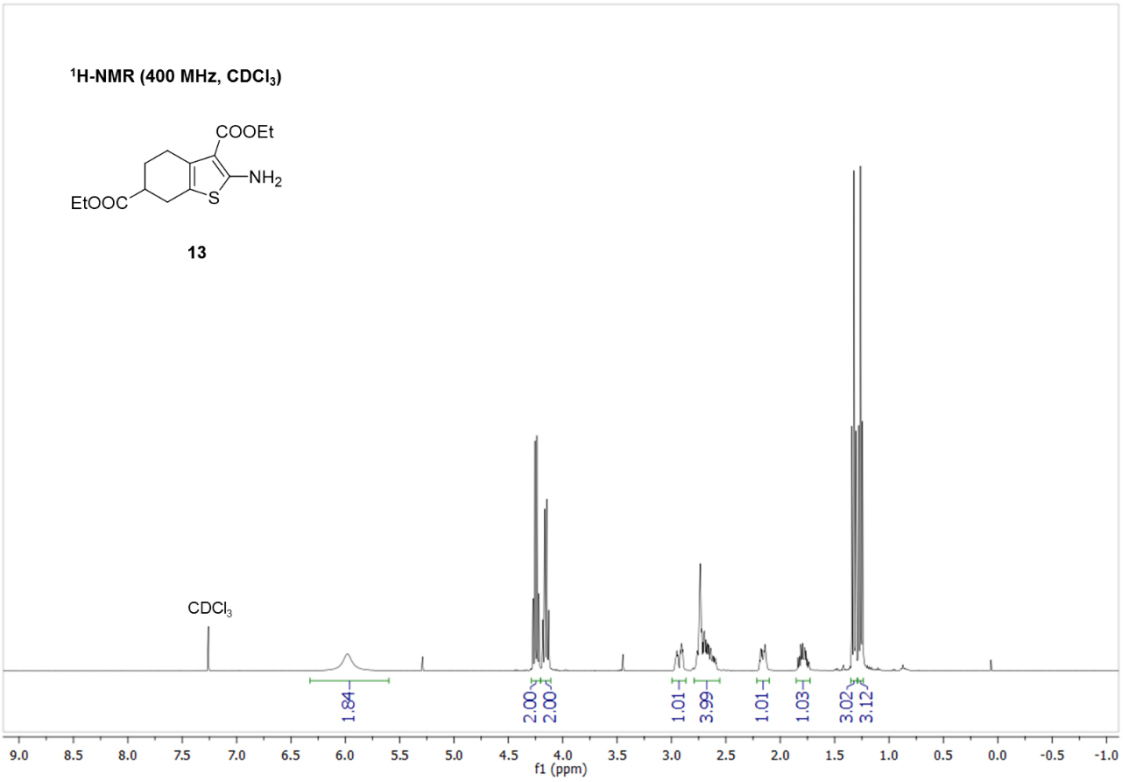
Compound 1B

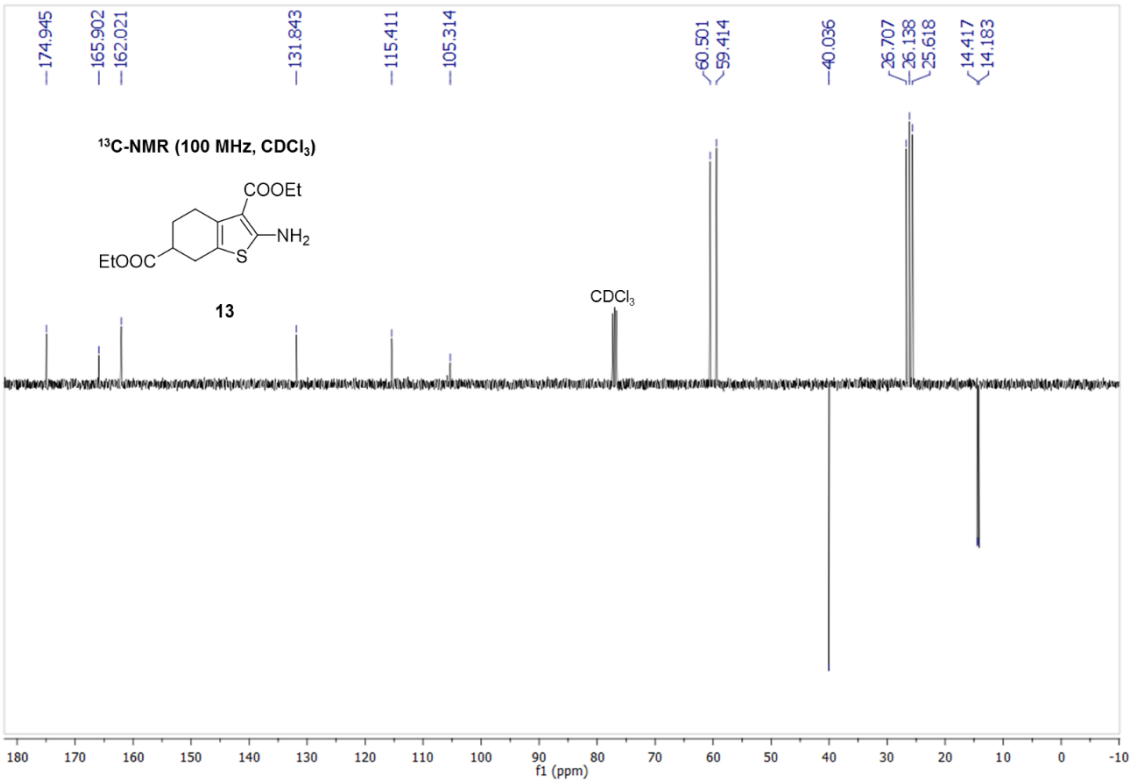




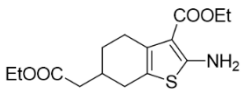
^{13}C-NMR (75 MHz, $CDCl_3$, 328 K)

Compound 1B

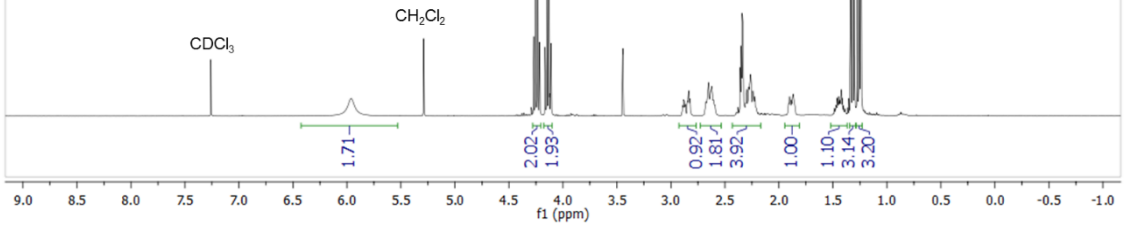

^{1}H-NMR (400 MHz, $CDCl_3$)
COOEt
NH_2
EtOOC
S
13
$CDCl_3$
1.84
2.00
2.00
1.01
3.99
1.01
1.03
3.02
3.12
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)

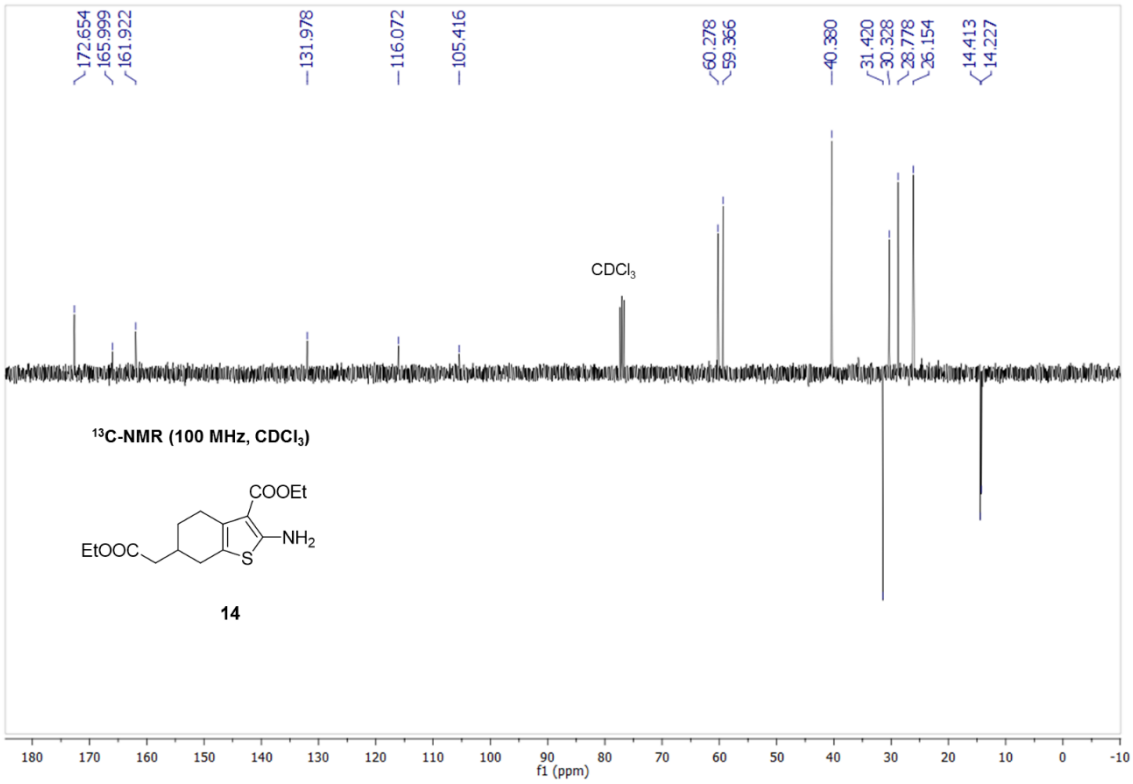

174.945
165.902
162.021
131.843
115.411
105.314
60.501
59.414
40.036
26.707
26.138
25.618
14.417
14.183
^{13}C-NMR (100 MHz, $CDCl_3$)
COOEt
NH_2
EtOOC
S
13
$CDCl_3$
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

^{1}H-NMR (400 MHz, $CDCl_3$)



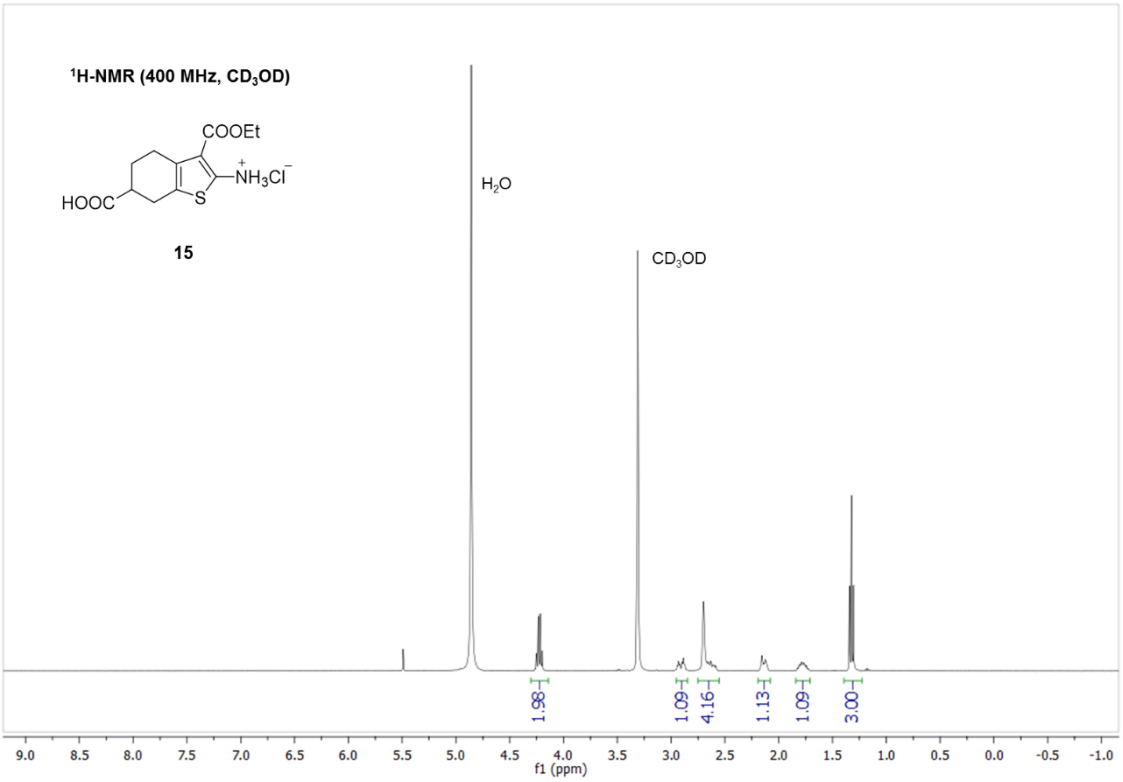
14

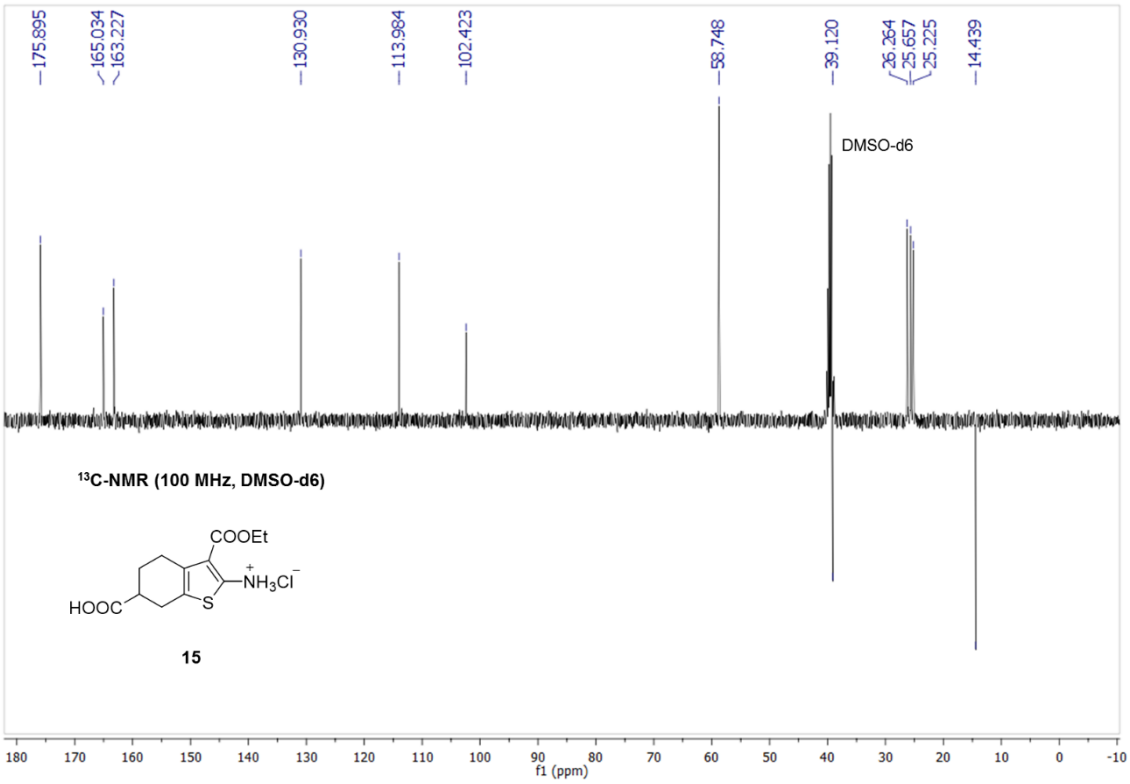




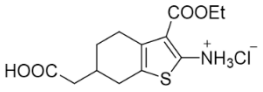
^{13}C-NMR (100 MHz, $CDCl_3$)

14

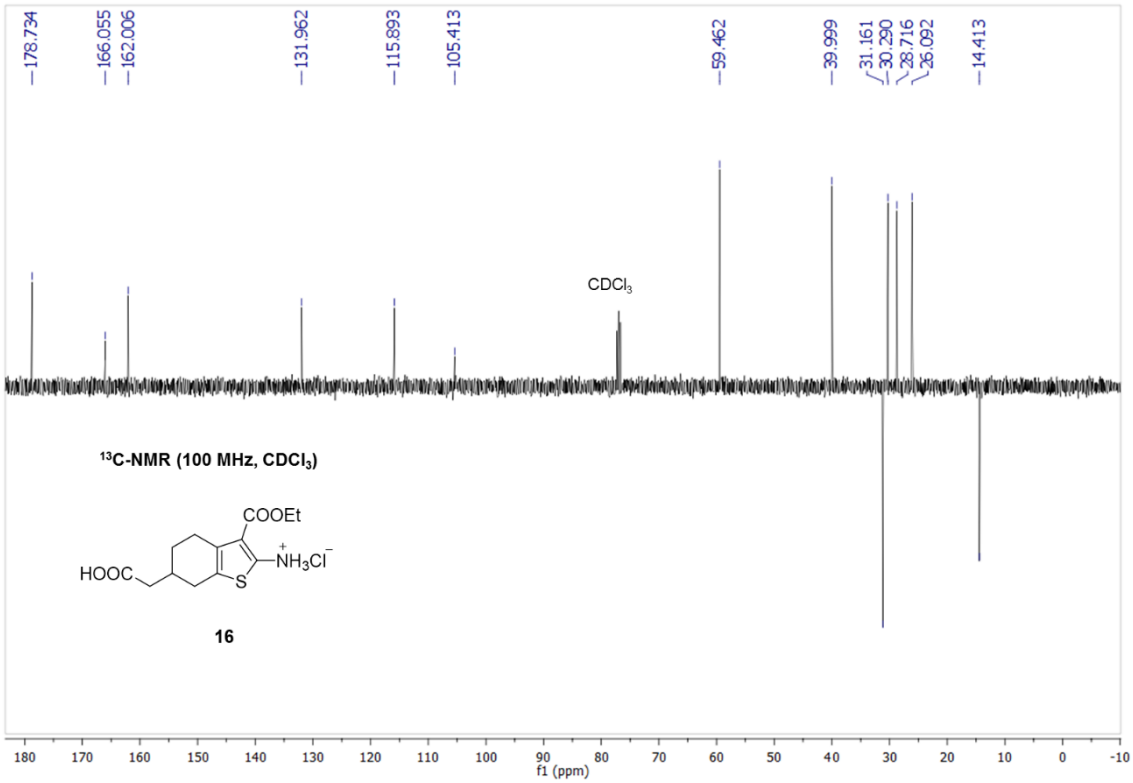

^{1}H-NMR (400 MHz, CD_3OD)
COOEt
NH_3Cl^-
HOOC
S
15
H_2O
CD_3OD
1.98
1.09
4.16
1.13
1.09
3.00
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


175.895
165.034
163.227
130.930
113.984
102.423
58.748
39.120
26.264
25.657
25.225
14.439
DMSO-d6
^{13}C-NMR (100 MHz, DMSO-d6)
COOEt
NH_3Cl^-
HOOC
S
15
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

^{1}H-NMR (400 MHz, $CDCl_3$)

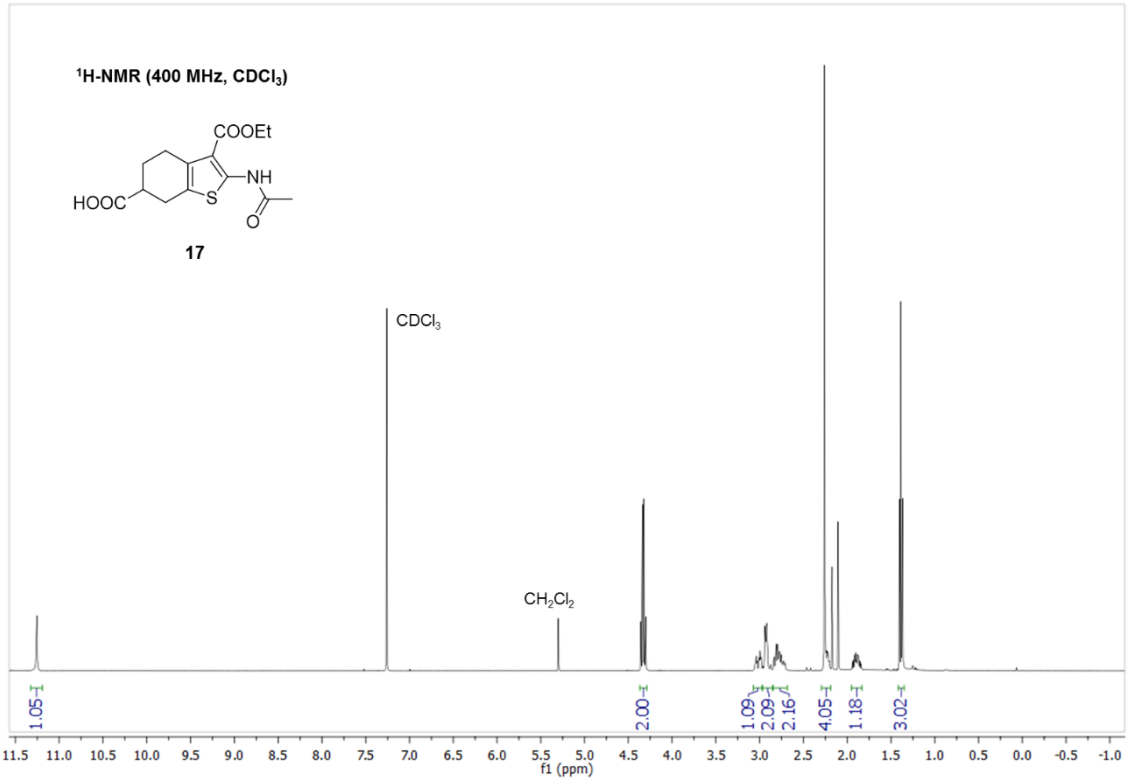


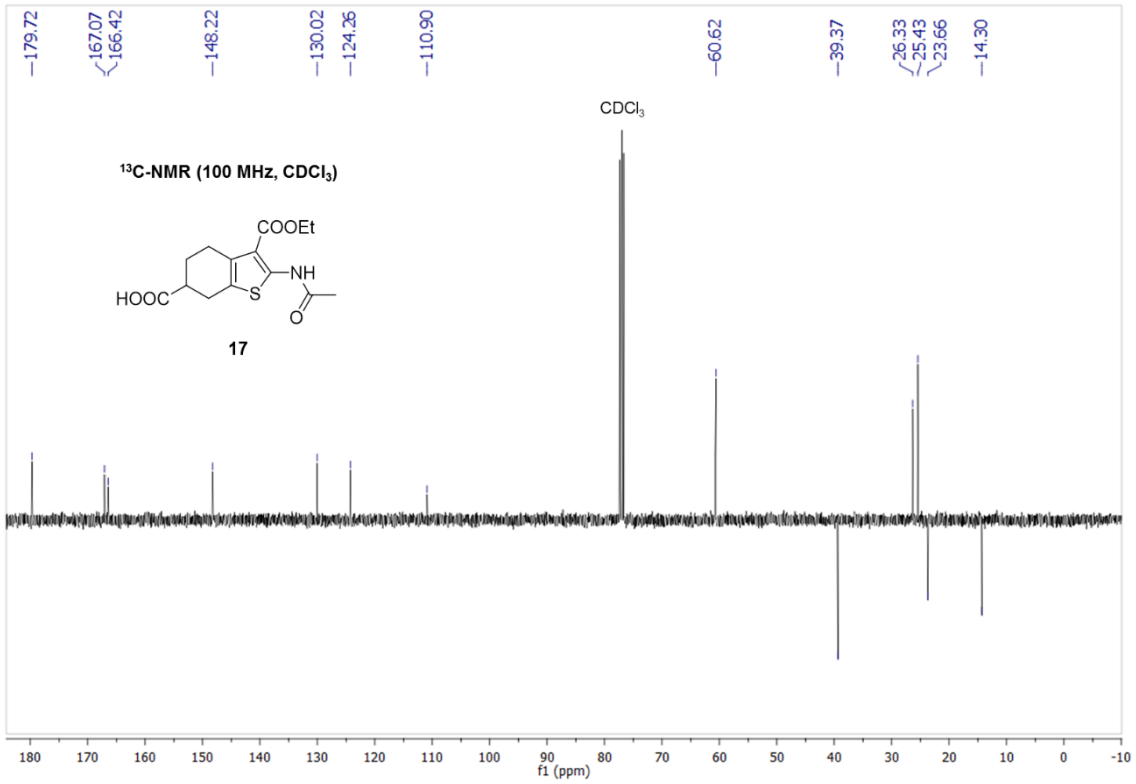
16



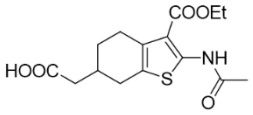
^{13}C-NMR (100 MHz, $CDCl_3$)

16

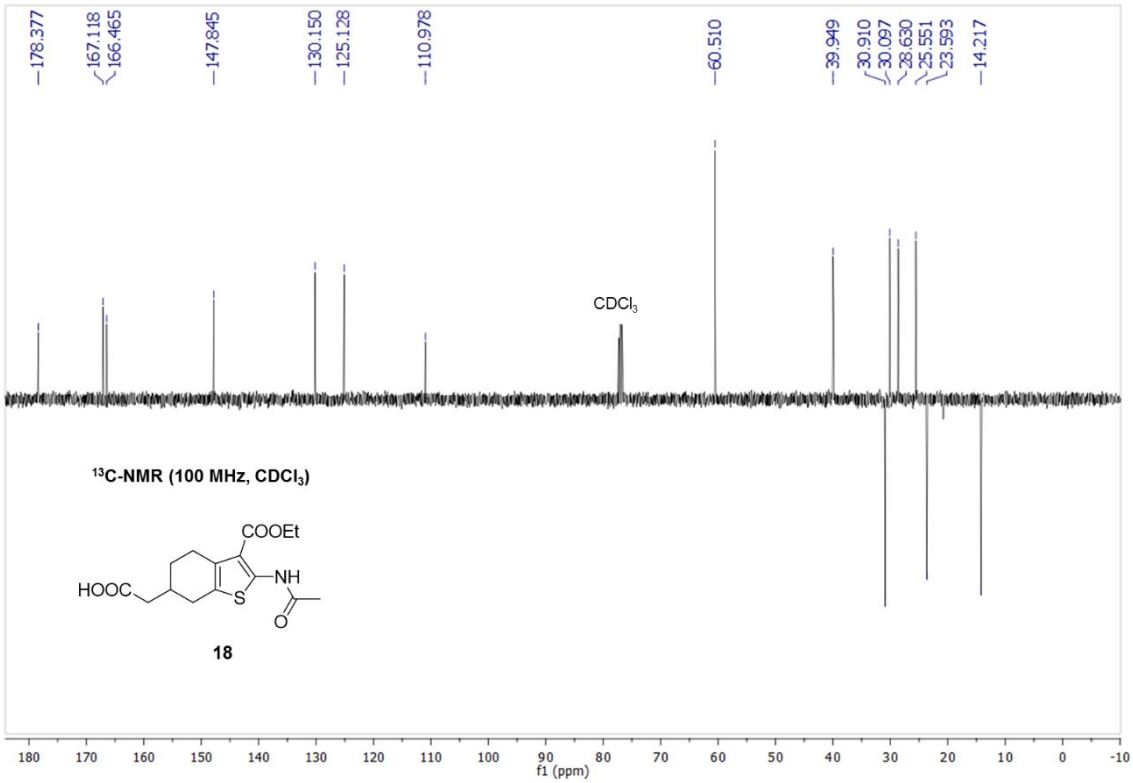

^{1}H-NMR (400 MHz, $CDCl_3$)
COOEt
NH
HOOC
S
O
17
$CDCl_3$
CH_2Cl_2
1.05
2.00
1.09
2.09
2.16
4.05
1.18
3.02
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


179.72
167.07
166.42
148.22
130.02
124.26
110.90
60.62
39.37
26.33
25.43
23.66
14.30
$CDCl_3$
^{13}C-NMR (100 MHz, $CDCl_3$)
COOEt
NH
HOOC
S
O
17
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

^{1}H-NMR (400 MHz, $CDCl_3$)

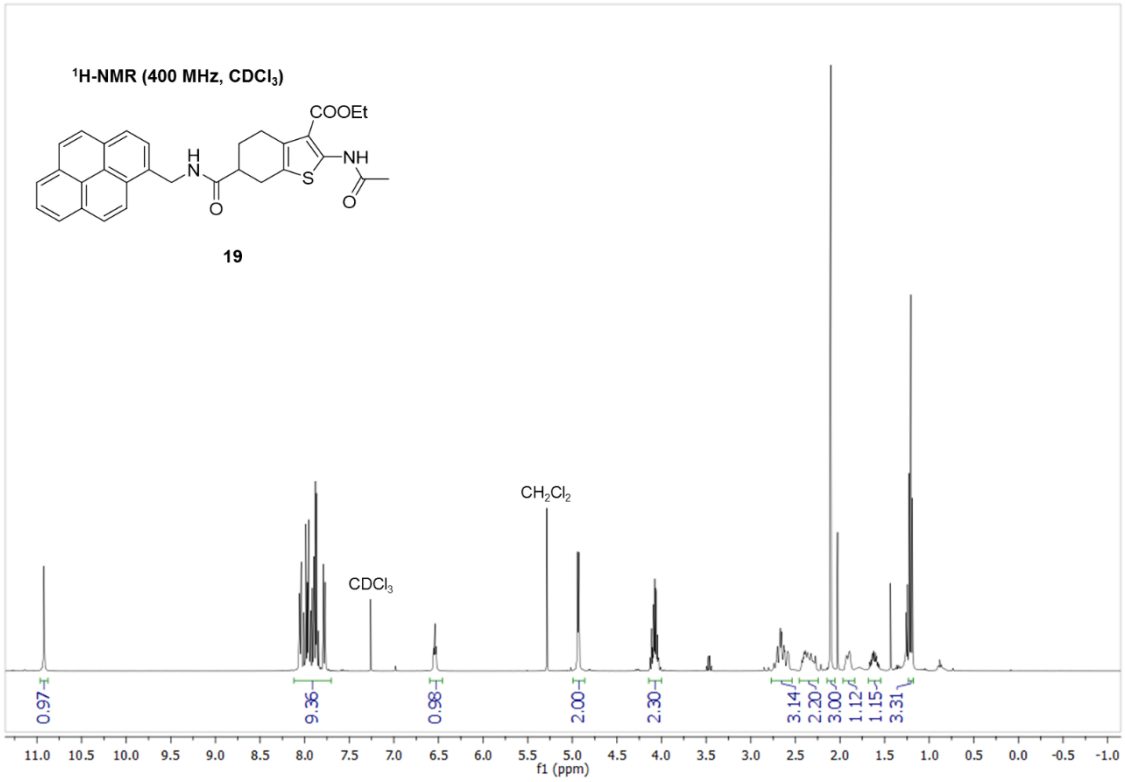


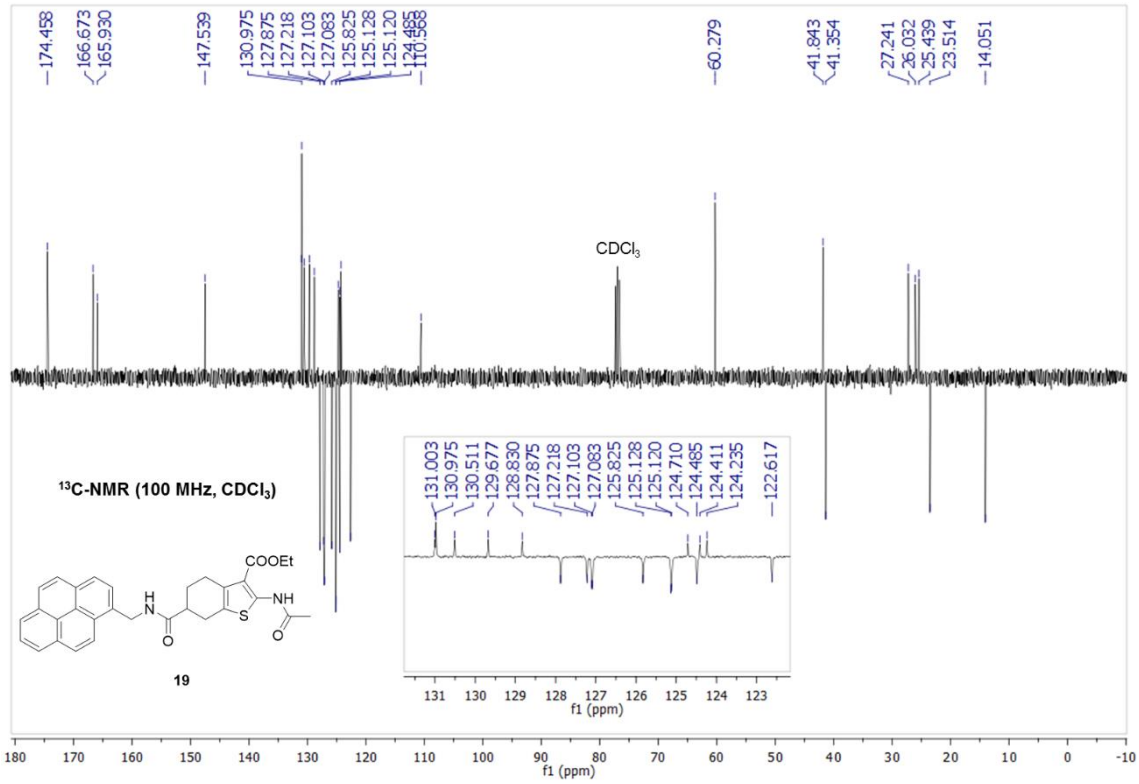
18

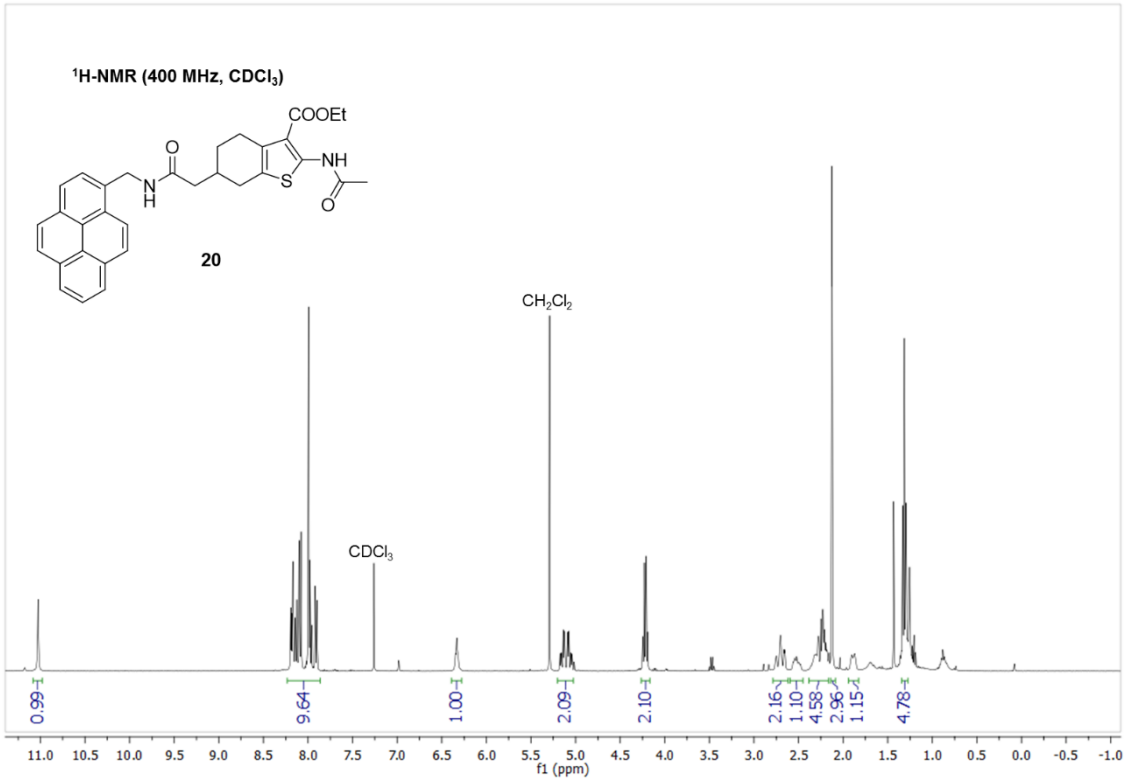


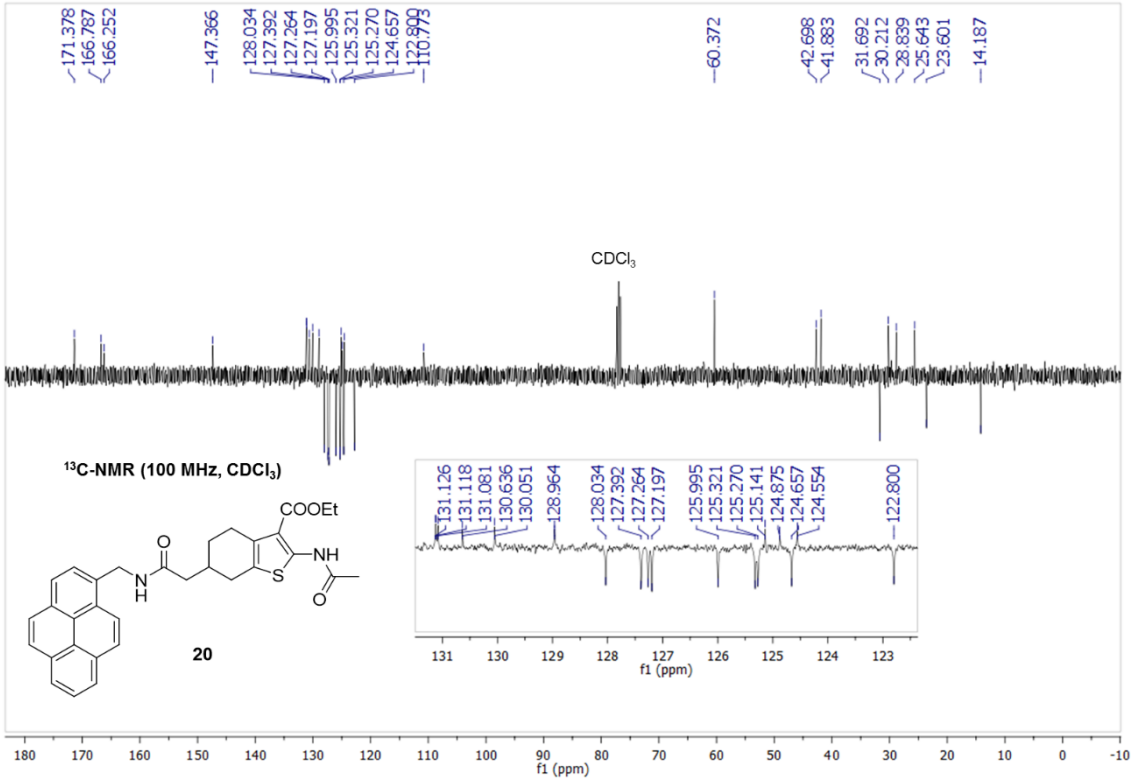
^{13}C-NMR (100 MHz, $CDCl_3$)

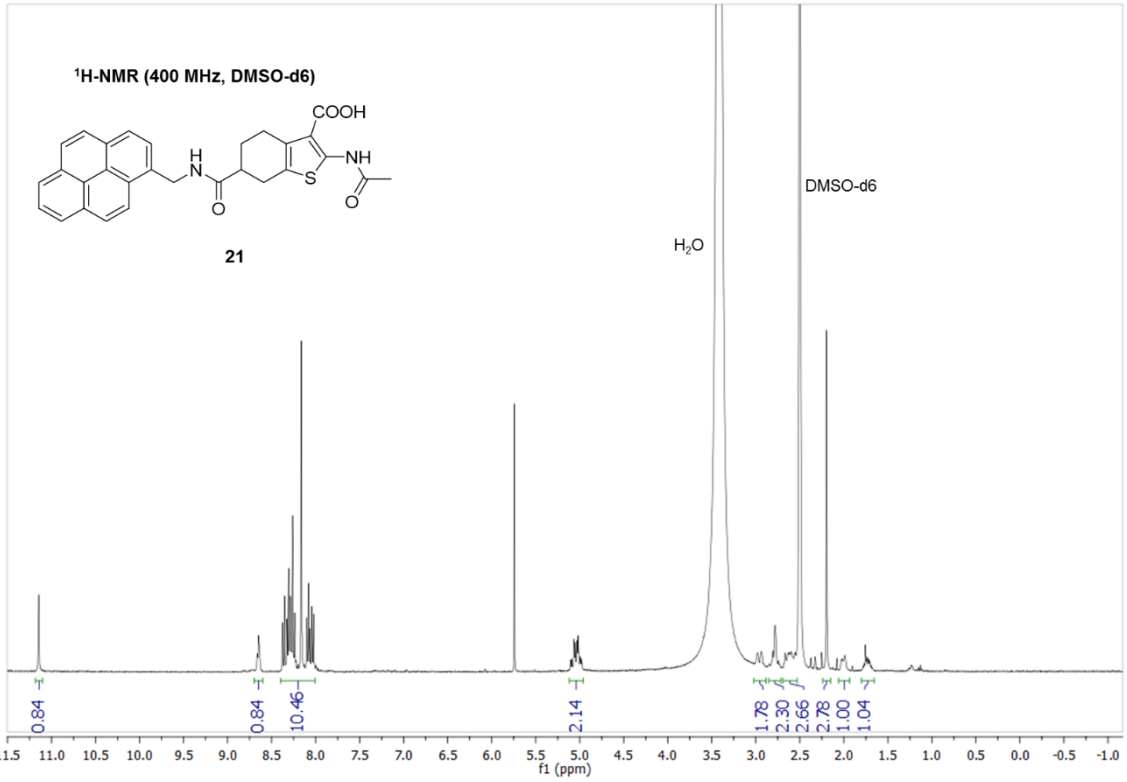
18

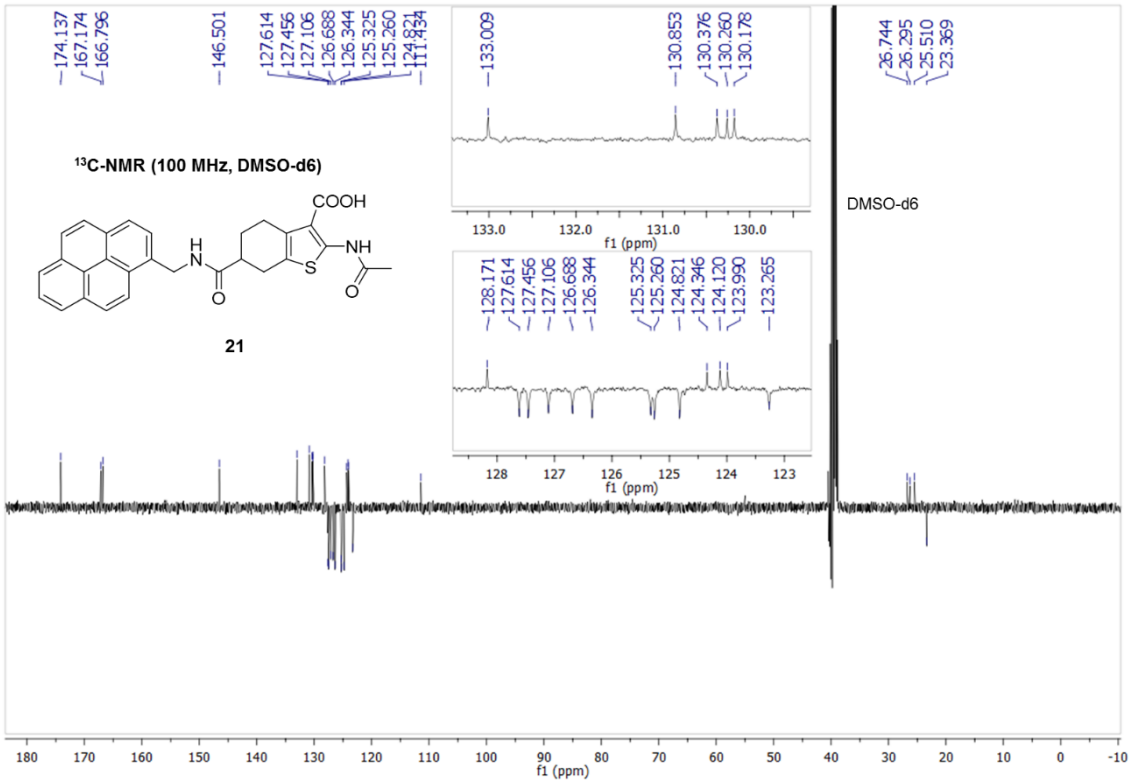

¹H-NMR (400 MHz, CDCl3)
COOEt
NH
H
N
S
O
O
19
CH2Cl2
CDCl3
0.97
9.36
0.98
2.00
2.30
3.14
2.20
3.00
1.12
1.15
3.31
11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)

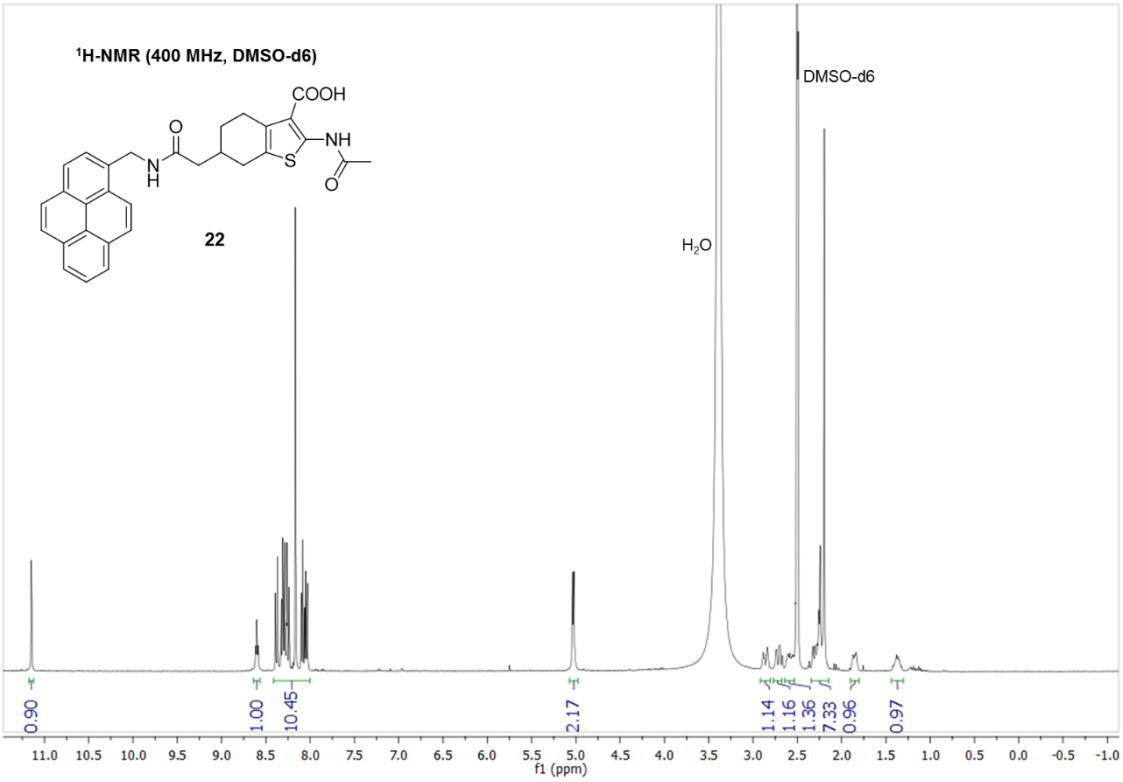

174.458
166.673
165.930
147.539
130.975
127.875
127.218
127.103
127.083
125.825
125.128
125.120
124.485
110.568
60.279
41.843
41.354
27.241
26.032
25.439
23.514
14.051
CDCl3
131.003
130.975
130.511
129.677
128.830
127.875
127.218
127.103
127.083
125.825
125.128
125.120
124.710
124.485
124.411
124.235
122.617
131 130 129 128 127 126 125 124 123
f1 (ppm)
13C-NMR (100 MHz, CDCl3)
COOEt
NH
H
N
S
O
O
19
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

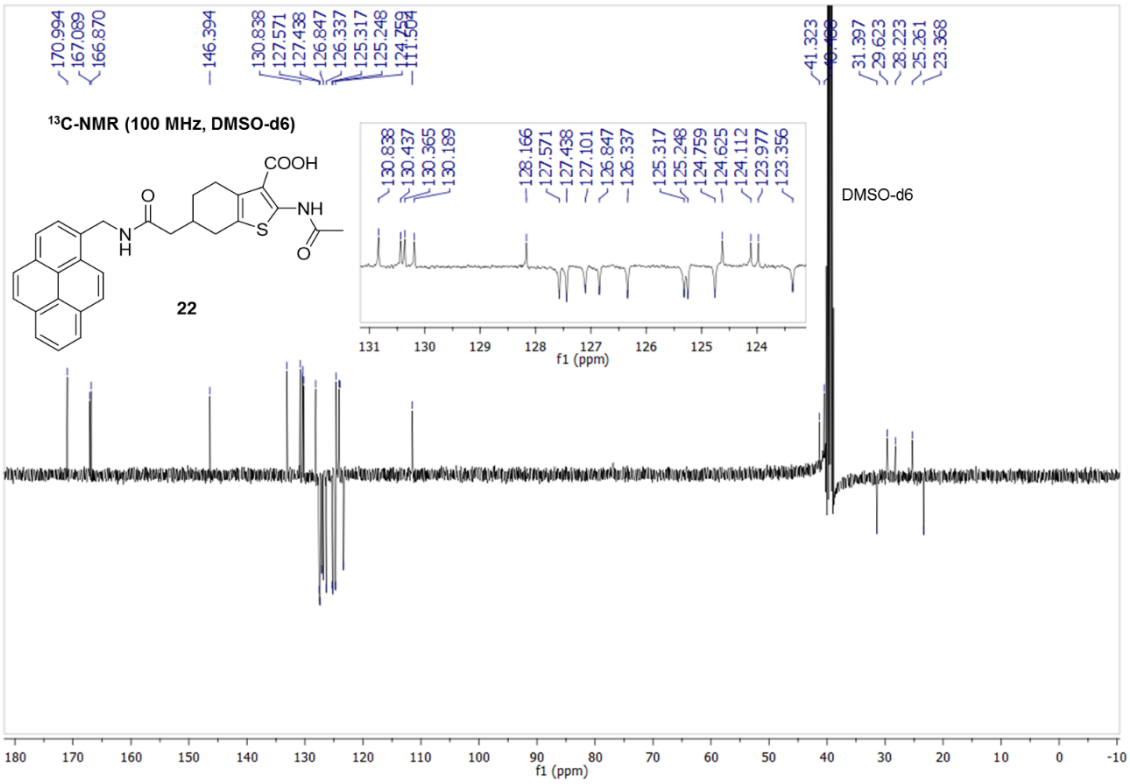

^{1}H-NMR (400 MHz, $CDCl_3$)
COOEt
NH
O
N
H
S
O
20
CH_2Cl_2
$CDCl_3$
0.99
9.64
1.00
2.09
2.10
2.16
1.10
4.58
2.96
1.15
4.78
f1 (ppm)

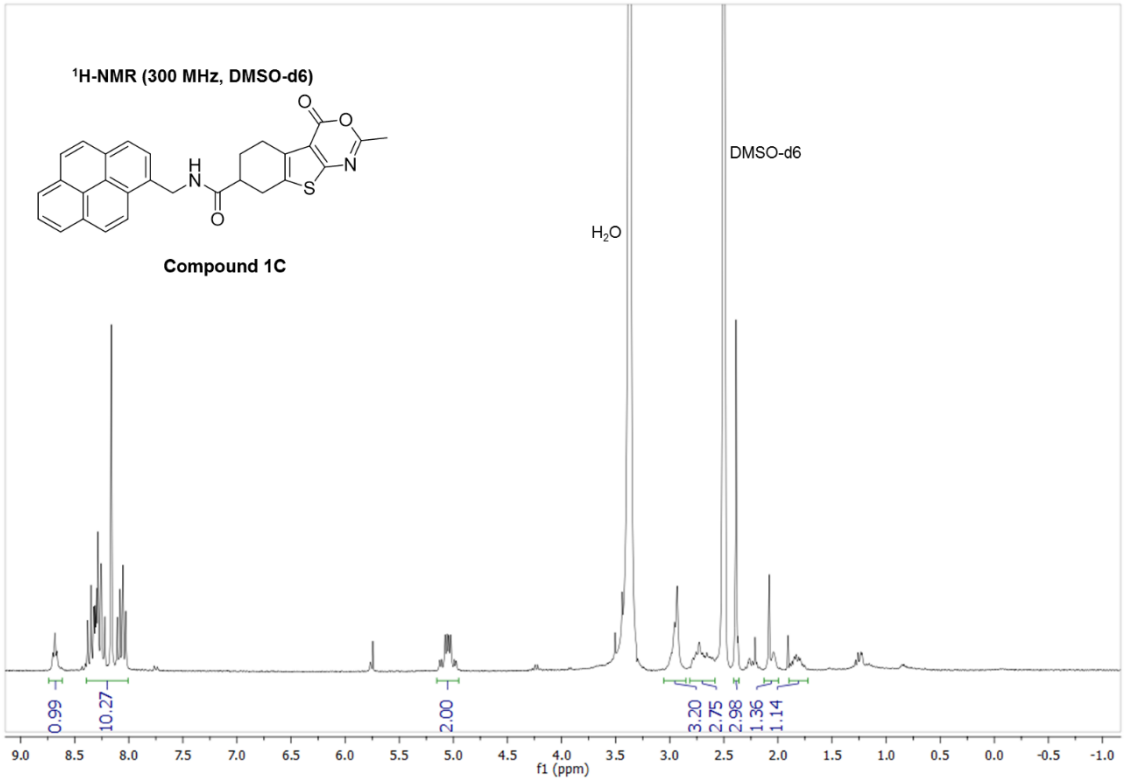

171.378
166.787
166.252
147.366
128.034
127.392
127.264
127.197
125.995
125.321
125.270
124.657
122.800
110.973
60.372
42.698
41.883
31.692
30.212
28.839
25.643
23.601
14.187
$CDCl_3$
^{13}C-NMR (100 MHz, $CDCl_3$)
COOEt
NH
O
N
H
S
O
20
131.126
131.118
131.081
130.636
130.051
128.964
128.034
127.392
127.264
127.197
125.995
125.321
125.270
125.141
124.875
124.657
124.554
122.800
f1 (ppm)

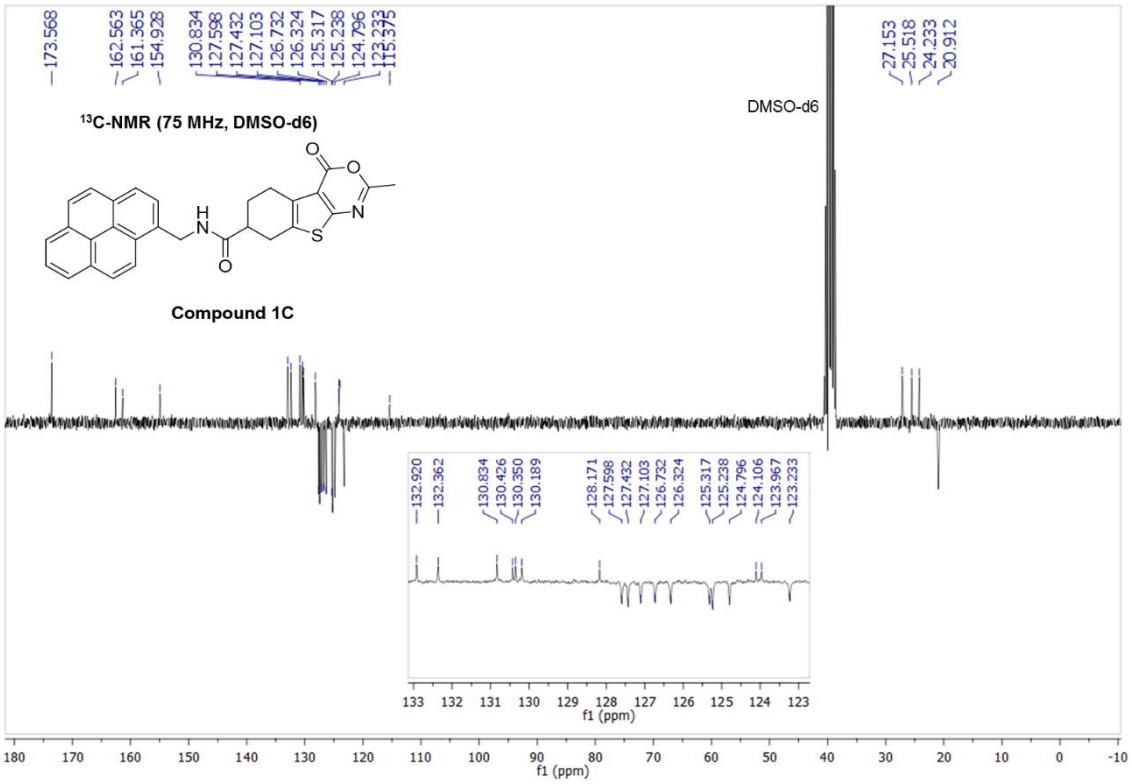

¹H-NMR (400 MHz, DMSO-d6)
COOH
NH
H
N
S
O
O
21
H2O
DMSO-d6
0.84
0.84
10.46
2.14
1.78
2.30
2.66
2.78
1.00
1.04
f1 (ppm)

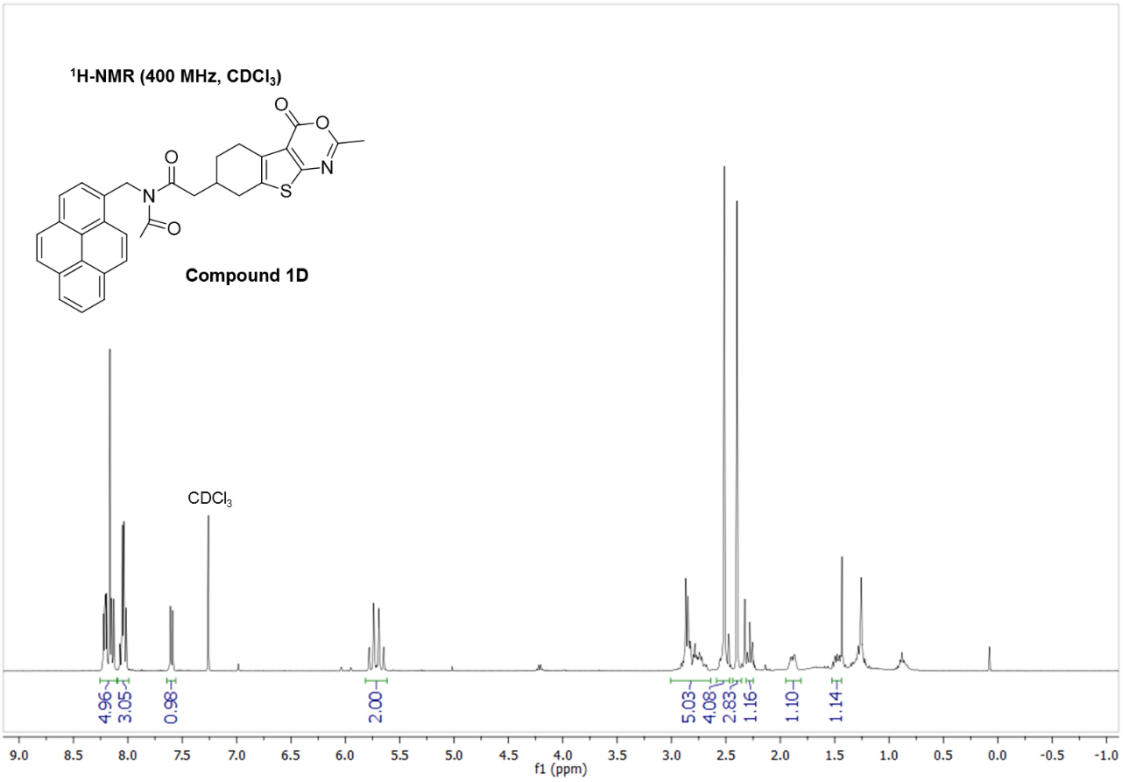

174.137
167.174
166.796
146.501
127.614
127.456
127.106
126.688
126.344
125.325
125.260
124.821
111.434
133.009
130.853
130.376
130.260
130.178
36.744
26.295
25.510
23.369
¹³C-NMR (100 MHz, DMSO-d6)
COOH
NH
H
N
S
O
O
21
DMSO-d6
128.171
127.614
127.456
127.106
126.688
126.344
125.325
125.260
124.821
124.346
124.120
123.990
123.265
f1 (ppm)

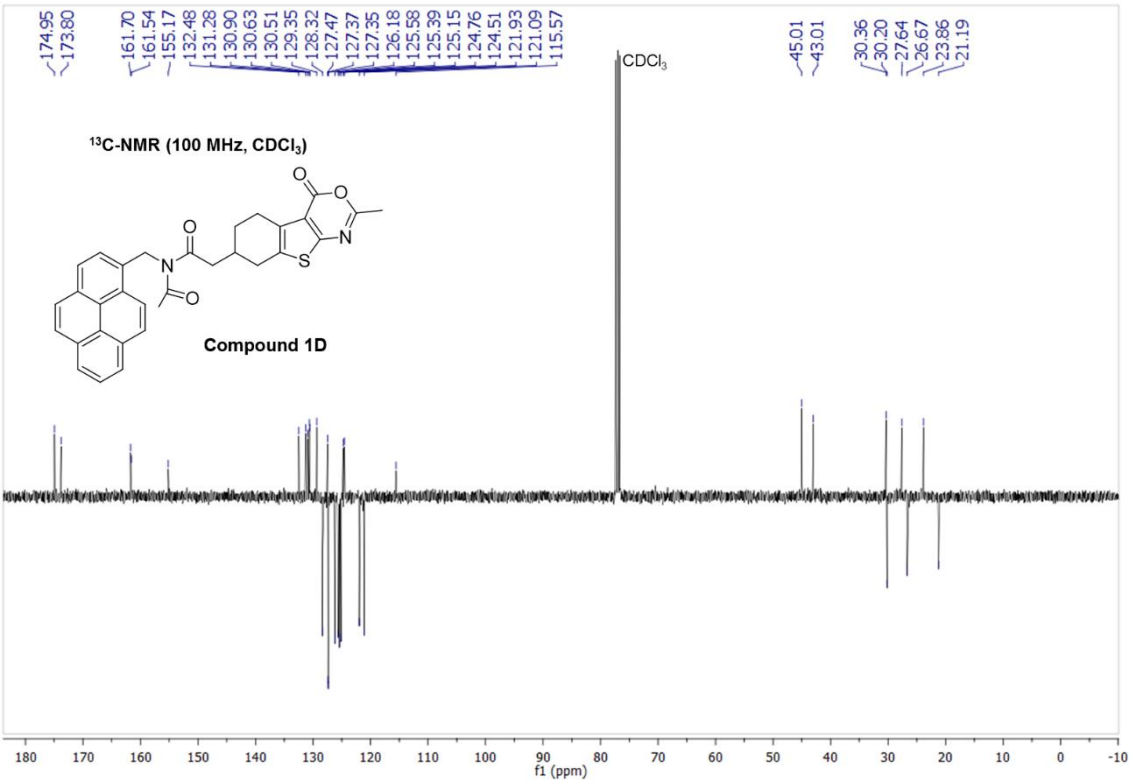

1H-NMR (400 MHz, DMSO-d6)
COOH
NH
O
N
H
S
O
22
H2O
DMSO-d6
0.90
1.00
10.45
2.17
1.14
1.16
1.36
7.33
0.96
0.97
11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)


170.994
167.089
166.870
146.394
130.838
127.571
127.438
126.847
126.337
125.317
125.248
124.759
111.504
41.323
31.397
29.623
28.223
25.261
23.368
13C-NMR (100 MHz, DMSO-d6)
COOH
NH
O
N
H
S
O
22
130.838
130.437
130.365
130.189
128.166
127.571
127.438
127.101
126.847
126.337
125.317
125.248
124.759
124.625
124.112
123.977
123.356
DMSO-d6
131 130 129 128 127 126 125 124
f1 (ppm)
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


¹H-NMR (300 MHz, DMSO-d6)
Compound 1C
H2O
DMSO-d6
0.99
10.27
2.00
3.20
2.75
2.98
1.36
1.14
f1 (ppm)


173.568
162.563
161.365
154.928
130.834
127.598
127.432
127.103
126.732
126.324
125.317
125.238
124.796
123.233
115.375
27.153
25.518
24.233
20.912
DMSO-d6
¹³C-NMR (75 MHz, DMSO-d6)
Compound 1C
f1 (ppm)


^{1}H-NMR (400 MHz, $CDCl_3$)
Compound 1D
$CDCl_3$
4.96
3.05
0.98
2.00
5.03
4.08
2.83
1.16
1.10
1.14
f1 (ppm)


174.95
173.80
161.70
161.54
155.17
132.48
131.28
130.90
130.63
130.51
129.35
128.32
127.47
127.37
127.35
126.18
125.58
125.39
125.15
124.76
124.51
121.93
121.09
115.57
45.01
43.01
30.36
30.20
27.64
26.67
23.86
21.19
$CDCl_3$
^{13}C-NMR (100 MHz, $CDCl_3$)
Compound 1D
f1 (ppm)

II. Supporting figures.

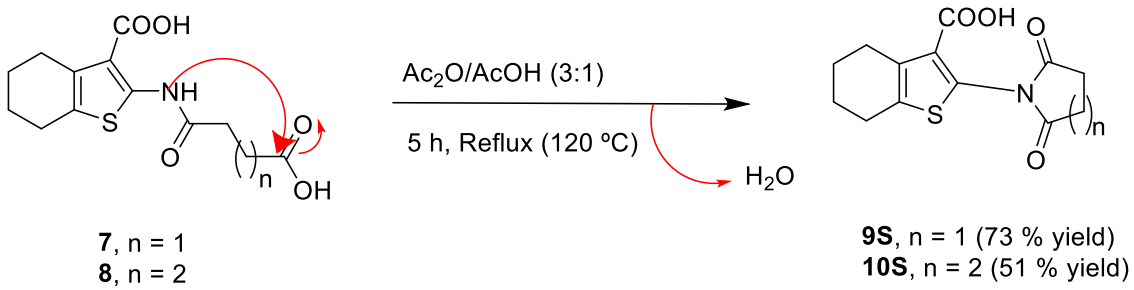


Fig. S1 Proposed reaction mechanism for the formation of 9S and 10S (byproducts)

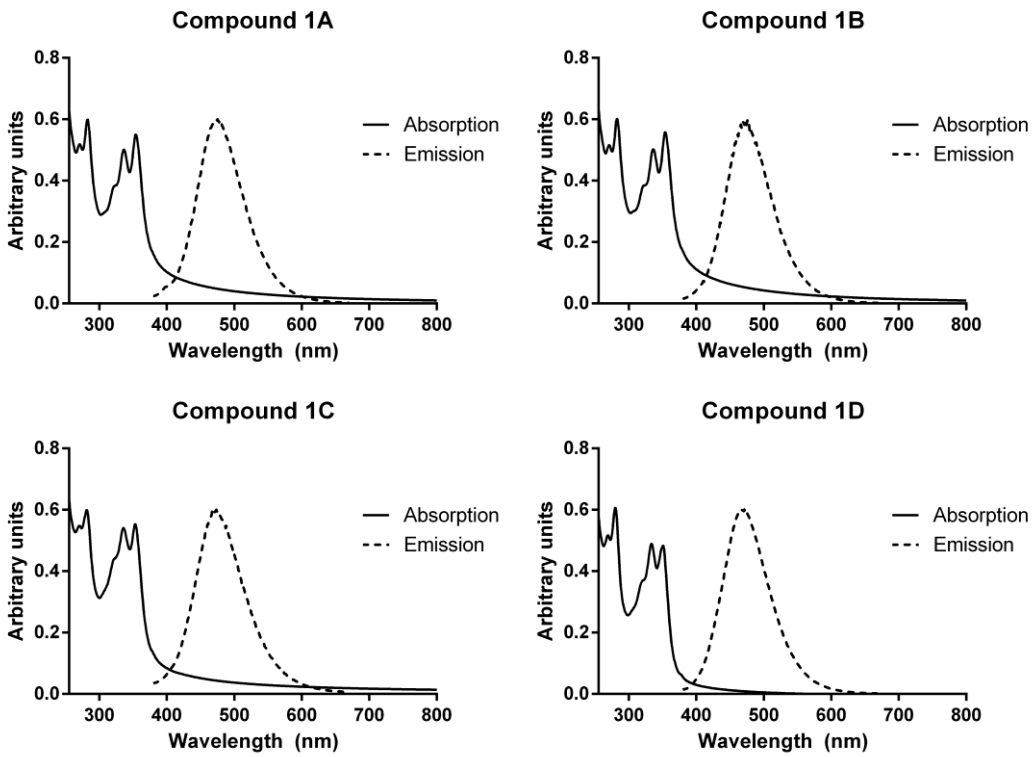


Fig. S2 Absorption and emission (excitation wavelength: 350 nm) spectra of synthesized compounds 1A, 1B, 1C and 1D in PBS buffer (30 μM, 5% DMSO)

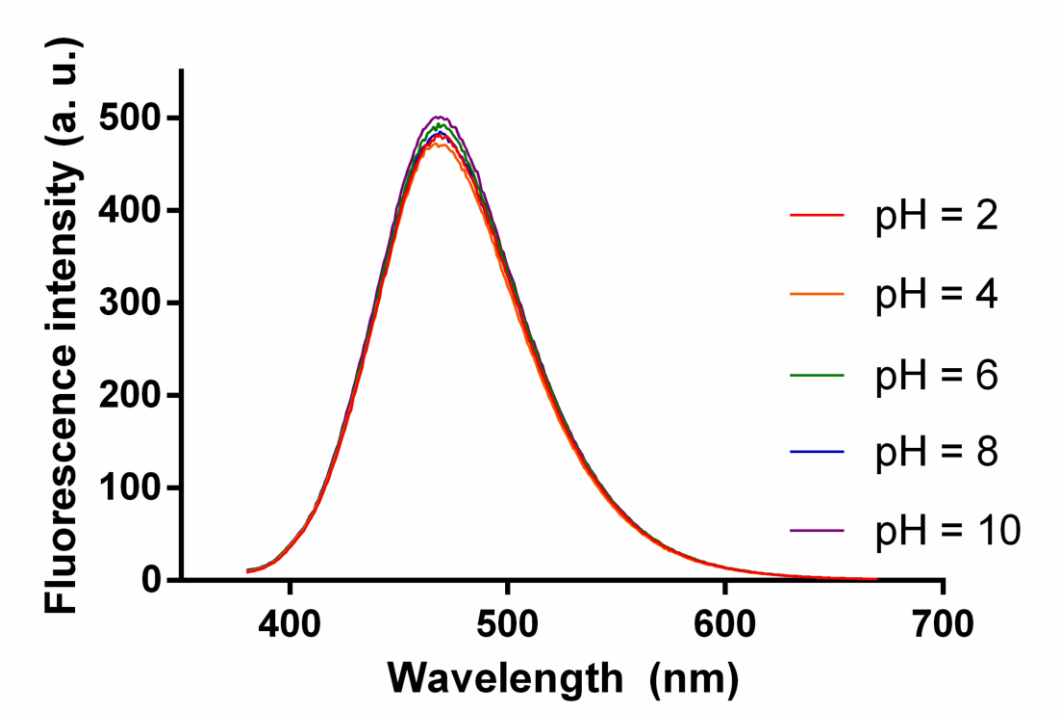


Fig. S3 Emission spectra (excitation wavelength: 350 nm) of compound 1D (30 μM, 5% DMSO) obtained in different pH buffer solutions: maleate (pH = 2.1; red line), benzoate (pH = 4.2; orange line), MES (pH = 6.2; green line), TRIS (pH = 8.1; blue line) and CHES (pH = 10.0; purple line)