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A FRET Probe for the Detection of Alkylating Agents

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Table S1. Optimizing reaction conditions for NMR studies using amine **3c**. 33

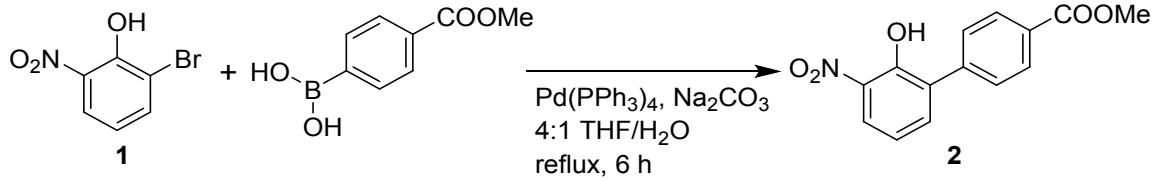
1. General information

Unless otherwise noted, all other commercially available reagents and solvents were used without further purification. The reaction progress was monitored on precoated silica gel TLC plates Macherey-Nagel ALUGRAM® SIL G/UV254 (0.2 mm silica gel 60). Spots were visualized under 254 nm/365 nm UV light. FT-ATR-IR spectra were recorded on a Perkin-Elmer UAR Two Spectrum spectrometer and are reported as wavelength numbers (cm^{-1}). Melting points were measured on a Büchi B-540 and are reported in °C. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) were measured on a Bruker Avance 400 MHz spectrometer Chemical shifts are reported in parts per million (ppm, δ) downfield from residual solvents peaks and coupling constants are reported as Hertz (Hz). Splitting patterns are designated as singlet (s), broad singlet (br), doublet (d), triplet (t). Splitting patterns that could not be interpreted or easily visualized are designated as multiplet (m).

LC-MS spectra were recorded on a Waters Acquity UPLC® equipped PDA eλ Detector and SQ Detector 2, mobile phase A: H_2O + 0.1% formic acid, mobile phase B: acetonitrile + 0.1% formic acid. Automatic chromatographies were performed with a COMBIFLASH RF 200i instrument equipped with UV detector and using flash cartridges Interchim silica 15 or 30 µm. High-resolution mass spectra (HRMS) were performed on a Bruker maXis mass spectrometer by the "Fédération de Recherche" ICOA/CBM (FR2708) platform", University of Orléans. Absorbances were measured on a Molecular Device SpectraMax® M5e. Fluorescence analysis was performed on Fluoromax-4 from Horiba Jobin Yvon.

2. Synthetic procedures and analytical data

Synthesis of methyl 2'-hydroxy-3'-nitro-[1,1'-biphenyl]-4-carboxylate (2)

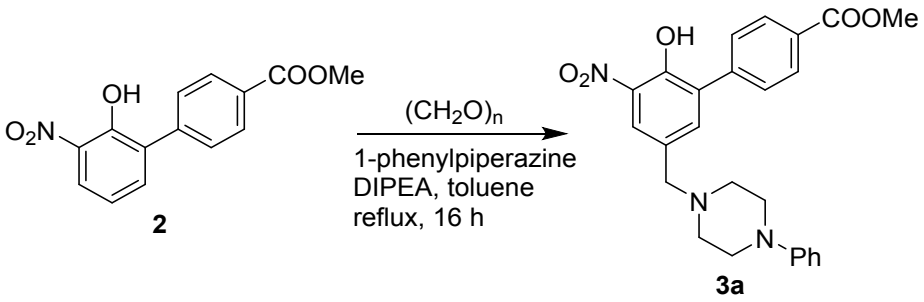


A mixture of compound **1** (1.00 g, 4.59 mmol, 1 equiv.), 4-methoxycarbonylphenylboronic acid (1.65 g, 9.17 mmol, 2 equiv.), tetrakis(triphenylphosphine)palladium(0) (0.53 g, 0.46 mmol, 0.1 equiv.), and sodium carbonate (0.97 g, 9.15 mmol, 2 equiv.) in 4:1 THF/H_2O (30 mL) was heated at reflux for 6 h in a sealed tube. At the end of the reaction, the solution was diluted with 50 mL distilled water and then acidified with 1 N HCl to pH 3, followed by extraction of the resulting suspension with 50 mL ethyl acetate (twice). The organic layers were then combined, washed with distilled water and dried over anhydrous magnesium sulfate. After filtration, the organic solvent was removed under vacuum. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in cyclohexane) to give compound **2** (pale yellow solid, 577 mg, 46%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H), 8.13–7.96 (m, 3H), 7.72 (dd, J = 7.5, 1.6 Hz, 1H), 7.69 (d, J = 8.4 Hz, 2H), 7.23–7.07 (m, 1H), 3.88 (s, 3H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.0 (C=O), 150.0, 141.2, 137.2, 136.5, 131.5, 129.8 (2C), 129.1 (2C), 128.8, 125.1, 120.1, 52.3.

IR (cm^{-1}): 3260, 3088, 2964, 1725, 1608, 1534, 1446, 1408, 1355, 1276, 1244, 1139, 1105, 749, 734, 703, 676.
HRMS (ESI) *m/z*: calcd. for $C_{14}H_{12}NO_5$ $[M+H]^+$ 274.0710, found 274.0706.
Mp: 193-194 °C

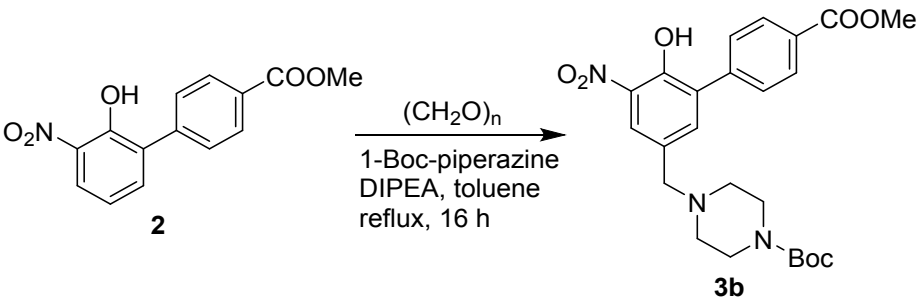
Synthesis of methyl 2'-hydroxy-3'-nitro-5'-((4-phenylpiperazin-1-yl)methyl)-[1,1'-biphenyl]-4-carboxylate (3a)



A suspension of 1-phenylpiperazine (112 µL, 0.733 mmol, 2 equiv.) and paraformaldehyde (22 mg, 0.733 mmol, 2 equiv.) in 10 mL anhydrous toluene was heated at reflux in a sealed tube until a clear solution was obtained. Afterwards, the reaction solution was cooled to room temperature, followed by the addition of compound **2** (100 mg, 0.366 mmol, 1 equiv.) and redistilled *N*,*N*-diisopropylethylamine (127 µL, 0.735 mmol, 2 equiv.) Then, the mixture was heated at reflux for 16 h. At the end of the reaction, the organic solvent was removed under vacuum. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **3a** (yellow powder, 129 mg, 79%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 8.4 Hz, 2H), 7.99 (d, J = 2.1 Hz, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 2.1 Hz, 1H), 7.23–7.15 (m, 2H), 6.91 (d, J = 8.0 Hz, 2H), 6.80–6.72 (m, 1H), 3.88 (s, 3H), 3.61 (s, 2H), 3.19–3.08 (m, 4H), 2.66–2.55 (m, 4H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.0 (C=O), 150.9, 150.4, 141.4, 137.5, 136.2, 131.6, 129.7 (2C), 129.0 (2C), 128.9 (3C), 128.6, 125.1, 118.8, 115.4 (2C), 60.1, 52.2, 48.1 (2C), 40.2 (2C).
IR (cm^{-1}): 2953, 2812, 1719, 1599, 1540, 1501, 1453, 1433, 1276, 1241, 1140, 1104, 761, 690.
HRMS (ESI) *m/z*: calcd. for $C_{25}H_{26}N_3O_5$ $[M+H]^+$ 448.1867, found 448.1861.
Mp: 60-61 °C

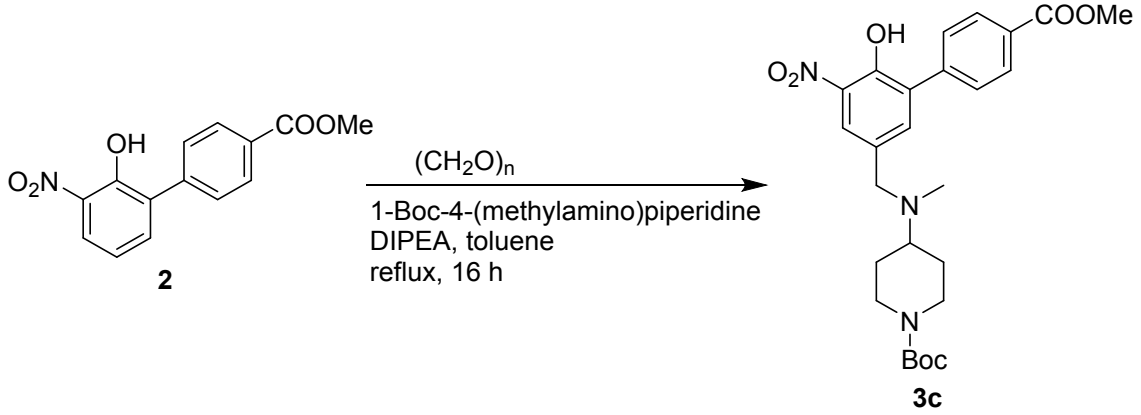
Synthesis of *tert*-butyl 4-((6-hydroxy-4'-(methoxycarbonyl)-5-nitro-[1,1'-biphenyl]-3-yl)methyl)piperazine-1-carboxylate (3b)



Compound **3b** was prepared from compound **2** (100 mg, 0.366 mmol, 1 equiv.) and 1-Boc-piperazine (136 mg, 0.730 mmol, 2 equiv.), analogously to compound **3a**. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **3b** (yellow powder, 126 mg, 73%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 7.0 Hz, 2H), 7.95 (s, 1H), 7.70 (d, J = 7.2 Hz, 2H), 7.61 (s, 1H), 3.88 (s, 3H), 3.53 (s, 2H), 3.38–3.23 (m, 4H), 2.42–2.28 (m, 4H), 1.38 (s, 9H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.0 (C=O), 153.8 (C=O), 150.0, 141.3, 137.6, 136.2, 131.5, 129.7 (2C), 129.0 (3C), 128.7, 125.0, 78.8, 60.1, 52.2, 52.1 (2C), 43.7, 42.8, 28.0 (3C).
IR (cm^{-1}): 2949, 1719, 1685, 1605, 1544, 1519, 1496, 1427, 1274, 1217, 1169, 1146, 1099, 1053, 957, 904.
HRMS (ESI) *m/z*: calcd. for $C_{24}H_{30}N_3O_7$ $[M+H]^+$ 472.2078, found 472.2079.
Mp: 155-156 °C

Synthesis of *tert*-butyl 4-(((6-hydroxy-4'-(methoxycarbonyl)-5-nitro-[1,1'-biphenyl]-3-yl)methyl)(methyl)amino)piperidine-1-carboxylate (3c)

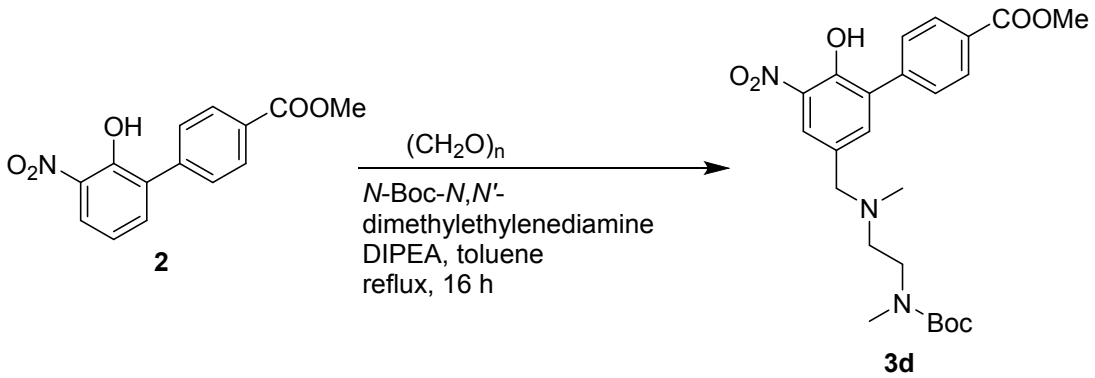


2 **3c**

Compound **3c** was prepared from compound **2** (100 mg, 0.366 mmol, 1 equiv.) and 1-Boc-4-(methylamino)piperidine (156 µL, 0.732 mmol, 2 equiv.), analogously to compound **3a**. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **3c** (yellow powder, 157 mg, 86%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 7.1 Hz, 2H), 7.91 (s, 1H), 7.68 (d, J = 7.1 Hz, 2H), 7.53 (s, 1H), 4.08–3.93 (m, 2H), 3.87 (s, 3H), 3.69 (s, 2H), 2.86–2.57 (m, 3H), 2.23 (s, 3H), 1.88–1.72 (m, 2H), 1.47–1.41 (m, 1H), 1.39 (s, 9H), 1.36–1.32 (m, 1H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.1 (C=O), 153.7 (C=O), 153.6, 142.3, 136.8, 136.4, 132.3, 129.7, 129.4 (2C), 128.8 (2C), 128.1, 125.6, 78.6, 60.5, 55.9, 52.1, 43.0, 42.4, 36.5, 28.1 (4C), 27.2.
IR (cm^{-1}): 2934, 2850, 1719, 1686, 1613, 1540, 1431, 1274, 1241, 1158, 1104, 861, 768, 704.
HRMS (ESI) *m/z*: calcd. for $C_{26}H_{34}N_3O_7$ $[M+H]^+$ 500.2391, found 500.2389.
Mp: 70-71 °C

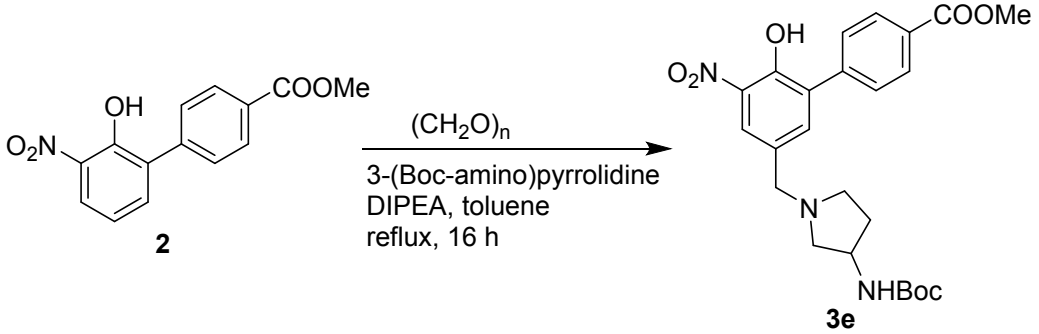
Synthesis of methyl 5'-(((2-((*tert*-butoxycarbonyl)(methyl)amino)ethyl)(methyl)amino)methyl)-2'-hydroxy-3'-nitro-[1,1'-biphenyl]-4-carboxylate (3d)



Compound **3d** was prepared from compound **2** (100 mg, 0.366 mmol, 1 equiv.) and *N*-Boc-*N*,*N*′-dimethylethylenediamine (138 µL, 0.733 mmol, 3 equiv.), analogously to compound **3a**. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **3d** (yellow powder, 118 mg, 68%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.11 (s, 1H), 8.12 (d, J = 8.4 Hz, 2H), 8.09 (s, 1H), 7.70–7.59 (m, 3H), 3.95 (s, 3H), 3.54 (s, 2H), 3.42–3.29 (m, 2H), 2.85 (s, 3H), 2.61–2.50 (m, 2H), 2.27 (s, 3H), 1.40 (s, 9H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.0 (C=O), 154.9 ((C=O), 154.7, 150.6, 141.6, 137.3, 136.2, 131.7, 129.6 (2C), 129.0 (2C), 128.5, 124.8, 78.3, 60.0 (rotamer 59.5), 53.9, 52.2, 45.8 (rotamer 45.0), 41.8 (rotamer 41.7), 34.1, 27.9 (3C).
IR (cm^{-1}): 2955, 1719, 1686, 1608, 1542, 1479, 1395, 1362, 1271, 1232, 1213, 1167, 1142, 1104, 939, 866, 772.
HRMS (ESI) *m/z*: calcd. for $C_{24}H_{32}N_3O_7$ $[M+H]^+$ 474.2235, found 474.2242.
Mp: 97-98 °C

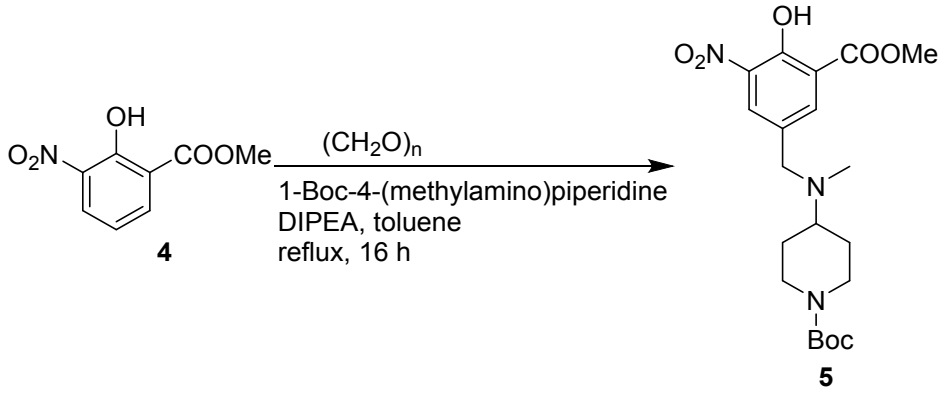
Synthesis of methyl 5'-((3-((tert-butoxycarbonyl)amino)pyrrolidin-1-yl)methyl)-2'-hydroxy-3'-nitro-[1,1'-biphenyl]-4-carboxylate (3e)



Compound **3e** was prepared from compound **2** (100 mg, 0.366 mmol, 1 equiv.) and 3-(Boc-amino)pyrrolidine (136 mg, 0.730 mmol, 2 equiv.), analogously to compound **3a**. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **3e** (yellow powder, 142 mg, 82%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (d, J = 8.5 Hz, 2H), 7.92 (d, J = 2.3 Hz, 1H), 7.68 (d, J = 8.5 Hz, 2H), 7.52 (d, J = 2.3 Hz, 1H), 7.11 (d, J = 6.8 Hz, 1H), 4.08–3.95 (m, 1H), 3.87 (s, 3H), 3.82 (s, 2H), 3.06–2.98 (m, 1H), 2.93–2.83 (m, 1H), 2.82–2.73 (m, 1H), 2.65–2.57 (m, 1H), 2.16–2.04 (m, 1H), 1.76–1.63 (m, 1H), 1.36 (s, 9H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.1 (C=O), 155.4, 155.1 (C=O), 142.6, 136.7, 136.6, 132.7, 129.3 (2C), 128.7 (2C), 128.0, 126.3, 122.5, 77.9, 58.8, 57.5, 52.1, 51.9, 49.0, 30.3, 28.2 (3C).
IR (cm^{-1}): 3356, 2971, 1707, 1613, 1541, 1501, 1276, 1245, 1164, 1104, 1069, 790, 770, 707.
HRMS (ESI) *m/z*: calcd. for $C_{24}H_{30}N_3O_7$ $[M+H]^+$ 472.2078, 472.2075.
Mp: 85-86 °C

Synthesis of *tert*-butyl 4-((4-hydroxy-3-(methoxycarbonyl)-5-nitrobenzyl)(methyl)amino)piperidine-1-carboxylate (5)

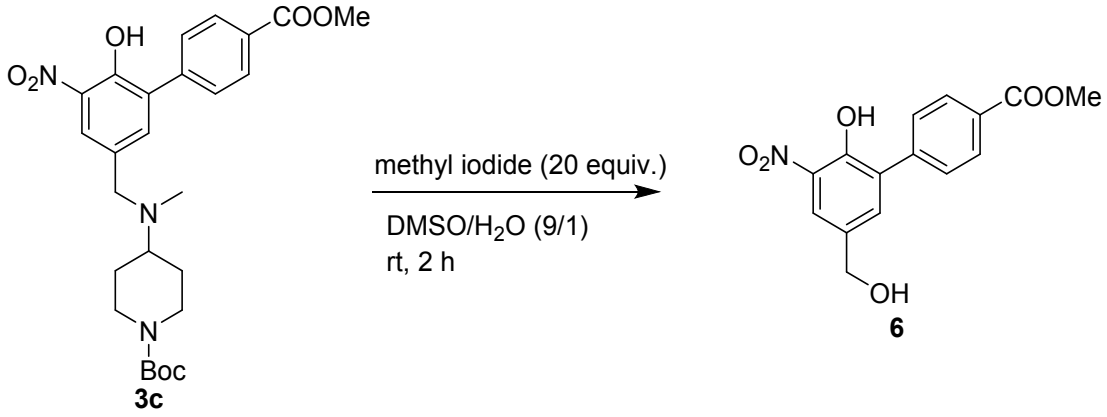


4 **5**

Compound **5** was prepared from compound **4** (200 mg, 1.014 mmol, 1 equiv.) and 1-Boc-4-(methylamino)piperidine (433 µL, 2.030 mmol, 2 equiv.), analogously to compound **3a**. The resulting residue was purified by flash chromatography on silica gel (0-60% ethyl acetate in dichloromethane) to give compound **5** (yellow powder, 245 mg, 57%)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (d, J = 2.3 Hz, 1H), 7.93 (d, J = 2.3 Hz, 1H), 4.09–3.94 (m, 2H), 3.87 (s, 3H), 3.67 (s, 2H), 2.82–2.58 (m, 3H), 2.20 (s, 3H), 1.80 (d, J = 11.5 Hz, 2H), 1.46–1.41 (m, 1H), 1.40 (s, 9H), 1.38–1.33 (m, 1H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 167.8 (C=O), 154.9, 153.7 (C=O), 138.6, 135.7, 130.5, 125.7, 118.7, 78.7, 60.6, 55.5, 52.6, 43.0, 42.4, 36.5, 28.1 (4C), 27.2.
IR (cm^{-1}): 2934, 2855, 1682, 1532, 1445, 1423, 1364, 1344, 1242, 1157, 1109, 1041, 798, 725.
HRMS (ESI) *m/z*: calcd. for $C_{20}H_{30}N_3O_7$ $[M+H]^+$ 424.2078, found 424.2076.
Mp: 60-61 °C

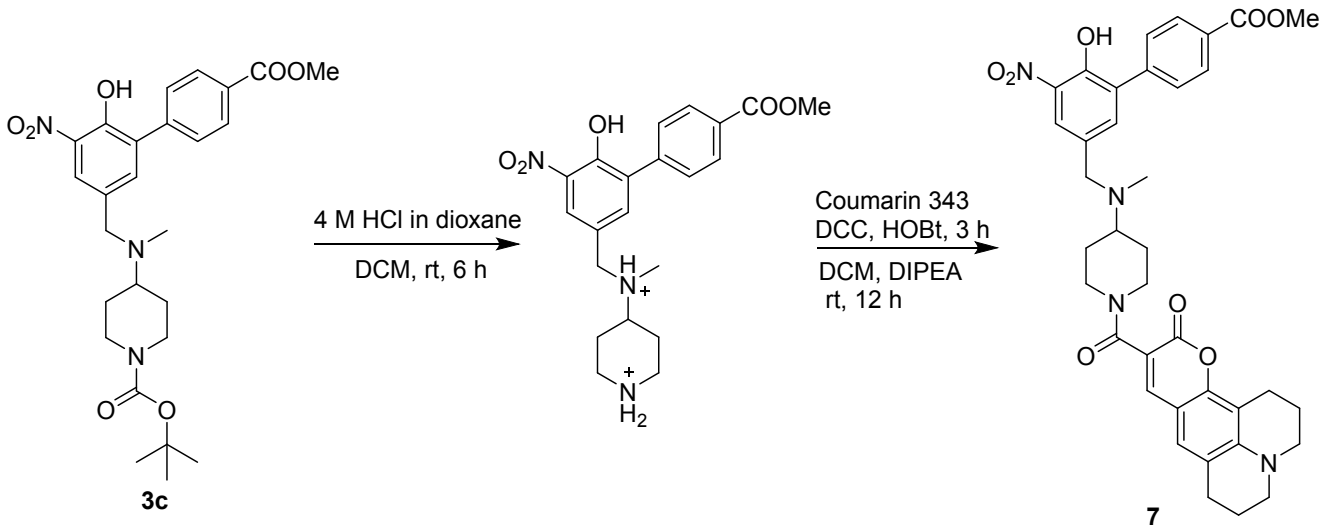
Synthesis of methyl 2'-hydroxy-5'-(hydroxymethyl)-3'-nitro-[1,1'-biphenyl]-4-carboxylate (6)



To a solution of compound **3c** (20 mg, 0.04 mmol, 1 equiv.) in 9:1 DMSO/H_2O (8 mL) was added methyl iodide (50 µL, 0.80 mmol, 20 equiv.). The mixture was stirred at room temperature for 2 h. At the end of the reaction, the mixture was diluted with 30 mL ethyl acetate and then washed with brine and water. The organic layer was dried over anhydrous magnesium sulfate. After filtration, the organic solvent was removed under vacuum. The residue was purified by flash chromatography on silica gel (0-3% ethyl acetate in dichloromethane) to give compound **6** (yellow solid, 9 mg, 74%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.61 (br, 1H), 8.04 (d, J = 8.5 Hz, 2H), 8.01 (d, J = 2.2 Hz, 1H), 7.69 (d, J = 8.5 Hz, 2H), 7.66 (d, J = 2.2 Hz, 1H), 5.50–5.34 (m, 1H), 4.54 (s, 2H), 3.88 (s, 3H).
^{13}C NMR (100 MHz, DMSO-d_6) δ 166.0 (C=O), 149.0, 141.3, 136.0, 135.5, 134.6, 131.2, 129.7 (2C), 129.1 (2C), 128.8, 122.3, 61.4, 52.3.
IR (cm^{-1}): 3384, 3280, 3080, 2961, 1710, 1613, 1540, 1435, 1402, 1282, 1105, 1019, 854, 778, 763, 703.
HRMS (ESI) *m/z*: calcd. for $C_{15}H_{14}NO_6$ $[M+H]^+$ 304.0816, found 304.0815.
Mp: 162-163 °C

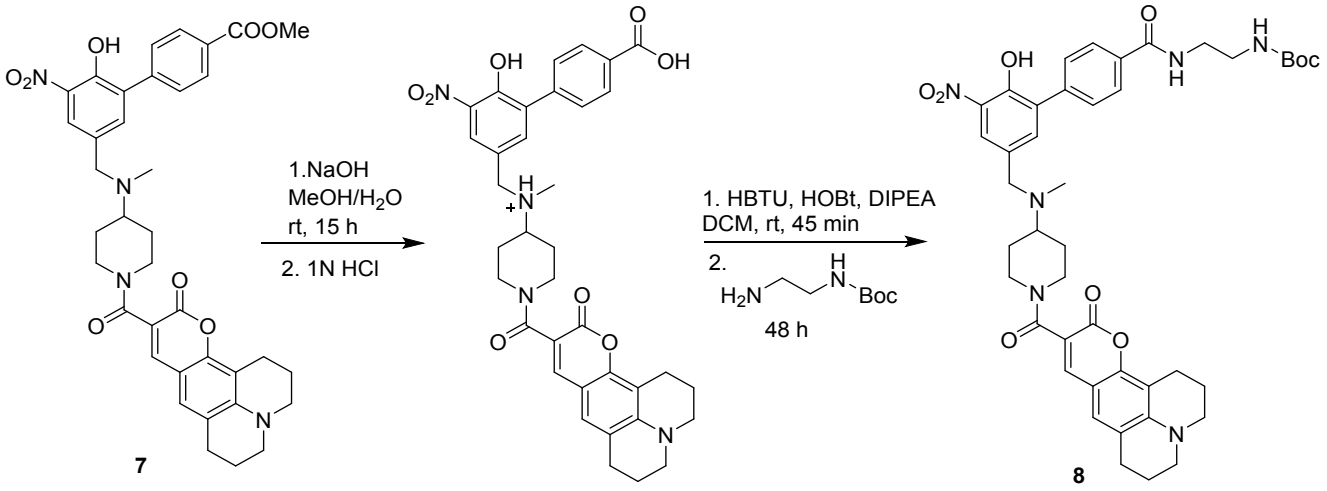
Synthesis of methyl 2'-hydroxy-5'-((methyl(1-(11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carbonyl)piperidin-4-yl)amino)methyl)-3'-nitro-[1,1'-biphenyl]-4-carboxylate (7)



To a solution of compound **3c** (500 mg, 1.0 mmol, 1 equiv) in dichloromethane (25 mL) was added 4 M HCl in dioxane (5 mL, 20 equiv.). The mixture was stirred at room temperature for 6 h. The reaction was checked for completion by UPLC-MS. At the end of the reaction, the organic solvent was removed under vacuum. The resulting residue was used for the next reaction without further purification.
To a mixture of coumarin 343 (342 mg, 1.2 mmol, 1.2 equiv.), *N*,*N*'-dicyclohexylcarbodiimide (310 mg, 1.5 mmol, 1.5 equiv.), and hydroxybenzotriazole (270 mg, 2.0 mmol, 2 equiv.) was added anhydrous dichloromethane (50 mL). The resulting solution was stirred at room temperature for 3 h, followed by the addition of a solution of the amine hydrochloride obtained in the previous step and *N*,*N*-diisopropylethylamine (350 µL, 2.0 mmol, 2 equiv.) in anhydrous dichloromethane (25 mL). The resulting solution was stirred at room temperature for another 12 h. At the end of the reaction, the solvent was removed under vacuum. The residue was purified by flash chromatography on silica gel (100% ethyl acetate) to give compound **7** (yellow solid, 527 mg, 79% over two steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, J = 8.3 Hz, 2H), 8.08 (d, J = 2.0 Hz, 1H), 7.74 (s, 1H), 7.68–7.57 (m, 3H), 6.87 (s, 1H), 4.85–4.68 (m, 1H), 3.94 (s, 3H), 3.79–3.67 (m, 1H), 3.65–3.54 (m, 2H), 3.37–3.21 (m, 4H), 3.16–3.00 (m, 1H), 2.86 (t, J = 6.4 Hz, 2H), 2.80–2.65 (m, 4H), 2.22 (s, 3H), 2.02–1.89 (m, 5H), 1.84–1.56 (m, 3H).
^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9 (C=O), 165.3 (C=O), 159.7 (C=O), 152.3, 151.8, 147.1, 145.0, 140.6, 138.7, 134.0, 132.4, 131.9, 129.7, 129.7 (2C), 129.5 (2C), 125.9, 124.3, 119.1, 115.6, 107.7, 106.2, 61.3, 56.6, 52.3, 50.2, 49.8, 47.3, 42.0, 37.8, 28.7, 27.6 (2C), 21.4, 20.4, 20.2.
IR (cm^{-1}): 2944, 2853, 1711, 1615, 1516, 1435, 1366, 1308, 1271, 1241, 1212, 1170, 1104.
HRMS (ESI) *m/z*: calcd. for $C_{37}H_{39}N_4O_8$ $[M+H]^+$ 667.2762, found 667.2753.
Mp: 138-139 °C

Synthesis of *tert*-butyl (2-(2'-hydroxy-5'-((methyl(1-(11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carbonyl)piperidin-4-yl)amino)methyl)-3'-nitro-[1,1'-biphenyl]-4-carboxamido)ethyl)carbamate (8)

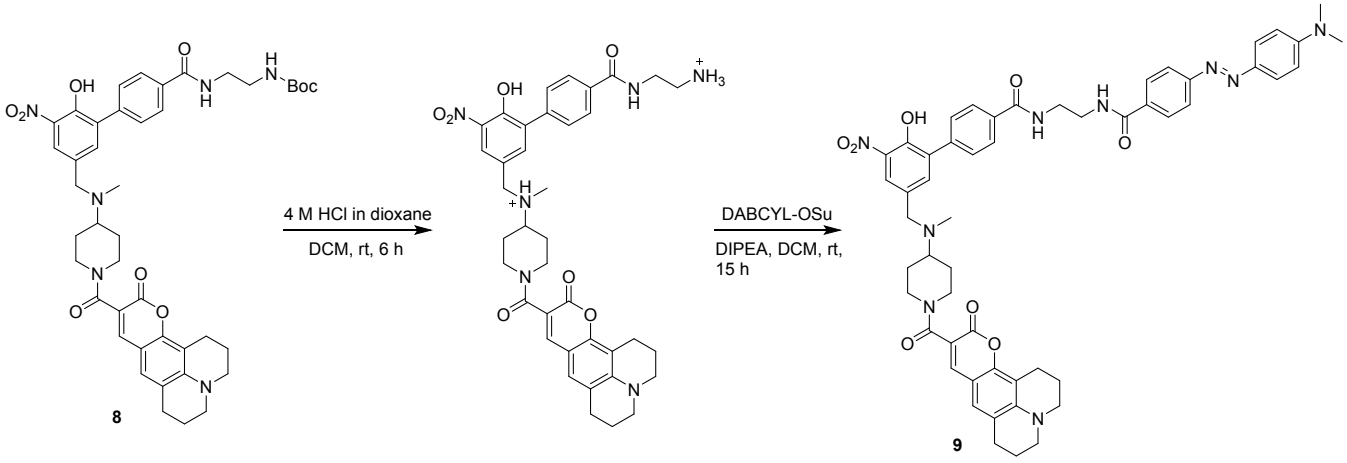


To a solution of compound **8** (500 mg, 0.75 mmol, 1 equiv.) in 100 mL methanol containing 5% H_2O was added NaOH (300 mg, 7.5 mmol, 10 equiv.) The mixture was stirred at room temperature for 15 h. The reaction was checked for completion by UPLC-MS. At the end of the reaction, the mixture was acidified with 1 N HCl to pH 2. Afterwards, the solvent was removed under vacuum. The resulting residue was dried by freeze dryer and then was used for the next reaction without further purification.

The residue obtained in the previous step was dispersed in 100 mL anhydrous dichloromethane and then (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (430 mg, 1.13 mmol, 1.5 equiv.), hydroxybenzotriazole (50 mg, 0.37 mmol, 0.5 equiv.), and *N*,*N*-diisopropylethylamine (523 µL, 3 mmol, 4 equiv.) were added successively. The mixture was stirred at room temperature for 45 min, then *N*-Boc-ethylenediamine (240 µL, 1.52 mmol, 2 equiv.) was added dropwise. The resulting solution was stirred at room temperature for another 48 h. At the end of the reaction, the solvent was removed under vacuum. The residue was purified by flash chromatography on silica gel (0-5% methanol in dichloromethane) to give compound **8** (yellow solid, 490 mg, 82% over two steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.08 (br, 1H), 8.08 (d, J = 2.0 Hz, 1H), 7.90 (d, J = 8.2 Hz, 2H), 7.74 (s, 1H), 7.68–7.55 (m, 3H), 7.33 (br, 1H), 6.87 (s, 1H), 5.08 (br, 1H), 4.84–4.67 (m, 1H), 3.81–3.66 (m, 1H), 3.65–3.51 (m, 4H), 3.48–3.37 (m, 2H), 3.35–3.22 (m, 4H), 3.16–2.99 (m, 1H), 2.86 (t, J = 6.4 Hz, 2H), 2.80–2.63 (m, 4H), 2.22 (s, 3H), 2.00–1.88 (m, 5H), 1.82–1.62 (m, 3H), 1.43 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5 (C=O), 165.4 (C=O), 159.7 (C=O), 157.7 (C=O), 152.3, 151.9, 147.2, 145.1, 139.1, 138.7, 134.0, 133.7, 132.4, 132.0, 129.7 (2C), 127.2 (2C), 125.9, 124.1, 119.1, 115.7, 107.8, 106.2, 80.2, 61.3, 56.6, 50.2, 49.8, 47.3, 42.3, 42.0, 40.1, 37.8, 28.7, 28.5 (3C), 27.6 (2C), 21.4, 20.5, 20.3.
IR (cm^{-1}): 3328, 2931, 2855, 1704, 1614, 1515, 1453, 1365, 1307, 1269, 1242, 1212, 1168, 1043, 730.
HRMS (ESI) *m/z*: calcd. for $C_{43}H_{51}N_6O_9$ $[M+H]^+$ 795.3712, found 795.3705.
Mp: 141-142 °C

Synthesis of *N*-(2-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)ethyl)-2'-hydroxy-5'-((methyl(1-(11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carbonyl)piperidin-4-yl)amino)methyl)-3'-nitro-[1,1'-biphenyl]-4-carboxamide (9)

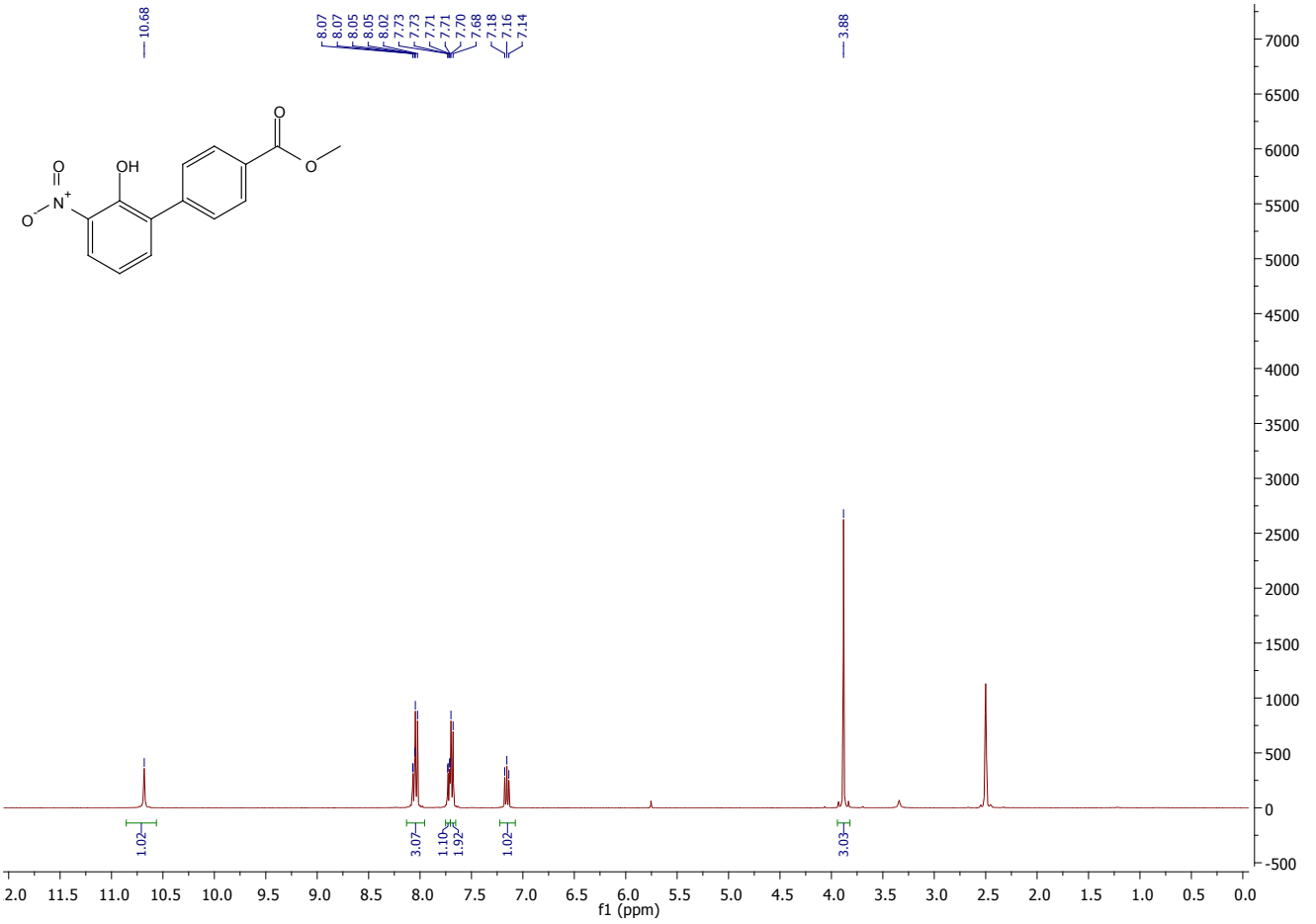


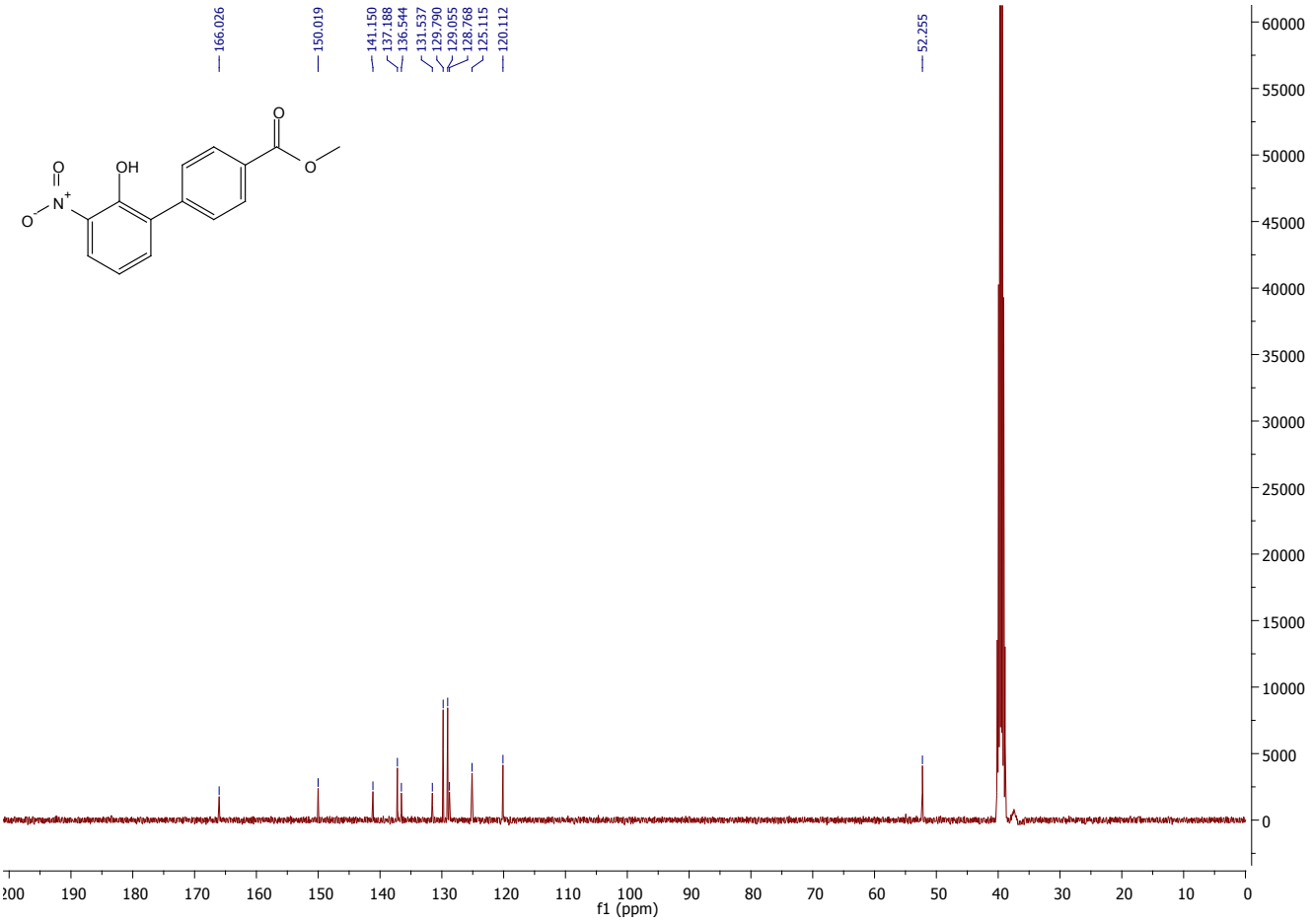
To a solution of compound **8** (100 mg, 0.126 mmol, 1 equiv) in dichloromethane (5 mL) was added 4 M HCl in dioxane (0.6 mL, 19 equiv.). The mixture was stirred at room temperature for 6 h. The reaction was checked for completion by UPLC-MS. At the end of the reaction, the organic solvent was removed under vacuum. The resulting residue was used for the next reaction without further purification.
The residue obtained in the previous step was dissolved in 50 mL dichloromethane containing *N*,*N*-diisopropylethylamine (132 µL, 0.758 mmol, 6 equiv.). Following the addition of 4-[4-(dimethylamino)phenylazo]benzoic acid *N*-succinimidyl ester (56 mg, 0.153 mmol, 1.2 equiv.), the resulting solution was stirred at room temperature for 15 h. At the end of the reaction, the solvent was

removed under vacuum. The residue was purified by flash chromatography on silica gel (0-5% methanol in dichloromethane) to give compound **9** (red powder, 101 mg, 85% over two steps).

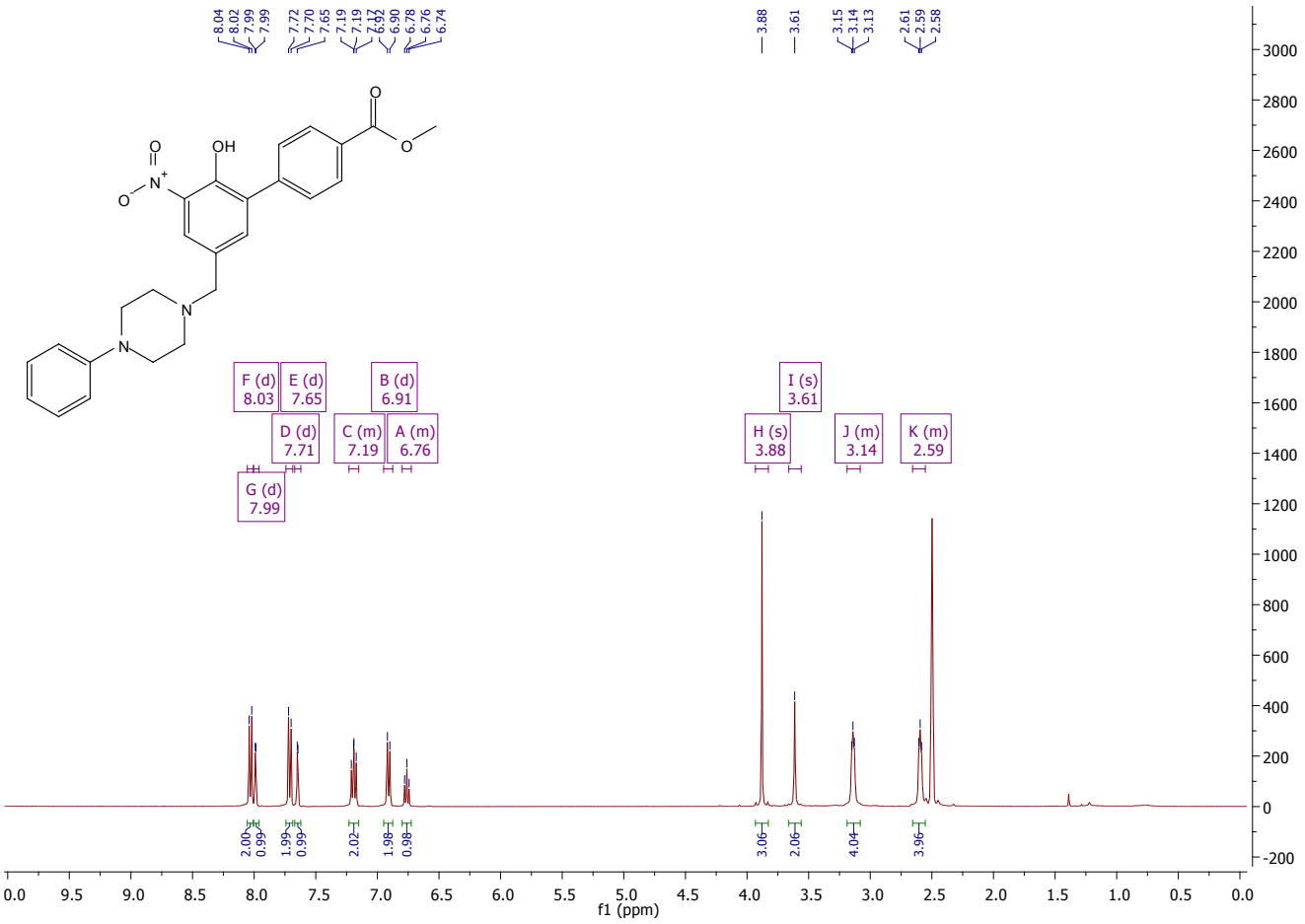
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, J = 1.3 Hz, 1H), 7.95–7.85 (m, 5H), 7.84–7.75 (m, 5H), 7.67 (s, 1H), 7.61–7.52 (m, 3H), 6.81 (s, 1H), 6.70 (d, J = 9.1 Hz, 2H), 4.77–4.62 (m, 1H), 3.79–3.64 (m, 5H), 3.57–3.45 (m, 2H), 3.31–3.19 (m, 4H), 3.11–2.99 (m, 7H), 2.79 (t, J = 6.2 Hz, 2H), 2.73–2.61 (m, 4H), 2.16 (s, 3H), 1.94–1.82 (m, 5H), 1.81–1.72 (m, 1H), 1.71–1.51 (m, 2H).
^{13}C NMR (100 MHz, $CDCl_3$) δ 168.5 (C=O), 168.3 (C=O), 165.4 (C=O), 159.7 (C=O), 155.1, 152.8, 152.2, 151.8, 147.1, 144.8, 143.6, 139.2, 138.7, 134.1, 133.9, 133.5, 132.3, 131.8, 129.6 (2C), 128.2(2C), 127.3 (2C), 125.9, 125.4 (2C), 124.1, 122.2 (2C), 119.1, 115.2, 111.5 (2C), 107.6, 106.1, 61.2, 56.5, 50.1, 49.7, 47.2, 41.9, 41.1, 41.0, 40.4 (2C), 37.7, 28.7, 27.5 (2C), 21.3, 20.4, 20.1.
IR (cm^{-1}): 3318, 2931, 2853, 1706, 1598, 1518, 1444, 1363, 1308, 1269, 1242, 1212, 1170, 1136, 945, 856, 821.
HRMS (ESI) *m/z*: calcd. for $C_{53}H_{57}N_9O_8$ $[M+2H]^{2+}$ 473.7160, found 473.7157; calcd. for $C_{53}H_{56}N_9O_8$ $[M+H]^{+}$ 946.4246, found 946.4239.
Mp: 180-181 °C

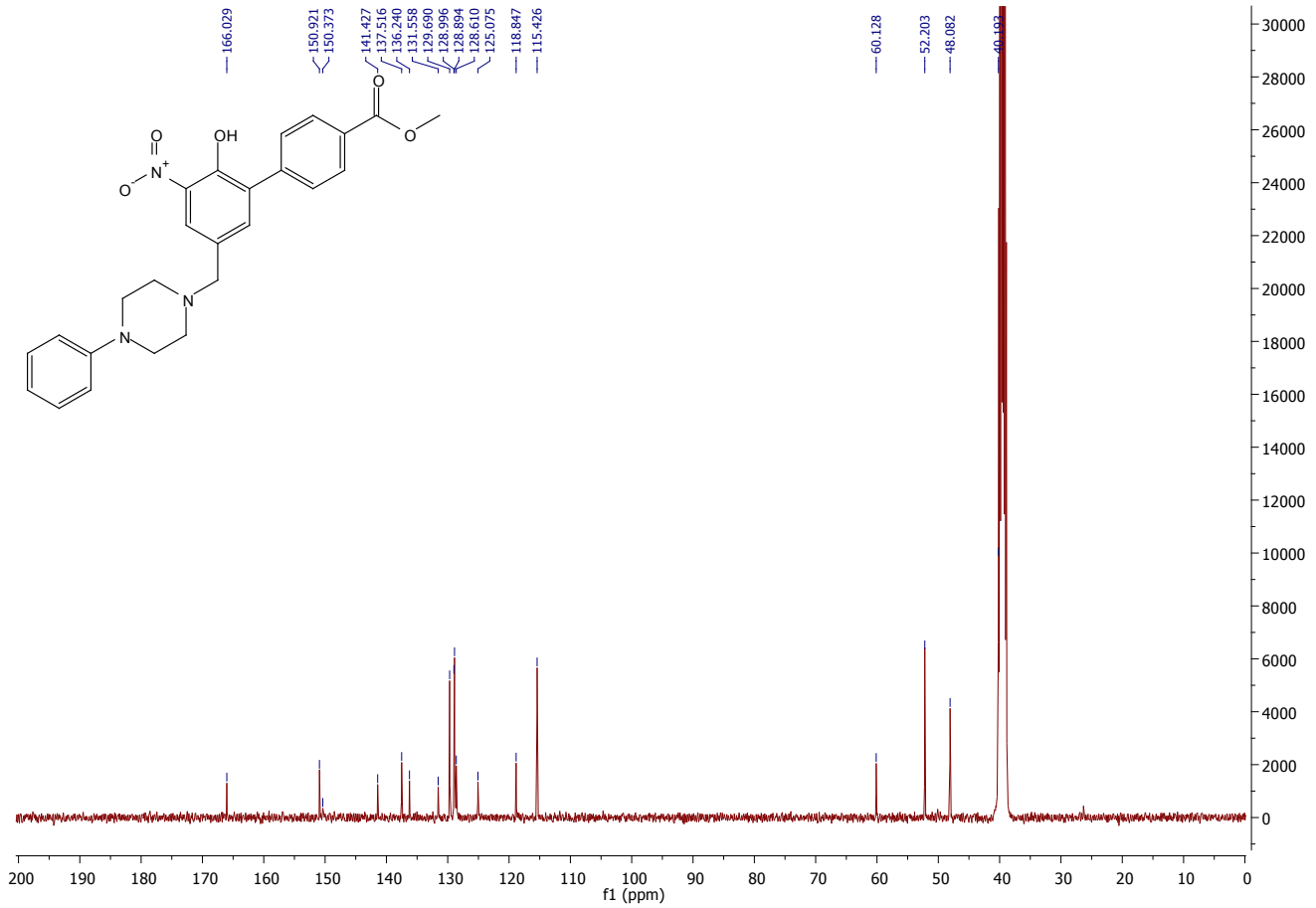
^{1}H and ^{13}C NMR spectra of **2** in DMSO-d_6



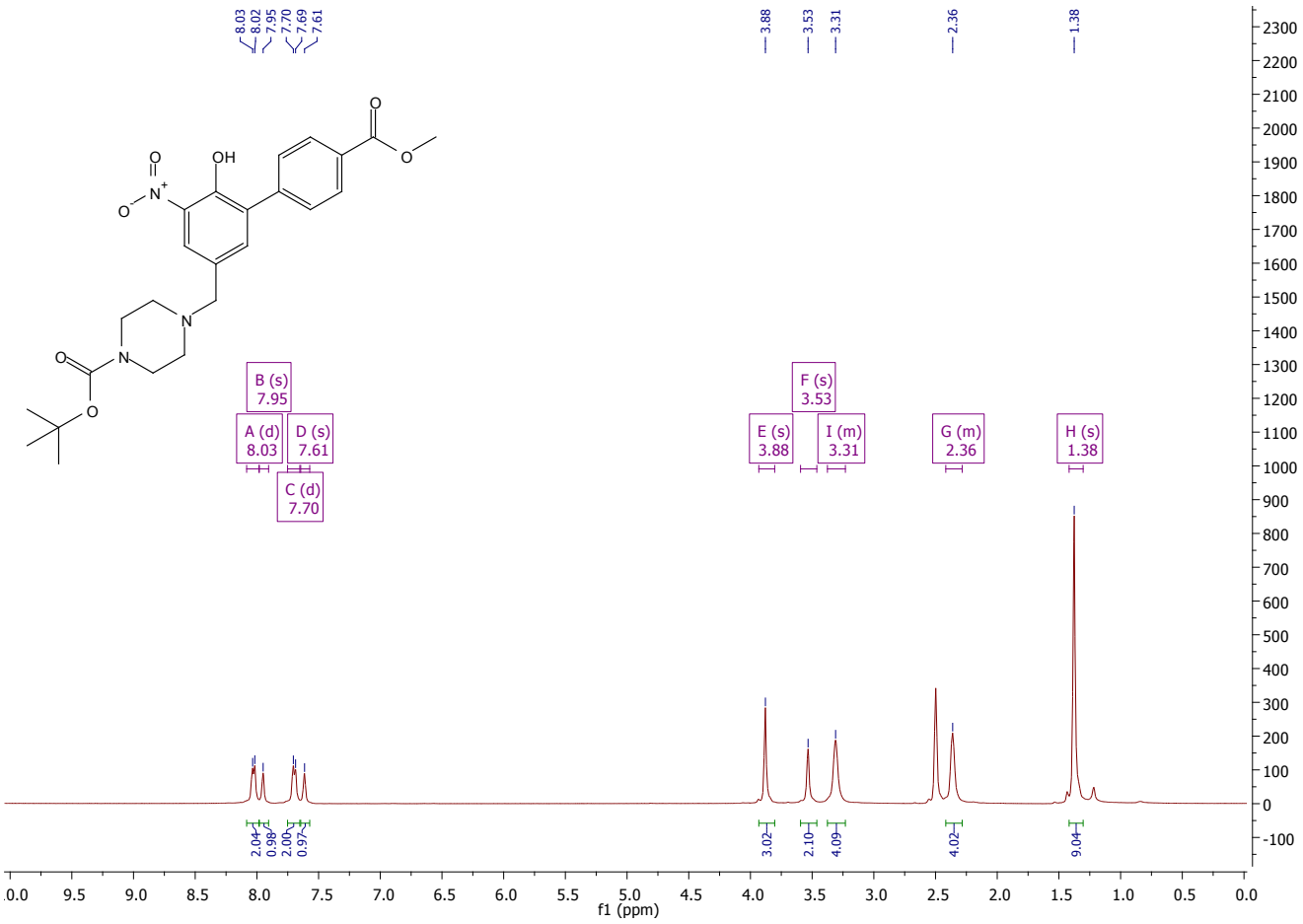


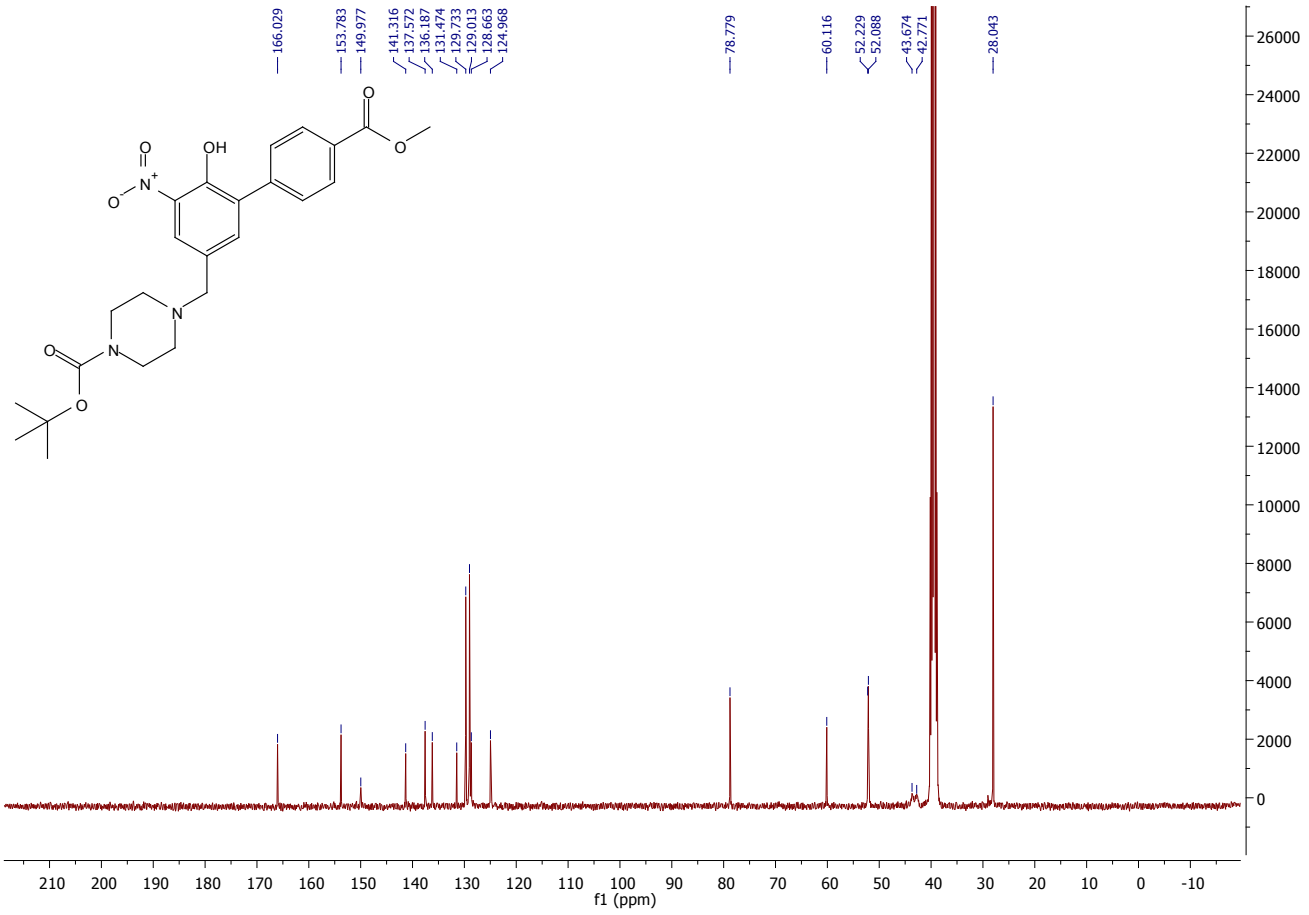
^{1}H and ^{13}C NMR spectra of **3a** in DMSO-d_6



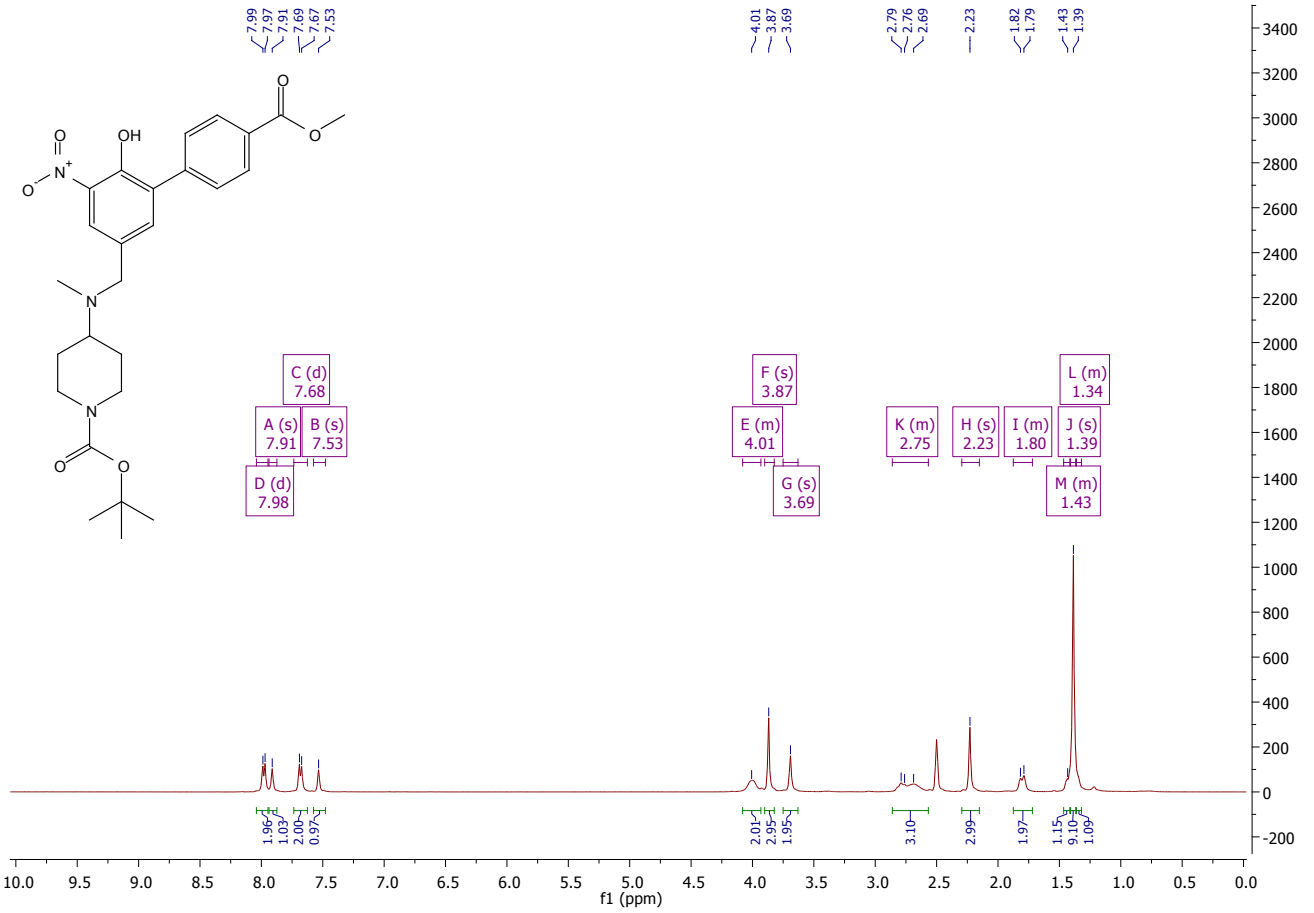


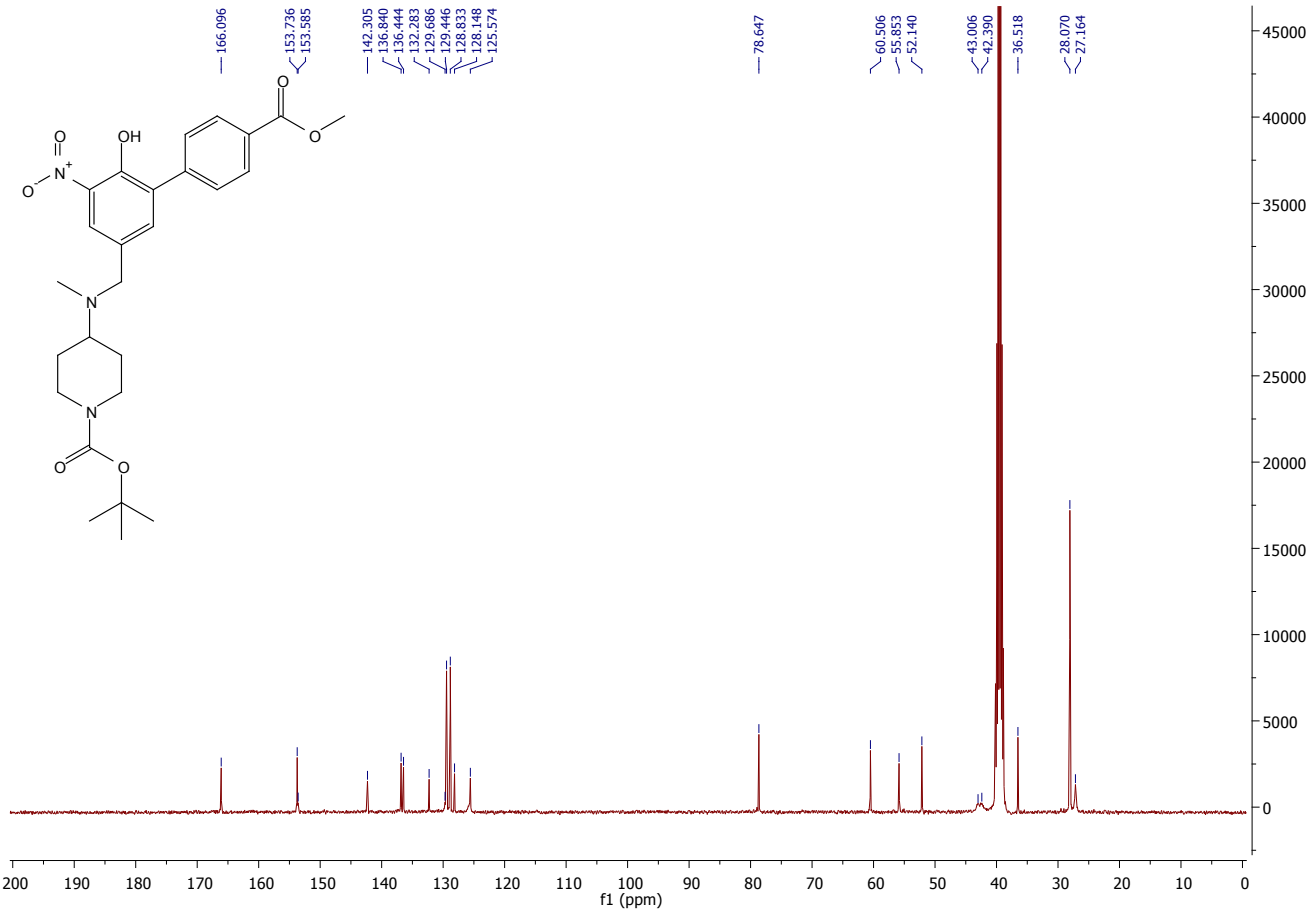
^{1}H and ^{13}C NMR spectra of **3b** in DMSO-d_6



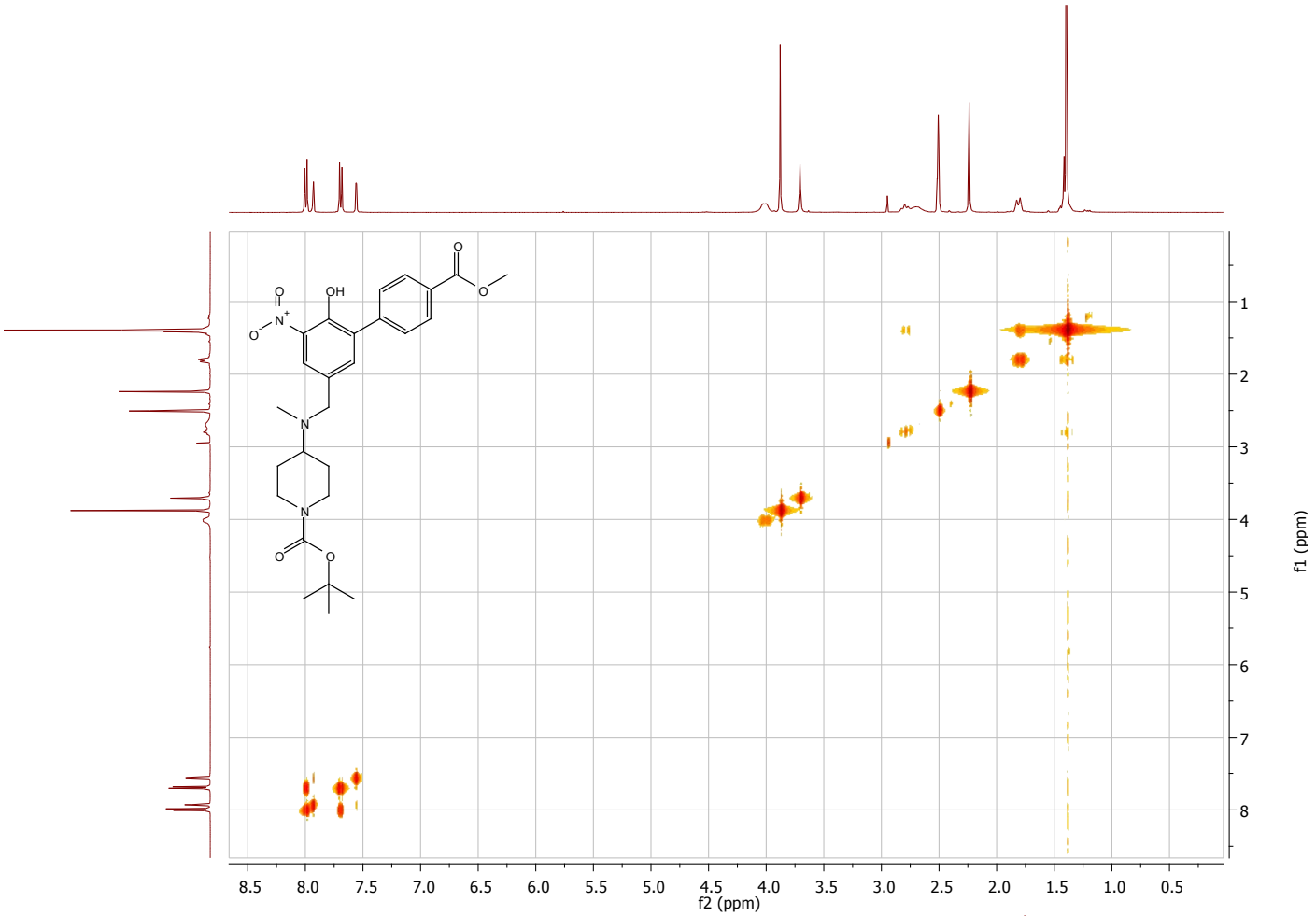


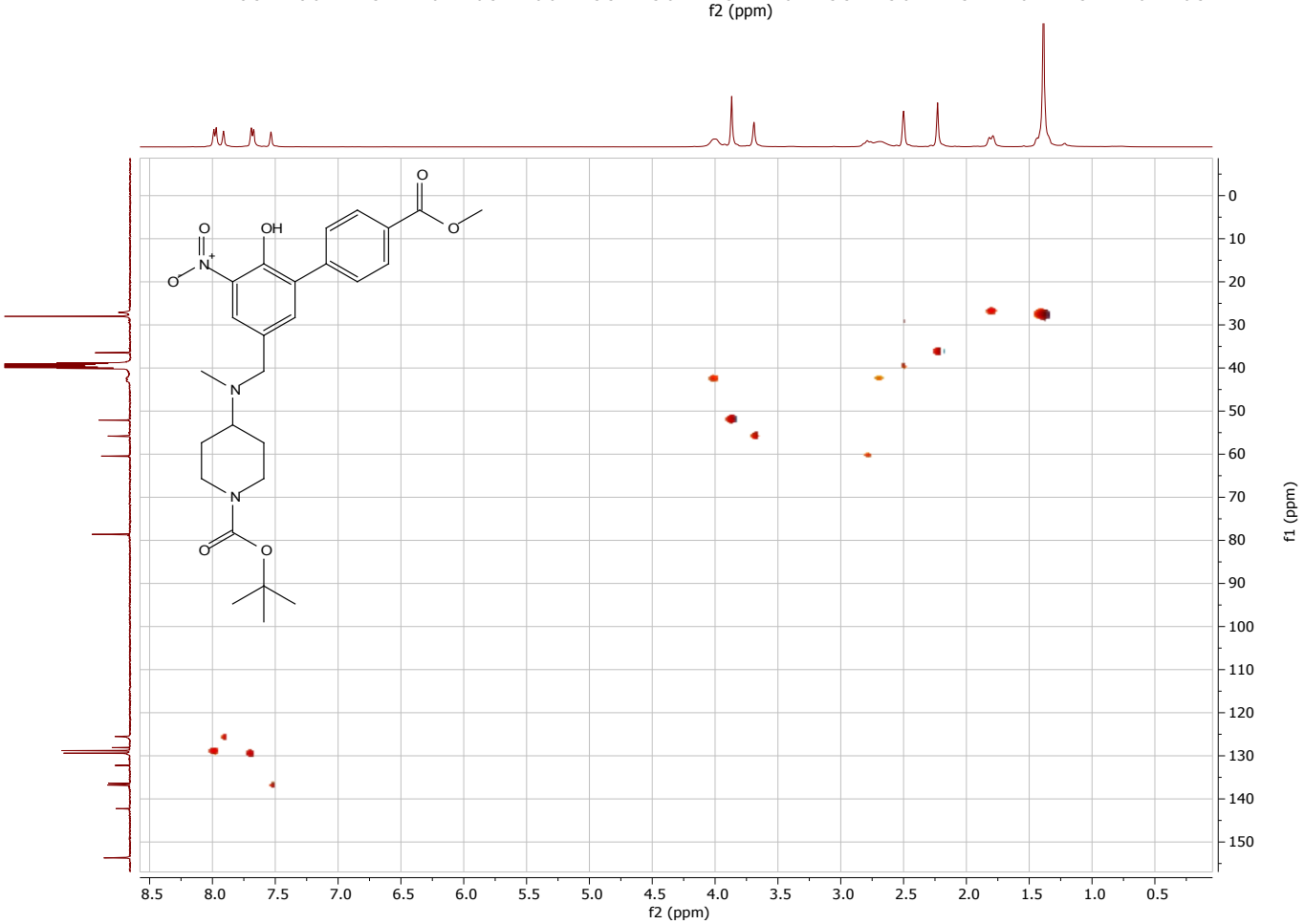
^{1}H and ^{13}C NMR spectra of **3c** in DMSO-d_6



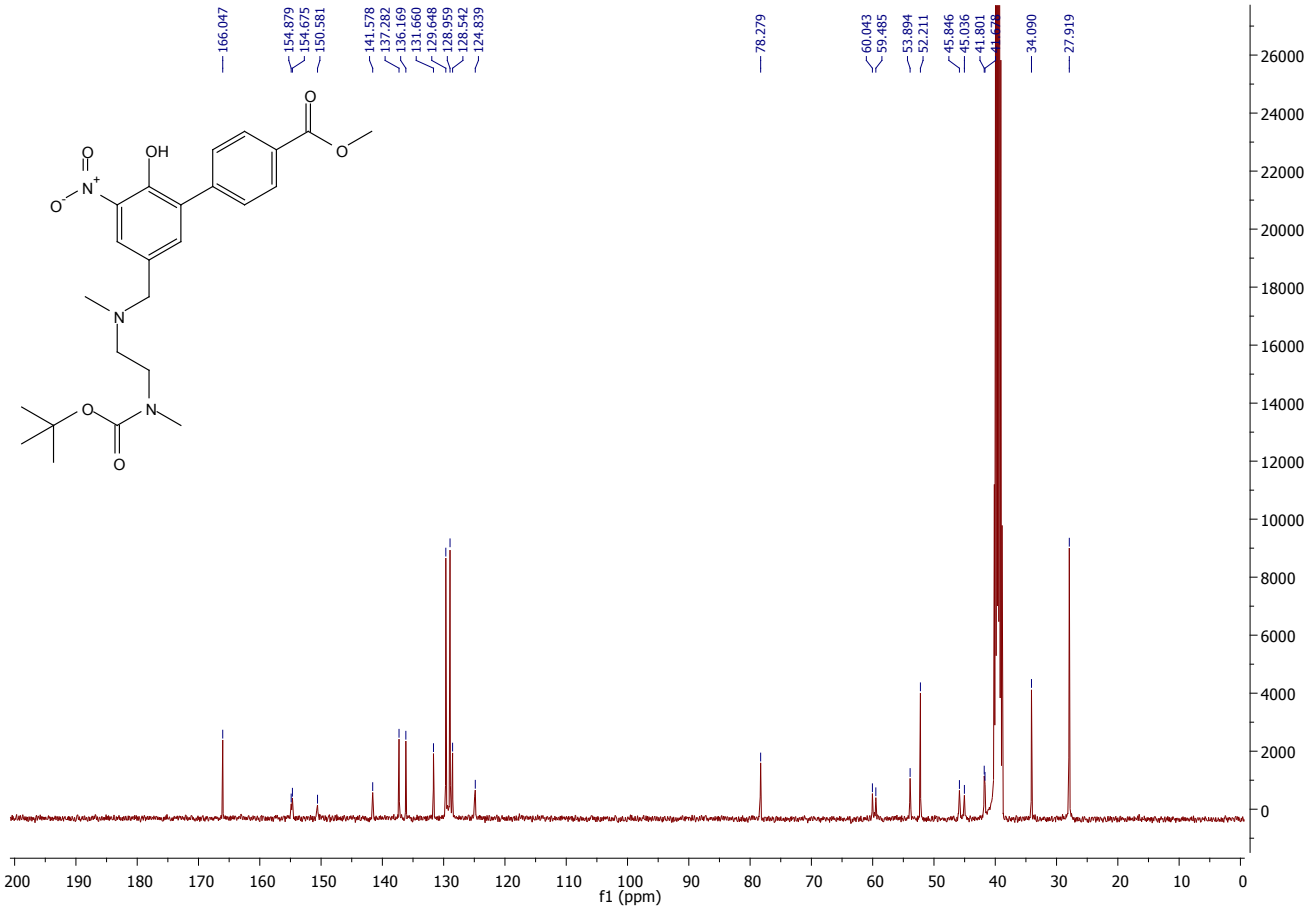


^{1}H-^{1}H COSY and HSQC spectra of **3c** in DMSO-d_6

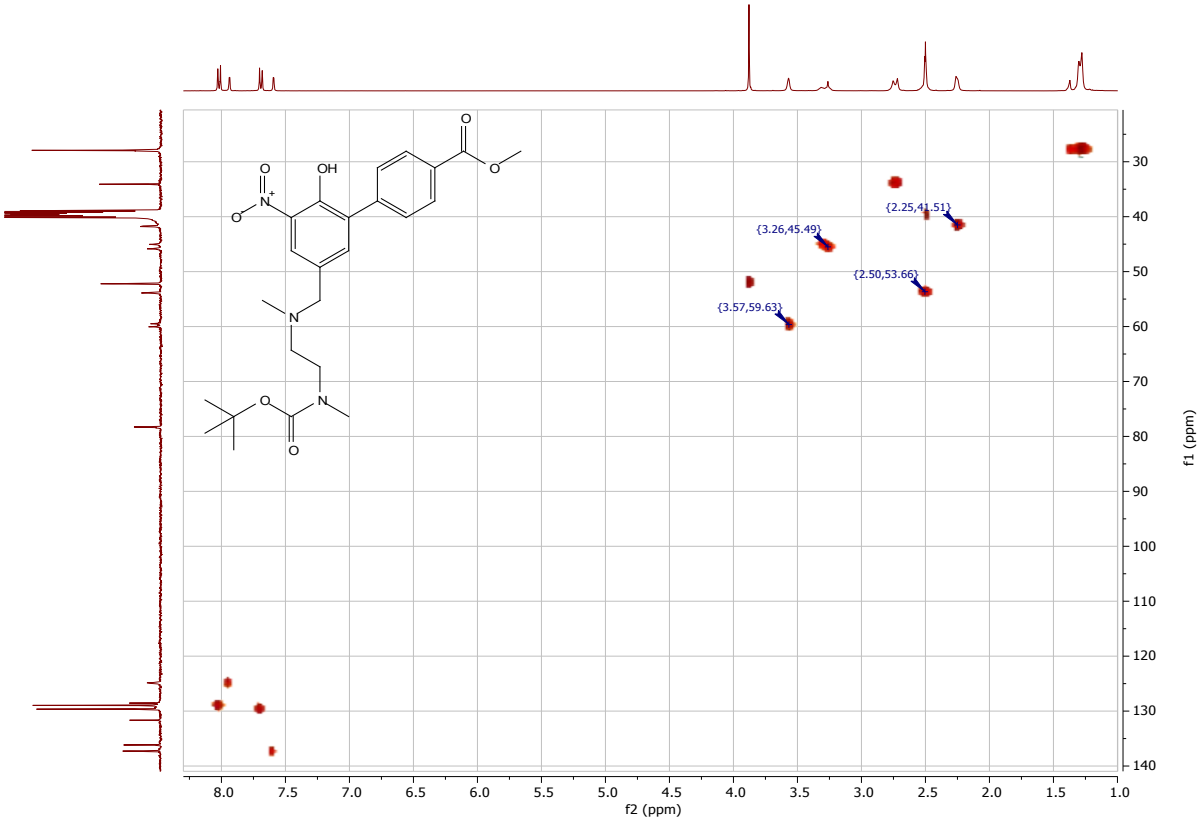




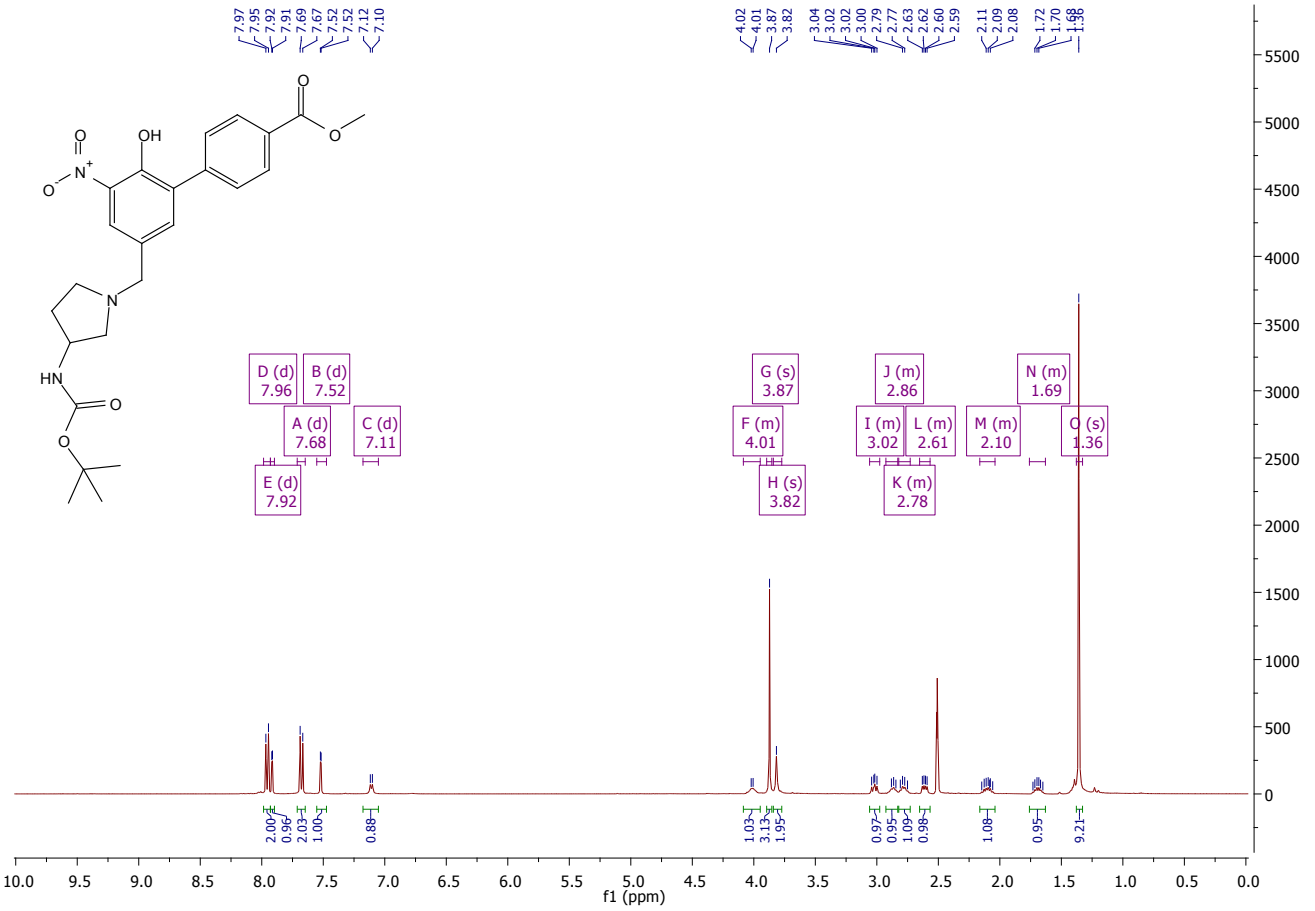
^{1}H NMR (in $CDCl_3$) and ^{13}C NMR (in DMSO-d_6) spectra of **3d**

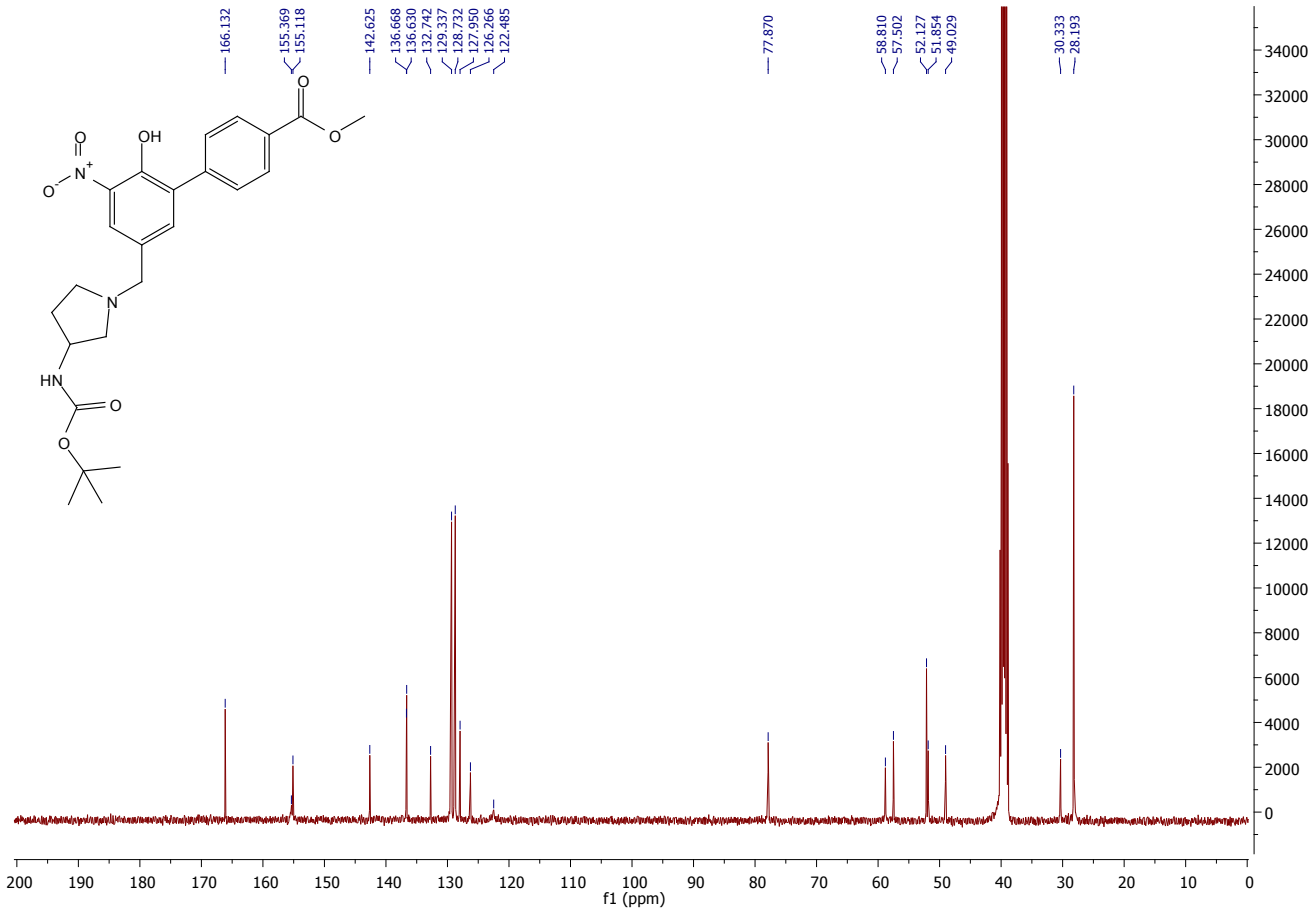


^{1}H NMR and HSQC of **3d** in DMSO-d_6

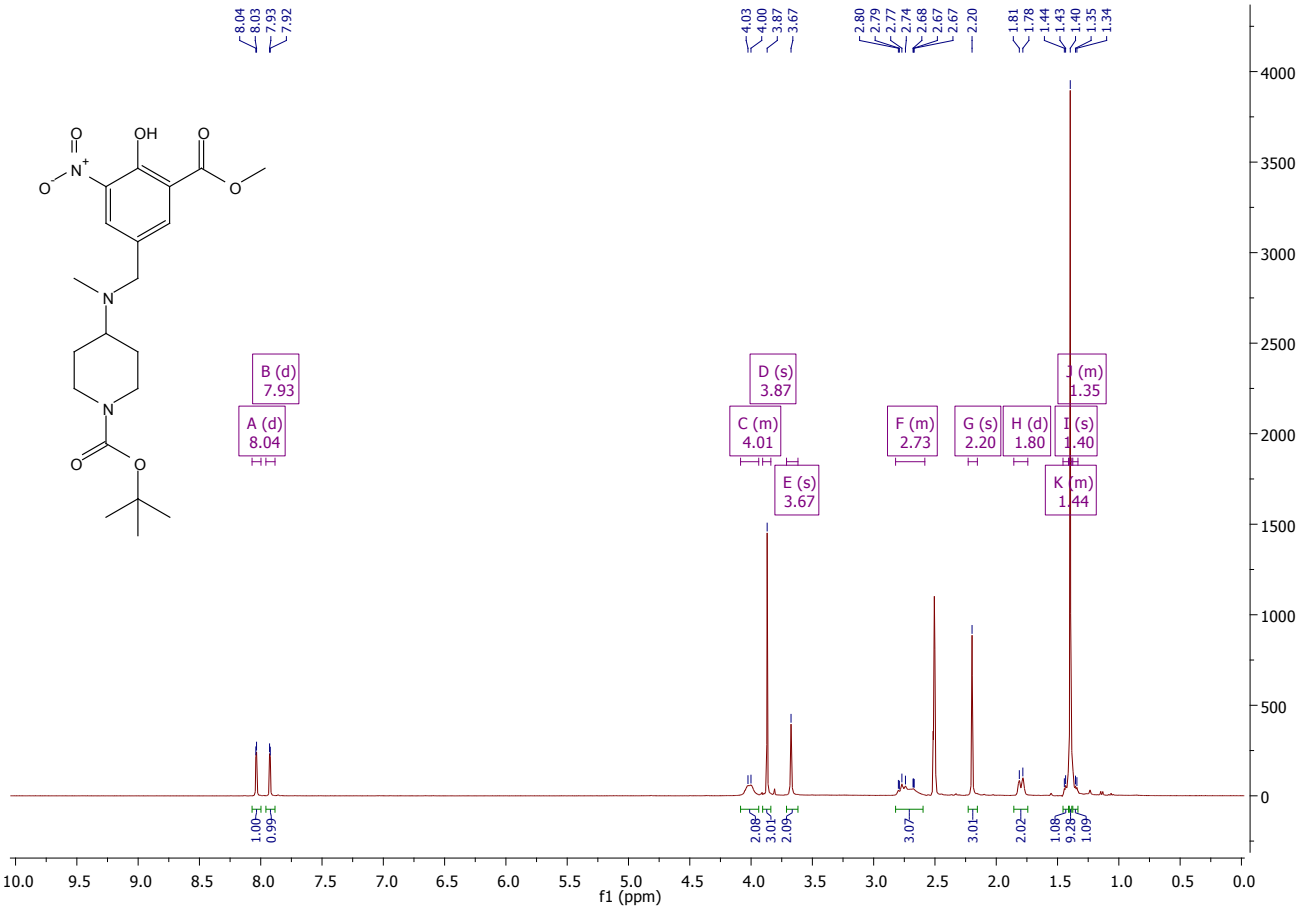


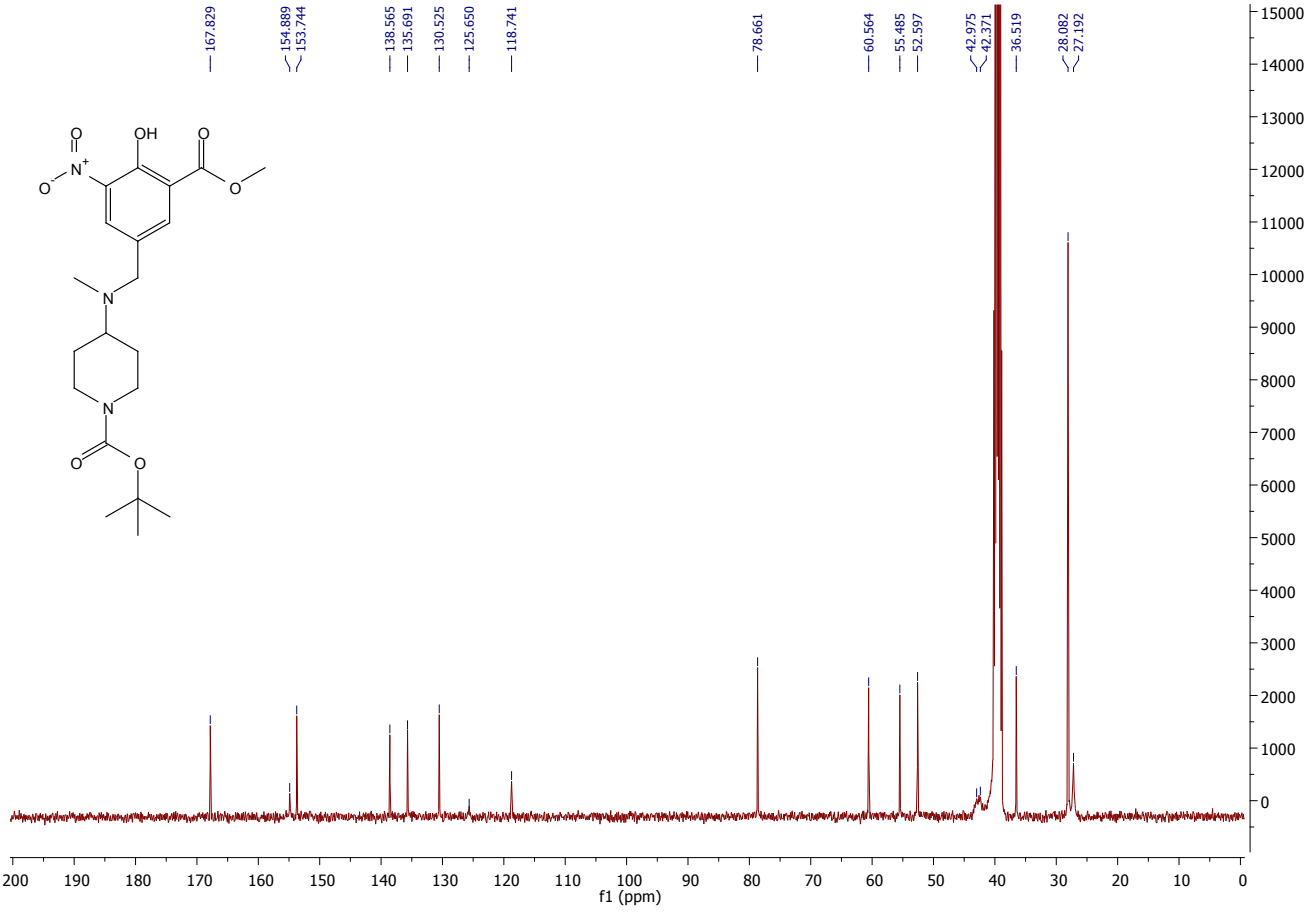
^{1}H and ^{13}C NMR spectra of **3e** in DMSO-d_6



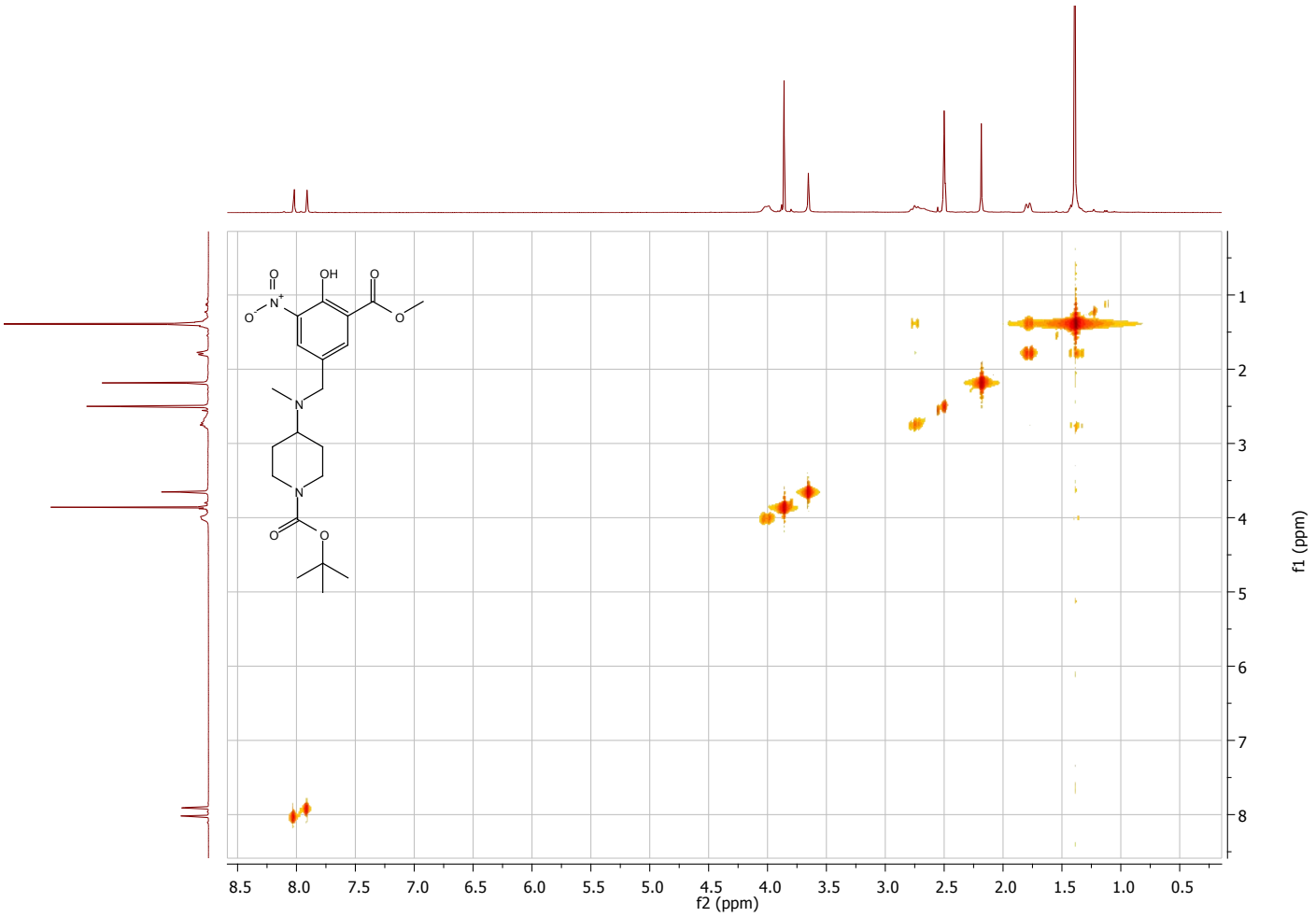


^{1}H and ^{13}C NMR spectra of **5** in DMSO-d_6

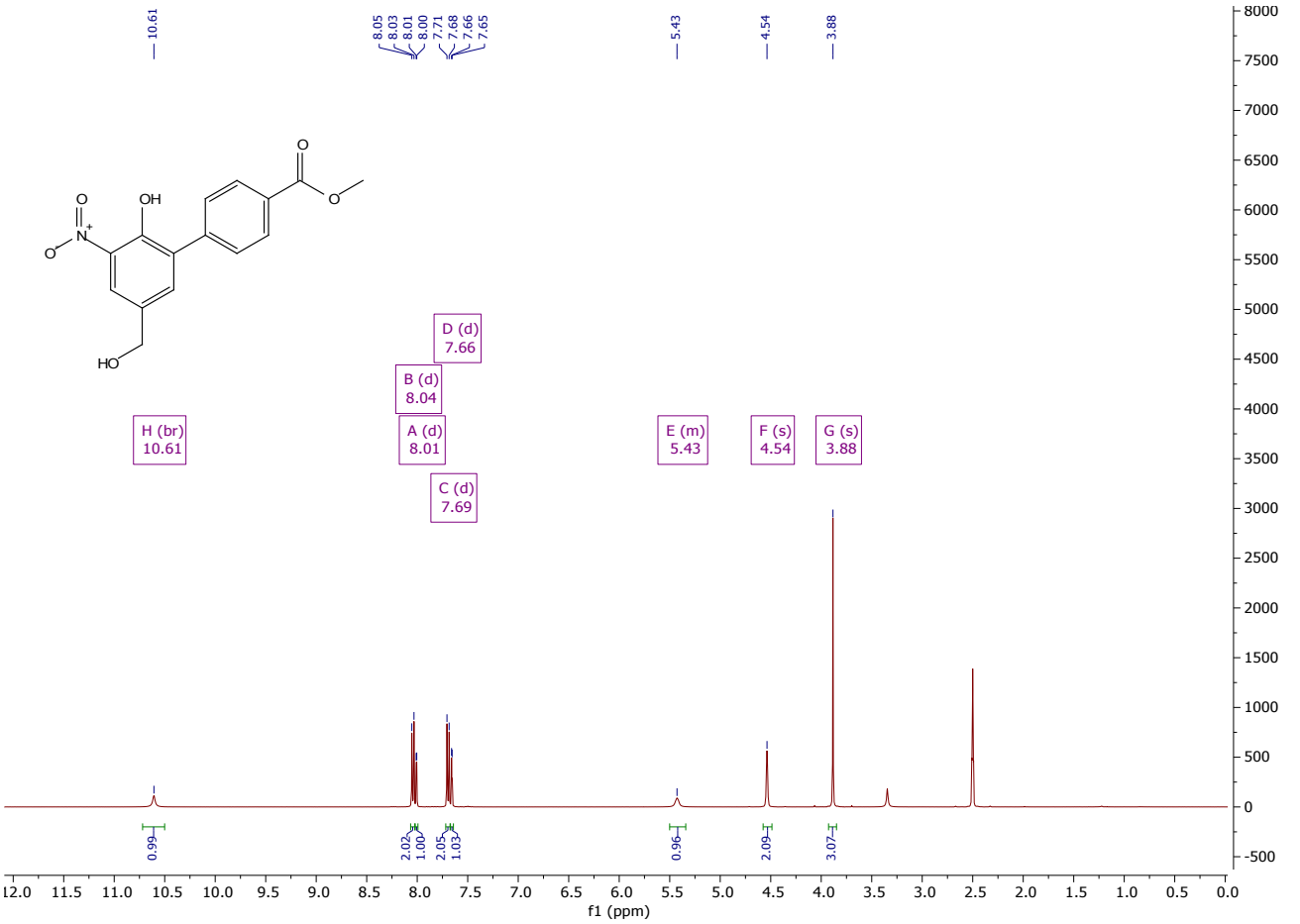


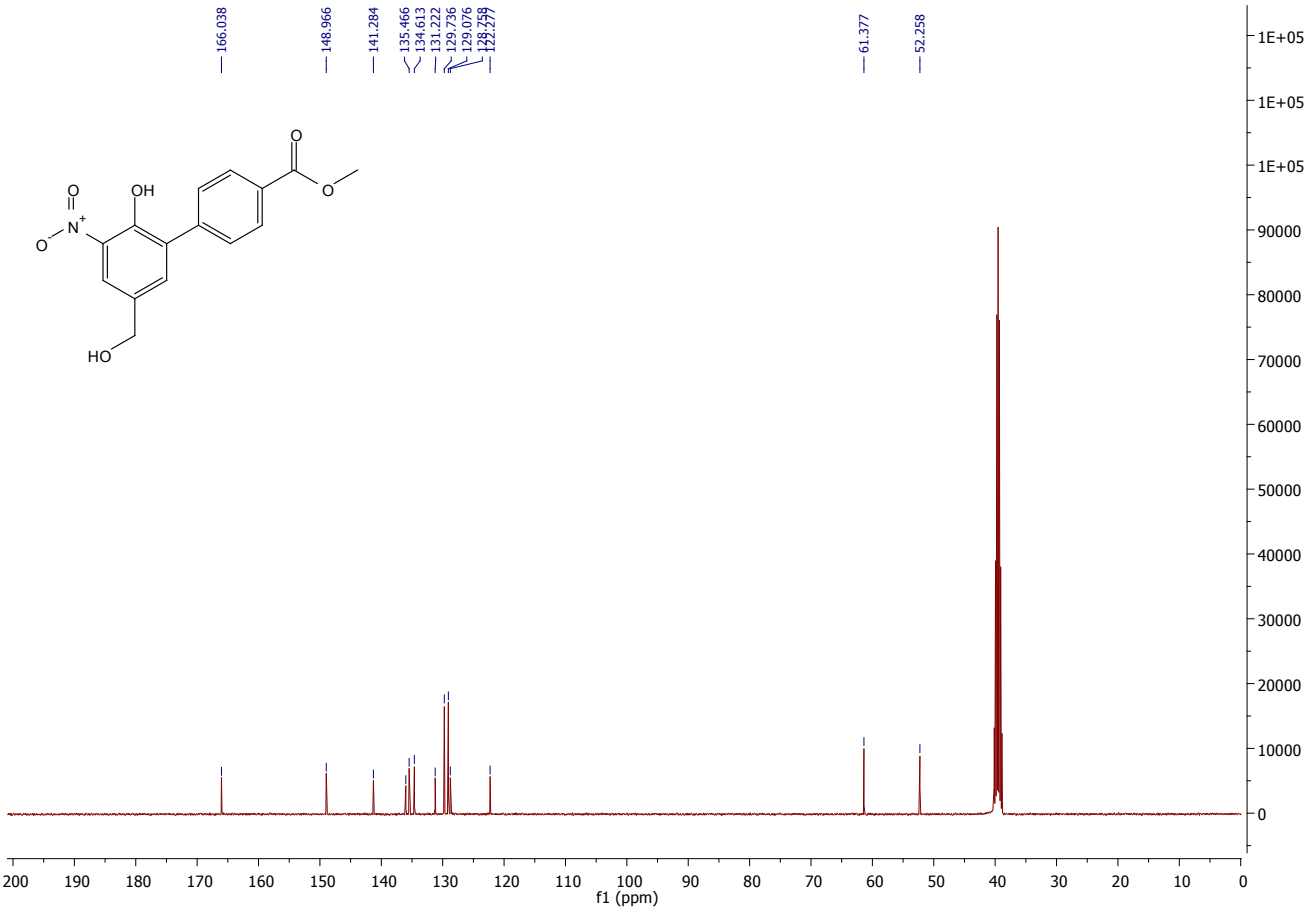


^{1}H-^{1}H COSY spectrum of **5** in DMSO-d_6

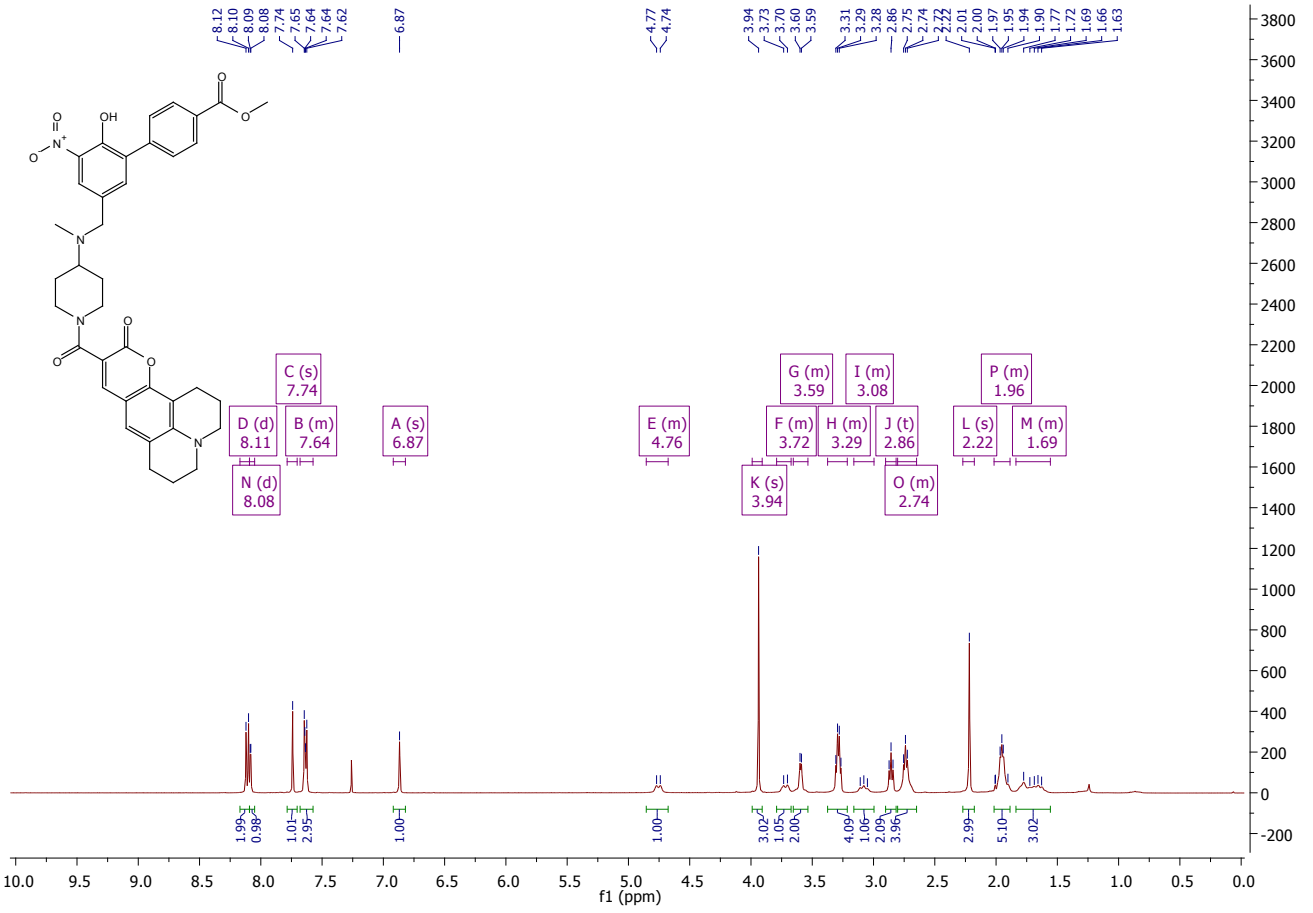


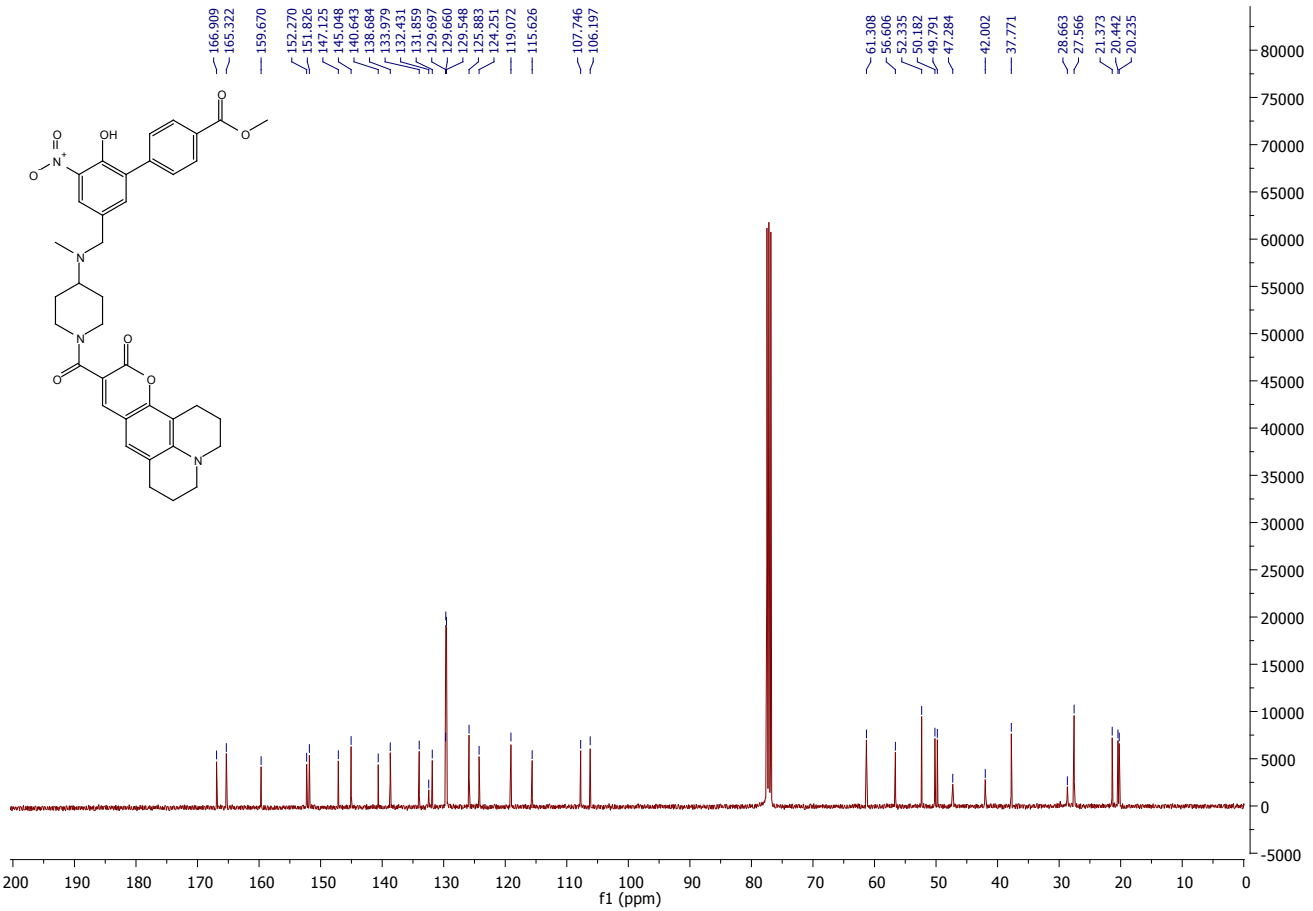
^{1}H and ^{13}C NMR spectra of **6** in DMSO-d_6



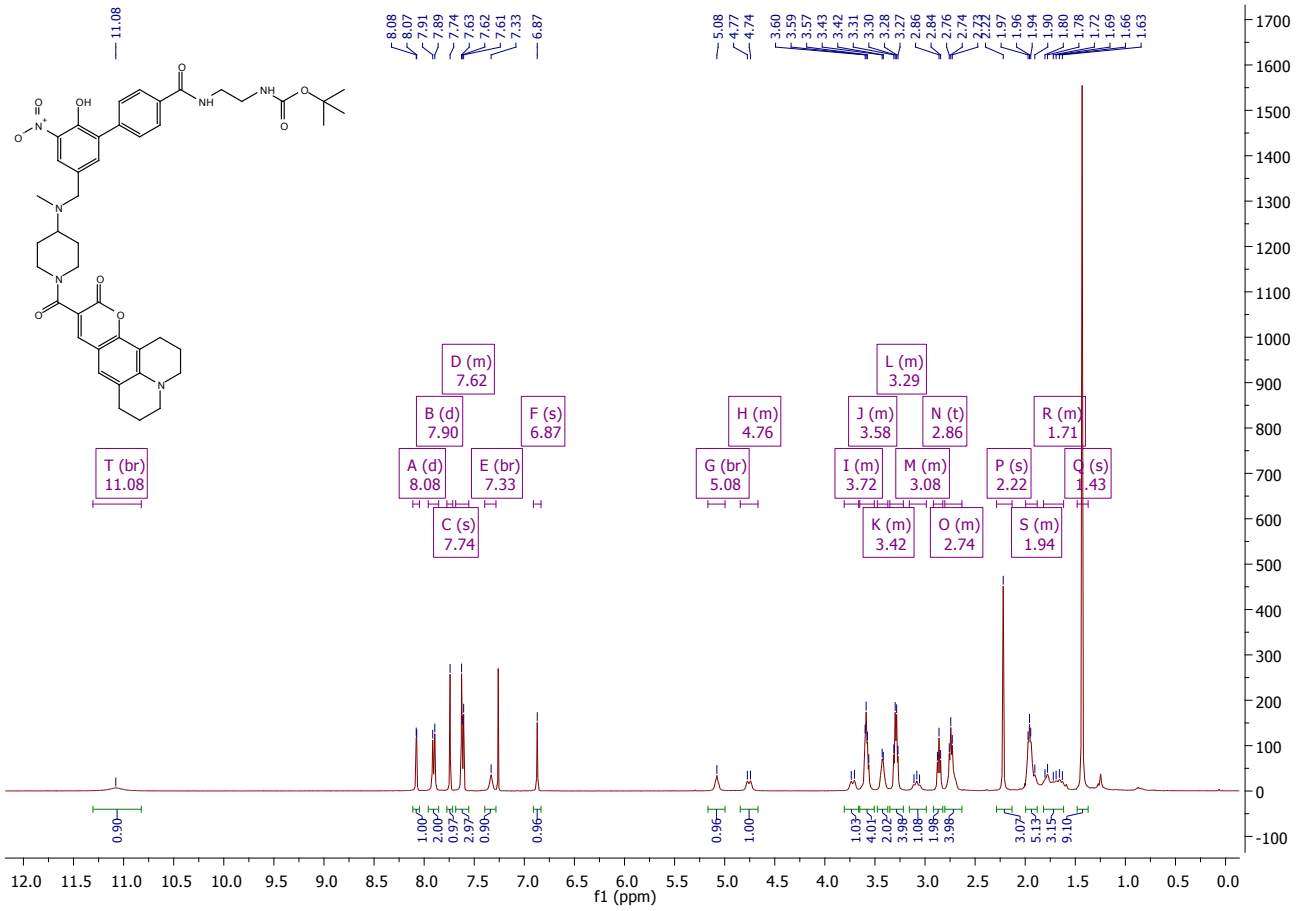


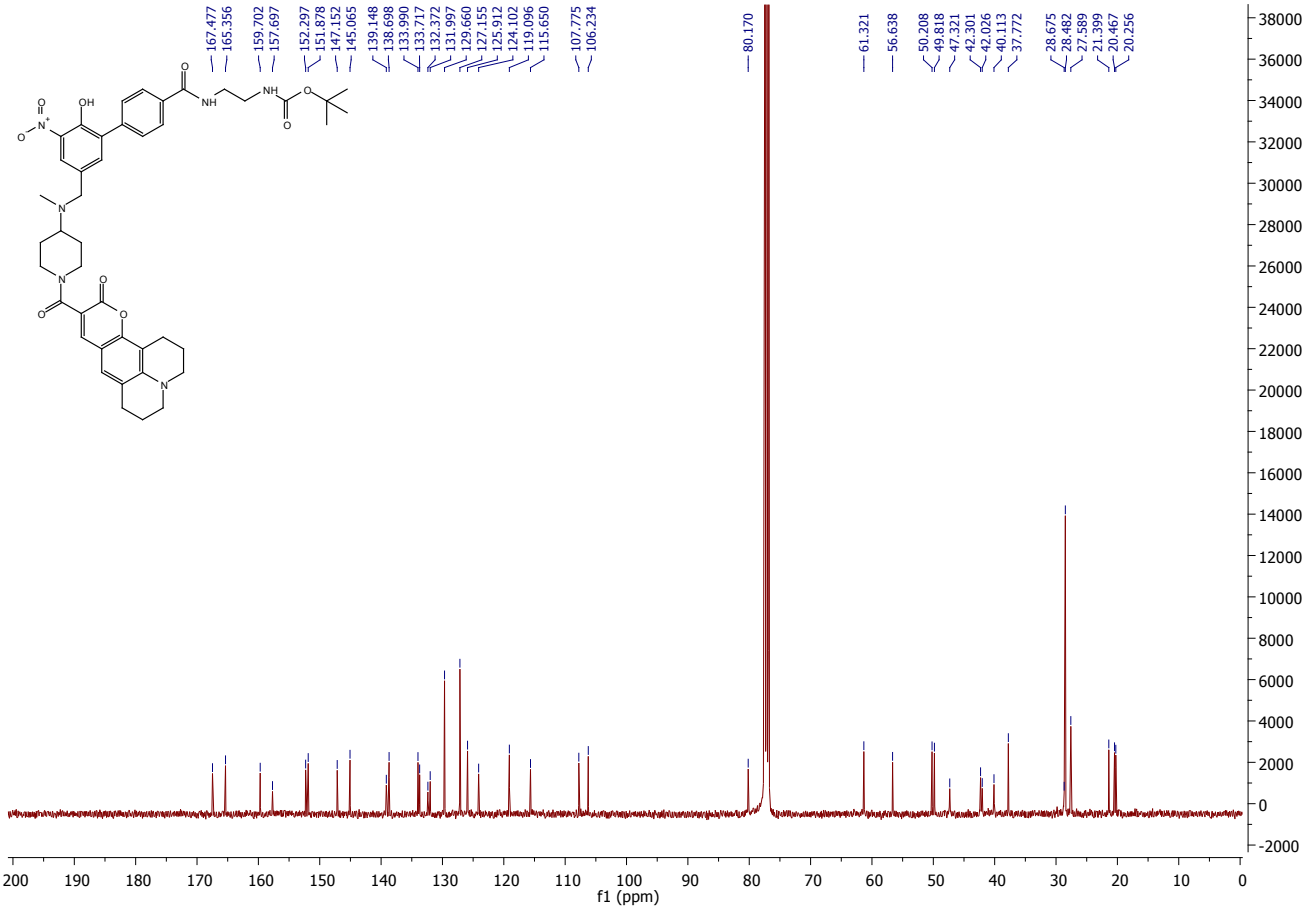
^{1}H and ^{13}C NMR spectra of **7** in $CDCl_3$



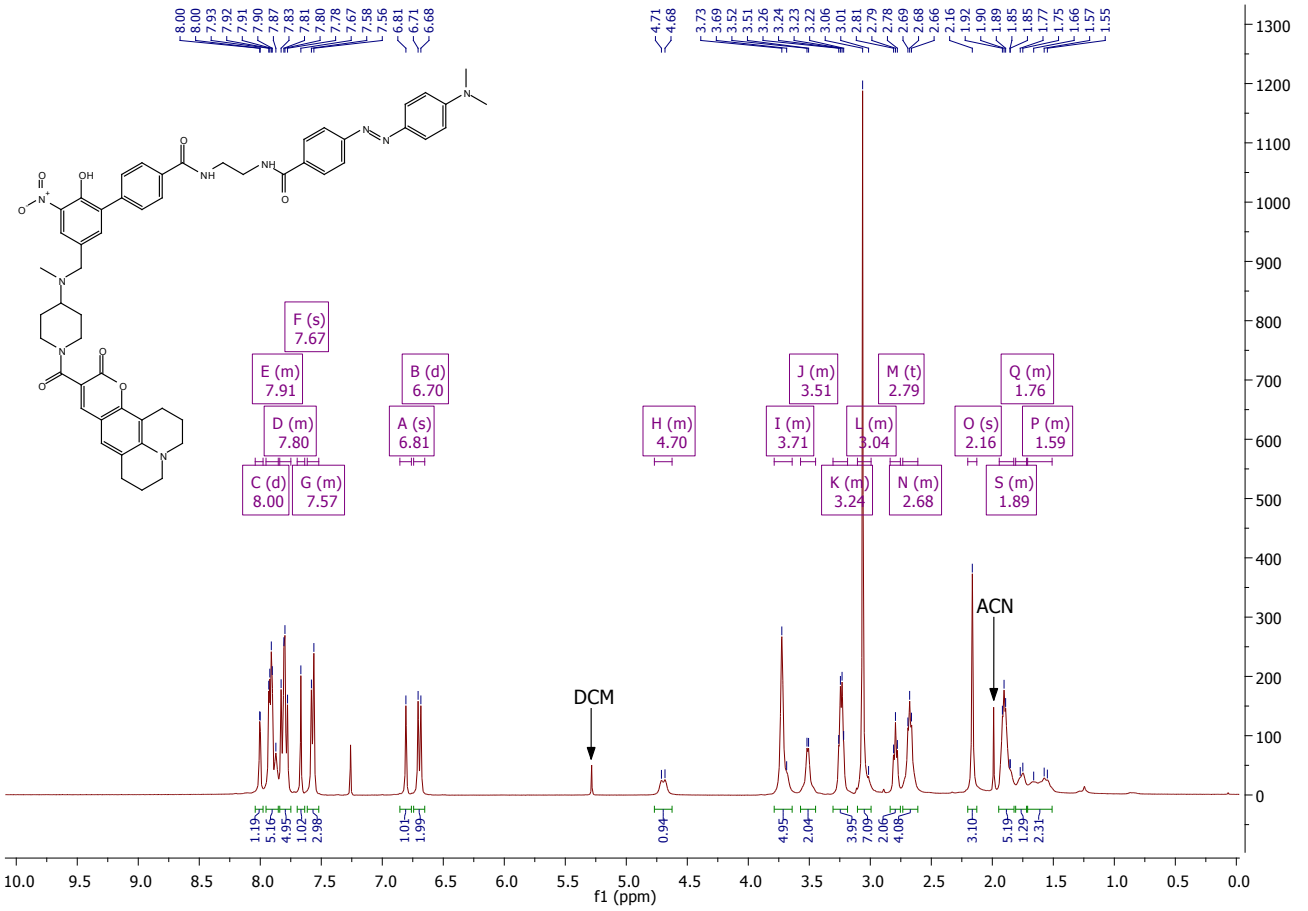


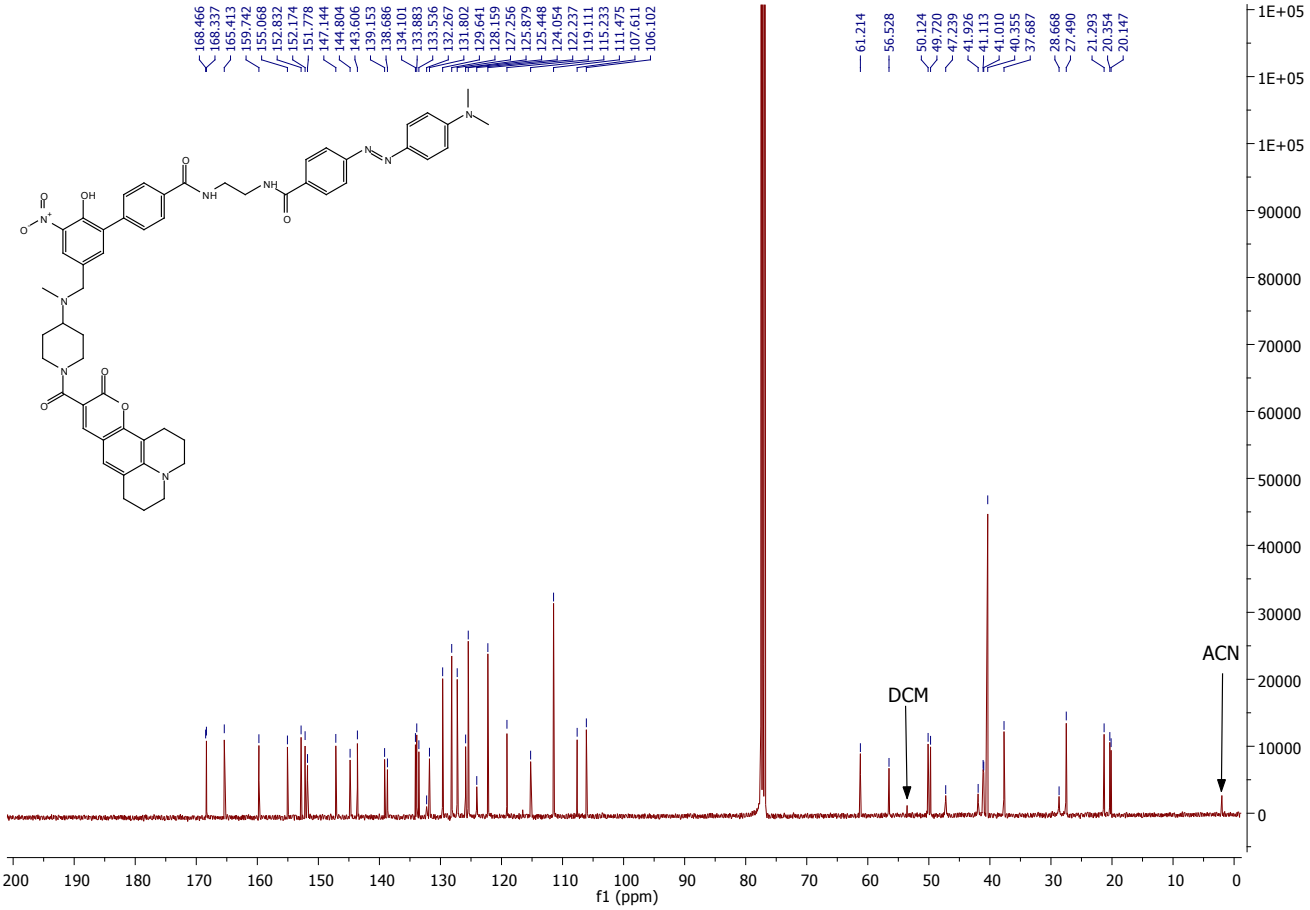
^{1}H and ^{13}C NMR spectra of **8** in $CDCl_3$



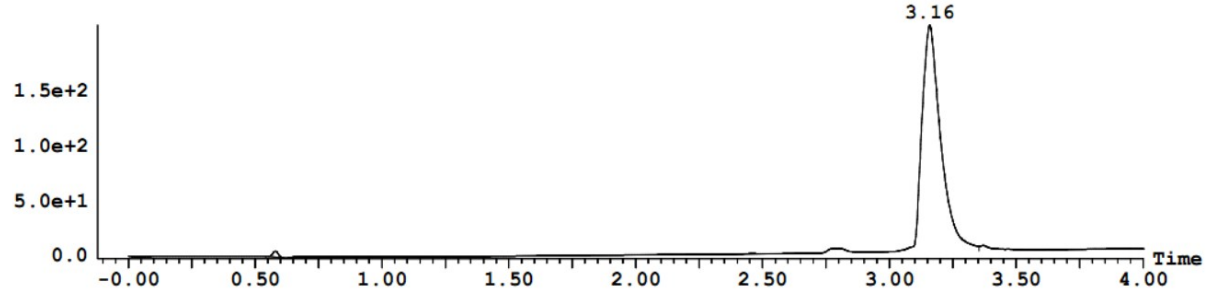


^{1}H and ^{13}C NMR spectra of **9** in $CDCl_3$





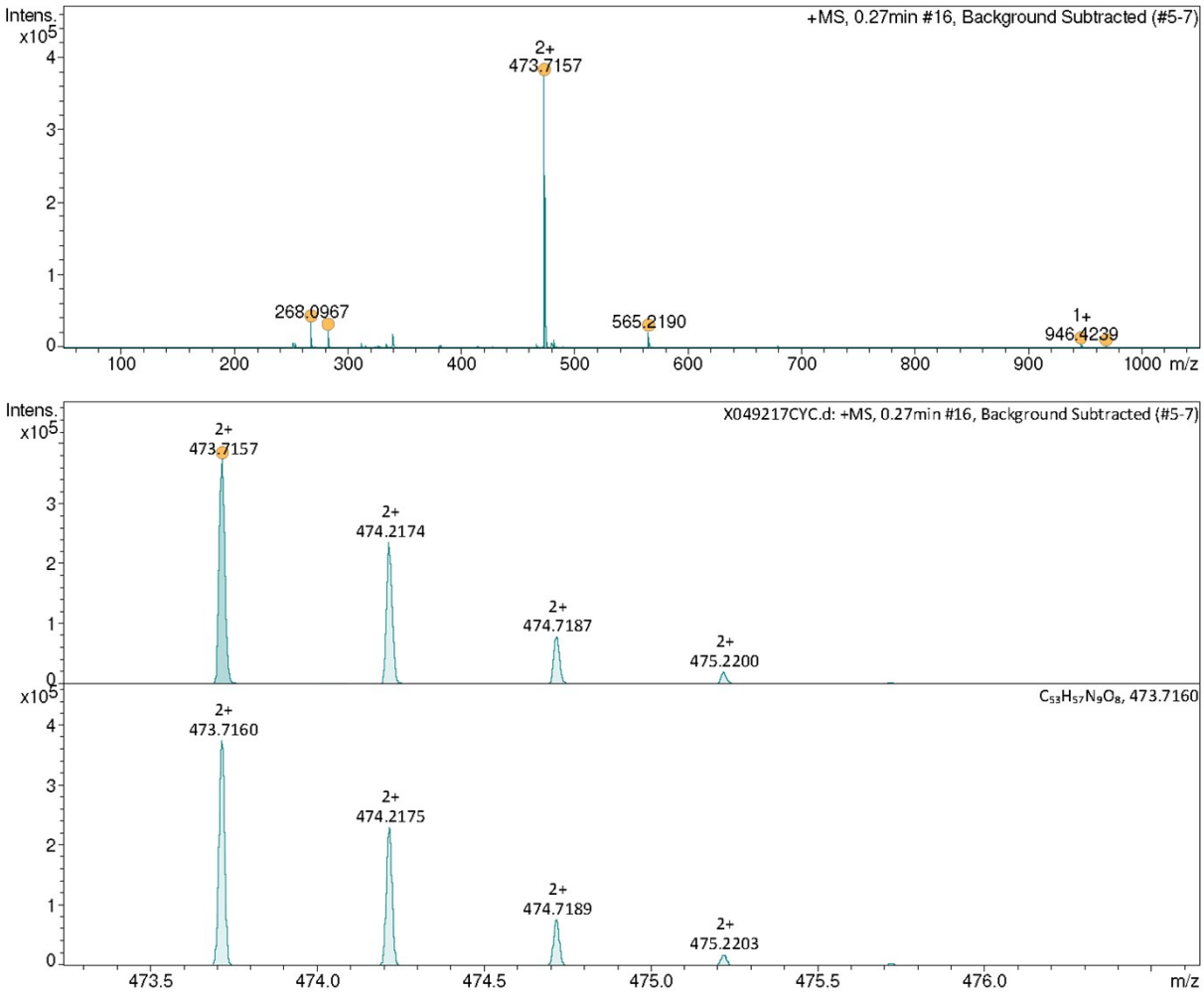
HPLC spectrum and HRMS analysis of probe **9**



HPLC spectrum of **9**

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.6 Bar
Scan Begin	50 m/z	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan End	2500 m/z	Set Collision Cell RF	1800.0 Vpp	Set Dry Gas	7.0 l/min



HRMS analysis of **9**

3. NMR studies of alkylating reaction with methyl iodide

(a) To a solution of compound **3a-e** or **5** (10 mM) in CD_3CN containing 10% D_2O was added methyl iodide (20 equiv.), ^1H NMR spectra were recorded before and after 2 h. (b) To a solution of compound **3a-e** (5 mM) in DMSO-d_6 containing 10% D_2O was added NaOD (1.2 equiv.) and methyl iodide (20 equiv.), successively, ^1H NMR spectra were recorded before and after 2 h. (c) To a solution of compound **3c** (5 mM) in DMSO-d_6 containing 10% D_2O was added NaOD (1.2 equiv.) and methyl iodide (20 equiv.), successively, ^1H NMR spectra were recorded before and after 5, 15, 27, 38, 49, 61, 72, 83, 96, 108, 120 min.

4. Fluorescence response of probe 9 in the presence of alkylating agents

(a) To a solution of **9** (1 mM, 1 equiv.) in DMSO containing 10% water was added methyl iodide (200 mM, 20 equiv.). Then, fluorescence response under UV lamp (Ex@365 nm) was recorded after 5 min. (b) To a solution of **9** (3 μM) in DMSO containing trace water was added alkylating agents (0-20 equiv.). The reaction solution was diluted 5 times with ethanol after 2 h and then fluorescence spectra were recorded (Ex@418 nm).

5. Figures, Tables and Schemes

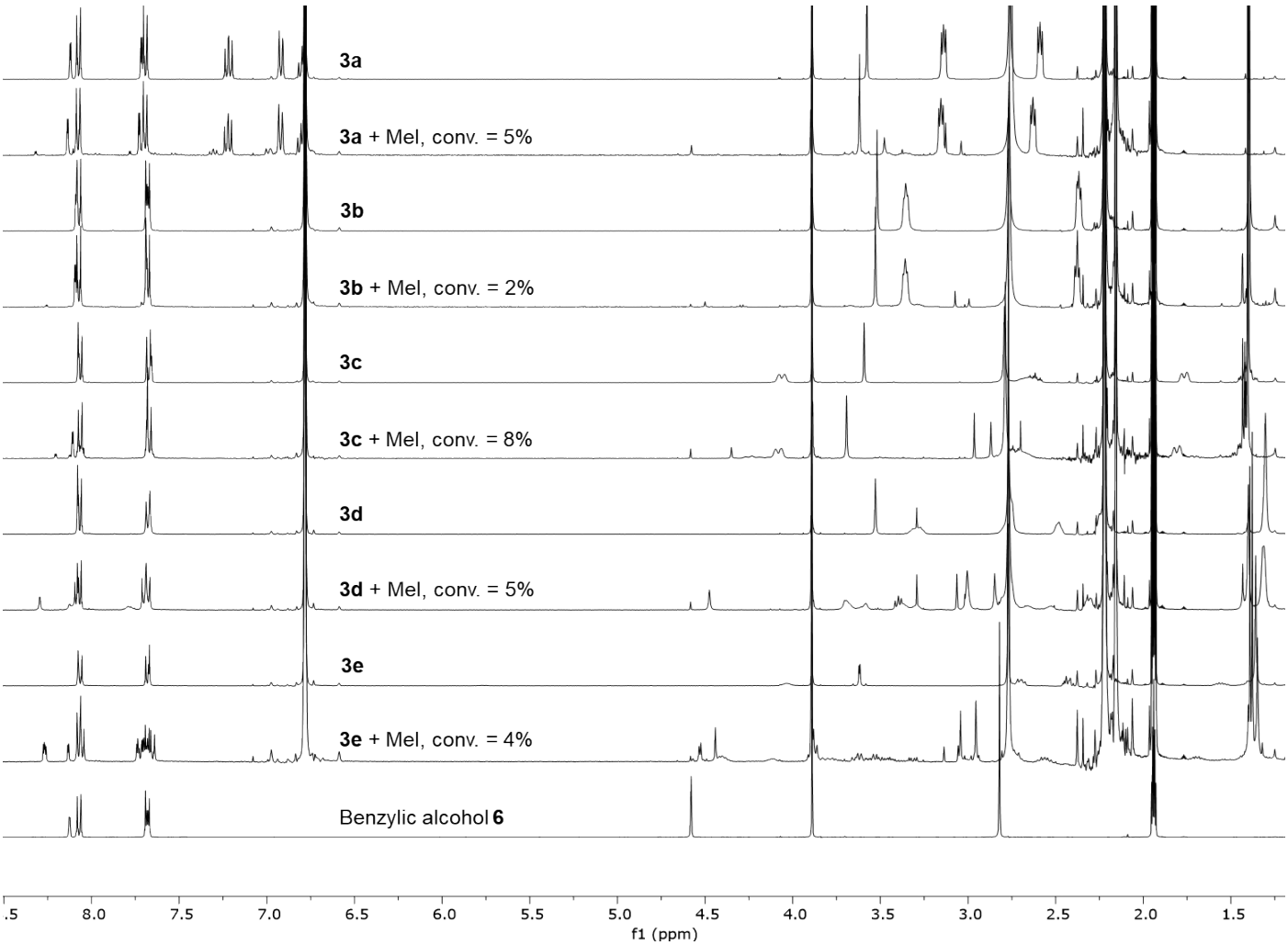


Figure S1. ^1H NMR spectra recorded 2 h after the addition of methyl iodide (20 equiv.) to a solution of amine **3a-e** (10 mM, 1 equiv.) in CD_3CN containing 10% D_2O. Some benzylic alcohol **6** was formed 2 h after adding methyl iodide.

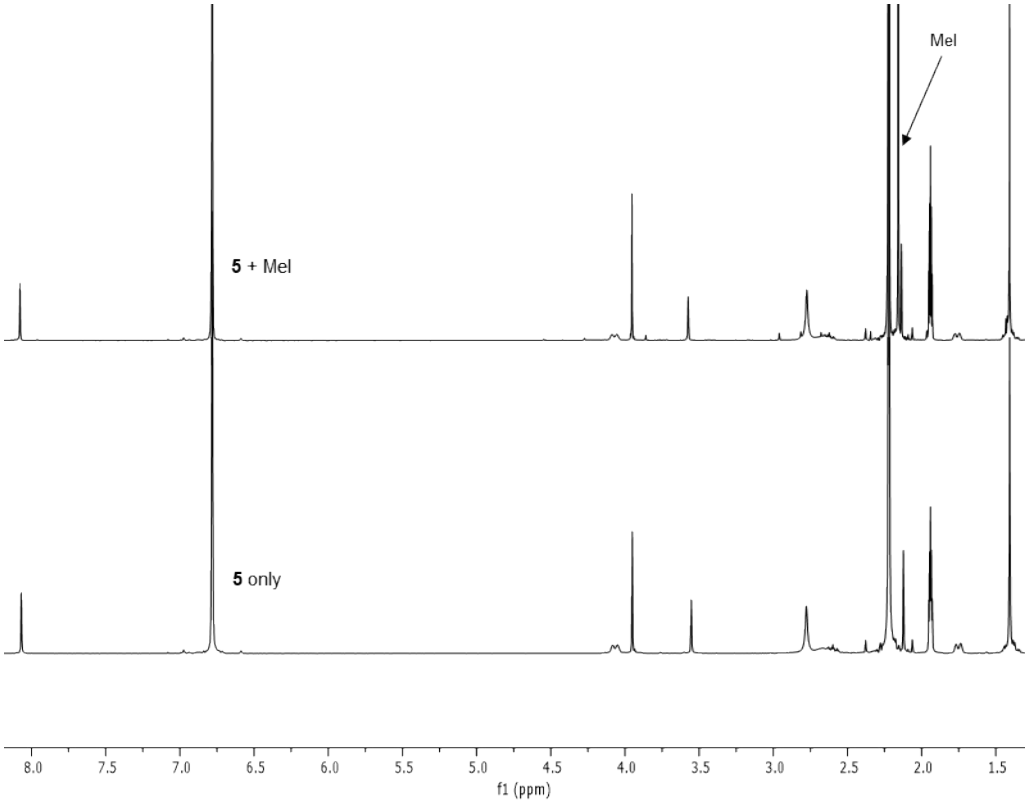


Figure S2. ^{1}H NMR spectra recorded before (lower) and 2 h after the addition of methyl iodide (20 equiv.) to a solution of amine **5** (10 mM, 1 equiv.) in CD_3CN containing 10% D_2O. No significant product was detected.

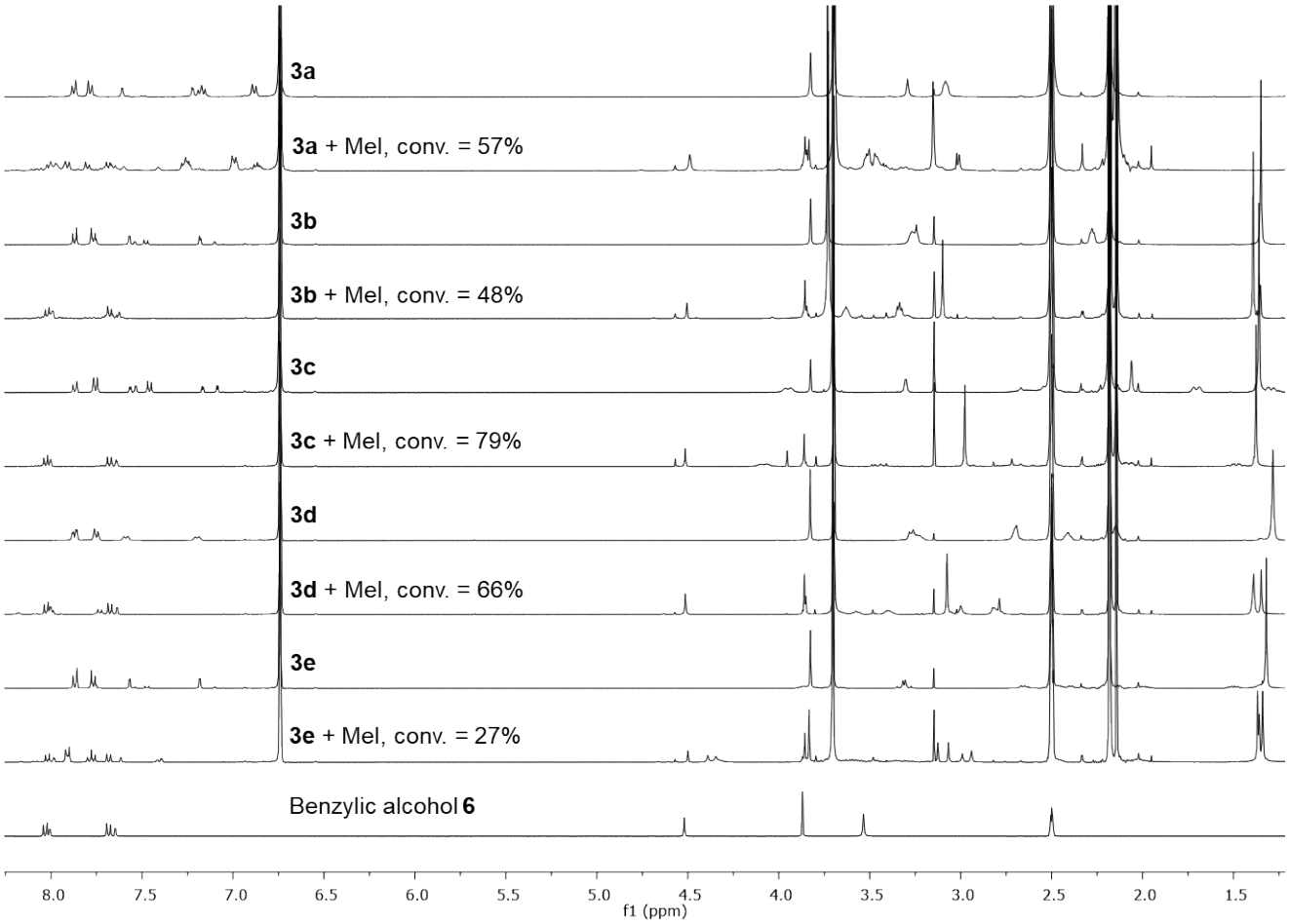


Figure S3. ^{1}H NMR spectra of **3a-e** (5 mM, 1 equiv.) recorded before and 2 h after adding methyl iodide (20 equiv.) in DMSO-d_6 containing 10% D_2O and NaOD (1.2 equiv.).

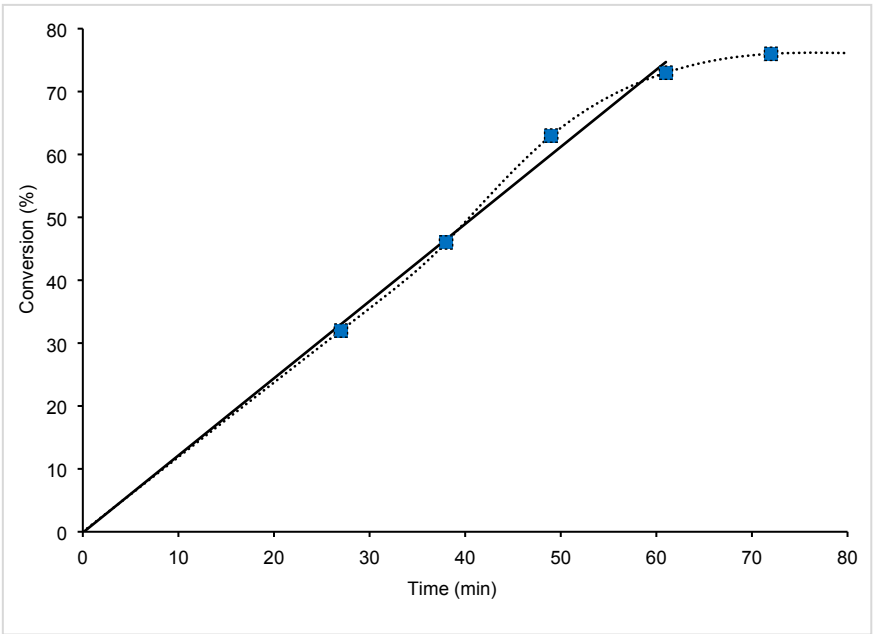


Figure S4. Kinetic study of the conversion of amine **3c** (5 mM) into benzylic alcohol **6** in DMSO-d_6 containing 10% D_2O at room temperature after addition of methyl iodide (20 equiv.) and NaOD (1.2 equiv).

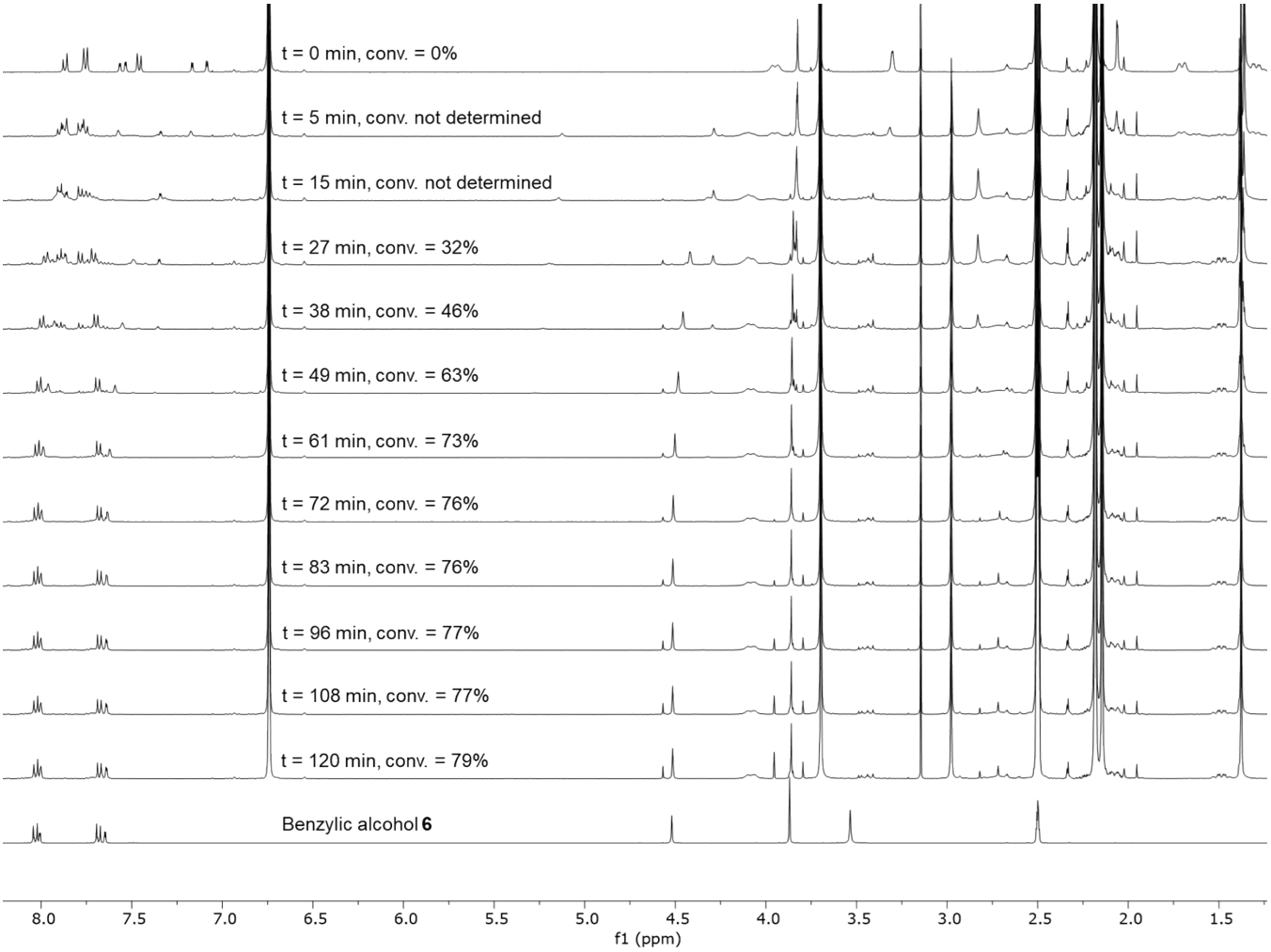


Figure S5. [1]H NMR spectra recorded overtime following the addition of methyl iodide (20 equiv.) to a solution of **5c** (5 mM, 1 equiv.) in DMSO-d_6 containing 10% D_2O and NaOD (1.2 equiv.).

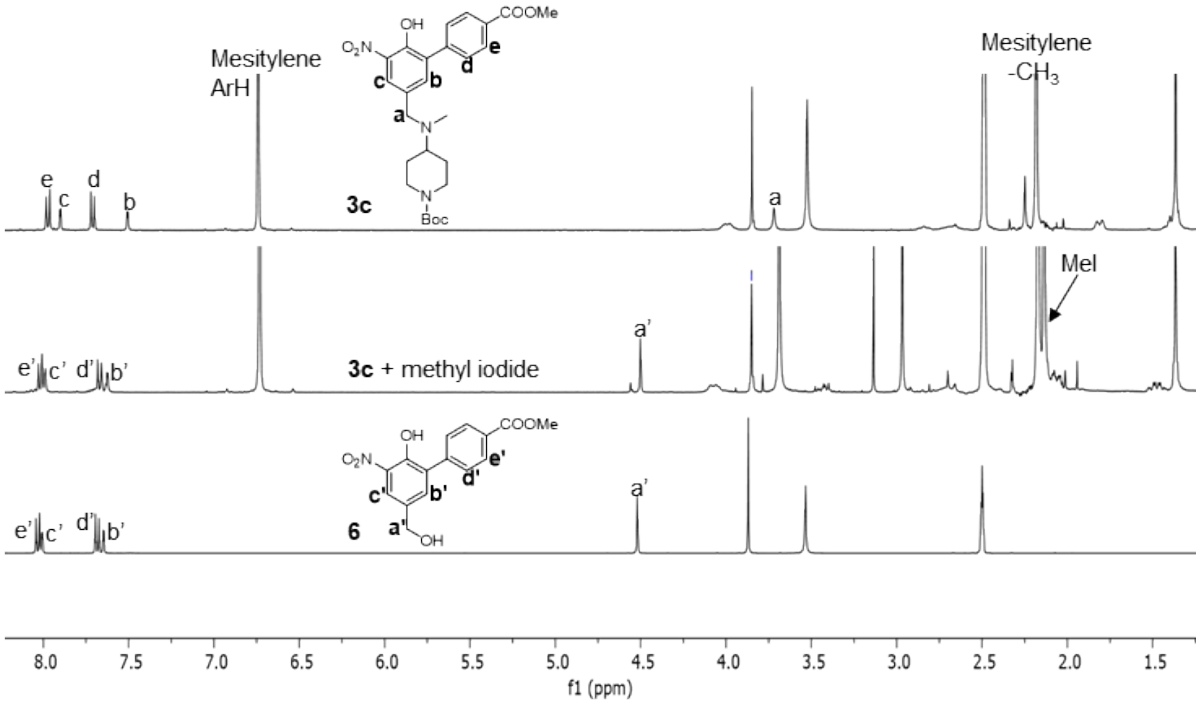


Figure S6. [1]H NMR spectra performed in DMSO-d_6 containing 10% D_2O at room temperature: compound **3c** (upper); compound **3c** after 72 min reaction with methyl iodide (20 equiv.) and NaOD (1.2 equiv.) (middle); (c) benzylic alcohol **6** (lower).

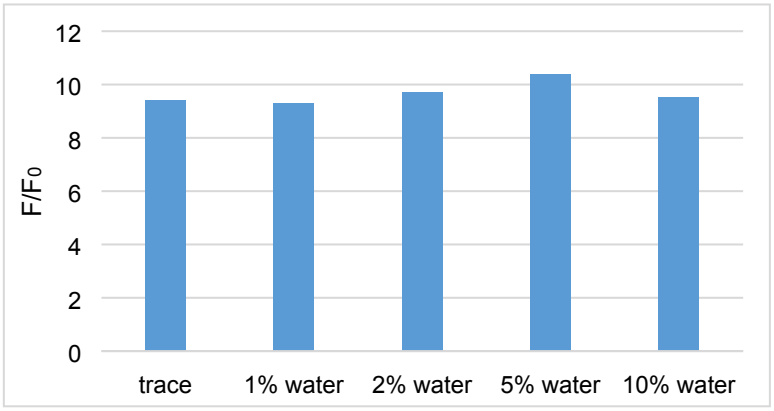


Figure S7. Fluorescence response (Ex@418 nm, Em@477 nm) recorded 2 h after treatment of probe **9** (3 μM in DMSO containing trace to 10% water) with methyl iodide (60 μM). Data are expressed as F/F_0.

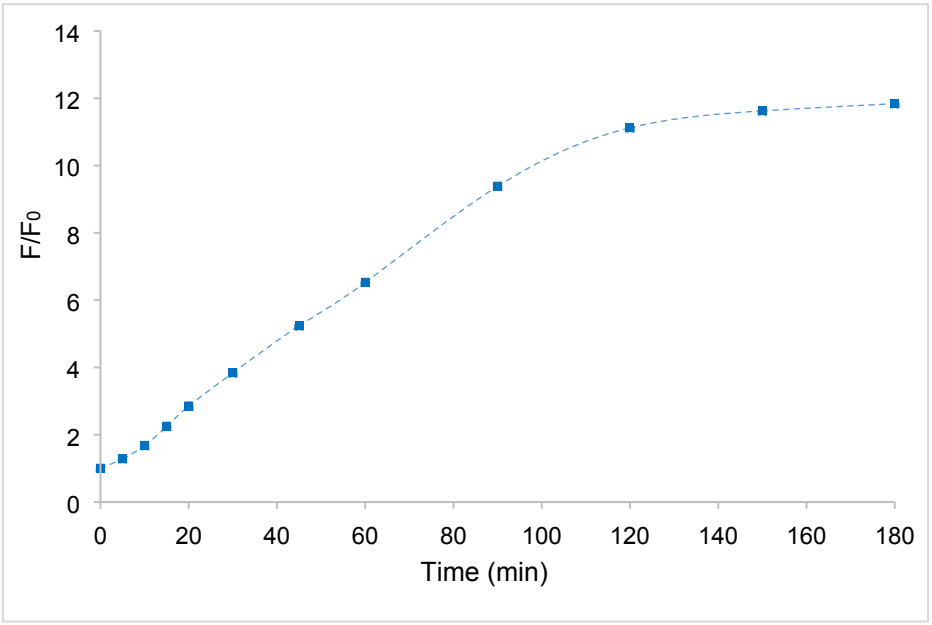


Figure S8. Fluorescence response (Ex@418 nm, Em@477 nm) recorded 0-180 min after treatment of probe **9** (3 μM in DMSO containing trace water) with methyl iodide (60 μM). Data are expressed as F/F_0.

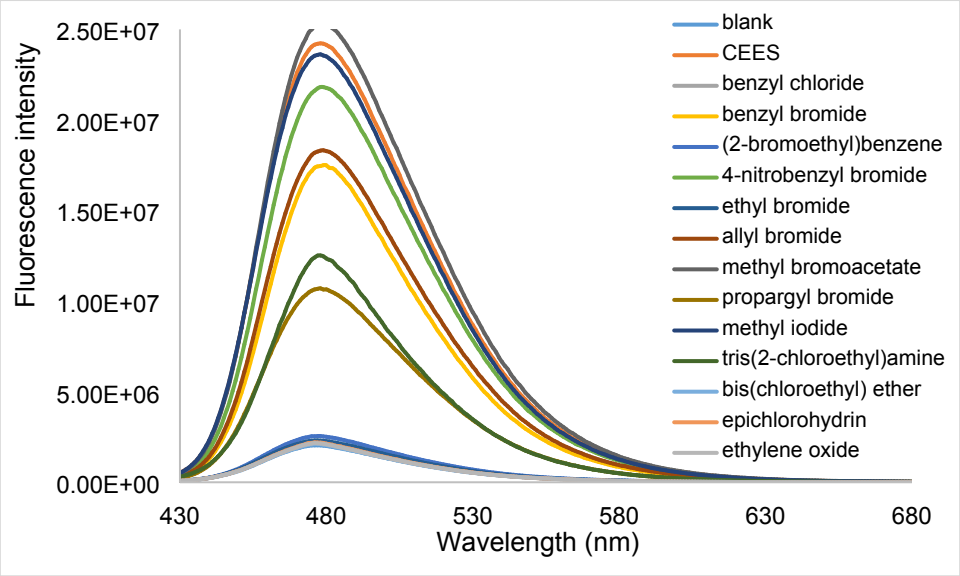
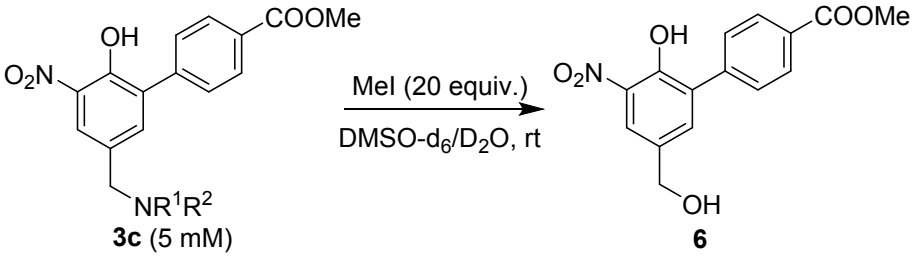


Figure S9. Fluorescence spectra (Ex@418 nm) recorded 2 h after treatment of probe **9** (3 μM in DMSO containing trace water) with different alkylating agents (60 μM).

Table S1. Optimizing reaction conditions for NMR studies using amine **3c**.



Reaction conditions	DMSO-d_6 + 5% D_2O	DMSO-d_6 + 10% D_2O	DMSO-d_6 + 15% D_2O	DMSO-d_6 + 20% D_2O	DMSO-d_6 + 10% D_2O + NaOD (1.2 equiv.)
Conversion (%)	61%	70%	67%	62%	79%