Supplementary Information

Fluorescent trimethoprim conjugate probes to assess drug accumulation in wild type and mutant *Escherichia coli*

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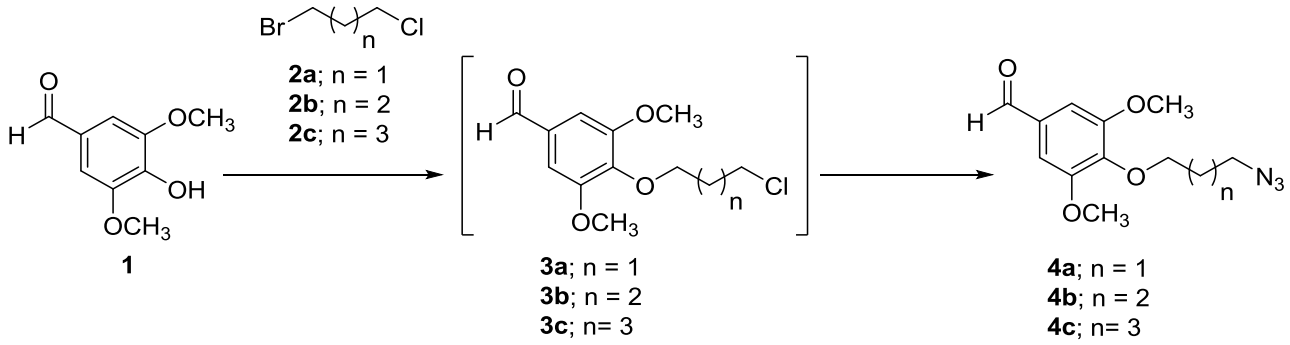
General

All materials, unless otherwise noted, were obtained from commercial suppliers and used without further purification. Non-aqueous reactions were conducted under an inert atmosphere of nitrogen. Analytical LCMS was performed on a Shimadzu LCMS 2020 using 0.05% formic acid in water as solvent A and 0.05% formic acid in acetonitrile as solvent B. LCMS conditions (solvent A = H_2O + 0.05% formic acid, solvent B = acetonitrile + 0.05% formic acid): Column Zorbax Eclipse XDB-Phenyl, 3.0 × 100 mm, 3.5 μ: Flow: 1 mL/min: Gradient timetable: 0.00 min, 5% B; 3.00 min, 100% B; 3.7 min, 100% B; 5.00 min, 5% B. Biotage Initiator microwave was used for Cu-catalyzed azide-alkyne cycloaddition. Column chromatography was performed using silica gel 60 (0.063–0.200 mm), 70–230 mesh ASTM. Gilson PLC 2020 and Grace Reveleris X2 chromatography systems were used for compound purification. Commercially available cartridges were used for MPLC chromatography (Reveleris C18 Reversed-Phase 12 g cartridge), while Gilson purifications used an XTerra® Prep RP18 5 μM, 19 × 100 mm column. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were obtained using a Bruker Avance-600 spectrometer equipped with a TXI cryoprobe. Chemical shifts are reported relative to the residual solvent signals in parts per million (δ) ($CDCl_3$: ^{1}H: δ 7.27, ^{13}C: δ 77.2; DMSO-d_6: ^{1}H: δ 2.50, ^{13}C: δ 39.5). High resolution mass spectrometry (HRMS) was performed on a Bruker Micro TOF mass spectrometer (Ultimate 3000) using (+)-ESI calibrated to HCOONa.

Synthesis

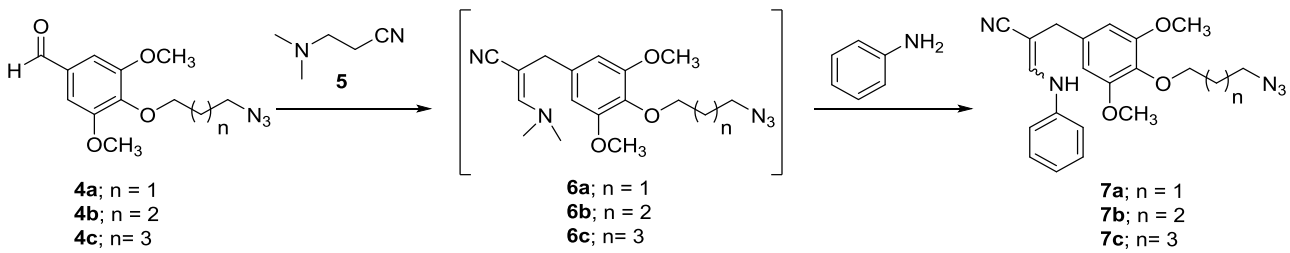
General procedure A for alkylation and azide replacement of syringaldehyde; 4a-c



A mixture of syringaldehyde **1** (5 g, 27.45 mmol) and K_2CO_3 (4.17 g, 30.19 mmol) in DMF (50 mL) was heated at 60 °C for 30 min. A solution of bromo-chloro alkane linker **2a-c** (1.5 eq.) in DMF (30 mL) was added dropwise to the reaction mixture (the mixture became cloudy) and the mixture was left stirring at 60 °C overnight. The mixture was neutralized with 5 M HCl and then extracted with CH_2Cl_2, dried over $MgSO_4$, and evaporated to dryness under reduced pressure to yield the crude product **3a-c**. The crude product was used for further reaction without purification.

The mixture of intermediate **3a-c**, sodium azide (5 eq.), and sodium iodide (1 eq.) in DMF (80 mL) was stirred at 100 °C for 16 h. The reaction mixture was concentrated under reduced pressure, and extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated under reduced pressure to yield the crude product **4a-c**. Purification by column chromatography (silica gel, 30% EtOAc/hexane as eluent) gave compounds **4a-c** as a yellow solid.

General procedure B for enamine; 7a-c



4a; n = 1
4b; n = 2
4c; n= 3

6a; n = 1
6b; n = 2
6c; n= 3

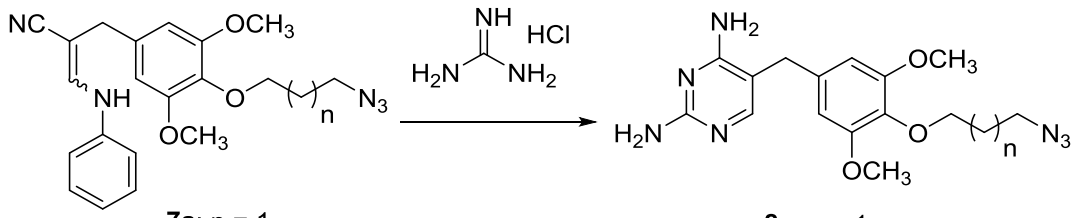
7a; n = 1
7b; n = 2
7c; n= 3

A mixture of DMSO (22 mL), methanol (2.2 mL) (10:1 v/v), solid potassium hydroxide (0.23 eq.) and 3-(dimethylamino)propanenitrile **5** (1.5 eq.) was heated to 35 °C. To this mixture was slowly added a solution of compound **4a-c** (21.23 mmol, 1 eq.) in DMSO (30 mL), and then the reaction solution was heated to 45 °C with vigorous stirring for 5 h or until compound **4a-c** was not observed on LCMS.

After generating the intermediate **6a-c**, the solution was cooled to 30 °C and diluted hydrochloric acid (2 N) was added slowly to the mixture to adjust the pH = 3.0-3.5. Aniline (1.02 eq.) was added, forming a basic solution, to which 2 M HCl was added to keep the pH at 3.0.

The mixture was then vigorously stirred for 1 h at 120 °C then cooled to ambient temperature. Water (14 mL) was added, and the reaction mixture was concentrated under reduced pressure to yield the crude product **7a-c**. Purification by column chromatography (silica gel, 30% EtOAc/hexane) gave compounds **7a-c** as yellow solids.

General procedure C for cyclisation of enamine; 8a-c



7a; n = 1
7b; n = 2
7c; n= 3

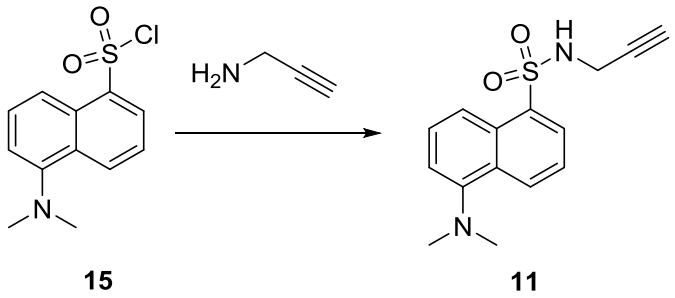
8a; n = 1
8b; n = 2
8c; n= 3

Sodium methoxide (15 eq.) was added to a suspension of guanidine hydrochloride (15 eq.) in ethanol (300 mL). The reaction mixture was heated to reflux for 30 min, cooled, and filtered into a reaction flask containing compound **7a-c** (14.97 mmol, 1 eq.) dissolved in DMSO (100 mL). The reaction mixture was stirred under reflux for 16 h, then diluted with water, extracted with chloroform, dried over $MgSO_4$, and concentrated under reduced pressure, to produce a white solid precipitate. The precipitate was filtered and then washed with chloroform to give compounds **8a-c** as white solids. The filtrate was collected and repurified by MPLC over C18 silica gel (Grace Reveleris, A: H_2O (0.1% TFA), B: ACN (0.1% TFA), 0–100% B) to give additional **8a-c**.

General procedure D for CuAAC reaction; 12a-c, 13a-c, 14a-c

To a solution of azide-TMP (1 eq.) and alkyne-fluorophore (1.2 eq.) in DMF (5 mL) was added aqueous $CuSO_4$ (0.2 eq. for NBD alkyne **9**, 0.5 eq. for DMACA and DNS alkynes **10** and **11**, dissolved in 1 mL of water), and aqueous sodium ascorbate (0.4 eq. for NBD, 1 eq. for DNS and DMACA, dissolved in 1 mL of water). The reaction mixture was stirred in a microwave reactor at 100 °C for 30 min for NBD alkyne **9** and 1 h for DMACA and DNS and DNS alkynes **10** and **11**. The reaction mixture was concentrated under reduced pressure to yield the crude product. The crude compounds were purified by MPLC over C18 silica gel (Grace Reveleris, A: H_2O (0.1% TFA), B: ACN (0.1% TFA), 0–100% B). Some compounds required repurification using the Gilson PLC 2020.

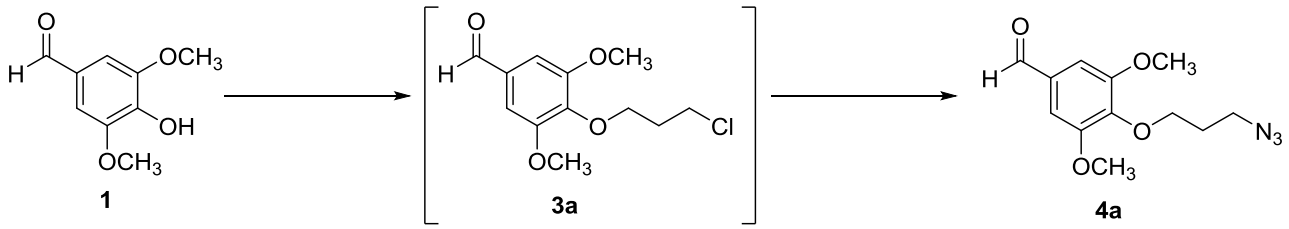
5-(Dimethylamino)-N-(prop-2-yn-1-yl)naphthalene-1-sulfonamide; **11**



15 **11**

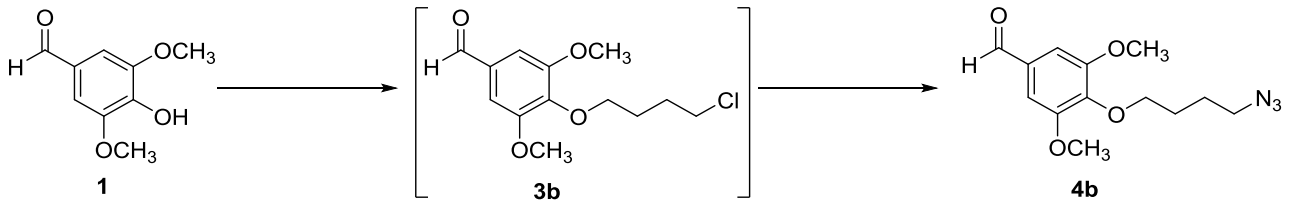
To a solution of 5-(dimethylamino)naphthalene-1-sulfonyl chloride **15** (900 mg, 3.34 mmol) at 4 °C was added propagylamine (231 μL, 3.61 mmol) and triethylamine (1.266 mL, 9.08 mmol). After 16 h at RT the reaction mixture was concentrated under reduced pressure to yield the crude product, which was purified by MPLC over C18 silica gel (Grace Reveleris, A: H_2O (0.1% TFA), B: ACN (0.1% TFA), 0–100% B) to give a green oil (930 mg, 97%). LCMS: R_t = 3.38 min, @ 254 nm, $[M + H]^+$ = 289.0; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.58 (d, *J* = 8.5 Hz, 1H), 8.31 (d, *J* = 8.7 Hz, 1H), 8.30 (dd, *J* = 7.4, 1.1 Hz, 1H), 7.61 (dd, *J* = 8.6, 7.7 Hz, 1H), 7.57 (dd, *J* = 8.5, 7.4 Hz, 1H), 7.26 (d, *J* = 7.6 Hz, 1H), 4.89 (t, *J* = 5.9 Hz, 1H), 3.79 (dd, *J* = 6.0, 2.5 Hz, 2H), 2.91 (s, 6H), 1.93 (t, *J* = 2.5 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 151.2, 134.5, 130.7, 130.2, 130.0, 129.8, 128.7, 123.7, 119.4, 115.7, 73.0, 45.7, 33.2; (+)-ESI-HRMS calc for $C_{15}H_{17}N_2O_2S$ $[M+H]^+$: 289.1011, found 289.1013.

4-(3-Azidopropoxy)-3,5-dimethoxybenzaldehyde; **4a**



General procedure **A**. Syringaldehyde **1** (5 g, 27.45 mmol) was reacted with 1-bromo-3-chloropropane **2a** to give intermediate **3a**; LCMS: R_t = 3.51 min, @ 254 nm, $[M + H]^+$ = 259.1, 261.0. Intermediate **3a** was reacted with sodium azide to give **4a** (6.38 g, 94 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.87 (s, 1H), 7.13 (s, 2H), 4.15 (t, *J* = 5.8 Hz, 2H), 3.92 (s, 6H), 3.61 (t, *J* = 6.6 Hz, 2H), 2.00 (tt, *J* = 6.3, 6.3 Hz, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 191.2, 154.0, 142.1, 132.1, 106.7, 70.2, 56.4, 48.4, 29.9; (+)-ESI-HRMS calc for $C_{12}H_{16}N_3O_4$ $[M+H]^+$: 266.1141, found 266.1146.

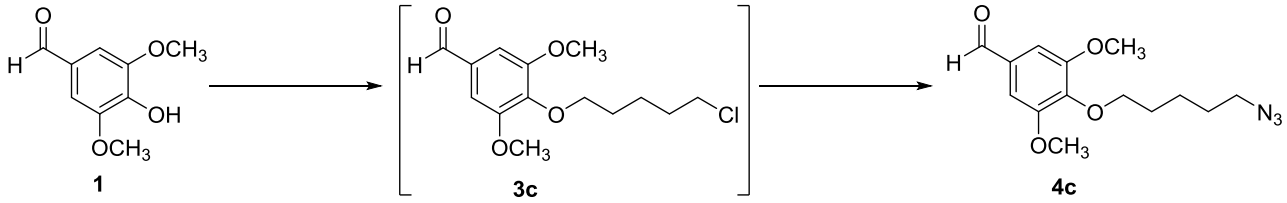
4-(4-Azidobutoxy)-3,5-dimethoxybenzaldehyde; **4b**



General procedure **A**. Syringaldehyde (5 g, 27.45 mmol) was reacted with 1-bromo-4-chloropropane **2b** to give intermediate **3b**; LCMS: R_t = 3.59 min, @ 254 nm, $[M + H]^+$ = 273.0, 274.7. Inthermedaite **3b** was reacted with sodium azide to give **4b** (6.20 g, 81 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.88 (s, 1H), 7.13 (s, 2H), 4.10 (t, *J* = 5.9 Hz, 2H), 3.92 (s, 6H), 3.38 (t, *J* = 6.6 Hz,

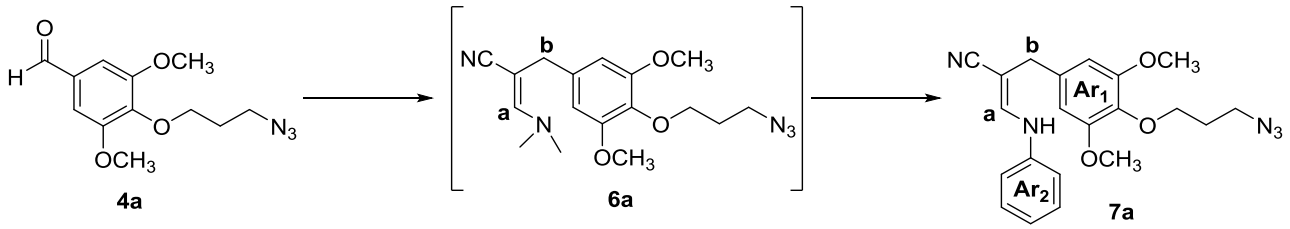
2H), 1.87-1.85 (m, 4H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 191.3, 154.0, 142.8, 131.9, 106.8, 72.8, 56.4, 51.3, 27.4, 25.6; (+)-ESI-HRMS calc for $C_{13}H_{18}N_3O_4$ $[M+H]^+$: 280.1297, found 280.1295.

4-((5-Azidopentyl)oxy)-3,5-dimethoxybenzaldehyde; **4c**



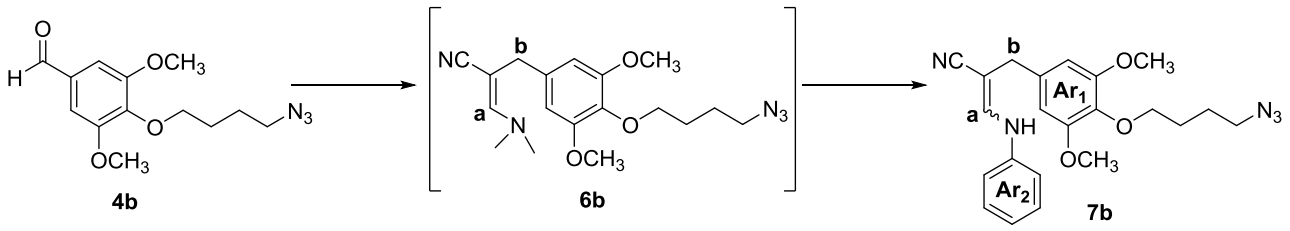
General procedure **A**. Syringaldehyde (5 g, 27.45 mmol) was reacted with 1-bromo-5-chloropropane **2c** to give intermediate **3c**; LCMS: R_t = 3.68 min, @ 254 nm, $[M + H]^+$ = 287.1, 288.8. Intermediate **3c** was reacted with sodium azide to give **4c** (5.91 g, 73 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.87 (s, 1H), 7.13 (s, 2H), 4.08 (t, *J* = 6.4 Hz, 2H), 3.92 (s, 6H), 3.30 (t, *J* = 6.8 Hz, 2H), 1.80 (tt, *J* = 6.8, 6.8 Hz, 2H), 1.68 (tt, *J* = 6.8, 6.8 Hz, 2H), 1.62-1.57 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 191.3, 154.0, 143.0, 131.8, 106.8, 73.3, 56.4, 51.6, 29.8, 28.8, 23.3; (+)-ESI-HRMS calc for $C_{14}H_{20}N_3O_4$ $[M+H]^+$: 294.1454, found 294.1443.

(E)-2-(4-(3-Azidopropoxy)-3,5-dimethoxybenzyl)-3-(phenylamino)acrylonitrile; **7a**



General procedure **B**. **4a** (6.29 g, 23.71 mmol) was converted to **7a** (6.08 g, 65 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.33-7.30 (m, 2H), 7.15 (d, *J* = 13.1 Hz, 1H), 7.02 (tt, *J* = 7.5, 1.0 Hz, 1H), 6.88-6.87 (m, 2H), 6.76 (d, *J* = 13.1 Hz, 1H), 6.47 (s, 2H), 4.04 (t, *J* = 5.8 Hz, 2H), 3.86 (s, 6H), 3.61 (t, *J* = 6.7 Hz, 2H), 3.45 (s, 2H), 1.99 (tt, *J* = 5.9, 5.9 Hz, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 153.7, 141.7, 140.3, 135.9, 134.3, 130.0, 122.8, 119.2, 115.3, 105.5, 82.3, 70.1, 56.3, 48.7, 37.0, 29.9; (+)-ESI-HRMS calc for $C_{21}H_{24}N_5O_3$ $[M+H]^+$: 394.1879, found 394.1859.

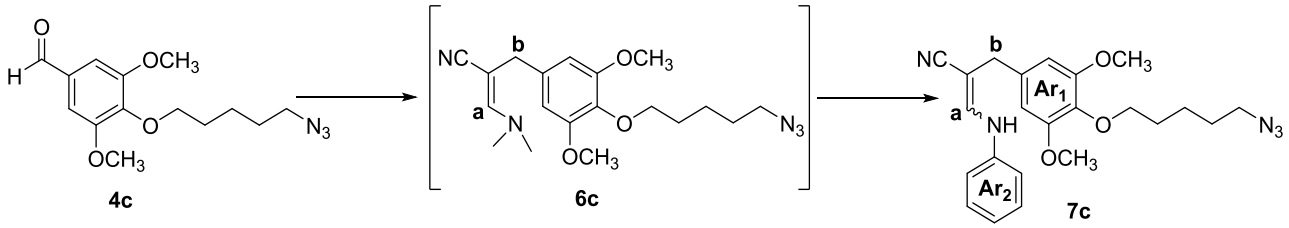
(E)-2-(4-(4-azidobutoxy)-3,5-dimethoxybenzyl)-3-(phenylamino)acrylonitrile; **7b**



General procedure **B**. **4b** (5.93 g, 21.23 mmol) was converted to **7b** (6.12 g, 71 %) as a mixture of *E* and *Z* isomers. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.13, 9.12 (d, *J* = 13.0, 12.6 Hz, 1H, NH), 7.66, 7.64 (t, *J* = 13.0, 12.6 Hz, 1H, H_a), 7.28-7.25 (m, 2H, Ar_2), 7.22-7.18 (m, 2H, Ar_2), 6.94-6.90 (m, 1H, Ar_2), 6.58, 6.57 (s, 2H, Ar_1), 3.83 (t, *J* = 6.1 Hz, 2H), 3.75, 3.74 (s, 6H, OCH_3), 3.57 (s, 1H,

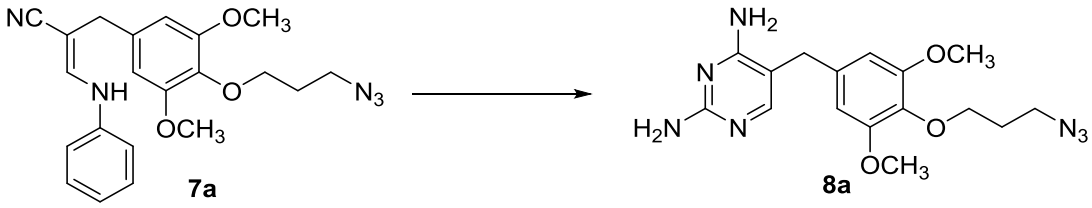
H_b), 3.43 (s, 1H, H_b), 3.39 (t, J = 6.9 Hz, 2H), 1.75-1.70 (m, 2H), 1.68-1.63 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 153.0, 142.7, 141.7, 141.5, 140.9, 135.5, 135.1, 135.0, 134.0, 129.4, 129.3, 122.9, 121.6, 121.3, 119.6, 115.2, 105.5, 81.9, 80.4, 71.8, 55.9, 50.5, 36.7, 31.7, 26.8, 25.1; (+)-ESI-HRMS calc for $C_{22}H_{25}N_5NaO_3$ $[M+Na]^+$: 430.1855, found 430.1836.

(E)-2-(4-((5-Azidopentyl)oxy)-3,5-dimethoxybenzyl)-3-(phenylamino)acrylonitrile; **7c**



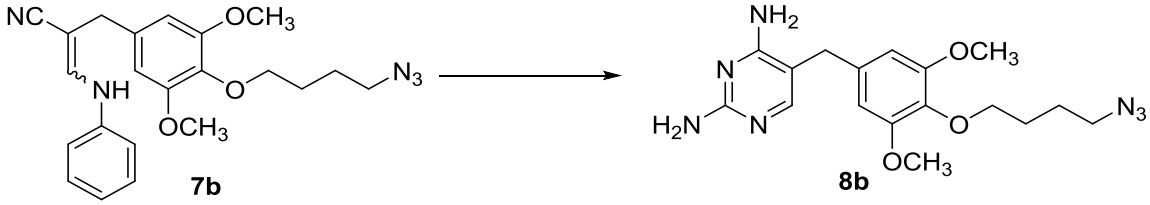
General procedure **B**. **4c** (5.78 g, 19.71 mmol) was converted to **7c** (5.42 g, 65 %) as a mixture of *E* and *Z* isomers. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.13, 9.11 (d, J = 12.4, 12.5 Hz, 1H, NH), 7.67, 7.64 (d, J = 12.9, 12.5 Hz, 1H, H_a), 7.28-7.25 (m, 2H, Ar_2), 7.22-7.18 (m, 2H, Ar_2), 6.94-6.90 (m, 1H, Ar_2), 6.58-6.57 (m, 2H, Ar_1), 3.81 (t, J = 6.3 Hz, 2H), 3.75, 3.74 (s, 6H, OCH_3), 3.57 (s, 1H, H_b), 3.43 (s, 1H, H_b), 3.34 (t, J = 6.9 Hz, 2H), 1.65-1.56 (m, 4H), 1.51-1.46 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 153.0, 142.7, 141.7, 141.5, 140.9, 135.4, 135.2, 135.1, 134.1, 129.4, 129.3, 122.9, 121.8, 121.3, 119.6, 115.2, 105.5, 81.8, 80.3, 72.1, 55.9, 50.7, 36.7, 31.7, 29.1, 28.0, 22.8; (+)-ESI-HRMS calc for $C_{23}H_{27}N_5NaO_3$ $[M+Na]^+$: 444.2012, found 444.1992.

5-(4-(3-Azidopropoxy)-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine; **8a**



General procedure **C**. **7a** (3.16 g, 8.03 mmol) was reacted with guanidine to give **8a** (2.55 g, 88% yield). LCMS: R_t = 2.91 min, @ 254 nm, $[M + H]^+$ = 360.1; ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.51 (s, 1H), 6.55 (s, 2H), 6.08 (s, 2H), 5.68 (s, 2H), 3.86 (t, J = 5.9 Hz, 2H), 3.72 (s, 6H), 3.54 (t, J = 6.8 Hz, 2H), 3.52 (s, 2H), 1.81 (tt, J = 6.1, 6.1 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.2, 162.1, 155.7, 152.7, 136.0, 134.4, 105.7, 105.6, 69.3, 55.8, 47.8, 33.0, 29.1; (+)-ESI-HRMS calc for $C_{16}H_{22}N_7O_3$ $[M+H]^+$: 360.1784, found 360.1790.

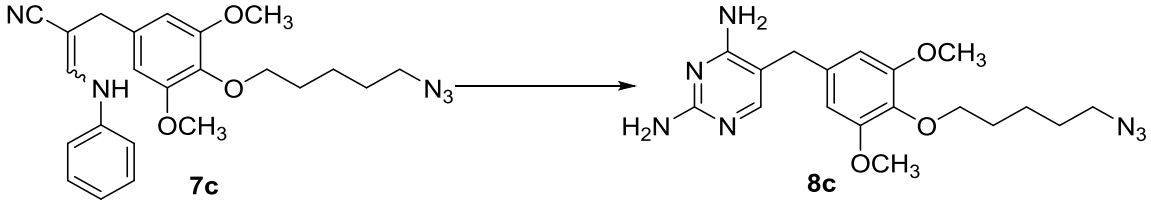
5-(4-(4-Azidobutoxy)-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine; **8b**



General procedure **C**. **7b** (6.1 g, 14.97 mmol) was reacted with guanidine to give **8b** (4.10 g, 73 %). LCMS: R_t = 2.98 min, @ 254 nm, $[M + H]^+$ = 374.1; ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.45 (s, 1H), 6.62 (s, 2H), 3.82 (t, J = 6.2 Hz, 2H), 3.73 (s, 6H), 3.59 (s, 2H), 3.39 (t, J = 6.7 Hz, 2H), 1.74-1.70 (m, 2H), 1.67-1.63 (m, 2H), ^{13}C NMR (150 MHz, DMSO-d_6): δ 164.0, 154.4, 153.0, 139.9,

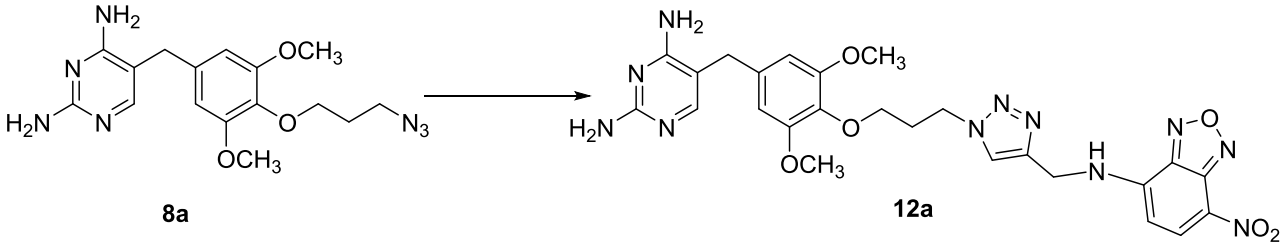
135.1, 133.0, 108.9, 106.2, 71.7, 55.9, 50.4, 32.1, 26.7, 25.1; (+)-ESI-HRMS calc for $C_{17}H_{24}N_7O_3$ $[M+H]^+$: 374.1941, found 374.1952.

5-(4-((5-Azidopentyl)oxy)-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine; **8c**



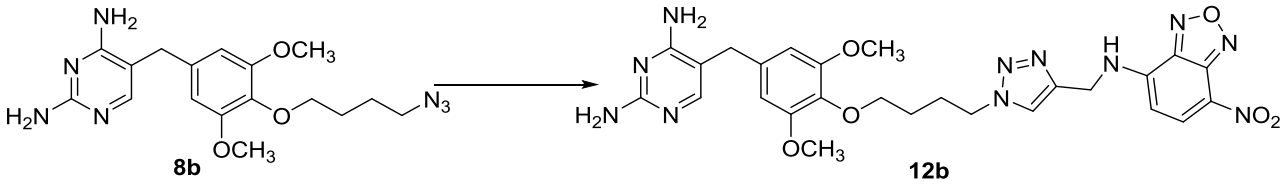
General procedure **C**. **7c** (5.4 g, 12.81 mmol) was reacted with guanidine to give **8c** (3.88 g, 78 %). LCMS: R_t = 3.07 min, @ 254 nm, $[M + H]^+$ = 388.2; ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.45 (s, 1H), 6.61 (s, 2H), 3.80 (t, J = 6.2 Hz, 2H), 3.73 (s, 6H), 3.59 (s, 2H), 3.33 (t, J = 6.8 Hz, 2H), 1.64-1.56 (m, 4H), 1.50-1.45 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 164.0, 154.4, 153.0, 139.9, 135.2, 132.9, 108.9, 106.2, 72.0, 55.9, 50.7, 32.1, 29.1, 28.0, 22.8; (+)-ESI-HRMS calc for $C_{18}H_{26}N_7O_3$ $[M+H]^+$: 388.2097, found 388.2103.

5-(3,5-Dimethoxy-4-(3-(4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)methyl)-1H-1,2,3-triazol-1-yl)propoxy)benzyl)pyrimidine-2,4-diamine; **12a**



General procedure **D**. **8a** (50 mg, 0.14 mmol) underwent cycloaddition with NBD alkyne **9** to give an orange solid **12a** (29 mg, 36 %). LCMS: R_t = 2.92 min, @ 254 nm, $[M + H]^+$ = 578.4; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.42 (d, J = 7.1 Hz, 1H), 8.28 (s, 1H), 8.10 (s, 1H), 7.51 (s, 1H), 6.53 (s, 2H), 6.45 (d, J = 8.9 Hz, 1H), 6.12 (s, 2H), 5.76 (s, 2H), 4.74 (s, 2H), 4.54 (t, J = 7.0 Hz, 2H), 3.80 (t, J = 5.8 Hz, 2H), 3.69 (s, 6H), 3.51 (s, 2H), 2.10 (tt, J = 6.6, 6.6 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.2, 162.1, 155.5, 152.7, 138.0, 136.1, 134.2, 123.5, 105.8, 105.7, 102.7, 69.1, 55.8, 46.7, 33.0, 30.4; (+)-ESI-HRMS calc for $C_{25}H_{28}N_{11}O_6$ $[M+H]^+$: 578.2224, found 578.2239.

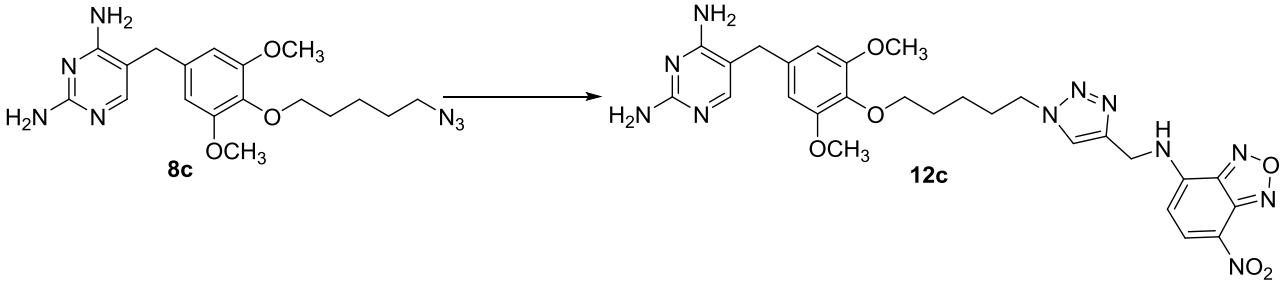
5-(3,5-Dimethoxy-4-(4-(4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)methyl)-1H-1,2,3-triazol-1-yl)butoxy)benzyl)pyrimidine-2,4-diamine; **12b**



General procedure **D**. **8b** (100 mg, 0.27 mmol) underwent cycloaddition with NBD alkyne **9** to give crude compound **12b**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give an orange solid (128 g, 80 %). LCMS: R_t = 3.02 min, @ 254 nm, $[M + H]^+$ = 592.4; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.50 (d, J = 9.0 Hz, 1H), 8.12 (s, 1H), 7.49 (s, 1H), 6.54

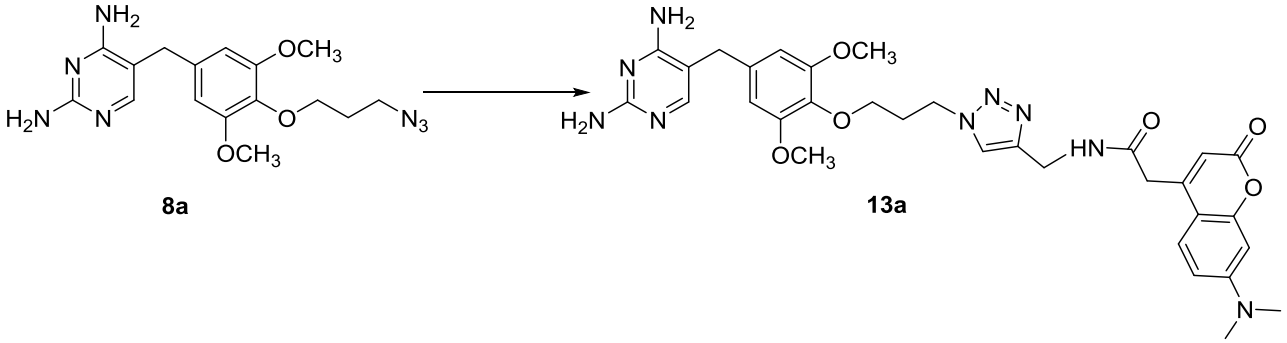
(s, 2H), 6.50 (d, *J* = 9.0 Hz, 1H), 6.32 (s, 2H), 6.02 (s, 2H), 4.75 (s, 2H), 4.40 (t, *J* = 7.1 Hz, 2H), 3.78 (t, *J* = 6.0 Hz, 2H), 3.68 (s, 6H), 3.51 (s, 2H), 1.96 (tt, *J* = 7.1, 7.1 Hz, 2H),1.54 (tt, *J* = 6.1, 6.1 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.4, 161.3, 153.6, 152.8, 412.6, 137.6, 135.5, 134.6, 123.3, 106.1, 105.8, 99.9, 71.5, 55.8, 49.1, 38.7, 32.9, 26.6, 26.5; (+)-ESI-HRMS calc for $C_{26}H_{30}N_{11}O_6$ $[M+H]^+$: 592.2381, found 592.2400.

5-(3,5-Dimethoxy-4-((5-(4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)methyl)-1H-1,2,3-triazol-1-yl)pentyl)oxy)benzyl)pyrimidine-2,4-diamine; **12c**



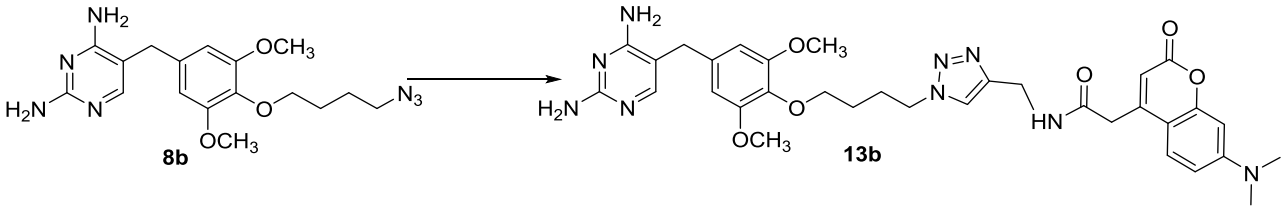
General procedure **D**. **8c** (104 mg, 0.27 mmol) underwent cycloaddition with NBD alkyne **9** to give crude compound **12c**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give an orange solid (140 mg, 86 %). LCMS: R_t = 3.07 min, @ 254 nm, $[M + H]^+$ = 606.4; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.50 (d, *J* = 8.9 Hz, 1H), 8.12 (s, 1H), 7.48 (s, 1H), 6.53 (s, 2H), 6.49 (d, *J* = 9.0 Hz, 1H), 6.42 (s, 2H), 6.20 (s, 2H), 4.75 (s, 2H), 4.33 (t, *J* = 7.0 Hz, 2H), 3.74 (t, *J* = 6.2 Hz, 2H), 3.68 (s, 6H), 3.52 (s, 2H), 1.83 (tt, *J* = 7.1, 7.1 Hz, 2H), 1.58 (tt, *J* = 6.7, 6.7 Hz, 2H), 1.40-1.35 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.6, 160.8, 152.9, 152.6, 142.6, 137.8, 135.2, 134.8, 123.3, 106.3, 105.8, 99.9, 72.0, 55.8, 49.5, 38.7, 32.8, 29.4, 29.0, 22.5; (+)-ESI-HRMS calc for $C_{27}H_{32}N_{11}O_6$ $[M+H]^+$: 606.2537, found 606.2549.

N-((1-(3-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide; **13a**



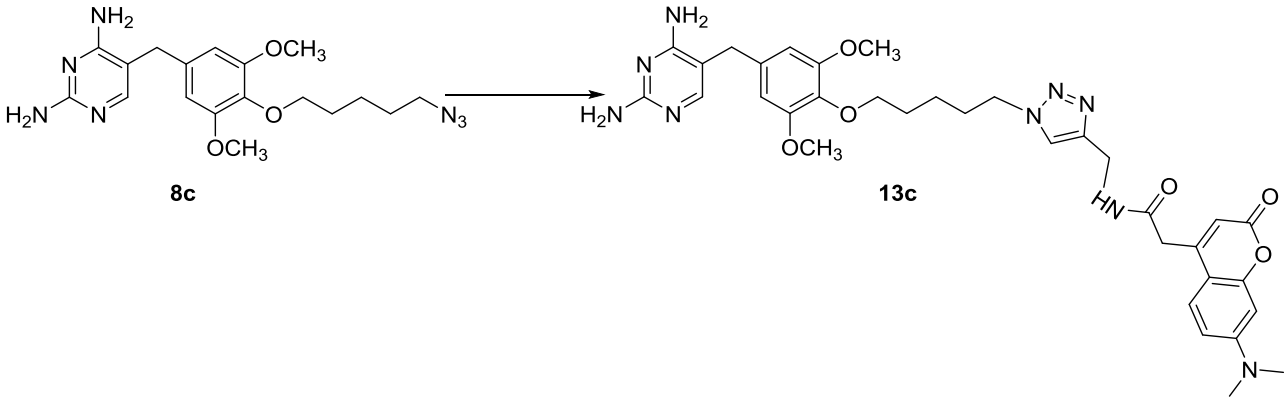
General procedure **D**. **8a** (36 mg, 0.1 mmol) underwent cycloaddition with DMACA alkyne **10** to give a green solid **13a** (26 mg, 41 %). LCMS: R_t = 2.94 min, @ 254 nm, $[M + H]^+$ = 644.6; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.70 (t, *J* = 5.6 Hz, 1H), 7.88 (s, 1H), 7.51 (s, 1H), 7.50 (d, *J* = 9.0 Hz, 1H), 6.68 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.56 (s, 2H), 6.54 (d, *J* = 2.5 Hz, 1H), 6.11 (s, 2H), 6.00 (s, 1H), 5.73 (s, 2H), 4.53 (t, *J* = 7.1 Hz, 2H), 4.31 (d, *J* = 5.6 Hz, 2H), 3.81 (t, *J* = 5.8 Hz, 2H), 3.71 (s, 6H), 3.63 (s, 2H), 3.52 (s, 2H), 3.00 (s, 6H), 2.10 (tt, *J* = 6.9, 6.9 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 167.4, 162.2, 162.1, 160.7, 155.4, 155.3, 152.8, 152.7, 151.1, 144.4, 136.0, 134.2, 126.0, 122.9, 109.5, 108.9, 108.2, 105.8, 105.7, 97.4, 69.1, 55.8, 46.5, 39.7, 38.6, 34.4, 33.0, 30.5; (+)-ESI-HRMS calc for $C_{32}H_{38}N_9O_6$ $[M+H]^+$: 644.2945, found 644.2924.

N-((1-(4-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)butyl)-1H-1,2,3-triazol-4-yl)methyl)-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide; **13b**



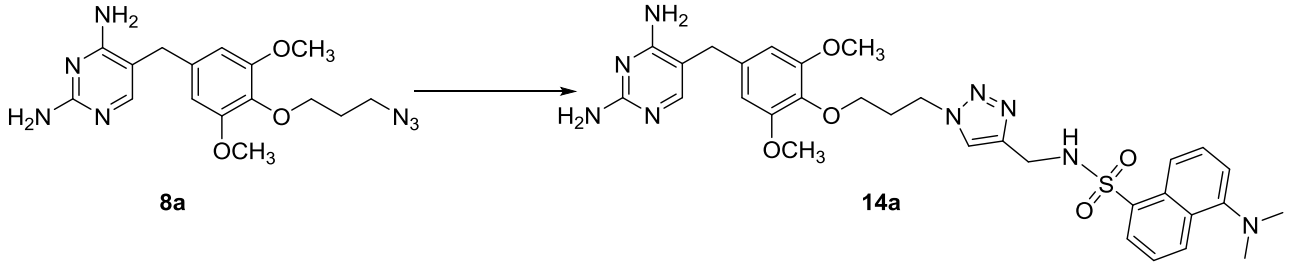
General procedure **D**. **8b** (50 mg, 0.13 mmol) underwent cycloaddition with DMACA alkyne **10** to give crude compound **13b**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give a green solid (10 mg, 11 %). LCMS: R_t = 2.97 min, @ 254 nm, $[M + H]^+$ = 658.5; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.72 (t, *J* = 5.6 Hz, 1H), 7.86 (s, 1H), 7.51 (d, *J* = 9.0 Hz, 1H), 7.50 (s, 1H), 6.68 (dd, *J* = 9.0, 2.4 Hz, 1H), 6.54 (s, 2H),6.53 (d, *J* = 2.5 Hz, 1H), 6.12 (s, 2H), 6.0 (s,1H), 5.73 (s, 2H), 4.38 (t, *J* = 7.0 Hz, 2H), 4.31 (d, *J* = 5.5 Hz, 2H), 3.78 (t, *J* = 6.2 Hz, 2H), 3.69 (s, 6H), 3.63 (s, 2H), 3.52 (s, 2H), 2.99 (s, 6H), 1.94 (tt, *J* = 7.2, 7.2 Hz, 2H), 1.54 (tt, *J* = 7.0 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 167.8, 162.2, 162.1, 160.7, 155.4, 155.3, 152.8, 151.2, 144.4, 135.9, 134.6, 126.1, 122.7, 109.5, 109.0, 108.2, 105.9, 105.8, 97.5, 71.6, 55.8, 49.0, 39.7, 38.7, 34.5, 33.0, 26.7, 26.5; (+)-ESI-HRMS calc for $C_{33}H_{40}N_9O_6$ $[M+H]^+$: 658.3102, found 658.3125.

N-((1-(5-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)pentyl)-1H-1,2,3-triazol-4-yl)methyl)-2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamide; **13c**



General procedure **D**. **8c** (52 mg, 0.13 mmol) underwent cycloaddition with DMACA alkyne **10** to give crude compound **13c**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give a green solid (18 mg, 20 %). LCMS: R_t = 3.04 min, @ 254 nm, $[M + H]^+$ = 672.5; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.72 (t, J = 5.7 Hz, 1H), 7.87 (s, 1H), 7.51 (d, J = 9.0 Hz, 1H), 7.49 (s, 1H), 6.69 (dd, J = 9.0, 2.6 Hz, 1H), 6.55 (s, 2H), 6.54 (d, J = 2.6 Hz, 1H), 6.49 (s, 2H), 6.17 (s, 2H), 6.0 (s, 1H), 4.33-4.30 (m, 4H), 3.76 (t, J = 6.2 Hz, 2H), 3.69 (s, 6H), 3.63 (s, 2H), 3.52 (s, 2H), 3.00 (s, 6H), 1.81 (tt, J = 7.4, 7.4 Hz, 2H), 1.60 (tt, J = 7.5, 7.5 Hz, 2H), 1.40-1.35 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 167.8, 162.6, 160.7, 160.6, 155.4, 152.9, 152.8, 151.2, 144.4, 135.2, 134.8, 126.0, 122.7, 109.5, 109.0, 108.2, 106.4, 105.9, 97.5, 72.0, 55.8, 49.3, 39.7, 38.6, 34.5, 32.8, 29.5, 29.0, 22.5; (+)-ESI-HRMS calc for $C_{34}H_{42}N_9O_6$ $[M+H]^+$: 672.3258, found 672.3279.

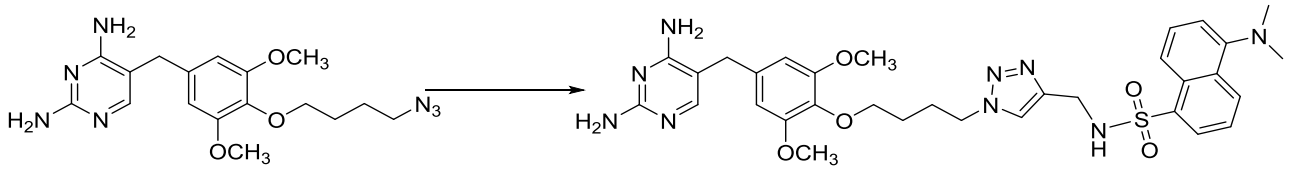
N-((1-(3-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)-1H-1,2,3-triazol-4-yl)methyl)-5-(dimethylamino)naphthalene-1-sulfonamide; **14a**



8a **14a**

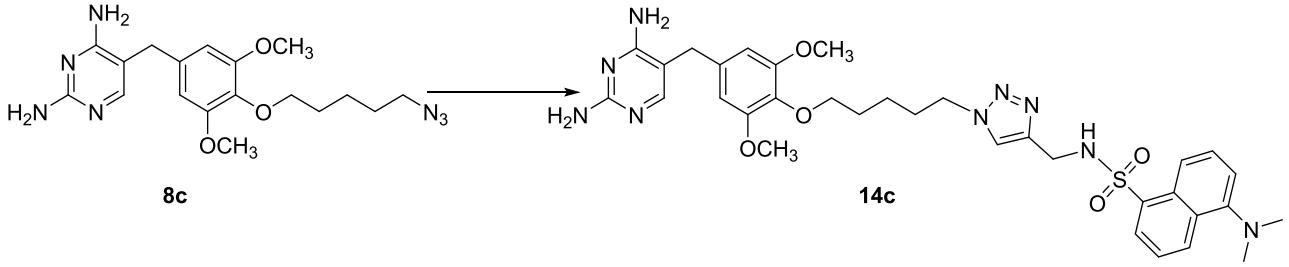
General procedure **D**. **8a** (96 mg, 0.27 mmol) underwent cycloaddition with DNS alkyne **11** to give crude compound **14a**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give a green solid (49 mg, 29 %). LCMS: R_t = 3.09 min, @ 254 nm, $[M + H]^+$ = 648.0; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.47 (t, *J* = 5.5 Hz, 1H), 8.39 (d, *J* = 8.4 Hz, 1H), 8.23 (d, *J* = 8.7 Hz, 1H), 8.07 (dd, *J* = 7.3, 0.9 Hz, 1H), 7.57-7.51 (m, 4H), 7.18 (d, *J* = 7.4 Hz, 1H), 6.58 (s, 2H), 6.28 (s, 2H), 5.88 (s, 2H), 4.37 (t, *J* = 7.0 Hz, 2H), 4.10 (d, *J* = 4.9 Hz, 2H), 3.71 (s, 6H), 3.70 (t, *J* = 5.9 Hz, 2H), 3.55 (s, 2H), 2.79 (s, 6H), 1.87 (tt, *J* = 6.4, 6.4 Hz, 2H), ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.4, 152.8, 151.2, 143.4, 136.0, 135.9, 134.0, 129.4, 129.0, 128.9, 128.4, 127.8, 123.5, 123.0, 119.1, 115.0, 105.7, 68.9, 55.8, 46.4, 45.0, 37.9, 33.0, 30.3; (+)-ESI-HRMS calc for $C_{31}H_{38}N_9O_5S$ $[M+H]^+$: 648.2717, found 648.2735.

N-((1-(4-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)butyl)-1H-1,2,3-triazol-4-yl)methyl)-5-(dimethylamino)naphthalene-1-sulfonamide; **14b**



General procedure **D**. **8b** (100 mg, 0.27 mmol) underwent cycloaddition with DNS alkyne **11** to give crude compound **14b**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give a green solid (161 mg, 90 %). LCMS: R_t = 3.13 min, @ 254 nm, $[M + H]^+$ = 662.0; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.48 (brs, 1H), 8.42 (d, *J* = 8.5 Hz, 1H), 8.26 (d, *J* = 8.6 Hz, 1H), 8.1 (dd, *J* = 7.3, 1.1 Hz, 1H), 7.59-7.54 (m, 3H), 7.51 (s, 1H), 7.22 (d, *J* = 7.5 Hz, 1H), 6.56 (s, 2H), 6.22 (s, 2H), 5.87 (s, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 4.10 (d, *J* = 3.3 Hz, 2H), 3.76 (t, *J* = 6.2 Hz, 2H), 3.70 (s, 6H), 3.53 (s, 2H), 2.80(s, 6H), 1.81 (tt, *J* = 7.2, 7.2 Hz, 2H), 1.45 (tt, *J* = 6.3, 6.3 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.3, 161.8, 154.6, 152.8, 151.3, 143.4, 136.0, 135.7, 134.6, 129.4, 129.0, 128.9, 128.4, 127.8, 123.5, 122.9, 119.1, 115.1, 106.0, 105.8, 71.5, 55.8, 48.8, 45.0, 37.9, 33.0, 26.5, 26.4; (+)-ESI-HRMS calc for $C_{32}H_{40}N_9O_5S$ $[M+H]^+$: 662.2873, found 662.2888.

N-((1-(5-(4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)pentyl)-1H-1,2,3-triazol-4-yl)methyl)-5-(dimethylamino)naphthalene-1-sulfonamide; **14c**



General procedure **D**. **8c** (104 mg, 0.27 mmol) underwent cycloaddition with DNS alkyne **11** to give crude compound **14c**, which was repurified (Gilson PLC 2020, A: H_2O (0.1% FA), B: ACN (0.1% FA), 5–100% B) to give a green solid (160 mg, 88 %). LCMS: R_t = 3.20 min, @ 254 nm, $[M + H]^+$ = 677.0; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.47 (t, *J* = 5.4 Hz, 1H), 8.42 (d, *J* = 8.5 Hz, 1H), 8.26 (d, *J* = 8.6 Hz, 1H), 8.09 (d, *J* = 7.3 Hz, 1H), 7.56-7.54 (m, 3H), 7.50 (s, 1H), 7.22 (d, *J* = 7.5 Hz, 1H), 6.55 (s, 2H), 6.22 (s, 2H), 5.88 (s, 2H), 4.17 (t, *J* = 7.1 Hz, 2H), 4.09 (d, *J* = 5.3 Hz, 2H), 3.74 (t, *J* = 6.2 Hz, 2H), 3.69 (s, 6H), 3.52 (s, 2H), 2.81 (s, 6H), 1.67 (tt, *J* = 7.3, 7.3 Hz, 2H), 1.58 (tt, *J* = 7.3, 7.3 Hz, 2H), 1.29 (tt, *J* = 7.5, 7.5 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.3, 161.8, 154.6, 152.8, 151.3, 143.4, 136.0, 135.6, 134.8, 129.4, 129.0, 128.9, 128.4, 127.8, 123.5, 122.8, 119.1, 115.1, 106.0, 105.8, 72.0, 55.8, 49.2, 45.0, 37.9, 33.0, 29.4, 28.9, 22.4; (+)-ESI-HRMS calc for $C_{33}H_{42}N_9O_5S$ $[M+H]^+$: 676.3030, found 676.3056.

Table S1. The *E. coli* strains.[1]

Strains	Strain description	Type
E. coli ATCC 25922	FDA control	Gram-negative
E. coli Clinical isolate	Timentin resistance	Gram-negative
E. coli Clinical isolate	Timentin resistance	Gram-negative
E. coli CGSC7319	Mutant DC2	Gram-negative
E. coli MB4827	Parent strain for *E. coli* mutants	Gram-negative
E. coli MB4902	Mutant lpxC	Gram-negative
E. coli MB5747	Mutant tolC (TolC deficient)	Gram-negative
E. coli MB5746	Mutant lpxC and mutant tolC (TolC deficient)	Gram-negative
S. aureus ATCC 25923	Methicillin-sensitive *S. aureus*	Gram-positive

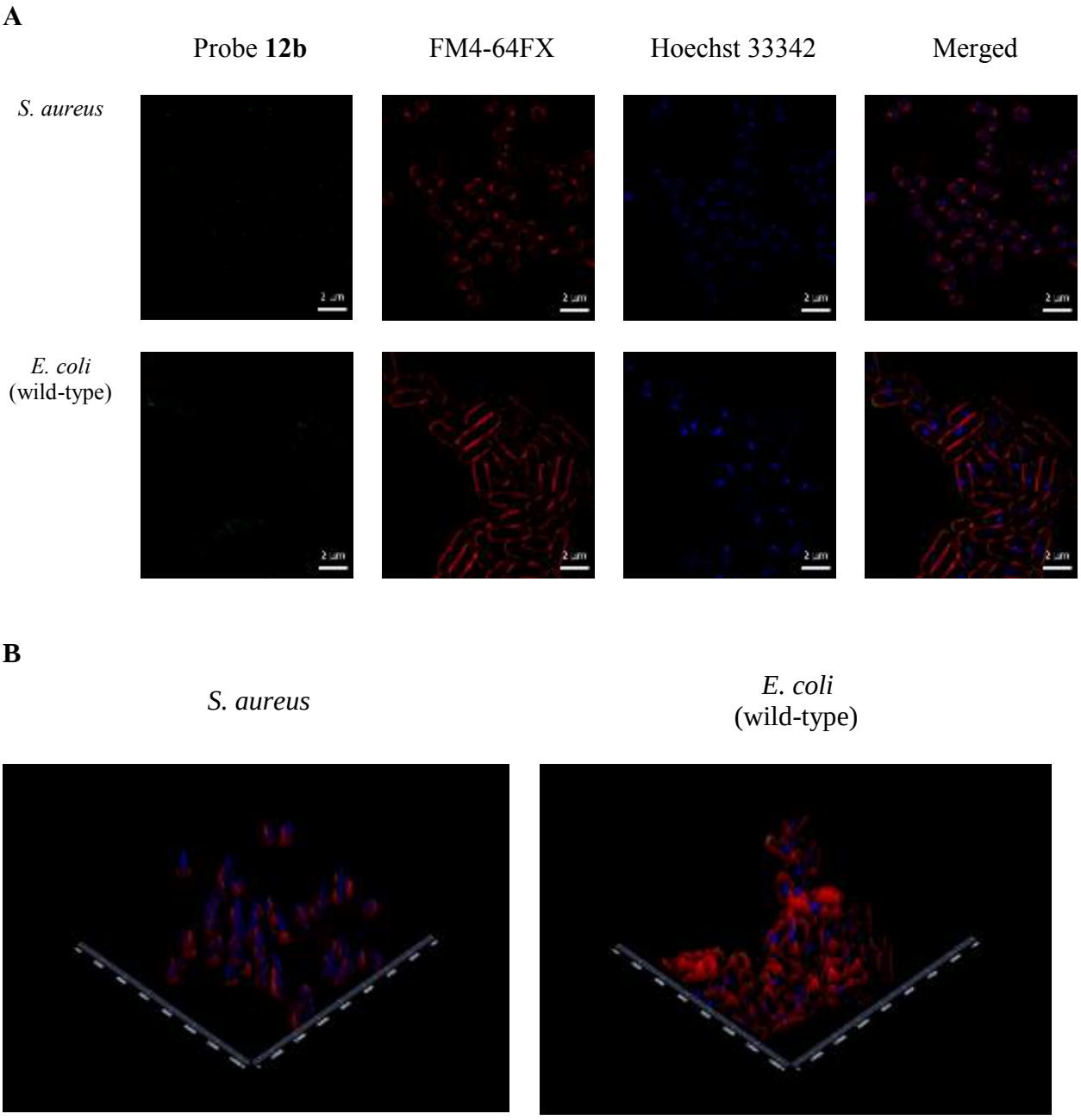


Fig S1. Fluorescence imaging of *S. aureus* and *E. coli* showing lack of staining by NBD-alkyne **9**. (A) Green, NBD alkyne **9** Red; FM4-64FX (bacterial membrane stain), Blue; Hoechst 33342 (nucleic acid stain). (B) Surface plot: XY axis indicated distance (nm). The scale bar shown represents 2 μm.

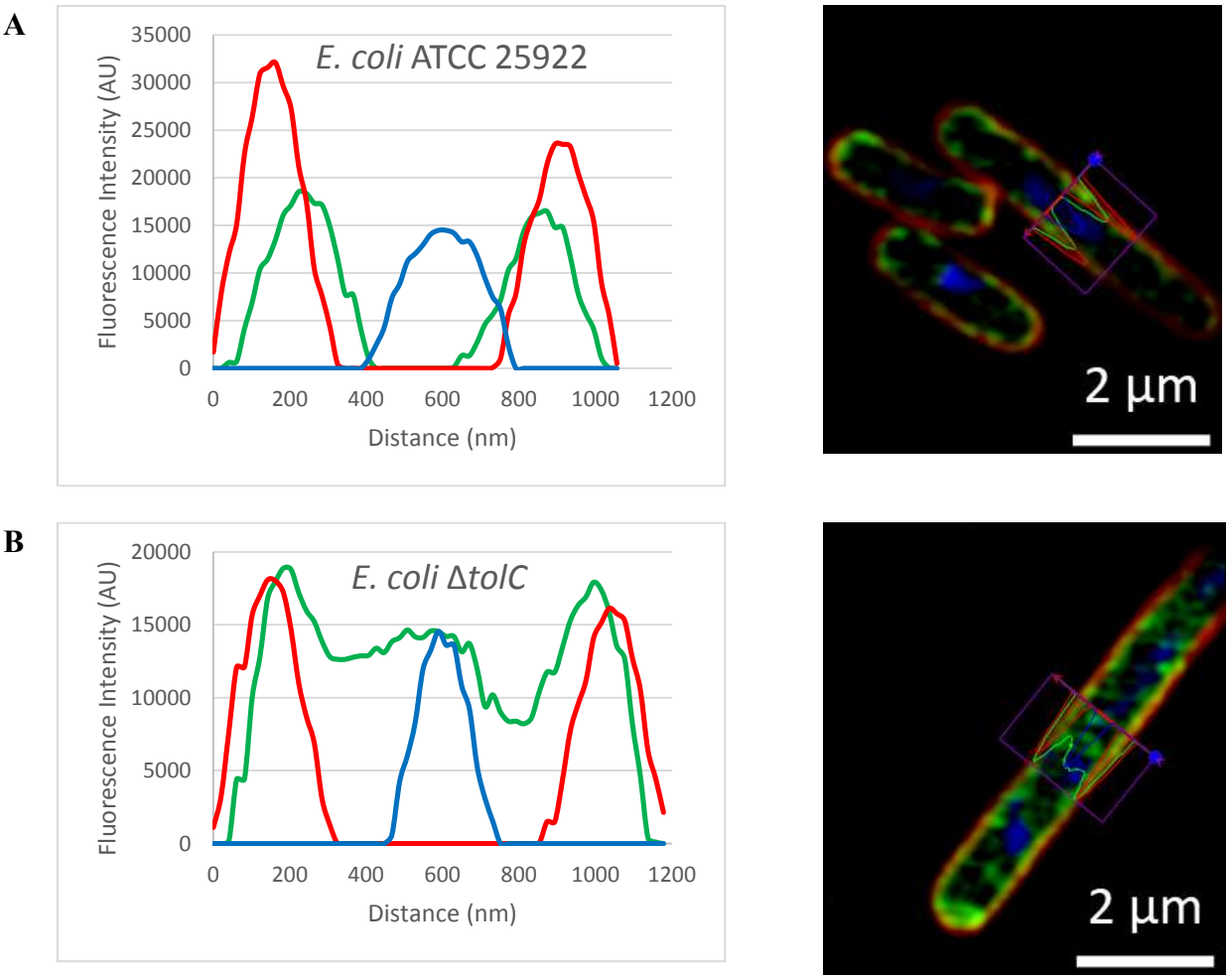


Figure S2. Cross section of fluorescent imaging of (A) *E. coli* (ATCC 25922 and **(B)** Δ*tolC E. coli*: Green = TMP-fluorophore **12b,** Red = FM4-64FX (bacterial membrane stain), Blue = Hoechst 33342 (nucleic acid stain). The images were process without raw scale applied.

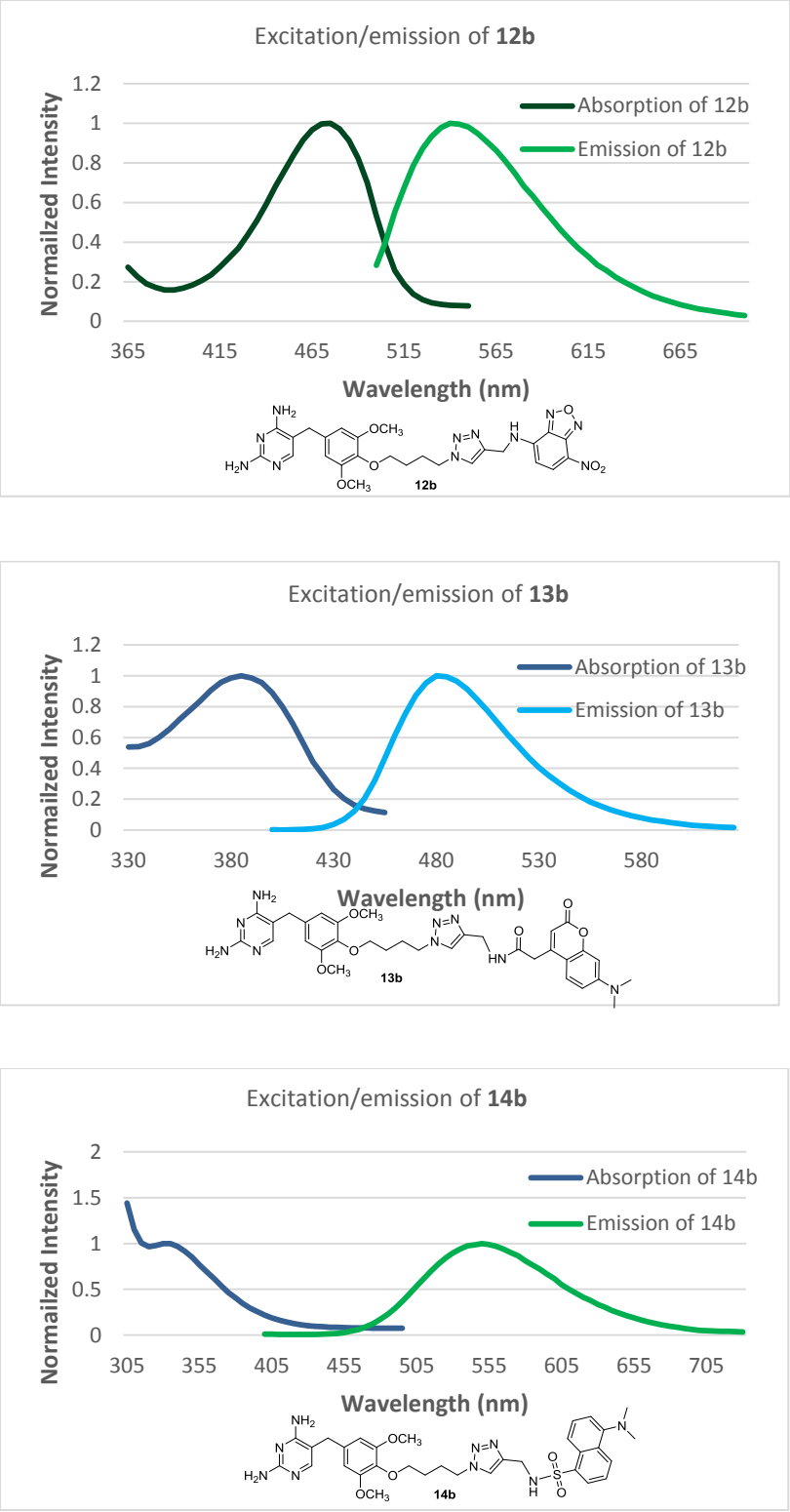


Fig S3. Excitation/emission spectra of TMP probes **12b, 13b, 14b.**

NMR spectra

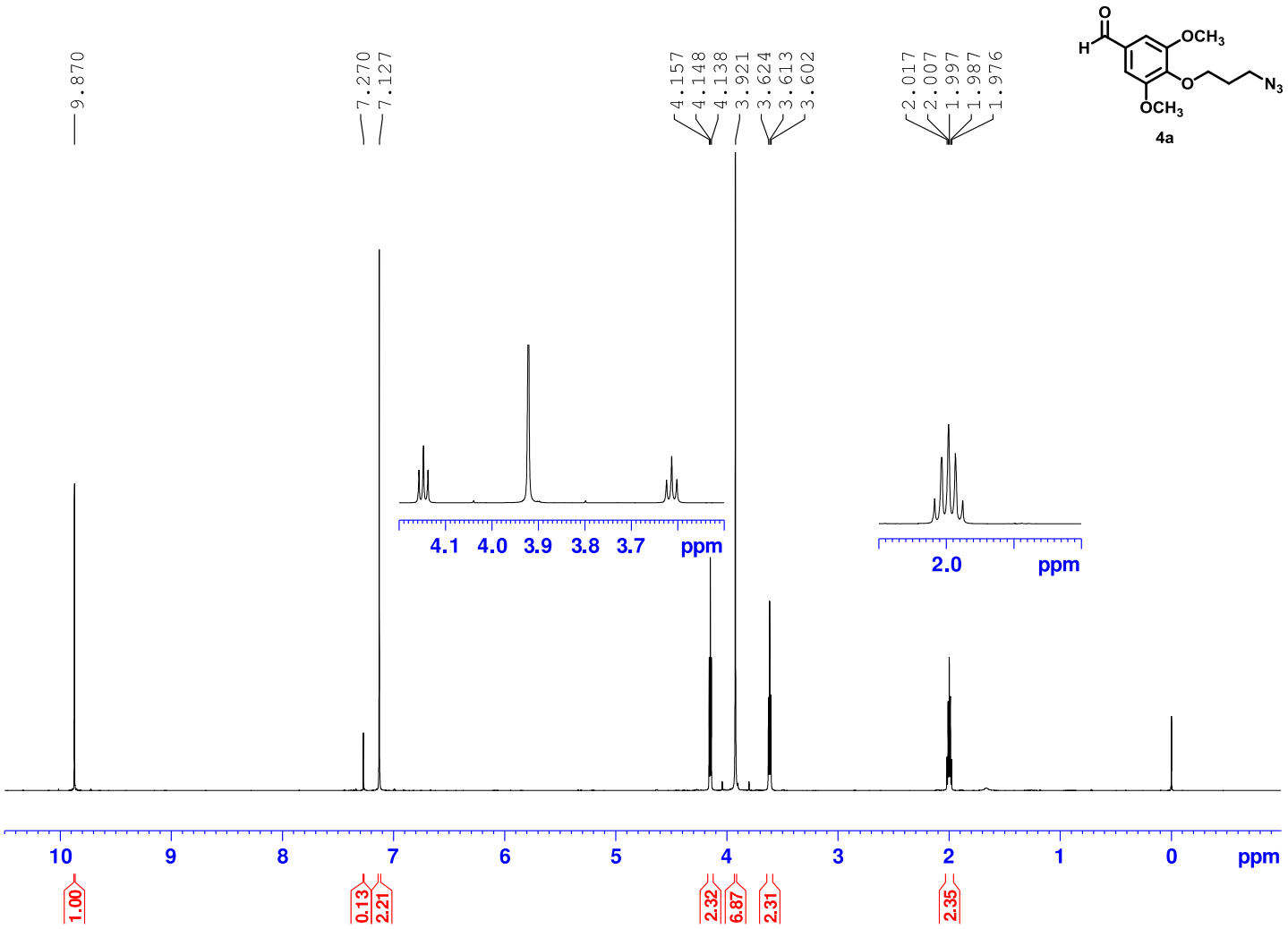


Fig S4. ^{1}H-NMR (600 MHz, $CDCl_3$) of **4a**.

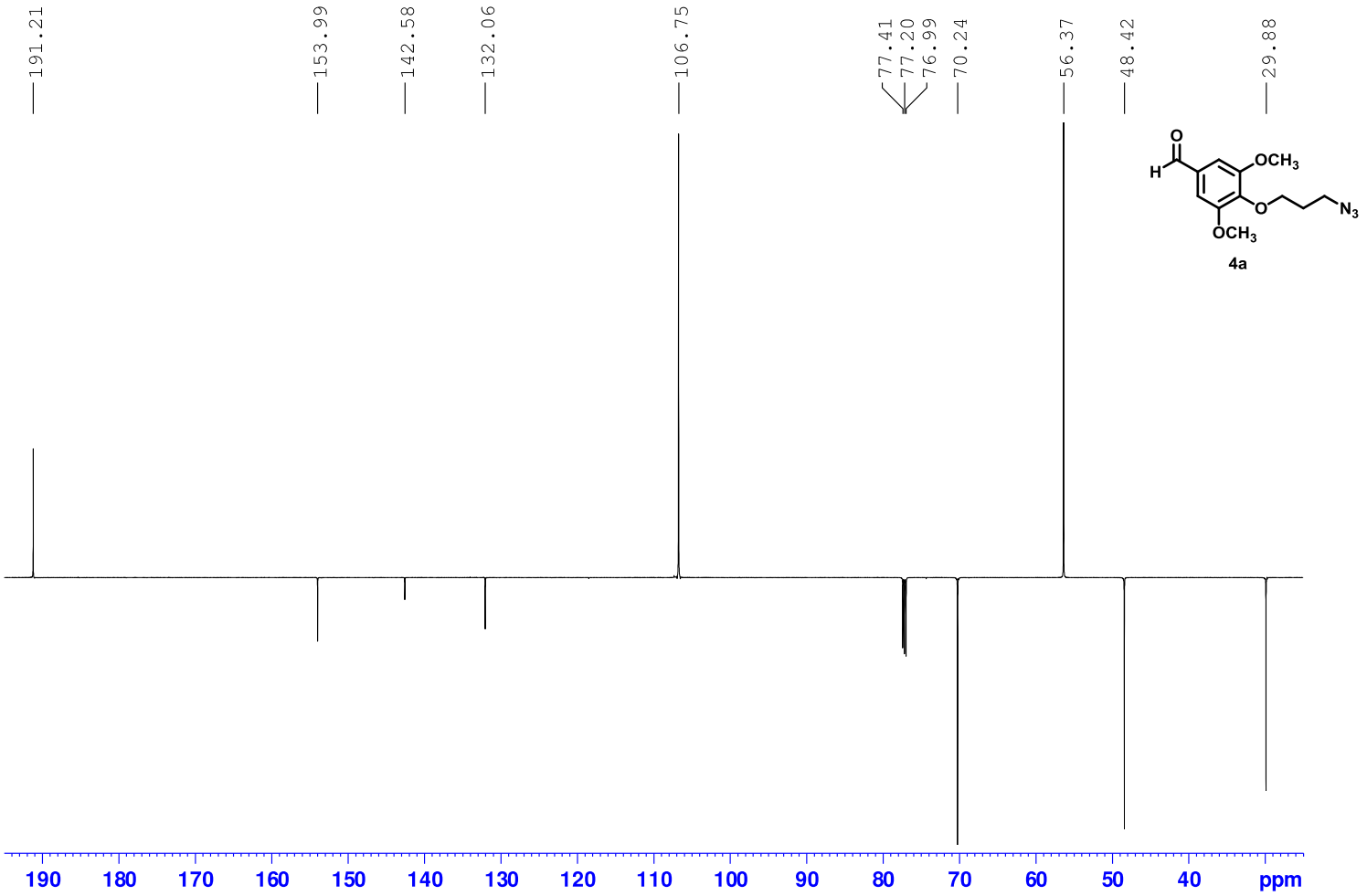


Fig S5. JMOD-NMR (150 MHz, $CDCl_3$) of **4a**.

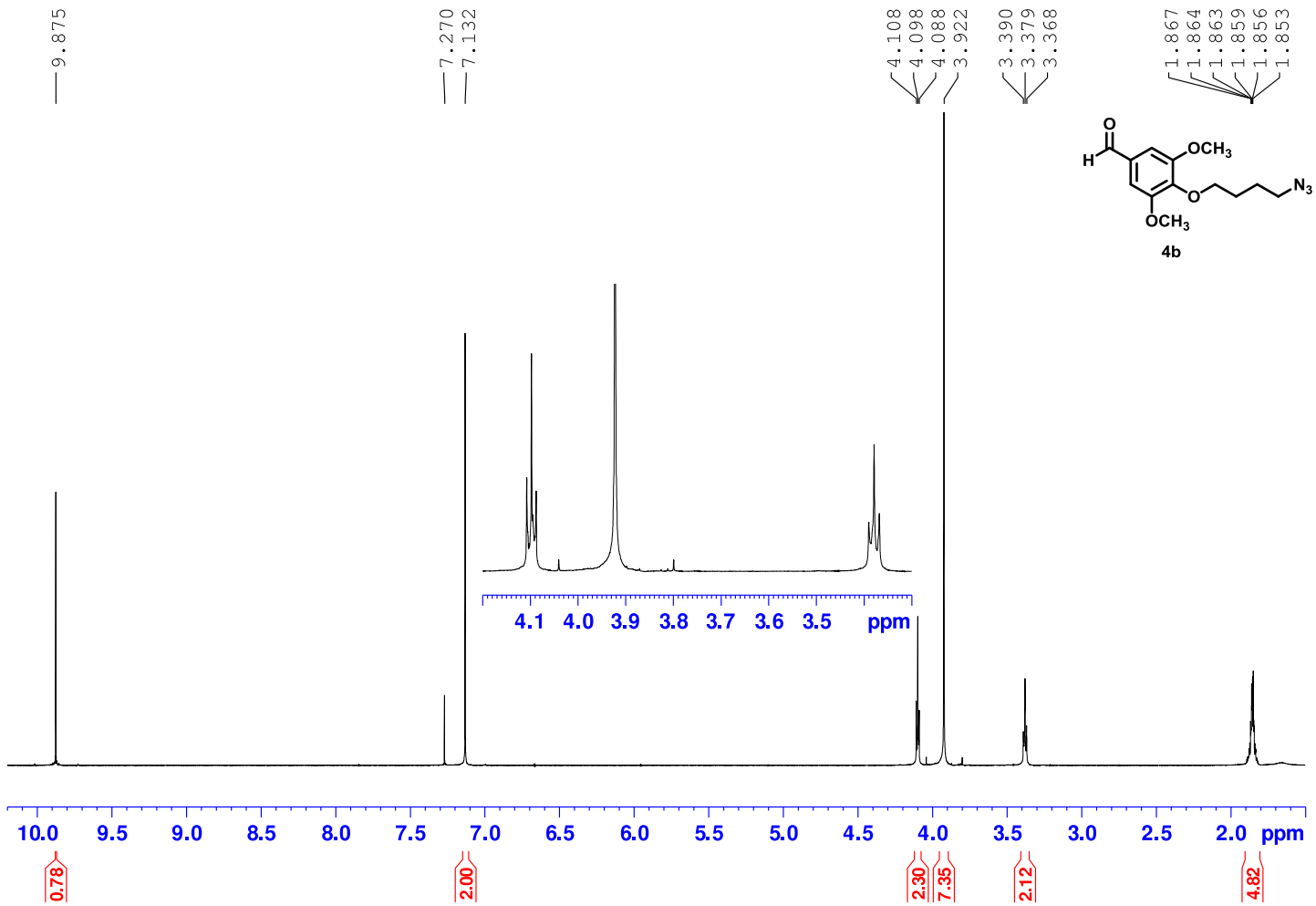


Fig S6. ^{1}H-NMR (600 MHz, $CDCl_3$) of **4b**.

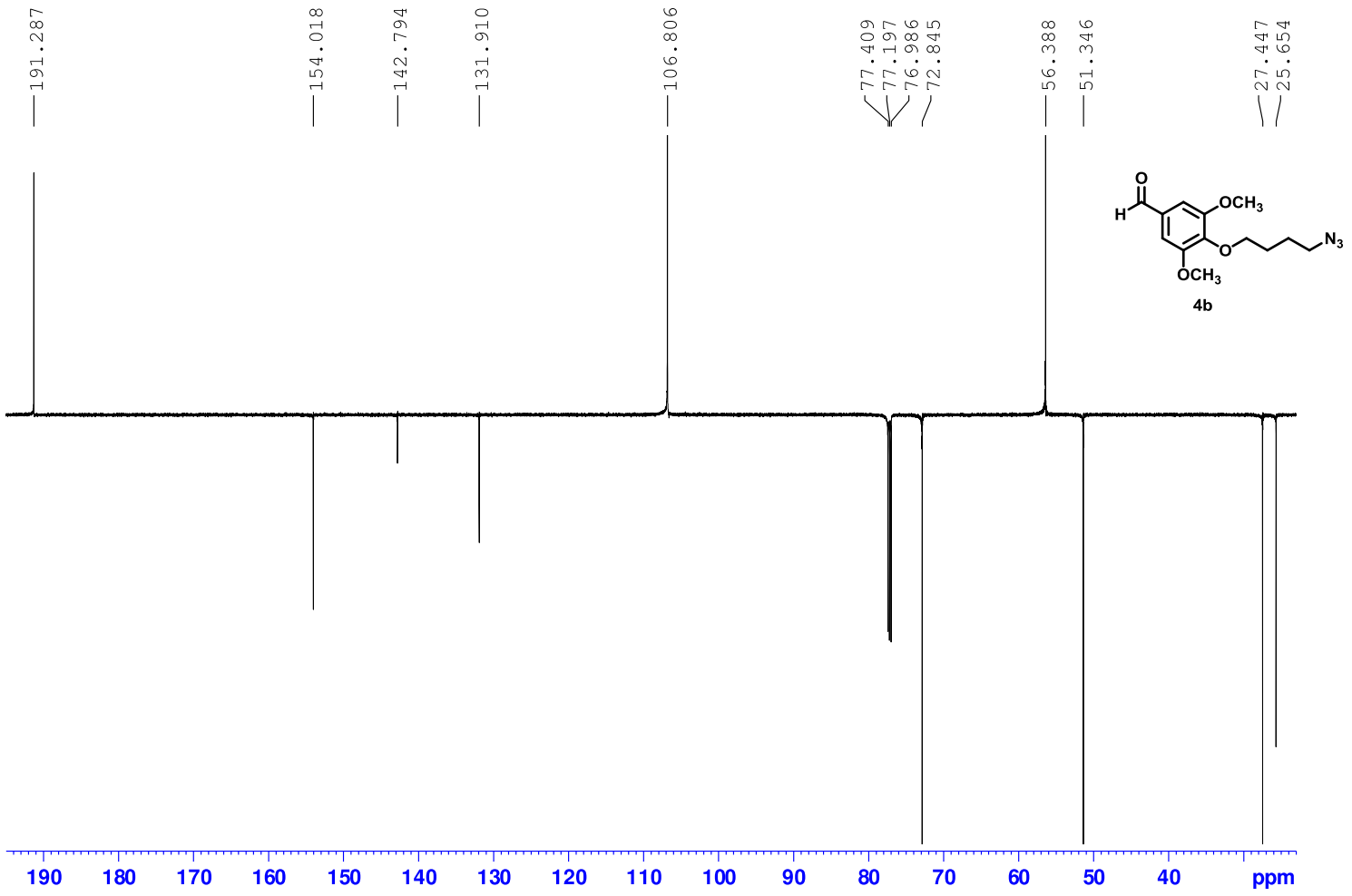


Fig S7. JMOD-NMR (150 MHz, $CDCl_3$) of **4b**.

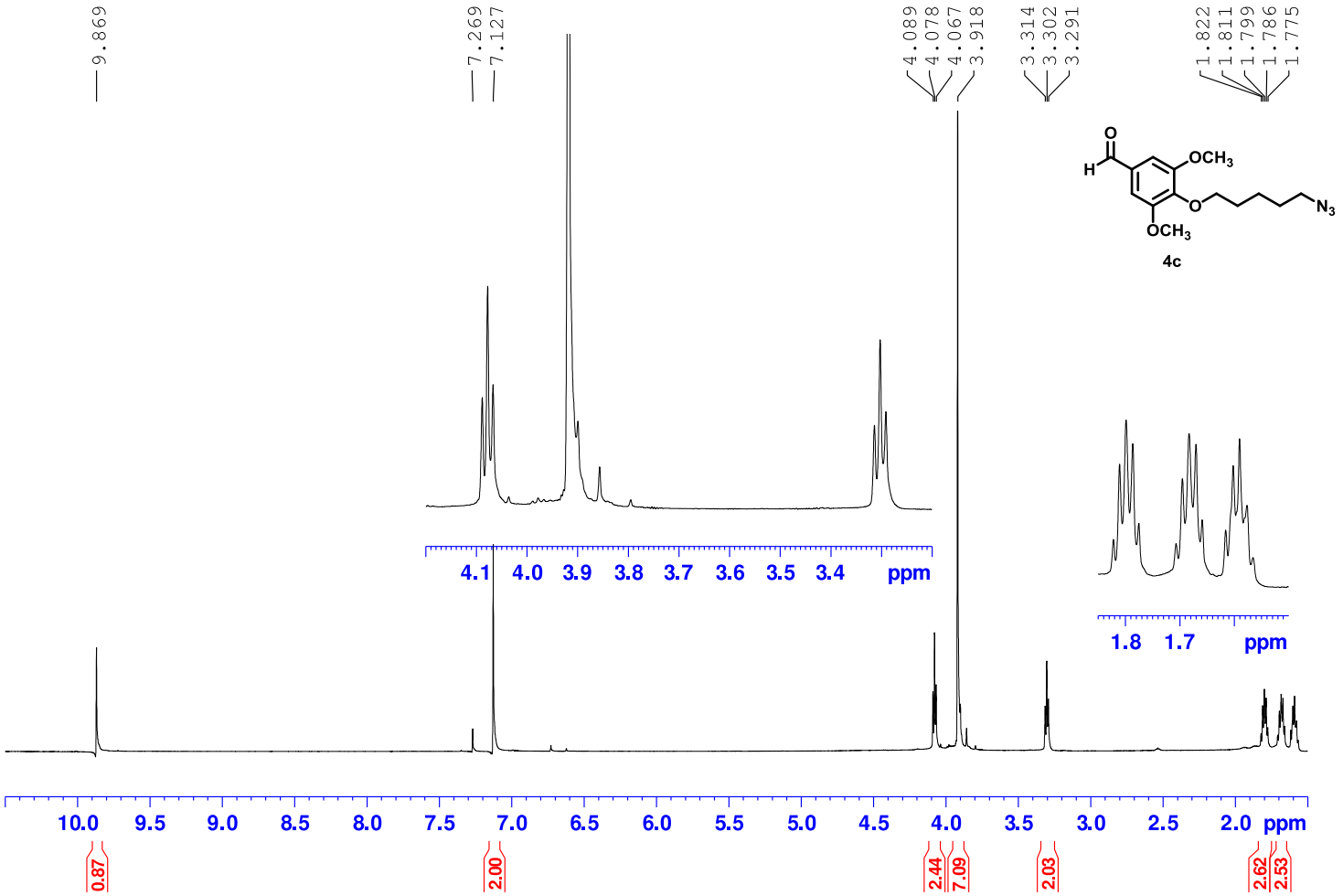


Fig S8. ^{1}H-NMR (600 MHz, $CDCl_3$) of **4c**.

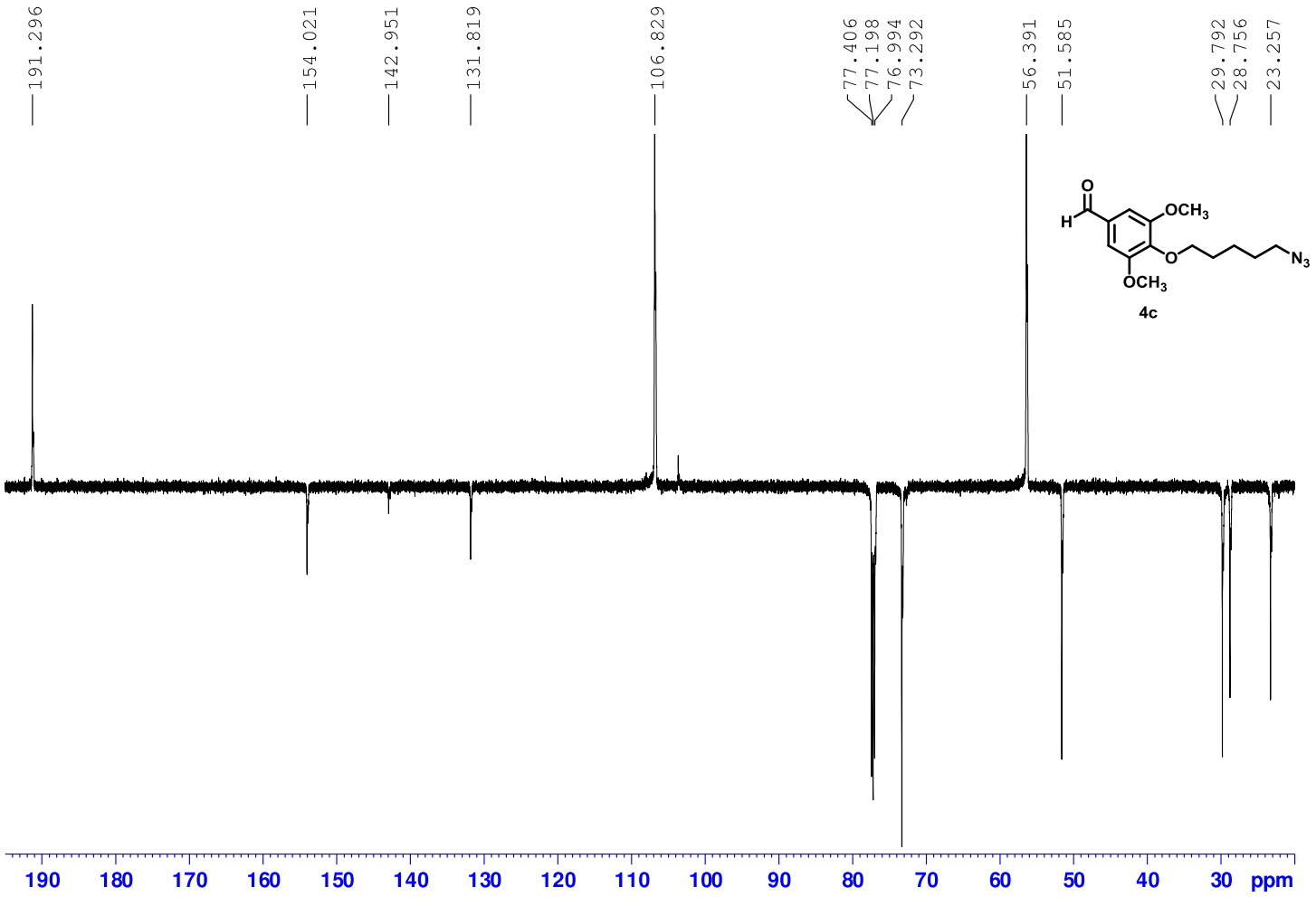


Fig S9. JMOD-NMR (150 MHz, $CDCl_3$) of **4c**.

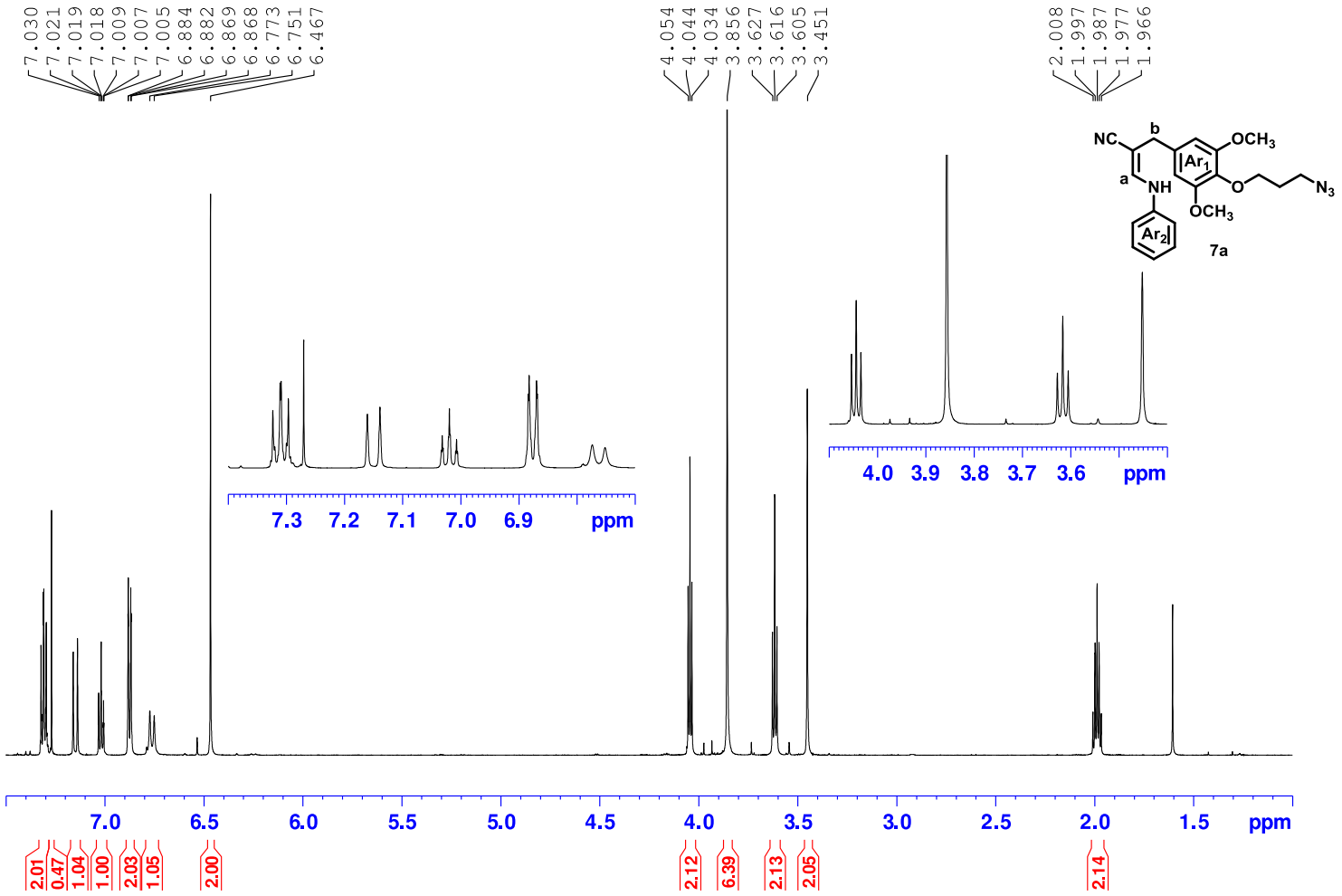


Fig S10. ^{1}H-NMR (600 MHz, $CDCl_3$) of **7a**.

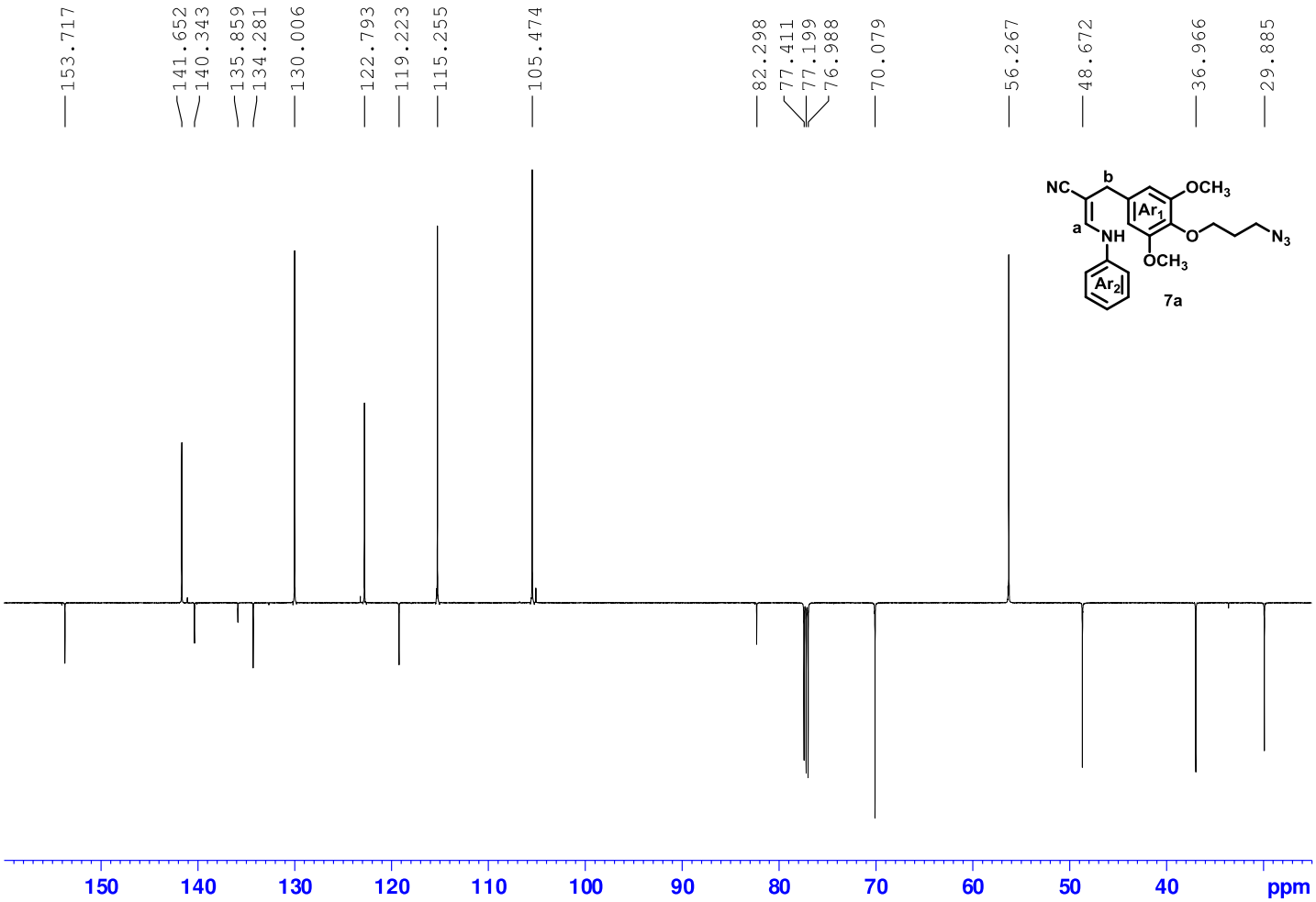


Fig S11. JMOD-NMR (150 MHz, $CDCl_3$) of **7a**.

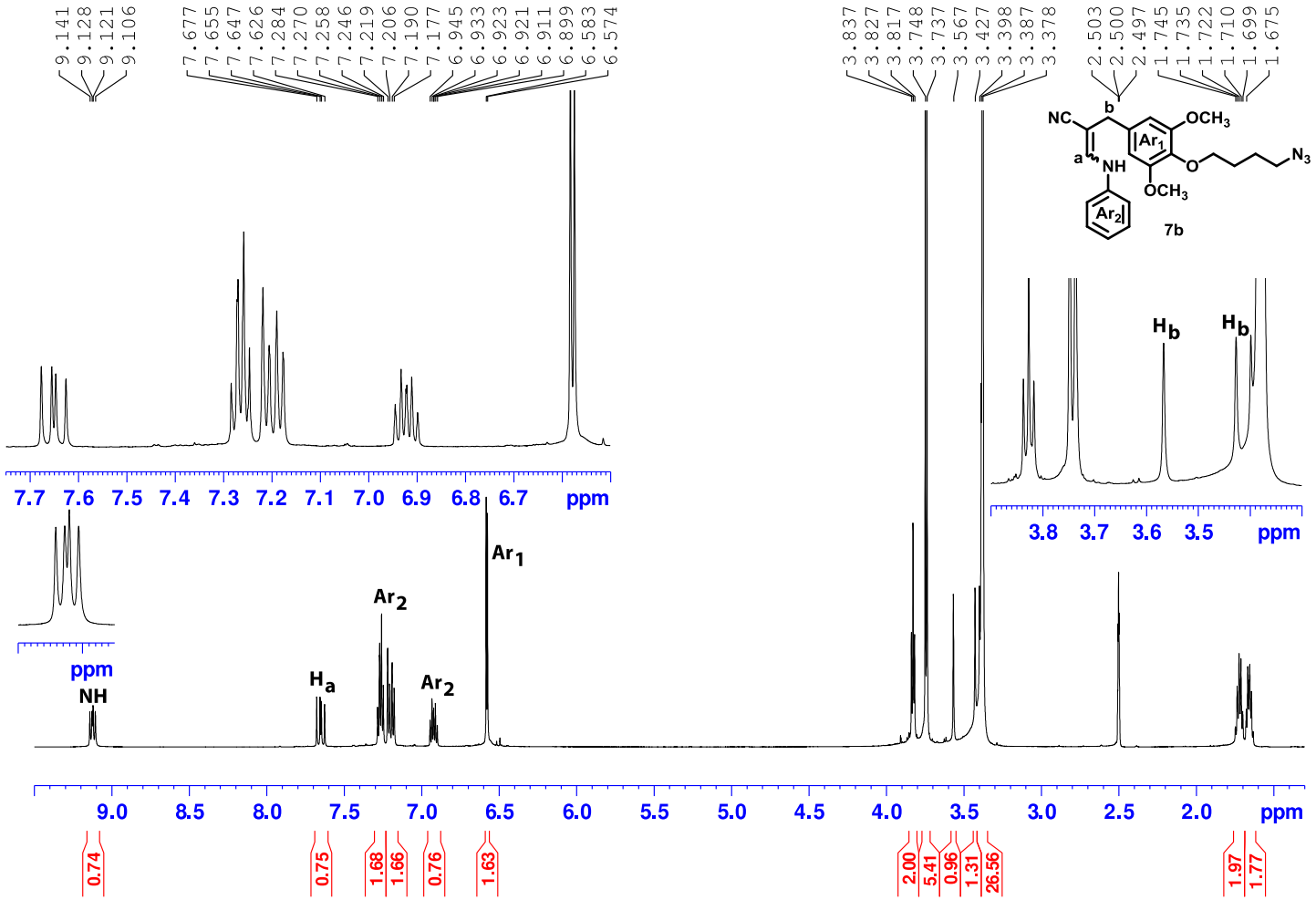


Fig S12. ^{1}H-NMR (600 MHz, DMSO-d_6) of **7b**.

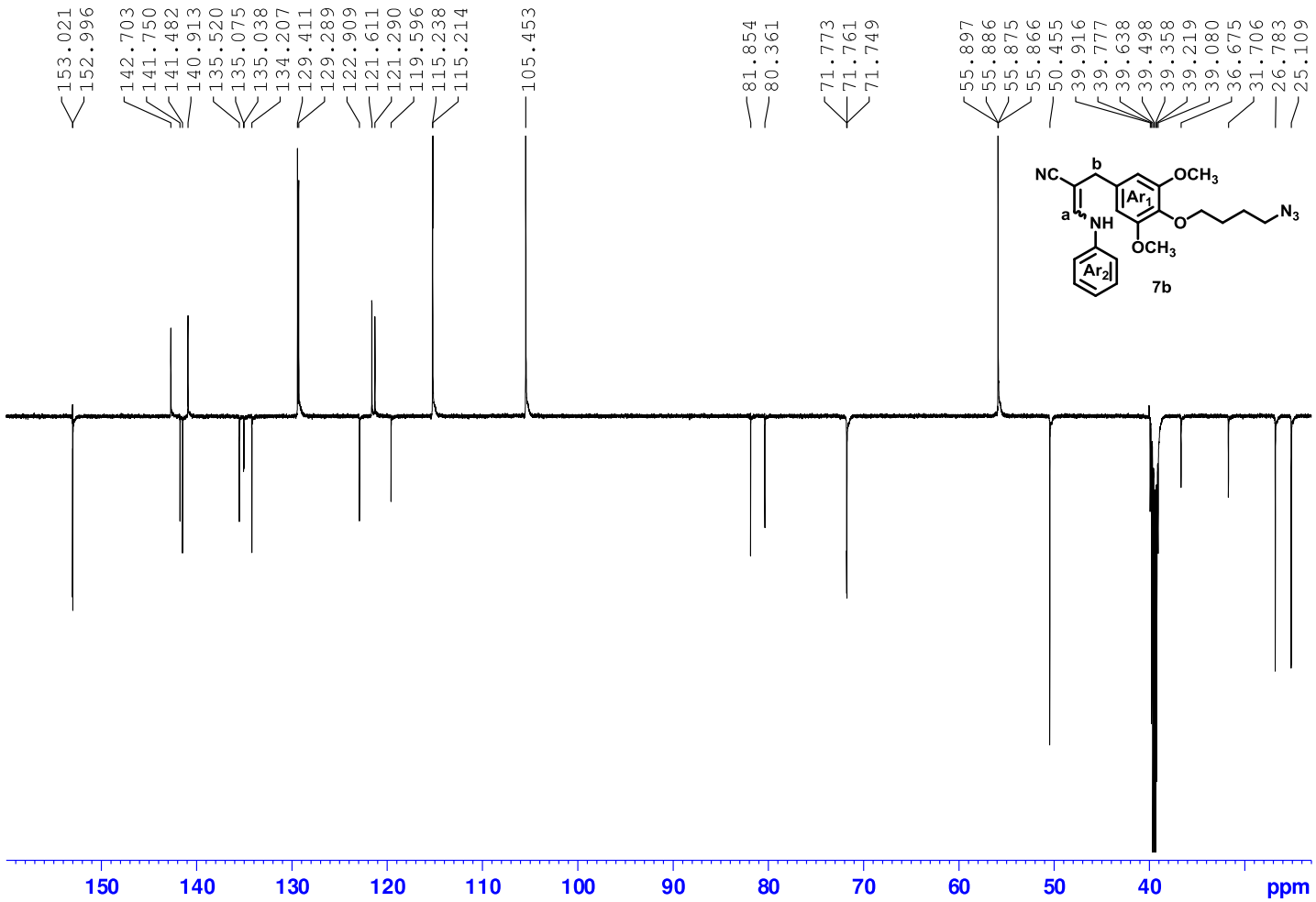


Fig S13. JMOD-NMR (150 MHz, DMSO-d_6) of **7b**.

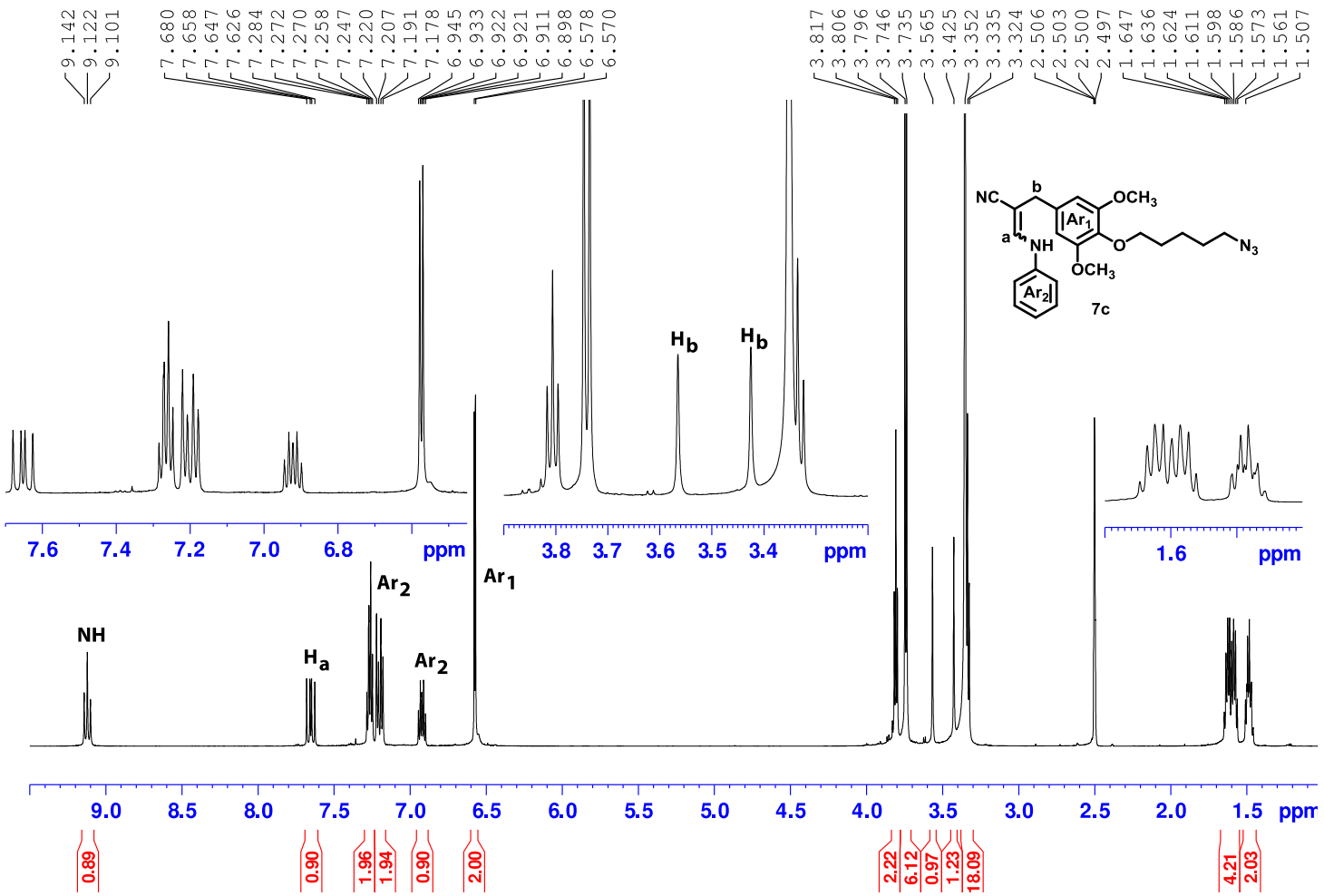


Fig S14. [1]H-NMR (600 MHz, DMSO-d_6) of **7c**.

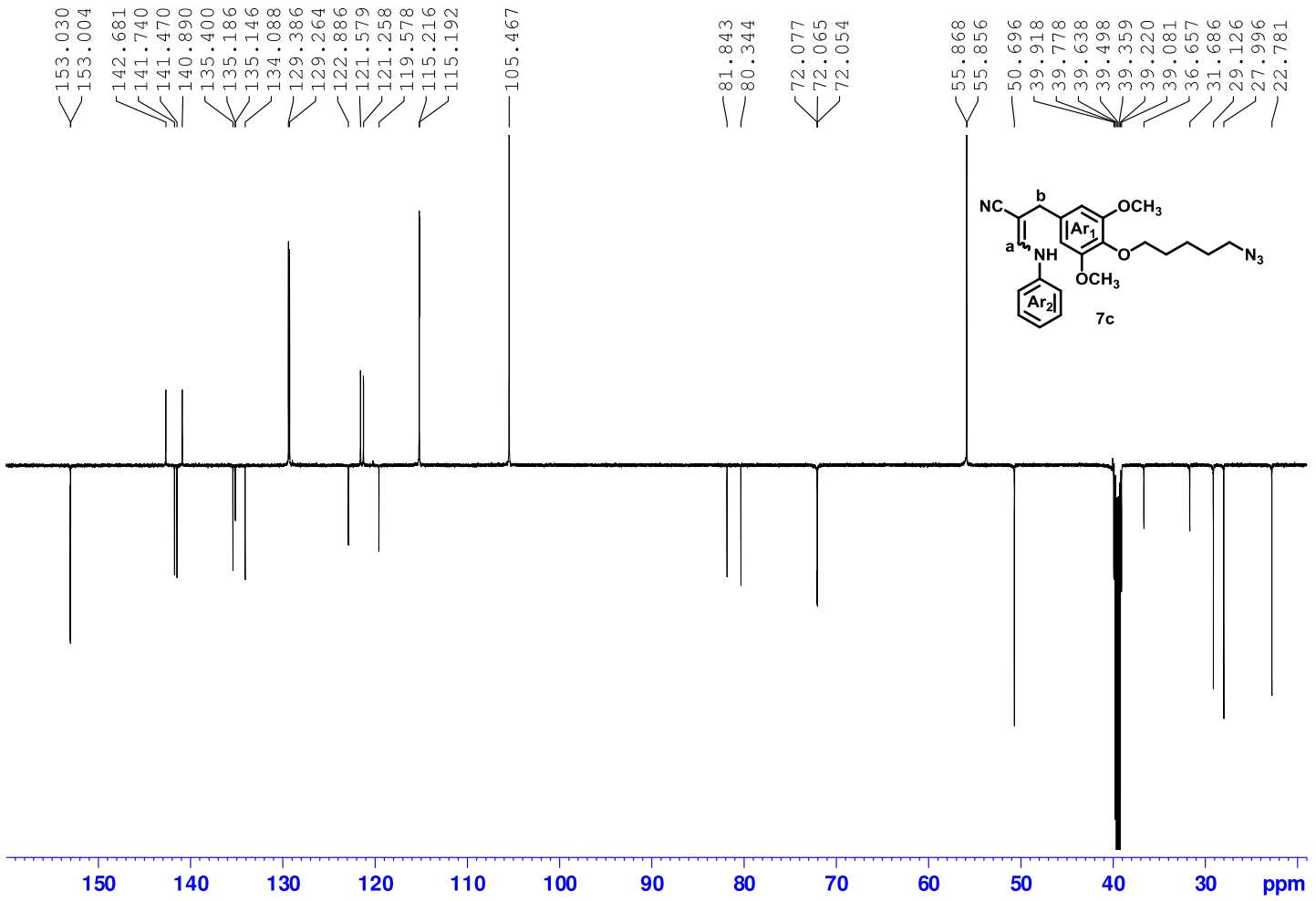


Fig S15. JMOD-NMR (150 MHz, DMSO-d_6) of **7c**.

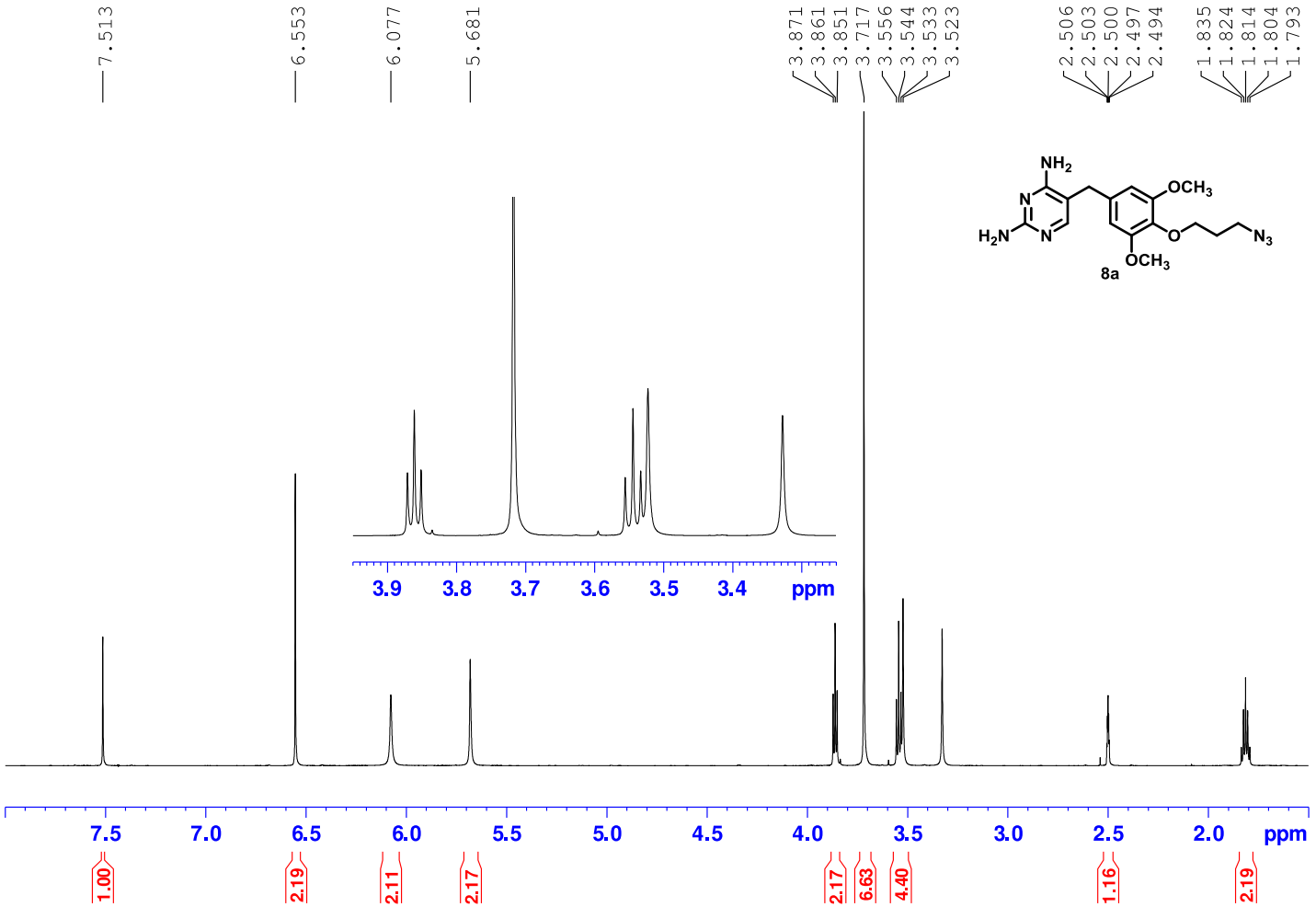


Fig S16. ^{1}H-NMR (600 MHz, DMSO-d_6) of **8a**.

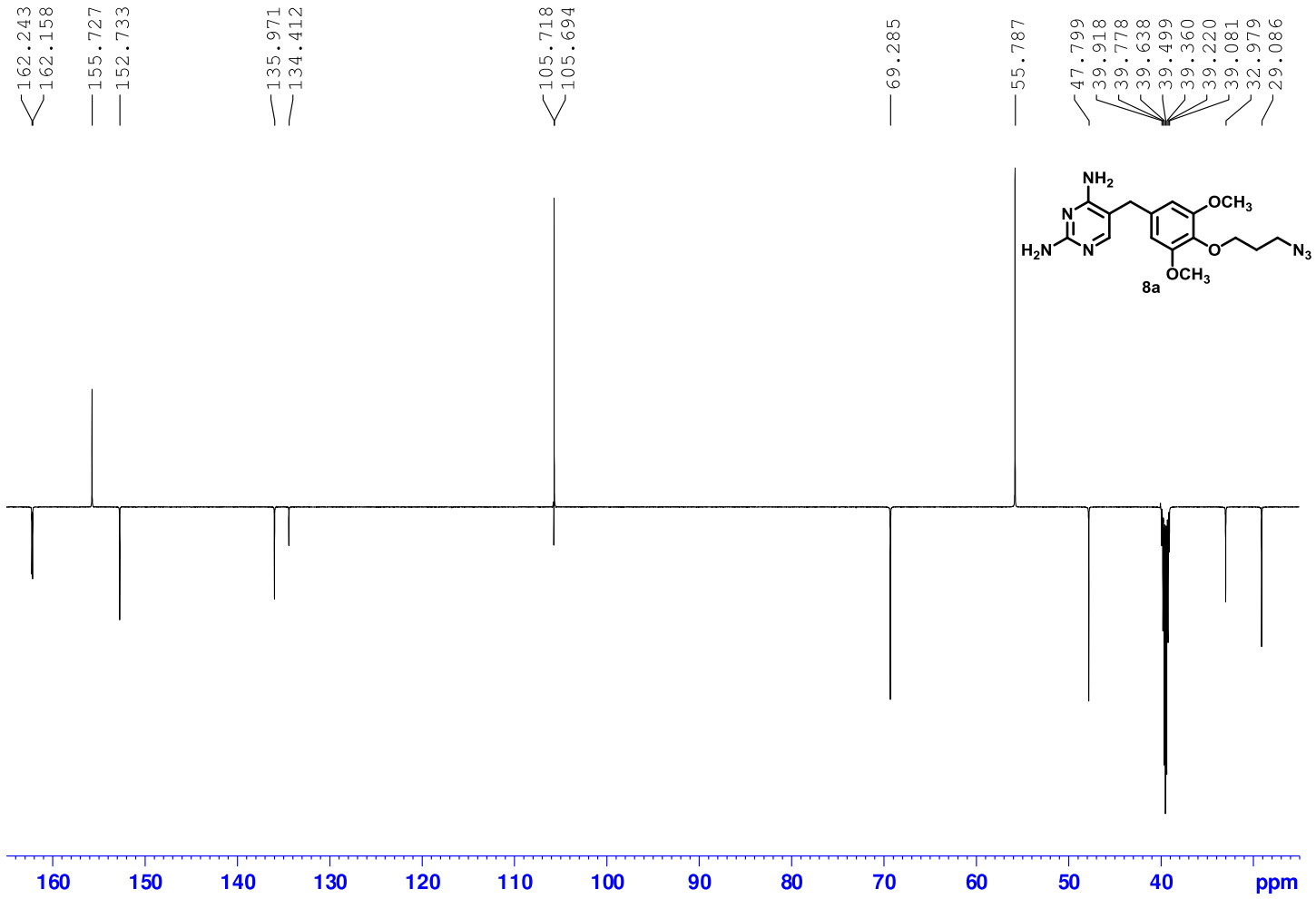


Fig S17. JMOD-NMR (150 MHz, DMSO-d_6) of **8a**.

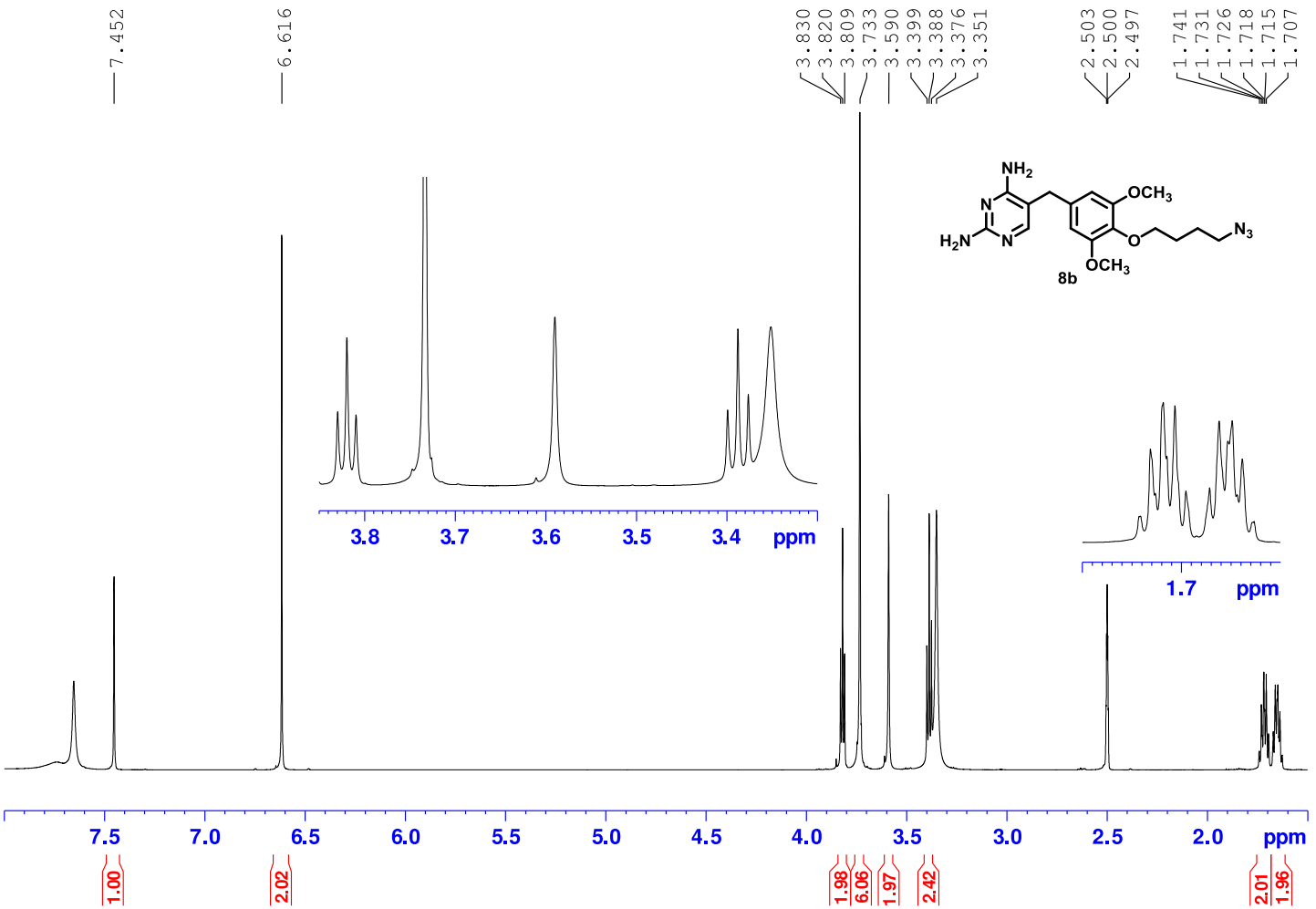


Fig S18. ^{1}H-NMR (600 MHz, DMSO-d_6) of **8b**.

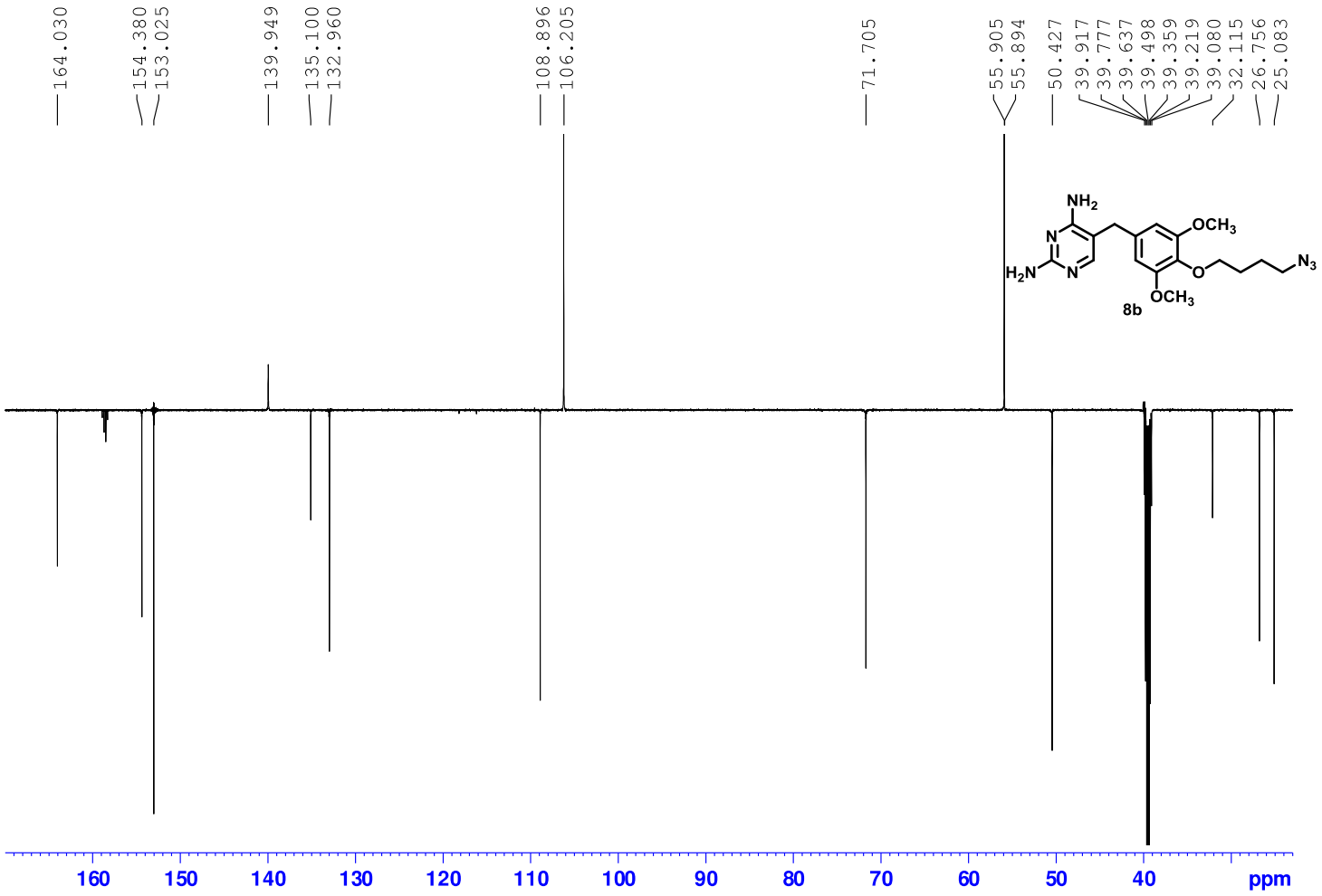


Fig S19. JMOD-NMR (150 MHz, DMSO-d_6) of **8b**.

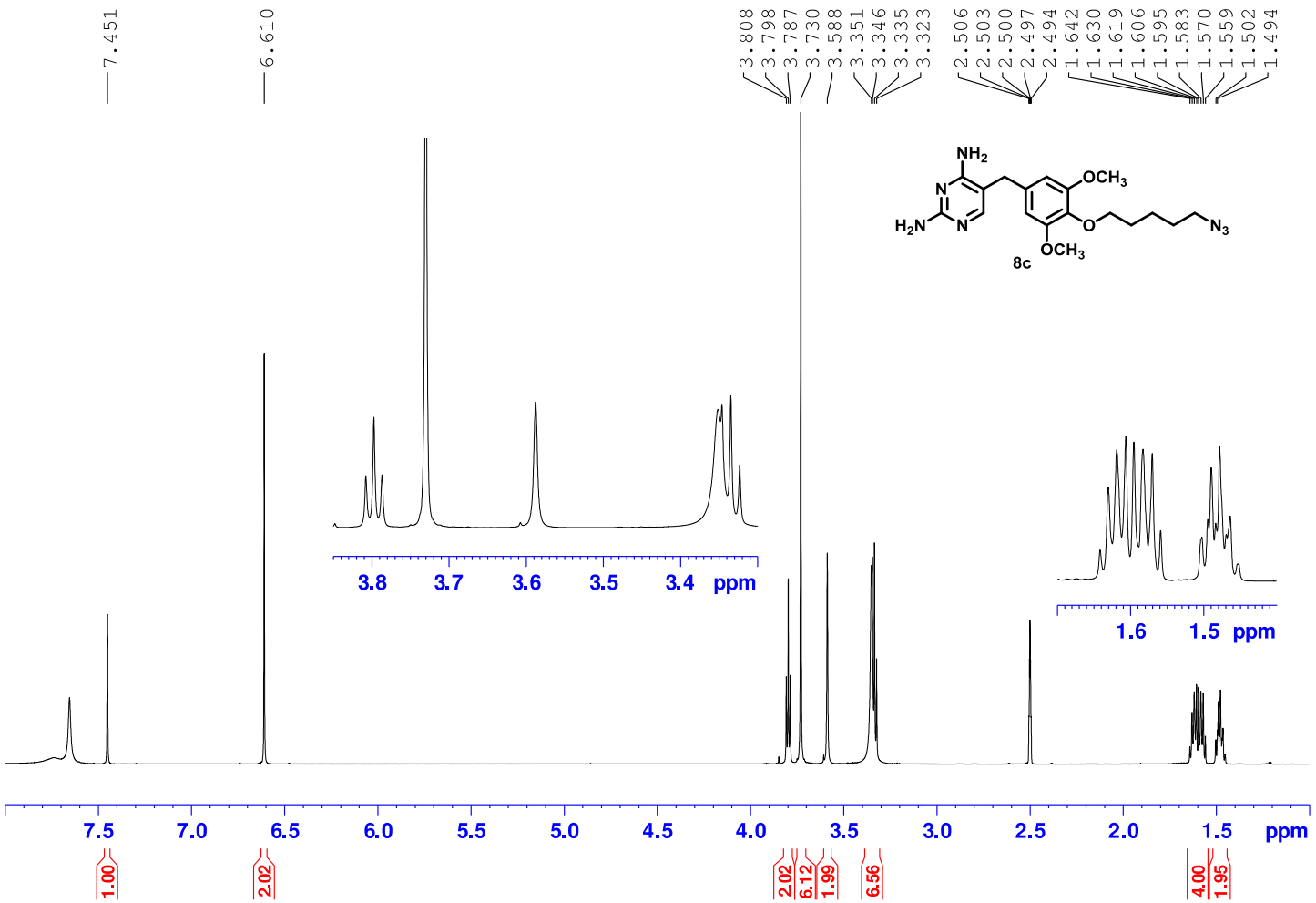


Fig S20. [1]H-NMR (600 MHz, DMSO-d_6) of **8c**.

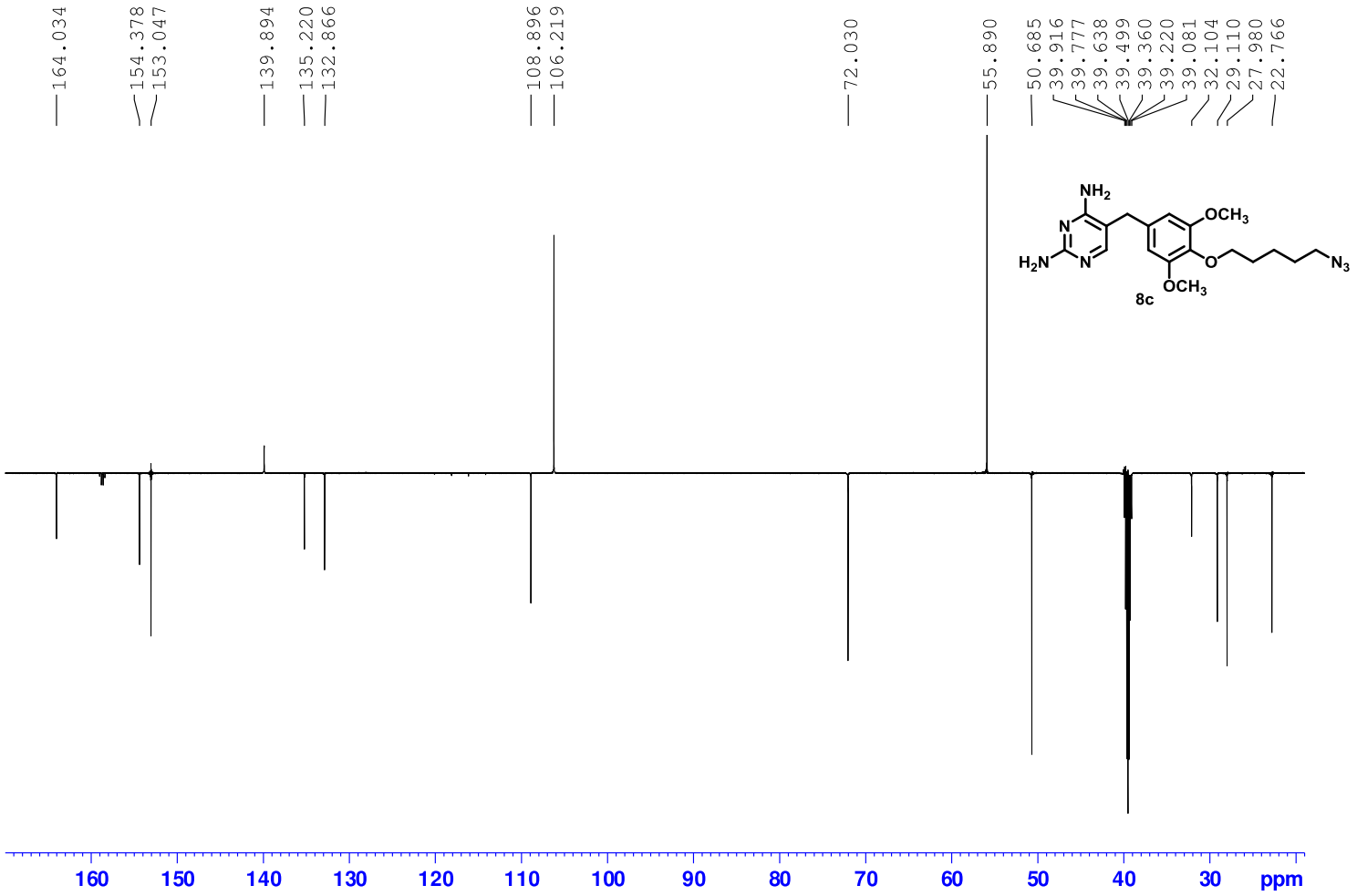


Fig S21. JMOD-NMR (150 MHz, DMSO-d_6) of **8c**.

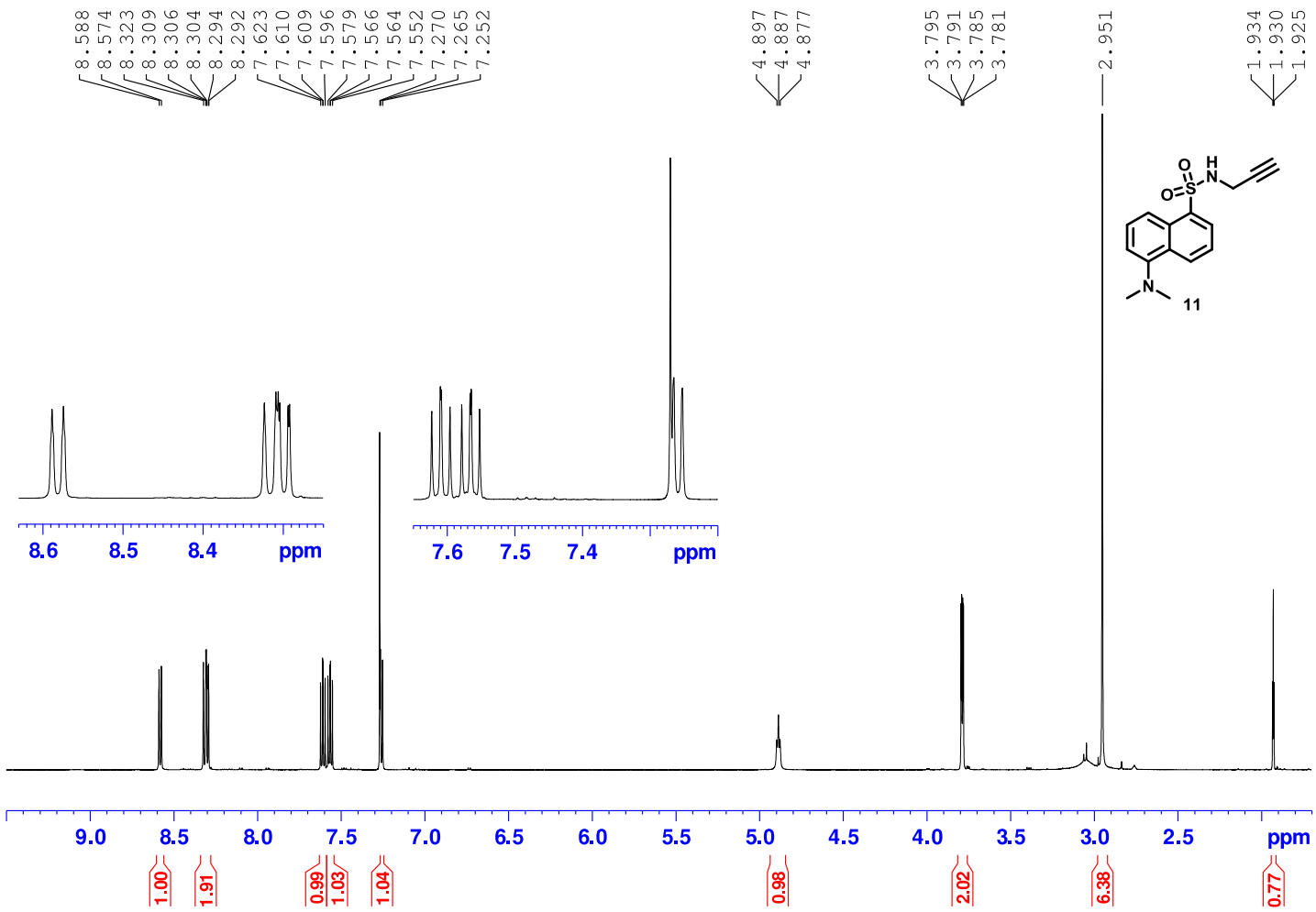


Fig S22. ^{1}H-NMR (600 MHz, $CDCl_3$) of **11**.

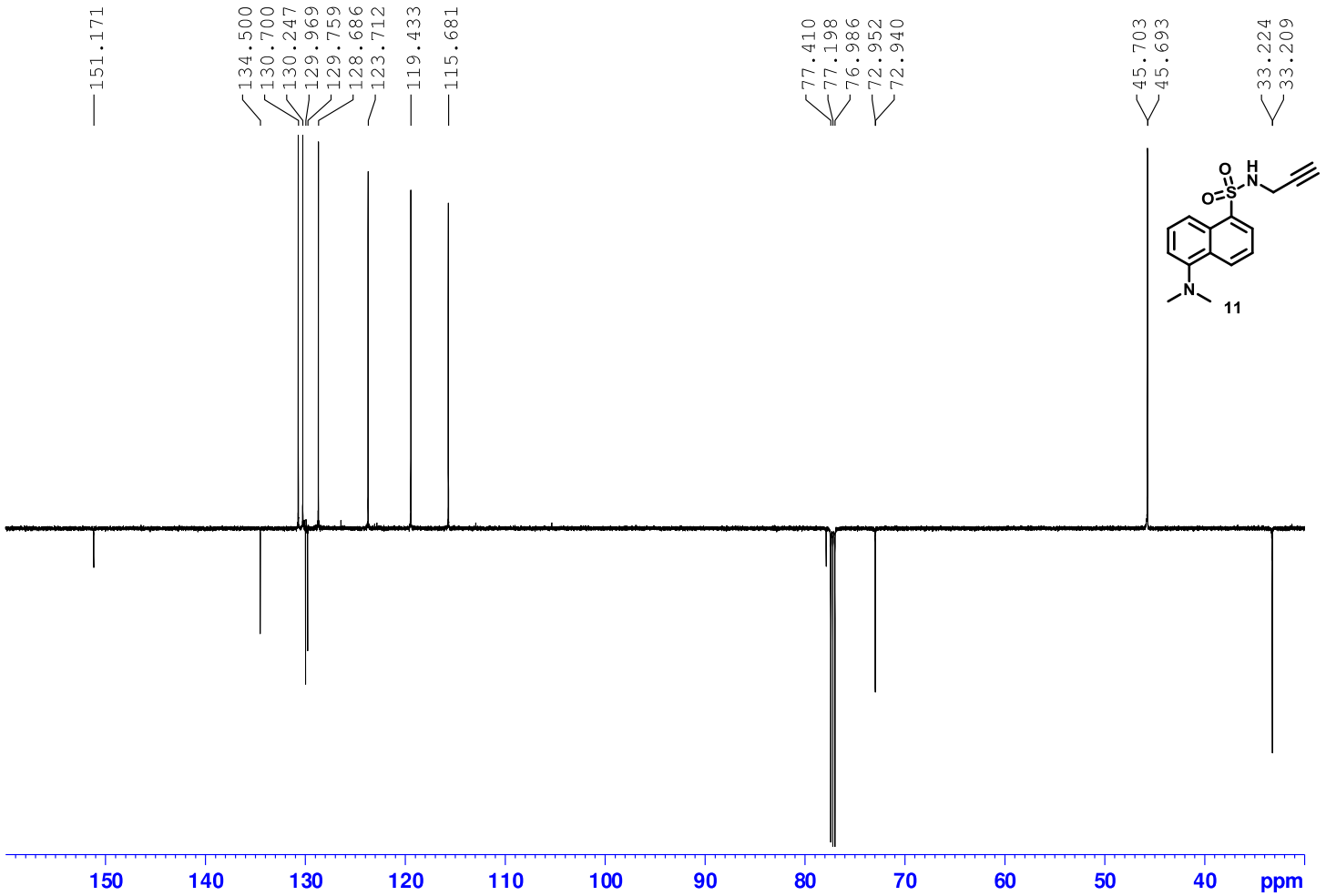


Fig S23. JMOD-NMR (150 MHz, $CDCl_3$) of **11**.

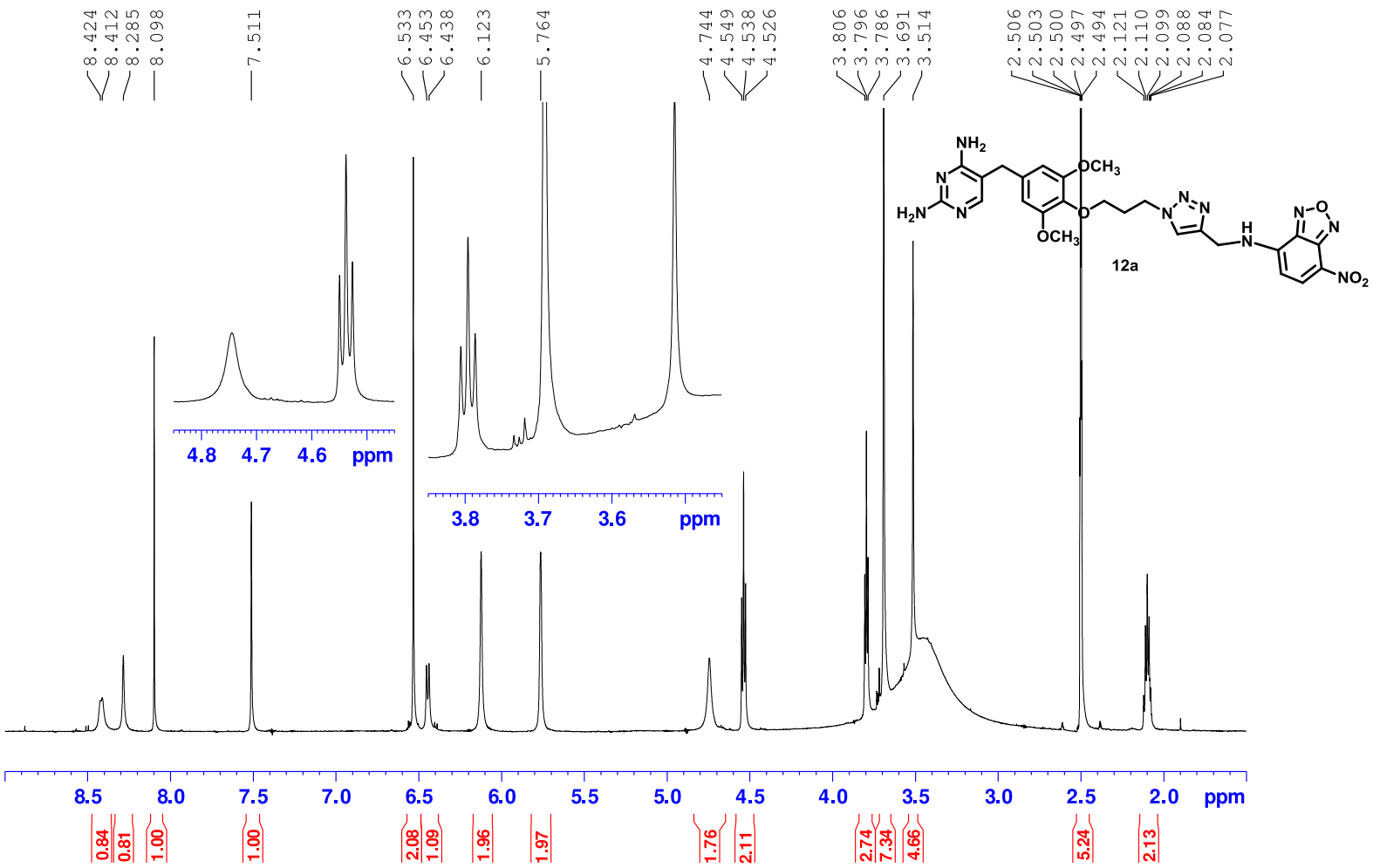


Fig S24. ^{1}H-NMR (600 MHz, DMSO-d_6) of **12a**.

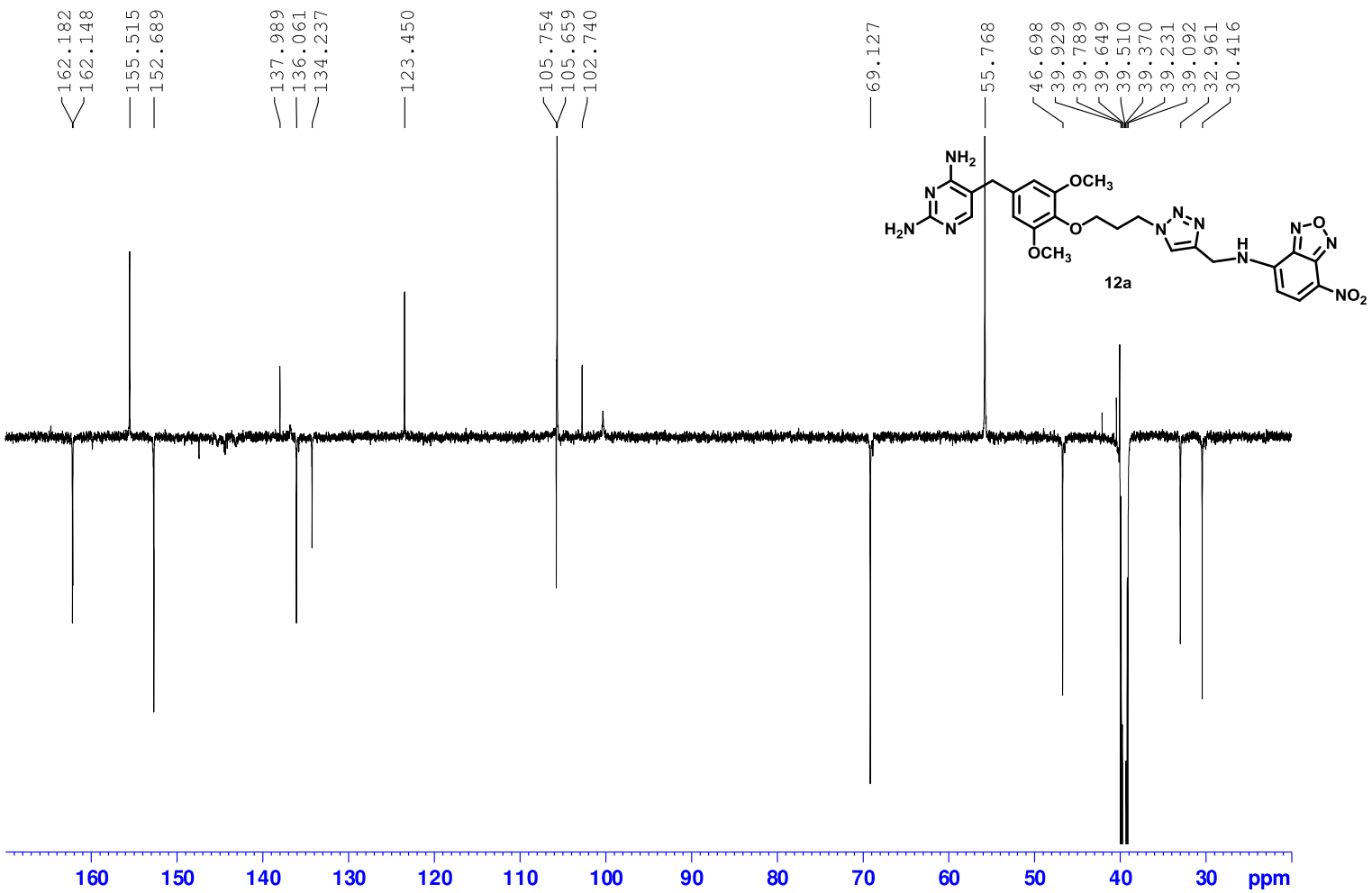


Fig S25. JMOD-NMR (150 MHz, DMSO-d_6) of **12a**.

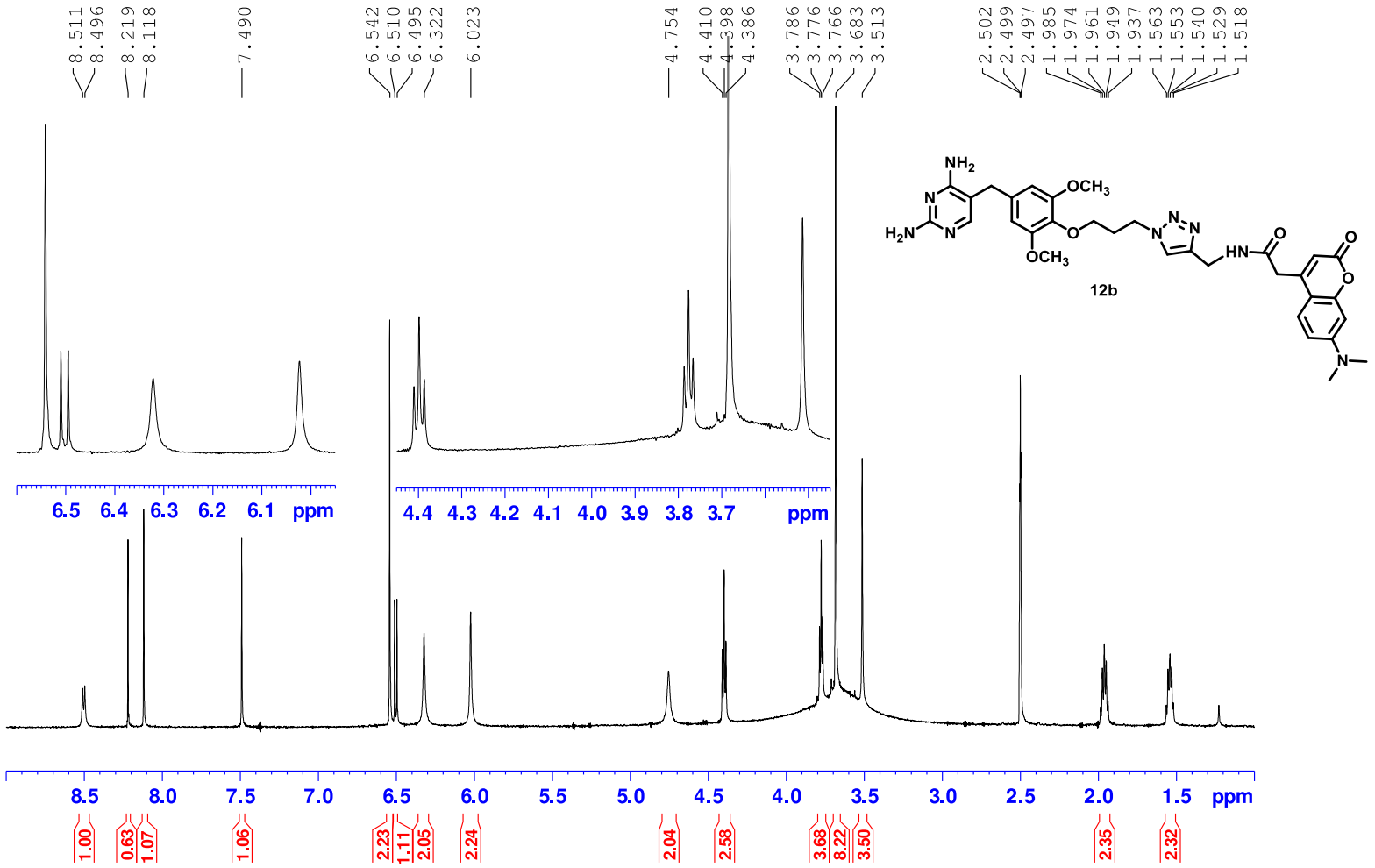


Fig S26. ^{1}H-NMR (600 MHz, DMSO-d_6) of **12b**.

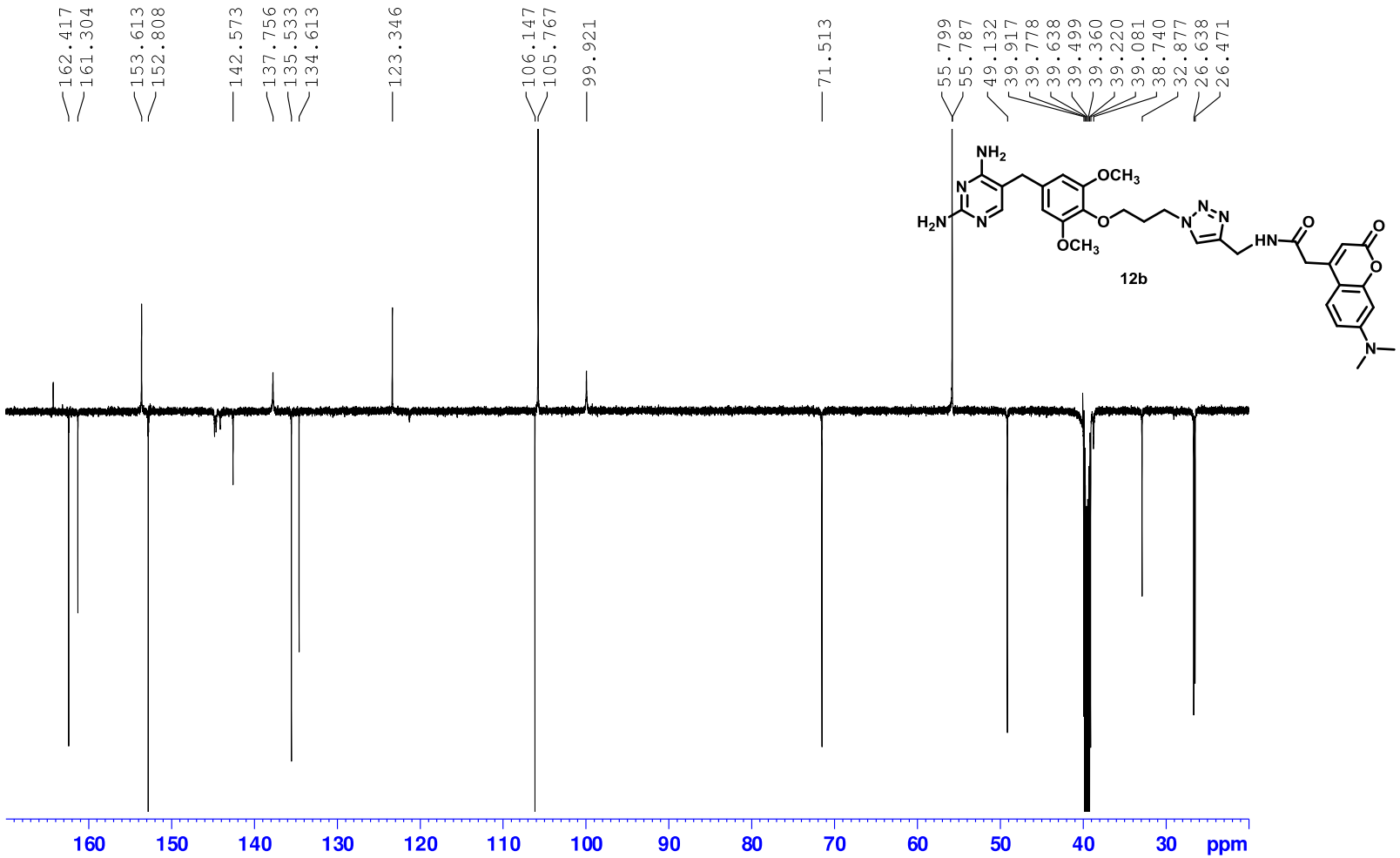


Fig S27. JMOD-NMR (150 MHz, DMSO-d_6) of **12b**.

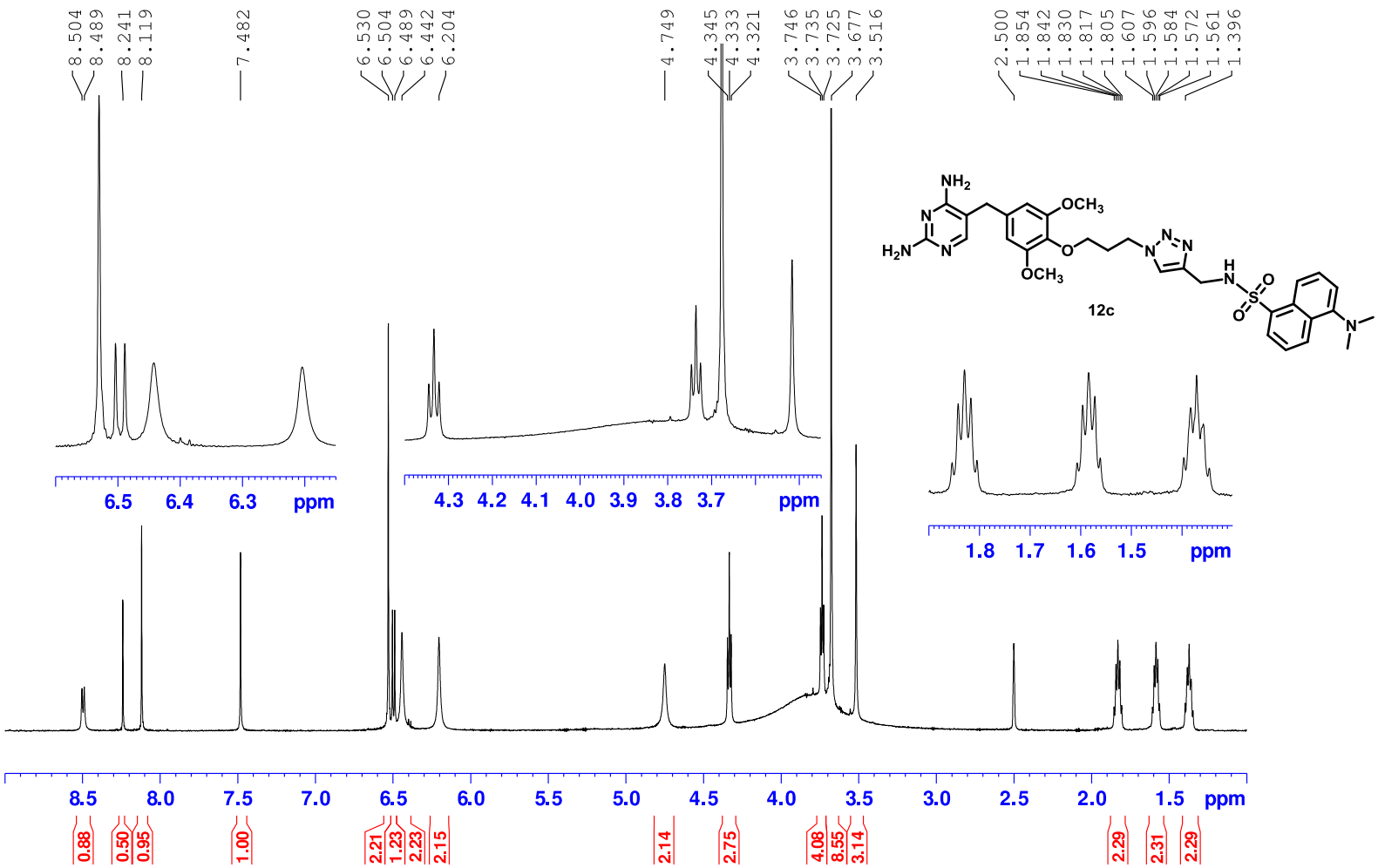


Fig S28. ^{1}H-NMR (600 MHz, DMSO-d_6) of **12c**.

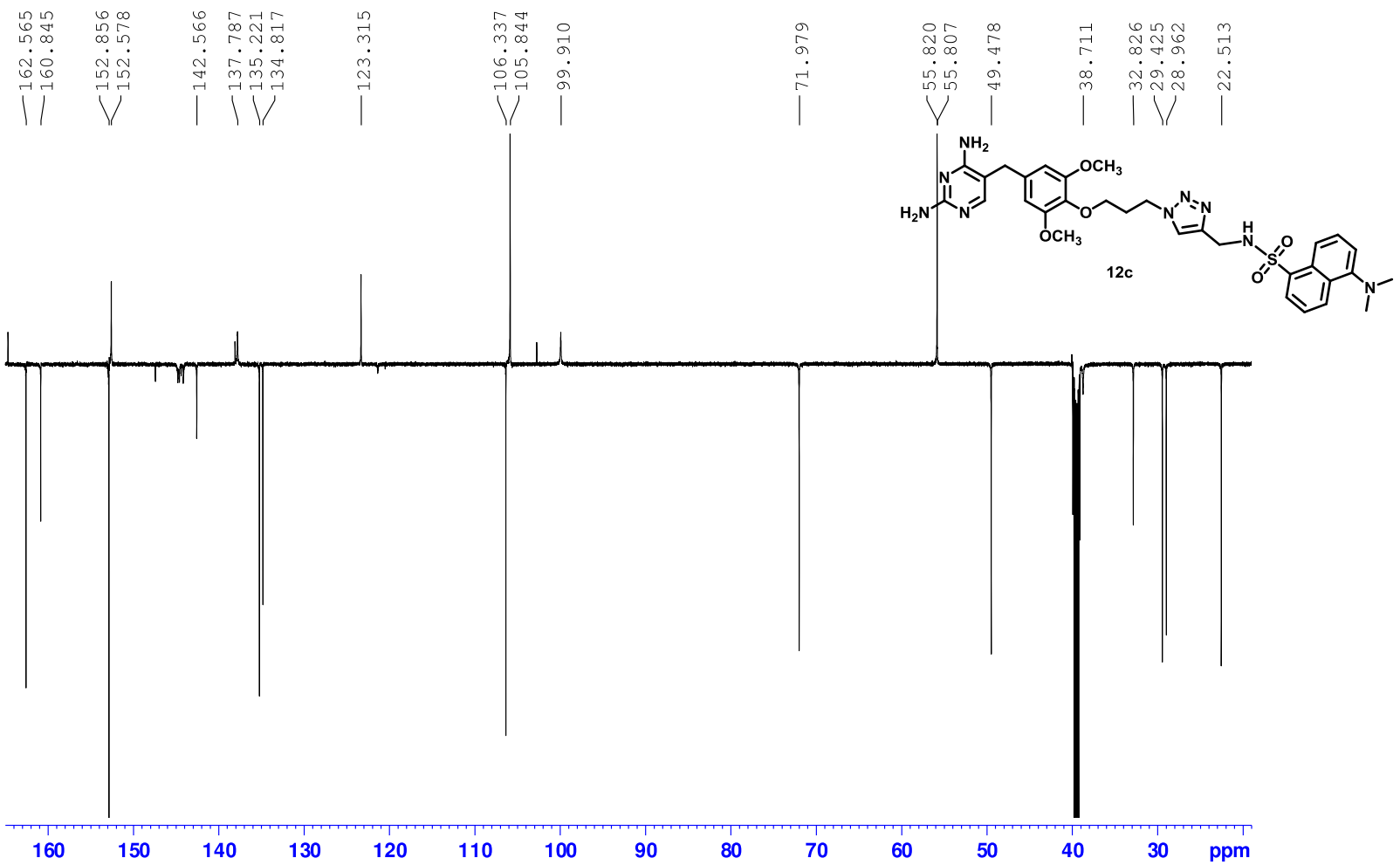


Fig S29. JMOD-NMR (150 MHz, DMSO-d_6) of **12c**.

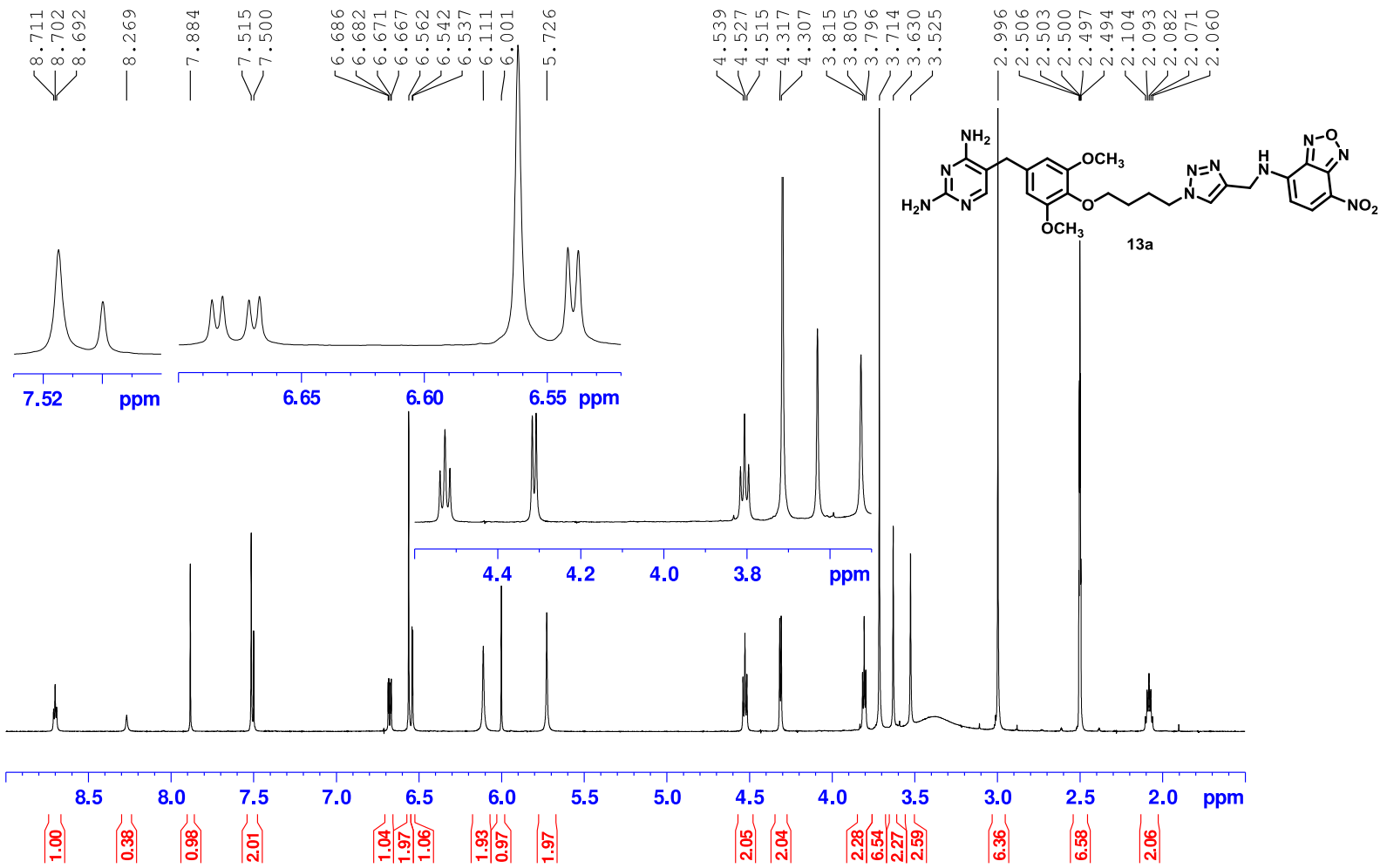


Fig S30. [1]H-NMR (600 MHz, DMSO-d_6) of **13a**.

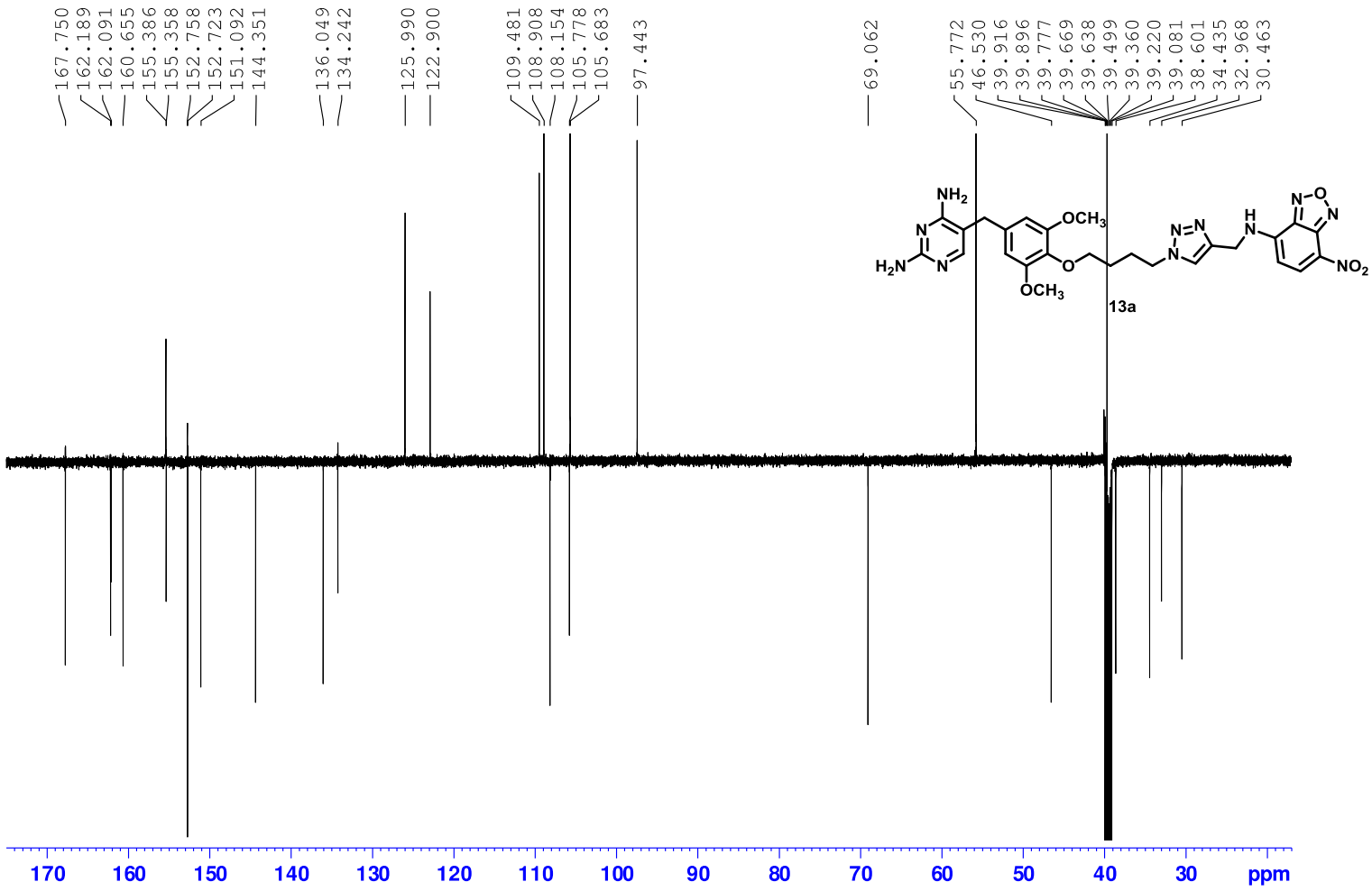


Fig S31. JMOD-NMR (150 MHz, DMSO-d_6) of **13a**.

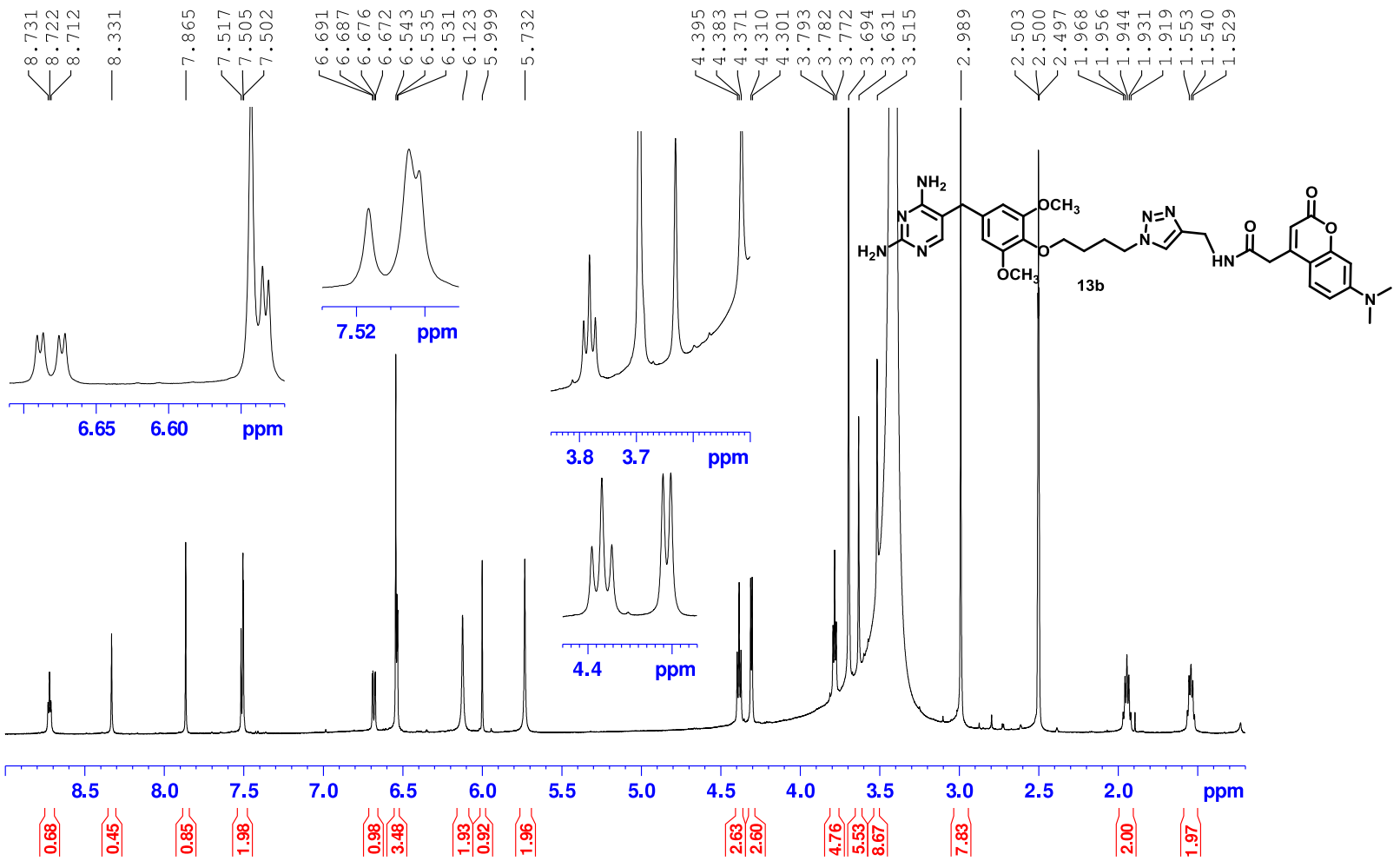


Fig S32. [1]H-NMR (600 MHz, DMSO-d_6) of **13b**.

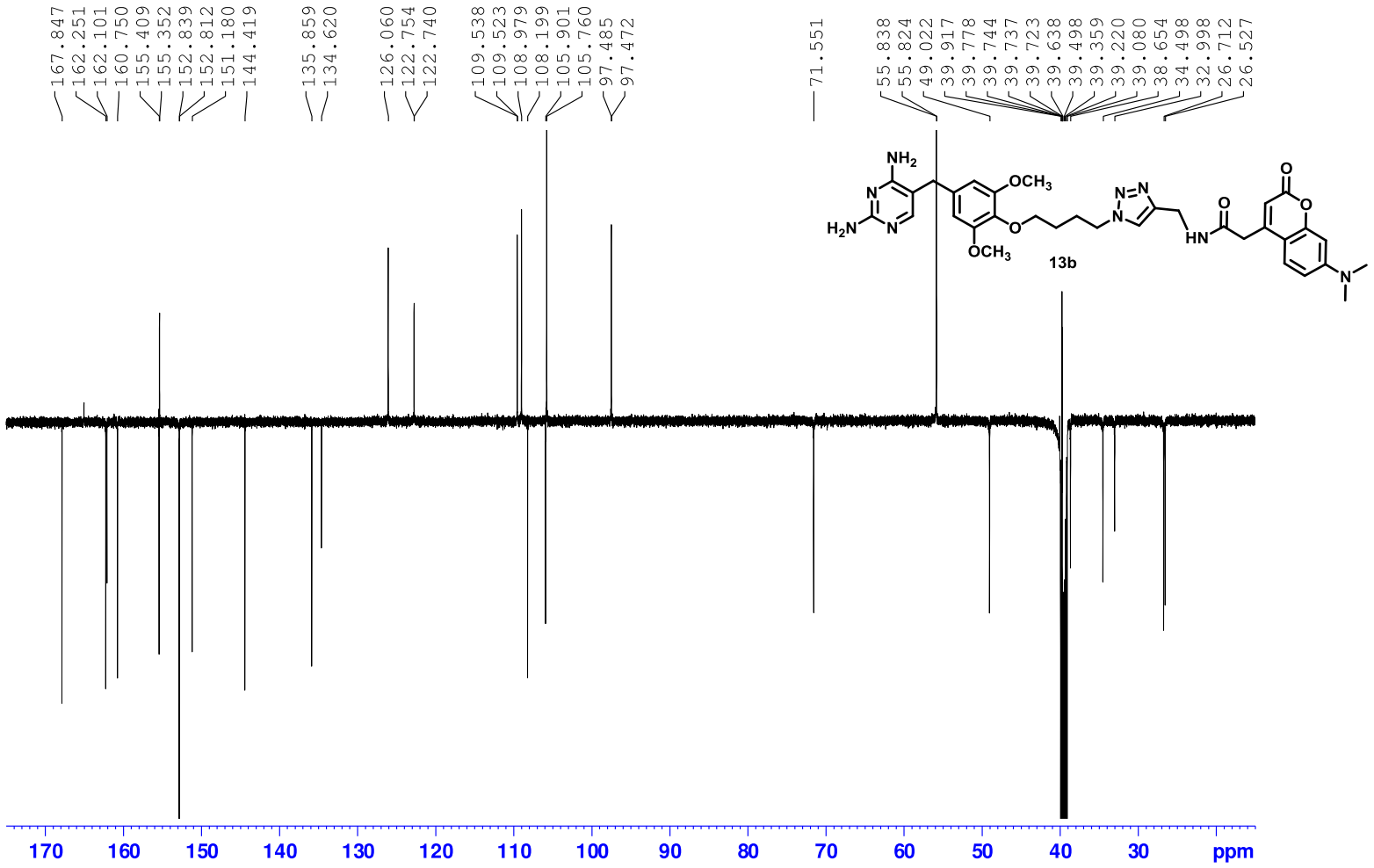


Fig S33. JMOD-NMR (150 MHz, DMSO-d_6) of **13b**.

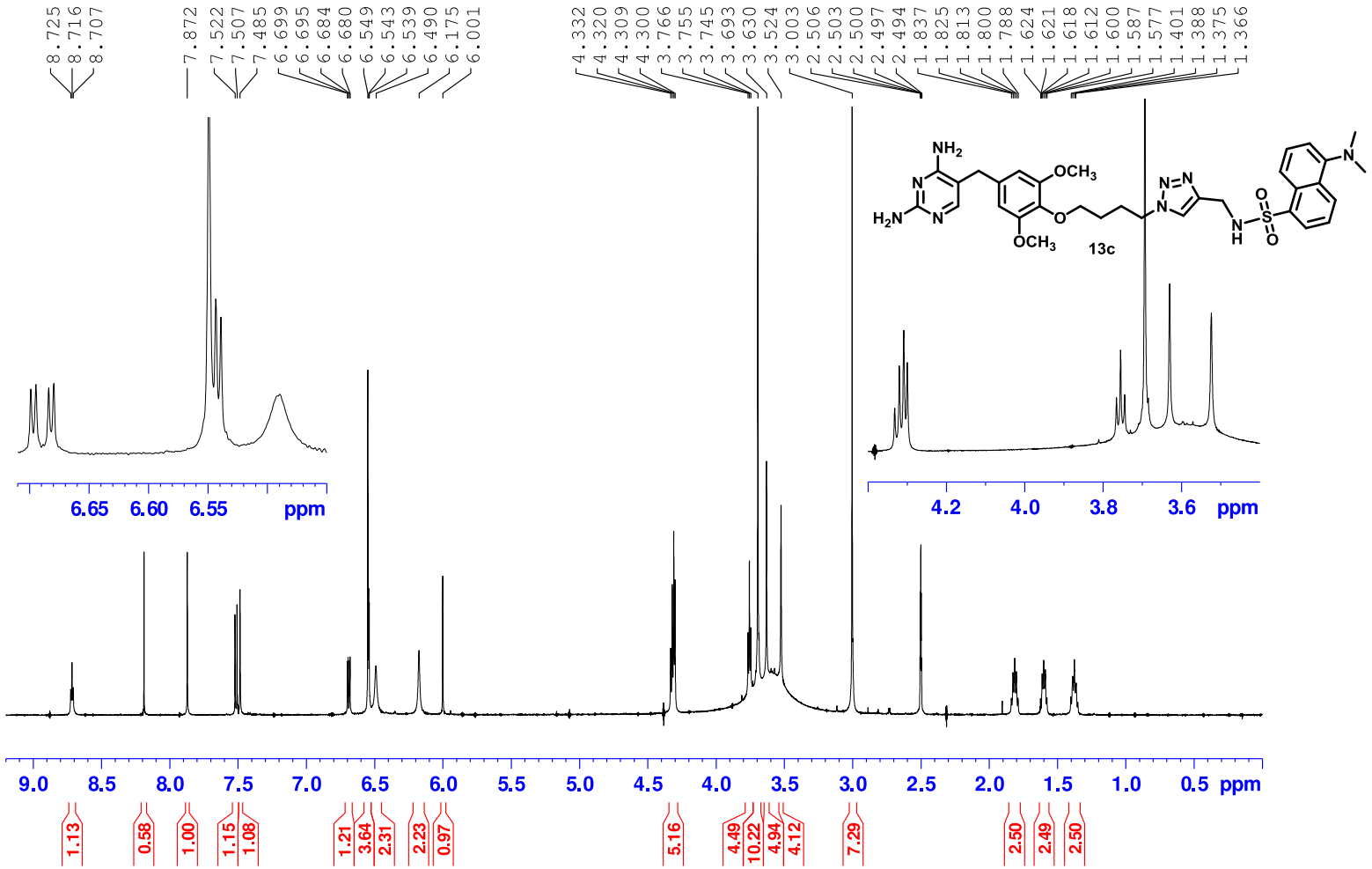


Fig S34. ^{1}H-NMR (600 MHz, DMSO-d_6) of **13c**.

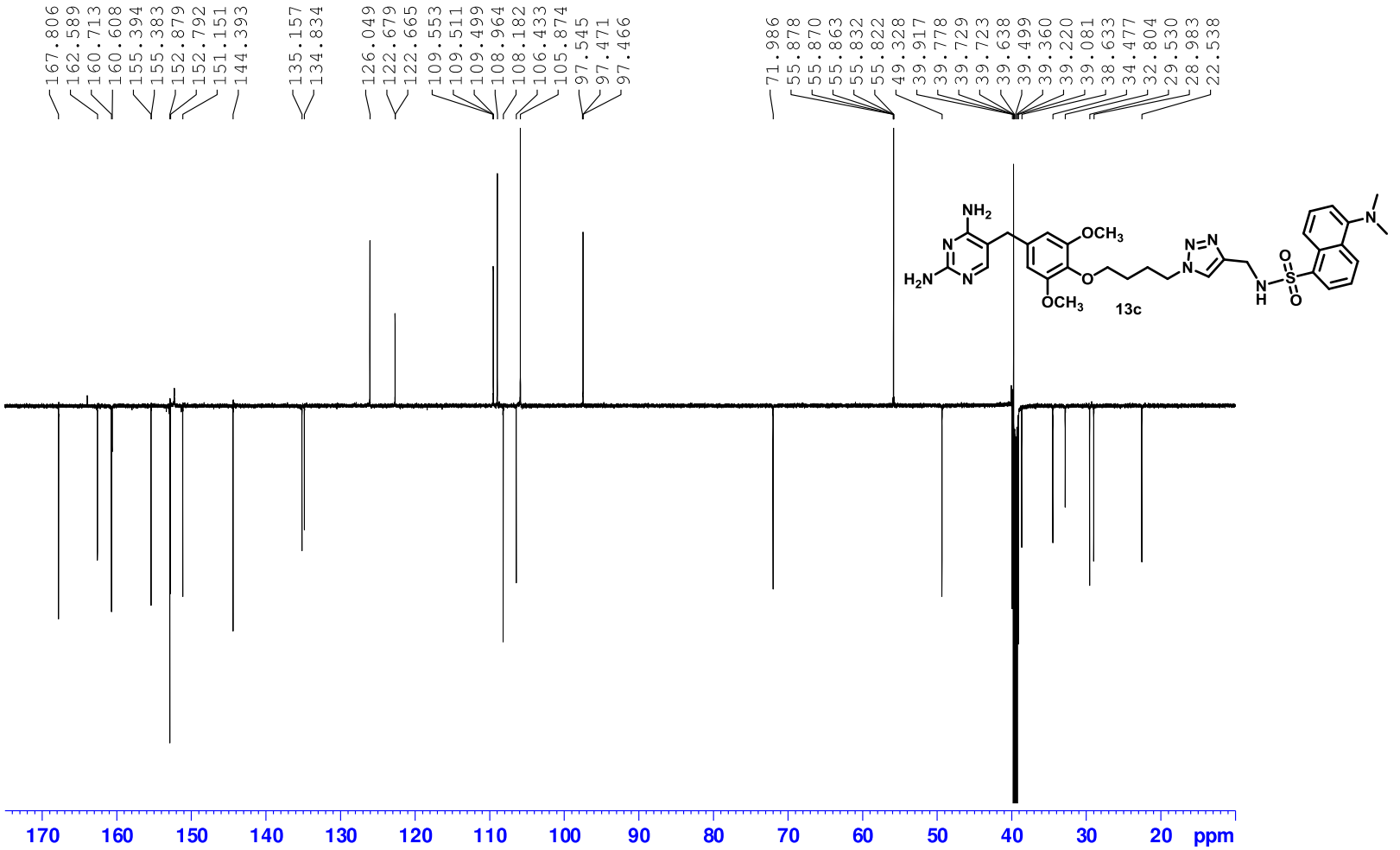


Fig S35. JMOD-NMR (150 MHz, DMSO-d_6) of **13c**.

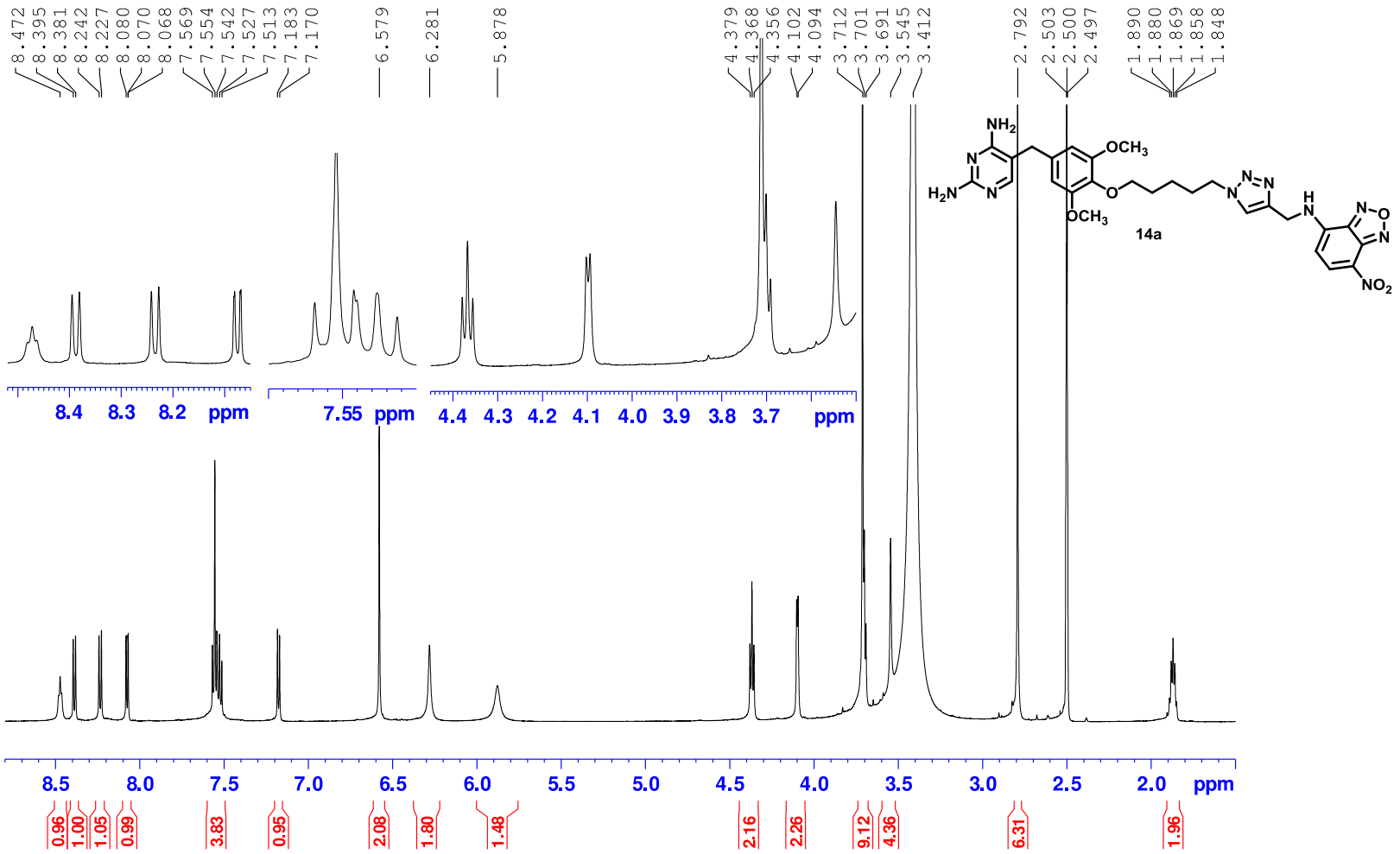


Fig S36. ^{1}H-NMR (600 MHz, DMSO-d_6) of **14a**.

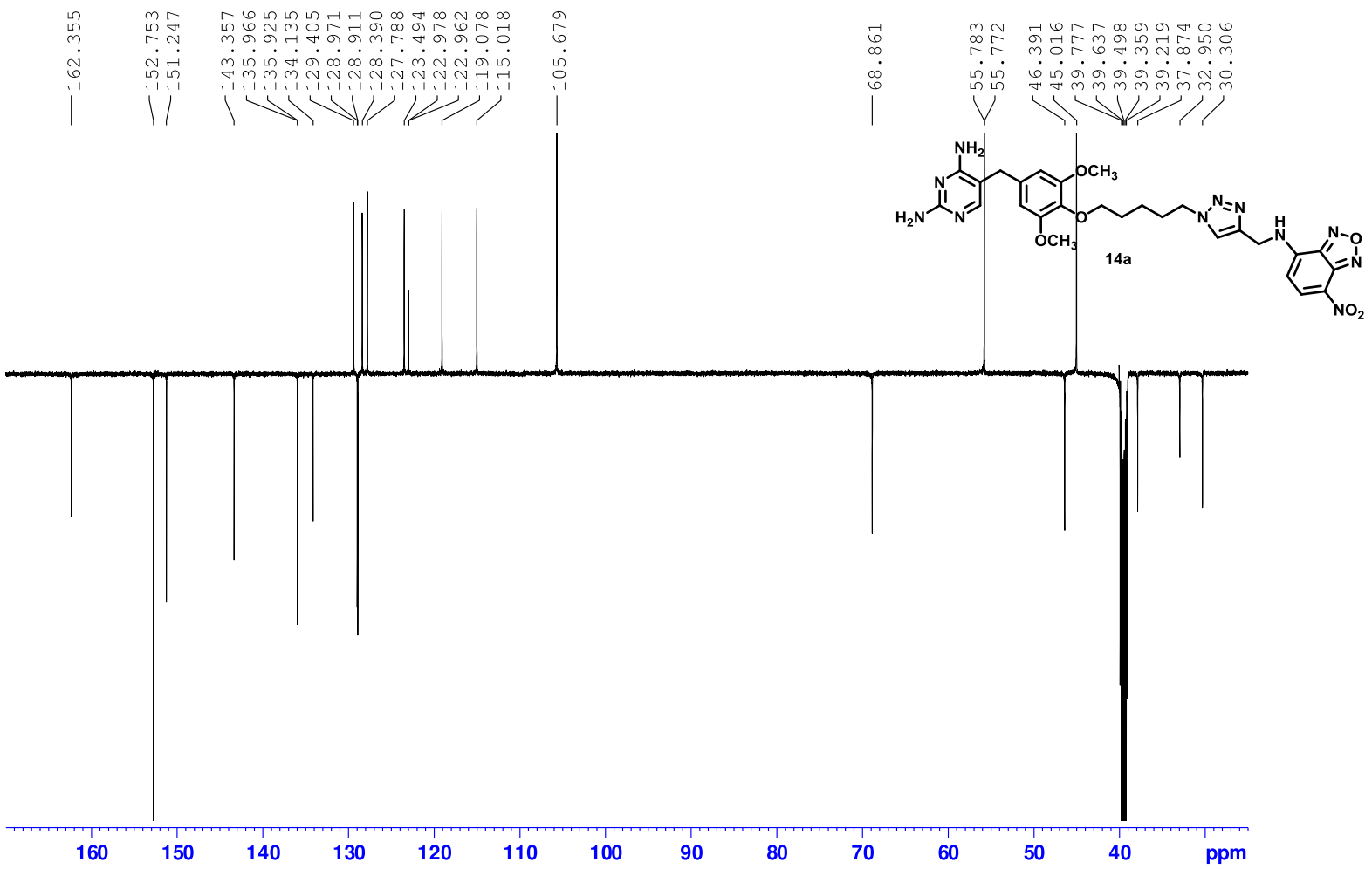


Fig S37. JMOD-NMR (150 MHz, DMSO-d_6) of **14a**.

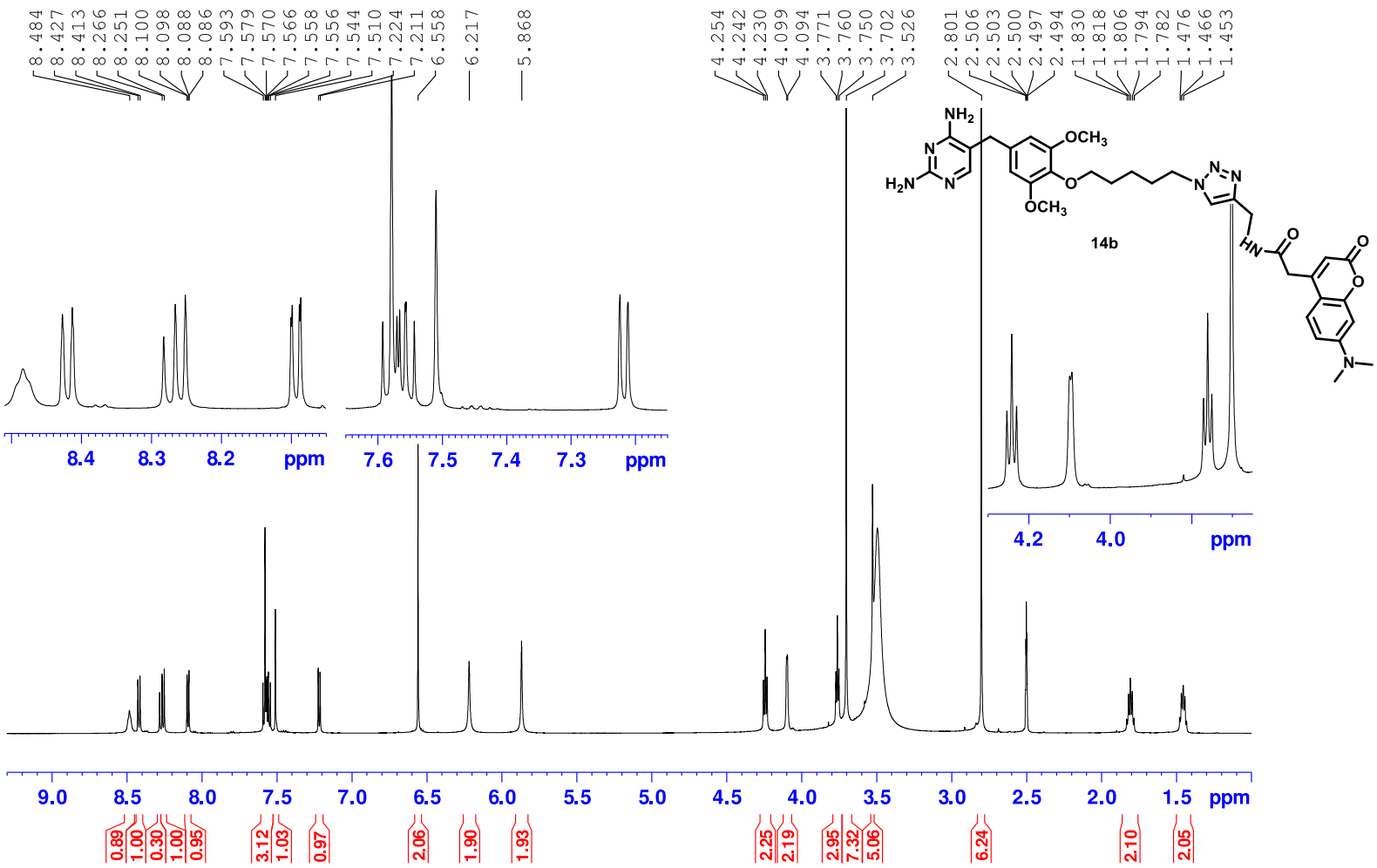


Fig S38. ^{1}H-NMR (600 MHz, DMSO-d_6) of **14b**.

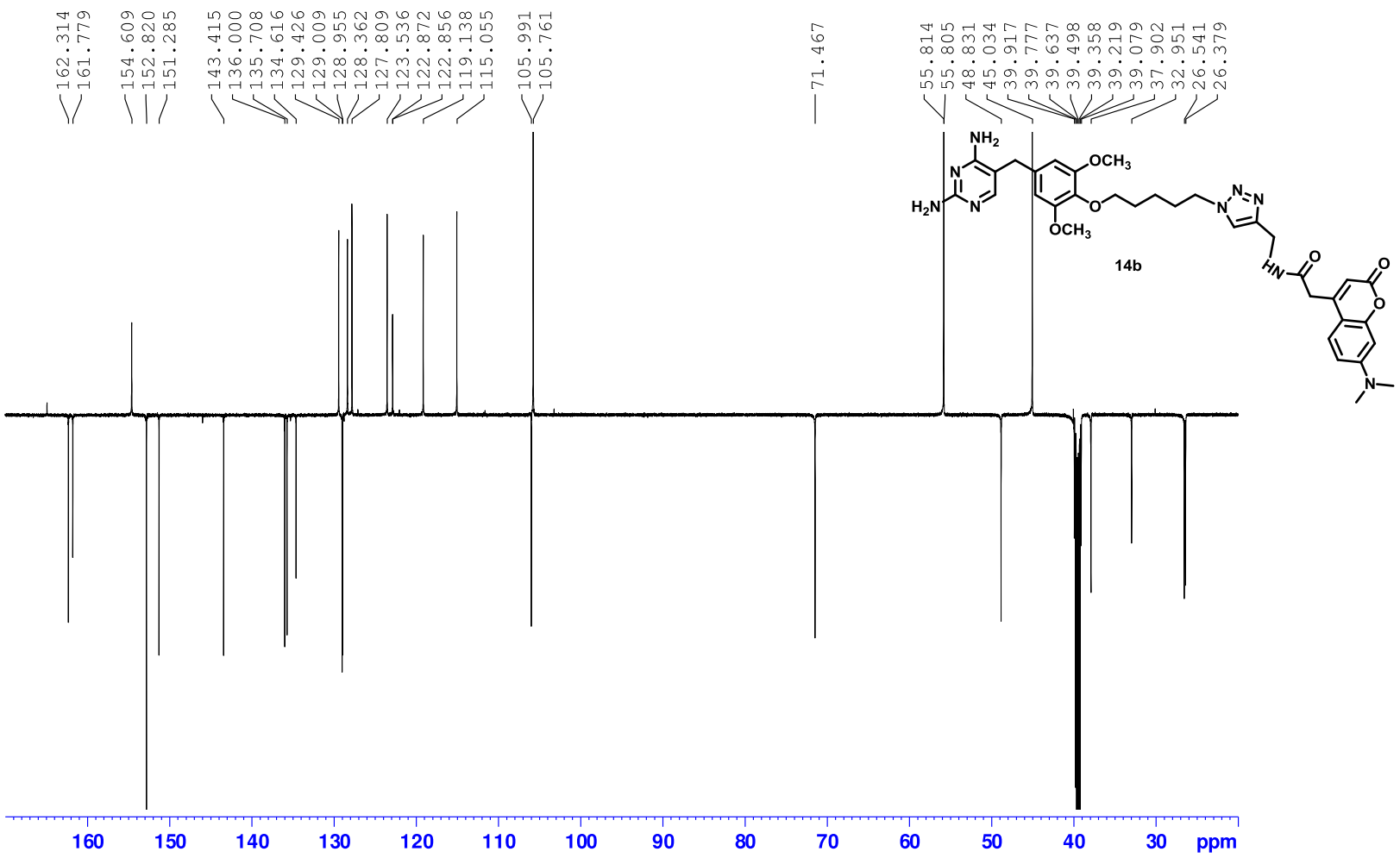


Fig S39. JMOD-NMR (150 MHz, DMSO-d_6) of **14b**.

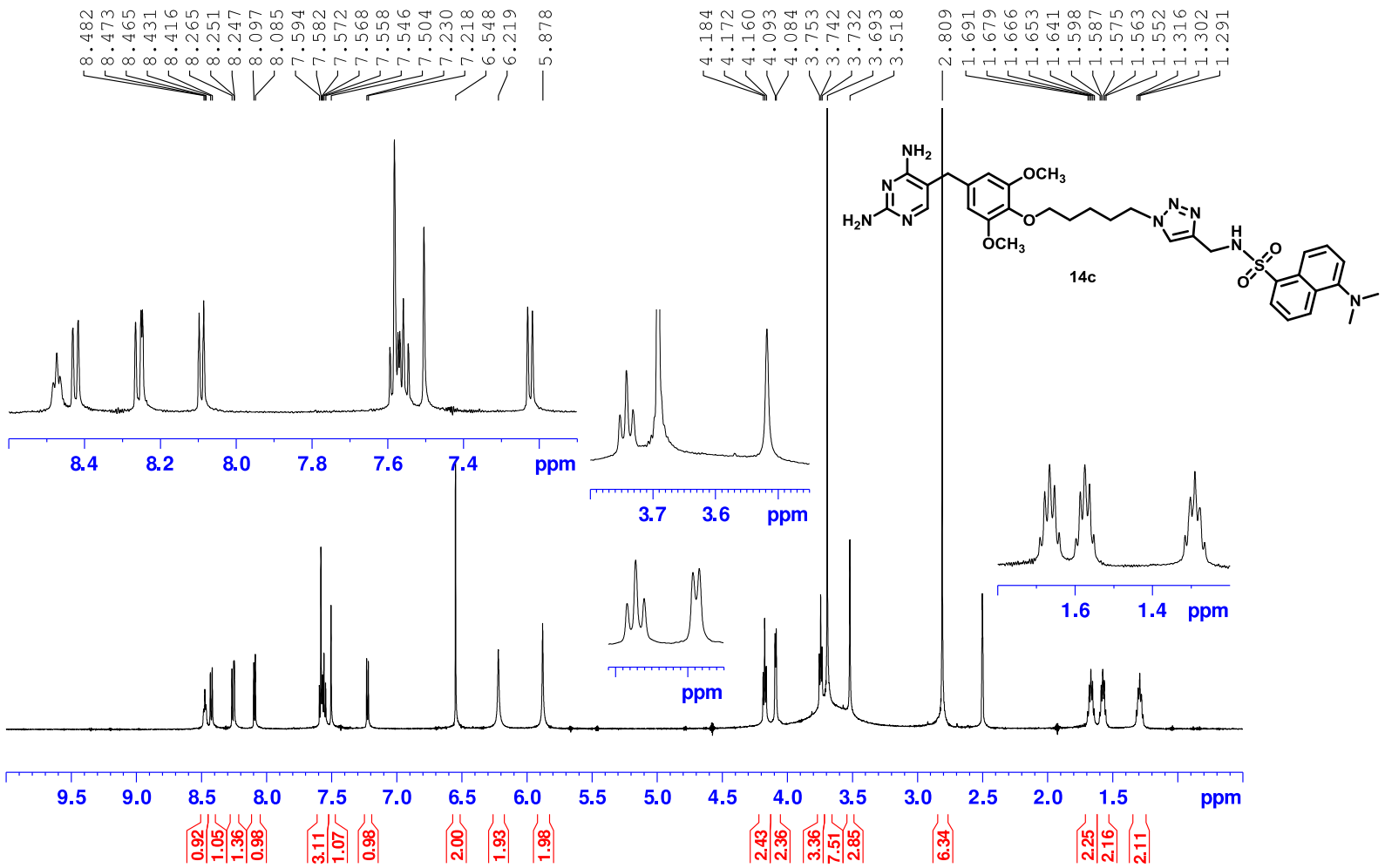


Fig S40. ^{1}H-NMR (600 MHz, DMSO-d_6) of **14c**.

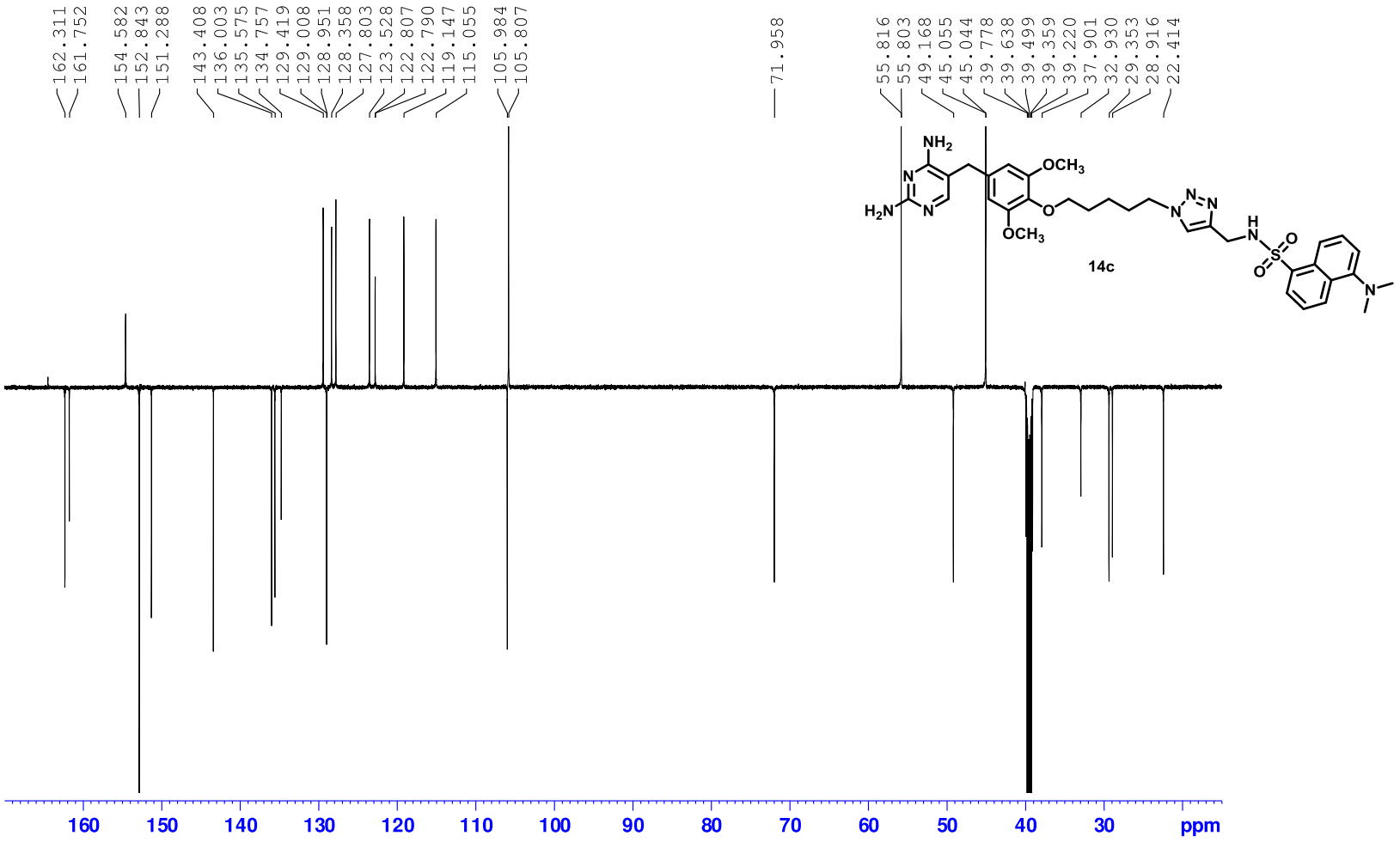


Fig S41. JMOD-NMR (150 MHz, DMSO-d_6) of **14c**.

References

1. Kodali, S., Galgoci, A., Young, K., Painter, R., Silver, L. L., Herath, K. B., Singh, S. B., Cully, D., Barrett, J. F., Schmatz, D., and Wang, J. (2005) Determination of Selectivity and Efficacy of Fatty Acid Synthesis Inhibitors, *J. Biol. Chem. 280*, 1669-1677.