Enhancing outer membrane permeability of tetracycline antibiotics in *P. aeruginosa* using TOB-CIP conjugates

Shiv Dhiman[a†], Danyel Ramirez[a†], Rajat Arora[a], Gilbert Arthur[b], and Frank Schweizer[a,c*]

[a]*Department of Chemistry, Faculty of Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada*

[b]*Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba R3E 0J9, Canada*

[c]*Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba R3E 0J9, Canada*

† *These authors equally contributed to this work.*

[*]*Corresponding author*

Email: Frank.Schweizer@umanitoba.ca

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Table S1. Antibacterial activity of hybrid **1a**, PMBN and tetracycline antibiotics in MDR-*P. aeruginosa* with CA-MHB

***P. aeruginosa* Strain**	**Minimum Inhibitory Concentration (MIC) (µg/mL)**									
	1a	**PMBN**	**MIN**	**DOX**	**TIG**	**ERV**	**CAZ**	**ATM**	**TOB**	**LVX**
PAO1	>128	16-32	32	64	32	>8	2	4	2	0.5
PA259	>128	16	128	64	32	32	512	32	512	>256
PA262	>128	>128	256	>512	64	64	16	32	1024	64
PA264	>128	8	128	>64	32	32	128	64	128	64
PA114228	>128	>128	256	64	64	16	8	32	2	4
PA200	32	8	0.5	1	1	0.5	2	0.25	0.5	0.031
PA095	8	2	64	64	32	32	>64	>64	>16	8
PA100036	>128	16	128	>128	64	32	8	16	64	128
PA101243	>128	>128	8	16	8	8	64	64	128	8

PMBN = polymyxin B nonapeptide; MIN = minocycline, DOX = doxycycline, and ERV = eravacycline; MDR strains = PA259, PA262, PA264, PA100036; Colistin-resistant strains = PA114228 and PA101243; Cystic fibrosis PA095 (non-mucoid); Effux pump deficient strain = PA200. Broth microdilution assay was performed in biological duplicates.

Table S2. Combination studies of hybrid **1a** with tetracyclines against Gram-negative bacteria in CAMHB.

GNB Organism	**AB**	**MIC $_{1a}$ [MIC$_{combo}$] (µg/mL)**	**MIC$_{AB}$ [MIC$_{combo}$] (µg/mL)**	**FICI**	**Interpretation**	**Absolute MIC (µg/mL)[a]**	**Fold Potentiation[b]**
E. coli EC107115	MIN	>128 [4]	128 [4]	0.0312<x<0.062	Synergy	4	32
	DOX	>128 [0.25]	64 [32]	0.5<x<0.562	Additive	32	2
	TIG	>128 [0.25]	1 [1]	1<x<1.02	Additive	1	1
	ERV	>128 [0.25]	1 [1]	1<x<1.02	Additive	1	1
E. coli EC94474	MIN	>128 [8]	64 [8]	0.125<x<0.1875	Synergy	8	8
	DOX	>128 [2]	128 [64]	0.5<x<0.515	Additive	64	2
	TIG	>128 [2]	2 [1]	0.5<x<0.515	Additive	1	2
	ERV	>128 [0.25]	1 [1]	1<x<1.02	Additive	1	1
A. baumannii AB031	MIN	>128 [0.25]	2 [1]	0.5<x<0.502	Additive	1	2
	DOX	>128 [0.25]	32 [16]	0.5<x<0.502	Additive	16	2

	TIG	>128 [1]	8 [4]	0.5<x<0.508	Additive	4	2
	ERV	>128 [0.25]	2 [1]	0.5<x<0.502	Additive	1	2

GNB = Gram-negative bacteria; AB = antibiotics; MIN = minocycline, DOX = doxycycline, and ERV = eravacycline; FICI = fractional inhibitory concentration index; MDR strains = *E. coli* EC107115 and *A. baumannii* AB031; Colistin-resistant strains = *E. coli* EC94474. The checkerboard assay was performed in biological duplicates.

Table S3. Combination studies of compounds **1a** with novobiocin and rifampicin against Gram-negative bacteria in CAMHB.

GNB Organism	**AB**	**MIC $_{1a}$ [MIC$_{combo}$] (µg/mL)**	**MIC$_{AB}$ [MIC$_{combo}$] (µg/mL)**	**FICI**	**Interpretation**	**Absolute MIC (µg/mL)[a]**	**Fold Potentiation[b]**
P. aeruginosa PA01	NOV	>128 [8]	1024 [4]	0.0039<x<0.066	Synergy	4	256-fold
	RIF	>128 [8]	32 [0.0312]	0.00097<x<0.063	Synergy	0.0312	1024-fold
P. aeruginosa PA259	NOV	>128 [8]	2048 [4]	0.002<x<0.064	Synergy	4	512-fold
	RIF	>128 [4]	32 [0.0625]	0.02<x<0.33	Synergy	0.062	512-fold
P. aeruginosa PA262	NOV	>128 [8]	2048 [8]	0.004<x<0.066	Synergy	8	256-fold
	RIF	>128 [8]	512 [16]	0.062<x<0.093	Synergy	16	32-fold
P. aeruginosa PA264	NOV	>128 [8]	1024[2]	0.002<x<0.064	Synergy	2	512-fold
	RIF	>128 [8]	32 [0.125]	0.004<x<0.035	Synergy	0.125	256-fold
P. aeruginosa PA114228	NOV	>128 [8]	2048 [2048]	1<x<1.062	Additive	2048	1-fold
	RIF	>128[4]	32 [16]	0.5<x<0.531	Additve	16	2-fold
A. baumannii ATCC 17978	NOV	>128 [8]	>16 [1]	0.062<x<0.125	Synergy	1	16-fold
	RIF	>128 [8]	>4 [0.125]	0.031<x<0.093	Synergy	0.125	32-fold
A. baumannii AB027	NOV	>128 [8]	>16 [16]	1<x<1.06	Additive	16	1-fold
	RIF	>128[8]	2 [0.062]	0.031<x<0.062	Synergy	0.062	32-fold
A. baumannii AB031	NOV	>128 [8]	>16 [16]	1<x<1.06	Additve	16	1-fold
	RIF	>128[8]	4 [0.25]	0.06<x<0.125	Synergy	0.25	16-fold
A. baumannii AB92247	NOV	>128 [8]	16 [2]	0.125<x<0.187	Synergy	2	8-fold
	RIF	>128[8]	1 [0.125]	0.125<x<0.187	Synergy	0.125	8-fold
E. coli ATCC 25922	NOV	>128 [4]	256 [16]	0.062<x<0.093	Synergy	4	16-fold
	RIF	>128 [4]	8 [2]	0.25<x<0.281	Synergy	2	4-fold
E. coli EC107115	NOV	>128 [1]	512 [32]	0.062<x<0.070	Synergy	32	16-fold
	RIF	>128 [2]	64 [2]	0.031<x<0.046	Synergy	2	32-fold
E. coli EC94474	NOV	>128 [2]	256 [64]	0.25<x<0.312	Synergy	64	4-fold
	RIF	>128 [8]	8 [4]	0.50<x<0.562	Additive	4	2-fold

GNB = Gram-negative bacteria; NOV = novobiocin, RIF = rifampicin, FICI = fractional inhibitory concentration index; Wild-type isolates = *P. aeruginosa* PAO1, *E. coli* ATCC 25922, *A.*

baumannii ATCC 17978; MDR strains = *P. aeruginosa* PA259, PA262, PA264; *E. coli* EC107115; *A. baumannii* AB027, and AB031; Colistin-resistant strains = *P. aeruginosa* PA114228, *E. coli* EC94474, and *A. baumannii* AB92247. The checkerboard assay was performed in biological duplicates.

Table S4. MIC of eravacycline (ERV) against *P. aeruginosa* PAO1 in varying concentrations of fetal bovine serum (FBS).

% FBS	**MIC of ERV (µg/mL)**
0%	8
5%	16
10%	32
25%	128
50%	128

HPLC Analysis

HPLC methodology:

Method-1: Synergy™ 2.5 µm Polar-RP 100 Å, LC column 50 x 2 mm (Phenomenex)

Buffer A: 0.1% TFA in water; Buffer B: 0.1% TFA in acetonitrile

Flow rate: 0.2 ml/min; run time: 20 min; UV-Visible detection at 275nm and 280

Table S5: Gradient used for method-1

Time duration (min)	% Buffer A	% Buffer B
0	85	15
3	85	15
4	80	20
6	80	20
7	70	30
9	70	30
10	60	40
13	60	40
14	50	50
15	50	50
18	85	15
20	85	15

Method-2: Synergy™ 2.5 µm Polar-RP 100 Å, LC column 50 x 2 mm (Phenomenex)

Buffer A: 0.1% TFA in water; Buffer B: 0.1% TFA in acetonitrile

Flow rate: 0.2 ml/min; run time: 20 min; UV-Visible detection at 280

Table S6: Gradient used for method-2

Time duration (min)	% Buffer A	% Buffer B
0	90	10
3	90	10
4	85	15
6	85	15
7	80	20
8	80	20
8.5	70	30
9	50	50
12	50	50
12.5	70	30
13	80	20
14	80	20
15	85	15
16	85	15
17	90	10
20	90	10

Method-3: Synergy™ 2.5 µm Polar-RP 100 Å, LC column 50 x 2 mm (Phenomenex)

Buffer A: 0.1% TFA in water; Buffer B: 0.1% TFA in acetonitrile

Flow rate: 0.1 ml/min; run time: 20 min; UV-Visible detection at 280 nm and 275 nm

Table S7: Gradient used for method-3

Time duration (min)	% Buffer A	% Buffer B
0	90	10
3	90	10
4	85	15
6	85	15
8	80	20
9	70	30
10	40	60
12	40	60
13	70	30
14	80	20
15	85	15
16	85	15
17	90	10
20	90	10

Method-4: Synergy™ 2.5 µm Polar-RP 100 Å, LC column 50 x 2 mm (Phenomenex)

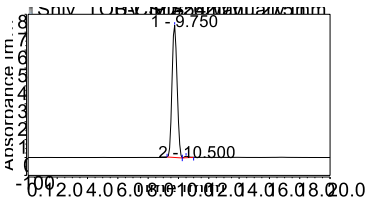
Buffer A: 0.1% TFA in water; Buffer B: 0.1% TFA in acetonitrile

Flow rate: 0.1 ml/min; run time: 20 min; UV-Visible detection at 265

Table S8: Gradient used for method-4

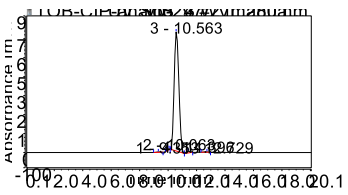
Time duration (min)	% Buffer A	% Buffer B
0	90	10
3	90	10
4	85	15
6	85	15
8	80	20
9	70	30
10	30	70
12	30	70
13	70	30
14	80	20
15	85	15
16	85	15
17	90	10
20	90	10

Chromatogram and Results			
Sample Name:	Compound 1a	Run Time (min):	20:00
Instrument Method:	Method-1	Channel:	UV-VIS-4
Injection date/Time:	09/Mar/23 11:01	Wavelength (nm):	275



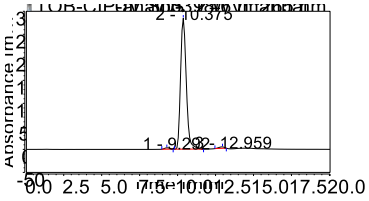
S.No	Retention Time (min)	Area (mAU*min)	Height (mAU)	Relative Area (%)
1	9.750	285.535	737.929	99.08
2	10.500	2.638	8.006	0.92

Chromatogram and Results			
Sample Name:	Compound 1b	Run Time (min):	20:00
Instrument Method:	Method-2	Channel:	UV-VIS-4
Injection date/Time:	06/Apr/23 16:48	Wavelength (nm):	280



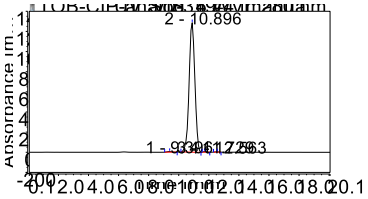
S.No	Retention Time (min)	Area (mAU*min)	Height (mAU)	Relative Area (%)
1	9.313	0.926	2.760	0.30
2	10.063	1.599	7.132	0.52
3	10.563	301.268	783.138	97.74
4	11.396	3.700	11.329	1.20
5	12.729	0.727	2.027	0.24

Chromatogram and Results			
Sample Name:	Compound 1c	Run Time (min):	20:00
Instrument Method:	Method-4	Channel:	UV-VIS-2
Injection date/Time:	22/Sep/23 13:47	Wavelength (nm):	265



S.No	Retention Time (min)	Area (mAU*min)	Height (mAU)	Relative Area (%)
1	9.292	1.152	2.978	0.92
2	10.375	123.188	284.337	98.05
3	12.959	1.303	3.005	1.04

Chromatogram and Results			
Sample Name:	SD-319	Run Time (min):	20:00
Instrument Method:	Method-3	Channel:	UV-VIS-4
Injection date/Time:	22/Sep/23 15:50	Wavelength (nm):	280



S.No	Retention Time (min)	Area (mAU*min)	Height (mAU)	Relative Area (%)
1	9.396	1.999	5.713	0.35
2	10.896	571.724	1285.659	99.30
3	11.729	1.367	5.255	0.24
4	12.563	0.687	2.330	0.12

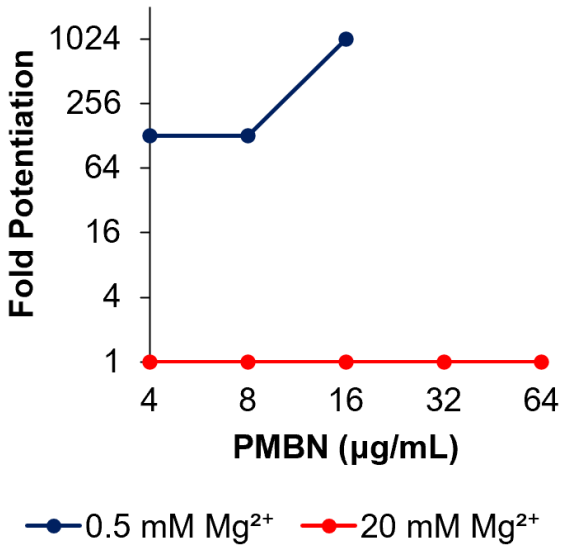


Figure S1. Fold potentiation of minocycline by PMBN against *P. aeruginosa* PAO1 in CAMHB and Mg^{2+} supplemented CAMHB. The checkerboard assay was performed in biological duplicates.

Synthetic procedures and characterizations of compounds 4-7.

Synthesis and characterization of 1, 3, 2′, 6′, 3″-penta-*N*-Boc- 4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (4).[1]

To solution of tobramycin (5.0 g, 10.69 mmol), water (70 mL), and methanol (MeOH) (140 mL) was added Boc-anhydride (23.34 g, 107 mmol) followed by trimethylamine (32.82 mL, 235 mmol) at ambient temperature. After addition, the mixture was heated at 55 ºC for 16 h. After the consumption tobramycin, solvent was evaporated from the reaction mixture under reduced pressure and dried under high vacuum for 24h to obtain crude Boc-protected tobramycin as a white solid in quantitative yield (10.23 g, 99%) and used in the next step without further purification. An oven-dried round bottom flask (RBF) was charged with crude Boc-protected tobramycin (9.0 g, 9.29 mmol) and dry *N,N*-dimethylformamide (DMF) (15 mL) and stirred at ambient temperature under nitrogen atmosphere. Then TBDMS-Cl (14.01 g, 92.97 mmol) was added portion-wise followed by 1-methylimidazole (11.43 ml, 139.46 mmol) dropwise. After addition, the mixture was continuously stirred for 4 days. After completion, DMF was evaporated under reduced pressure, and resulting residue was dissolved in ethyl acetate (500 mL), washed with ice-cold water (x2) and then washed with brine solution. The collected organic layer was dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated to obtain the crude compound as an off-white

solid. The crude compound was purified by flash column chromatography using P60 silica gel and the pure compound was eluted in 12-15% ethyl acetate:hexanes (EtOAc:Hex) (v/v) to give desired product **4** (11.50 g, 77%) as a crystalline white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.40 (s, 1H), 5.26 (s, 1H), 4.99 – 4.90 (m, 2H), 4.51 (s, 1H), 4.32 (s, 1H), 3.88 – 3.83 (m, 2H), 3.70 – 3.14 (m, 13H), 2.74 – 2.72 (m, 1H), 2.04– 2.02 (m, 1H), 1.45 – 1.42 (m, 45H, Boc-^{t}Bu), 0.92 – 0.87 (m, 36H, TBDMS-^{t}Bu), 0.13 – -0.05 (m, 24H, TBDMS-$SiMe_2$). MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{67}H_{133}N_5O_{19}Si_4Na^+$, 1446.8564; observed 1446.7999.

General procedure C for C_5-*O*- alkylation of 1, 3, 2′, 6′, 3″-penta-*N*-Boc- 4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (5a-d).[1]

To a solution of compound **4** (1 equiv.) and toluene (2 mL) were added potassium hydroxide (3 equiv.), tetrabutylammonium hydrogen sulfate (TBAHS) (0.1 equiv.), 1,*n*-dibromoalkylating reagent (3 equiv.) and catalytic amount of water (2-3 drops). The mixture was continuously stirred at ambient temperature for 20 h. After completion the reaction, toluene was evaporated under reduced pressure. The resulting residue was diluted with ethyl acetate (100 mL), washed with water (50 mL) and then brine solution (30 mL). The collected organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to obtain the crude compound as an off-white solid. The crude compound was then purified by flash column chromatography using P60 silica gel and the pure compound was eluted in 10-12% EtOAc:Hex (v/v) to afford desired product **5a-d** (52-76%) as a crystalline white solid.

5-*O*-(12-bromododecyl)-1, 3, 2′, 6′, 3″-penta-*N*-Boc-4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (5a).

This compound was synthesized by following general procedure C using compound **4** (1 g, 0.701 mmol), 1, 12-dibromooctane (0.690 g, 2.1 mmol), potassium hydroxide (0.118 g, 2.1 mmol), tetrabutylammonium hydrogen sulfate (TBAHS) (0.023 g, 0.07 mmol), and toluene (3 mL) to give desired product **5a** (0.841 g, 70%) as a crystalline white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.21 (s, 1H), 5.15 (s, 1H), 5.06 – 5.04 (m, 1H), 4.77 (s, 1H), 4.50 (s, 1H), 4.26 (s, 1H), 4.17 – 4.13 (m, 1H), 4.08 – 4.06 (m, 1H), 3.81 – 3.68 (m, 4H), 3.65 – 3.48 (m, 5H), 3.41 – 3.33 (m, 4H), 3.27 – 3.17 (m, 3H), 2.48 – 2.45 (m, 1H), 2.02 – 1.98 (m, 1H), 1.88 – 1.82 (m, 2H), 1.57 – 1.53 (m, 2H), 1.45 – 1.41 (m, 45H, Boc-^{t}Bu), 1.31 – 1.23 (m, 18H, CH_2 linker), 0.94 – 0.86 (m, 36H, TBDMS-

^{t}Bu), 0.15 – 0.02 (m, 24H, TBDMS-SiMe$_2$). MALDI TOF-MS *m/e* [M+Na]$^+$ calcd for $C_{79}H_{156}BrN_5O_{19}Si_4Na^+$, 1692.9547; observed *m/e*, 1692.9785.

Synthesis and characterization of 5-*O*-(8-bromooctyl)-1, 3, 2′, 6′, 3″-penta-*N*-Boc- 4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (5b).

This compound was synthesized by following general procedure C using compound **9** (1 g, 0.701 mmol), 1,8-dibromooctane (0.572 g, 2.1 mmol), potassium hydroxide (0.118 g, 2.1 mmol), tetrabutylammonium hydrogen sulfate (TBAHS) (0.023 g, 0.07 mmol), and toluene (3 mL) to give desired product **10** (0.862 g, 76%) as a crystalline white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.22 (s, 1H), 5.14 (s, 1H), 5.03 – 5.01 (m, 1H), 4.76 (s, 1H), 4.50 (s, 1H), 4.24 (s, 1H), 4.15 (s, 1H), 4.08 (s, 1H), 3.82 – 3.68 (m, 4H), 3.62 – 3.53 (m, 5H), 3.43 – 3.34 (m, 4H), 3.27 – 3.20 (m, 3H), 2.48 – 2.45 (m, 1H), 2.02 – 1.98 (m, 1H), 1.87 – 1.81 (m, 2H), 1.56 – 1.41 (m, 49H, Boc-^{t}Bu and CH_2 linker), 1.31 – 1.27 (m, 6H, CH_2 linker), 0.94 – 0.86 (m, 36H, TBDMS-^{t}Bu), 0.15 – 0.02 (m, 24H, TBDMS-SiMe$_2$). MALDI TOF-MS *m/e* [M+Na]$^+$ calcd for $C_{75}H_{148}BrN_5O_{19}Si_4Na^+$, 1636.8921; observed 1636.9015.

5-*O*-(2-bromo-PEG3)-1, 3, 2′, 6′, 3″-penta-*N*-Boc-4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (5c).

This compound was synthesized by following general procedure C using compound **4** (0.600g, 0.421 mmol), bromo-PEG3-bromide (0.404 g, 1.26 mmol), potassium hydroxide (0.071 g, 1.26 mmol), tetrabutylammonium hydrogen sulfate (TBAHS) (0.014 g, 0.042 mmol), and toluene (3 mL) to give desired product **5c** (0.368 g, 52%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.31 (s, 1H), 5.17 (s, 2H), 4.76 (s, 1H), 4.51 (s, 1H), 4.18 – 4.03 (m, 3H), 3.79 (d, J = 6.4 Hz, 2H), 3.77 – 3.72 (m, 2H), 3.69 – 3.50 (m, 16H), 3.46 (t, J = 6.4 Hz, 2H), 3.42 – 3.24 (m, 4H), 3.11 (s, 1H), 2.45 – 2.43 (m, 1H), 1.96 – 1.94 (m, 1H), 1.58 – 1.39 (m, 46H, Boc-^{t}Bu), 0.93 – 0.86 (m, 36H, TBDMS-^{t}Bu), 0.15 – -0.02 (m, 24H, TBDMS-SiMe$_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.46, 154.81, 154.65, 96.31, 92.08, 85.44, 79.84, 79.38, 79.25, 78.93, 75.13, 72.51, 71.98, 71.42, 71.18, 70.89, 70.54, 70.50, 70.45, 70.29, 68.18, 67.22, 63.24, 57.22, 50.54, 49.06, 48.39, 41.72, 36.63, 36.07, 35.45, 30.23, 28.62, 28.49, 28.44, 26.11, 26.01, 25.98, 25.80, 24.67, 23.37, 18.50, 18.29, 18.07, 17.93, -3.50, -3.78, -4.18, -4.84, -4.91, -5.04, -5.13, -5.19. MALDI TOF-MS *m/e* [M+Na]$^+$ calcd for $C_{75}H_{148}BrN_5O_{22}Si_4Na^+$, 1684.8769; observed *m/e*, 1684.9650.

5-*O*-((4-bromomethyl)biphenyl)-1, 3, 2′, 6′, 3″-penta-*N*-Boc-4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (5d).

This compound was synthesized by following general procedure C using compound **4** (0.584 g, 0.409 mmol), 4,4-bis(bromomethyl)biphenyl (0.417 g, 1.22 mmol), potassium hydroxide (0.069 g, 1.22 mmol), tetrabutylammonium hydrogen sulfate (TBAHS) (0.014 g, 0.041 mmol), and toluene (2 mL) to give desired product **5d** (0.764 g, 65%) as a crystalline white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, J = 7.9 Hz, 2H), 7.49 – 7.43 (m, 6H), 5.35 (s, 1H), 5.24 – 5.23 (m, 2H), 5.11 – 5.10 (m, 1H), 5.00 (s, 1H), 4.83 (s, 1H), 4.63 – 4.50 (m, 4H), 4.29 – 4.24 (m, 2H), 3.87 – 3.85 (m, 2H), 3.73 – 3.58 (m, 5H), 3.52 – 3.48 (m, 1H), 3.45 – 3.32 (m, 4H), 3.18 (s, 1H), 2.53 – 2.51 (m, 1H), 2.03 – 2.01 (m, 1H), 1.59 – 1.25 (m, 46H, Boc-^{t}Bu), 0.98 – 0.81 (m, 36H, TBDMS-^{t}Bu), 0.15 – -0.11 (m, 24H, TBDMS-$SiMe_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.64, 154.81, 154.63, 141.30, 139.55, 137.23, 136.55, 129.42, 129.15, 127.42, 126.95, 98.23, 96.22, 85.76, 79.95, 79.44, 79.08, 78.54, 75.75, 74.44, 72.62, 71.52, 68.02, 67.14, 63.66, 57.42, 50.50, 48.90, 48.34, 41.61, 35.98, 35.63, 33.39, 28.62, 28.52, 28.49, 28.26, 26.12, 26.04, 25.95, 25.78, 18.42, 18.31, 18.11, 17.92, -3.33, -3.61, -4.23, -4.92, -5.20, -5.27. MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{81}H_{144}BrN_5O_{19}Si_4Na^+$, 1704.8608; observed 1704.8703.

General procedure D for preparation of terminal amine-tethered 1, 3, 2′, 6′, 3″-penta-*N*-Boc- 4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (6a-d).[2]

An oven-dried clean RBF was charged with bromoalkylated tobramycin (**5a-d**) (1.0 equiv.), sodium azide (20 equiv.), dry DMF (5 mL) and mixture was stirred and heated up to 90 °C for 5 h. After completion, DMF was evaporated, and resulting residue was diluted with ethyl acetate (100 mL), washed with ice-cold water (x2) followed with brine solution. The collected organic layer, dried over anhydrous Na_2SO_4, filtered, and concentrated to obtain the crude azido compound as an off-white solid which was used in the next step without further purification. The azido intermediate (1.0 equiv.) was dissolved in methanol (20 mL) and then $Pd(OH)_2/C$ (0.1 equiv., 20 wt% loading) was added. The mixture was stirred under H_2-gas balloon at ambient temperature for 5 h. After completion, resulting reaction mixture was filtered through a celite bed, washed with methanol (x2), and concentrated the filterate. The crude compound was purified by flash column

chromatography (P60 silica gel) and the pure compound and was eluted in 5-10% methanol:dichloromethane (MeOH:DCM) (v/v) to give corresponding amino appended tobramycin derivatives (**6a-d**) as white solid (62-77%).

5-*O*-(12-aminododecyl)-1, 3, 2', 6', 3"-penta-*N*-Boc-4',2",4",6"-tetra-*O*-TBDMS-tobramycin (6a).

Compound 18 was synthesized by follow general procedure D using compound **5a** (0.780 g, 0.466 mmol), NaN_3 (0.606 g, 9.33), $Pd(OH)_2/C$ (0.033 g, 0.047 mmol, 20 wt% loading) and obtained the pure product **6a** (0.590 g, 77%) as white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.22 (s, 1H), 5.16 (s, 1H), 5.08 – 5.03 (m, 1H), 4.80 (s, 1H), 4.55 (s, 1H), 4.28 – 3.95 (m, 5H), 3.82 – 3.69 (m, 4H), 3.63 – 3.35 (m, 8H), 3.28 – 3.21 (m, 3H), 2.79 (t, J = 7.5 Hz, 2H), 2.49 – 2.46 (m, 1H), 2.03 – 2.00 (m, 1H), 1.59 – 1.53 (m, 3H), 1.46 – 1.42 (m, 45H, Boc-^{t}Bu), 1.31 – 1.24 (m, 16H), 0.96 – 0.87 (m, 36H, TBDMS-^{t}Bu), 0.16 – -0.03 (m, 24H, TBDMS-$SiMe_2$). MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{79}H_{158}N_6O_{19}Si_4Na^+$, 1628.0395; measured *m/e*, 1628.0399.

Synthesis and characterization of 5-*O*-(8-aminooctyl)-1, 3, 2′,6′, 3″-penta-*N*-Boc- 4′, 2″, 4″,6″-tetra-*O*-TBDMS-tobramycin (6b).

The compound **6b** was synthesized by following general procedure xx using compound **5b** (0.600 g, 0.371 mmol), sodium azide (0.482 g, 7.42 mmol), $Pd(OH)_2/C$ (0.025 g, 0.038 mmol, 20 wt% loading) to give desired product **6b** as white solid (0.413 g, 70%). ^1H NMR (500 MHz, $CDCl_3$) δ 5.17 (s, 1H), 5.09 (s, 1H), 5.04 – 4.99 (m, 1H), 4.73 (s, 1H), 4.51 (s, 1H), 4.20 – 4.02 (m, 5H), 3.78 – 3.64 (m, 4H), 3.59 – 3.36 (m, 6H), 3.23 – 3.11 (m, 4H), 2.72 (s, 2H), 2.51 – 2.40 (m, 1H), 1.97 – 1.88 (m, 1H), 1.51 – 1.36 (m, 49H, Boc-^{t}Bu), 1.26 – 1.12 (m, 8H), 0.90 – 0.81 (m, 36H, TBDMS-^{t}Bu), 0.10 – -0.03 (m, 24H, TBDMS-$SiMe_2$). MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{75}H_{150}N_6O_{19}Si_4Na^+$, 1573.9925; observed 1573.9843.

Synthesis and characterization of 5-*O*-(2-amino-PEG3)-1, 3, 2′,6′, 3″-penta-*N*-Boc- 4′, 2″, 4″,6″-tetra-*O*-TBDMS-tobramycin (6c).

The compound **6c** was synthesized by following general procedure D using compound **5c** (0.360 g, 0.216 mmol), sodium azide (0.281 g, 4.32 mmol), $Pd(OH)_2/C$ (0.016 g, 0.022 mmol, 20 wt% loading) and gave desired product **6c** (0.226 g, 64%) as white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.34 (s, 1H), 5.20 (s, 1H), 5.10 (s, 1H), 4.99 – 4.96 (m, 1H), 4.39 (s, 1H), 3.80 – 3.53 (m, 26H),

3.32 – 3.13 (m, 5H), 2.35 – 2.31 (m, 1H), 1.97 – 1.91 (m, 1H), 1.70 – 1.57 (m, 2H), 1.50 – 1.39 (m, 45H, Boc-^{t}Bu), 0.98 – 0.83 (m, 36H, TBDMS-^{t}Bu), 0.25 – 0.05 (m, 24H, TBDMS-SiMe$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.85, 155.41, 154.81, 96.13, 79.39, 79.28, 79.19, 79.03, 75.21, 72.17, 71.66, 71.39, 70.82, 70.24, 69.95, 67.70, 67.19, 63.10, 56.80, 50.60, 48.94, 48.30, 41.68, 39.67, 35.54, 35.29, 29.71, 28.66, 28.63, 28.59, 28.53, 28.48, 28.43, 26.17, 26.12, 26.05, 26.00, 25.88, 18.56, 18.34, 18.13, 17.98, 14.13, -3.80, -4.81, -4.91, -5.13, -5.25. MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{75}H_{150}N_6O_{19}Si_4Na^+$, 1621.9772; observed 1621.9472.

5-*O*-((4-aminomethyl)biphenyl)-1, 3, 2′, 6′, 3″-penta-*N*-Boc-4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (6d). [3]

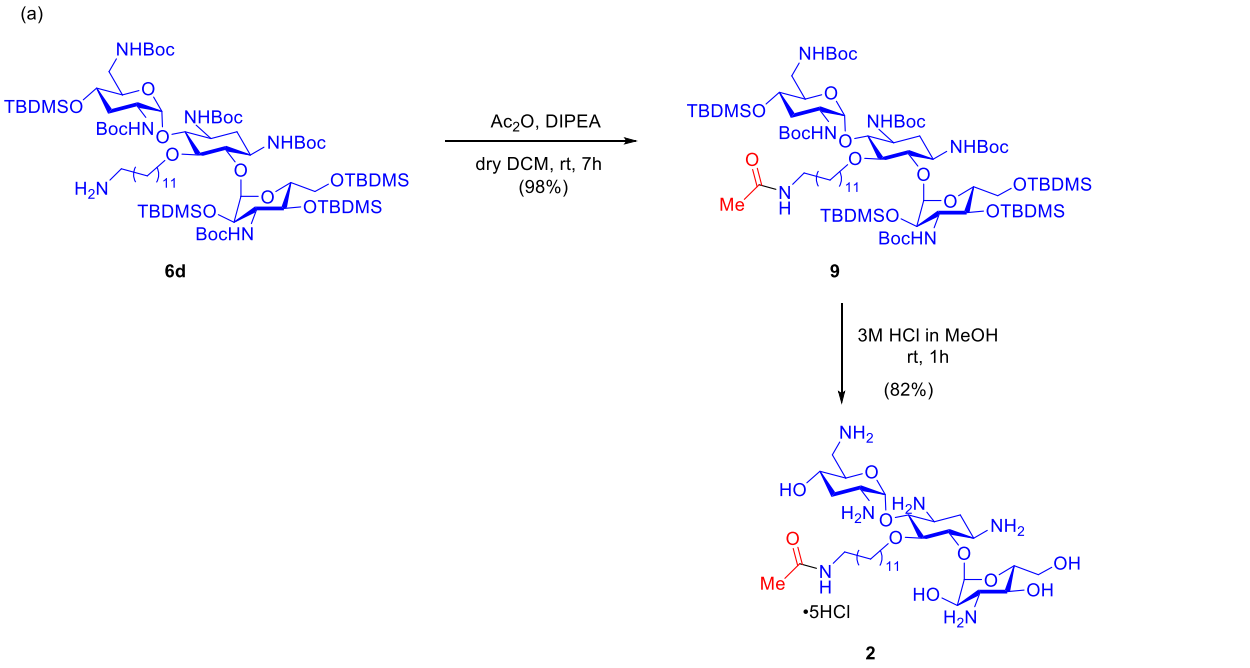
To a solution of corresponding azido compound of **5d** prepared by aforementioned procedure D (0.300 g, 0.182 mmol) in THF (5 mL) was added PMe_3 (1.82 mL, 1.82 mmol, 1.0 M solution in THF) under inert atmosphere at 0 °C. After addition, reaction mixture was stirred at ambient temperature for 5 h. After consumption of 5d, concentrated the reaction mass under reduced pressure and crude residue was subjected to purification by column chromatography (P60 silica) using 5-10 % MeOH/DCM (v/v) to afford desired product **6d** (0.183 g, 62%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (d, J = 7.7 Hz, 2H), 7.43 (d, J = 7.4 Hz, 2H), 7.39 – 7.34 (m, 4H), 5.31 (s, 1H), 5.20 – 5.18 (m, 2H), 5.10 – 5.08 (m, 1H), 4.99 (s, 1H), 4.79 (s, 1H), 4.59 – 4.51 (m, 2H), 4.24 – 4.18 (m, 2H), 3.88 (s, 2H), 3.82 – 3.74 (m, 3H), 3.66 – 3.28 (m, 10H), 3.12 (s, 1H), 2.70 (s, 3H), 2.47 – 2.44 (m, 1H), 1.98 – 1.95 (m, 1H), 1.49 – 1.26 (m, 75H, Boc-^{t}Bu + Me_3P=O), 0.90 – 0.76 (m, 36H, TBDMS-^{t}Bu), 0.10 – -0.16 (m, 24H, TBDMS-SiMe$_2$). MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{75}H_{150}N_6O_{19}Si_4Na^+$, 1641.9612; observed 1641.9322. Compound **6d** was used as such without further purification in the next step.

7-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**7**).[4, 5]

In an oven-dried RBF, ciprofloxacin (0.5 g, 1.50 mmol) was dissolved in dioxane:1N NaOH (2:1, 20 mL) solution and stirred at 0 °C. Then Boc-anhydride (0.416 mL, 1.81 mmol) was added at 0 °C. Then reaction mixture was warm to ambient temperature and stirred for 5 h. After consumption of ciprofloxacin, mixture was concentrated under reduced pressure and crude residue was subjected to purification by column chromatography (P60 silica) using 10-20% MeOH:DCM (v/v) to afford desired product **7** (0.520 g, 80%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.93

(s, 1H, COOH), 8.75 (s, 1H, H-2), 8.01 (d, J = 12.9 Hz, 1H H-5), 7.36 (d, J = 7.0 Hz, 1H, H-5), 3.67 (t, J = 5.0 Hz, 4H, CH_2 *piperazine*), 3.56 – 3.51 (m, 1H, CH-^{c}Pr$_{Cip}$), 3.29 (t, J = 5.0 Hz, 4H, CH_2 *piperazine*), 1.50 (s, 9H, Boc-^{t}Bu), 1.42 – 1.37 (m, 2H, CH_2-^{c}Pr$_{Cip}$), 1.22 – 1.18 (m, 2H, CH_2-^{c}Pr); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.10, 176.07, 165.89, 153.88, 153.54, 151.39, 146.49, 144.83, 144.72, 138.01, 119.17, 119.09, 111.67, 111.44, 107.20, 104.00, 103.96, 79.35, 48.74, 34.27, 27.39, 7.25. MS-ESI $[M+H]^+$ calcd for $C_{22}H_{27}FN_3O_5$, 432.1929; observed,432.1916.

Synthesis, procedure, and characterizations of compounds 2 – 3 and 9 – 10



5-*O*-(*N*-dodecylacetamide)-1, 3, 2′, 6′, 3″-penta-*N*-Boc-4′, 2″, 4″, 6″-tetra-*O*-TBDMS-tobramycin (9).

To a solution of compound **6a** (0.200 g, 0.124 mmol) in dry DCM (5 mL) DIPEA (0.05 mL, .310 mmol) was added followed by acetic anhydride (0.023 mL, 0.248 mmol) at 0 °C and after addition,

mixture was stirred at room temperature for 3 h. Then mixture was diluted with DCM (50 mL) and washed with water. Collected the DCM layer and washed with sat. solution of $NaHCO_3$ (10 mL) followed by brine solution (10 mL). The collected organic layer, dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was further subjected to purification by column chromatography (P60 silica) using 3-5% MeOH:DCM (v/v) to afford desired product **9** (0.200 g, 98%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 – 7.14 (m, 1H), 5.50 – 5.41 (m, 1H), 5.21 – 5.03 (m, 4H), 4.77 (s, 2H), 4.51 (s, 1H), 4.26 – 3.97 (m, 3H), 3.78 – 3.33 (m, 12H), 3.26 – 3.10 (m, 5H), 2.45 – 2.36 (m, 1H), 2.06 – 1.84 (m, 5H), 1.53 – 1.12 (m, 60H), 0.95 – 0.74 (m, 36H), 0.15 – -0.08 (m, 24H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.89, 155.27, 154.74, 154.58, 96.55, 85.68, 79.92, 79.22, 75.20, 73.02, 71.58, 67.99, 66.86, 63.11, 57.17, 50.51, 48.36, 41.71, 39.70, 35.64, 30.60, 29.99, 29.59, 29.48, 29.30, 29.30, 28.64, 28.50, 28.40, 26.92, 26.13, 26.02, 25.99, 25.77, 23.35, 18.48, 18.33, 18.10, 17.90, -3.41, -3.80, -4.21, -4.88, -5.24. MALDI TOF-MS *m/e* $[M+Na]^+$ calcd for $C_{75}H_{150}N_6O_{19}Si_4Na^+$, 1672.0657; observed 1672.1345

5-*O*-(*N*-dodecylacetamide)-tobramycin (2): Compound **2** was synthesized by following general procedure B using compound **9** (0.200 g, 0.167 mmol) and methanolic HCl solution (10 mL) to give desired compound **3** (0.087 g, 82%) as an off-white solid. ^{1}H NMR (500 MHz, D_2O) δ 5.41 (d, J = 2.6 Hz, 1H, H-1′), 5.20 (d, J = 3.5 Hz, 1H, H-1″), 4.33 – 4.30 (m, 1H, H-5′), 4.24 (t, J = 9.8 Hz, 1H, H-4), 3.98 – 3.94 (m, 3H, H-6, H-5, H-4′), 3.93 – 3.79 (m, 6H, H-4″, H-5″, H-6″, *O*-CH_2-linker), 3.76 – 3.73 (m, 2H, H-2′,H-6″), 3.69 – 3.59 (m, 3H, H-1, H-3, H-3″), 3.43 (dd, J = 14.0, 9.2 Hz, 1H, H-6′), 3.37 – 3.32 (m, 1H, H-6′), 3.17 (t, J = 6.9 Hz, 2H, *N*-CH_2-linker), 2.57 (dt, J = 12.7, 4.4 Hz, 1H, H-2), 2.31 – 2.23 (m, 2H, H-3′), 2.08 – 2.00 (m, 1H, H-2), 1.99 (s, 3H, $CH_{3\ acetyl}$), 1.68 – 1.63 (m, 2H, CH_2-linker), 1.54 – 1.48 (m, 2H, CH_2-linker), 1.30 (s, 16H, CH_2-linker); ^{13}C NMR (125 MHz, D_2O) δ 173.92, 101.38, 92.71, 81.86, 81.84, 76.66, 75.79, 73.86, 73.19, 68.58, 64.85, 63.29, 59.30, 54.80, 49.81, 48.50, 47.35, 39.61, 38.59, 29.49, 28.98, 28.88, 28.78, 28.76, 28.74, 28.42, 28.25, 28.14, 27.74, 26.08, 25.33, 21.97. MALDI TOF-MS *m/e* calcd for $C_{32}H_{64}N_6O_{10}Na^+$ $[M+Na]^+$, 715.4576; found 715.4826.

Synthesis of tert-butyl 4-(1-cyclopropyl-3-(dodecylcarbamoyl)-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)piperazine-1-carboxylate (10).

This compound was synthesized by follow general procedure A for amide coupling using compound **7** (0.100g, 0.231 mmol), dodecylamine (0.043 g, 0.347 mmol), HATU (0.132 g, 0.0347

mmol), and DIPEA (0.100 ml, 0.579 mmol) to afford the desired product (0.110 g, 68%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.96 (s, 1H), 8.84 (s, 1H), 8.06 (d, *J* = 13.0 Hz, 1H), 7.35 (d, *J* = 8.0 Hz, 1H), 3.68 (s, 4H), 3.46 (s, 3H), 3.25 (s, 4H), 1.70 – 1.56 (m, 2H), 1.51 (s, 9H), 1.41 – 1.02 (m, 22H), 0.89 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 175.47, 175.44, 164.76, 161.62, 154.58, 152.10, 146.67, 144.77, 144.66, 138.40, 122.11, 122.04, 112.70, 112.47, 111.47, 104.98, 104.95, 80.19, 49.92, 45.61, 39.26, 34.67, 31.89, 29.64, 29.61, 29.53, 29.37, 29.33, 28.39, 27.14, 22.66, 14.11, 8.17. MS-ESI $[M+H]^+$ calcd for $C_{34}H_{51}FN_4O_4H$, 599.396; observed 599.3934.

1-cyclopropyl-*N*-dodecyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxamide (3): Compound **3** was synthesized by following general procedure B using compound **10** (0.100 g, 0.167 mmol) and methanolic HCl solution (5 mL) to give desired compound **3** (0.081 g, 91%) as a light yellow solid. ^{1}H NMR (500 MHz, D_2O) *δ* 9.93 (bs, 1H), 8.22 (s, 1H), 7.28 (d, *J* = 12.4 Hz, 1H), 7.11 (s, 1H), 3.40 – 3.20 (m, 11H), 1.52 (b, 2H), 1.30 – 1.29 (m, 20H), 0.96 (bs, 3H), 0.72 (bs, 2H); ^{13}C NMR (125 MHz, D_2O) *δ* 173.97, 165.11, 151.36, 145.82, 143.01, 137.57, 121.55, 111.89, 109.81, 105.64, 46.74, 43.39, 39.40, 35.00, 32.27, 32.25, 30.24, 29.88, 29.82, 29.40, 27.54, 22.88, 14.03, 7.65. MALDI TOF-MS *m/e* calcd for $C_{29}H_{44}FN_4O_2$ $[M+H]^+$, 499.3443; found 499.3964 and $[M+Na]^+$ 521.3855.

NMR spectra of compounds 1 – 10

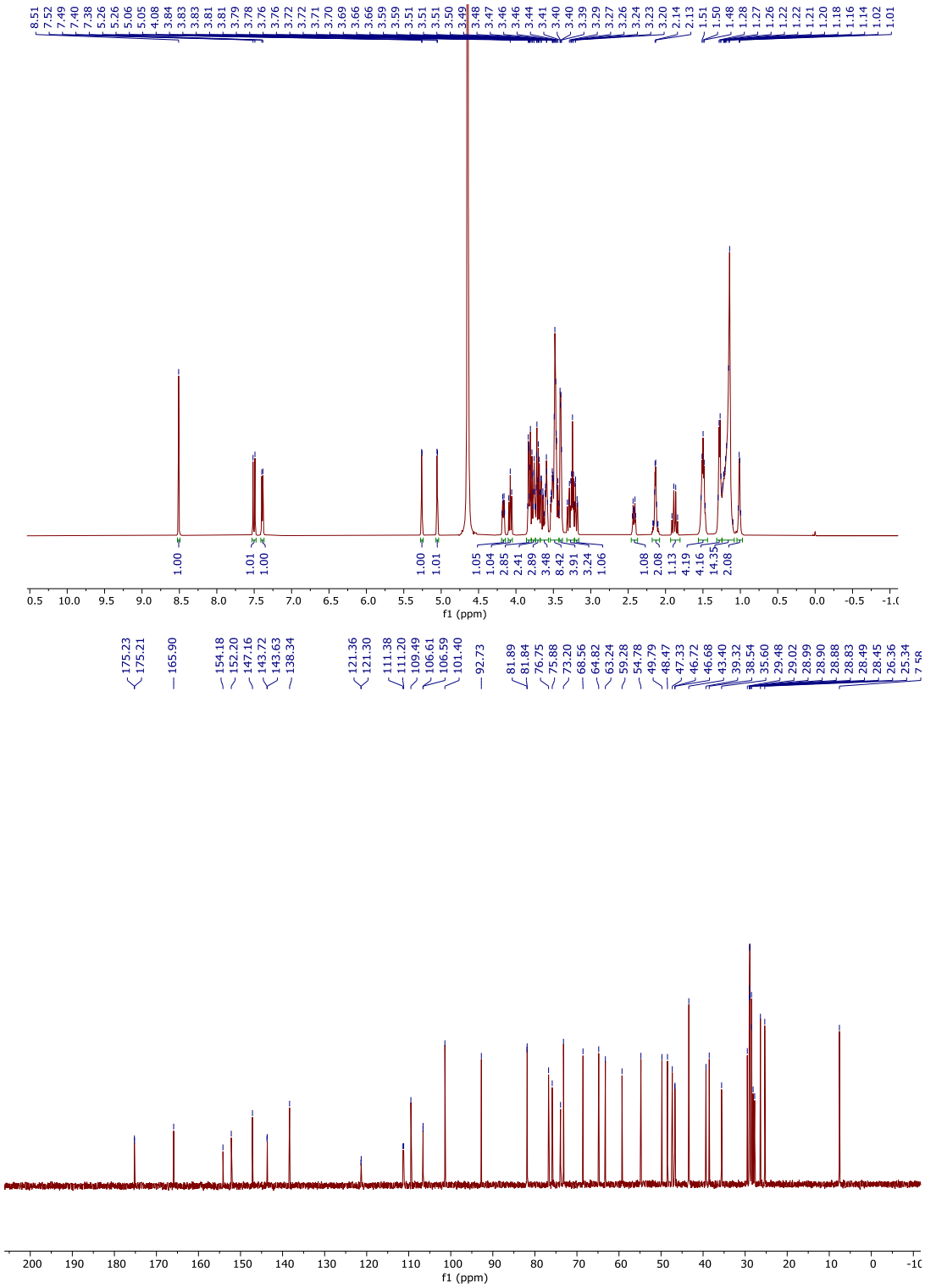


Figure S2. ^{1}H and ^{13}C NMR of compound **1a** in D_2O

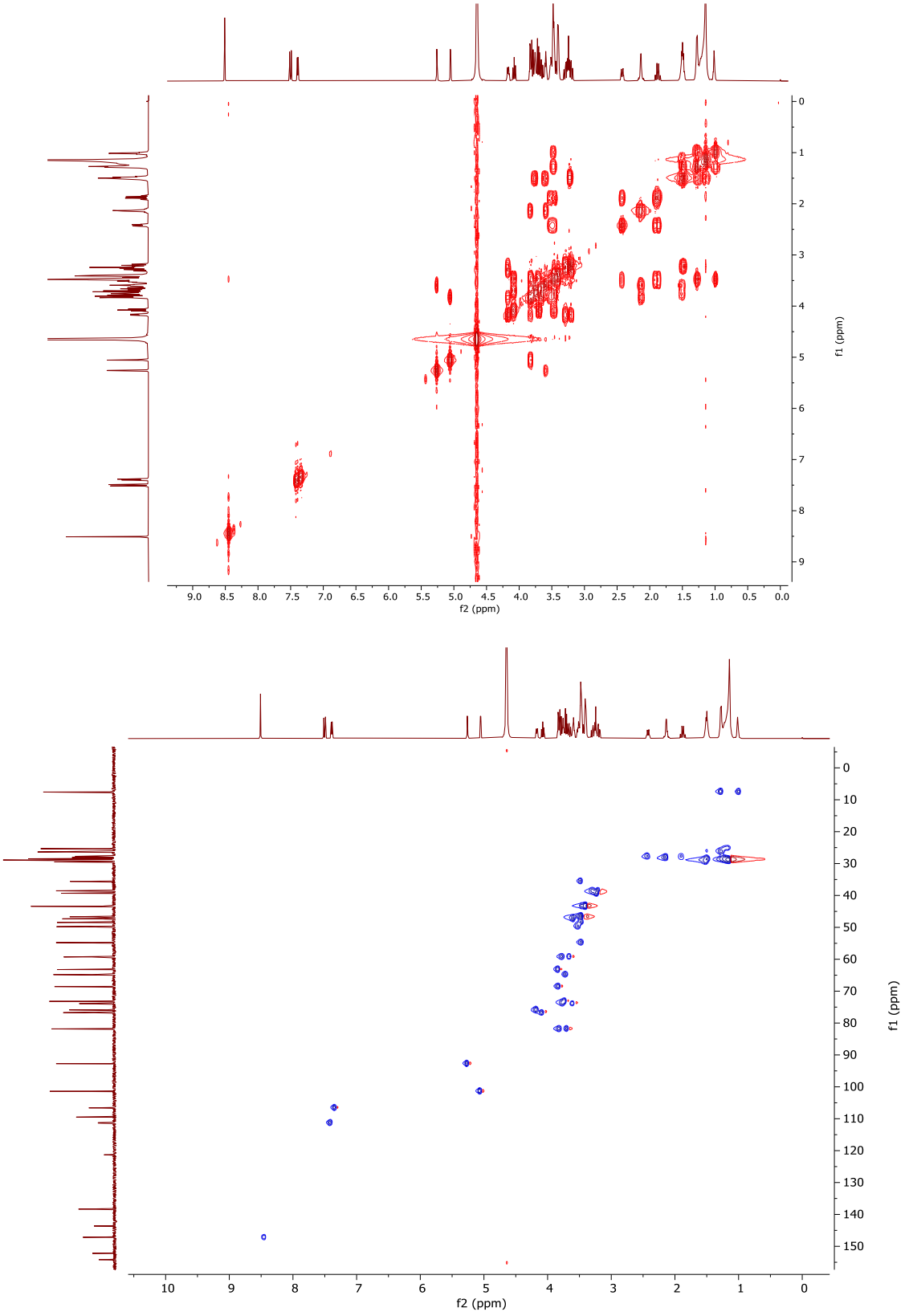


Figure S3. COSY and HSQC 2D-NMR of compound **1a** in D_2O

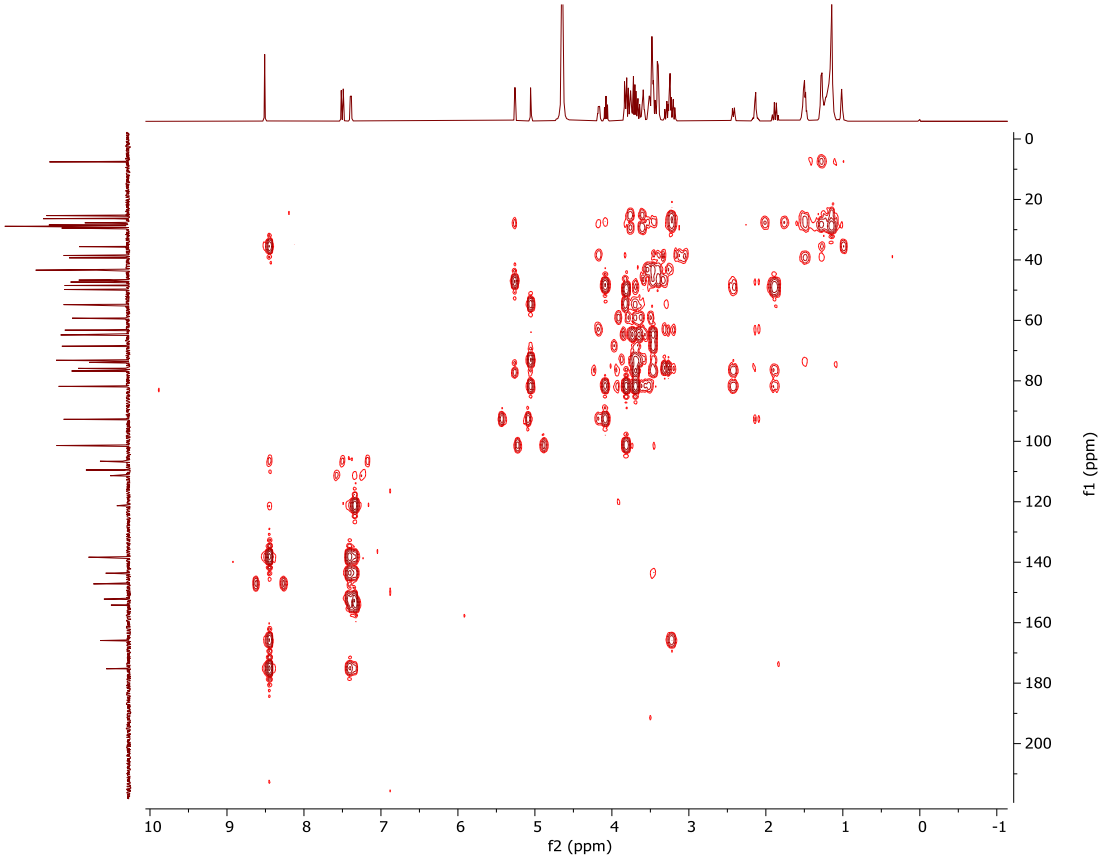


Figure S4. HMBC 2D-NMR of compound **1a** in D_2O

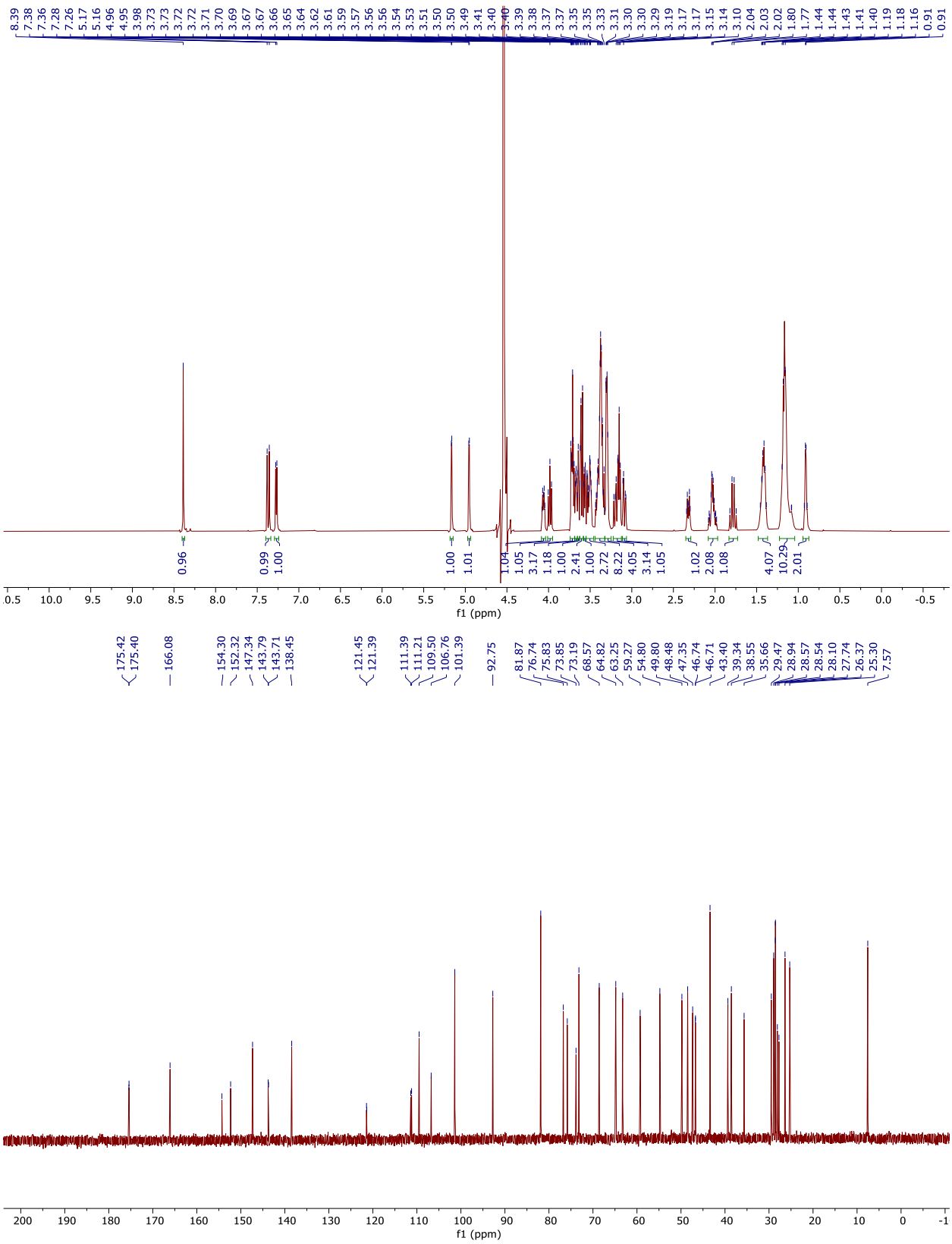


Figure S5. ^{1}H and ^{13}C NMR of compound **1b** in D_2O

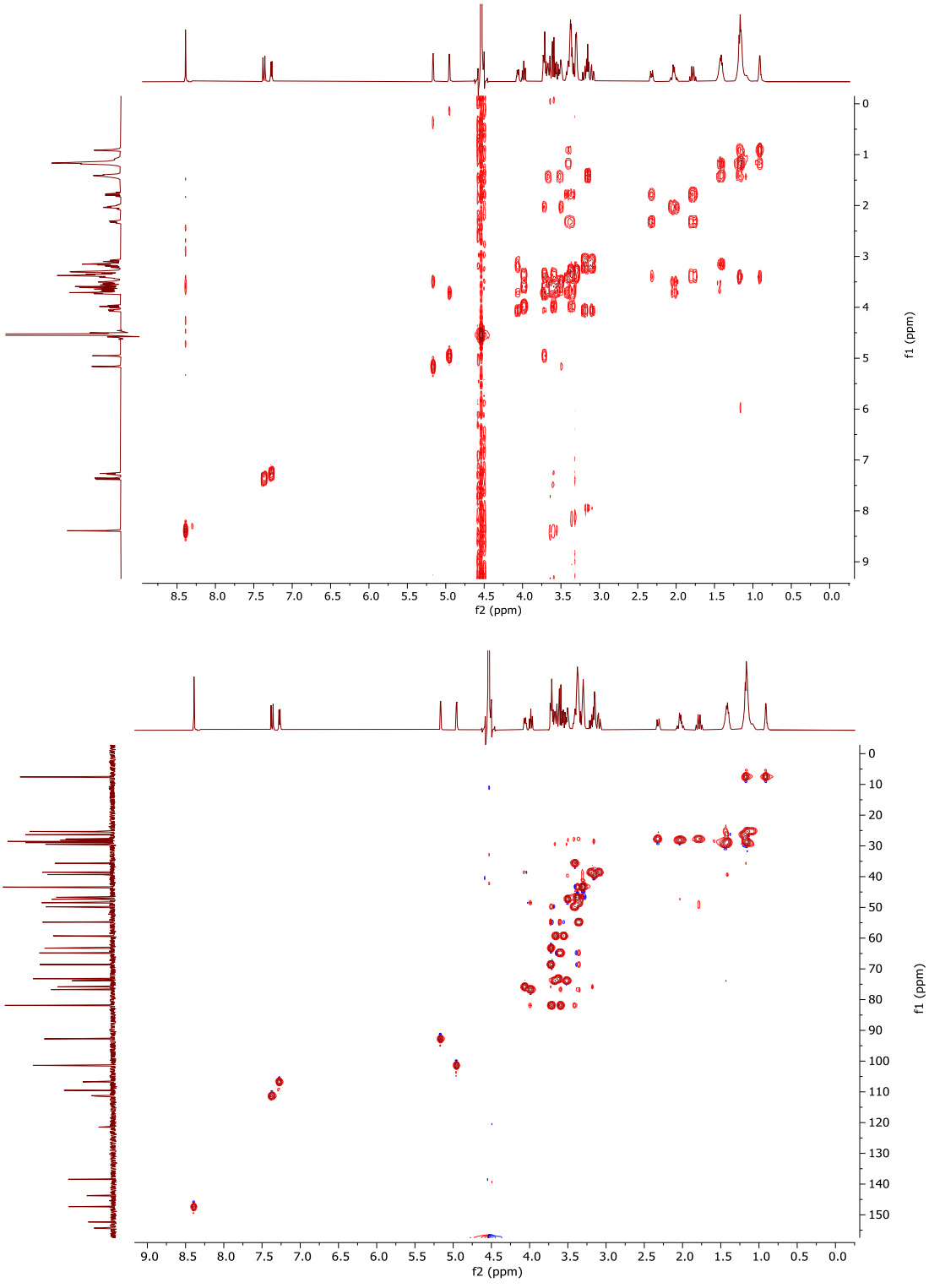


Figure S6. COSY and HSQC 2D-NMR of compound **1b** in D_2O

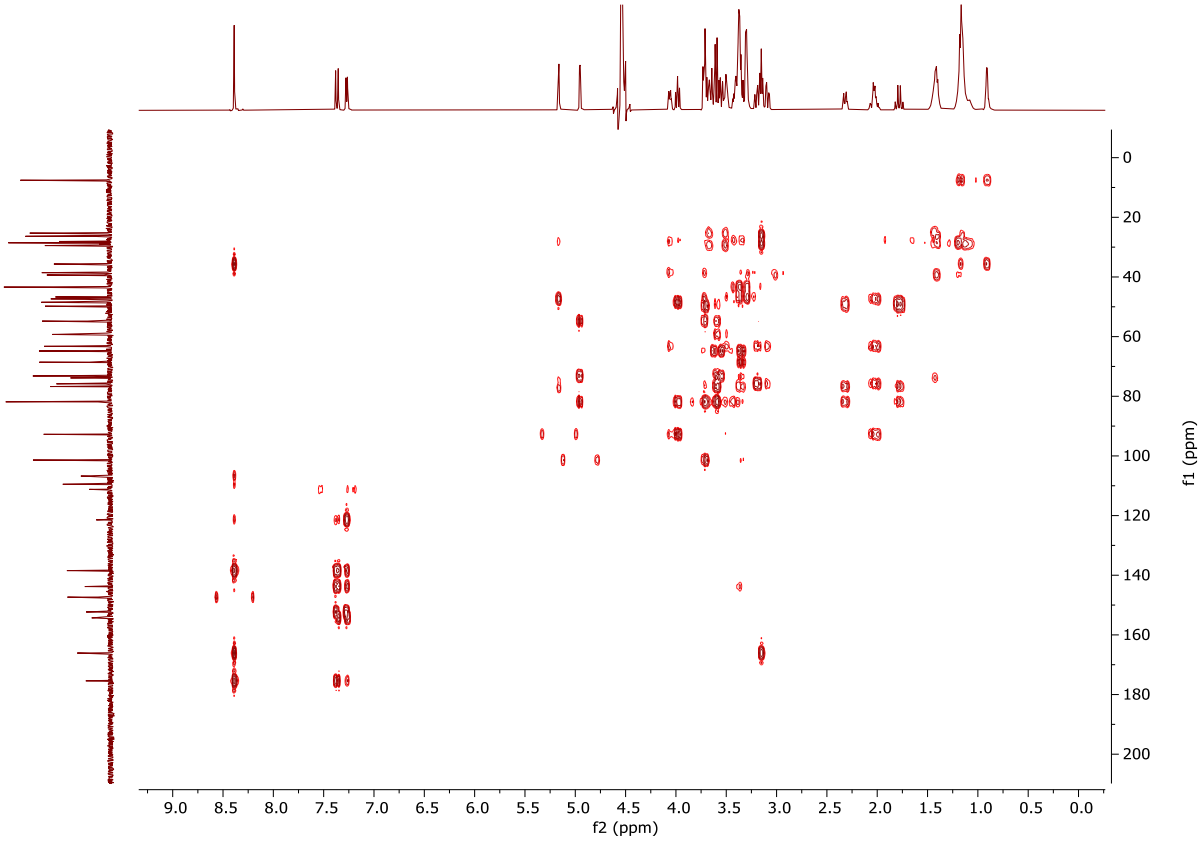


Figure S7. HMBC 2D-NMR of compound **1b** in D_2O

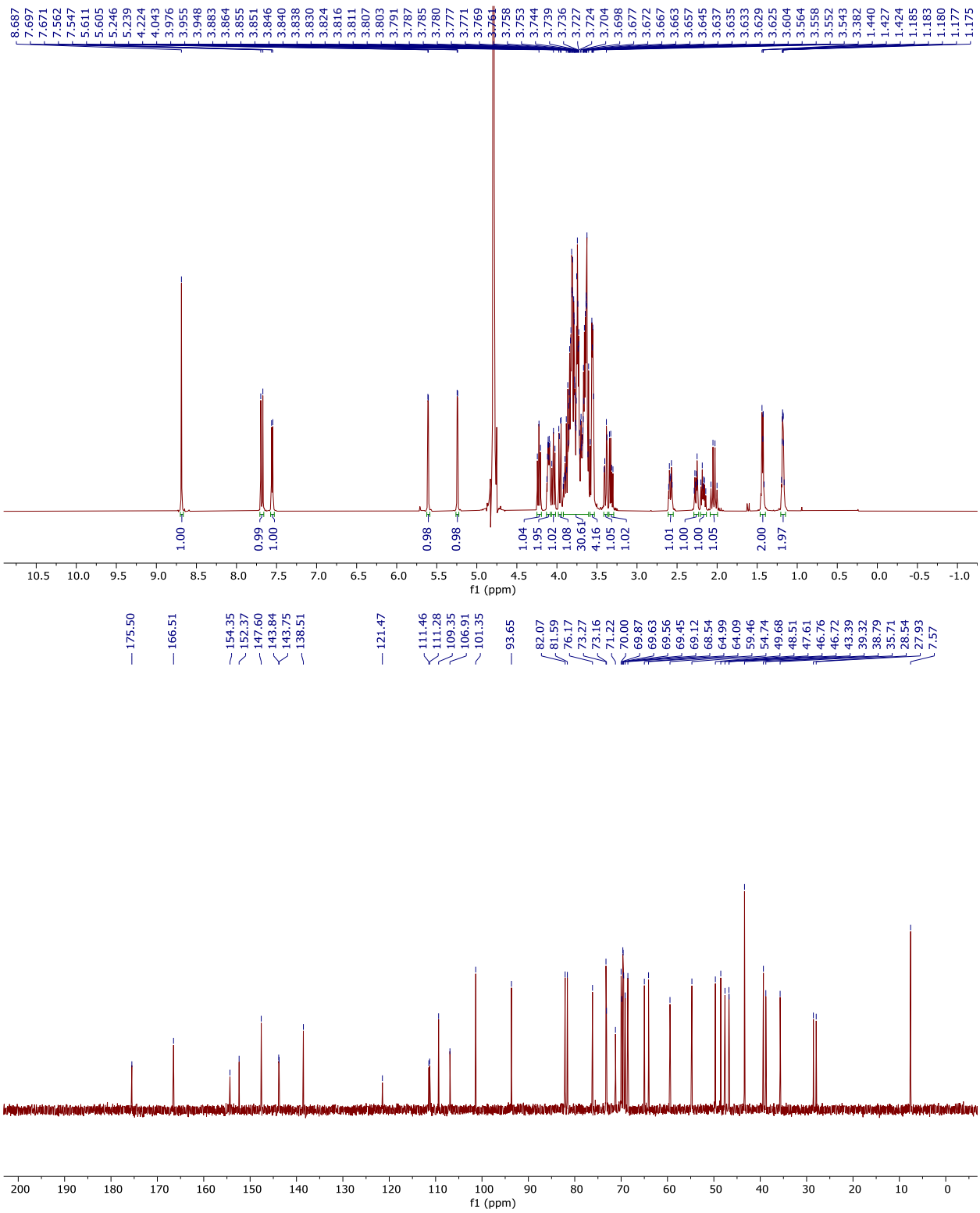


Figure S8. ^{1}H and ^{13}C NMR of compound **1c** in D_2O

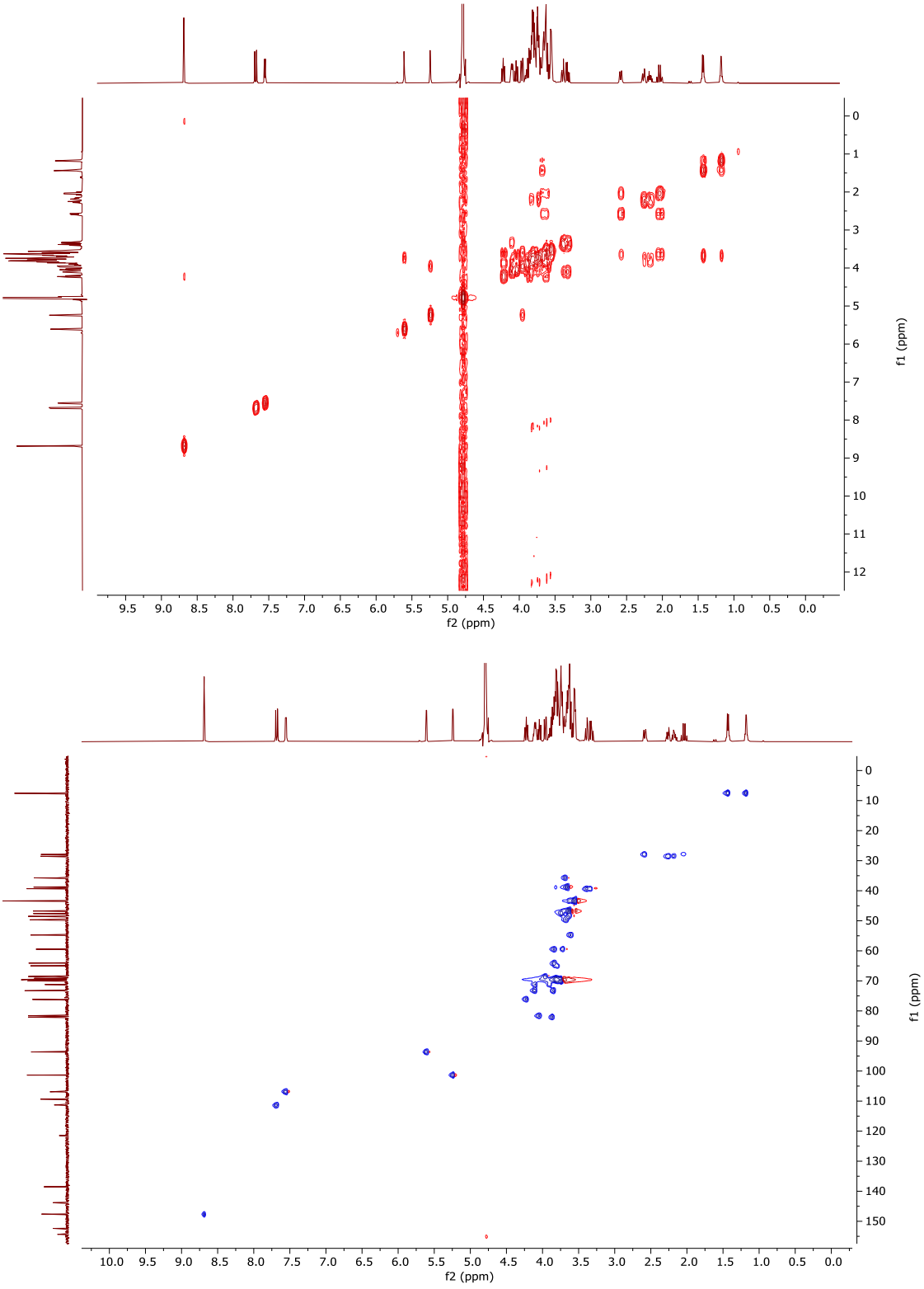


Figure S9. COSY and HSQC 2D-NMR of compound **1c** in D_2O

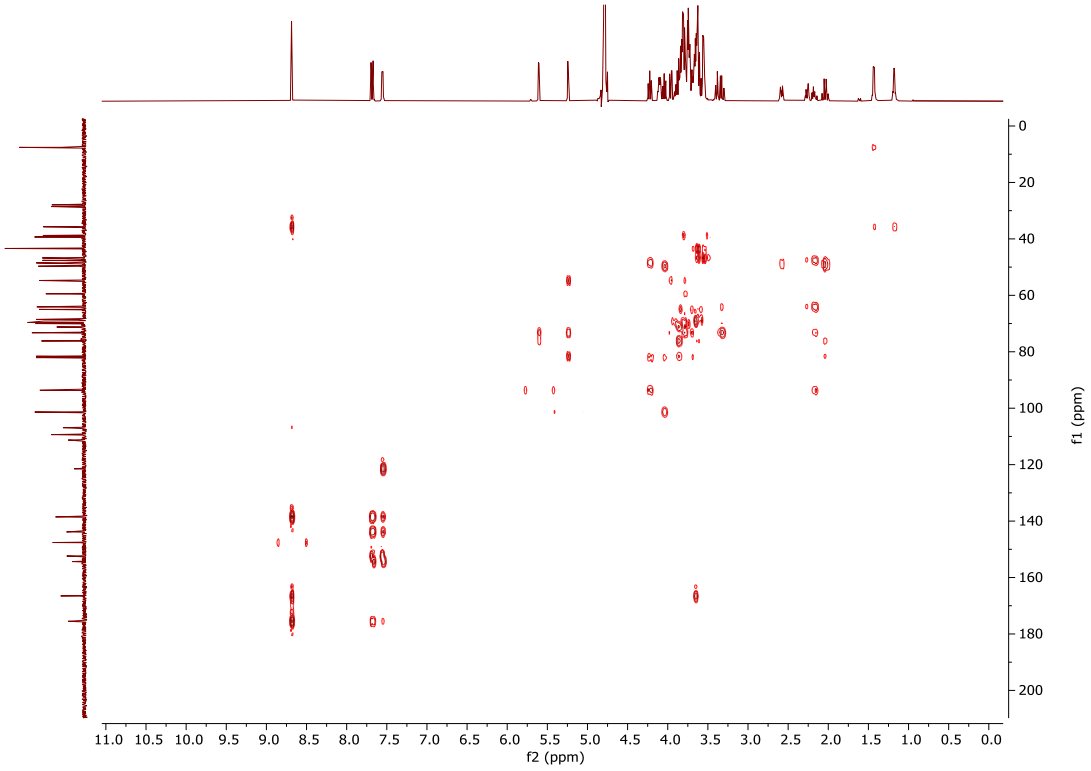


Figure S10. HMBC 2D-NMR of compound **1c** in D_2O

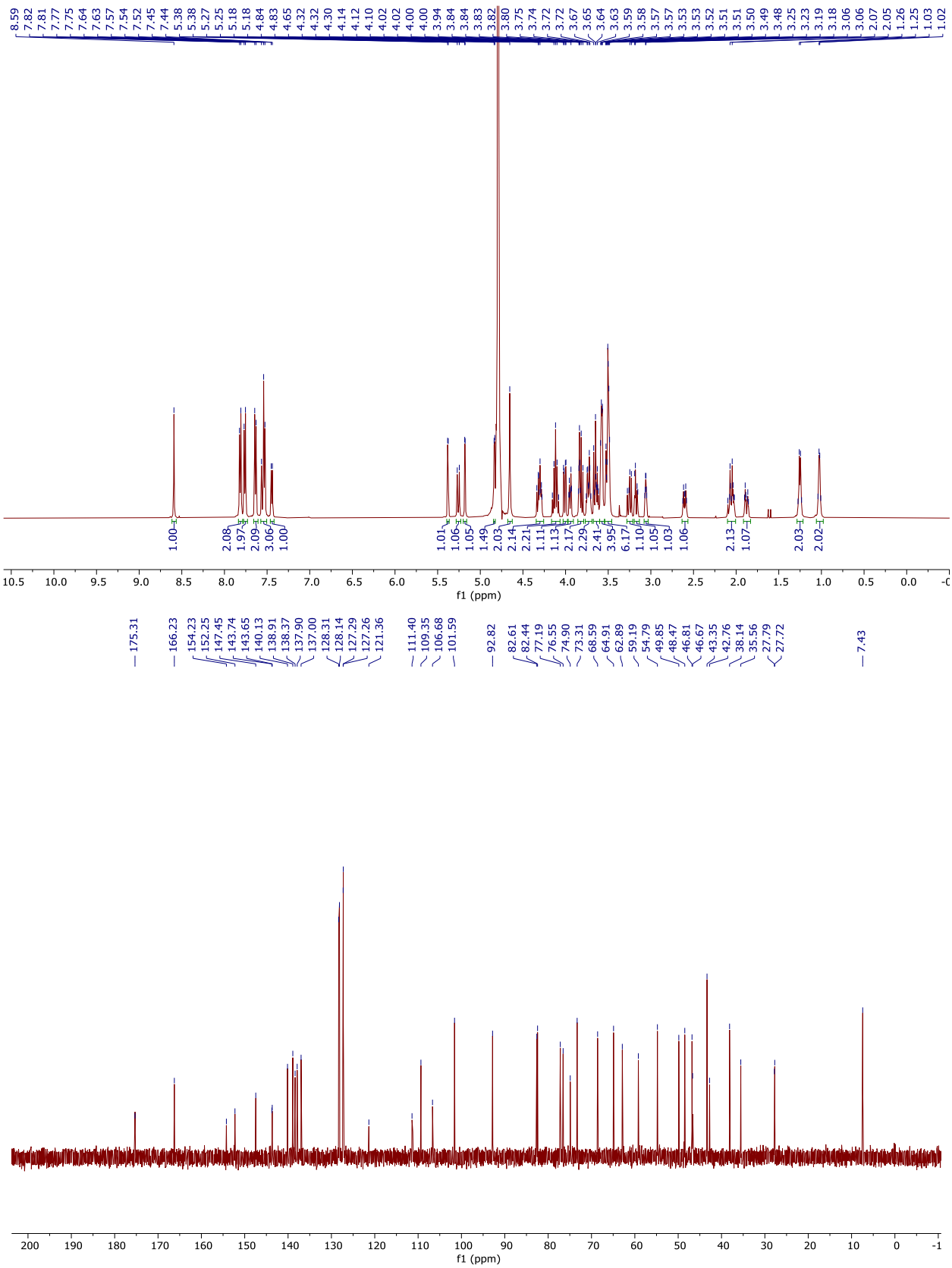


Figure S11. ^{1}H and ^{13}C NMR of compound **1d** in D_2O

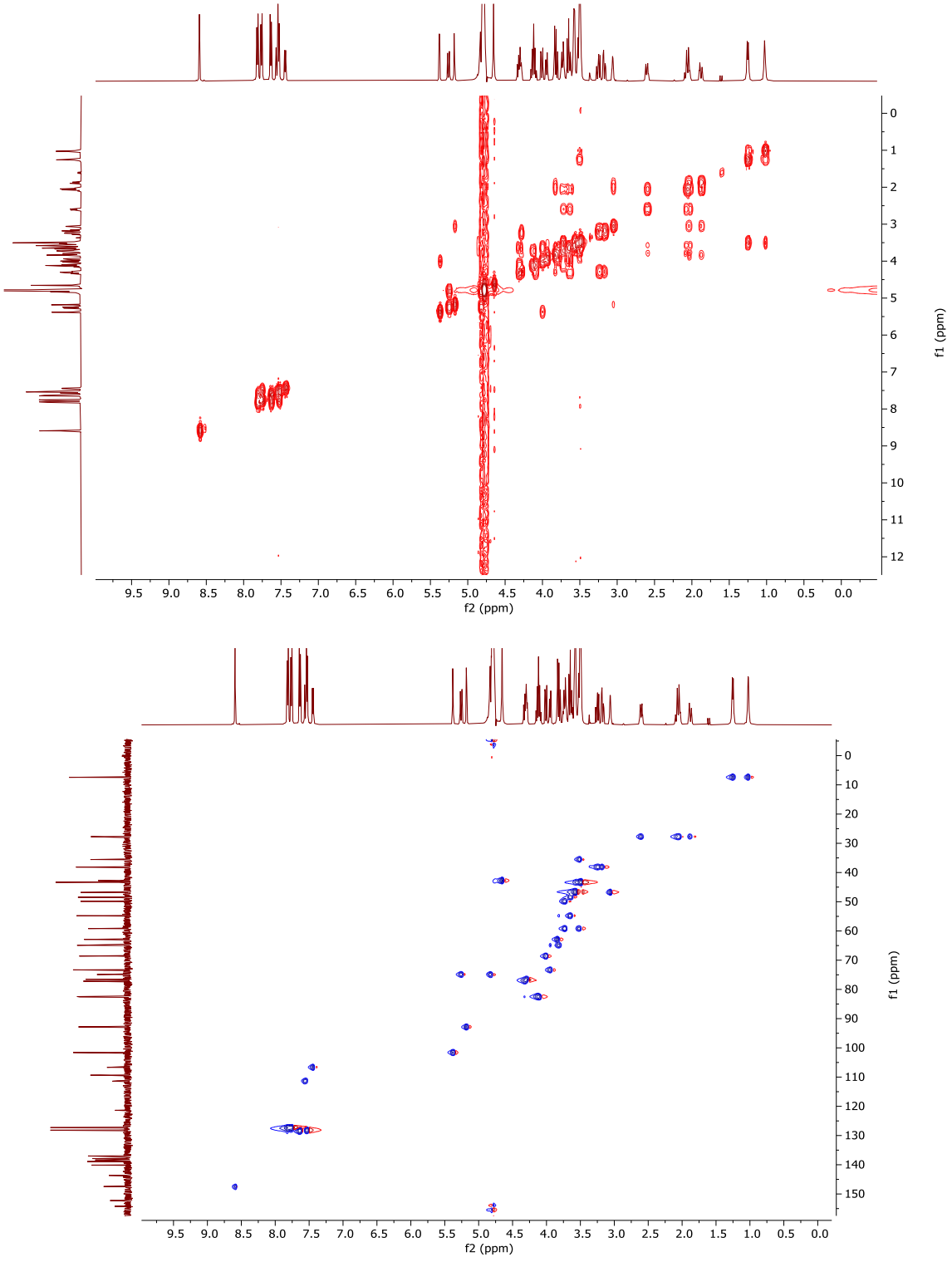


Figure S12. COSY and HSQC 2D-NMR of compound **1d** in D_2O

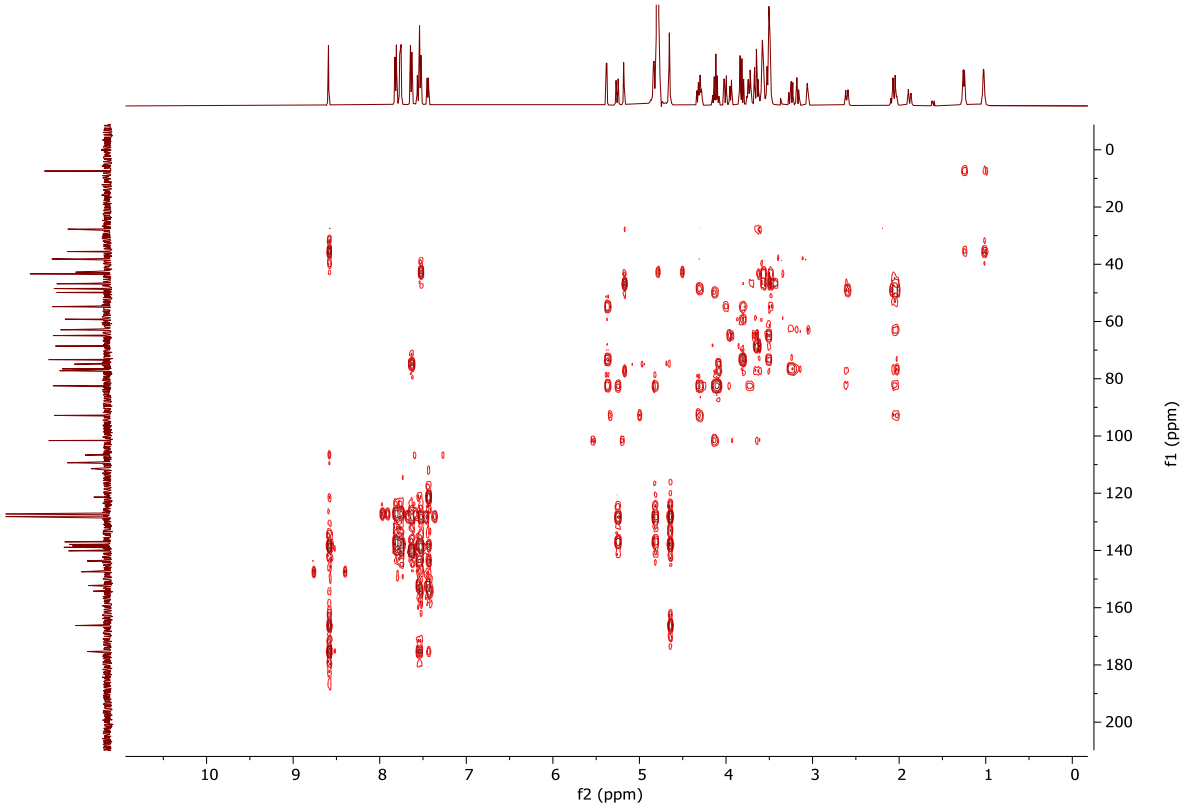


Figure S13. HMBC 2D-NMR of compound **1d** in D_2O

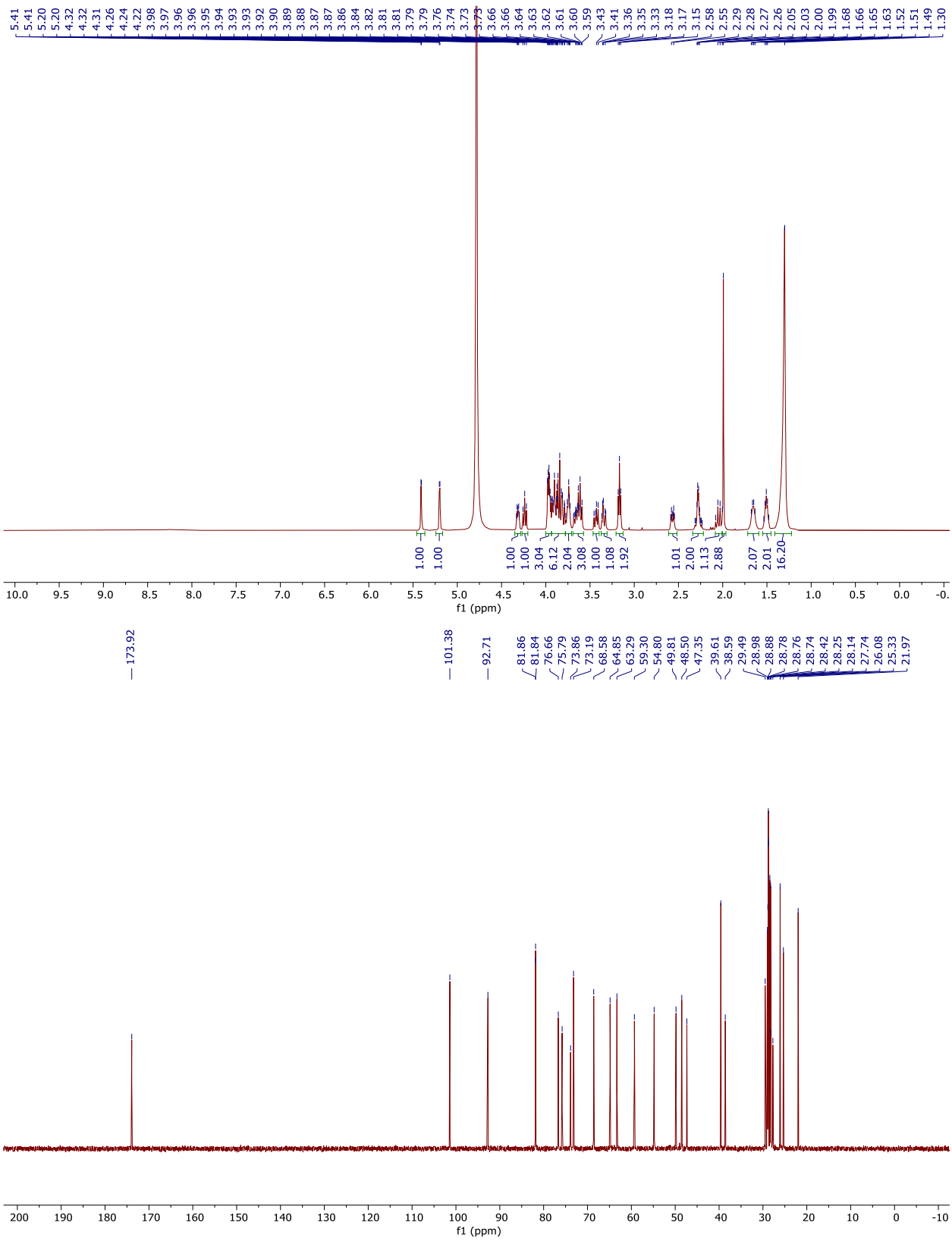


Figure S14. ^{1}H and ^{13}C NMR of compound **2** in D_2O

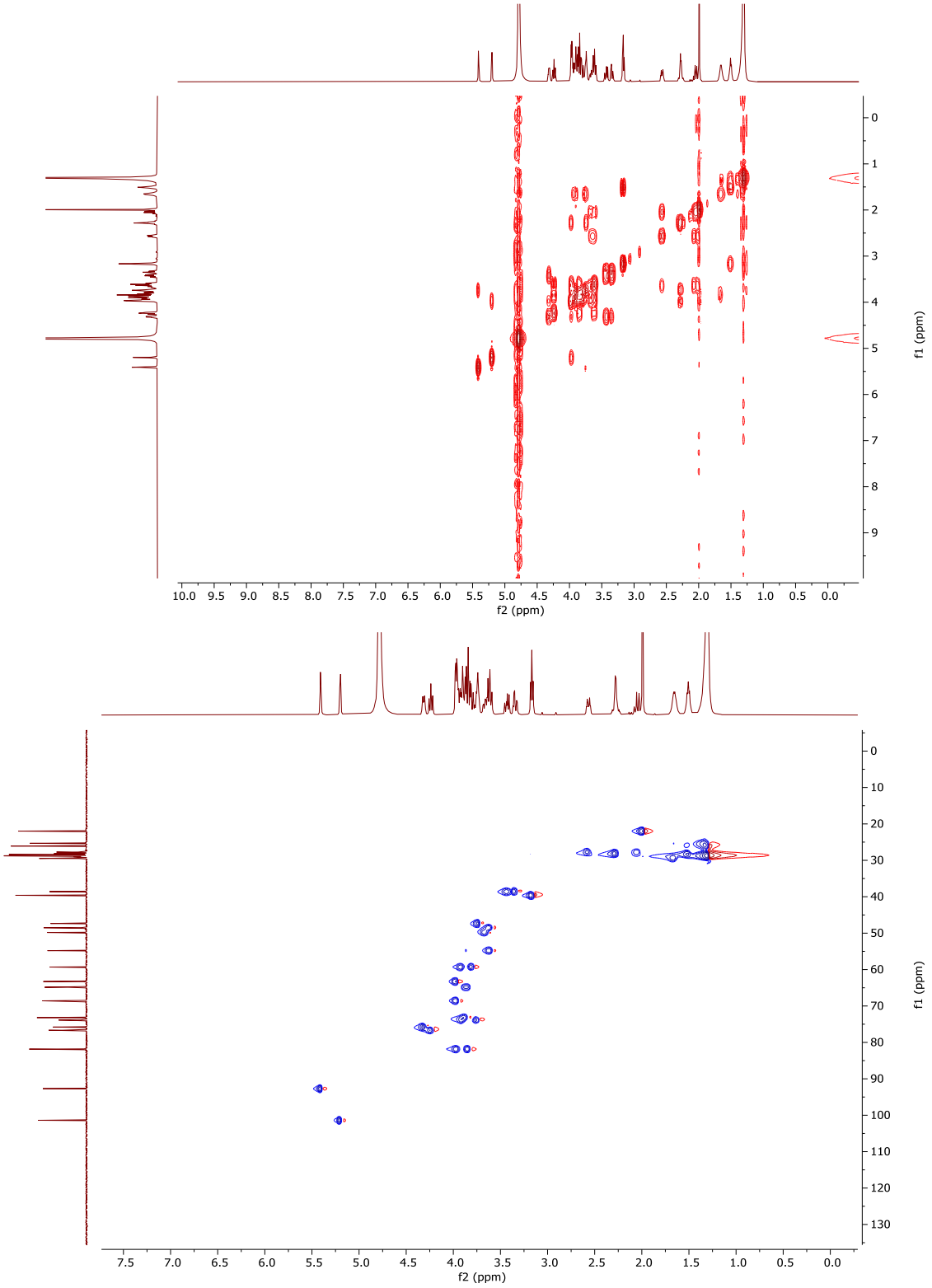


Figure S15. COSY and HSQC 2D-NMR of compound **2** in D_2O

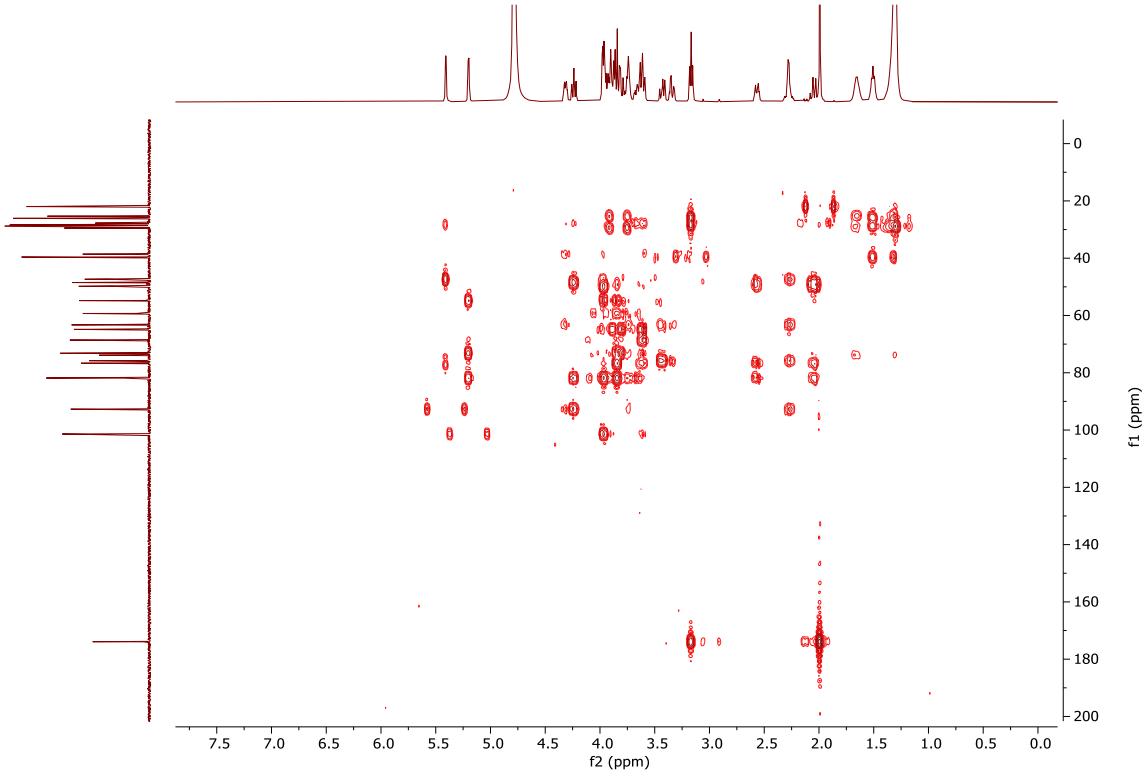


Figure S16. HMBC 2D-NMR of compound **2** in D_2O

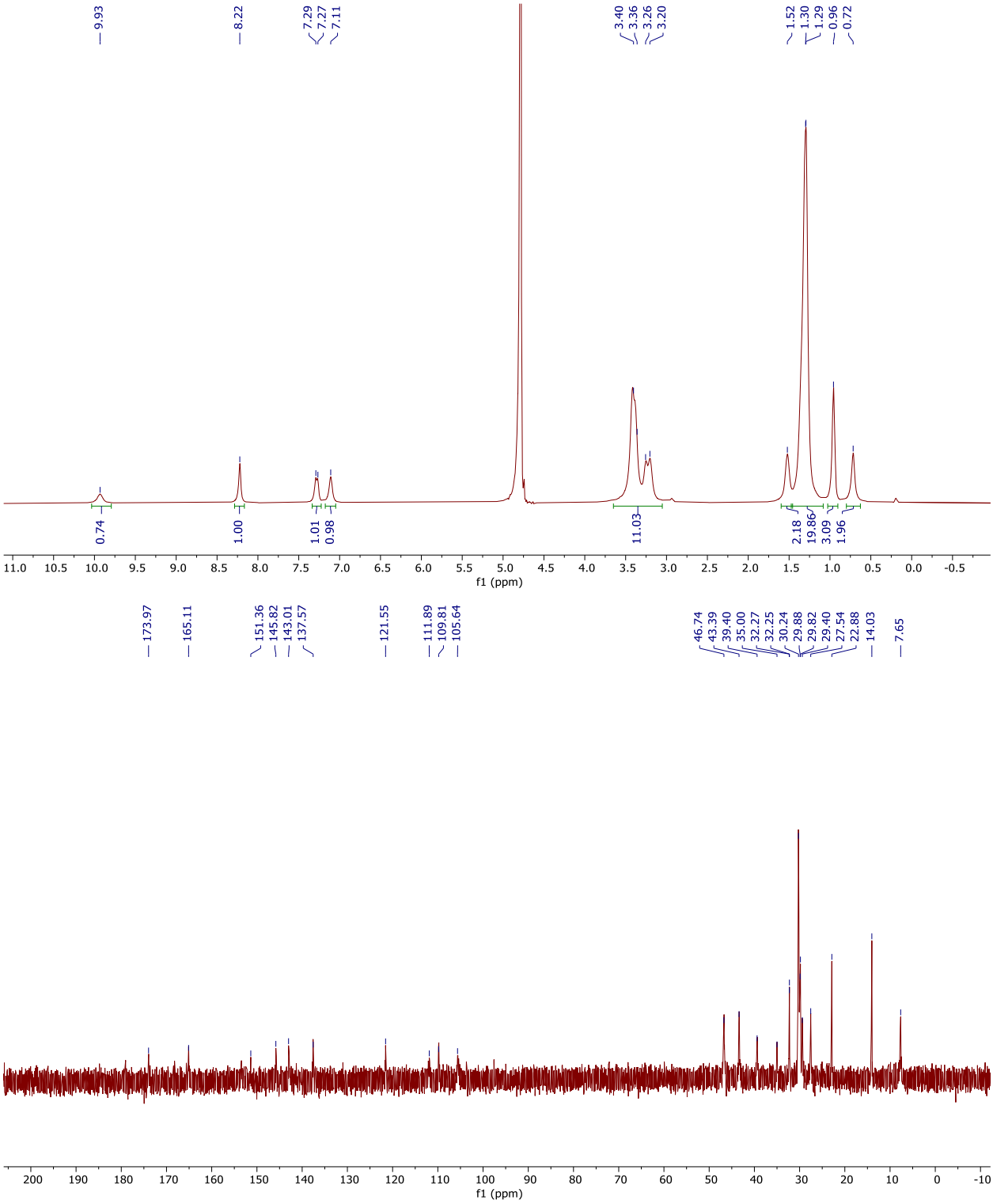


Figure S17. ^{1}H and ^{13}C NMR of compound **3** in D_2O

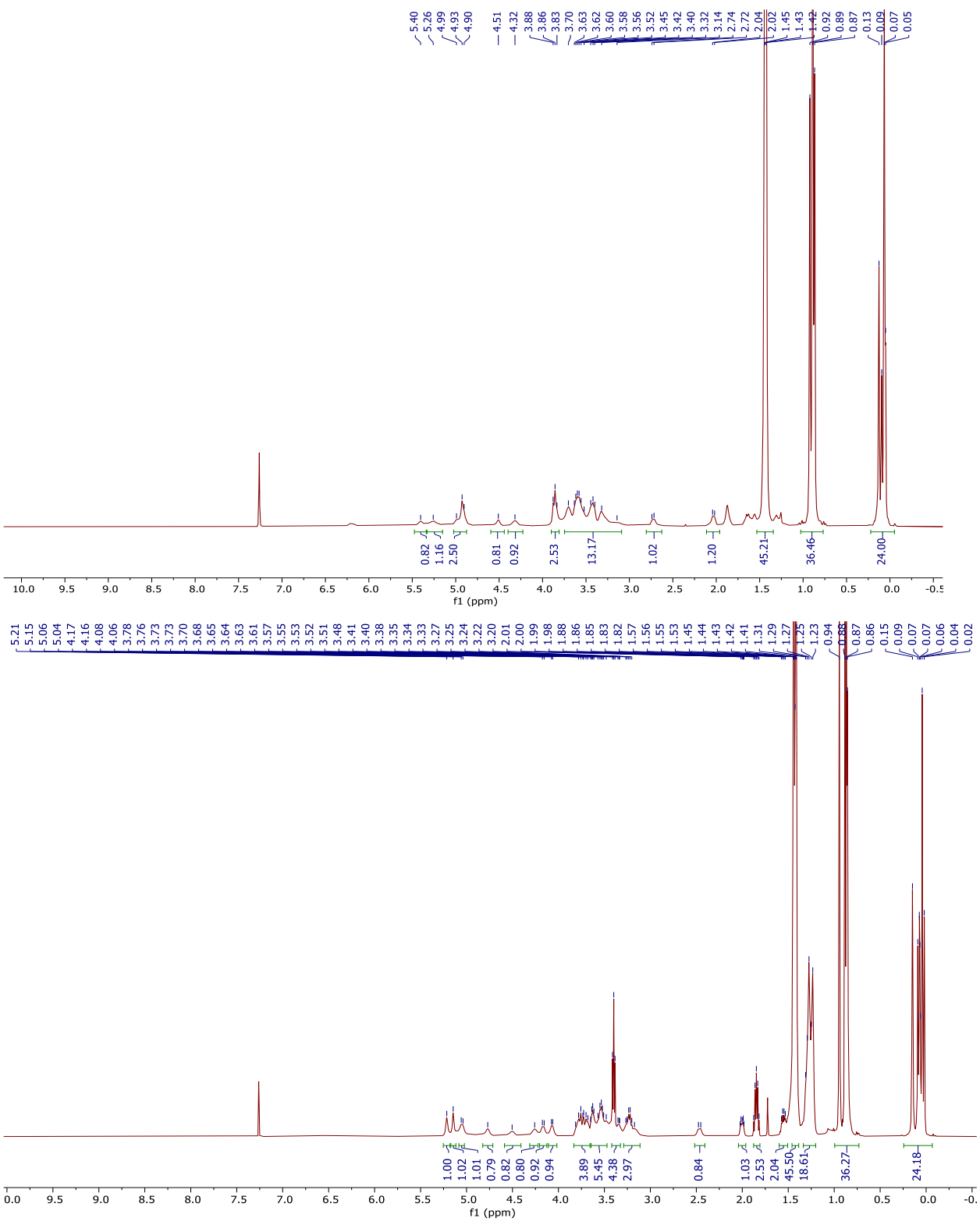


Figure S18. ^{1}H of compound **4** and **5a** in $CDCl_3$

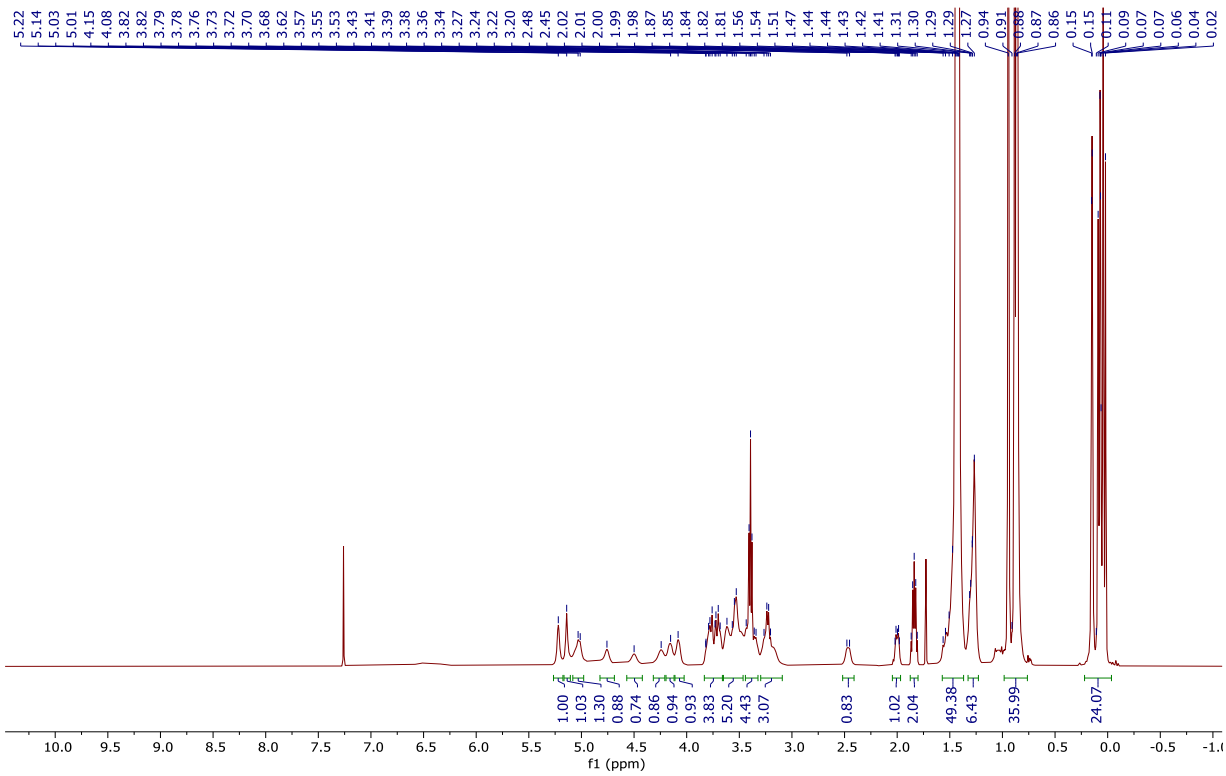


Figure S19. ^{1}H of compound **5b** in $CDCl_3$

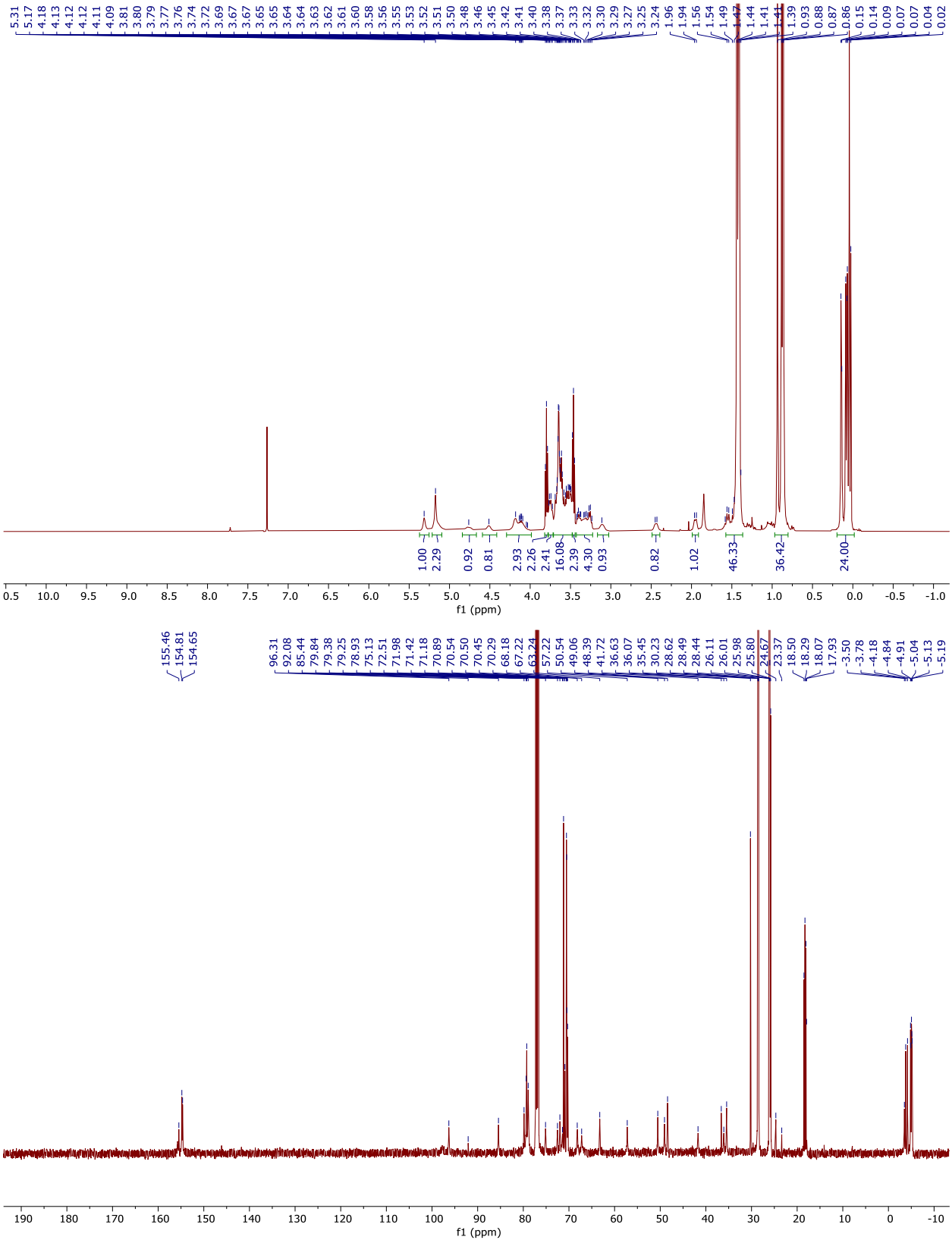


Figure S20. ^{1}H and ^{13}C NMR of compound **5c** in $CDCl_3$

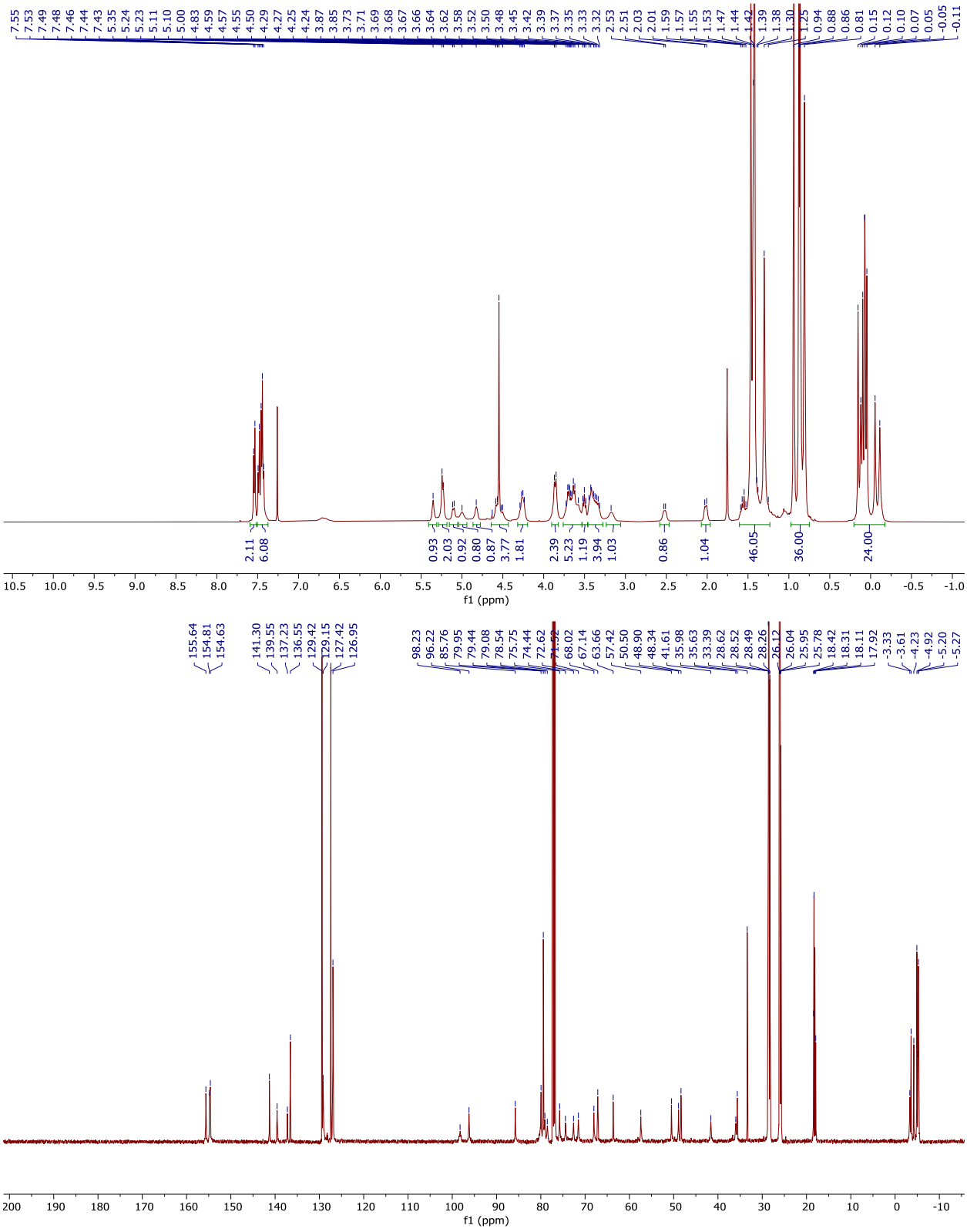


Figure S21. ^{1}H and ^{13}C NMR of compound **5d** in $CDCl_3$

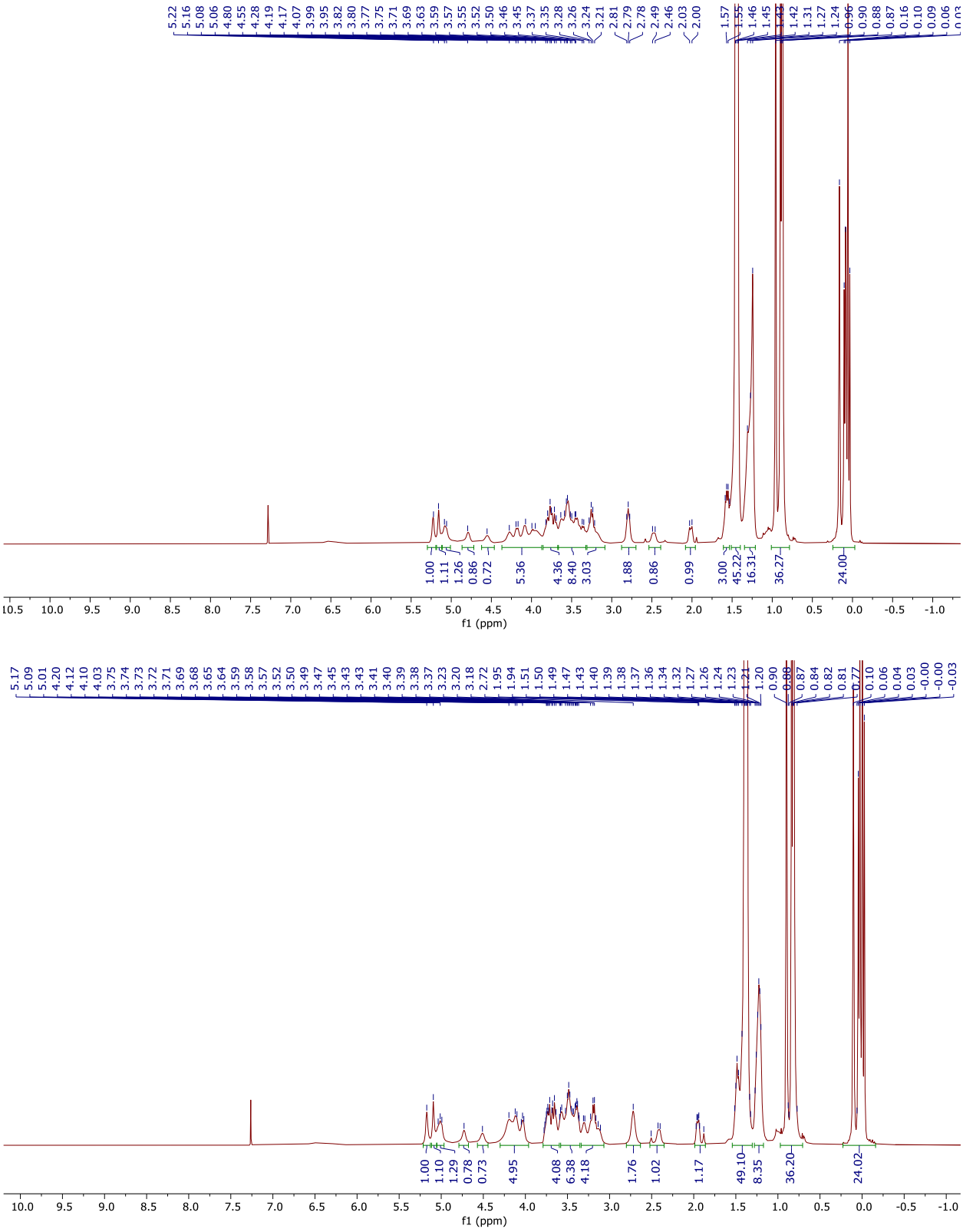


Figure S22. ^{1}H of compound **6a** and **6b** in $CDCl_3$

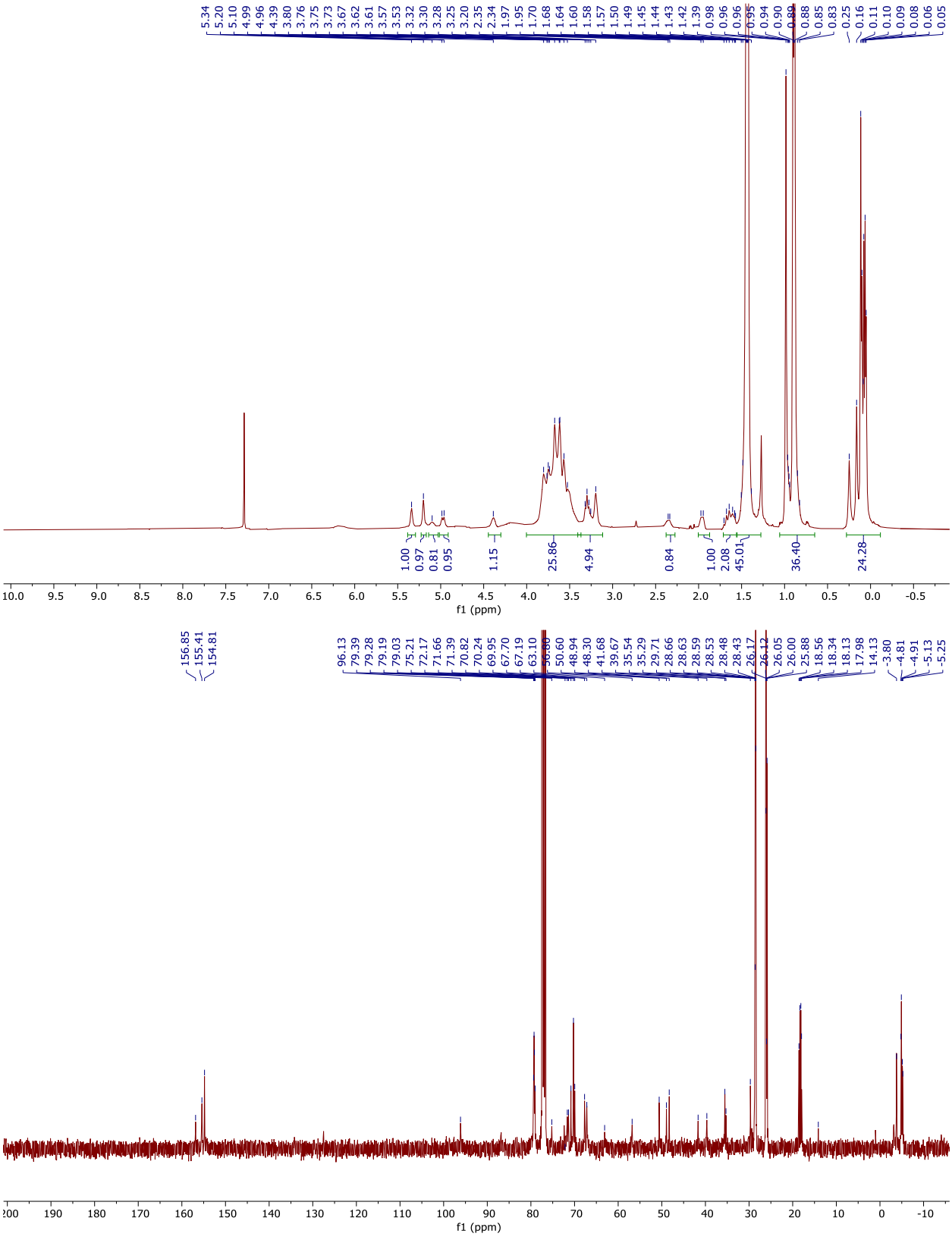


Figure S23. ^{1}H and ^{13}C NMR of compound **6c** in $CDCl_3$

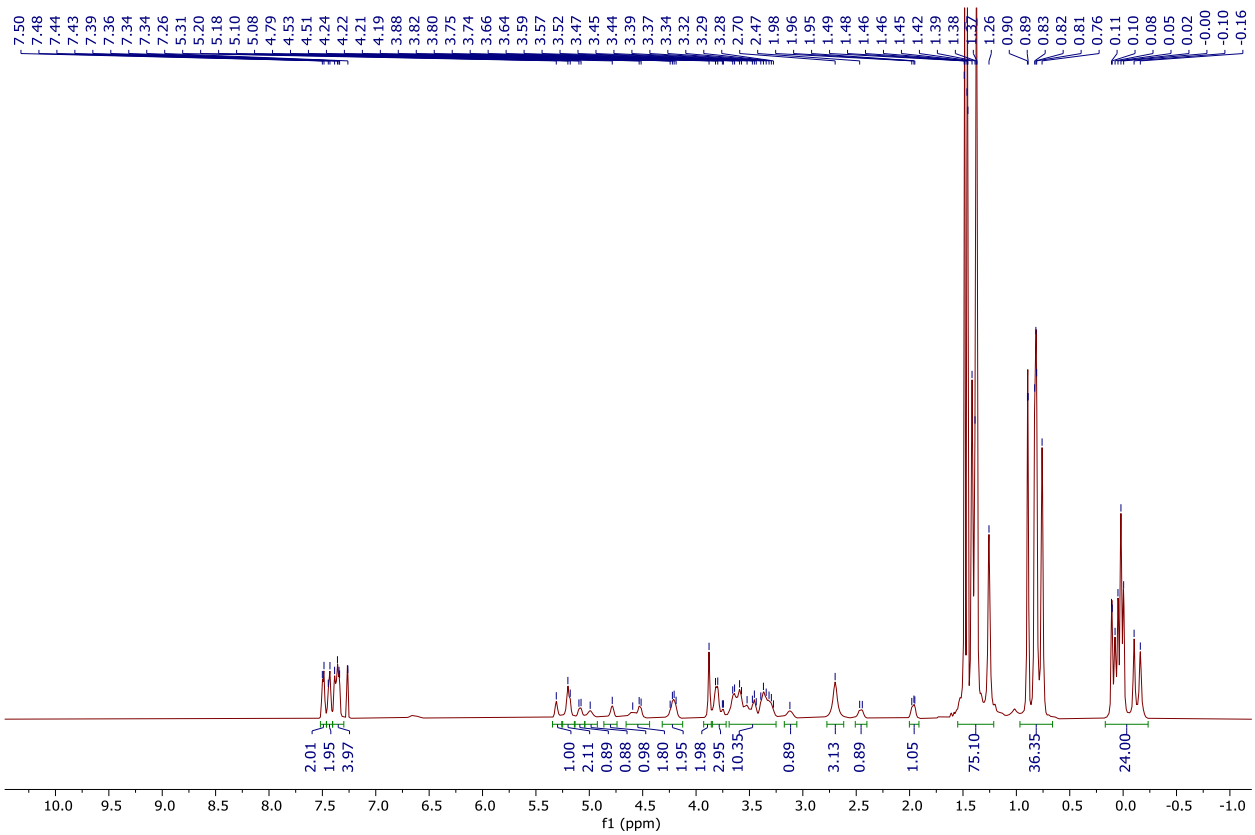


Figure S24. ^{1}H NMR of compound **6d** in $CDCl_3$

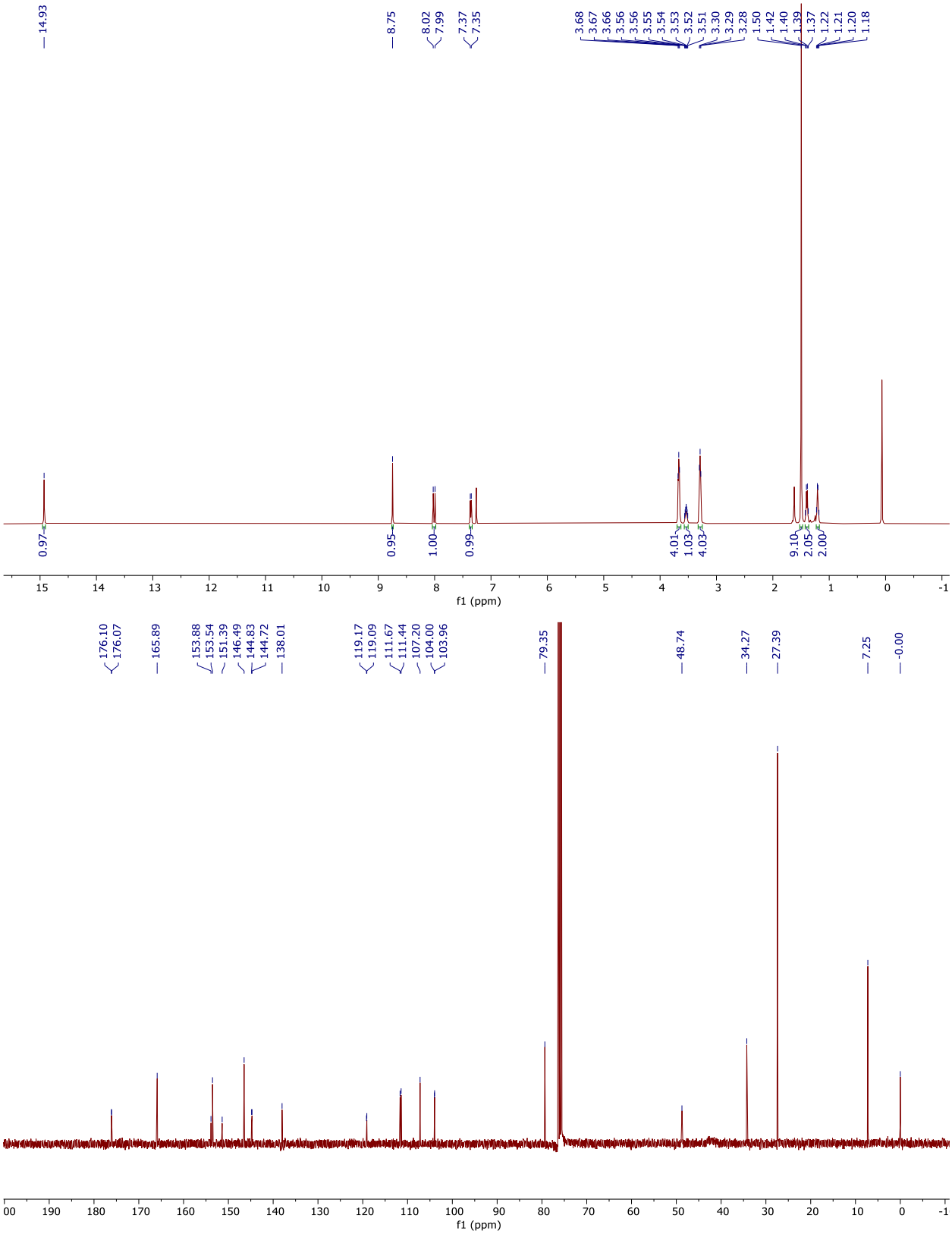


Figure S25. ^{1}H and ^{13}C NMR of compound **7** in $CDCl_3$

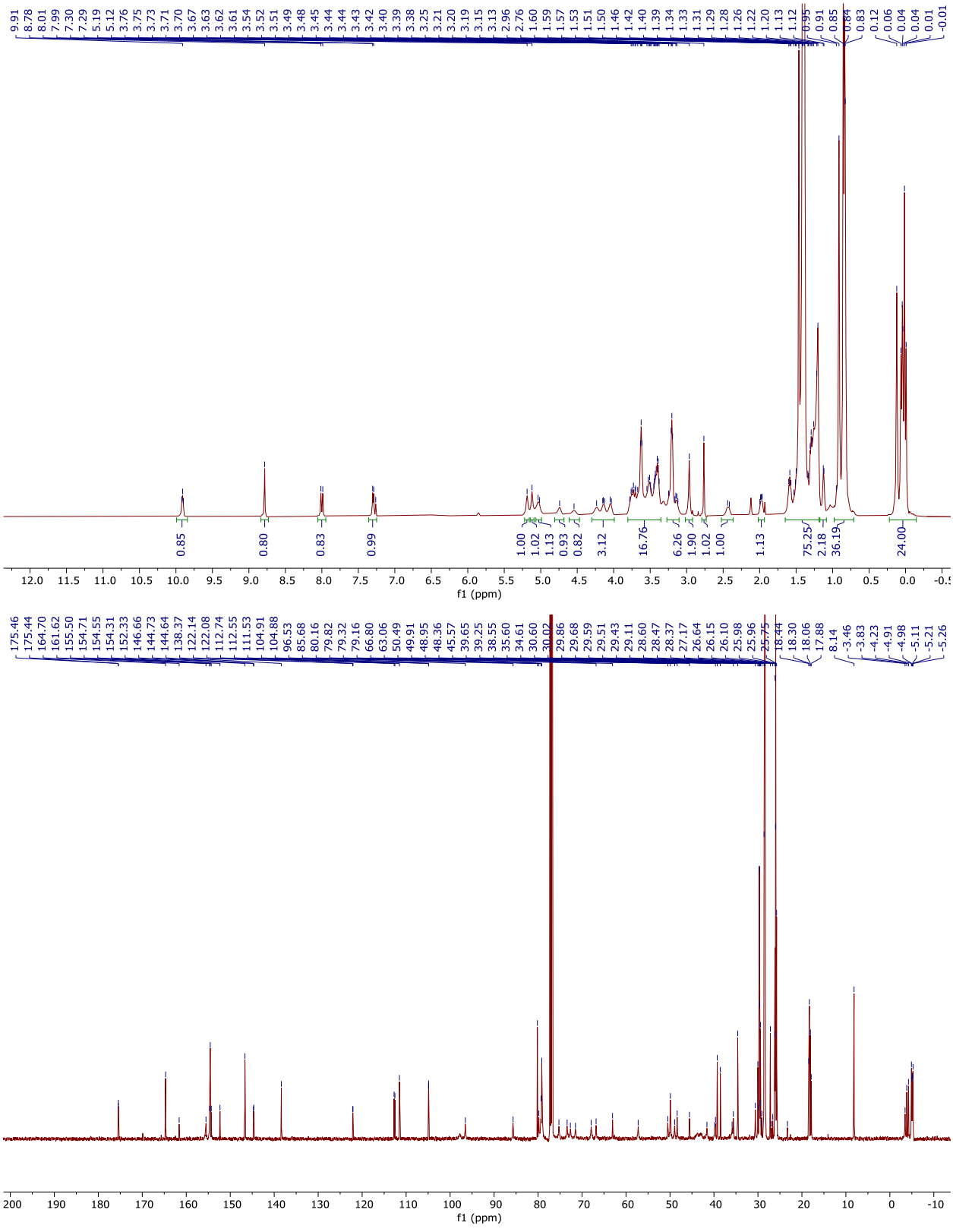


Figure S26. ^{1}H and ^{13}C NMR of compound **8a** in $CDCl_3$

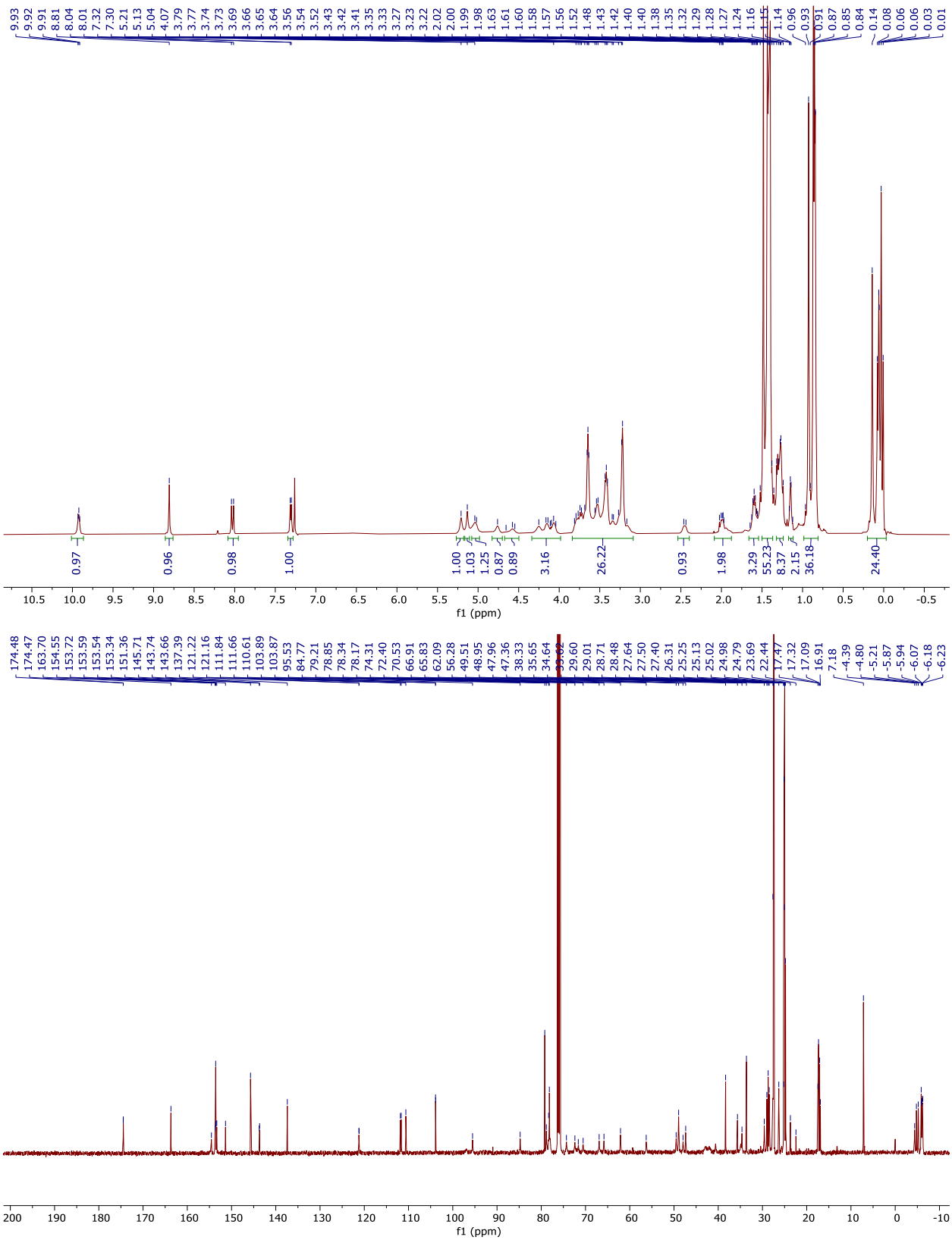


Figure S27. ^{1}H and ^{13}C NMR of compound **8b** in $CDCl_3$

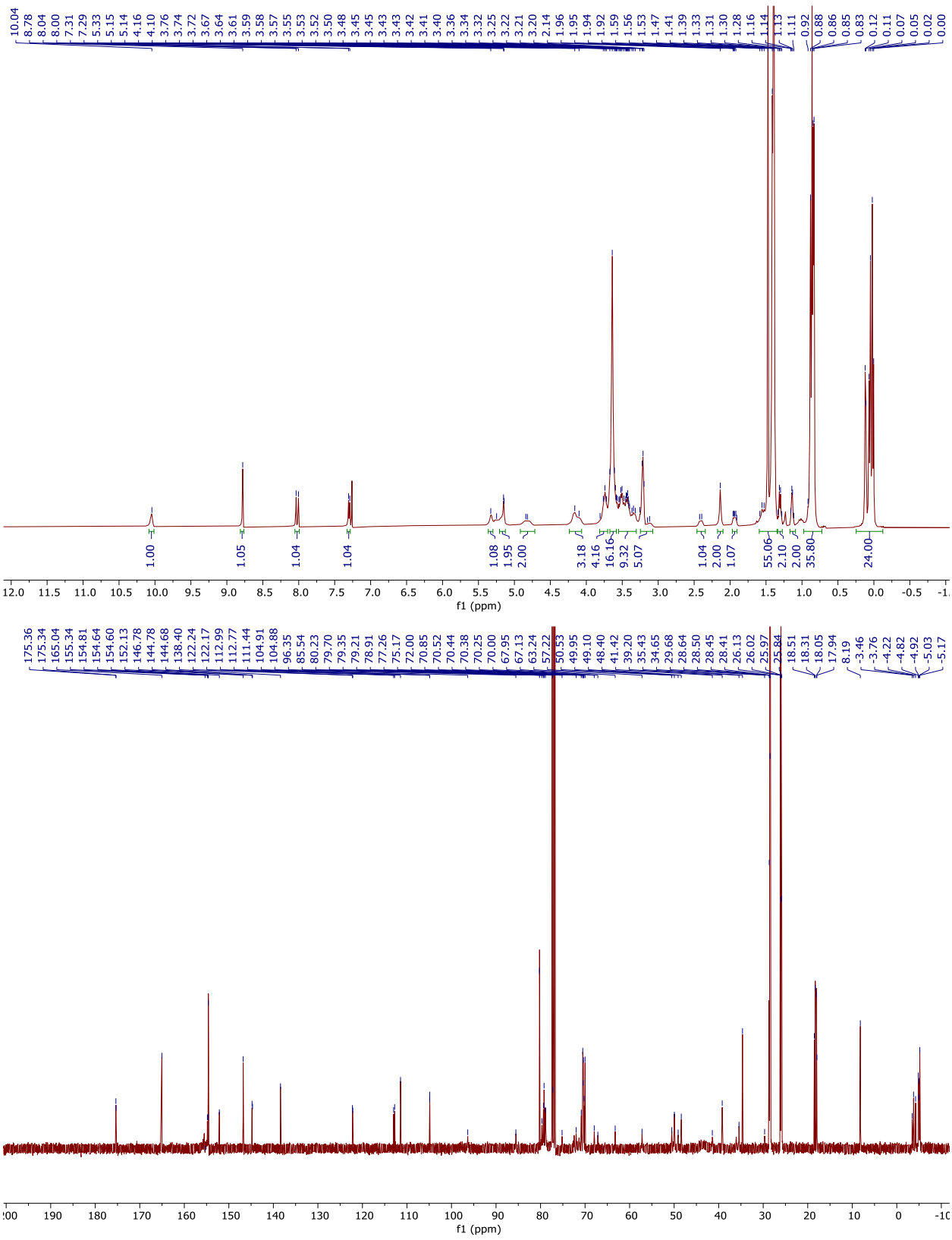


Figure S28. ^{1}H and ^{13}C NMR of compound **8c** in $CDCl_3$

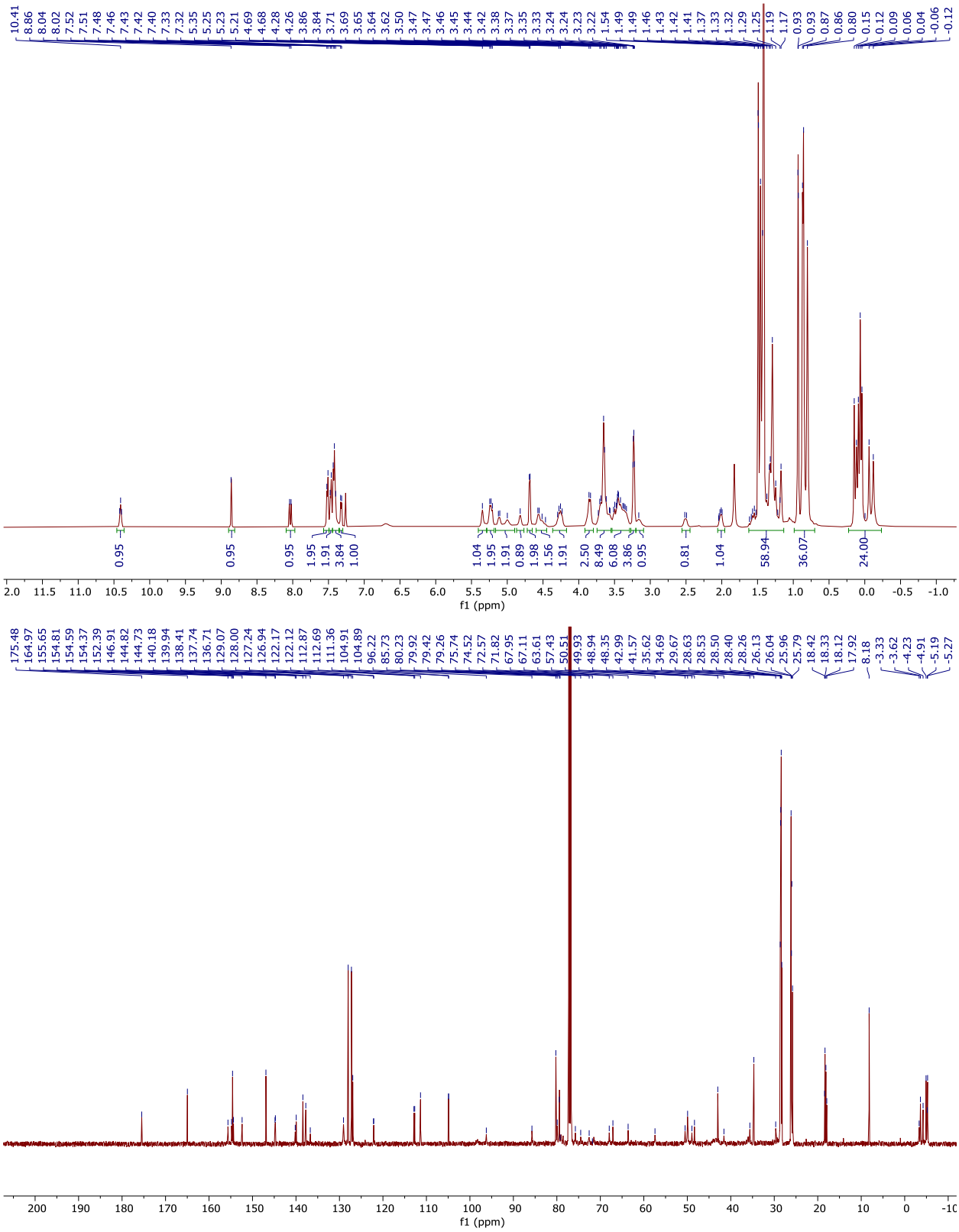


Figure S29. ^{1}H and ^{13}C NMR of compound **8d** in $CDCl_3$

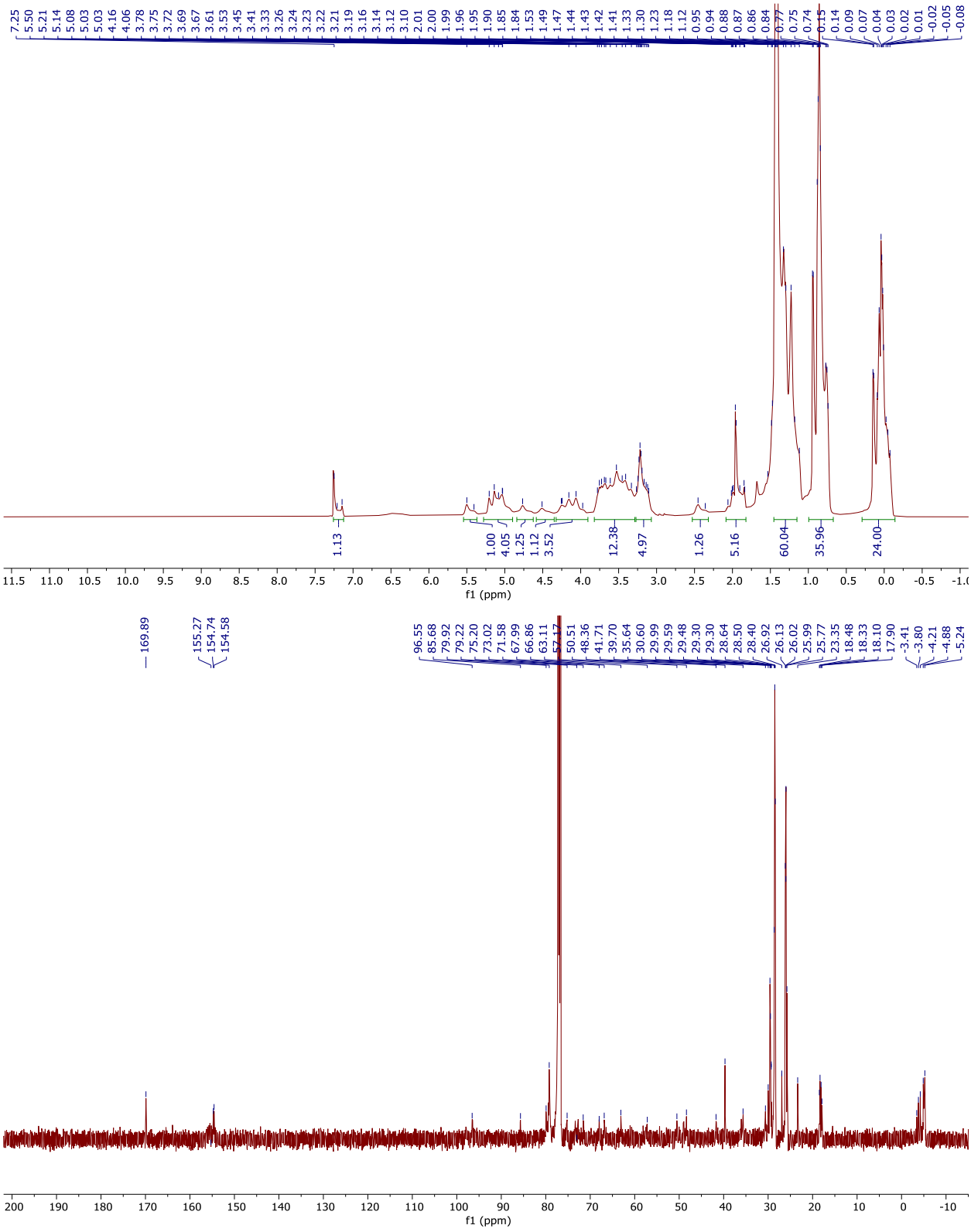


Figure S30. ^{1}H and ^{13}C NMR of compound **9** in $CDCl_3$

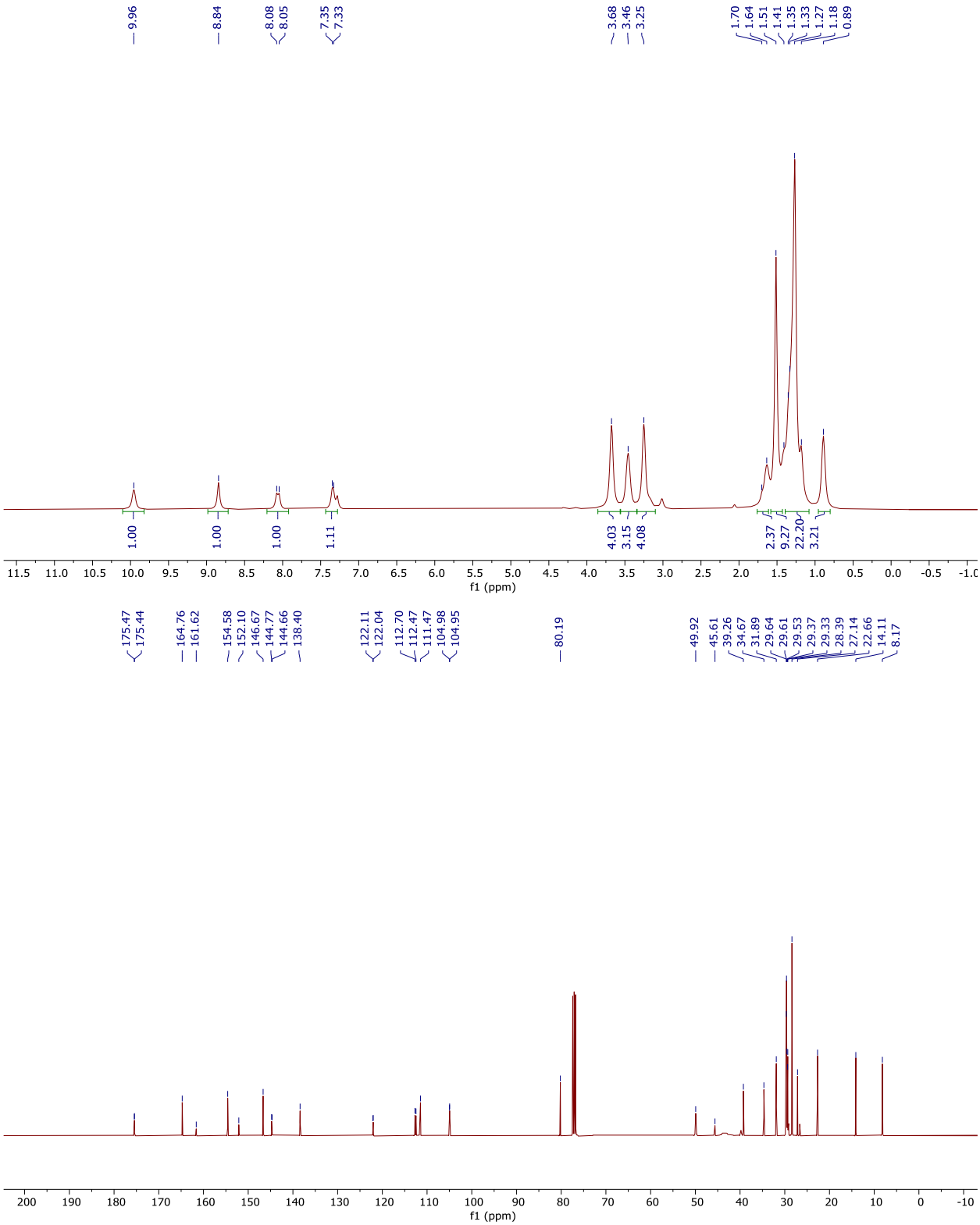


Figure S31. ^{1}H and ^{13}C NMR of compound **10** in $CDCl_3$

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