Supporting Information

Calixarene-decorated liposomes for intracellular cargo delivery

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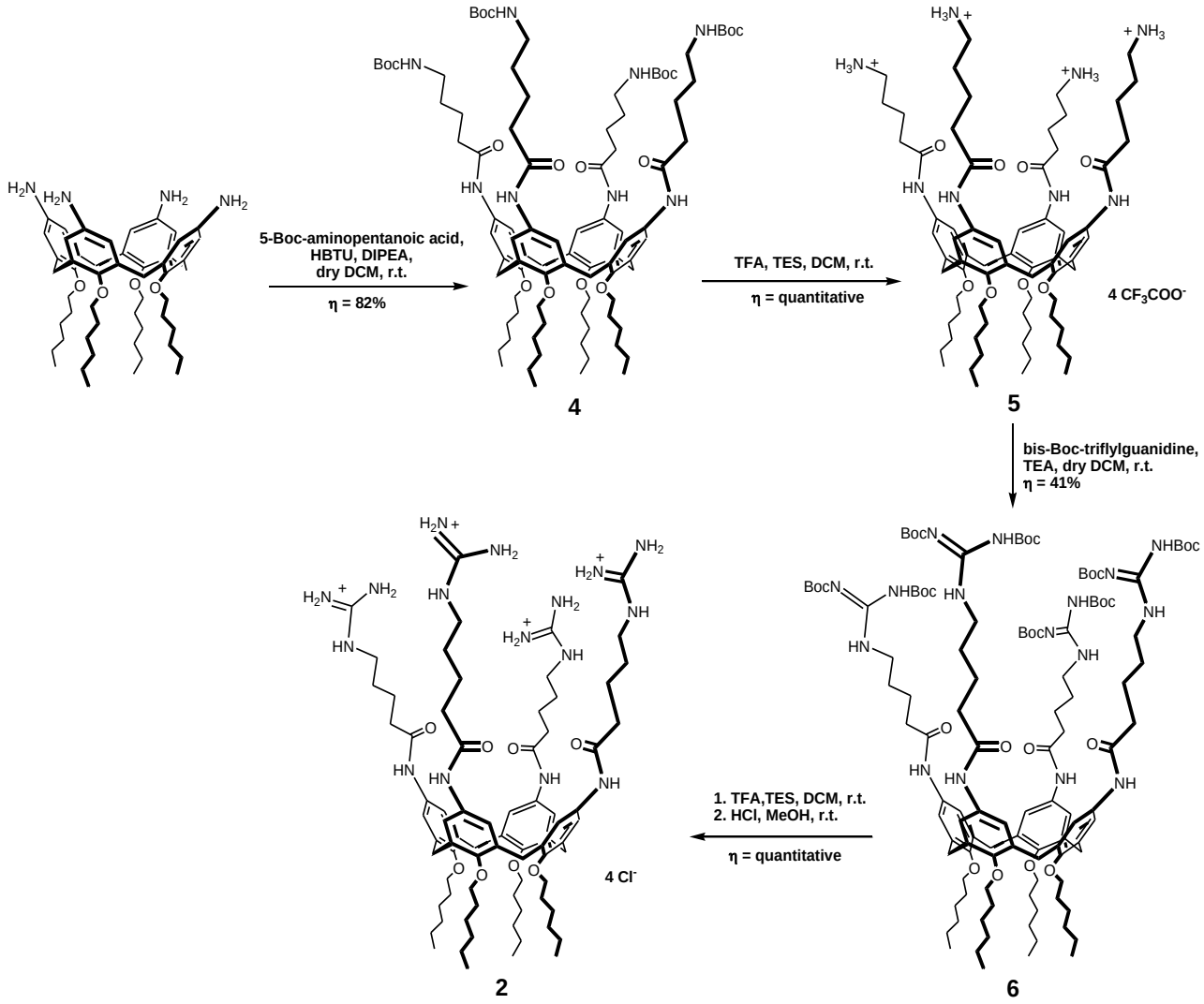
Scheme S1


5-Boc-aminopentanoic acid,
HBTU, DIPEA,
dry DCM, r.t.
η = 82%
TFA, TES, DCM, r.t.
η = quantitative
4 CF3COO-
bis-Boc-triflylguanidine,
TEA, dry DCM, r.t.
η = 41%
1. TFA,TES, DCM, r.t.
2. HCl, MeOH, r.t.
η = quantitative
4 Cl-
4
5
6
2

Experimental

General information. Calixarenes **1**[1] and **3**[2] were synthesized as reported in literature. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phospho-ethanolamine), and cholesterol were purchased from Avanti Polar Lipids. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV300 and Bruker AV400 spectrometers (observation of ^{1}H nucleus at 300 MHz and 400 MHz, respectively, and of ^{13}C nucleus at 75 MHz and 100 MHz, respectively) and partially deuterated solvents were used as internal standards to calculate the chemical shifts (δ values in ppm). All ^{13}C NMR spectra were performed with proton decoupling. For ^{1}H NMR spectra recorded in D_2O at values higher than the room temperature, the correction of chemical shifts was performed using the expression $\delta = 5.060 - 0.0122 \times T(°C) + (2.11 \times 10^{-5}) \times T^2(°C)$ to determine the resonance frequency of water protons.[3] HRMS-ESI mass analyses were performed with a LTQ Orbitrap XL spectrometer. Melting points were determined on an Electrothermal apparatus in closed capillaries. Absorption measurements were performed using an Agilent 8453 spectrometer. Fluorescence spectroscopy measurements were performed using a Horiba fluorimeter. Particle size, polydispersity, and surface charge of the lipid vesicles were measured by dynamic light scattering on a Zetasizer Nano ZS (model ZEN3600 from Malvern Instruments).

5,11,17,23-Tetrakis[5-(Boc-amino)pentanoyl]amido-25,26,27,28-tetra-*n*-hexyloxycalix[4]arene (4) To a solution in dry DCM of 5-(Boc-amino)pentanoic acid (153 mg, 0.72 mmol) and DIPEA (245 μL, 1.43 mmol), HBTU (308 mg, 0.81 mmol) and tetraamino-tetrahexyloxycalixarene (100 mg, 0.12 mmol) were added. The mixture was stirred at room temperature for 24 h. The reaction was quenched with water; the organic layer was washed with brine, and then dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the pure product was isolated as white solid (160 mg, 82% yield) by flash column chromatography (CH_2Cl_2/MeOH, 96/4, v/v) and subsequent centrifugation with hexane of the solid obtained from chromatography. Mp: 118.5-120.2 °C. ^{1}H-NMR (300 MHz, CD_3OD): δ = 6.89 (s, 8H, ArH), 6.61 (bt, N*H*Boc), 4.44 (d, *J* = 12.9 Hz, 4H, ArCHH_{ax}Ar), 3.88 (t, *J* = 7.2 Hz, 8H, OCH_2), 3.17-2.96 (m, 12H, ArCHH_{eq}Ar and CH_2NHBoc), 2.26 (t, *J* = 7.2 Hz, 8H, NHCOCH_2), 2.06-1.88 (m, 8H, OCH_2CH_2), 1.72-1.57 (m, 8H, CH_2CH_2NHBoc) 1.55-1.28 (m, 68H, $COCH_2CH_2$, $O(CH_2)_2(CH_2)_3$ and $C(CH_3)_3$), 0.95 (t, *J* = 6.6 Hz, 12H, CH_2CH_3). ^{13}C-NMR (100 MHz, CD_3OD): δ = 173.8 and 158.6 (C=O), 154.5, 136.3, 133.8 and 122.0 (Ar), 79.9 ($C(CH_3)_3$), 76.6 (OCH_2), 41.0 (CH_2NHBoc), 37.5 ($COCH_2$), 33.5 ($OCH_2CH_2CH_2$), 32.2 (ArCH_2Ar), 31.6 (OCH_2CH_2), 30.7 (CH_2CH_2NHBoc), 29.0 ($C(CH_3)_3$) and $CH_2CH_2CH_3$), 27.0 ($COCH_2CH_2$), 24.1 (CH_2CH_3), 14.6 (CH_2CH_3). HRMS-ESI calcd for $[C_{92}H_{144}O_{16}N_8 + Na]^+$ 1640.059, found 1640.061.

5,11,17,23-Tetrakis(5-aminopentanoyl)amido-25,26,27,28-tetra-*n*-hexyloxycalix[4]arene, tetrahydrotrifluoroacetate (5). Compound **4** (150 mg, 93 μmol) was dissolved in a TFA/TIS/H_2O mixture (95/2.5/2.5, v/v/v, 2 mL). The reaction was then stirred at room temperature and its progression monitored by ESI-MS. After completion, the volatiles were removed under reduced pressure. The residue was washed with ethyl acetate (3×5 mL) to remove the exceeding TFA. Product **5** was isolated as white solid (148 mg, quantitative yield). Mp: 225 °C dec. ^{1}H-NMR (400 MHz, CD_3OD) δ 6.89 (s, 8H, Ar*H*), 4.46 (d, *J* = 13.0 Hz, 4H, ArCHH_{ax}Ar), 3.89 (t, *J* = 7.2 Hz, 8H, OCH_2), 3.12 (d, *J* = 13.0 Hz, 4H, ArCHH_{eq}Ar), 2.93 (t, *J* = 6.9 Hz, 8H, C$H_2NH_3^+$), 2.33 (t, *J* = 6.3 Hz, 8H, NHCOCH_2),

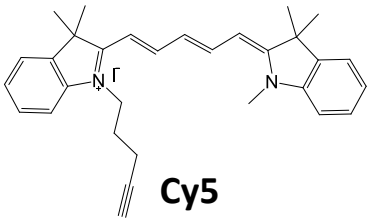
2.03-1.88 (m, 8H, OCH_2CH_2), 1.80-1.61 (m, 16H, $CH_2CH_2NH_3^+$ and $COCH_2CH_2$), 1.55-1.30 (m, 24H, $O(CH_2)_2(CH_2)_3$), 1.02- 0.89 (m, 12H, CH_2CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ 173.1 (C=O), 162.9 (q, *J* = 36 Hz, CF_3C=O) 154.6, 136.2, 133.4 and 121.9 (Ar), 118.2 (q, *J* = 288 Hz, CF_3) 76.7 (OCH_2), 40.4 ($CH_2NH_3^+$), 36.8 ($COCH_2$), 33.4 ($CH_2CH_2CH_3$), 32.2 ($ArCH_2Ar$), 31.5 (OCH_2CH_2), 28.1 and 23.4 ($CH_2CH_2NH_3^+$ and $COCH_2CH_2$), 27.4 ($OCH_2CH_2CH_2$), 24.0 (CH_2CH_3), 14.7 (CH_2CH_3). HRMS-ESI calcd for $[C_{72}H_{112}N_8O_8 + 2H]^{2+}$ 609.4374, found 609.4381.

5,11,17,23-Tetrakis[(5-(N,N'-di-Boc-guanidyl)pentanoylamido]-25,26,27,28-tetra-*n*-hexyloxycalix[4]arene (6). To a solution of calix[4]arene **5** (100 mg, 63 μmol) in dry CH_2Cl_2 (10 mL) and Et_3N (86 μL, 626 μmol), *N*,*N'*-Bis(tert-butoxycarbonyl)-*N''*-triflylguanidine (122.6 mg, 313 μmol) was added and the mixture was stirred for 16 h. The mixture was transferred to a separatory funnel and washed with 2 M aqueous potassium bisulfate (10 mL) and with saturated sodium bicarbonate (10 mL). Each aqueous layer was extracted with CH_2Cl_2 (2×5 mL). The combined organic phases were washed with brine (15 mL), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Pure product was isolated by flash column chromatography (gradient from CH_2Cl_2 to CH_2Cl_2/MeOH, 95/5, v/v) as yellowish solid (56 mg, 41% yield). Mp: 135-137 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ 6.91 (s, 8H, Ar*H*), 4.45 (d, *J* = 12.9 Hz, 4H, $ArCH_{ax}Ar$), 3.89 (t, *J* = 6.9 Hz, 8H, OCH_2), 3.38 (t, *J* = 6.6 Hz, 8H, CH_2NH), 3.12 (d, *J* = 12.9 Hz, 4H, $ArCH_{eq}Ar$), 2.29 (bt, 8H, $COCH_2$), 2.07-1.86 (m, 8H, OCH_2CH_2), 1.55-1.34 (m, 112H, $COCH_2$ CH_2CH_2, $O(CH_2)_2(CH_2)_3$ and $C(CH_3)_3$), 0.97 (t, *J* = 6.9 Hz, 12H, CH_2CH_3). ^{13}C-NMR (75 MHz, CD_3OD) δ 173.5 (C=O), 164.6 (C=N), 157.6 and 154.4 (C=O), 154.2, 136.2, 133.8 and 121.0 (Ar), 84.5 and 80.4 ($C(CH_3)_3$), 76.6 (OCH_2), 41.5 (CH_2NH), 37.3 ($COCH_2$), 33.5 ($OCH_2CH_2CH_2$), 32.2 ($ArCH_2Ar$), 31.6 (OCH_2CH_2), 29.8 (CH_2CH_2NH), 28.8 and 28.4 ($C(CH_3)_3$), 27.5 ($CH_2CH_2CH_3$), 24.1 ($COCH_2CH_2$ and CH_2CH_3), 14.7 (CH_2CH_3). HRMS-ESI calcd for $[C_{116}H_{184}N_{16}O_{24} + 2Na]^{2+}$ 1115.673, found 1115.675.

5,11,17,23-Tetrakis(5-guanidinopentanoylamido)-25,26,27,28-tetra-*n*-hexyloxycalix[4]arene, tetrahydrochloride (2) A solution of calix[4]arene **6** (53 mg, 24.2 μmol) in DCM/TFA/TES (92.5/5/2.5, 10 mL) was stirred at 0 °C. The progression of the reaction was followed using mass spectrometry. After completion, the volatiles were removed under reduced pressure. The crude material was suspended in distilled diethyl ether (5 mL) and centrifuged (3×). The trifluoroacetate anion of the resulting TFA salt was exchanged with chloride by adding 10 mM HCl solution (3 mL) followed by evaporation under reduced pressure (3×). The pure product **2** was isolated as white solid (37 mg, quantitative yield). Mp: 190 °C dec. ^{1}H-NMR (400 MHz, CD_3OD) δ 6.91 (s, 8H, Ar), 4.48 (d, *J* = 13.2 Hz, 4H, $ArCH_{ax}Ar$), 3.91 (t, *J* = 7.6 Hz, 8H, OCH_2), 3.21 (t, *J* = 6.8 Hz, 8H, CH_2NH), 3.13 (d, *J* = 13.2 Hz, 4H, $ArCH_{eq}Ar$), 2.33 (t, *J* = 6.8 Hz, 8H, $COCH_2$), 2.04-1.91 (m, 8H, OCH_2CH_2) 1.78-1.57 (m, 16H $COCH_2CH_2CH_2$), 1.55-1.32 (m, 24H, $O(CH_2)_2(CH_2)_3$), 0.97 (t, *J* = 6.8 Hz, 12H, CH_2CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ 173.6 (C=O), 158.6 (C=N), 154.5, 136.2, 133.6 and 122.1 (Ar), 76.6 (OCH_2), 42.3 (CH_2NH), 37.1 ($COCH_2$), 33.4 ($OCH_2CH_2CH_2$), 32.2 ($ArCH_2Ar$), 31.5 (OCH_2CH_2), 29.4 (CH_2CH_2NH), 27.4 ($CH_2CH_2CH_3$), 24.0 (CH_2CH_3), 23.8 ($COCH_2CH_2$), 14.5 (CH_2CH_3). HRMS-ESI calcd for $[C_{76}H_{122}N_{16}O_8 + 2H]^{2+}$ 693.4810, found 693.4823.

Preparation of liposomes

Lipid films were prepared from DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), and cholesterol (73:11:16) dissolved in chloroform. The solvent was evaporated and the residue further dried under high vacuum to form a lipid film. The resulting film was rehydrated with an aqueous solution of the cargo to be encapsulated (100 µM Cy5). The lipid suspension was subjected to sonication, freeze and thaw cycles, and extrusion through 100 nm polycarbonate membranes. Extravesicular cargo was removed by gravitational gel filtration (Sephadex G-50 for small molecules) eluting with water. Lipid concentration was determined by adapting the Stewart method.[4]



Cy5

For the preparation of the calixarene decorated liposomes, an aqueous solution of the calixarene (20 µL, 2.7 mol %) was added to a suspension of plain liposomes (2 mg/mL, 1 mL) and after a stirring of 1 h at room temperature the unincorporated calixarene was removed via centrifuge gel filtration (Sephadex G-50).[5]

All the liposomes suspensions were stored at 4 °C and tested on cells within three days from their preparation.

Determination of lipid concentration in liposomal suspension

Lipid concentration was determined by adapting the Stewart method.[4] Briefly, chloroform (2 mL) and ammonium ferrothiocyanate (2 mL, 0.1 M) were added to diluted liposomes (100 µL). The biphasic system was vortexed for 20 s then centrifuged for 1 min. The absorbance of the organic phase was measured at 480 nm against chloroform as a blank. The amount of lipids present was estimated by comparison to a calibration curve generated using liposomal suspensions with a known lipid content.

C5y encapsulation efficiency

Encapsulation efficiency was calculated as the ratio between the fluorescence intensity (640/672) of 1 mL of a methanolic solution of liposomes (0.1 mg/mL) before and after size exclusion purification.

Biological tests

General information. Materials obtained from commercial suppliers were used without further purification. EDTA/Trypsin was purchased from VWR; PBS and F-12 media were purchased from Life Technologies. CellTiter-Blue was purchased from Promega. 35 mm glass bottom culture dishes were

purchased from MatTek. Fluorescence spectroscopy measurements were performed using a Horiba fluorimeter. Flow cytometry studies were performed on a BD FACSCalibur.

Cell Culture

All cells were grown at 37 °C under an atmosphere of 5% CO_2 in air and 100% relative humidity. Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61), and pgsA-745 cells were prepared as previously reported.[6,7] CHO-K1 and pgsA-745 cells were grown in F-12 medium (Life Technologies) supplemented with fetal bovine serum (10% v/v), streptomycin sulfate (100 µg/mL), and penicillin G (100 units/mL).

Cellular Uptake

Wild-type CHO-K1 and mutant pgsA cells were seeded onto 24-well tissue culture plates (100 000 cells/well, 0.4 mL) and grown for 24 h to about 80% confluence. Cells were washed with PBS and incubated with 300 µL of liposomal suspension diluted in F-12 growth medium to the desired concentration and incubated at 37 °C for 20 minutes or 1 hour under an atmosphere of 5% CO_2. The cells were washed twice with 300 µL of PBS, detached with 50 µL of trypsin-EDTA at 37 °C for 5 min, diluted with PBS containing 1% BSA, and analyzed by flow cytometry. Cellular uptake was quantified by the mean fluorescence intensity; raw data was interpreted by using FlowJo v8.8.6.

Cell Viability

CHO-K1 cells were seeded in a 96-well plate at a density of 20 000 cells per well. After growing overnight, the cells were treated with plain liposomes, **Lipo-1**, **Lipo-2** and **Lipo-3** at the indicated concentrations in serum-free medium and incubated for 24 h. Cells were washed, and the growth medium was replaced. Cell Titer Blue (20 µL) was added to each well, and the cells were incubated for 4 h at 37 °C. Fluorescence was measured in a plate reader with excitation/emission wavelengths set at 530/580. Fluorescence intensity was normalized to that of untreated cells.

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2 V. Bagnacani, V. Franceschi, M. Bassi, M. Lomazzi, G. Donofrio, F. Sansone, A. Casnati, R. Ungaro, *Nat. Commun.* 2013, **4**, 1721.

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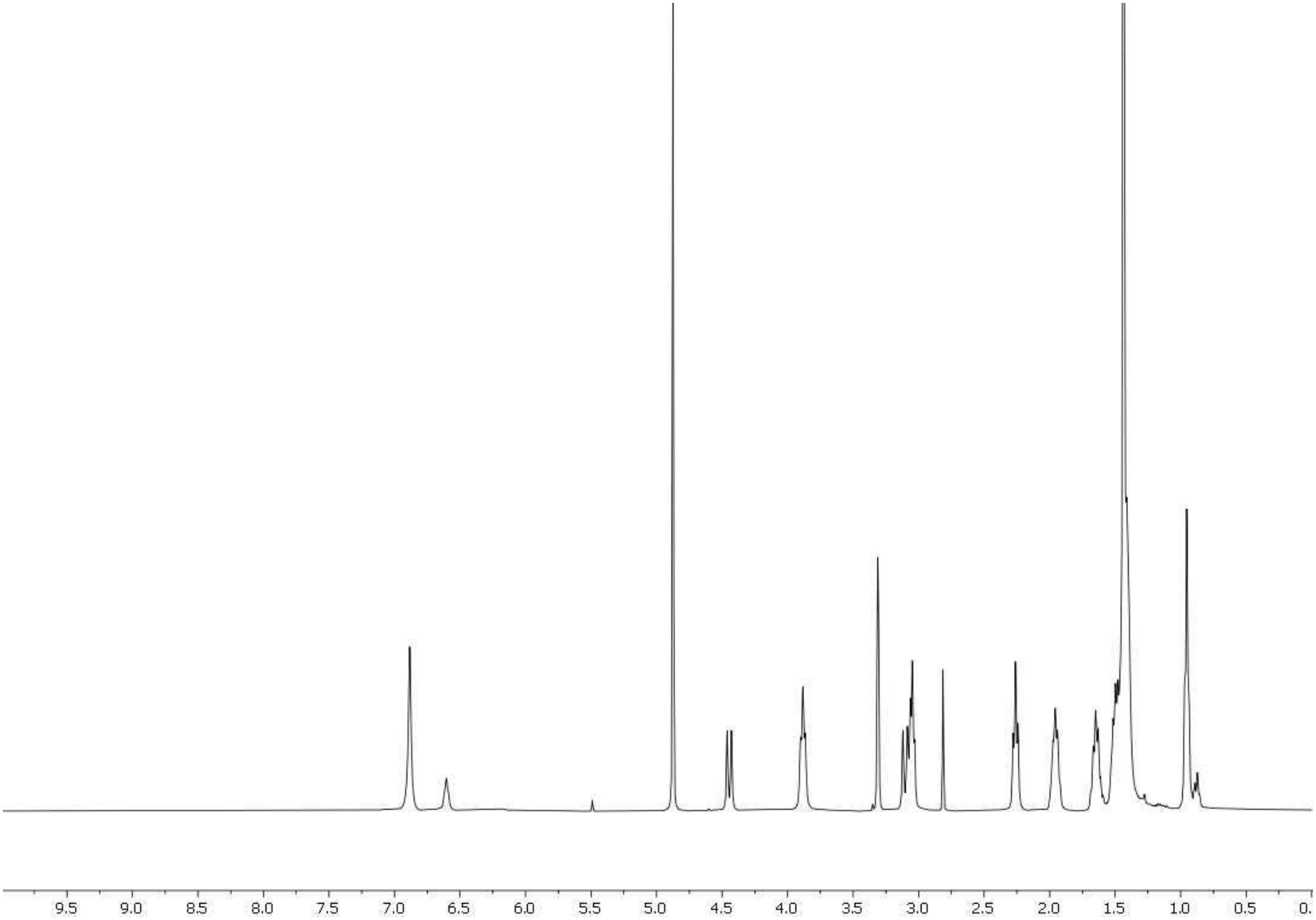

Fig. S1. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of compound **4**.

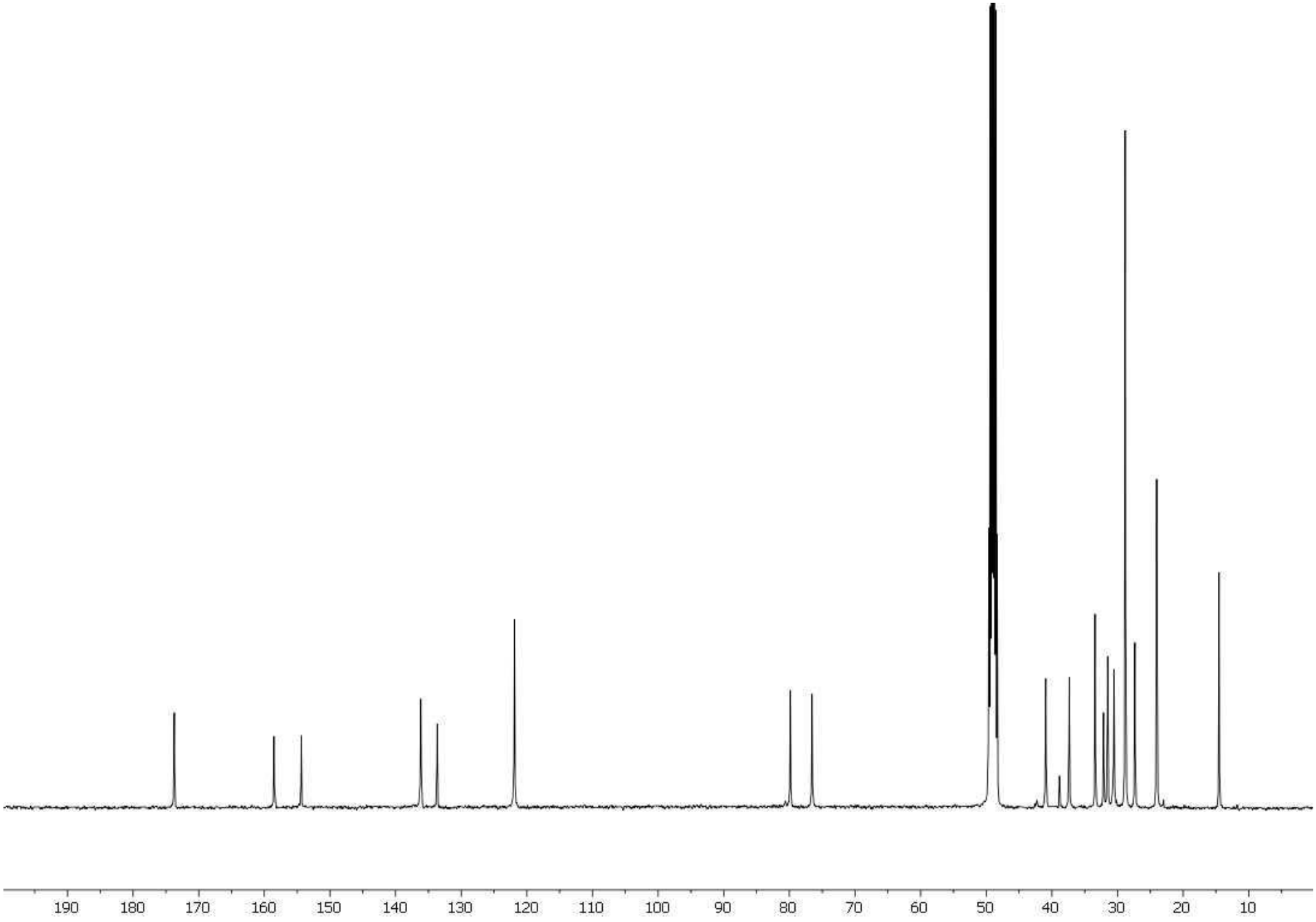

Fig. S2. ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound **4**.

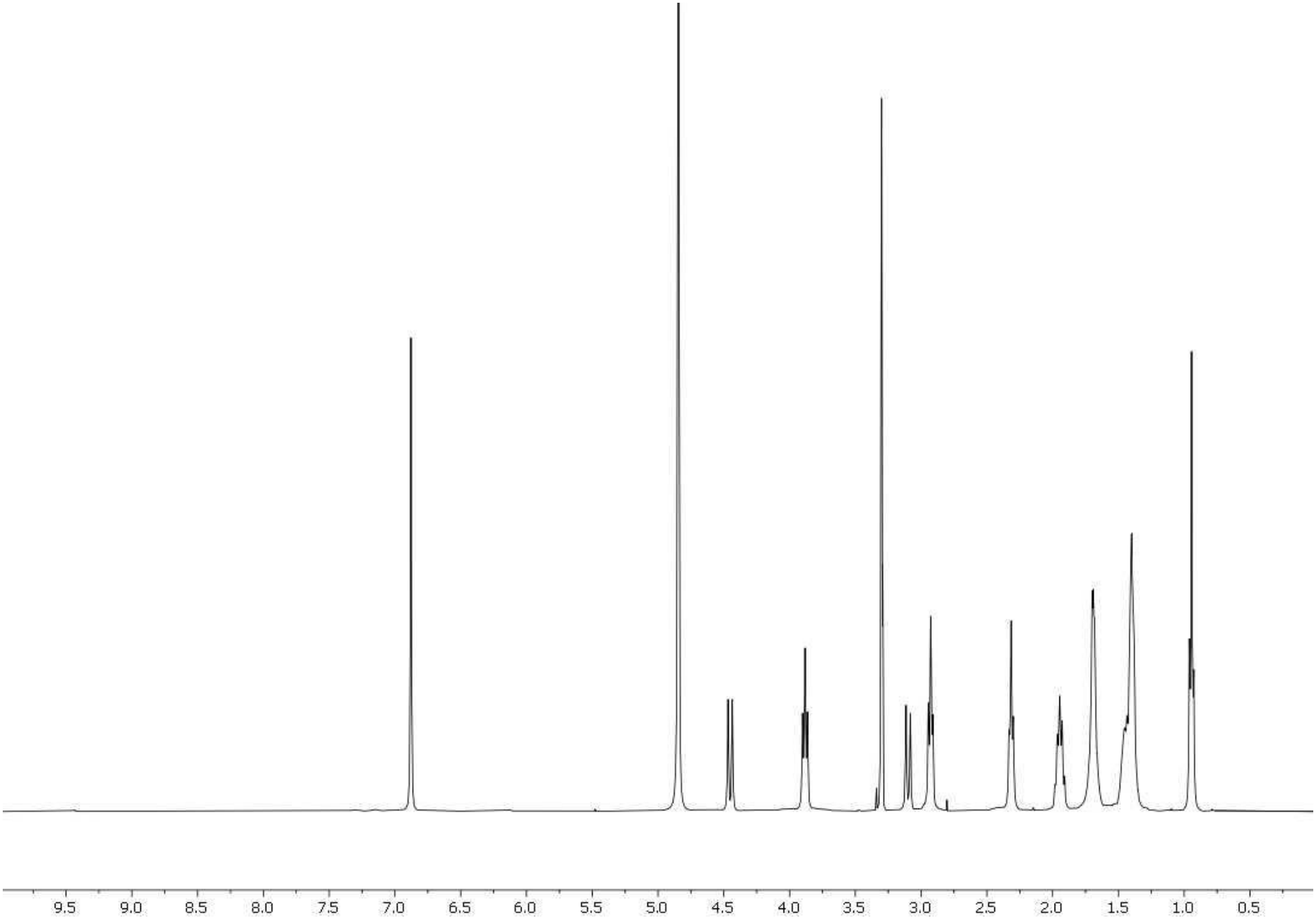

Fig. S3. ^{1}H NMR spectrum (CD_3OD, 400 MHz) of compound **5**.

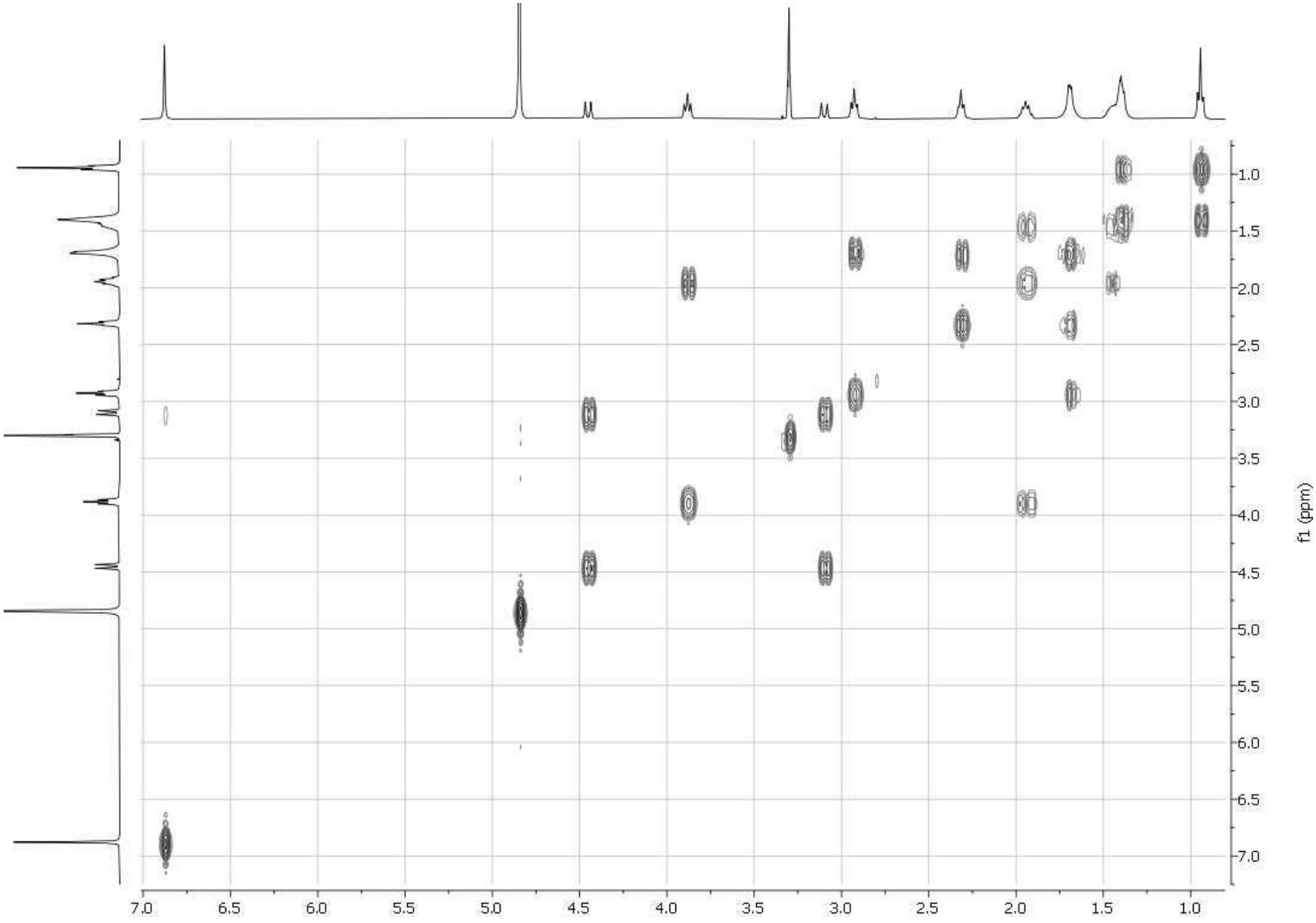

Fig. S4. ^{1}H COSY NMR map (400 MHz, CD_3OD) of compound **5**.

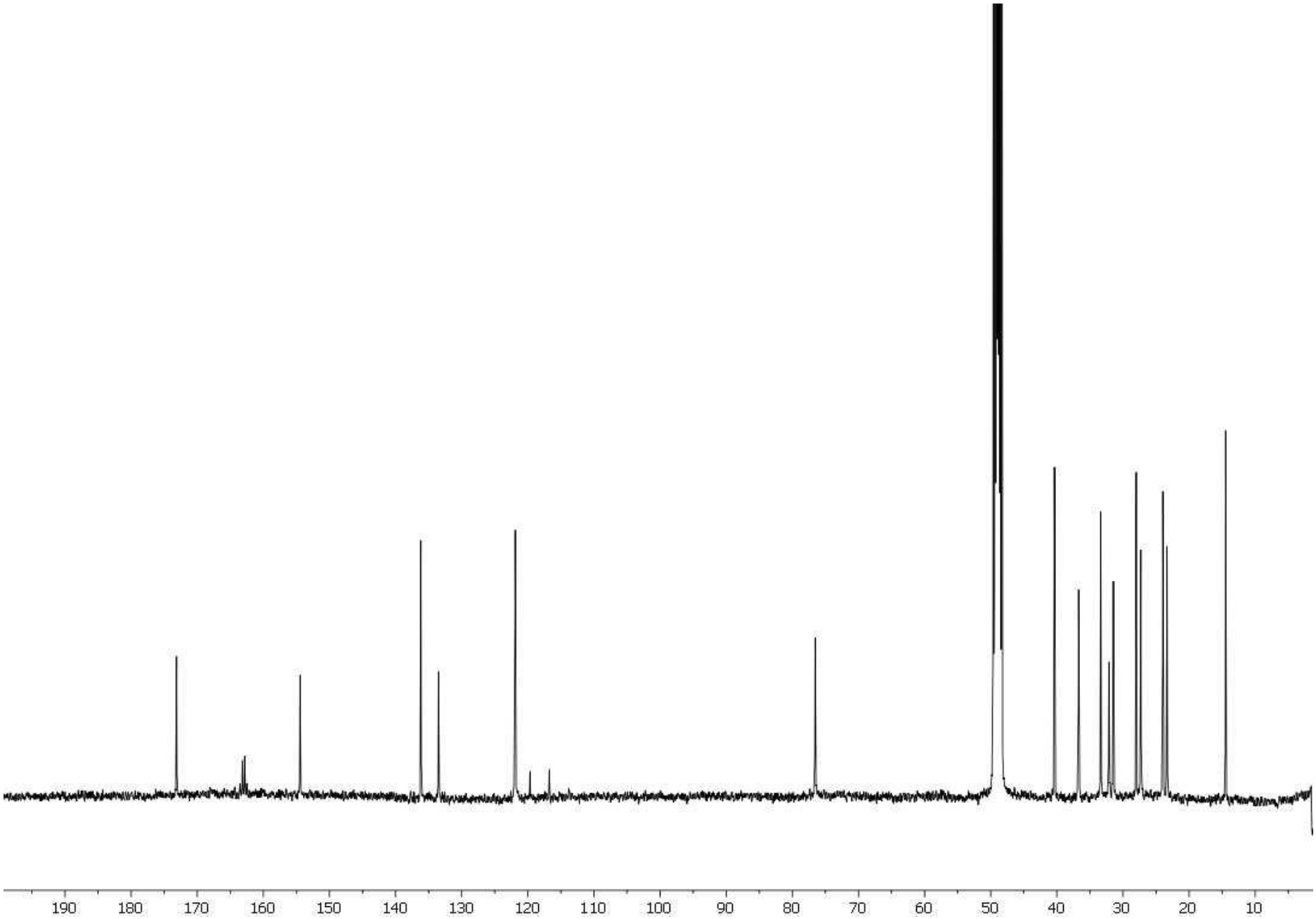

Fig. S5. ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound **5**.

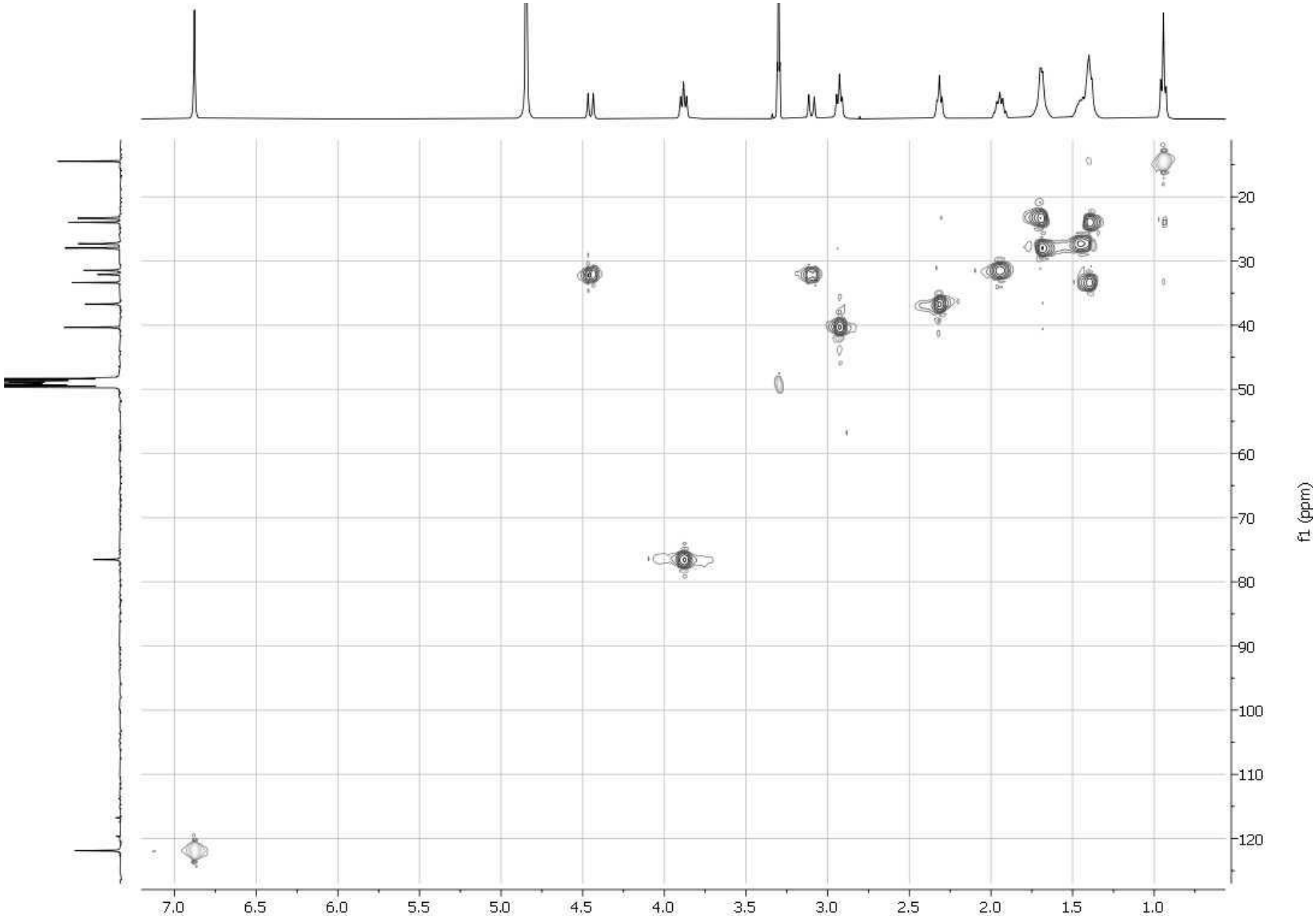

Fig. S6. ^{1}H-^{13}C HSQC NMR spectrum in CD_3OD of compound **5**.

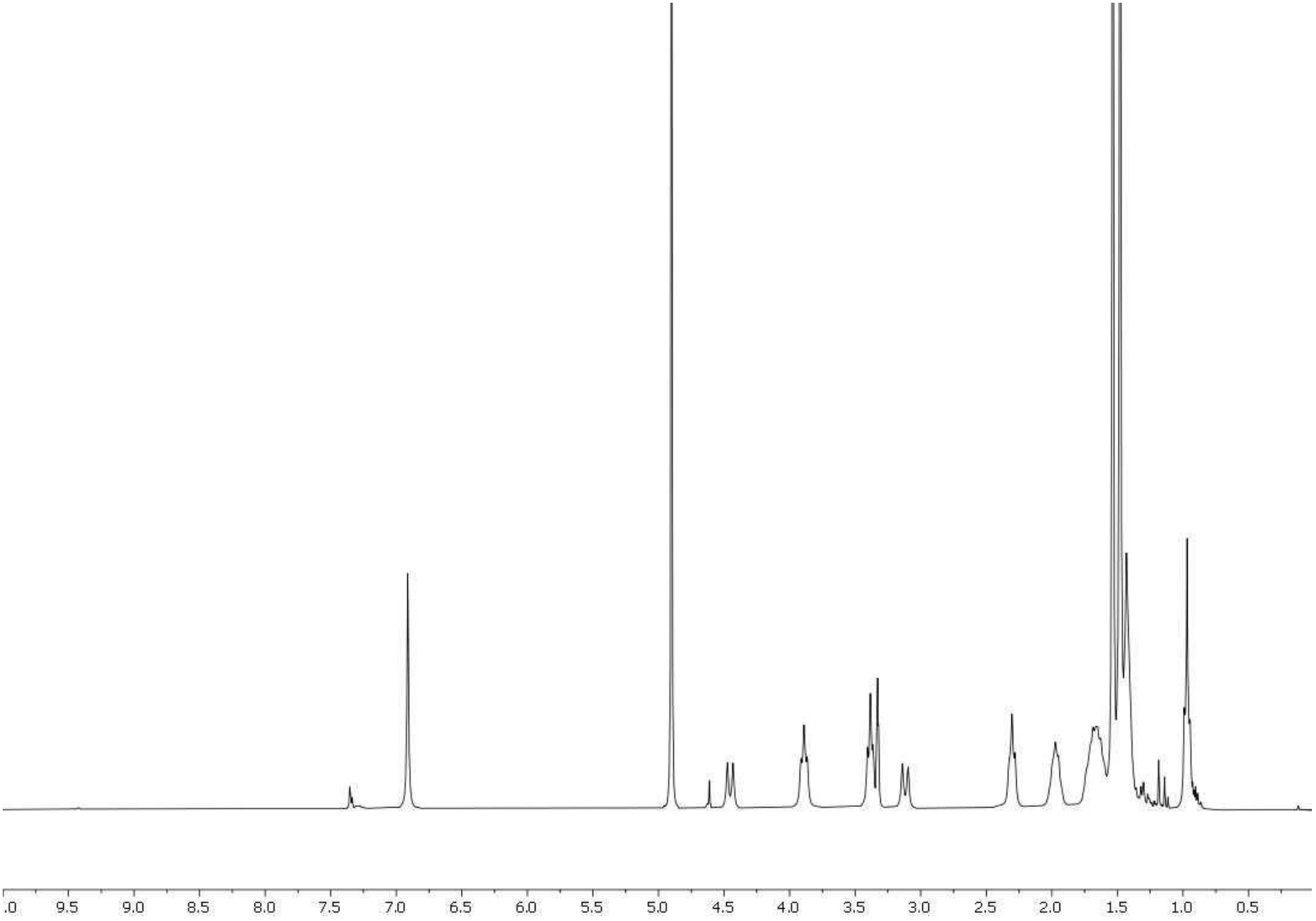

Fig. S7. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of compound **6**.

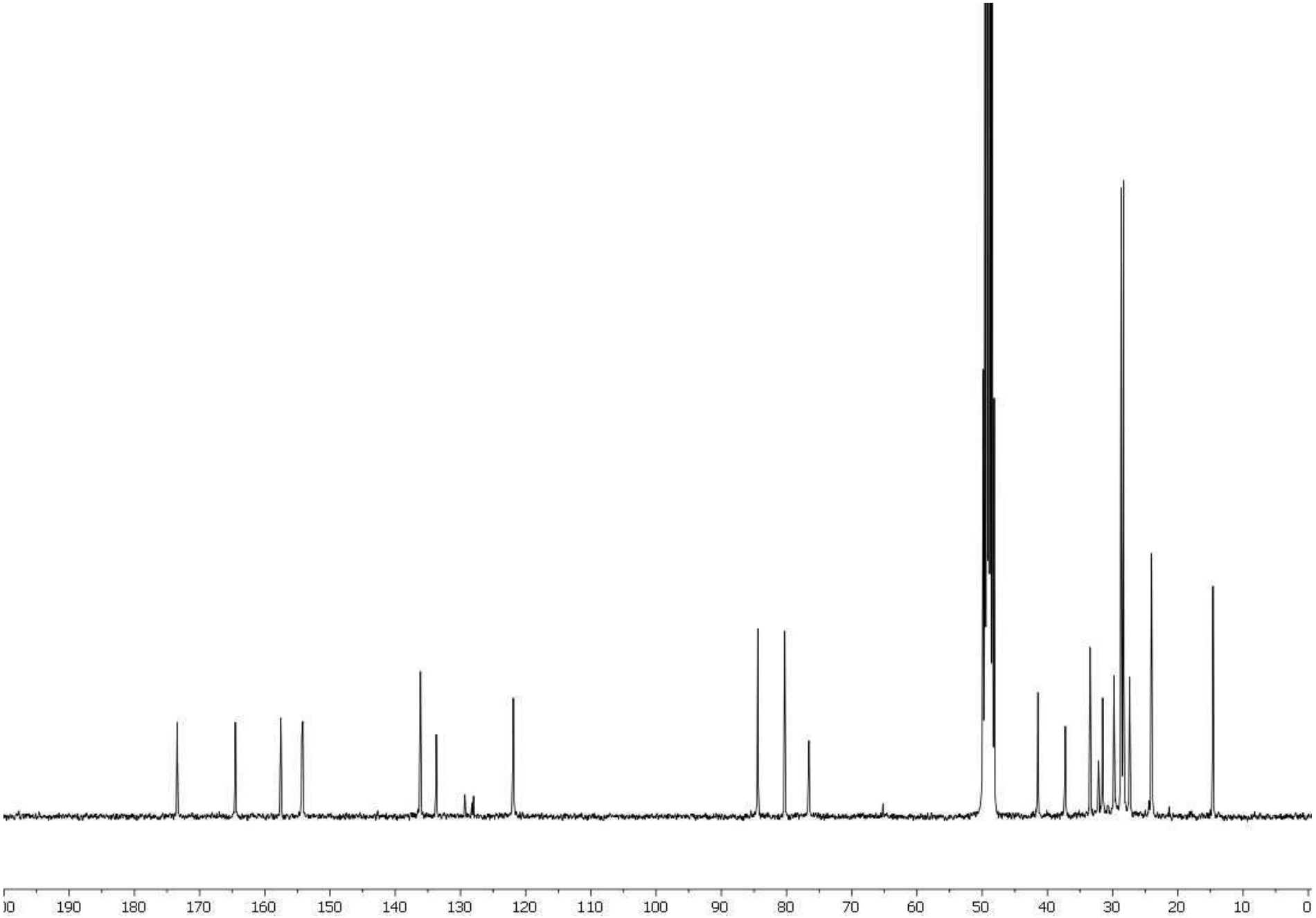

Fig. S8. ^{13}C NMR spectrum (CD_3OD, 75 MHz) of compound **6**.

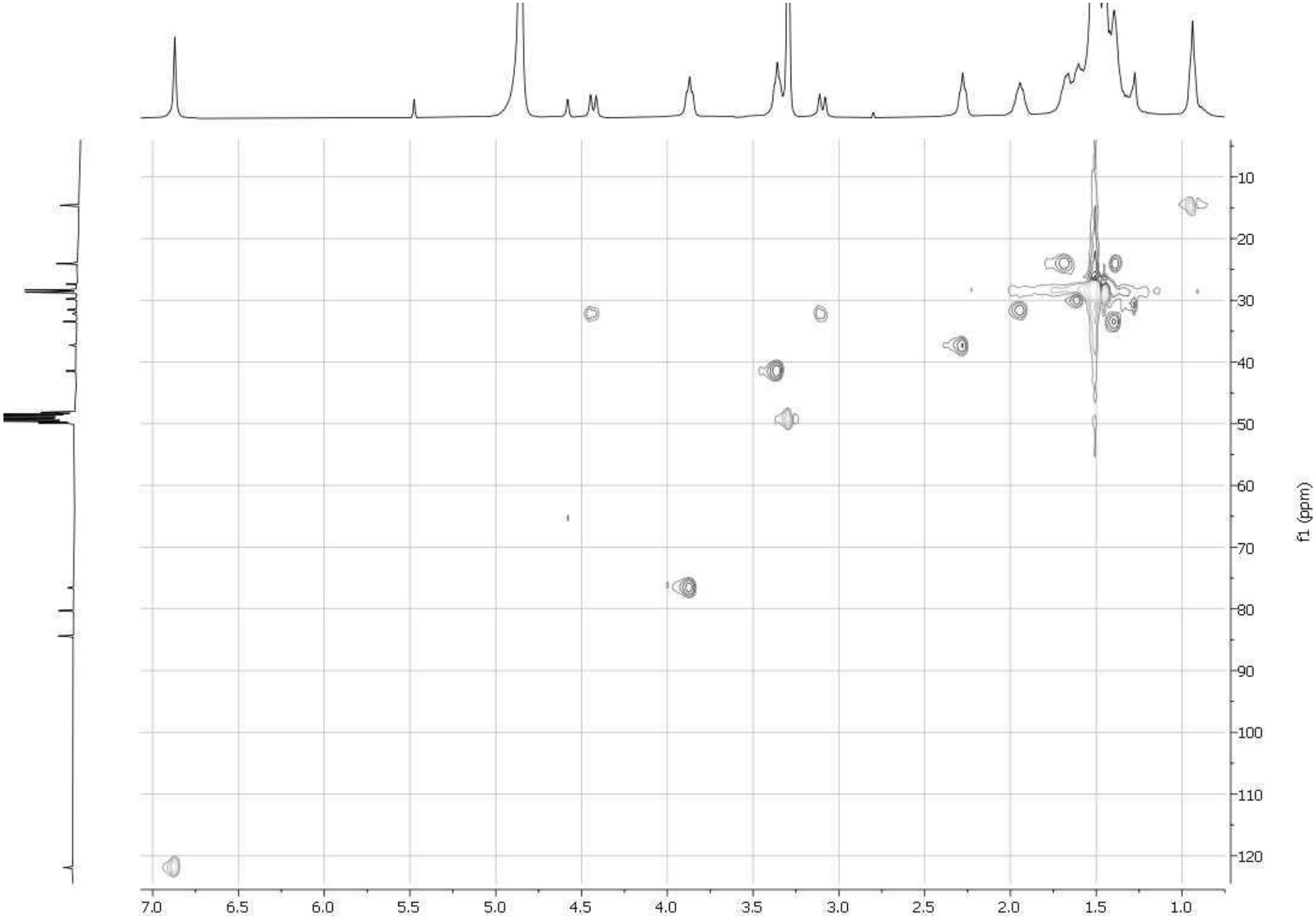

Fig. S9. ^{1}H-^{13}C HSQC NMR spectrum in CD_3OD of compound **6**.

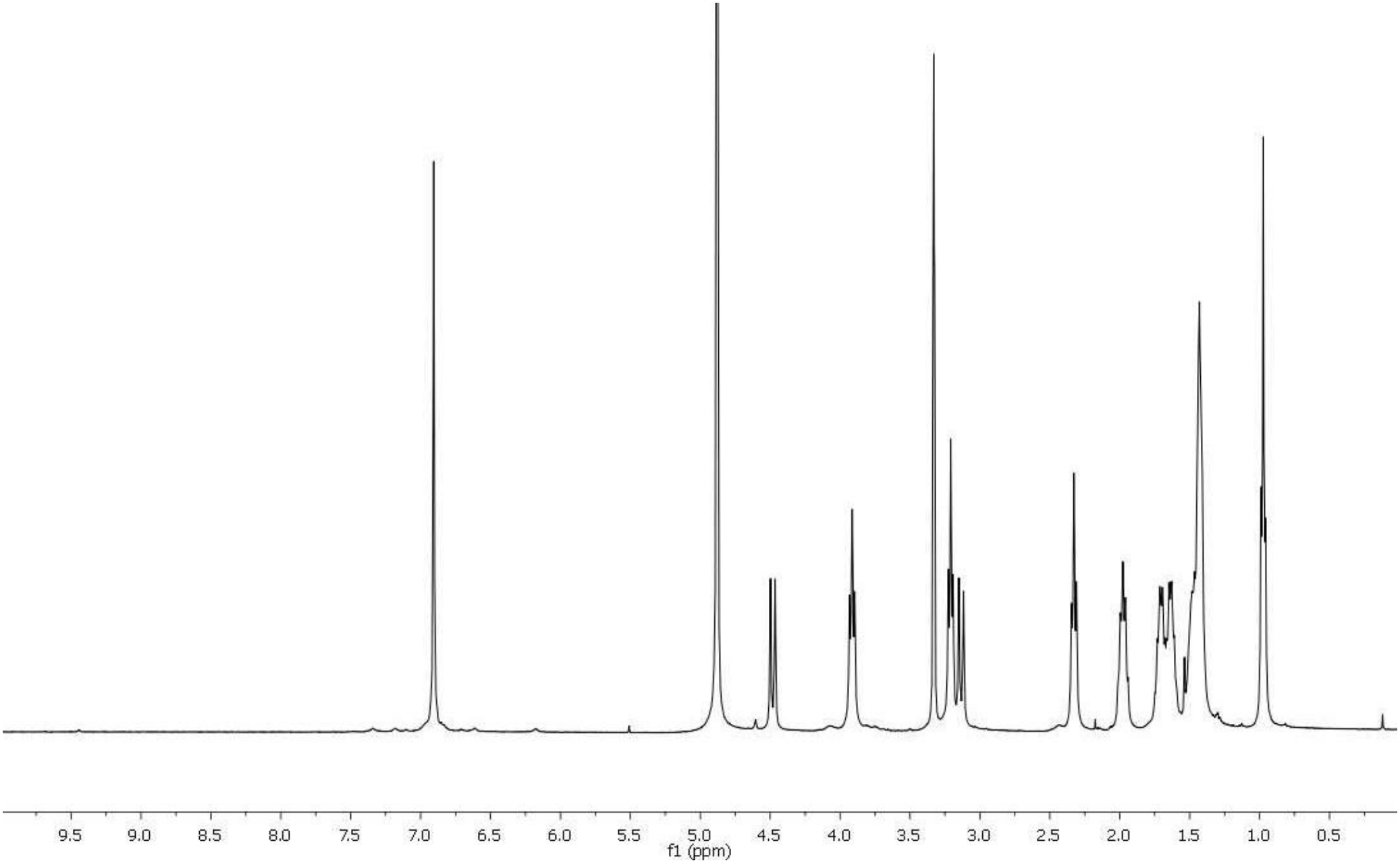

Fig. S10. ^{1}H NMR spectrum (CD_3OD, 400 MHz) of compound **2**.

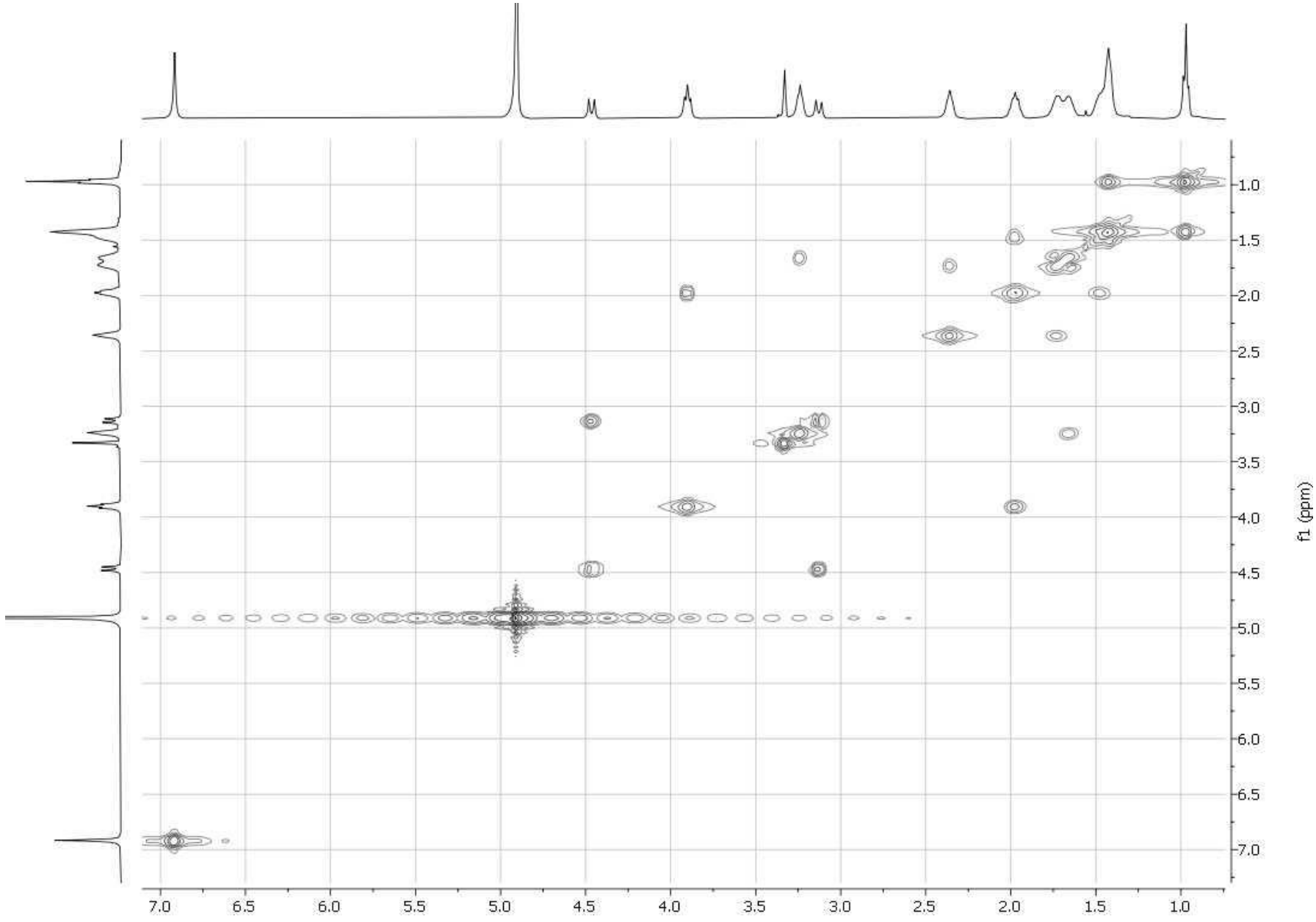

Fig. S11. ^{1}H COSY NMR map (400 MHz, CD_3OD) of compound **2**.

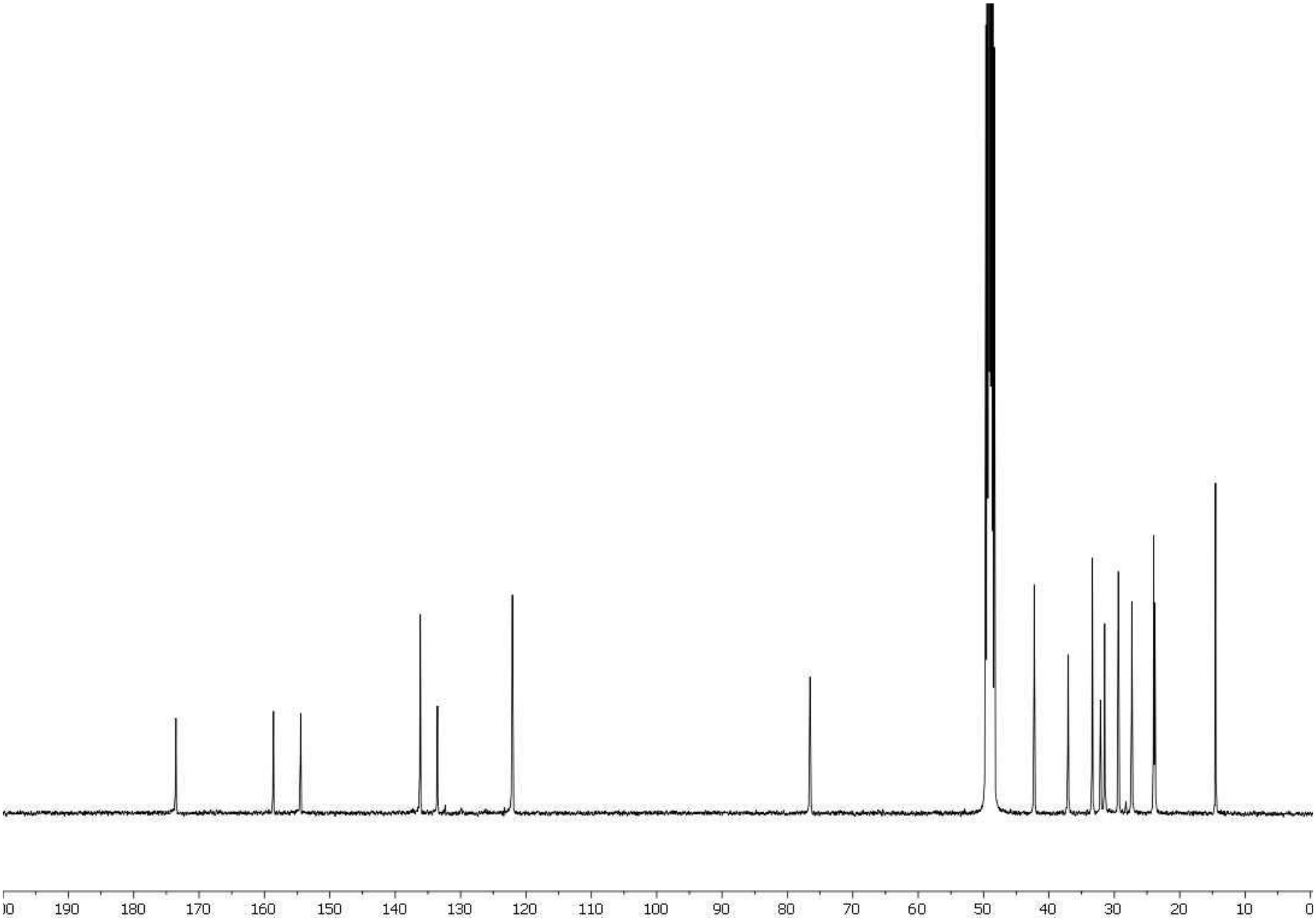

Fig. S12. ^{13}C NMR spectrum (CD_3OD, 100 MHz) of compound **2**.

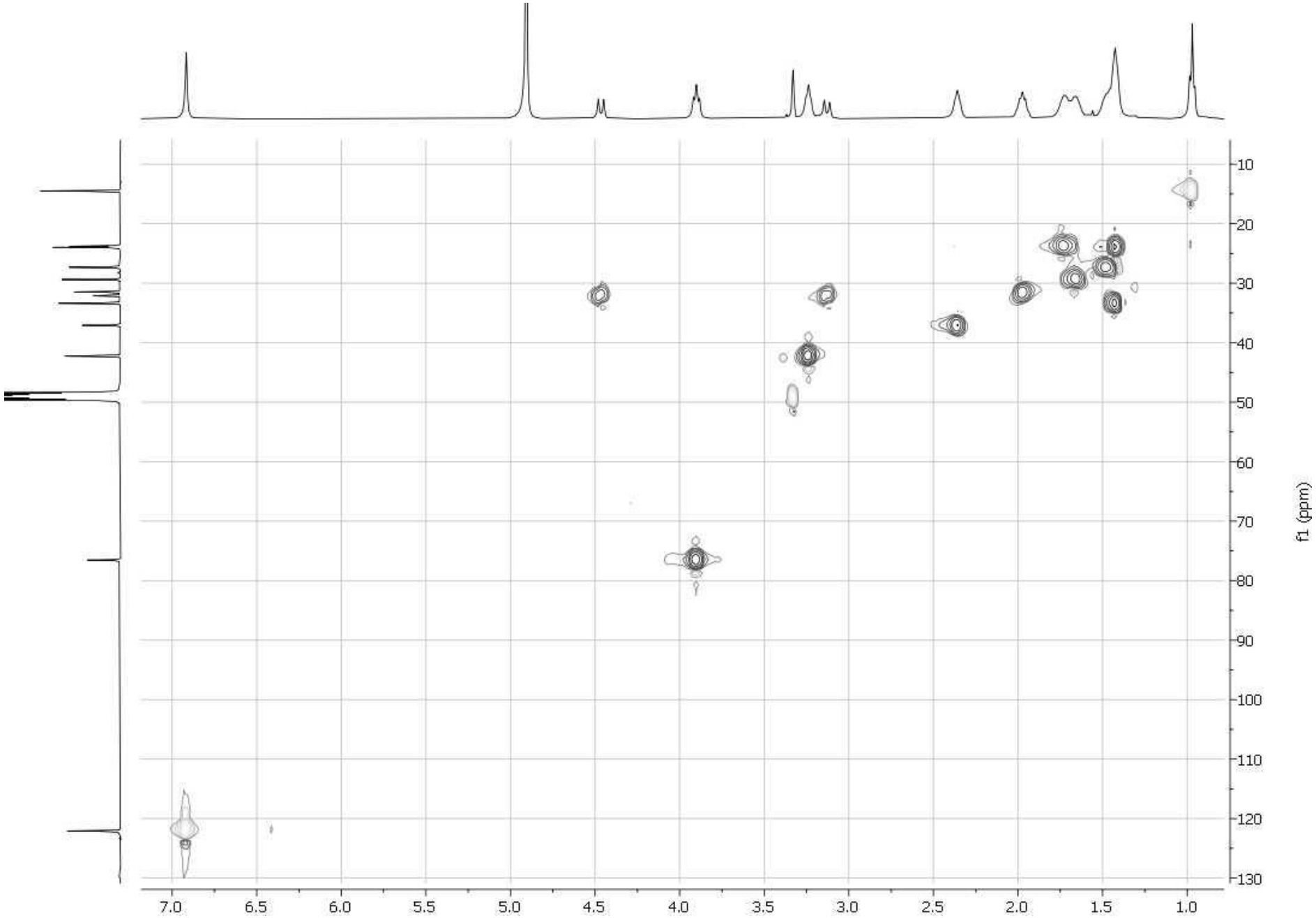

Fig. S13. ^{1}H-^{13}C HSQC NMR map in CD_3OD of compound **2**.

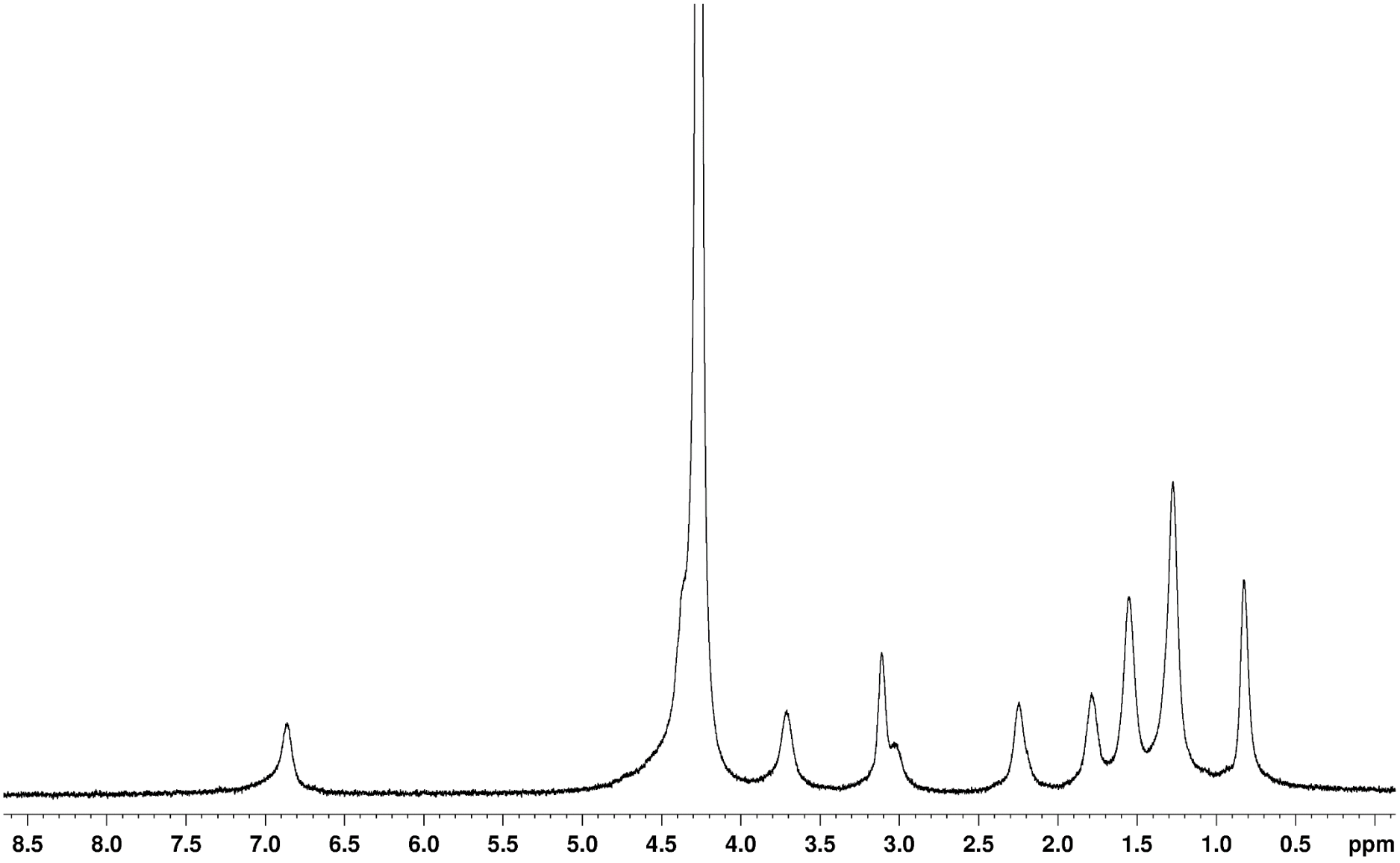

Fig. S14. ^{1}H NMR spectrum (D_2O, 300 MHz, 353 K) of compound **2** (1 mM).

	diameter (± SD) / nm	*PDI (± SD)*	*Z-potential (± SD) /mV*
Plain	146 (4)	0.04 (0.03)	3.5 (0.9)
Lipo-1	154 (5)	0.07 (0.01)	43 (5)
Lipo-2	158 (3)	0.07 (0.01)	49 (5)
Lipo-3	149 (3)	0.06 (0.02)	34 (3)

Table S1. Hydrodynamic diameter, polydispersity and Z-potential of liposomes

	Plain	***Lipo-1***	***Lipo-2***	***Lipo-3***	***MFI Lipo-n/MFI Plain***		
[Lipid] (µg/mL)	***MFI (SD)***				***Lipo-1***	***Lipo-2***	***Lipo-3***
100	1.0 (0.4)	45.5 (2.3)	55.4 (13.5)	42.2 (13.9)	45.5	55.4	42.2
300	2.3 (0.5)	276.9 (29.0)	328.8 (56.9)	94.2 (19.8)	120.4	143.0	41.0
500	3.8 (0.9)	280.9 (36.6)	435.9 (83.4)	147.5 (20.1)	73.9	114.7	38.8

Table S2. Mean fluorescence intensity (MFI) values of CHO-K1 cells upon treatment with liposomes (columns 2–5) and ratio between the signals from modified and plain liposomes (columns 6–8). The background signal from untreated cells was subtracted.

	Lipo-1	***Lipo-2***	***Lipo-3***	***MFI pgsA-745/ MFI CHO-K1 (%)***		
[Lipid] (µg/mL)	***MFI (±SD) pgsA-745***			***Lipo-1***	***Lipo-2***	***Lipo-3***
100	2.4 (0.4)	1.3 (0.1)	1.0 (0.3)	3.3	2.3	2.4
300	7.6 (1.9)	12.0 (0.4)	3.1 (0.9)	2.7	3.6	3.3
500	14.1 (0.6)	15.3 (0.7)	6.7 (2.3)	5.0	3.5	4.6

Table S3. Mean fluorescent intensity (MFI) of pgsA-745 cells upon treatment with liposomes (columns 2–4) and ratio between MFI values for pgsA-745 cells and CHO-K1 cells expressed as a percentage. The background signal from untreated cells was subtracted.

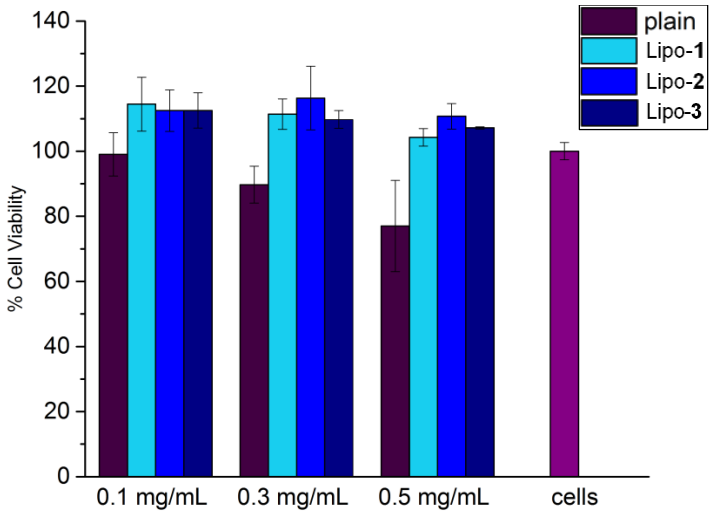

Figure S15. Cell viability in CHO-K1 cells treated with plain liposomes (purple), **Lipo-1** (light blue), **Lipo-2** (blue) and **Lipo-3** (dark blue) after 24 hours of incubation.