Supporting Information

Dual Targeting of Intracellular Pathogenic Bacteria with a Cleavable Conjugate of Kanamycin and an Antibacterial, Cell Penetrating Peptide

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1. Experimental Procedures

Synthesis and characterization of kanamycin derivatives

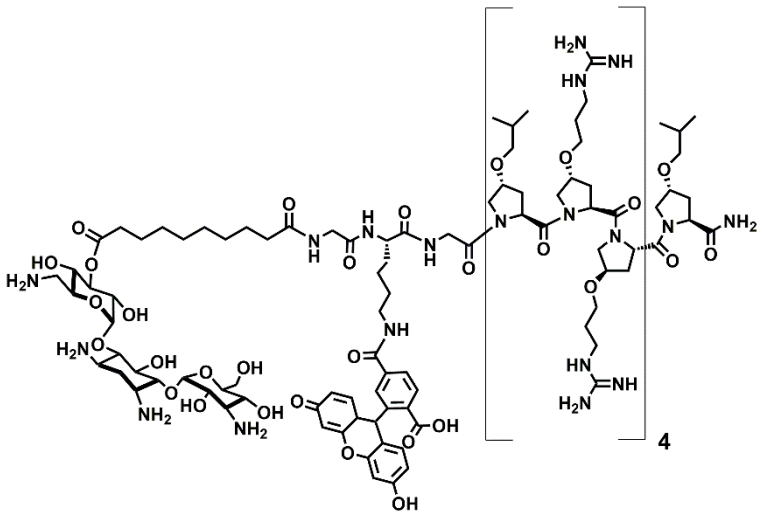
*General Synthesis of **2a-c***

To a solution of 4,4'-dithiodibutyric acid or sebacic acid (5.9 mmol) in 5 mL of dry dimethylformamide (DMF), at room temperature, was added O-(7-azabenzotriazole-1-yl)-N,N,N,N'-tetramethyluronium hexafluorophosphate (HATU) (5.2 mmol) and diisopropylethylamine (DIEA) (27 mmol). After 20 min at RT, **1a** or **1b** (2.3 mmol) was added. The mixture was stirred overnight. The solvent was removed *in vacuo,* and the resulting material was dissolved in dimethylsulfoxide (DMSO) and purified by reversed phase HPLC using a C_8 and C_{18} semi-preparative column (Phenomenix), for **2a-b** and **2c**, respectively. An eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 60 min gradient of 35-95% solvent A, a flow rate of 10mL/min and ELS detection for compounds **2a-b** (see Figure S1a for a representative HPLC trace) and a 60 min gradient of 45-95% solvent A, a flow rate of 12 mL/min and UV detection at 214 nm for compound **2c** (see Figure S1b for representative HPLC trace). Fractions consisting of the desired products were collected and lyophilized to obtain **2a-c** (approximately a 10% yield for each). The structures of **1a-b** and **2a-2c** were elucidated using COSY (Figures S15, S21, S27 and S32), TOCSY (Figures S16, S22, S28 and S33) and HMBC (Figures S17, S23, S29 and S34). Chemical shifts for the 2'- 4' protons for **2a**, for example, were found to shift by 0.242, 1.579 and 0.256 ppm, respectively, as compared to **1a** (Figures S2, S3, S12 and S18).

Synthesis and characterization of conjugates

General synthesis of resin-bound conjugates: **P14LRR** on resin was synthesized using Fmoc-based, solid phase strategy on the Rink amide ChemMatrix resin (45 μmol), as previously described. [1-2] This procedure was repeated until all amino acids were coupled to the resin. The resin was washed with dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), DCM and DMF (2 x 5 mL). Compounds **2a**, **b** or **c** (2 equiv., 0.12 mmol) in DMF (5 mL), HATU (2 equiv., 0.12 mmol) and diisopropylethylamine (DIEA) (4 equiv., 0.24 mmol) were added to the resin and the reaction flask was agitated for 24 h. The resin was washed with DMF, DCM, MeOH, and DCM (2 x 5 mL).

In order to make the fluorescein labeled analog of **P14KanC** (**P14KanC-Fl**), Mtt-protected lysine and glycine residues were added to the amino terminus of the peptide on resin. Once these residues were in place, the Mtt group was deprotected using 1.8% TFA in DCM. The (5 and 6)-carboxyfluorescein succinimidyl ester was coupled to the free amino terminus in presence of DIEA to provide the fluorescein labeled peptide on resin.



P14KanC-Fl

*Cleavage of **P14KanS, P14KanC, P14KanC-Fl** and **P14KanZ$_4$S** from resin and purification:* A trifluoroacetic acid (TFA) cleavage cocktail TFA:triisopropylsilane:water (95:2.5:2.5) (10 mL) was added to the resin and the mixture was agitated for 1h. The solution was filtered through glass wool into a 50 mL centrifuge tube. The resin was washed with DCM (4 x 4 mL), and the filtrate was collected into the same tube. The resulting solution was concentrated *in vacuo* to remove the TFA. The residue was dissolved in cold diethyl ether and placed in the freezer to precipitate the desired peptide conjugates. The precipitate was collected by centrifugation and washed with cold diethyl ether. The collected materials was purified to homogeneity by reversed phase HPLC using C_{18} semi-preparative column (Phenomenix). An eluent consisting of solvent A (acetonitrile and 0.1% TFA) and solvent B (water and 0.1% TFA) with a flow rate of 12mL/min, UV detection at 214 nm and 60 min solvent gradient of 15-60% for **P14KanS** and **P14KanC**, 25-55% for **P14KanC-Fl,** and 30-70% for **P14KanZ$_4$S**. Fractions consisting of the

desired products were collected and lyophilized to obtain **P14KanS, P14KanC, P14KanC-Fl** and **P14KanZ$_4$S.**

Characterization of the conjugates - Analytical HPLC

Purity was determined by analytical RP-HPLC using a C_{18} reverse phase analytical column (5 µm, 4 mm x 250 mm; Phenomenex Luna), a flow rate of 1.2 mL/min, UV detection at 214 nm, and a 30 min gradient of 15-55%, 25-55% and 30-70% solvent A (A: acetonitrile and 0.05% trifluoroacetic acid (TFA) ; B: water and 0.05% TFA) for **P14KanS, P14KanC-Fl** and **P14KanZ$_4$S,** respectively (Figure S6). For **P14KanC**, a C_8 reverse phase analytical column (5 µm, 4 mm x 250 mm; Phenomenex Luna) was used with a flow rate of 1.2 mL/min, UV detection at 214 nm, and a 30 min gradient of 15-50% solvent A (A: acetonitrile and 0.05% trifluoroacetic acid (TFA) ; B: water and 0.05% TFA).

Characterization of the conjugates - Mass spectrometry

Peptides were further characterized using matrix associated laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry (Voyager DE, Applied Biosystems) (Figure S7).

A) MALDI for **P14KanS** Expected Mass 3302.87, Observed Mass 3303.46

B) MALDI for **P14KanZ$_4$S** Expected Mass 3839.02, Observed Mass 3844.22

C) MALDI for **P14KanC** Expected Mass 3266.96, Observed Mass 3268.59

D) MALDI for **P14KanC-Fl** Expected Mass 3814.5, Observed Mass 3810.17

Method for reduction of the conjugate

To a solution of previously degassed phosphate buffered saline (PBS), pH 7.4 and dimethylformamide (DMF), (500 µL PBS and 470 µL DMF) was added 10 µL of 10 mM **P14KanZ$_4$S** (to give a final concentration of 100 µM) and 10 µL of 100 mM dithiothreitol (DTT) in PBS, pH 7.4 (to give a final concentration of 1 mM). 10 µL of 50 mM N-α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Alfa Aesar A18181) (to give final concentration 500 µM) was also added as an internal standard. The mixture was incubated at 37^{o}C. At different time points, aliquots from the reaction mixture were removed and immediately injected on an HPLC equipped with a C_{18} reverse phase analytical column (5 µm, 4 mm

x 250 mm; Phenomenex Luna) with an eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 5-95% solvent A, a flow rate of 1.2mL/min and UV detection at 214nm. The peak area corresponding to $KanZ_4$ released was quantified and half-life were obtained by fitting the time-dependent data using GraphPad Prism (Figure S8). Each experiment was performed in duplicate.

Method for stability studies using porcine liver esterase

To a solution of PBS, pH 7.4 (690 µL PBS) was added, 10 µL of 10 mM **P14KanS** (to give a final concentration of 100 µM) and 300 µL of porcine liver esterase in PBS, pH 7.4 (to give a final concentration of 100 unit/ml). The mixture was incubated at 37^{o}C. At different time points, 50 µL aliquots from the reaction mixture was removed and 50 µL of cold acetonitrile was added to deactivate the proteins. The solution was then vortexed for 20 sec and centrifuged for 10 min at 2000 rpm. Next, 50 µL of the supernatant was removed, added to 50 µL of 100 mM N-α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Alfa Aesar A18181) (to give final concentration 500 µM) as an internal standard, and was immediately injected on an HPLC equipped with a C_{18} reverse phase analytical column (5 µm, 4 mm x 250 mm; Phenomenex Luna) with an eluent consisting of solvent A (acetonitrile and 0.1% trifluoroacetic acid (TFA)) and solvent B (water and 0.1% TFA) with a 30 min gradient of 15-65% solvent A, a flow rate of 1.2 mL/min and UV detection at 214nm. The appearance of the peak corresponding to P14LRR modified with 4,4'-dithiodibutyric acid was monitored and only ~15% release was observed after 120 hrs.

Method for *in vitro* antimicrobial activity assessment

a)* Against *Escherichia coli* and *Staphylococcus aureus

Escherichia coli (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923) was grown to the mid-exponential phase in Tryptic Soy Broth (TSB) at 37 °C with shaking. An aliquot of the bacterial suspension was centrifuged for 5 min at 3000 rpm, the supernatant aspirated and the pellet re-suspended

in Muller Hinton Broth (MHB) to a final optical density of 0.001 as measured by absorbance at 600nm (OD_{600}). Next 90 µL of this culture was added in a sterile 96-well plate (Cellstar 655180) and then supplemented with 10 µL of sterile water or two-fold serial dilutions of the drugs in water. Melittin was used as positive control for both bacterial strains. The plate was then incubated for 6 h at 37 °C. The OD_{590} was determined using a microplate reader (TECAN SpectraFluor Plus). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug at which no growth was observed. Data was obtained in duplicates from at least two independent experiments.

b) Against pathogenic bacteria

The broth microdilution technique was used to determine the minimum inhibitory concentrations (MIC) of drugs according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) .[3] *Salmonella enteritidis*, *Shigella flexneri*, *Brucella abortus*, or *Mycobacterium smegmatis* were diluted to a bacterial inoculum of 5×10^5 colony forming unit (CFU/ml) in Mueller-Hinton broth (MHB) (*S. enteritidis*, and *S. flexneri*), Brain Heart Infusion broth (BHI) (*B. abortus)*, and Tryptic Soy Broth (TSB) (*M. smegmatis*). Drugs were added to polystyrene 96 well plates (CytoOne, CC7672-7596) at desired concentrations and plates were incubated at 37^{o}C for 18 h (*S. flexneri*), 24 h (*S. enteritidis*) or 48 h (*B. abortus* and *M. smegmatis)*. MIC was defined as the lowest concentration of drug which inhibited the visible growth of bacteria.

The resazurin microtiter assay (REMA) was used to determine the minimum inhibitory concentrations (MIC) of drugs against *Mycobacterium tuberculosis* H37Ra, as described previously.[4] Briefly, 100 µl of Middlebrook 7H9 broth supplemented with Oleic Albumin Dextrose Catalase Growth Supplement (OADC) enrichment and glycerol was added to wells of 96-well plate, and serial two fold dilutions of each drug were prepared directly in the plate. One hundred microliters of inoculum was added to each well. A growth control and a sterile control were also included for each isolate. The plate was covered, sealed in a plastic bag, and incubated at 37°C under a normal atmosphere. After 7 days of incubation, 30 µl of resazurin solution was added to each well, and the plate was reincubated for 24 hr. A change in color

from blue to pink indicated bacterial growth, and the MIC was determined as the minimum concentration of drug that prevented color change.

c) **Determination of minimum inhibitory concentration (MIC) values against biofilm-forming clinical isolates of *S. aureus* and *S. epidermidis*.**

Biofilm-forming clinical isolates of *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 35984) were grown overnight in TSB. After incubation, cultures were diluted to 5×10^5 colony forming unit (CFU/ml) in MHB. The minimum inhibitory concentrations of compounds were determined via the broth microdilution technique according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Compounds were added to polystyrene 96-well plates at desired concentrations and subsequently serially diluted. The MIC was defined as the lowest concentration of peptide or antibiotic that inhibited bacterial growth visually.

Method for cytotoxicity assessment

Cellular toxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Briefly, 1.5 x 10^4 J774A.1 cells suspended in 200 µL, of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1% L-glutamine and 1% penicillin/ streptomycin were seeded in 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere. The cells were cultured for 24 h (60% confluency) before the assays. At the end of the pre-incubation period, the media was discarded and the cells were washed twice with 100 µL PBS. The J774A.1 cells were further incubated with 100 µL of dilutions ranging from 2.5-30 µM of drugs in DMEM for 9h. Following incubation, 20 µL of 5 mg/mL MTT (Sigma M5655) in PBS was added per well and the plate was returned to the incubator for an additional 1.5 h. Next, the MTT solution was aspirated and 100 µL of dimethyl sulfoxide (DMSO) per well was added to dissolve formazan crystals formed. For each experiment, a negative control of untreated cells was also analyzed. All samples were

run in duplicate, and each experiment was duplicated. The intensity of color was quantified at 590 nm using a 96-well ELISA plate reader (SoftMax ProInc., USA). Results were expressed as the percentage mean absorbance of treated cells in respect to incubation with control (Figure S10).

Method for rescue of J774A.1 cells infected with pathogenic bacteria

Macrophage-like cell line (J774A.1) was seeded at a density of 1.5 x 10^4 per well in a tissue culture 96-well plate (CytoOne, CC7682-7596) in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% Fetal Bovine Serum (FBS), and incubated at 37°C in a 5% CO_2 atmosphere for 24 h (to 95 % confluence). Following incubation, the cells were washed once with DMEM media. Then the cells were infected with *Salmonella enteritidis*, *Shigella flexneri* or B*rucella abortus* (at multiplicity of infection 1:10 for *S. enteritidis*, and 1:100 for *S. flexneri* or B. *abortus*) in DMEM and 10% FBS for 1 h. While for *Mycobacterium tuberculosis* cells were infected (at multiplicity of infection 1:20) in DMEM and 10% FBS for 4 hr, and for *Mycobacterium smegmatis* cells were infected (at multiplicity of infection 1:50) in DMEM and 10% FBS for 1 h. After infection the wells were washed three times with 200 µL media with gentamicin (final concentration 50 µg/mL) and further incubated for 30 minutes (or 1 h for *M. tuberculosis)* with gentamicin to kill extracellular bacteria. Drugs were diluted in DMEM and 10 FBS% to the desired concentrations. Subsequently, after washing, wells were treated with 100 µL of DMEM and 10% FBS containing drugs for 9 h (*S. enteritidis* and *S. flexneri)*, 12 h (*M. smegmatis)*, 21 h (*B. abortus)* and 7 days (*M. tuberculosis)*. Ciprofloxacin (5 µM) was used as a positive control for *S. enteritidis* and B. *abortus,* and isoniazid (0.12 µM) for *M. tuberculosis and M. smegmatis*. Medium alone used as a negative control. After incubation, the media was aspirated and the wells were washed twice with PBS. Then 100 µL of PBS with 0.01% triton X was added to each well to lyse the macrophage cells. Subsequently, bacteria were diluted 10 fold serially in PBS and plated on Tryptic Soy Agar (TSA) plates (*M. tuberculosis* was plated on Middlebrook 7H10 Agar plates supplemented with OADC Enrichment). Plates were incubated at 37^{o}C for 20 h (*S. enteritidis* and *S. flexneri*), 48 h (B.*abortus*), 72 h (*M. smegmatis)* and 4-5 weeks (*M. tuberculosis)*. After incubation, bacteria were counted and analyzed by Graph pad

software. Each drug treatment was done with three biological replicates. Experiments were repeated independently twice except *M. tuberculosis* was done once with three biological replicates.

Antimicrobial efficacy of treatment *in vivo* using a *Salmonella*-infected *C. elegans* animal model.

The infection and treatment of *Caenorhabditis elegans* were performed as reported previously. [5] A pathogen sensitive strain of *C. elegans* {glp-4(bn2) I; sek-1(km4)} was used in this study. Bacterial lawns used for the *C. elegans* infection assays were prepared by spreading 200 µL of the overnight culture of *Salmonella enteritidis* on the modified Nematode Growth Media (NGM) (0.35% peptone) agar plates. The plates were incubated at 37°C overnight before being seeded with young adult hermaphrodite nematodes, grown at 25°C, from a synchronized culture. The infections were performed at 25°C for 24 h. Subsequently, the worms were collected and washed with PBS to remove extracellular bacteria and diluted to reach 1 worm per 1 µL of PBS. Approximately 100 worms (100 µL PBS) were transferred to 1.5 mL microcentrifuge tubes. Drugs at concentration equal to 50 µM (except ciprofloxacin 10 µM) were added to tubes in triplicates. Control negative tubes contain only PBS. After 24 h, worms were checked for survival under a microscope to assess the toxicity of drugs. The tubes were centrifuged and the supernatant was removed. Worms were washed twice with 1 ml of PBS then 200 µg of autoclaved silica carbide were added to each tube, and the worms were vortexed for 1 minute at high speed. One hundred microliter from each tube was diluted 10 fold serially in PBS. Bacteria were plated in Tryptic Soy Agar (TSA) plates containing spectinomycin (18 µg/mL) to allow for selective growth of *S. enteritidis* over *Escherichia coli.* Plates were incubated for 16 h at 37 °C and colonies were counted. Statistical analysis was analyzed by two tailed student t test. ($P \leq 0.05$ was considered significant).

Method for *in vivo* toxicity assessment in *C. elegans* animal model

Bacterial lawns used for *C. elegans* infection assays were prepared by spreading 200 ul of the overnight culture of biofilm strains of *S. aureus or S. epidermidis* on modified NGM (0.35% peptone) agar plates. The plates were incubated at 37°C overnight before being seeded with young adult hermaphrodite

nematodes, grown at 25°C, from a synchronized culture. The infections were performed at 25°C for 12 hours. After infection of synchronized worms, around 25-30 adult worms were transferred to each well in 96 well plate, Drugs at concentration equal to 50 μM were added in triplicates. Control negative contains only PBS. Worms were checked for survival under microscope at different time points. [6] Living nematodes maintain a sinusoidal shape, whereas dead nematodes appear as straight, rigid rods as the corpse becomes filled with bacteria (Figure S11).

Method for leakage of β-Galactosidase from *E. coli* treated with P14KanC and P14KanS

In a sterile 250 mL erlenmeyer flask *E. coli* (ATCC 25922) was grown to mid-exponential phase (OD_{590}≈0.6) in TSB (~ 50 mL) at 37 °C with shaking. β-Galactosidase expression was induced by the addition of freshly prepared isopropyl-β-D-thiogalactopyranoside (IPTG) (Gold Biotechnology I2481C5) in PBS (1 mM final concentration). A 4 mL aliquot of the bacterial suspension was centrifuged, washed twice with fresh TSB and plated into a sterile 96-well plate (90 μL). Next, 10 μL aliquots of **P14KanS or P14KanC** in sterile water were added to give final concentrations of 10 μM. Bacteria treated with sterile water and melittin (40 μM final concentration) served as controls. The plate was then incubated for 1 h at 37 °C. At the end of the incubation period, the plate was centrifuged at 3000 rpm at 4 °C for 10 min. 80 μL of the supernatant from each well was carefully transferred to a new sterile 96-well plate. Next, 20 μL of freshly prepared 2-Nitrophenyl- β-D-galactopyranoside (ONPG) (Sigma 73660) in PBS was added to each well (0.8 mg/ml final concentration). The β- Galactosidase activity was monitored at OD_{405} every five minutes for a period of 1 h using a micro-plate reader (Figure S9). Data was obtained in duplicates from at least two independent experiments.

Flow Cytometry Analysis of P14KanC-Fl

J774A.1 murine macrophage-like cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (Cambrex bioscience, WalkersVille, Inc.), 5 mM L-glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (Cellgro Mediatech). Cells were grown at

37 °C in 5% carbon dioxide atmosphere. 125,000 cells were counted and collected in round bottom tubes (BD Falcon). Next 400 μL of FBS supplemented DMEM containing the peptides at 10 μM concentration were added to the tubes and incubated for 1 h at 37 °C. Media containing no peptide served as control. The samples were centrifuged at 1500 rpm at 4 °C, the media was aspirated and re-suspended in 400 μL fresh DMEM. Finally, the cells were centrifuged, media aspirated and re-suspended in 400 μL PBS. Samples were then analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser. Emissions for fluorescein labeled peptides were collected in the FL1. Ten thousand cells were counted for each data point.

Confocal Microscopy: Uptake of P14KanC-Fl in J774A.1 Cells

Murine J774A.1 macrophage-like cells were seeded into 4-well LabTek chambered slides at a density of 200,000 cells/well in 1 mL of DMEM media and incubated for 18 h at 37 °C. The media was aspirated, cells washed with 400 μL PBS and 10 μM **P14KanC-Fl** and **P14LRR** in 400 μL DMEM was added. The cells were incubated for 1 h at 37 °C. Excess peptide was then aspirated and the cells washed with 400 μL PBS thrice. Next, 400 μL of DMEM supplemented with 100 nm MitoTracker (Invitrogen 7512) was added. The cells were incubated for 30 min at 37 °C. The excess dye was aspirated, the cells washed with 400 μL PBS twice and 400 μL DMEM added. Imaging was performed using a Nikon A1R Multiphoton inverted Confocal Microscope using a 60X oil objective. 488 nm and 561 nm lines for fluorescein and MitoTracker excitation were employed, respectively.

NMR Spectroscopy

NMR spectra were obtained using a Bruker AV-III-800 spectrometer operating at 800.13 MHz for ^{1}H and 201.21 MHz for ^{13}C and equipped with a 5mm TXI Z-gradient probe. Bruker TopSpin 2.1 software was used for both data acquisition and processing. Samples were made up in conventional 5mm NMR tubes and were run at either ca. 300 or 323K (see text). Samples which were run at 323K were also run at 300K

after being heated to confirm that no irreversible changes took place. Spectra were referenced to the chemical shift of the residual DMSO-d6 solvent peak (2.49 ppm for ^{1}H, 39.5 ppm for ^{13}C)

1D ^{1}H spectra were obtained using the following parameters: pulse program, zg30; sweep width, ca. 15 ppm; acquisition time, ca. 2.7 seconds; relaxation delay, 2 seconds; digital resolution of final spectrum, ca. 0.4 Hz.; number of scans, 8.

1D ^{13}C and ^{13}C DEPT-135 spectra were obtained using the following parameters: pulse program, zgpg30 and dept (respectively); sweep width, ca. 250 ppm; acquisition time, ca. 0.7 seconds; relaxation delay, 2 seconds; digital resolution of final spectrum, ca. 1.5 Hz.; number of scans, 1k – 24k, (depending on experiment and sample concentration), $1J_{(C-H)}$ optimization (DEPT), 135 – 140 Hz.

2D ^{1}H COSY: pulse program, cosygpqf; number of scans per increment, 2; number of increments, 128; data acquisition mode, QF (magnitude mode).

2D ^{1}H TOCSY: pulse program, mlevph; mixing time, 80 milliseconds; number of scans per increment, 4; number of increments, 256; data acquisition mode, States-TPPI.

2D ^{1}H DQF-COSY (not obtained for all molecules): pulse program, cosydfetgp.2; number of scans per increment, 8; number of increments, 512; data acquisition mode, echo-antiecho.

2D ^{1}H-^{13}C HSQC: pulse program, hsqcetgpsi2; $1J_{(C-H)}$ optimization, 135 Hz.; number of scans per increment, 4 or 8; number of increments, 256; data acquisition mode, echo-antiecho.

2D ^{1}H-^{13}C HMBC: pulse program, hmbcgplpndqf; $1J_{(C-H)}$ optimization, 135 Hz.; $nJ_{(LR)}$ optimization, 6 Hz.; number of scans per increment, 8; number of increments, 256; data acquisition mode, QF.

For all 2D experiments the sweep widths were optimized according to the locations of the relevant peaks as observed in the corresponding 1D spectra.

2. Supplementary Figures and Tables

Table S1: Antibacterial activity of **P14KanC-Fl**

Bacteria	MIC [µM]
	P14KanC-Fl
Escherichia coli	4
Staphylococcus aureus	32
Salmonella enteritidis	8
Mycobacterium smegmatis	4

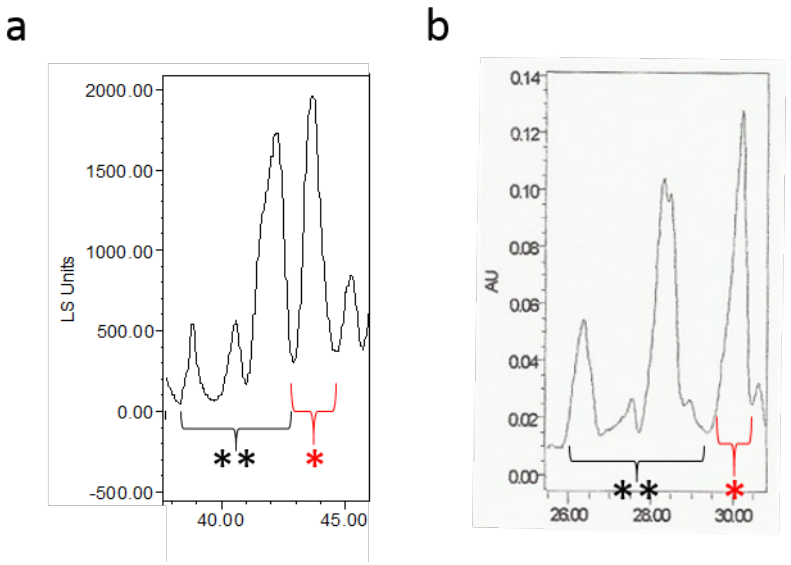


Figure S1: Representative HPLC traces for purification of compounds: a) **2a** and b) **2c** *Peak corresponding to single isomer which was collected and lyophilized to give pure **2a** and **2c** ** Peaks corresponding to mixture of isomers.

	^{13}C	^{1}H	
1	49.98	3.347	
2	34.69B	1.388	1.793
3	49.04	3.299	
4	83.98	3.284	
5	74.96	3.435	
6	80.29	3.400	
1'	101.11	4.892	
2'	72.12	3.258	
3'	72.65	3.368	
4'	70.46	3.058	
5'	70.08	3.576	
6'	41.40	3.244	
1''	97.79	4.905	
2''	70.30	3.217	
3''	55.88	3.468	
4''	67.36	3.210	
5''	72.88	3.805	
6''	60.25	3.476	3.509
A	155.36		
B	154.90		
C	156.11		
D	156.34		
N1		6.873	
N3		6.551	
N'		6.313	
N''		6.478	
OH2'		5.32	
OH4'		4.85	
OH4''		4.67	
OH6''		4.17	

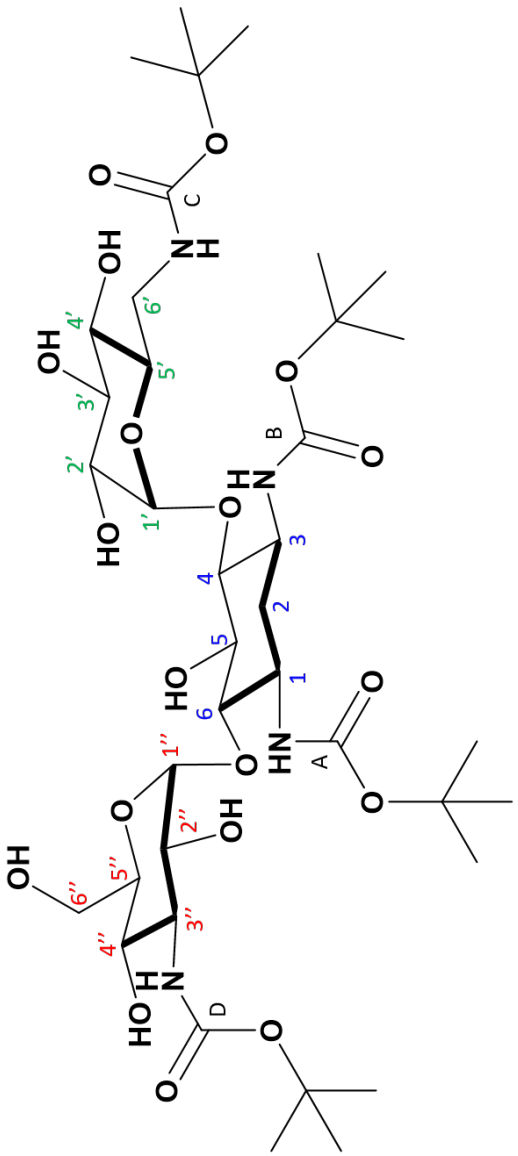
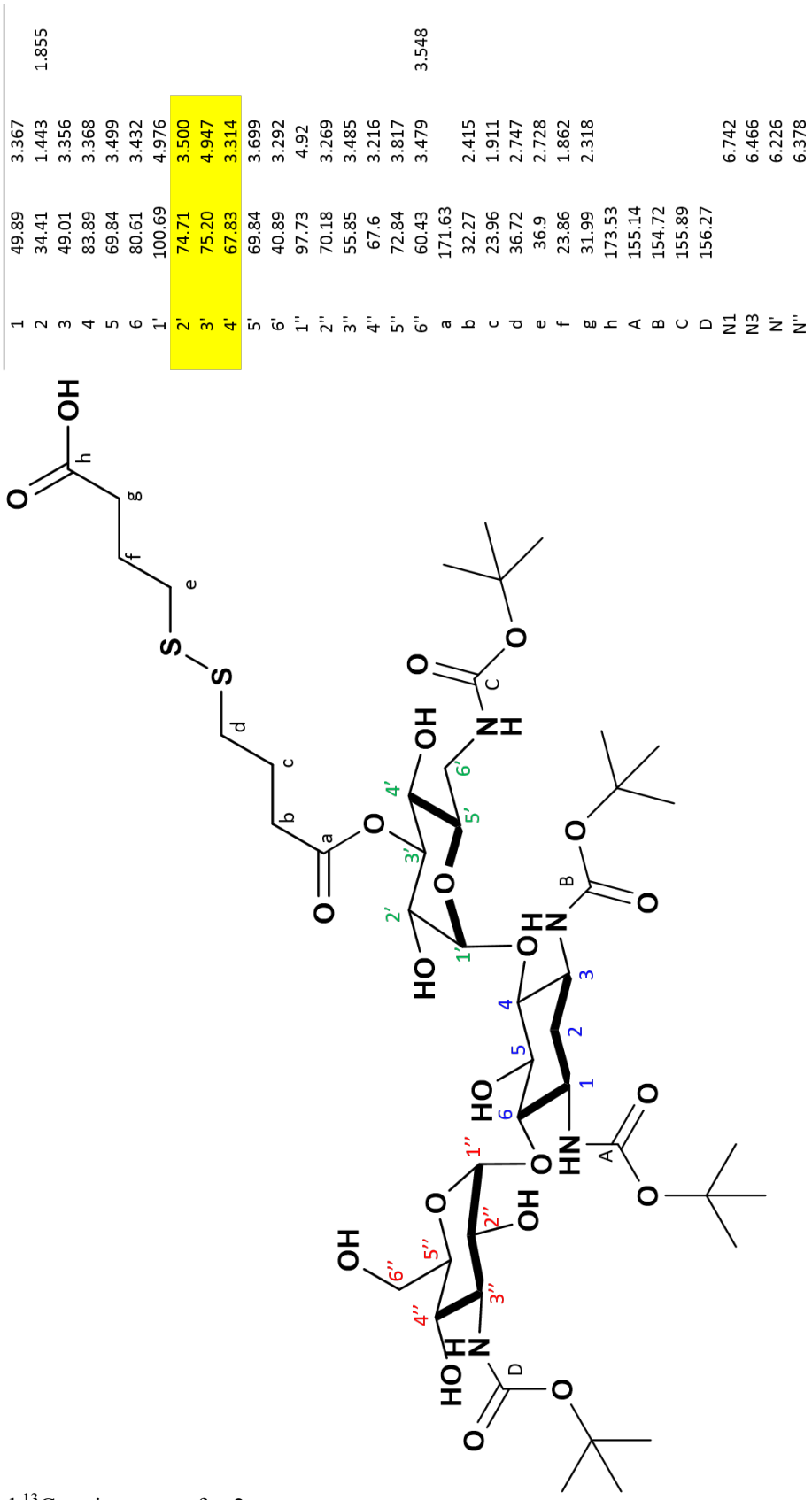


Figure S2: ^{1}H and ^{13}C assignments for **1a**



	^{13}C	^{1}H	
1	49.89	3.367	
2	34.41	1.443	1.855
3	49.01	3.356	
4	83.89	3.368	
5	69.84	3.499	
6	80.61	3.432	
1'	100.69	4.976	
2'	74.71	3.500	
3'	75.20	4.947	
4'	67.83	3.314	
5'	69.84	3.699	
6'	40.89	3.292	
1''	97.73	4.92	
2''	70.18	3.269	
3''	55.85	3.485	
4''	67.6	3.216	
5''	72.84	3.817	
6''	60.43	3.479	3.548
a	171.63		
b	32.27	2.415	
c	23.96	1.911	
d	36.72	2.747	
e	36.9	2.728	
f	23.86	1.862	
g	31.99	2.318	
h	173.53		
A	155.14		
B	154.72		
C	155.89		
D	156.27		
N1		6.742	
N3		6.466	
N'		6.226	
N''		6.378	

Figure S3: ^{1}H and ^{13}C assignments for **2a**

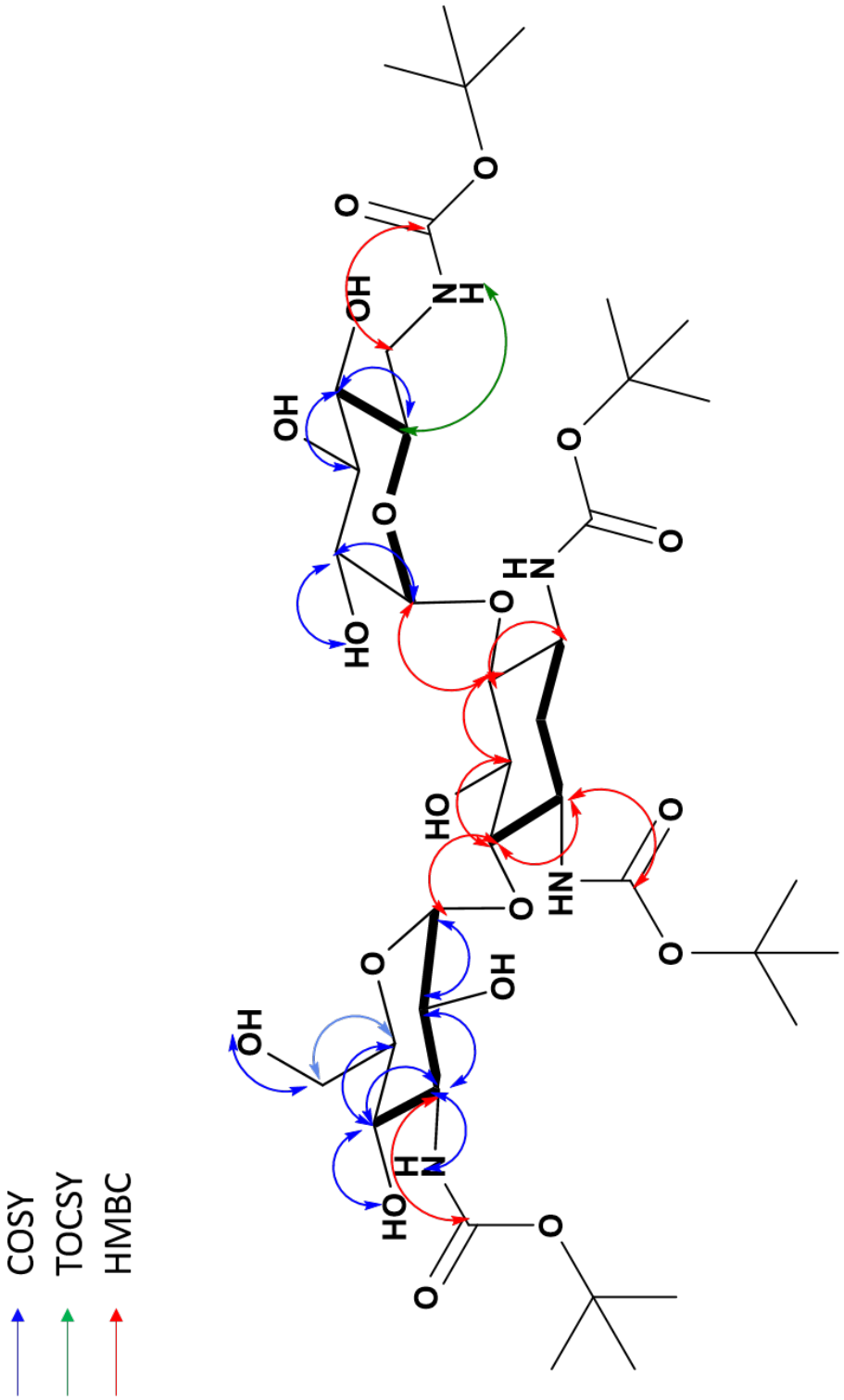


Figure S4: 2D correlation based on COSY, TOCSY and HMBC data for **1a**

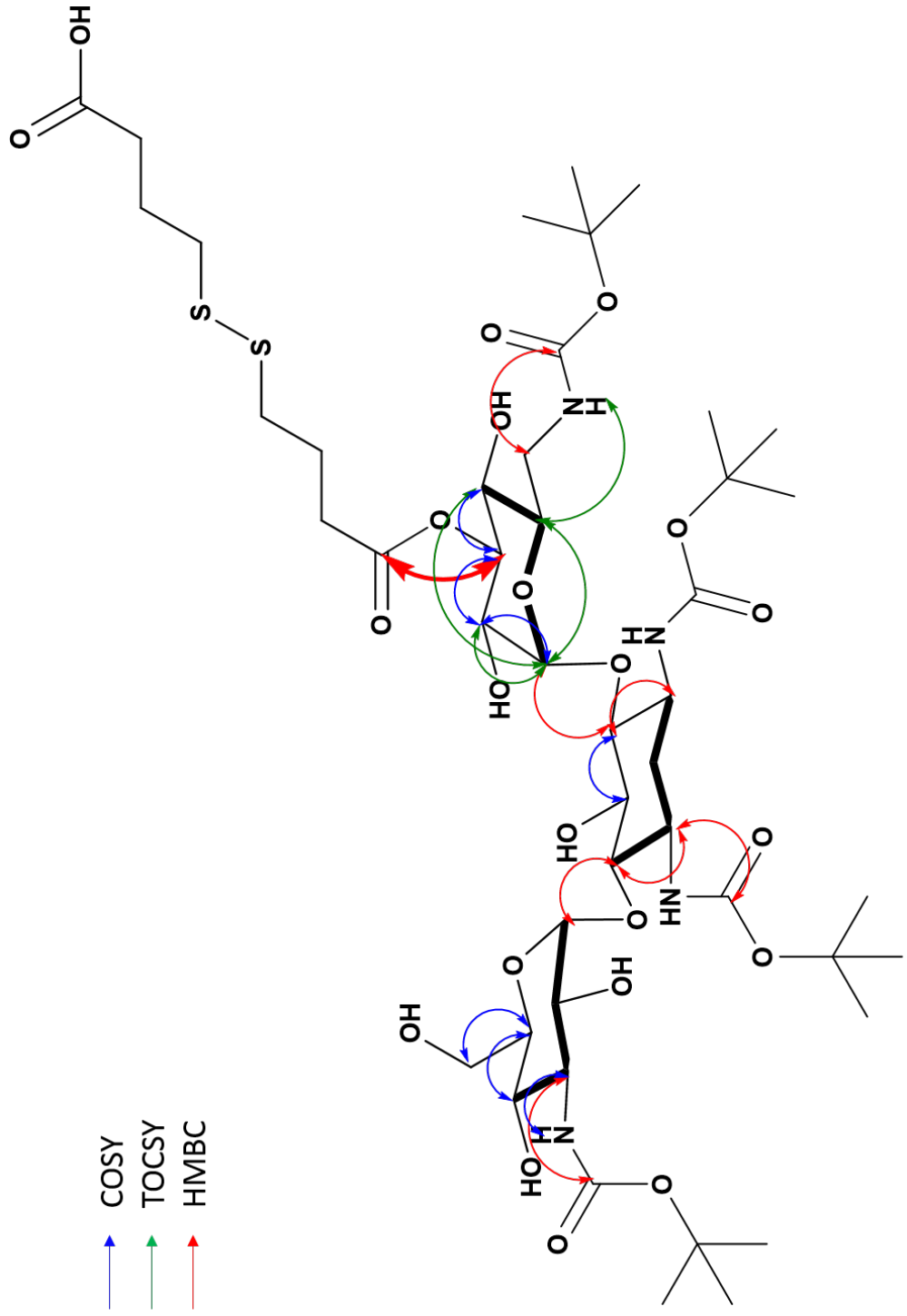
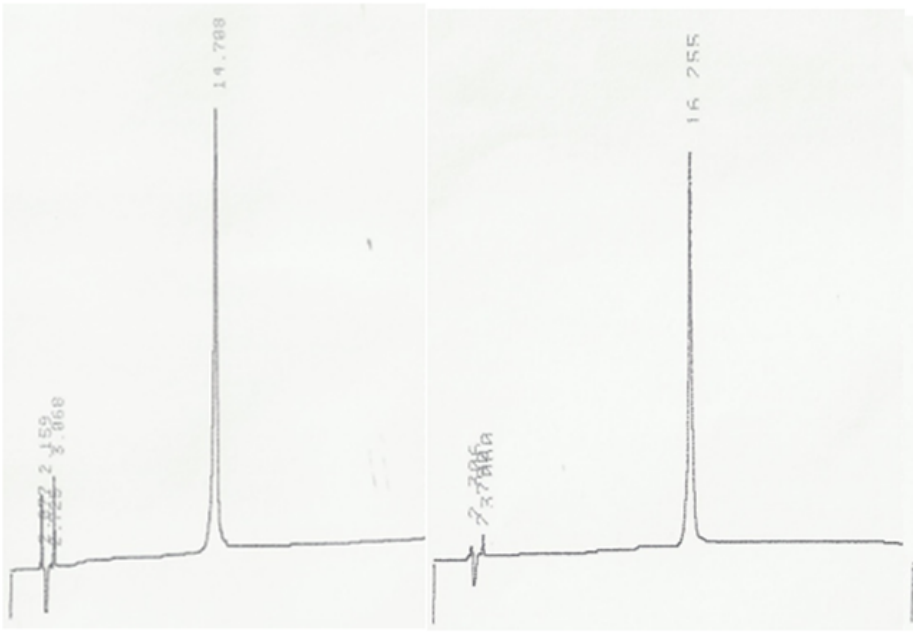


Figure S5: 2D correlation based on COSY, TOCSY and HMBC data for **2a**

A B



C D

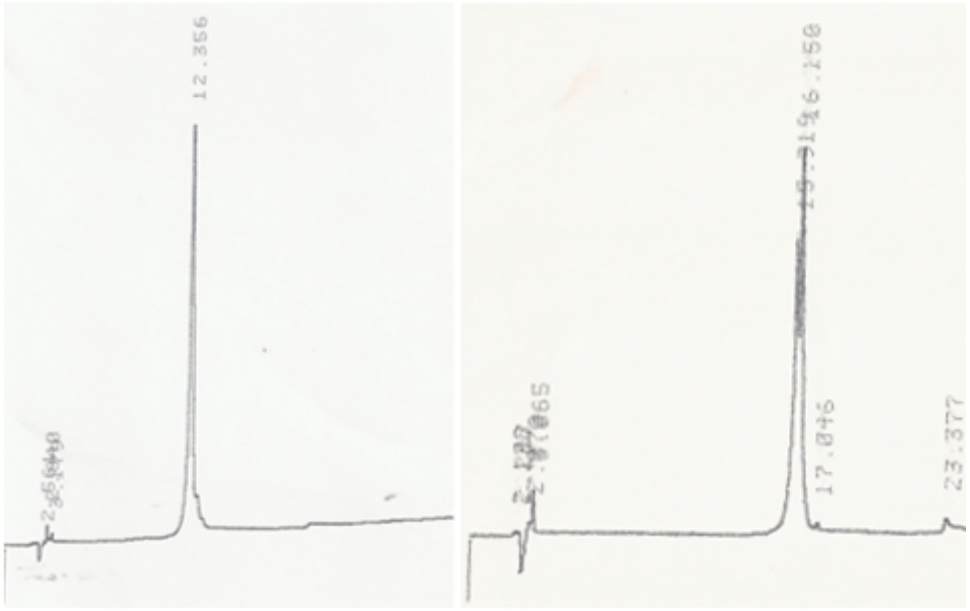
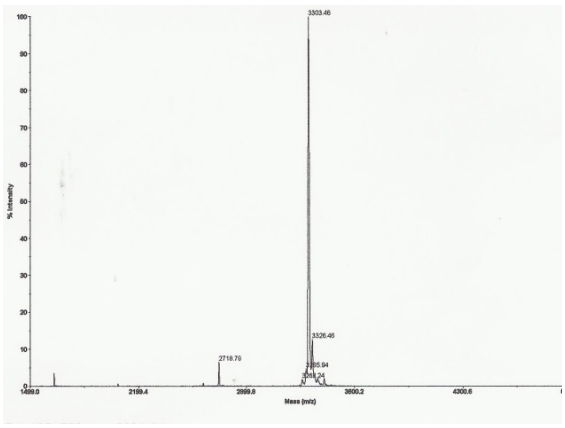
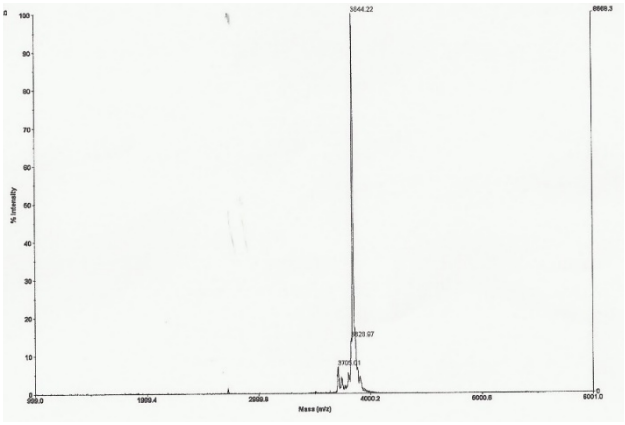


Figure S6: Analytical HPLC traces for A) **P14KanS**, B) **P14KanZ$_4$S**, C) **P14KanC** D) **P14KanC-Fl** at 100 μM concentration.

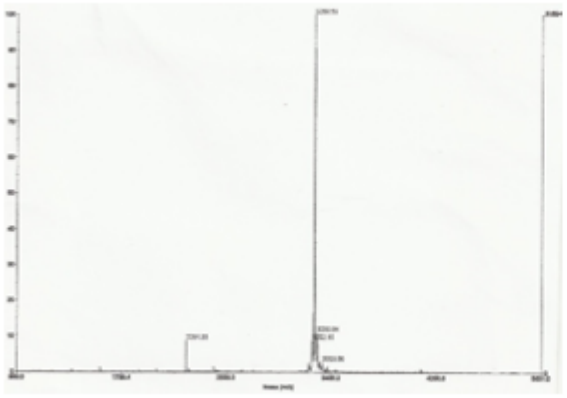
A



B



C



D

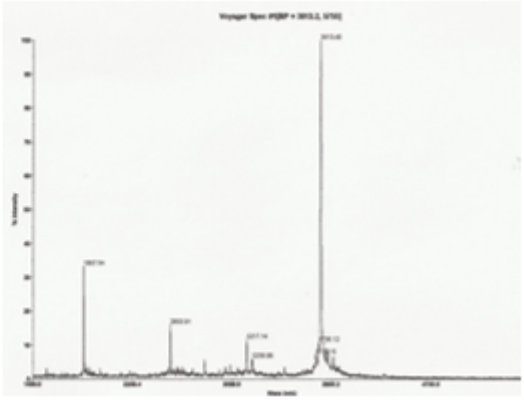


Figure S7: MALDI spectra of A) **P14KanS**, B) **P14KanZ$_4$S**, C) **P14KanC**, D) **P14KanC-Fl**.

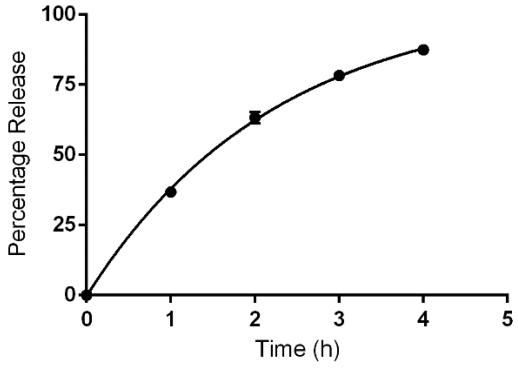


Figure S8: Percent release of KanZ$_4$ upon reduction of **P14KanZ$_4$S**.

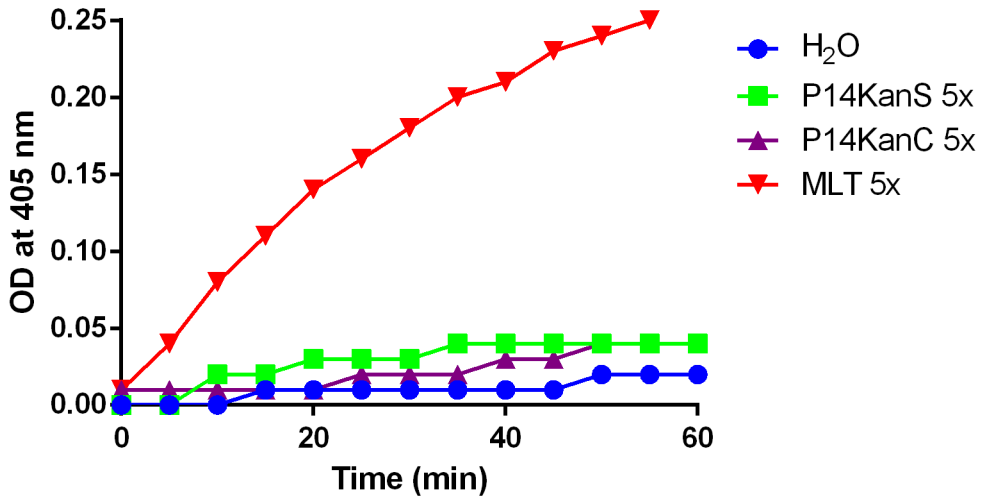


Figure S9: Peptide-induced leakage of β-galactosidase from E. coli treated with melittin, control and P14KanS and P14KanC. Absorbance due to ONPG cleavage was monitored at 405 nm.

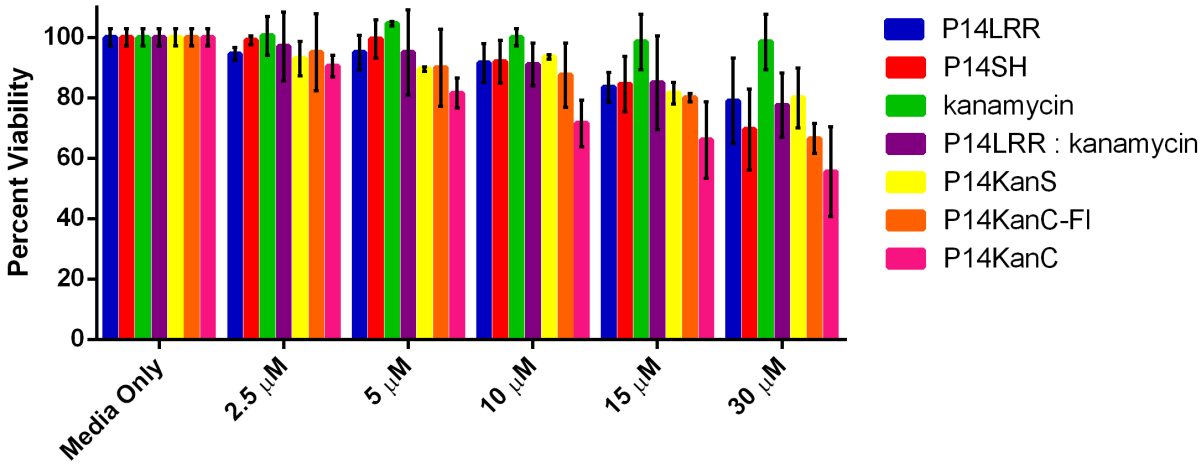


Figure S10: Cell viability after incubating J774A.1 cells with **P14LRR**, **P14SH,** kanamycin, **P14LRR** : kanamycin (1:1 mixture) and **P14KanS, P14KanC, P14KanC-Fl** at 2.5 - 30 μM. Buffer was used as a control.

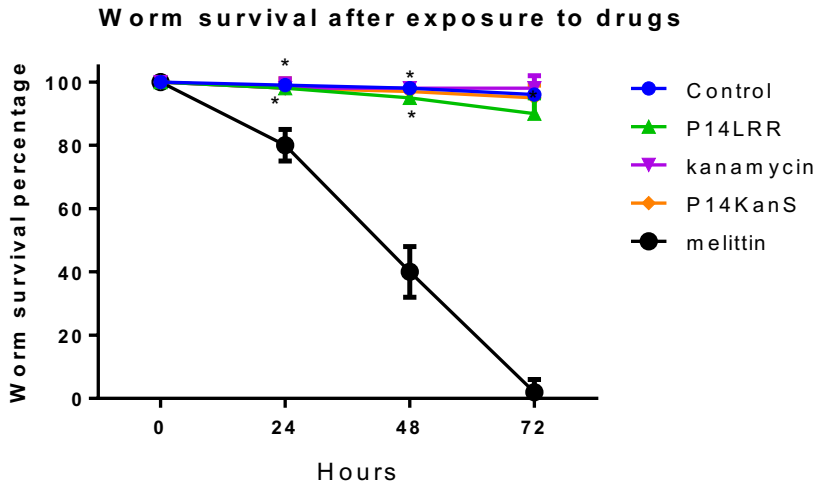


Figure S11: Evaluation of toxicity in *Caenorhabditis elegans* model. *C. elegans* strain glp-4;sek 1(L4-stage) were grown for four days in the presence of drugs at 50 µM concentrations. Worms were monitored daily and the live worms were counted. Results were expressed as percent live worms in relative to the untreated control groups. Triplicate wells were used for each group and the results were means ± SD (n = 3).

3. Supplementary References

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[S2] Geisler, I.; Chmielewski, J., Probing length effects and mechanism of cell penetrating agents mounted on a polyproline helix scaffold. *Bioorg. Med. Chem. Lett.,* **2007**, 17, 2765-8.

[S3] **CLSI.** 2007. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7. CLSI, Wayne, PA.

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[S5] Alajlouni, RA, Seleem MN. Targeting listeria monocytogenes rpoA and rpoD genes using peptide nucleic acids. *Nucleic acid therapeutics*, **2013**, 23, 363-7.

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4. NMR Spectra

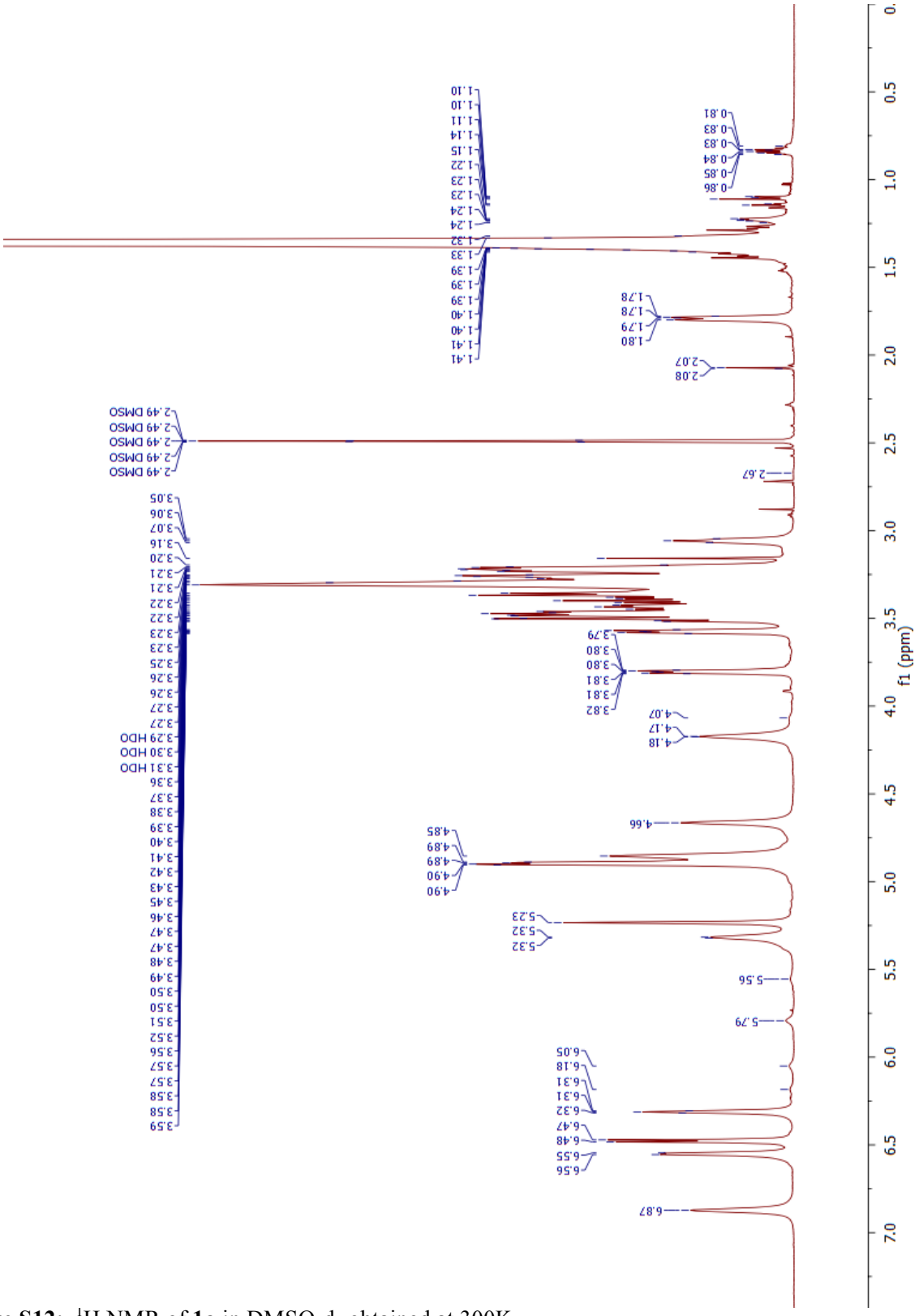

Figure S12: ^{1}H NMR of **1a** in DMSO-d_6 obtained at 300K

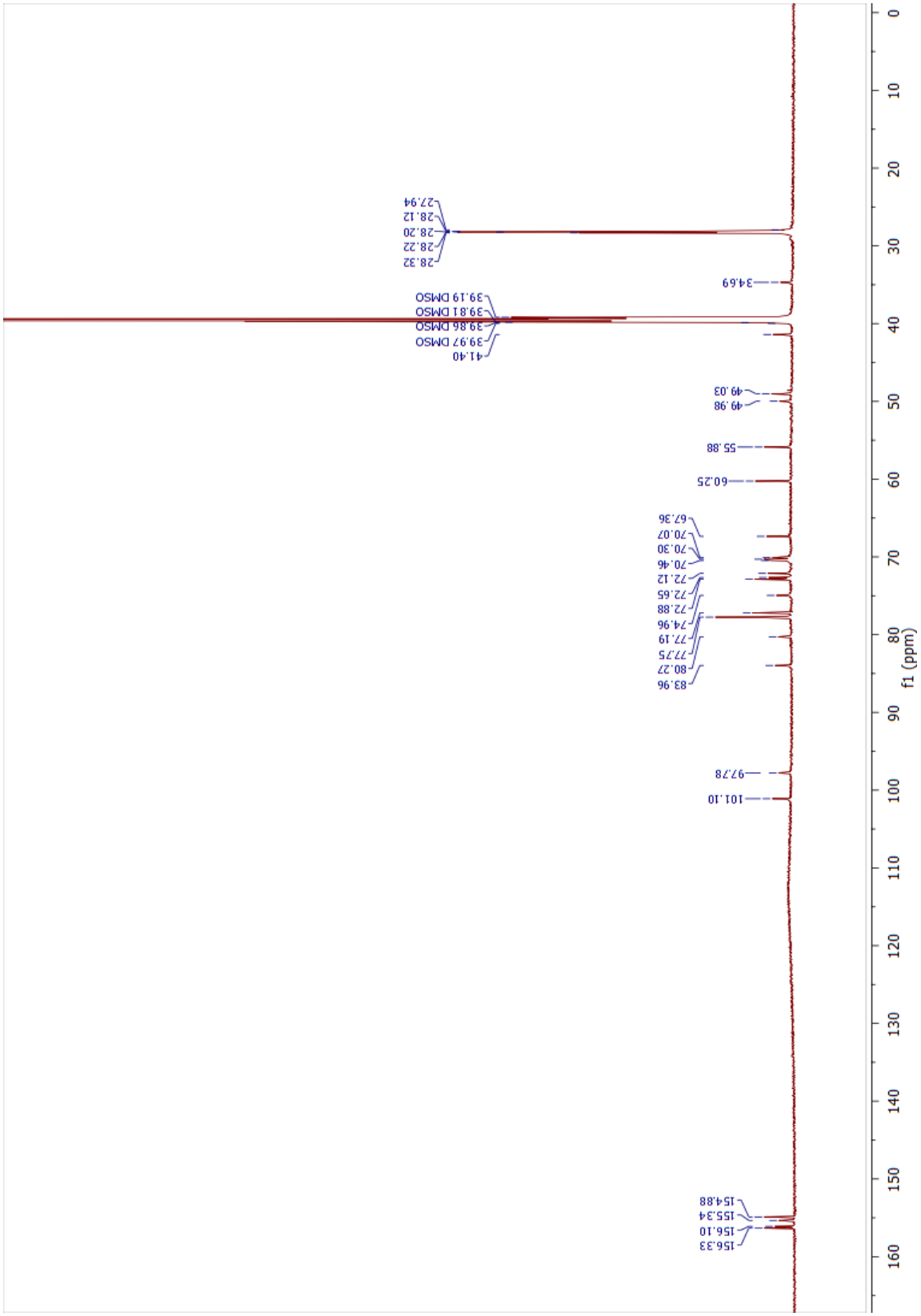

Figure S13: ^{13}C NMR of **1a** in DMSO-d_6 obtained at 300K

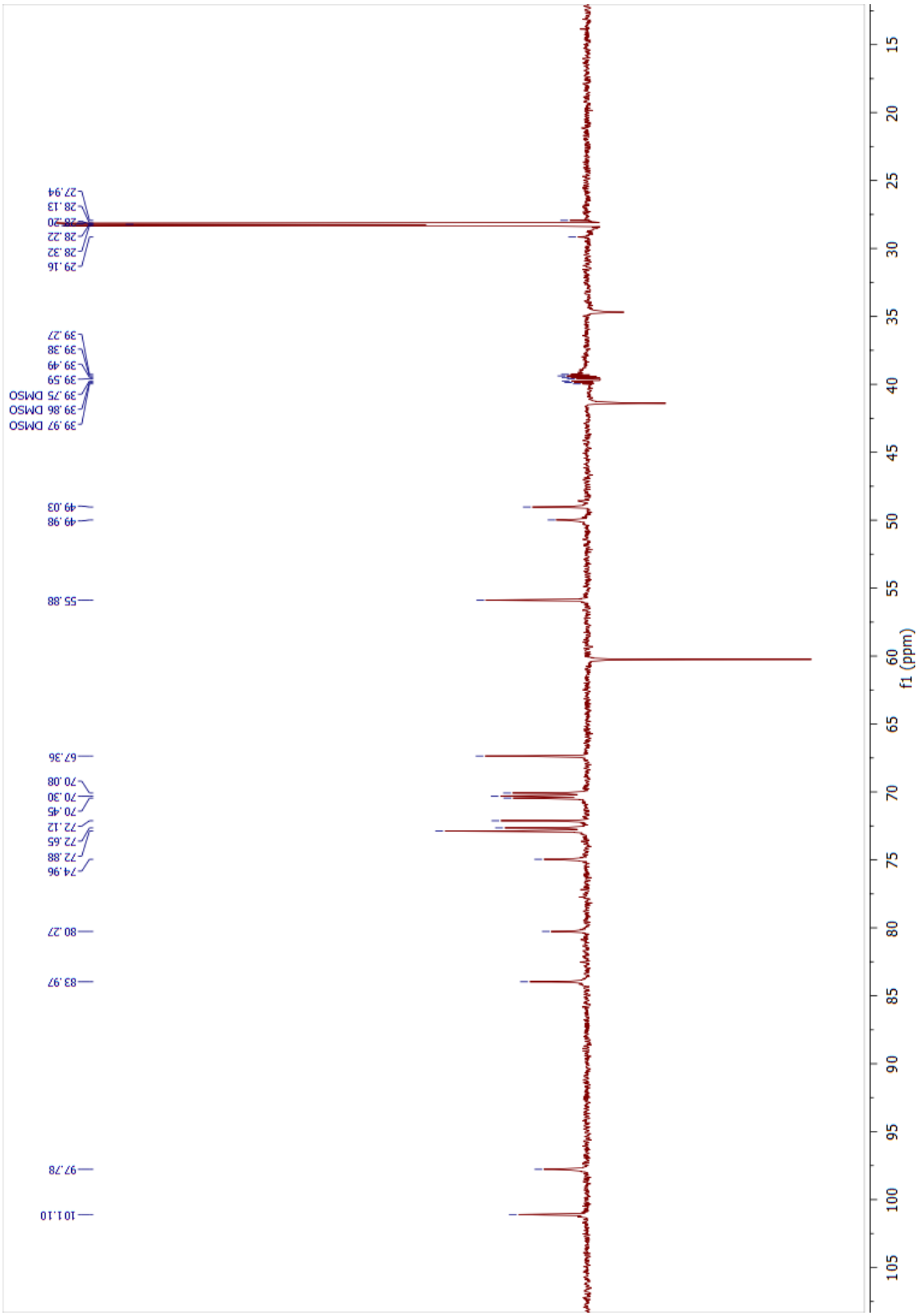

Figure S14: ^{13}C DEPT of **1a** in DMSO-d_6 obtained at 300K

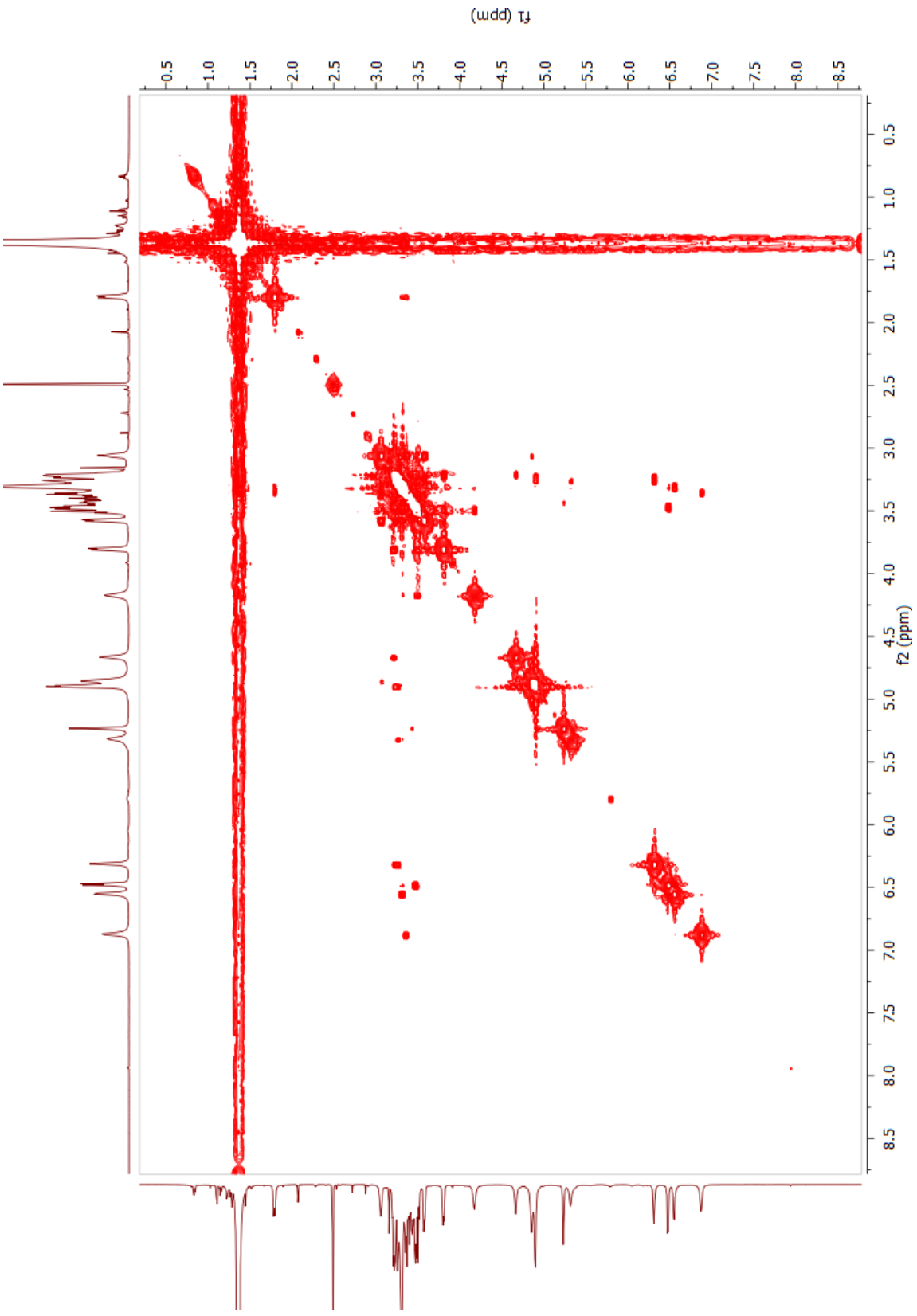

Figure S15: ^{1}H COSY of **1a** in DMSO-d_6 obtained at 300K

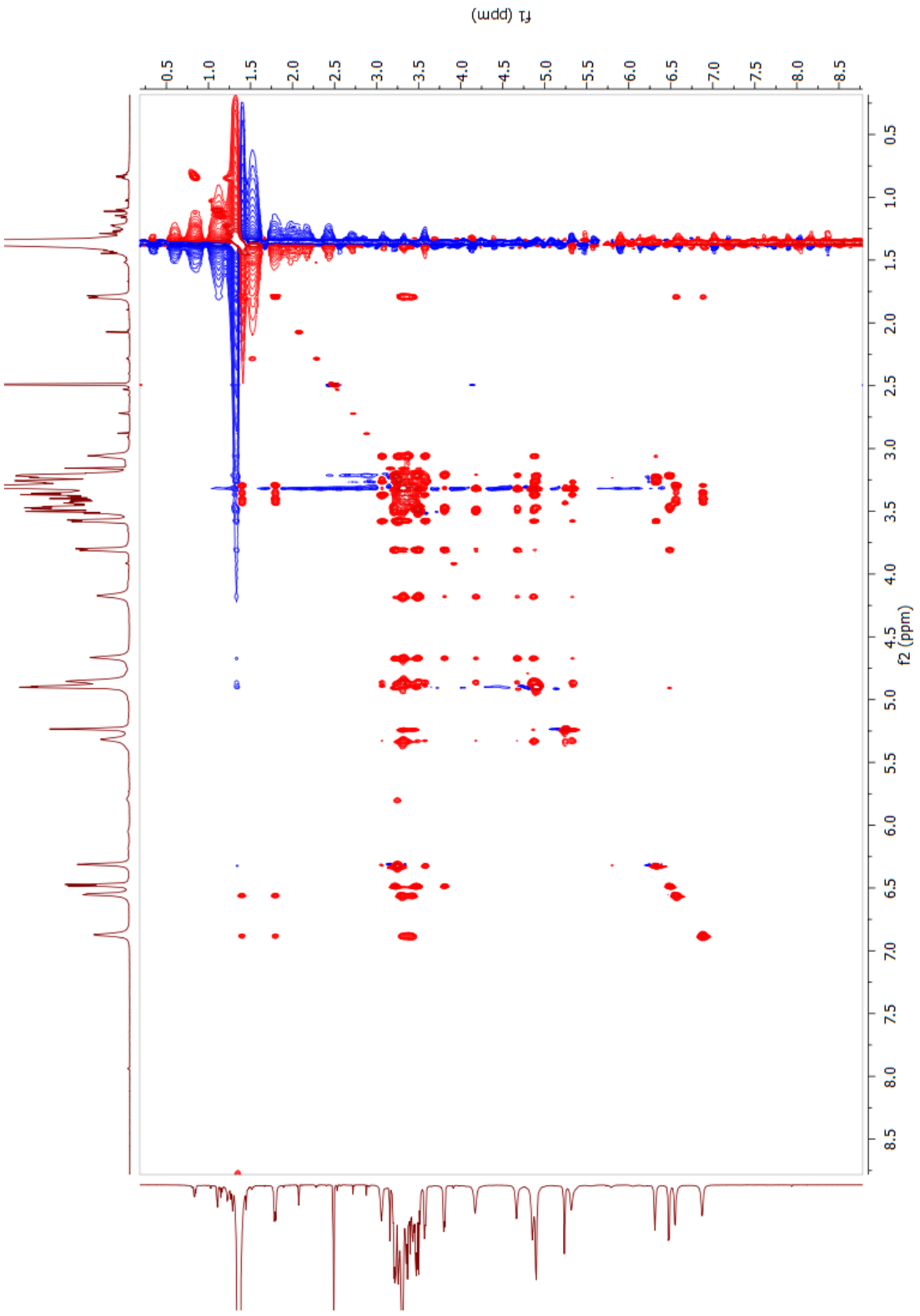

Figure S16: ^{1}H TOCSY of **1a** in DMSO-d_6 obtained at 300K

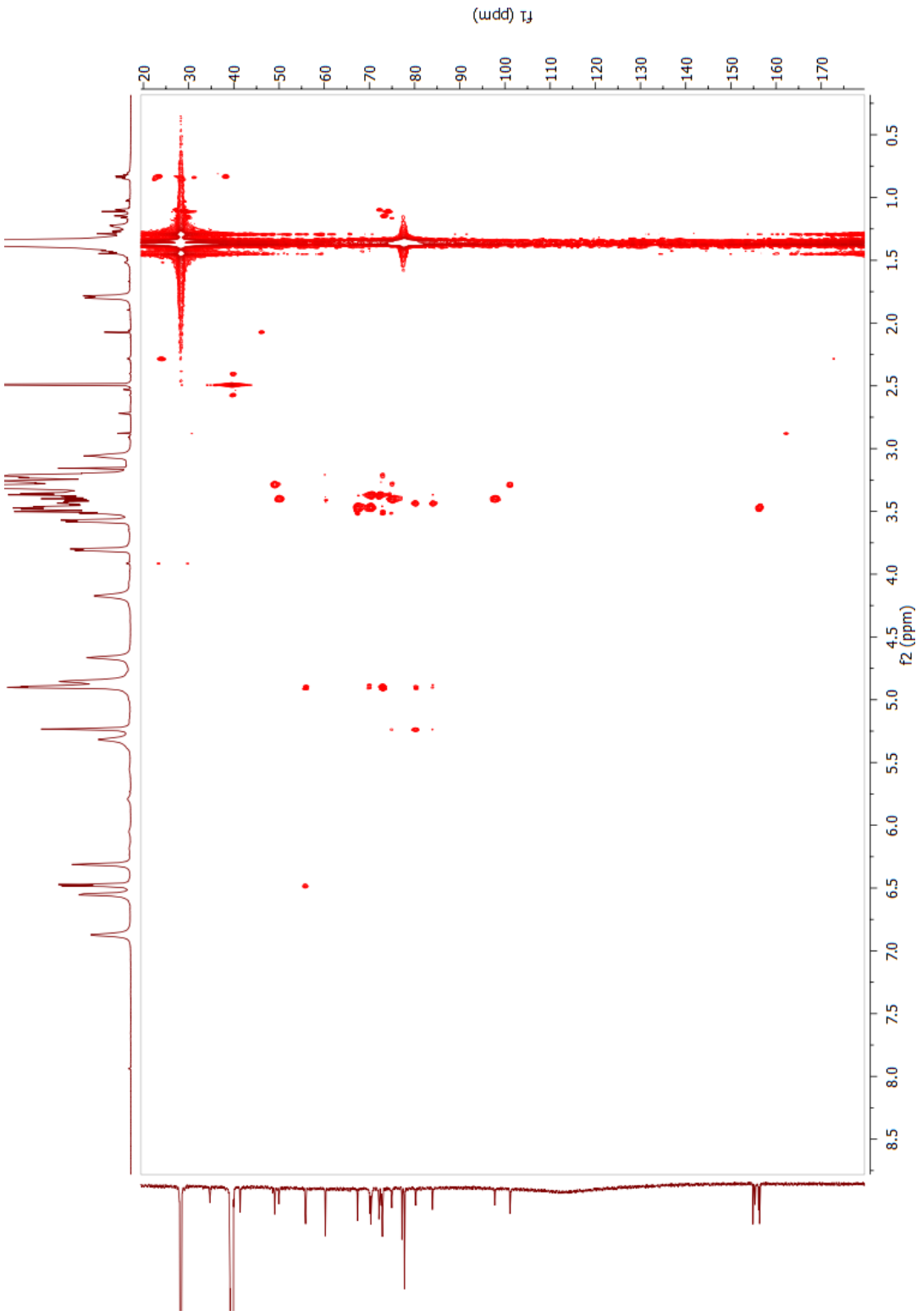

Figure S17: ^{1}H-^{13}C HMBC of **1a** in DMSO-d_6 obtained at 300K

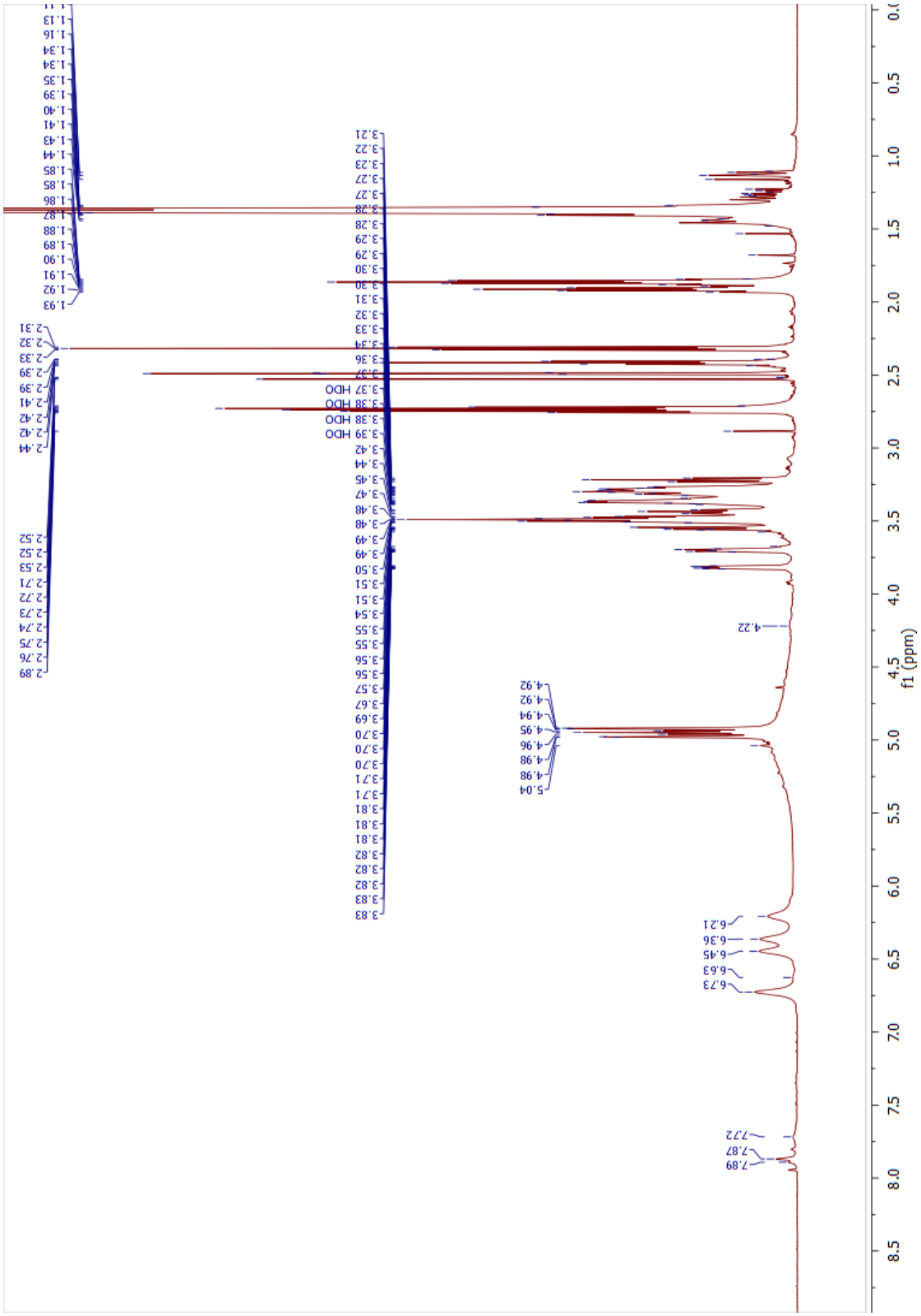

Figure S18: ^{1}H NMR of **2a** in DMSO-d_6 obtained at 323K

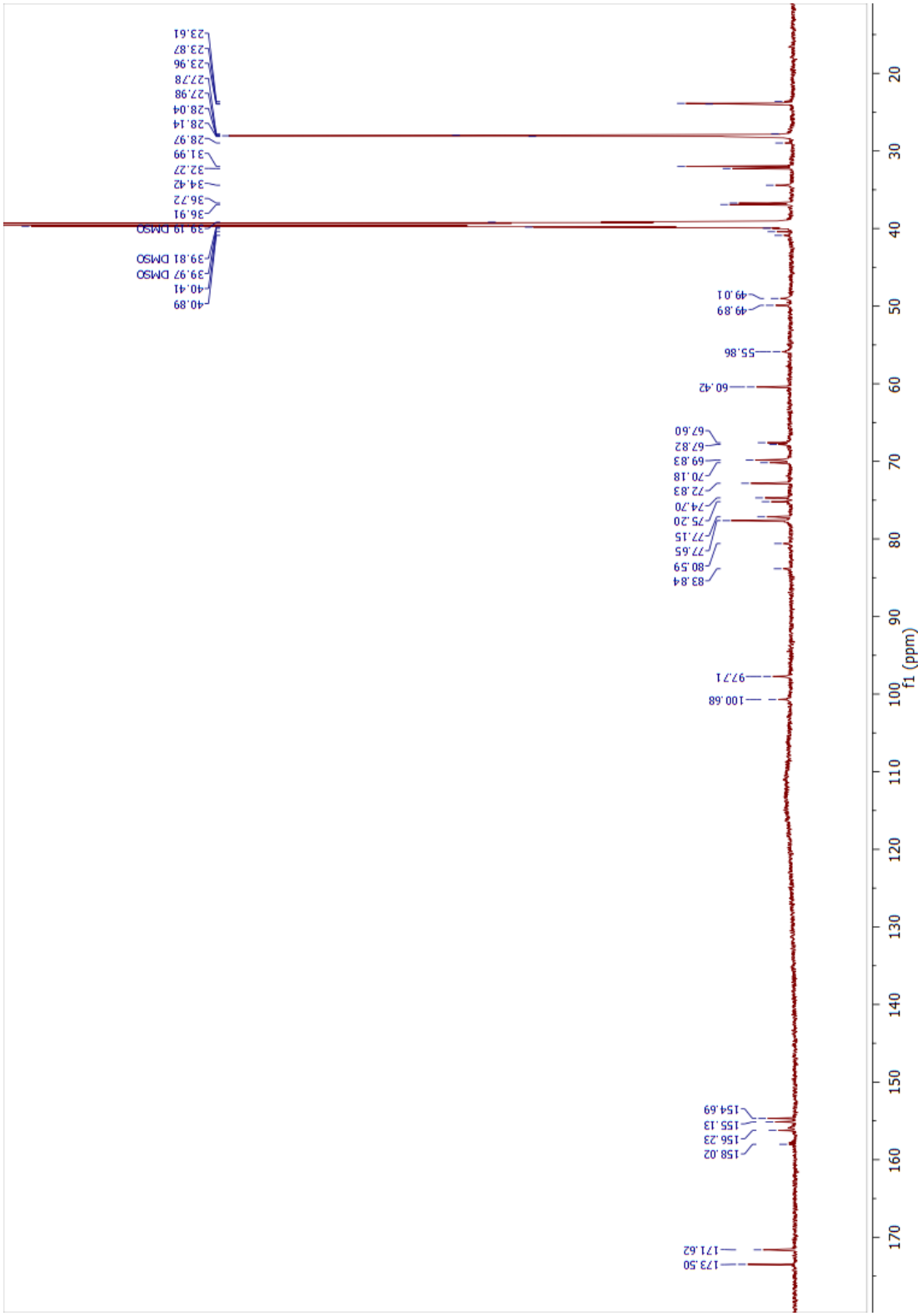

Figure S19: ^{13}C NMR of **2a** in DMSO-d_6 obtained at 323K

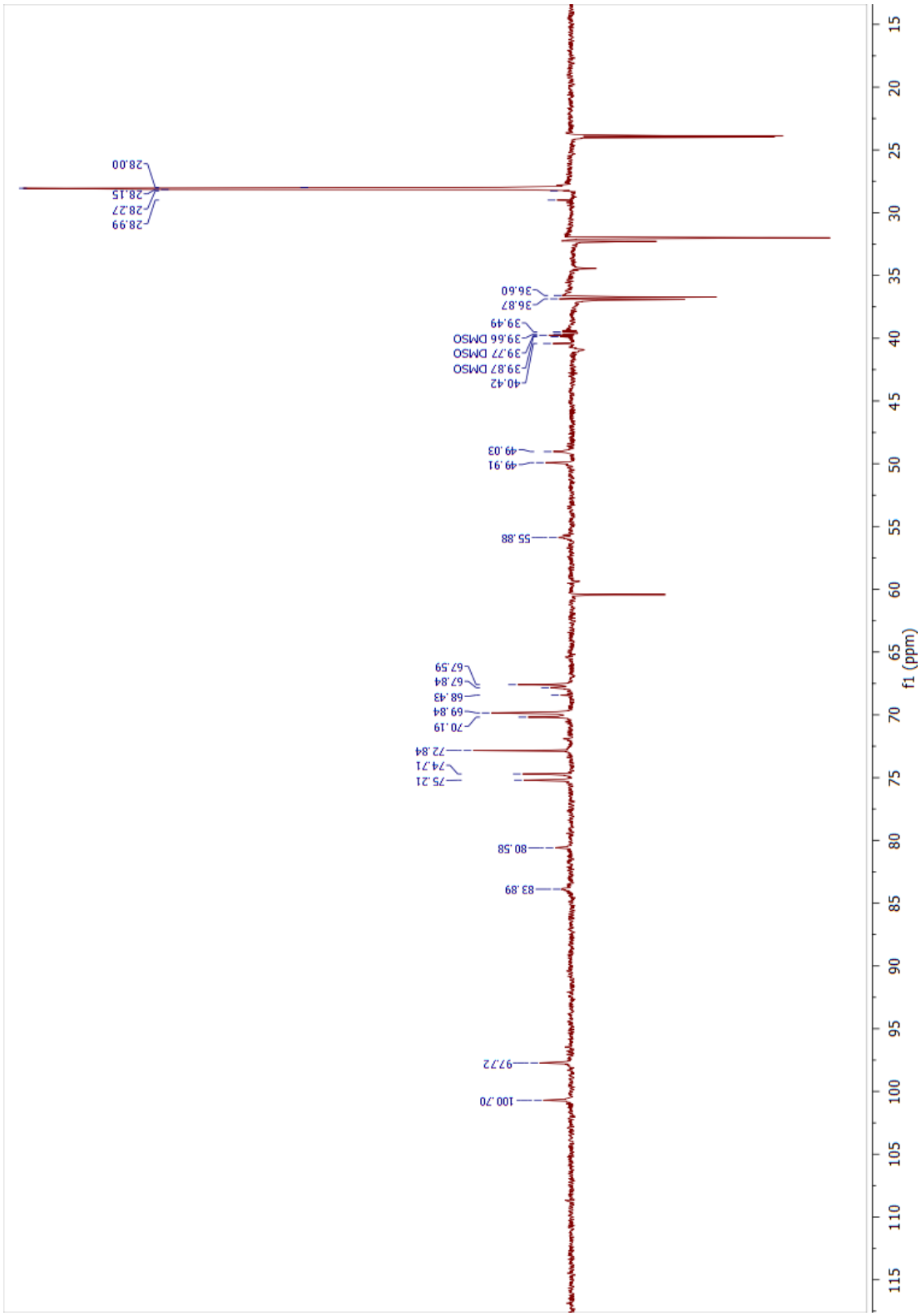

Figure S20: ^{13}C DEPT of **2a** in DMSO-d_6 obtained at 323K

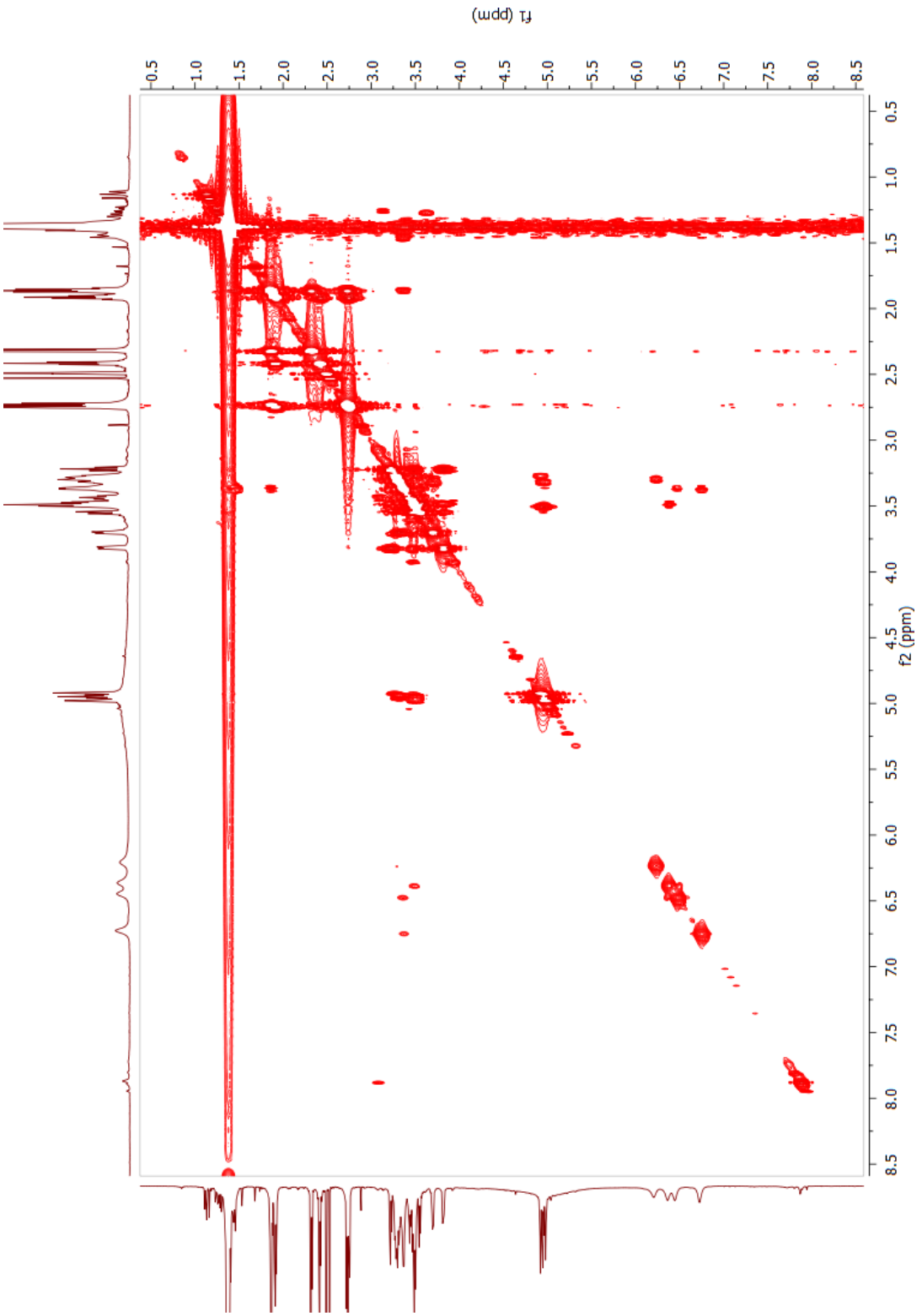

Figure S21: ^{1}H COSY of **2a** in DMSO-d_6 obtained at 323K

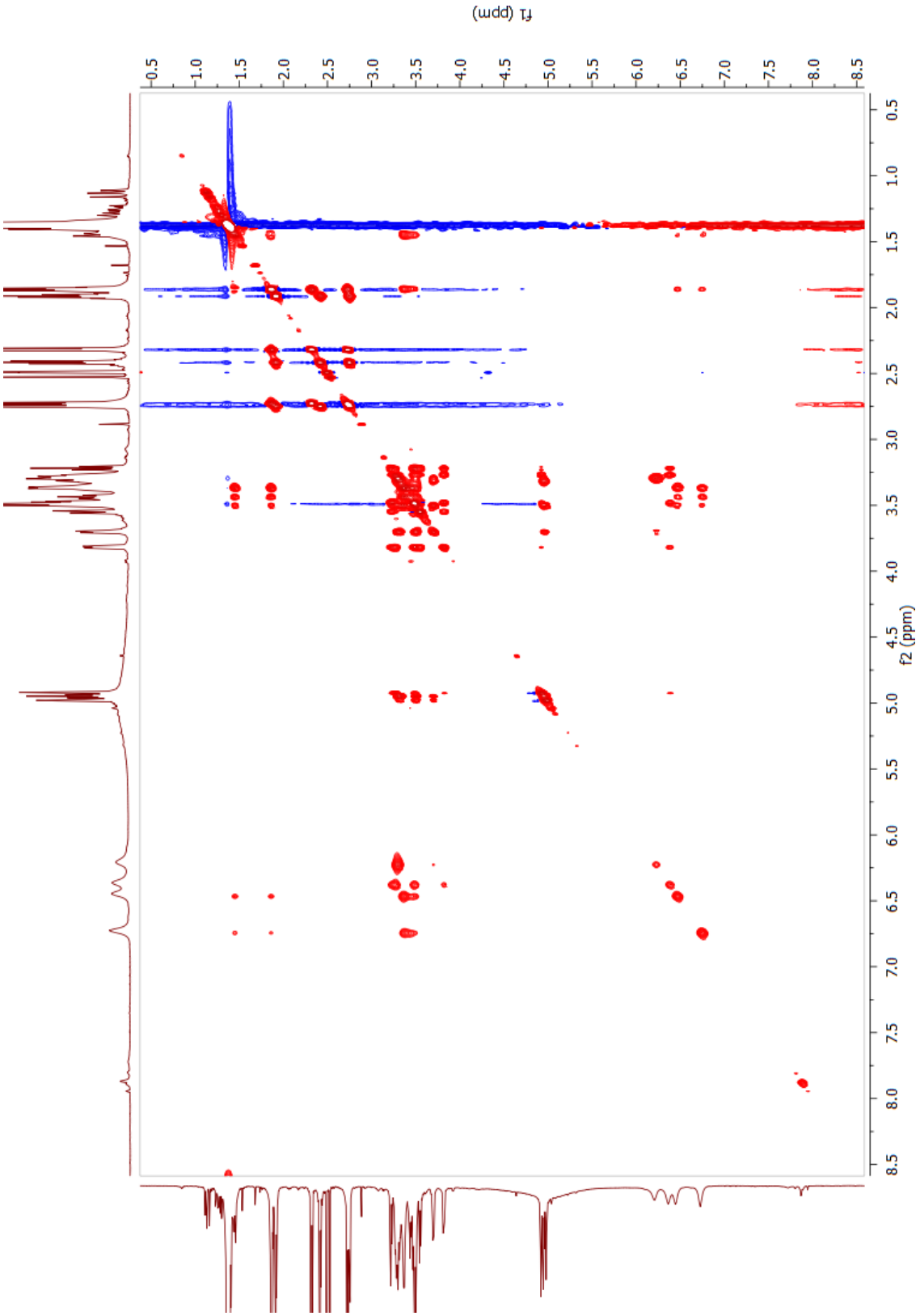

Figure S22: ^{1}H TOCSY of **2a** in DMSO-d_6 obtained at 323K

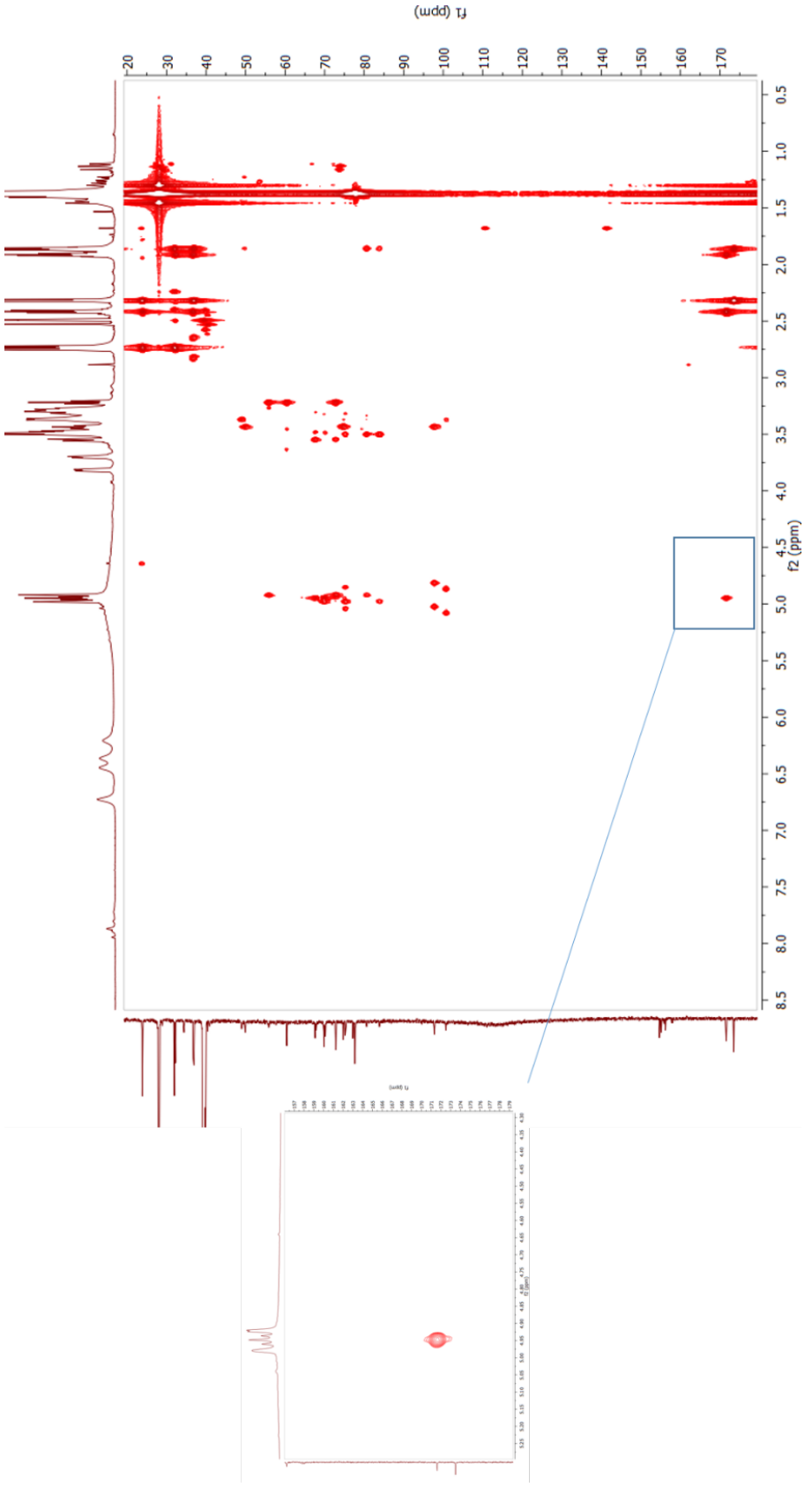

Figure S23: ^{1}H-^{13}C HMBC of **2a** in DMSO-d_6 obtained at 323K

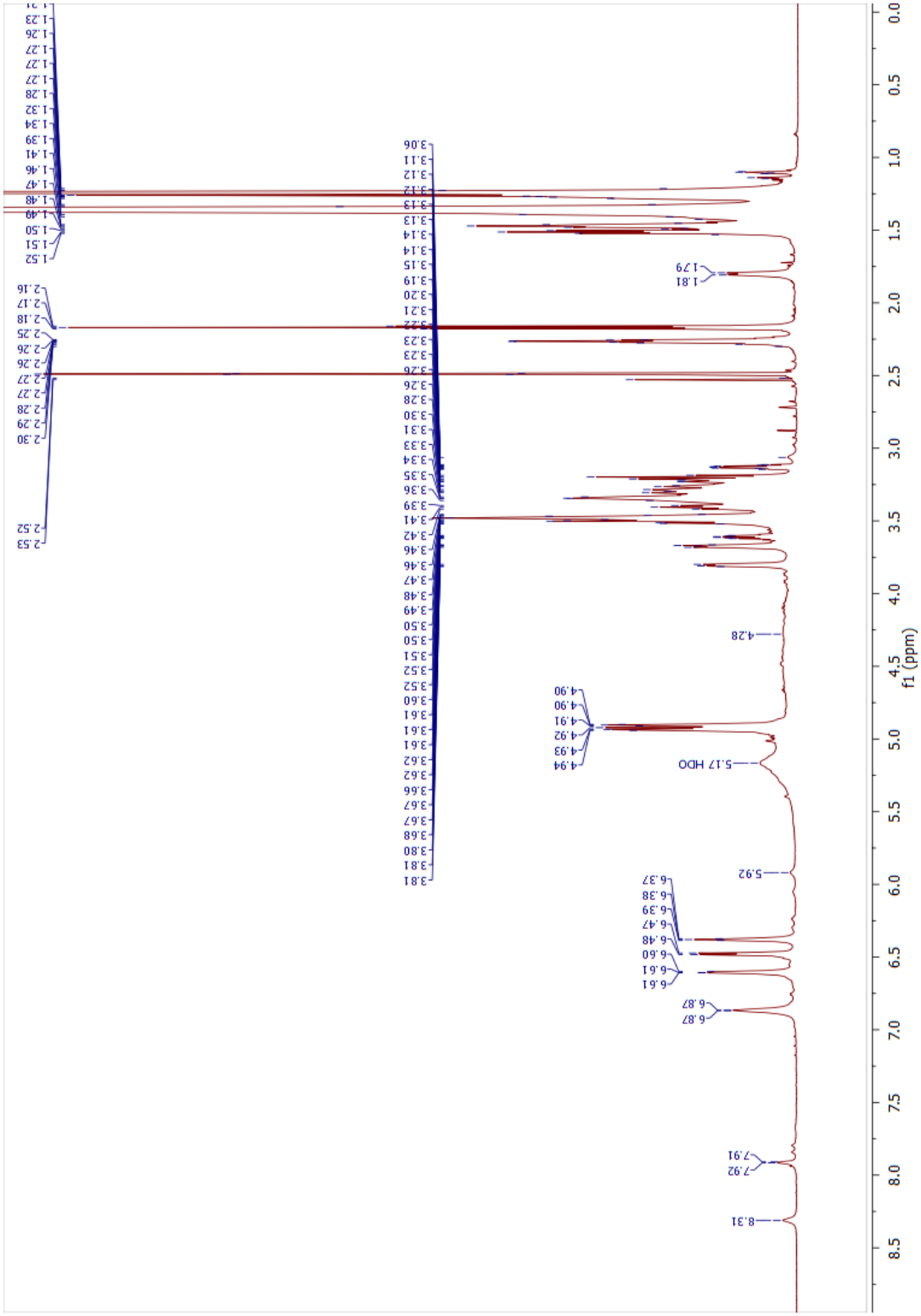

Figure S24: ^{1}H NMR of **2b** in DMSO-d_6 obtained at 323K

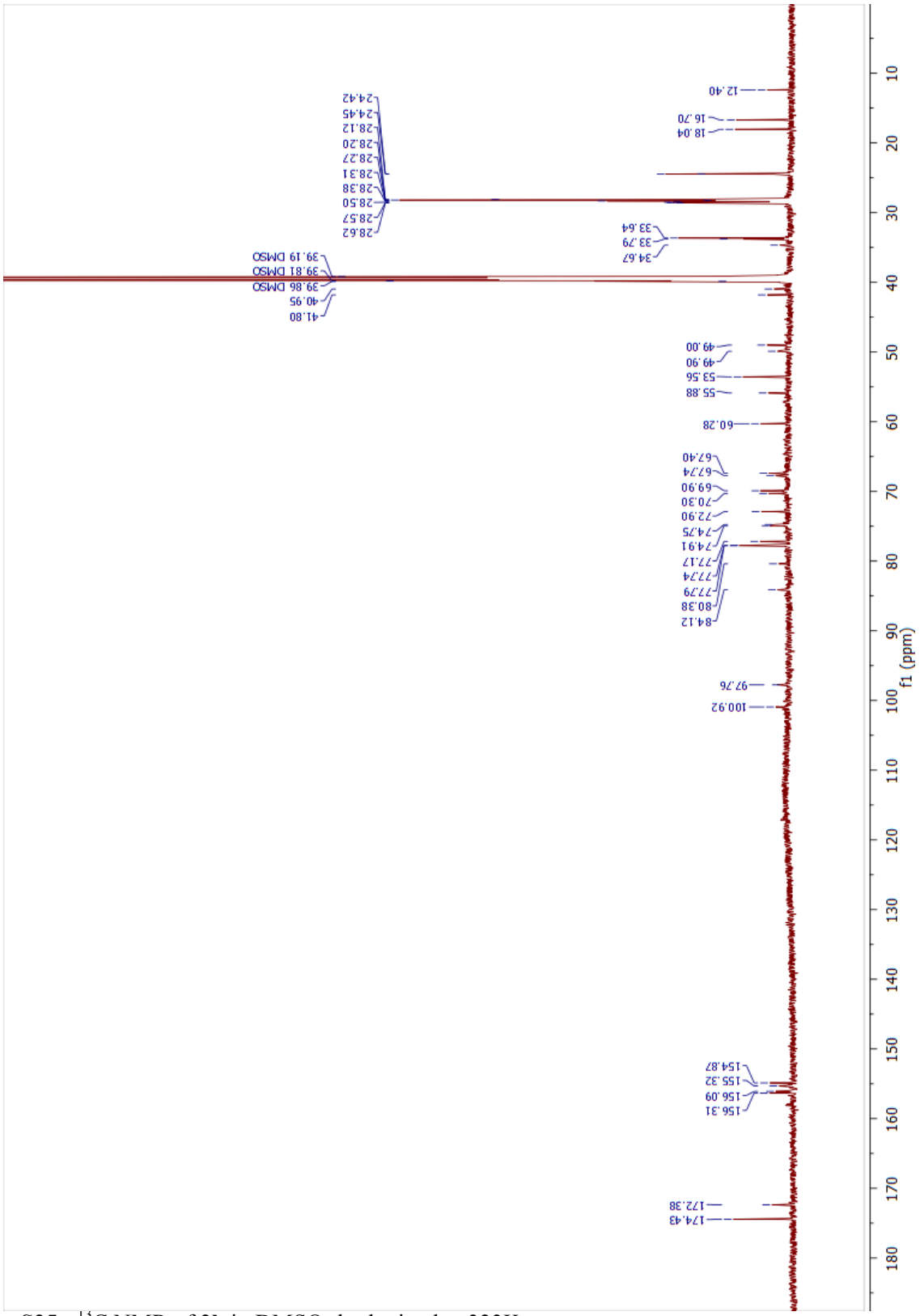

Figure S25: ^{13}C NMR of **2b** in DMSO-d_6 obtained at 323K

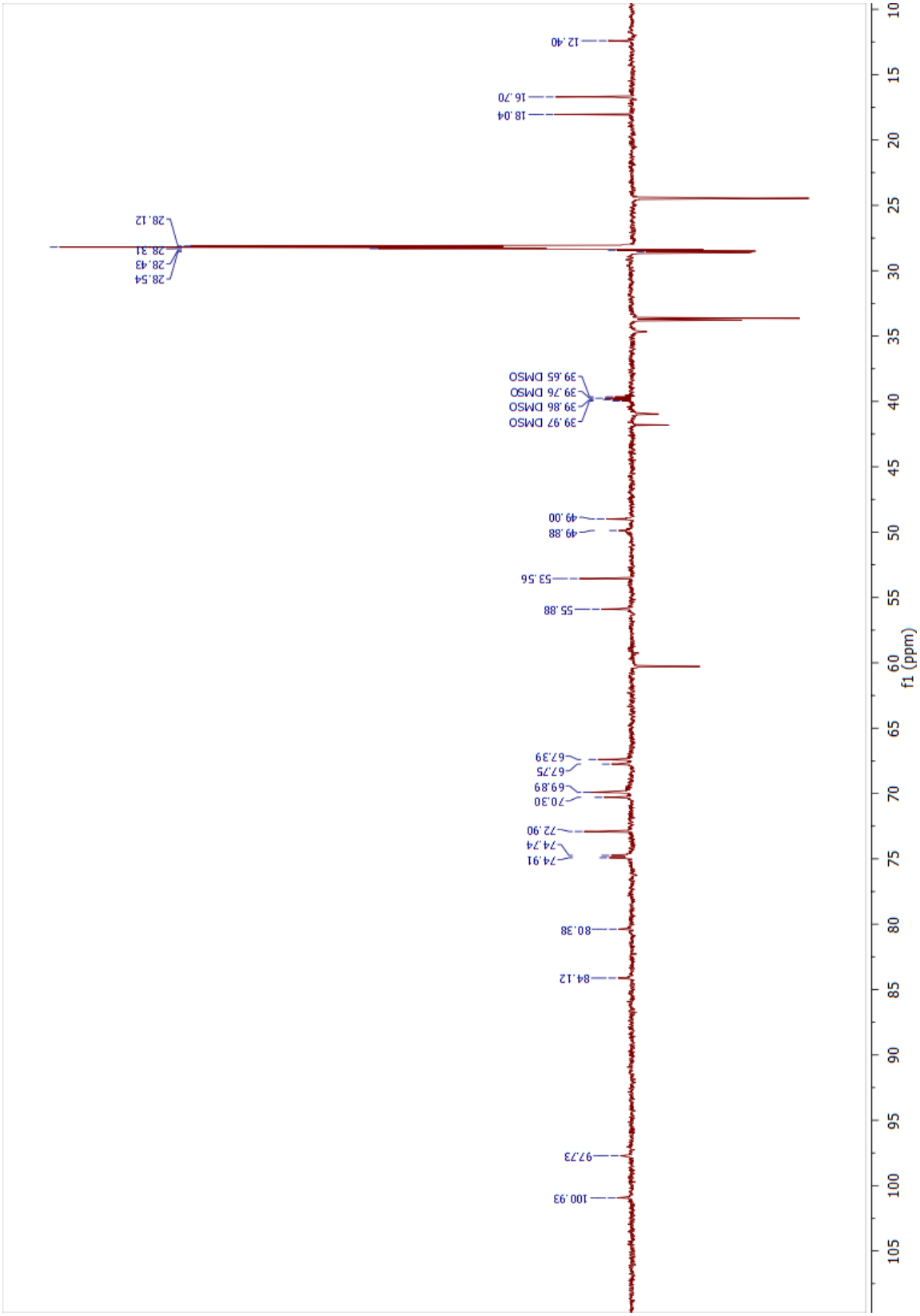

Figure S26: ^{13}C DEPT of **2b** in DMSO-d_6 obtained at 323K

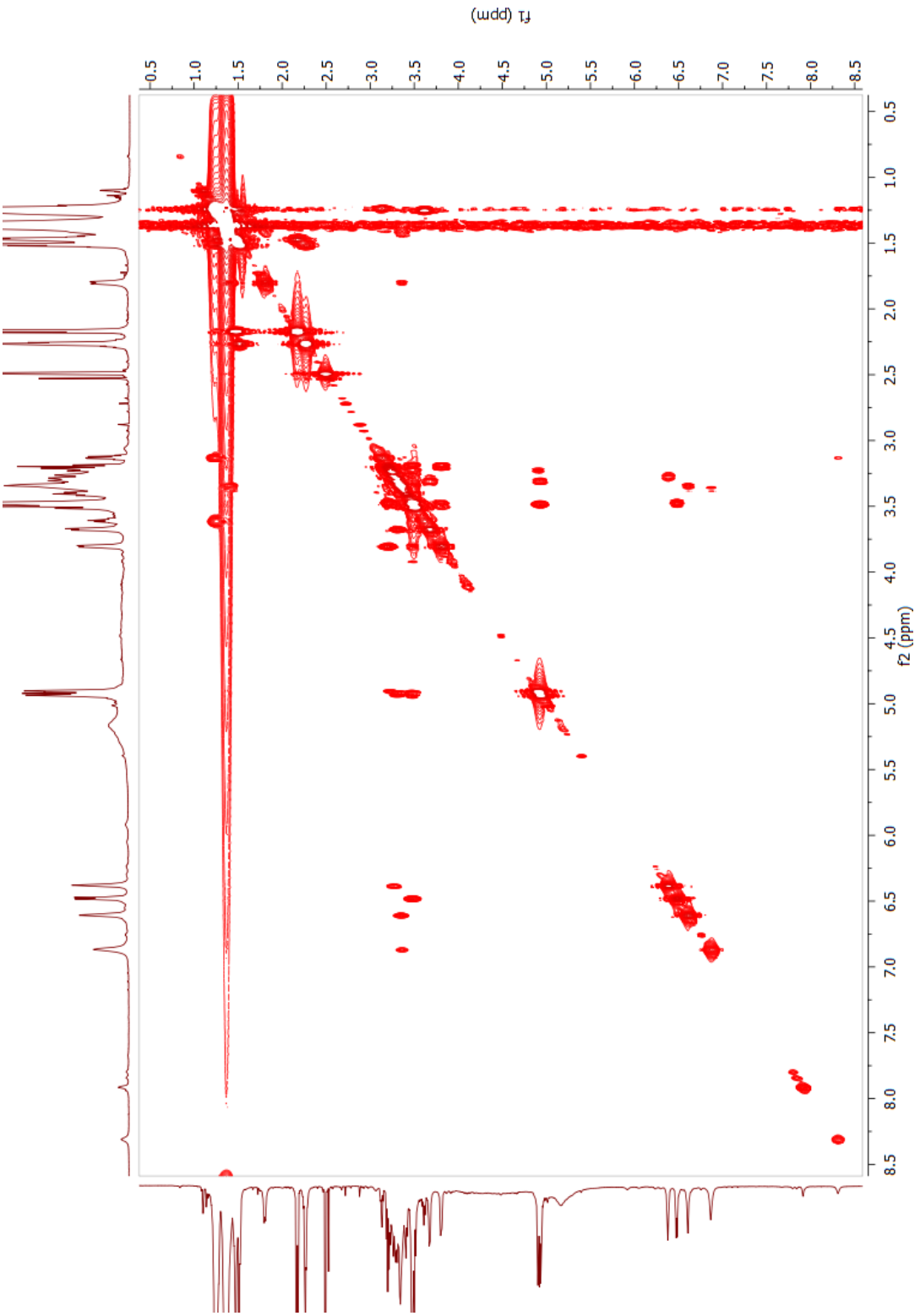

Figure S27: ^{1}H COSY of **2b** in DMSO-d_6 obtained at 323K

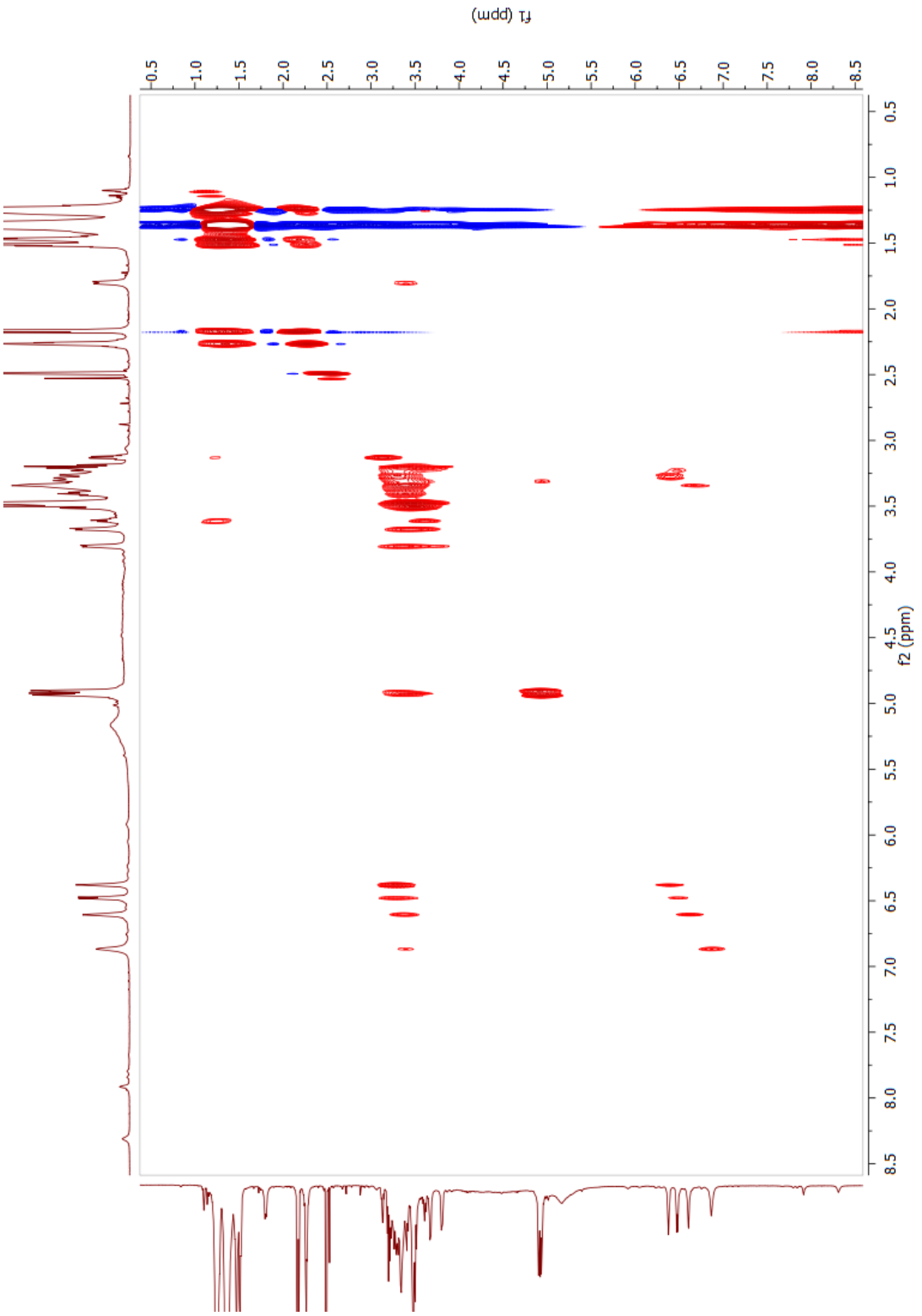

Figure S28: ^{1}H TOCSY of **2b** in DMSO-d_6 obtained at 323K

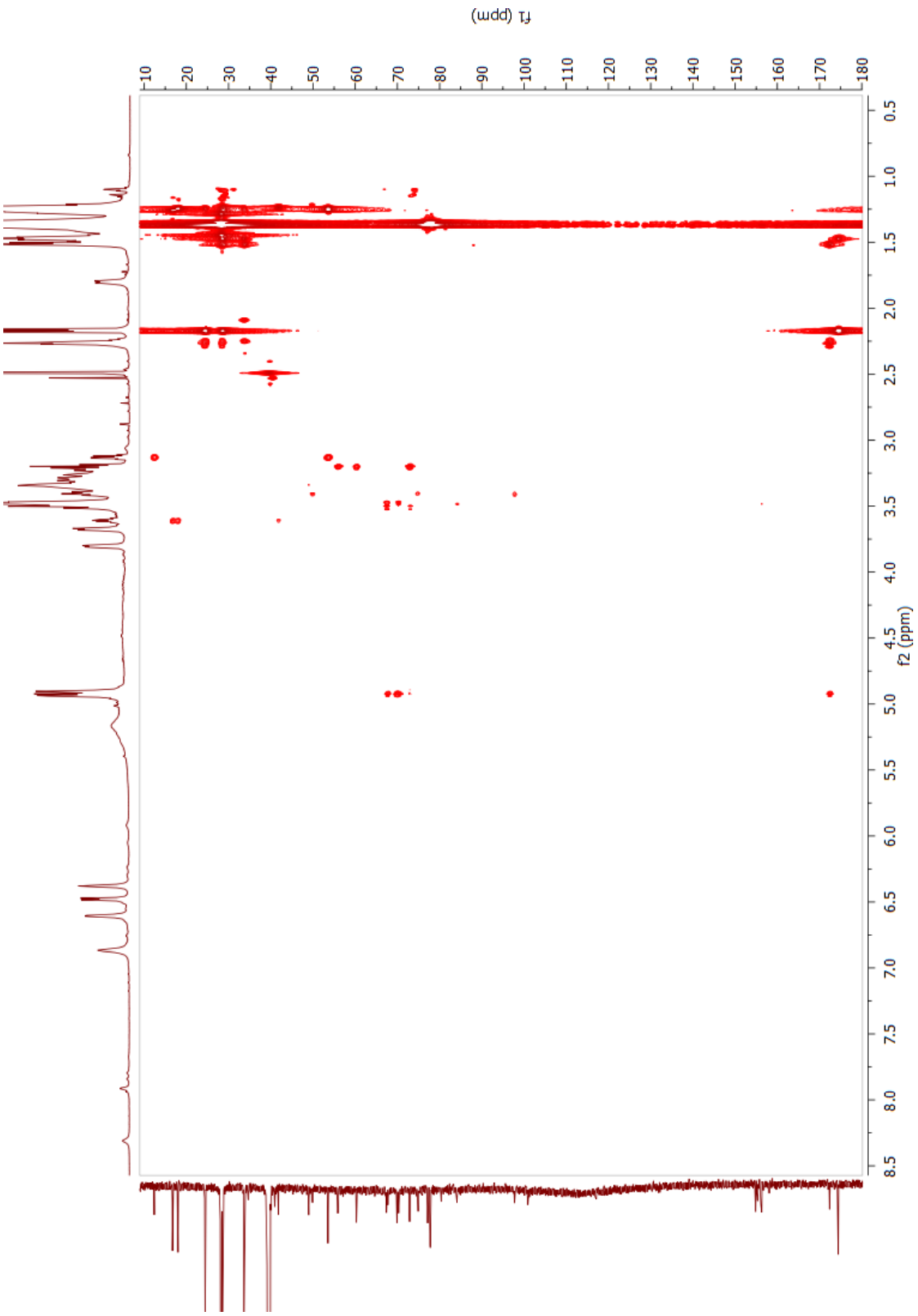

Figure S29: ^{1}H-^{13}C HMBC of **2b** in DMSO-d_6 obtained at 323K

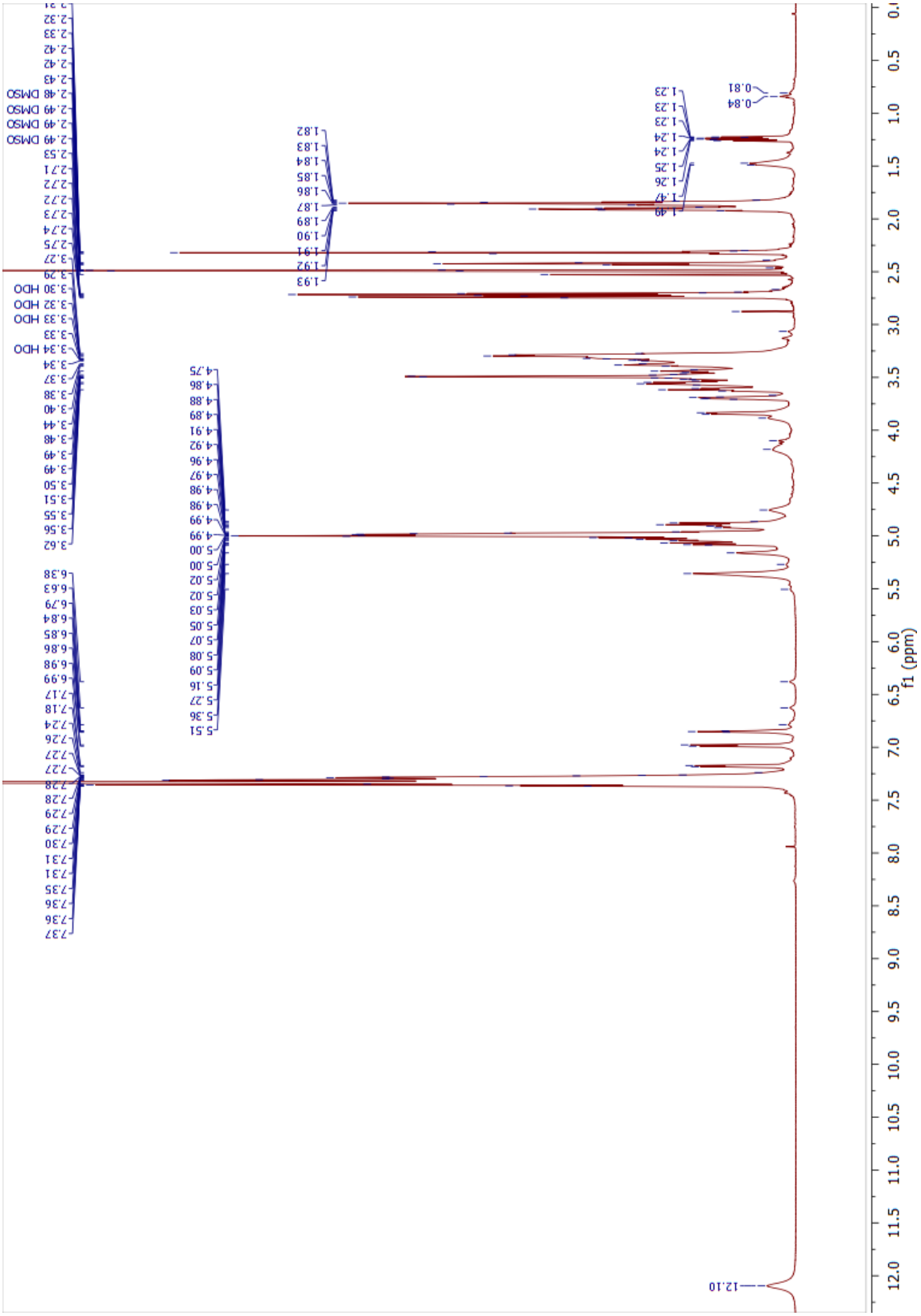

Figure S30: ^{1}H NMR of **2c** in DMSO-d_6 obtained at 323K

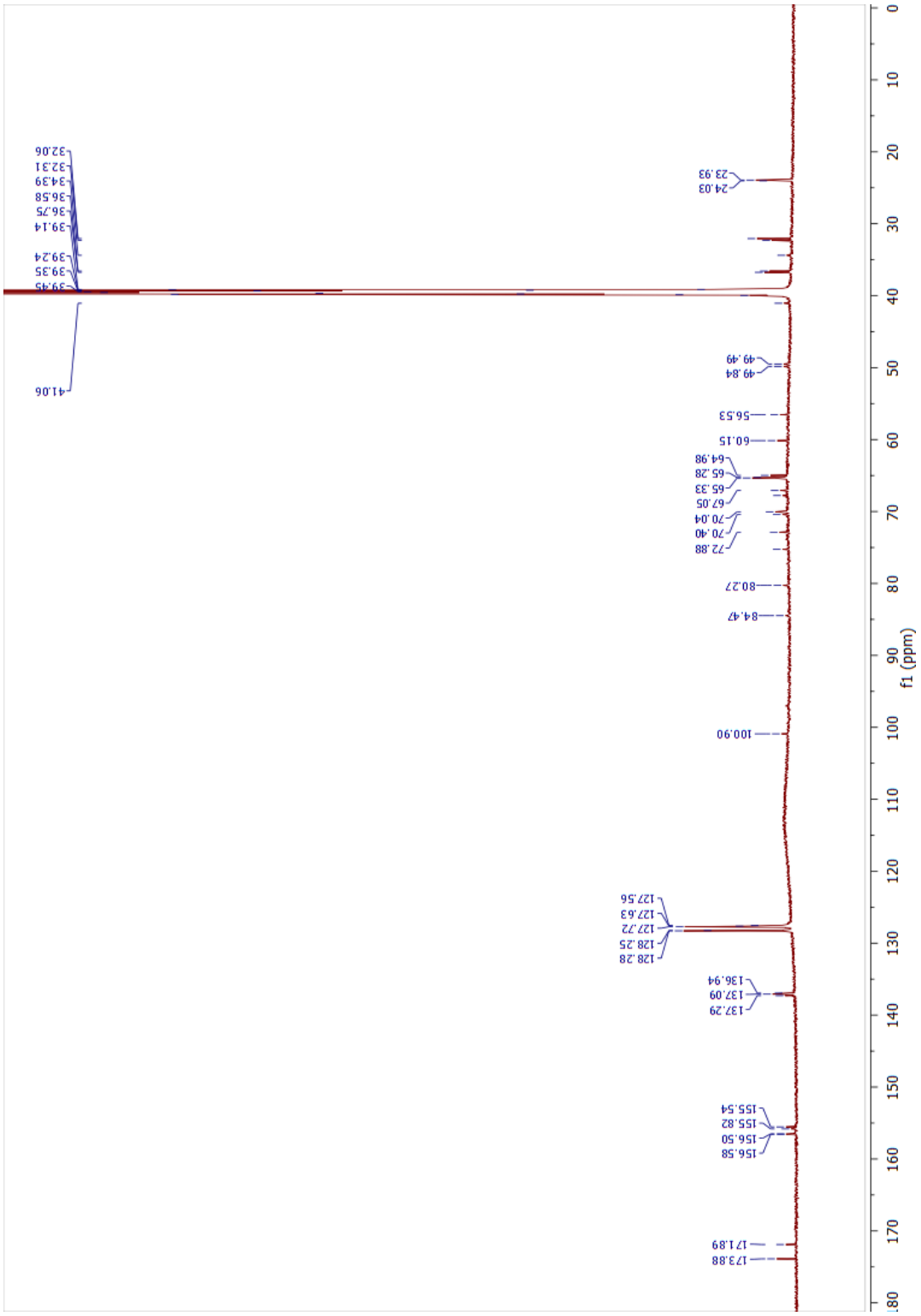

Figure S31: ^{13}C NMR of **2c** in DMSO-d_6 obtained at 323K

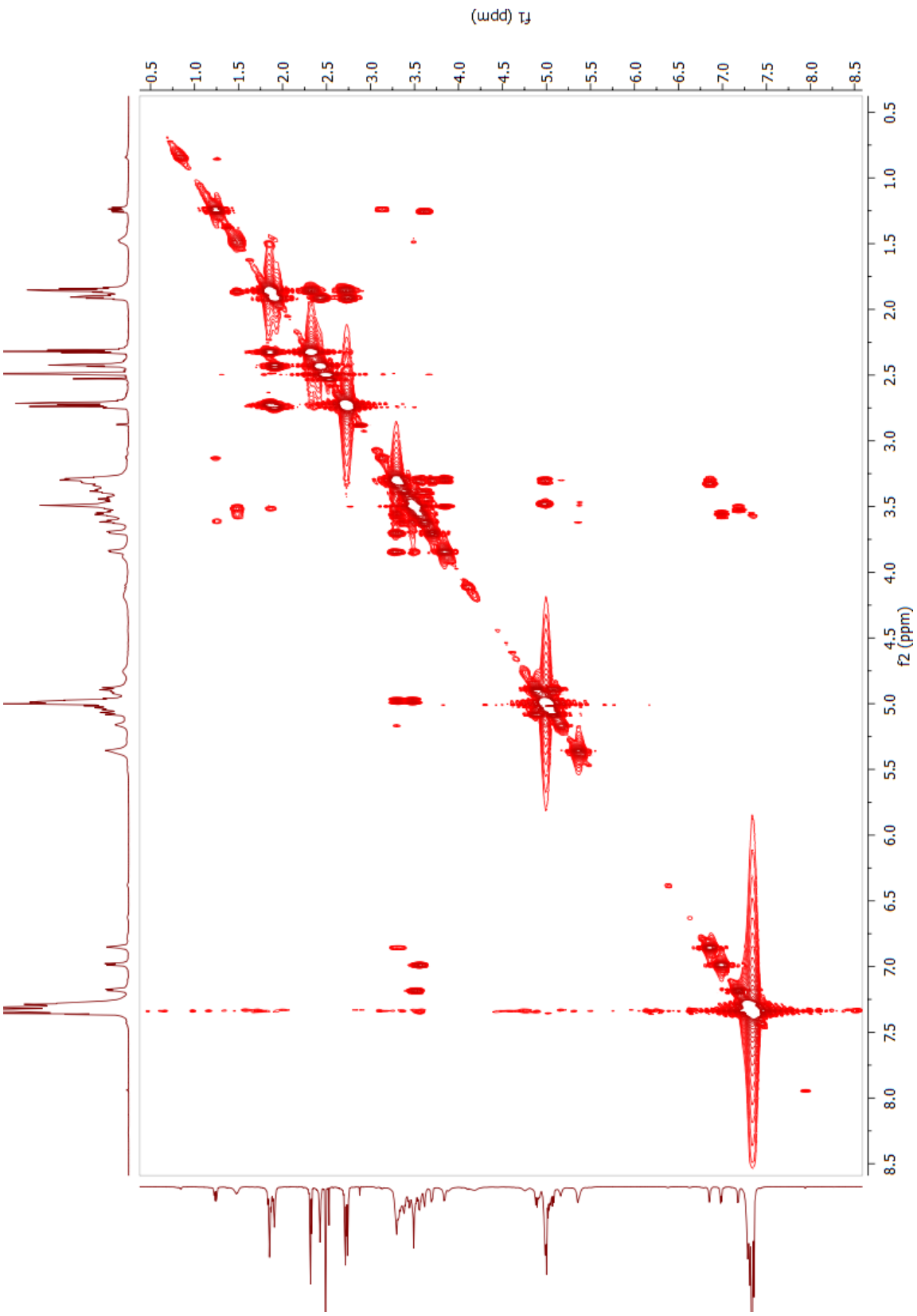

Figure S32: ^{1}H COSY of **2c** in DMSO-d_6 obtained at 323K

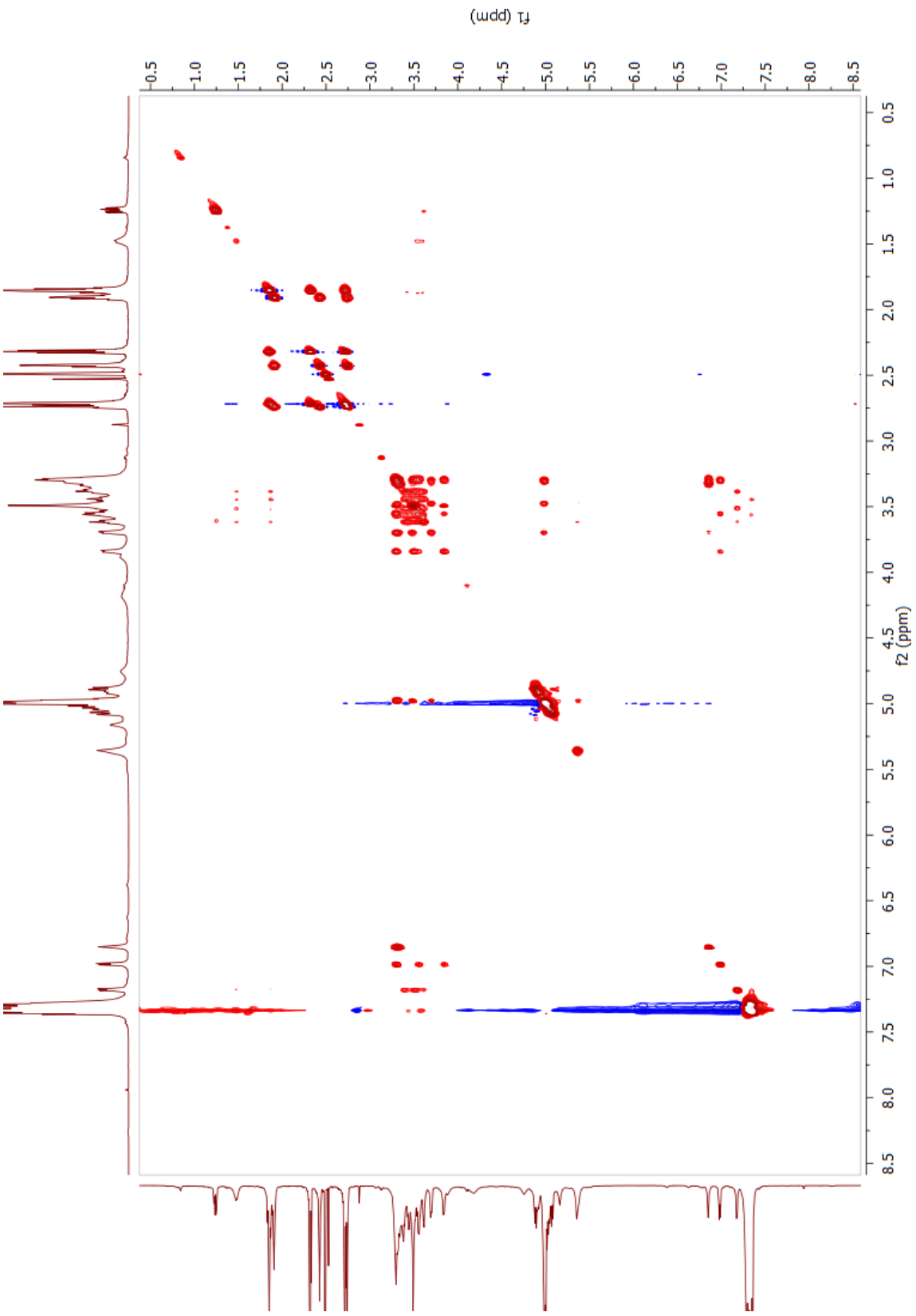

Figure S33: ^{1}H TOCSY of **2c** in DMSO-d_6 obtained at 323K

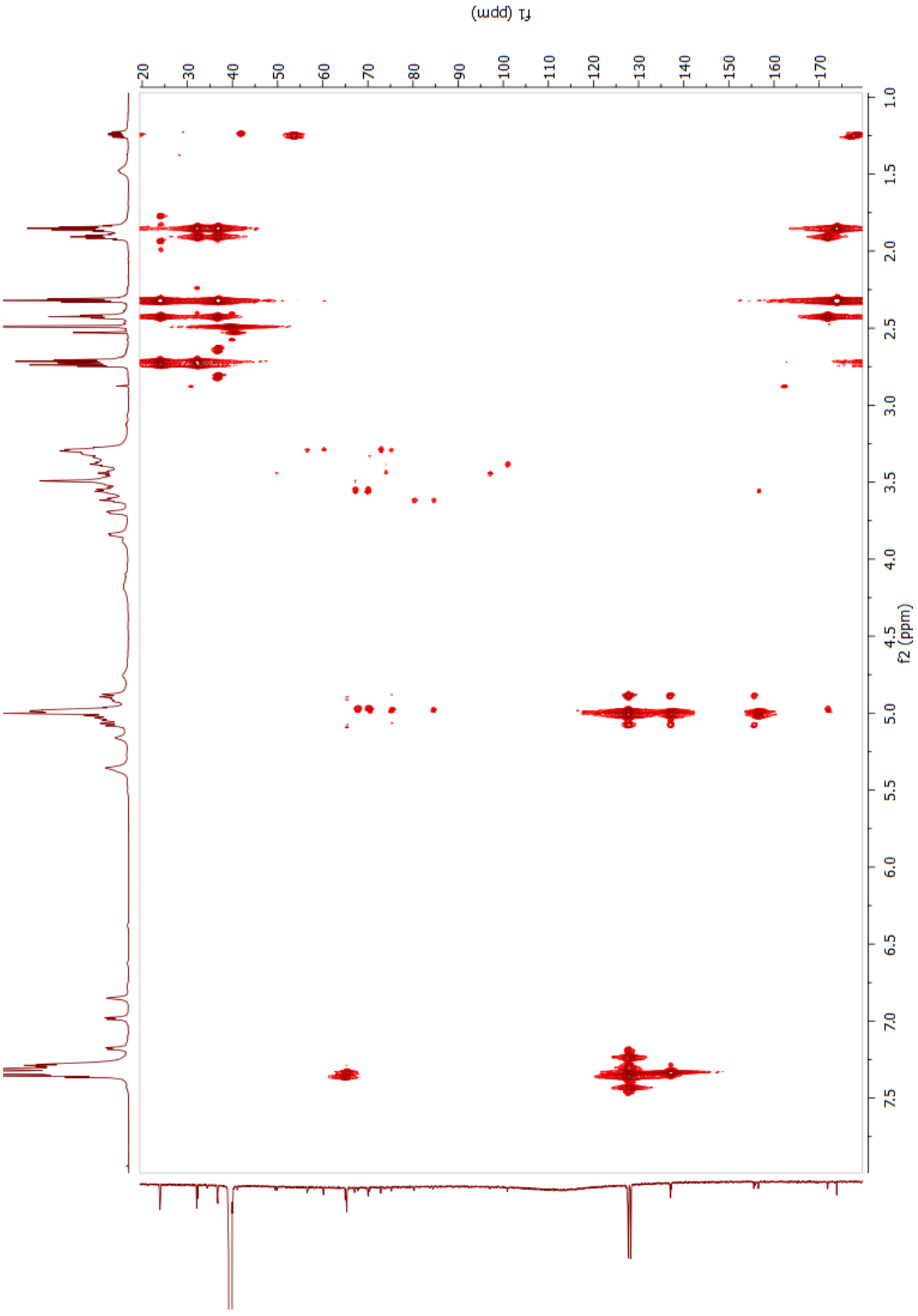

Figure S34: ^{1}H-^{13}C HMBC of **2c** in DMSO-d_6 obtained at 323K