Allene Functionalized Azobenzene Linker Enables Rapid and Light-Responsive Peptide Macrocyclization

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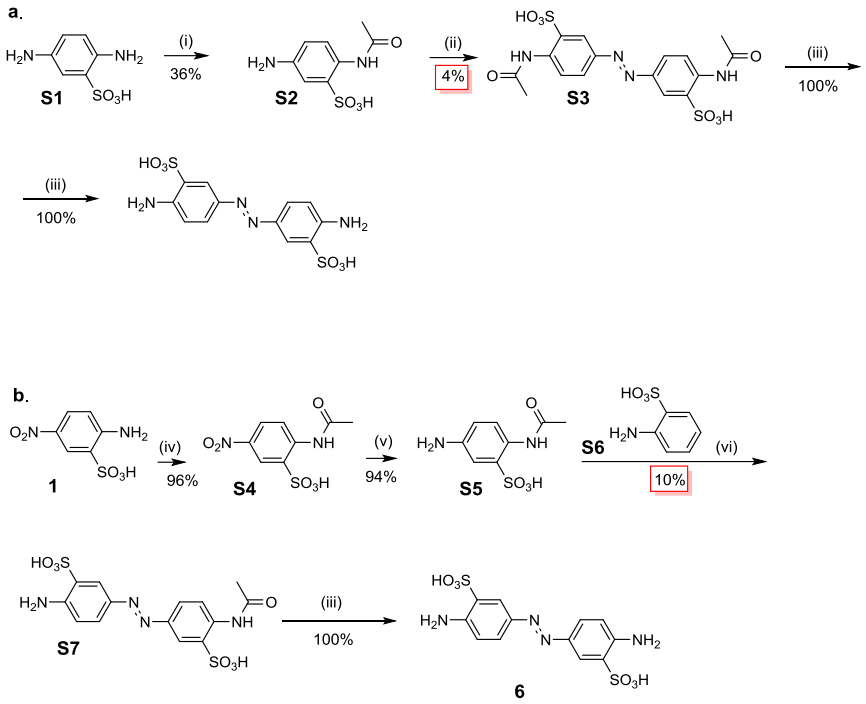
List of abbreviations

DMF	dimethylformamide
THF	tetrahydrofuran
DCM	dichloromethane
TFA	trifluoroacetic acid
DIPEA	*N*,*N*-diisopropylethylamine
r.t.	room temperature
MOPS	3-(*N*-morpholino)propanesulfonic acid,
BIA	Biotin-PEG_2-iodoacetamide
TCEP	tris(2-carboxyethyl)phosphine

General Information:

Chemical reagents and solvents were purchased from Sigma-Aldrich or TIC chemicals unless otherwise noted. 3-butynoic acid and[1] 2-azidoacetic acid[2] were synthesized according a previously published protocols and their spectroscopic characteristics matched to the previously reported. 3-butyne-1-ol was purchased from AKScientific. **BSBCA** (**10**) was synthesized from 3,3'-bis(sulfonato)-4,4'-bis(chloroacetamido)azobenzene in 89% yield according to previous protocols and the spectroscopy characteristics matched to the previously reported compound.[3] Peptide sequence ACGFERERTCG was purchased from Lifetein and was purified by HPLC before use. Proton (^{1}H-NMR) and Carbon (^{13}C-NMR) nuclear magnetic resonance spectra were recorded on an Agilent/Varian VNMRS two channel 500 MHz or Agilent/Varian Inova two-channel 400 MHz spectrometer. The chemical shifts are given in part per million (ppm). The solvent peak was used as a reference. The following abbreviations were used: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad; d, doublet of doublet of doublets. High resolution ESI mass spectra (HRMS) were recorded by Agilent Technologies 6220 oaTOF. Preparative HPLC was conducted using Waters 1525 Binary pump equipped with a Waters Symmetryprep 19×50 mm C18 Columns and Waters 2489 UV detector. We used following method for HPLC purification unles otherwise noted: A gradient of solvent A (0.1% TFA in MQ water) and solvent B (0.1% TFA in MeCN) was run at a flow rate of 8 mL/min (0-2 min 5% B; 2-18 min 5%→50% B; 18-21 min 50%→100% B; 21-24 100% →2% B). LCMS data was obtained on Agilent Technologies 6130

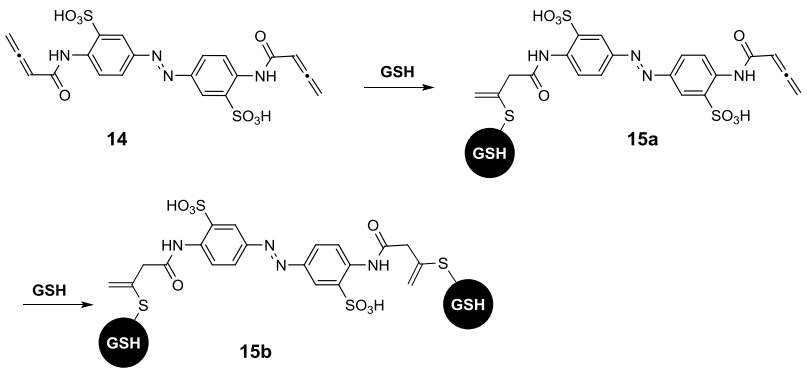
LCMS. A gradient of solvent A (MQ water) and solvent B (MeCN/H_2O 95/5) was run at a flow rate of 0.5 mL/min (0-0.4 min 5% B; 0.4-5 min 5%→60% B; 5-5.5 min 60%→100% B; 5.50-7.50 100% B, 7.50-11 min 100%→5% B).



Scheme S1. a) The original synthetic route to **6** by Zhang *et al.*[4] (i) Acetic acid, acetic anhydride, 2 h, reflux. (ii) NaClO, pH 8.5, 3 days, 4 °C, dark. (iii) 2 M HCl, 2 h reflux. b) Synthesis of **6** through diazo coupling by Jafari *et al.*[5] (iv) acetic anhydride, acetic acid, 2 h, reflux. (v) H_2, Pd/C, MeOH, 3 h. (vi) 1. 1 M HCl/MeOH = 1/1, $NaNO_2$, 0 °C, 1 h. 2. Sodium 2-aminobenzenesulfonate (aq), pH 8.5, overnight. Highlighted steps exhibit low yields and/or difficulties in purification of the product.

Measuring the kinetics of reaction of glutathione (GSH) with BSBDA

To 150 µL of 0.1% TFA in H_2O solution, we added 2 µL of a fresh solution of 16 mM GSH in water, followed by 10 µL of 100 µM solution of BSBDA in H_2O. The reaction was triggered by addition of 25 µL of 500 mM MOPS buffer (pH 8.5) to adjust the pH of the mixture to 8.0 and the reaction was mixed by pipetting. We added 30 µL of reaction mixture to 5 µL of glacial acetic acid to stop the reaction at 0.5, 1, 2, 4, 10 and 20 min.



The data was processed using MATLAB to fit the reading to consecutive pseudo first order kinetic process:

$$\mathbf{14} \overset{k1}{\rightarrow} \mathbf{15}\boldsymbol{a} \overset{k2}{\rightarrow} \mathbf{15}\boldsymbol{b}$$

$A_t = A_0 e^{k_1 t}$

$B_t = \frac{k_1 A_0}{k_2 - k_1} \left(e^{-k_1 t} - e^{-k_2 t}\right)$

$C_t = A_0 \left[1 + \frac{1}{k_1 - k_2} \left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)\right]$

where A_t, B_t and C_t are area under the curve (AUC) for **14**, **15a** and **15b**, respectively, at time t. A_0 is initial AUC and the k_1 and k_2 are the rate constants of the first and second reaction. (Figure S1)

Measuring the efficiency of switching of 16

We prepared a 0.2 mM solution of **16** in 50 mM NH_4OAc (pH 7.2) in water. We transferred 100 µL of this solution to a 300 µL glass vial insert and irradiated the solution with 365 nm LED for 2 min. A 10 µL of the solution was then injected to LCMS system to separate the *cis* and *trans* isomers on C8 column at 15 minutes intervals. The absorption of each isomer at 322 nm (isosbestic point) showed the amount of each isomer in the solution. The data was fitted into first order kinetics: $A_t = A_0 + A(1\text{-}e^{-kCt})$ using MatLab software. The A_0 was then interpolated to give the amount of *cis* and *trans* isomer at t_0 (Figure S2).

Measuring the biotinylation of the phage with BIA

To 96.5 μL of a solution of 10^{11} pfu/mL of phage (sequence ACPARSPLEC/WT = 5/1) in 50 mM Tris buffer (pH 8.5), we added 2.5 μL of 20 mM TCEP followed by 1 μL of 100 mM BIA. We incubated this reaction for 30 min at r.t. and then stopped the reaction by diluting it 10^7 times in PBS (i.e., three serial 1:100 dilutions performed by mixing of 10 μL of the reaction mixture and 990 μL PBS, and one 1:10 dilution). The reaction was split in two aliquots of A and B. We added aliquot A to 10 μL of pre-washed streptavidin coated magnetic beads (Promega) and agitated the mixture for 10 min at 1000 rpm using IKA MS 3 digital orbital shaker. Then we captured the beads on a magnetic stand and titered the supernatant of A and the aliquot B. The amount of biotinylation was calculated as:

%Biotinylated phage = [(titer of B – titer of A) / titer of B] × 100%

Measuring the reaction of the phage with BSBDA

To 96.5 μL of a solution of 10^{11} pfu/mL of phage (sequence ACPARSPLEC/WT = 5/1) in 50 mM Tris buffer (pH 8.5), we added 2.5 μL of 20 mM TCEP followed by 2.5 μL of 20 mM BSBDA. We incubated this reaction for 30 min at r.t. and then added 1 μL of a 100 mM solution of BIA in DMF. We stopped the reaction by diluting it 10^7 times in PBS. The reaction was split in two aliquots of A and B. We added aliquot A to 10 μL of pre-washed streptavidin coated magnetic beads (Promega) and agitated the mixture for 10 min at 1000 rpm using IKA MS 3 digital orbital shaker. Then we captured the beads on a magnetic stand and titered the supernatant of A and the aliquot B. The amount of biotinylation was calculated as:

%Biotinylated phage = [(titer of B – titer of A) / titer of B] × 100%

Reacted Phage = 100% - %biotinylated phage

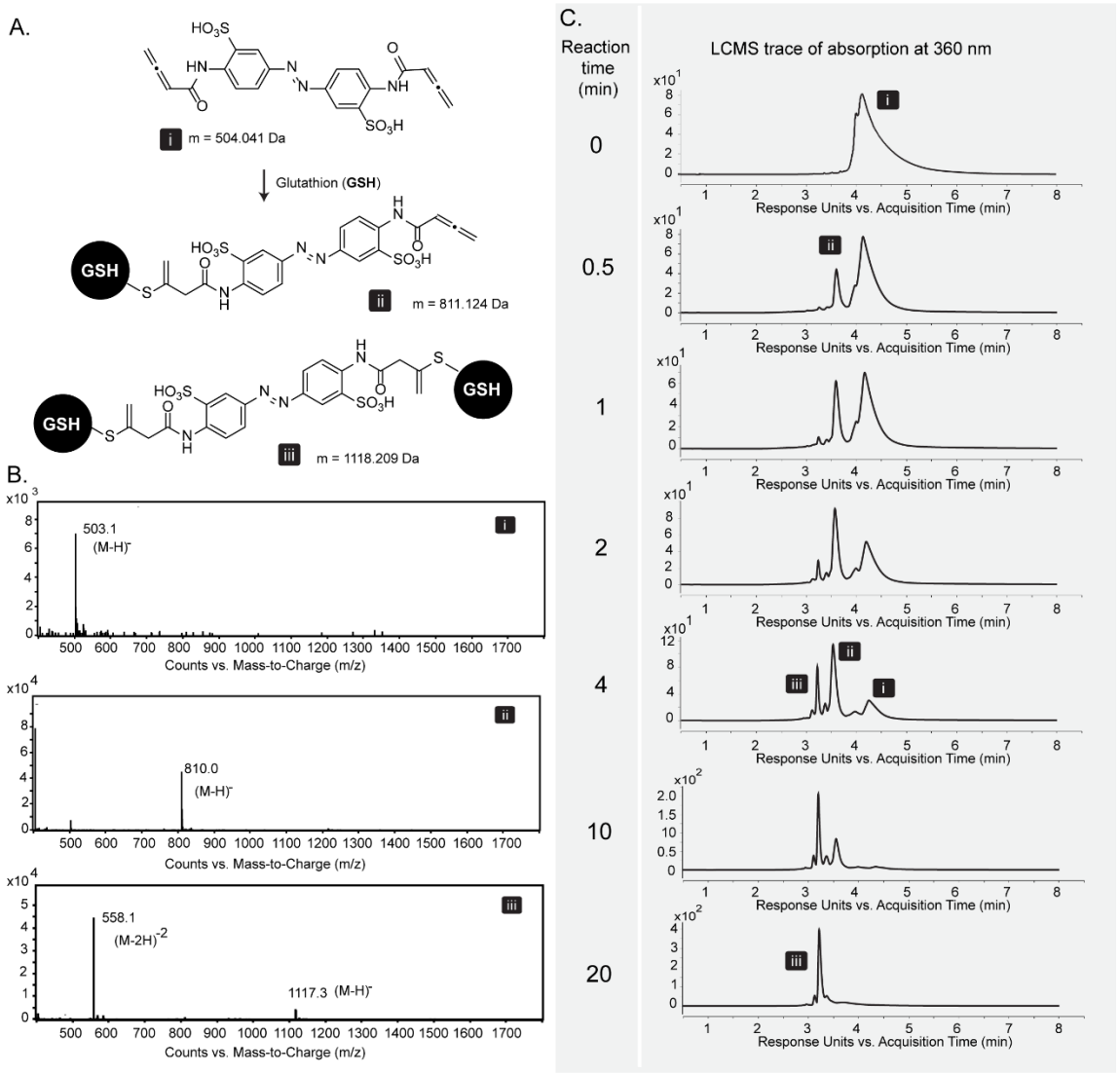


Figure S1. Kinetics of reaction of BSBDA with GSH. A) Reaction of BSBDA (**14**) with glutathione can result in formation of mono- and di-substituted product. B) Mass spectra of BSBDA (i), mono-substituted product (ii) and di-substituted product (iii). C) LCMS trace of the reaction of BSBDA with GSH shows the consumption of BSBDA and formation of di-substituted product in 20 min.

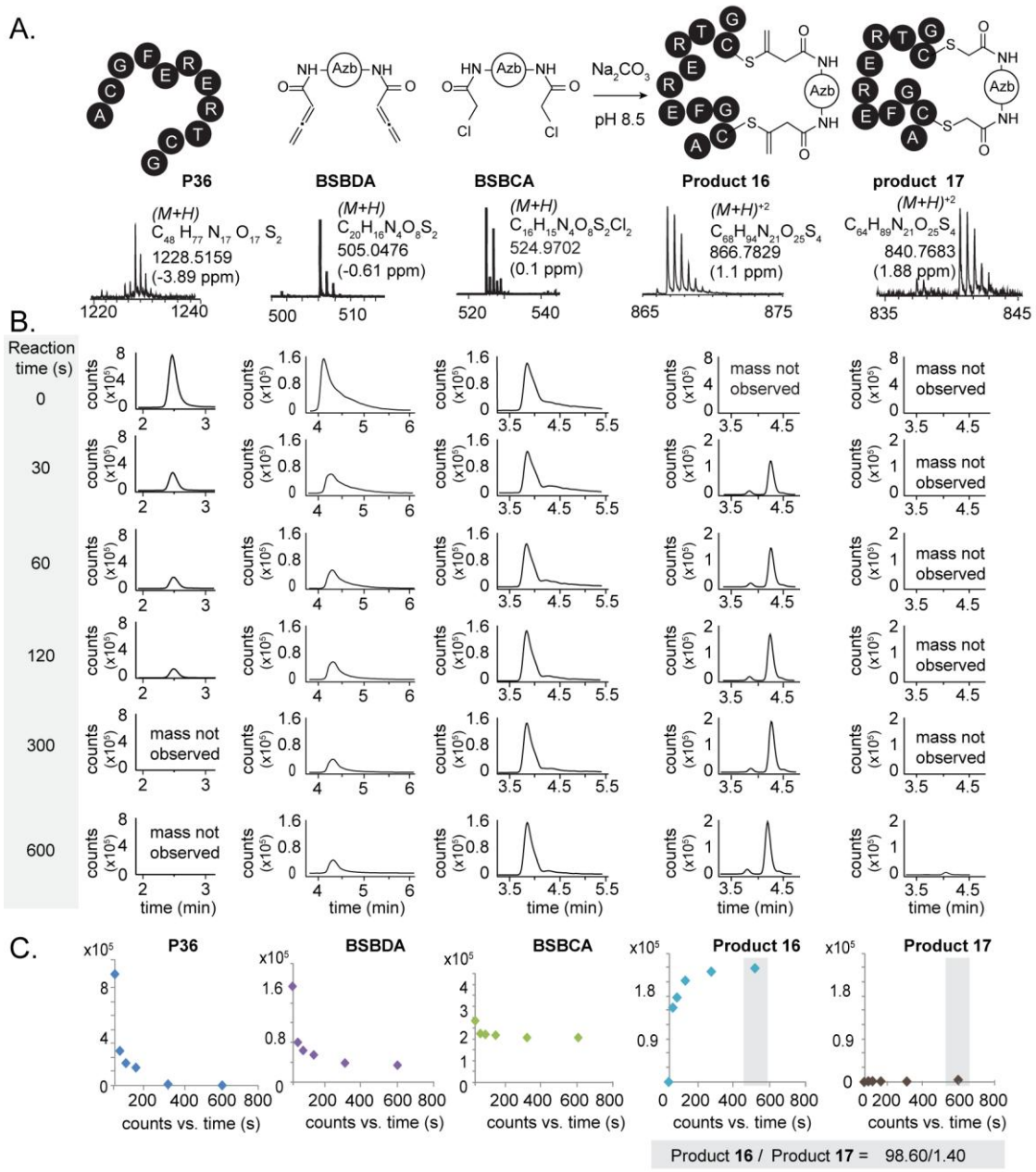


Figure S2. A) Cyclization of **P36** (100 µM) with BSBDA (160 µM) in the presence of BSBCA (160 µM). Over 85% of the product **16** was formed in 2 min. All of the peptide was consumed in 5 min. The ratio of product **16** to product **17** was 98.60/1.40 after 10 min. B) To monitor this reaction, at the indicated time points (0, 30, 600, 120, 300 and 600 s), we sampled 15 µL of the reaction, quenched it with 5 µL of acetic acid and injected 12 µL of this mixture onto Agilent Technologies nano-LC high-resolution-MS equipped with 6220 oaTOF detector. C) In each spectrum, we identified desired high-resolution MS peak (A), extracted and integrated the total ion count (EIC) elution profile for this fragment

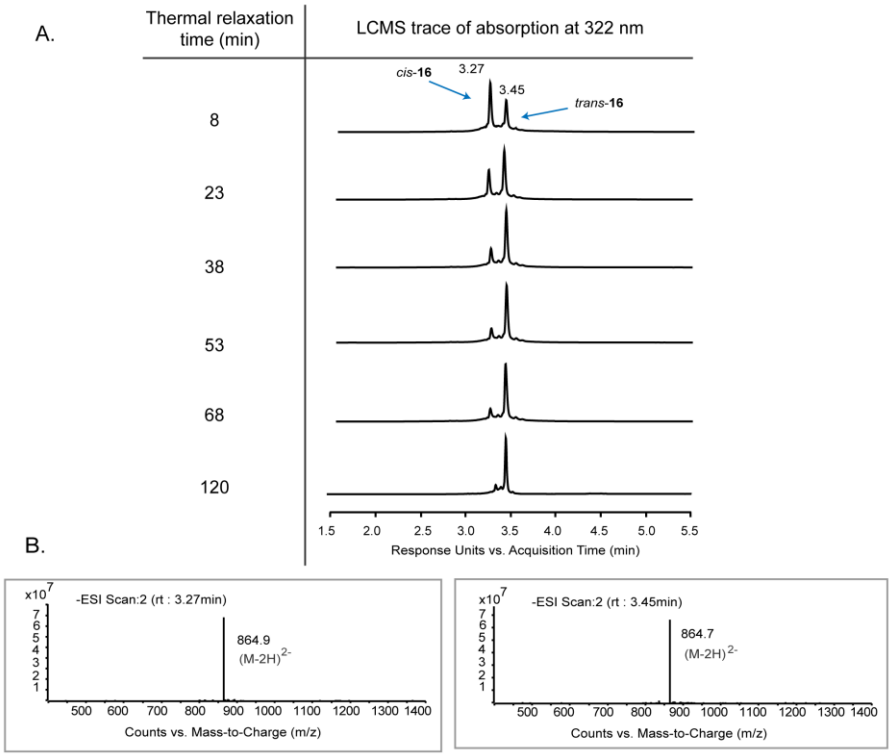
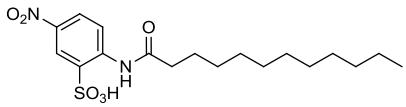


Figure S3. Kinetic of thermal relaxation of **16.** A) *Cis*- and *trans*-**16** can be isolated on C8 column using LCMS instrument after thermal relaxation. Changes in absorption of **16** at 322 nm (isosbestic point) 8, 23, 38, 53, 68 and 120 min of thermal relaxation after irradiation with 365 nm LED. B) Mass spectra of *cis* and *trans*-**16**.

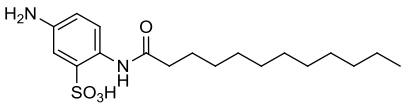
Synthesis of 2-(dodecanoylamino)-5-nitrobenzenesulfonic acid (**2**)



To a solution of sodium 2-amino-5-nitrobenzenesulfonate (1.2 g, 5 mmol, TCI Chemicals) in ice-cold dry DMF, we added lauroyl chloride (1.1 mL, 5.5 mmol, TCI Chemicals) drop wise. The reaction was warmed up to room temperature and then stirred at 110 °C for 4 h. The reaction was cooled down to r.t., the solvent was evaporated under vacuum and the resulting crude mixture was re-dissolved in 50 mL of DCM. The DCM solution was washed with 25 mL of 2 M HCl,

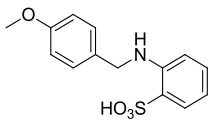
organic layer was separated and the DCM was removed by rotary evaporator. The resulting viscose liquid was purified by column chromatography and the product was eluted using DCM/MeOH = 8/2. The solvent was removed by rotary evaporator and the resulting pale yellow oil was incubated at room temperature under vacuum for 24 h to yield compound **9** as pale yellow solid (1.89 g, 92%). **^{1}H-NMR** (399.8 MHz, DMSO) δ 10.66 (br, 1 H, NH), 8.56 (d, *J* = 9.2 Hz, 2 H), 8.43 (d, *J* = 2.8 Hz, 2 H), 8.19 (d, *J* = 2.8 Hz, *J* = 9.2 Hz, 2 H), 2.34 (t, *J* = 7.6 Hz, 2 H), 1.59 (m, 2 H), 1.2 (m, 16 H), 0.81 (t, *J* = 6.8 Hz, 3 H) ppm. **^{13}C-NMR** (100.5 MHz, DMSO) δ 171.8, 141.4, 141.4, 135.6, 125.9, 122.9, 119.5, 38.1, 31.7, 29.5, 29.4, 29.2, 28.9, 25.1, 225.5, 14.3 ppm. **HRMS-ESI** (*m/z*)**:** $(M-H)^-$ calc. for: $C_{18}H_{27}N_2O_6S$: 399.1595, observed: 399.1594, 0.29 ppm.

Synthesis of 5-amino-2-(dodecanoylamino)benzenesulfonic acid (**3**)



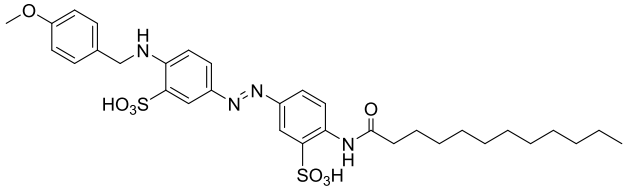
To a solution of **2** (1.2 g, 3 mmol) in 100 mL of MeOH, we added Pd/C (100 mg, 0.1 mmol) with vigorous stirring. We put a white septum on the flask and passed hydrogen through a needle for 2 min. Then we attached a balloon on the septum, filled it with hydrogen (~2 L) and let the reaction mixture to stir for 2 h. After completion of the reaction, we filtered the reaction mixture through celite and removed the solvent by rotary evaporator. The resulting solid was purified on silica by column chromatography and eluted by DCM/MeOH = 8/2 to yield **3** as pale yellow solid (1.09 g, 96%). **^{1}H-NMR** (399.8 MHz, DMSO) δ 10.0 (s, 1 H), 7.99 (s, *J* = 8.4 Hz, 2 H), 7.1 (d, *J* = 2.4 Hz, 2 H), 6.61 (dd, *J* = 2.4 Hz, *J* = 8.4 Hz, 2 H), 2.20 (t, *J* = 7.2 Hz, 2 H), 1.57 (t, *J* = 6.8 Hz, 2 H), 1.27-1.17 (m, 16 H), 0.85 (t, *J* = 6.4 Hz, 3 H) ppm. **^{13}C-NMR** (100.5 MHz, DMSO) δ 170.0, 141.2, 136.5, 126.8, 121.3, 116.4, 114.3, 55.5, 46.2, 38.2, 31.7, 29.5, 29.4, 29.4, 29.2, 29.2, 29.0, 25.5, 22.5, 14.4, 9.0. **HRMS-ESI** (*m/z*): $(M-H)^-$ calc. for: $C_{18}H_{29}N_2O_4S$: 369.1854, observed: 369.1847; 1.84 ppm.

Synthesis of sodium 2-[(4-methoxybenzyl)amino]-benzenesulfonate (**4**)



To 3.0 mL of anisaldehyde (30 mmol, TCI chemicals), we added 1.54 g (10 mmol) of 2-aminobenzenesulfonic acid. The resulting suspension was stirred overnight at 110 °C in the presence of activated molecular sieves. We then cooled down the reaction mixture to r.t. and diluted it with 30 mL DCM. The suspension was vacuum filtered through Whatman grade 1 filter paper and the yellow precipitate was washed 5 times with DCM. We then collected the solid residue and re-suspended it in 50 mL MeOH. The suspension was stirred on ice for 30 min and then 370 mg (10 mmol) of sodium borohydride was added in four portions. We stirred the suspension for 2 h on ice followed by 6 h at r.t. After quenching the excess of sodium borohydride with slow addition of ice-cold water to the mixture, we added HCl until the pH of the solution reached ~1 (as determined by universal pH paper). Cooling the solution on ice resulted in precipitation of white solid flakes, which were collected by vacuum filtration. Drying the solid *in vacuo* yielded the product **4** as a white solid (1.9 g, 65% over two steps). **^{1}H-NMR** (300.0 MHz, DMSO) δ 7.47 (dd, *J* = 2.4 Hz, *J* = 10 Hz, 1 H), 7.27 (dd, *J* = 2.8 Hz, *J* = 8.8 Hz, 2 H), 7.01 (t, *J* = 8.8 Hz, 1 H), 6.87 (dd, *J* = 2.8 Hz, *J* = 8.8 Hz, 2 H), 6.74 (t, *J* = 7.6 Hz, 1 H), 6.49-6.43 (m, 2 H), 4.24 (d, J = 7.6 Hz, 2 H), 3.72 (s, 3 H) ppm. **^{13}C-NMR** (100.5 MHz, DMSO) δ 158.6, 145.2, 132.0, 131.2, 130.4, 128.8, 127.6, 114.6, 114.2, 111.0, 55.5, 46.4 ppm. **HRMS-ESI** (*m/z*): $(M-H)^-$ calc. for: $C_{14}H_{14}NO_4S$ = 292.0649, observed: 292.0649; -0.5 ppm.

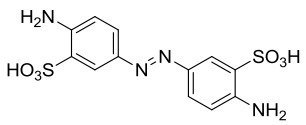
Synthesis of 3,3'-bis(sulfonato)-(4-methoxybenzyl)amido-(4'-dodecanoylamido)-azobenzene (**5**)



To an ice cold solution of 2-amino-5-nitrobenzenesulfonate (**3**) (1.85 g, 5 mmol) in 2 M HCl (4 mL) and MeOH (50 mL), we added 5 mL of a solution of 1 M $NaNO_2$ drop-wise. Stirring this solution for 1 h yielded the diazonium salt of **3**. We dissolved the PMB-2-ABS (**4**, 1.7 g, 6

mmol) in 2 mL of 2 M aqueous NaOH solution and diluted this solution by adding 20 mL of MeOH. This methanolic solution of **4** was stirred on ice for 20 min. We then added the diazonium salt suspension to the ice cold solution of **4** slowly with vigorous stirring. After stirring for 15 min, we added 100 mL of ice-cold DCM to the mixture and continued stirring vigorously on ice bath for 1 h. We noticed that adding the DCM was critical possibly because of higher solubility of the fatty acid protected diazonium salt in DCM. A 10 cm magnetic stir bar and high stirring speed (2000 rpm) were used to ensure proper mixing of the aqueous and organic phase. We then warmed up the reaction mixture to room temperature and increased the pH to 5.0 by slowly adding saturated solution of $NaHCO_3$ to the reaction. The reaction turned pale orange in 1 h and deep red after 18 h of stirring. We transferred the reaction mixture to a separatory funnel, separated the orange-colored organic layer, extracted the aqueous layer twice with 25 mL of DCM, combined organic layers and then removed the DCM by rotary evaporator. The resulting red crude solid was purified by column chromatography on silica and the product eluted in DCM/MeOH = 3/1. Removing of the solvent by a rotary evaporator yielded compound **5** as a dark orange solid (1.8 g, 55%). **^{1}H-NMR** (399.8 MHz, DMSO) δ 10.6 (s, 1 H), 8.48 (d, *J* = 8.4 Hz, 1 H), 8.12 (d, *J* = 2.0 Hz, 1 H), 8.08 (d, *J* = 2.0 Hz, 1 H), 7.78 (dd, *J* = 8.8 Hz, *J* = 2.0 Hz, 1 H), 7.72 (dd, *J* = 8.8 Hz, *J* = 2.0 Hz, 1 H), 7.31 (d, *J* = 8.4 Hz, 2 H), 6.92 (d, *J* = 8.4 Hz, 2 H), 6.70 (d, *J* = 8.8 Hz, 1 H), 4.42 (s, 2 H), 3.74 (s, 3 H), 2.31 (t, *J* = 7.6 Hz, 2 H), 1.62 (t, *J* = 7.6, 2 H), 1.29-1.14 (m, 16 H), 0.84 (t, *J* = 6.8, 3 H) ppm. **^{13}C-NMR** (100.5 MHz, DMSO) δ 171.2, 158.8, 147.9, 147.2, 142.0, 136.8, 136.1, 131.5, 1313.1, 128.9, 127.7, 125.4, 121.1, 120.2, 119.3, 114.4, 110.9, 55.5, 46.2, 38.1, 29.5, 29.4, 29.4, 29.3, 29.2, 29.2, 29.0, 25.3, 22.5, 14.4 ppm. **HRMS-ESI** (*m/z*): $(M-2H)^{2-}$ calc. for: $C_{32}H_{40}N_4O_8S_2$: 336.1149, observed: 336.1154; -1.52 ppm.

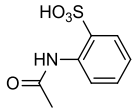
Synthesis of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6**)



To a suspension of 1.0 g (1.5 mmol) of compound **5** in 30 mL of 30% aqueous methanol, we slowly added 8 mL of 37% HCl in H_2O followed by 0.81 mL of anisole. The resulting solution was heated to reflux for 2 h at 110 °C and then cooled down to r.t. The solvent was removed us-

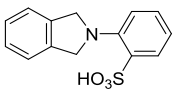
ing rotary evaporator and the resulting solid was washed 5 times with 50 mL DCM. Recrystallization of the product in EtOH/H_2O = 95/5 yielded the product as dark purple crystals (520 mg, 92%). **^{1}H-NMR** (400.0 MHz, D_2O) δ 7.99 (s, 2 H), 7.54 (d, *J* = 8.4 Hz, 2 H), 6.82 (d, *J* = 8.4 Hz, 2 H) ppm. **^{13}C-NMR** (125.7 MHz, 0.1% TFA in D_2O) δ 147.9, 143.8, 126.5, 126.2, 124.7, 118.9 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for: $C_{12}H_{11}N_4O_6S_2$, 371.0125, observed: 371.0135; -2.42 ppm.

Synthesis of 2-(acetylamino)benzenesulfonic acid (**7)**



To a solution of 2-aminobenzene sulfonic acid (0.7 g, 4 mmol) in 50 mL of acetic acid, we slowly added 0.36 mL of acetyl chloride (5 mL) and stirred the resulting mixture for 2 h at 100 °C. Removing the solvent by rotary evaporator yielded product **7** as a pale pink solid (0.86 g, quantitative). **^{1}H-NMR** (400.0 MHz, DMSO) δ 10.31 (s, 1 H), 8.21 (d, *J* = 8.0 Hz, 1 H), 7.62 (dd, *J* = 7.6 Hz, *J* = 1.6 Hz, 1 H), 7.24 (dt, *J* = 8.4, *J* = 1.6 Hz, 1 H), 6.96 (dt, *J* = 1.6 Hz, *J* = 7.6 Hz, 1 H), 1.99 (s, 3 H) ppm. **^{13}C-NMR** (100.6 MHz, DMSO) δ 168.1, 135.4, 130.2, 127.3, 122.7, 120.15, 25.3 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for $C_8H_8NO_4S$: 214.018, observed: 214.0176; 1.85 ppm.

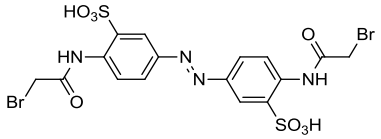
Synthesis of 2-(1,3-dihydro-2*H*-isoindol-2-yl)benzenesulfonic acid (**9)**



To a suspension of 2-aminobenzene sulfonic acid (0.34 g, 2 mmol) in 15 mL DMF, we added of dibromomethylbenzene (0.58 g, 2.2 mmol) and NaOH (100 mg, 2.2 mmol). We warmed up the mixture to 90 ° C using oil bath and then stirred the resulting solution for 1 h. After removal of the solvent by rotary evaporator we purified the product by column chromatography. The product was eluted by DCM/MeOH = 95/5. Removing the solvent by rotary evaporator yielded the product as grey powder (0.37 g, 68%). **^{1}H-NMR** (400.0 MHz, DMSO) δ 7.85 (dd, *J* = 2.4, *J* = 5.6 Hz, 1 H), 7.65 (dd, *J* = 2.4 Hz, *J* = 5.6 Hz, 1 H), 7.56 (m, 2 H), 7.43 (m, 4 H), 5.20 (s, 4 H)

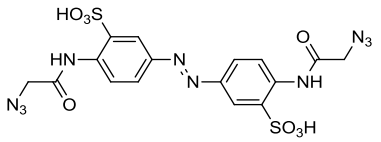
ppm. **^{13}C-NMR** (100.5 MHz, DMSO) δ 139.3, 137.6, 134.4, 132.4, 130.1, 129.3, 128.2, 123.4, 121.6, 63.4 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for $C_{14}H_{12}NO_3S$: 274.0543, observed: 274.0541; -0.99 ppm.

Synthesis of 3,3'-bis(sulfonato)-4,4'-bis(bromoacetamido)azobenzene (**11**)



To an ice cold solution of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6**, 50 mg, 0.13 mmol) in 5 mL of dry DMF, we added 35 μL (0.39 mmol) of bromoacetyl bromide, followed by addition of 135 μL of DIPEA (0.78 mmol) with vigorous stirring. The solution was warmed up to r.t. in 15 min and was stirred for an additional 30 min in that temperature. The progress of the reaction was monitored by LCMS. The reaction mixture was purified by HPLC on a C18 silica column. The product was eluted in H_2O/MeCN:70/30. After collecting the fractions, the solvent volume was reduced to 1.0 mL on a rotary evaporator and the solution was lyophilized to yield product **11** as a dark red powder (63 mg, 79%). We note that the same reaction did not yield the desired product when bromoacetyl chloride was used as the acylating agent, because the chloride ion displaced the bromide during the course of the reaction. **^{1}H-NMR** (400.0 MHz, D_2O) δ 8.30 (d, *J* = 2 Hz, 2 H), 8.20 (d, *J* = 8.8 Hz, 2 H), 7.97 (d, *J* = 8.8 Hz, *J* = 2 Hz, 2 H), 4.25 (s, 4 H) ppm. **^{13}C-NMR** (125.6 MHz, D_2O) δ 168.6, 149.1, 136.7, 134.9, 127.0, 124.9, 122.8, 30.2 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for: $C_{16}H_{13}Br_2N_4O_8S_2$: 610.8547, observed: 610.8555; 1.3 ppm.

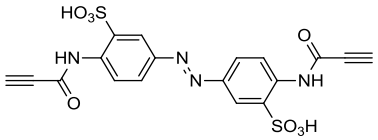
Synthesis of 3,3'-bis(sulfonato)-4,4'-bis(azidoacetamido)azobenzene (**12**)



To an ice cold solution of azidoacetic acid (0.16 mL, 2.1 mmol) in 0.4 mL of chloroform, we added 1.8 mL (2.1 mmol) oxalyl chloride. After stirring for 15 minutes at 0 °C, we added 10 μL of DMF to the mixture. The reaction mixture was stirred for 15 minutes on ice, followed by warming up to r.t. and stirring for an additional 90 minutes until the effervescence was stopped.

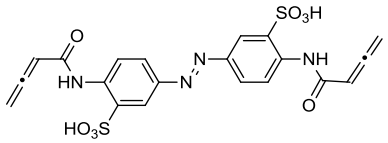
We then added this solution to an ice cold solution of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6,** 50 mg, 0.13 mmol) in 5 mL of dry DMF followed by addition of 0.7 mL of DIPEA. The reaction was warmed up to r.t. and stirred for 15 min. The reaction mixture was purified by HPLC on a C18 silica column. The product was eluted in H_2O/MeCN = 70/30. After collecting the fractions, the solvent volume was reduced to 1.0 mL on a rotary evaporator and the solution was lyophilized to yield product **12** as a dark red solid (64 mg, 89%). **^{1}H-NMR** (400.0 MHz, DMSO) δ 11.06 (s, 2 H), 8.55 (d, *J* = 8.8 Hz, 2 H), 8.20 (d, *J* = 2.4 Hz, 2 H), 7.96 (dd, *J* = 8.8 Hz, *J* = 2.4 Hz, 2 H), 4.25 (s, 4 H) ppm. **^{13}C-NMR** (125.7 MHz, DMSO) δ 166.1, 146.8, 136.8, 136.4, 126.0, 120.16, 119.4, 52.3 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for: $C_{16}H_{13}N_{10}O_8S_2$: 537.0365, observed: 537.0369; -0.86 ppm.

Synthesis of 3,3'-bis(sulfonato)-4,4'-bis(prop-2-ynoylamido)azobenzene (**13**)



To 0.10 mL of ice cold propiolic acid (0.01 g, 1.4 mmol), we slowly added 0.11 mL (0.16 g, 1.4 mmol) of oxalyl chloride at r.t. After stirring for 15 min, we cooled down the reaction to 0 °C on ice bath and added 10 μL of dry DMF. The reaction mixture was stirred on ice for 15 min and then for an additional 45 min at r.t. until effervescence stopped. We then added this solution drop-wise, using a Gilson P200 pipette, to a solution of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6**, 20 mg, 0.05 mmol) in 2 mL of 1.0 M sodium carbonate in water, at r.t. over 15 min. We stirred the reaction mixture for an additional 30 min at r.t. The reaction mixture was purified by HPLC on a C18 silica column. The product was eluted in H_2O/MeCN = 60/40. After collecting the fractions, the solvent volume was reduced to 1.0 mL on a rotary evaporator and the solution was lyophilized to yield product as a yellow-orange solid (17.4 mg, 69%). **^{1}H-NMR** (400.0 MHz, DMSO) δ 8.45 (d, *J* = 8.8 Hz, 2 H), 8.20 (d, *J* = 2.4 Hz, 2 H), 7.98 (dd, *J* = 8.8 Hz, *J* = 2.4 Hz, 2 H), 4.57 (s, 2 H) ppm. **^{13}C-NMR** (125.7 MHz, D_2O, 10% DMSO-d_6) δ 152.8, 149.2, 136.2, 134.8, 127.0, 125.2, 122.9, 79.1, 77.3 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for: $C_{18}H_{11}N_4O_8S_2$: 475.0024, observed: 475.0035; 2.4 ppm.

Synthesis of 3,3'-bis(sulfonato)-4,4'-bis(buta-2,3-dienoylamido)azobenzene (**14**)



To a suspension of 2-butynoic acid (250 mg, 3 mmol) in 0.5 mL chloroform, we slowly added 0.26 mL of oxalyl chloride. After stirring for 15 min at r.t., we cooled down the reaction to 0 °C on ice bath and added 20 μL of dry DMF. The reaction mixture was stirred on ice for 15 min and then for an additional 90 min at r.t. until effervescence stopped and solids dissolved completely. We then added this solution drop-wise, using a Gilson P200 pipette to a solution of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6,** 100 mg, 0.27 mmol) in 20 mL of saturated sodium bicarbonate in water, at r.t. over 15 min. The reaction was stirred for an additional 30 min at r.t. The reaction mixture was washed with 2 × 20 mL DCM, concentrated to 5 mL on a rotary evaporator and purified by HPLC on a C18 silica column. The product was eluted in H_2O/MeCN = 70/30. After collecting the fractions, the solvent volume was reduced to 1.0 mL on a rotary evaporator and the solution was lyophilized to yield product **14** as a yellow-orange solid (88 mg, 65%). **^{1}H-NMR** (399.8 MHz, D_2O) δ 8.25 (d, *J* = 2.4 Hz, 2 H), 8.23 (d, *J* = 9.2 Hz, 2 H), 7.85 (dd, *J* = 2.4 Hz, *J* = 9.2 Hz, 2 H), 5.86 (t, *J* = 6.4 Hz, 2 H), 5.58 (d, *J* = 6.4 Hz, 4 H) ppm. **^{13}C-NMR** (125.7 MHz, D_2O) δ 213.8, 167.0, 148.3, 137.3, 133.4, 127.1, 123.4, 122.8, 91.5, 83.2 ppm. **HRMS-ESI** (*m/z*): (M-H) calc. for $C_{20}H_{15}N_4O_8S_2$: 503.0345, observed: 503.0337; 1.57 ppm.

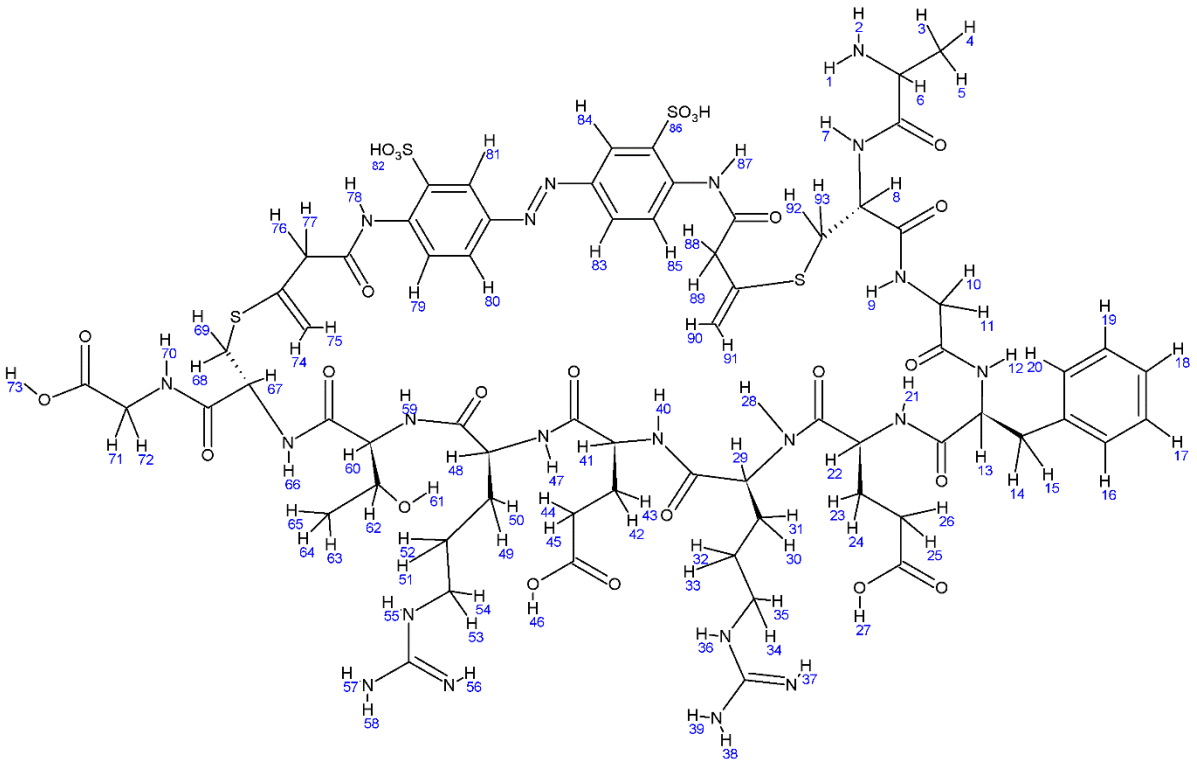
Synthesis of cyclized peptide ACGFERETCG (**16**)

To a 1 mL solution of 5.5 mg of peptide 36 (with amino acid sequence of NH_2-ACGFERERTCG-CO_2H, 0.004 mmol) in Tris buffer (50 mM, pH 8.5) we added 2.2 mg of BSBDA (**14**), followed by 40 μL of 100 mM aqueous TCEP solution. The solution was incubated for 15 minutes in dark at r.t. before purification by HPLC over C18 column. Collected fractions were concentrated to 1 mL using rotary evaporator and then lyophilized to yield the product as a yellow powder (4.2 mg, 82%). **HRMS-ESI** (*m/z*): $(M-2H)^{-2}$ calc. for $C_{68}H_{91}N_{21}O_{25}S_4$: 864.7694, observed: 864.7715; 2.37 ppm.

Residue	NH (#)	NH (δ)	Hα (#)	Hα (δ)	Hβ (#)	Hβ (δ)	Hγ (#)	Hγ (δ)	Hδ (#)	Hδ (δ)
Ala	1,2	---	6	4.1	3,4,5	1.48	---	---	---	---
Cys	7	8.77	8	4.65	92,93	3.22	---	---	---	---
Gly	9	8.33	10,11	3.95	---	---	---	---	---	---
Phe	12	7.64	13	4.35	14,15	2.82	---	---	---	---
Glu	21	8.04	22	4.12	23,24	1.89, 1.78	25,26	2.23	---	---
Arg	28	7.87	29	4.13	30,31	1.56, 1.65	32,33	1.4	34,35	2.98
Glu	40	8.18	41	4.22	42,43	1.9, 2.0	44,45	2.4	---	---
Arg	47	8.2	48	4.27	49,50	1.62, 1.73	51,52	1.41	53,54	2.96
Thr	59	7.96	60	4.25	62	4.2	63-65	1.1	---	---
Cys	66	8.21	67	4.65	68,69	3.21	---	---	---	---
Gly	70	8.37	71,72	3.67	---	---	---	---	---	---
BSBDA	78	9.95	---	---	---	---	---	---	---	---
BSBDA	87	9.93	---	---	---	---	---	---	---	---

Residue	functionality	H (#)	H (δ)
Arg	guanidine	55-58 ,36-39	---
Phe	aromatic	16,17,18,19,20,	7.15, 7.15, 7.17
BSBDA	aromatic	79-81, 83-85	8.28, 8.27, 8.21, 7.88, 7.85
BSBDA	alkene	74,75	5.51, 5.54
BSBDA	alkene	90,91	5.29,5.31
BSBDA	sulfonate	82,86	---
BSBDA	methylene	76,77	3.40, 3.45
Gly	carboxylic acid	73	---
Thr	Hydroxyl	61	---
Glu	carboxylic acid	27,46	---

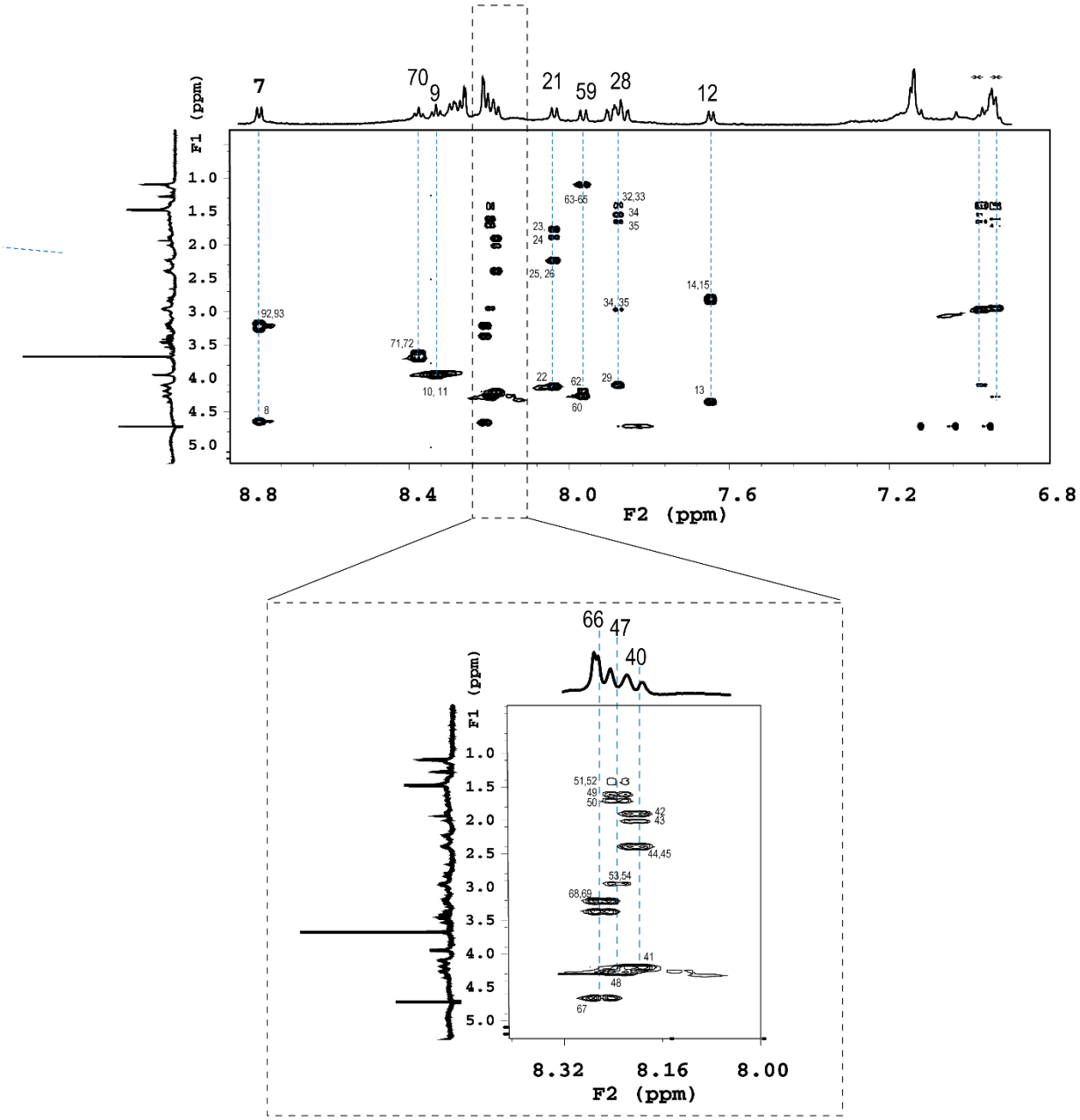
Table S1. NMR signal of protons in the cyclized peptide **16.** The hydrogens highlighted in grey in table were exchanged with deuterium and hence were not detectable. Chemical shifts are reported in ppm. 1D-NMR, TOCSY, COSY and ROESY were run using a 3.5 mM peptide solution sample in H_2O/D_2O (90/10) supplemented with 0.1% trifluoroacetic acid. For the structure of the peptide and hydrogen numbering see Scheme 2 (next page).



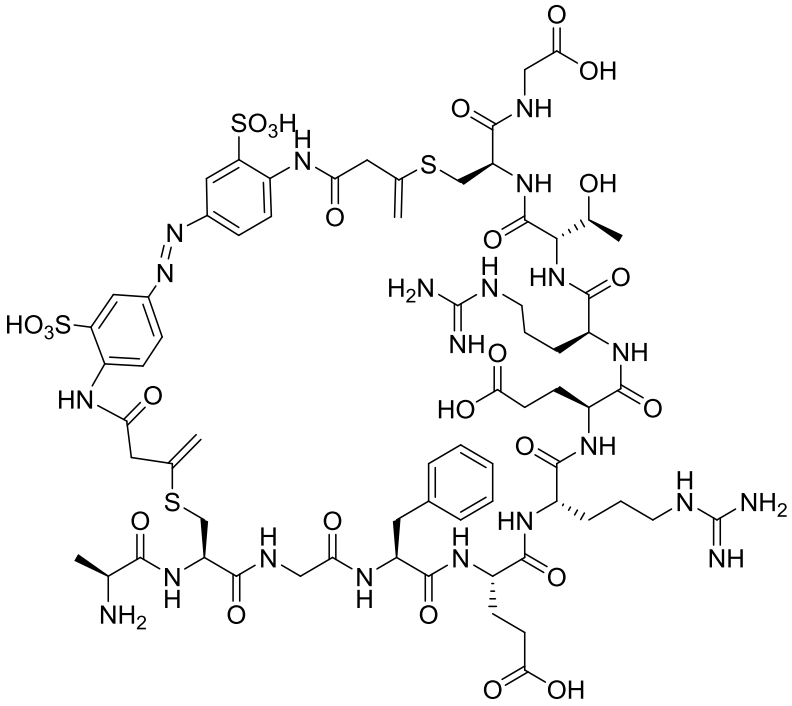
Scheme 2. Structure of **16** with explicit hydrogens.

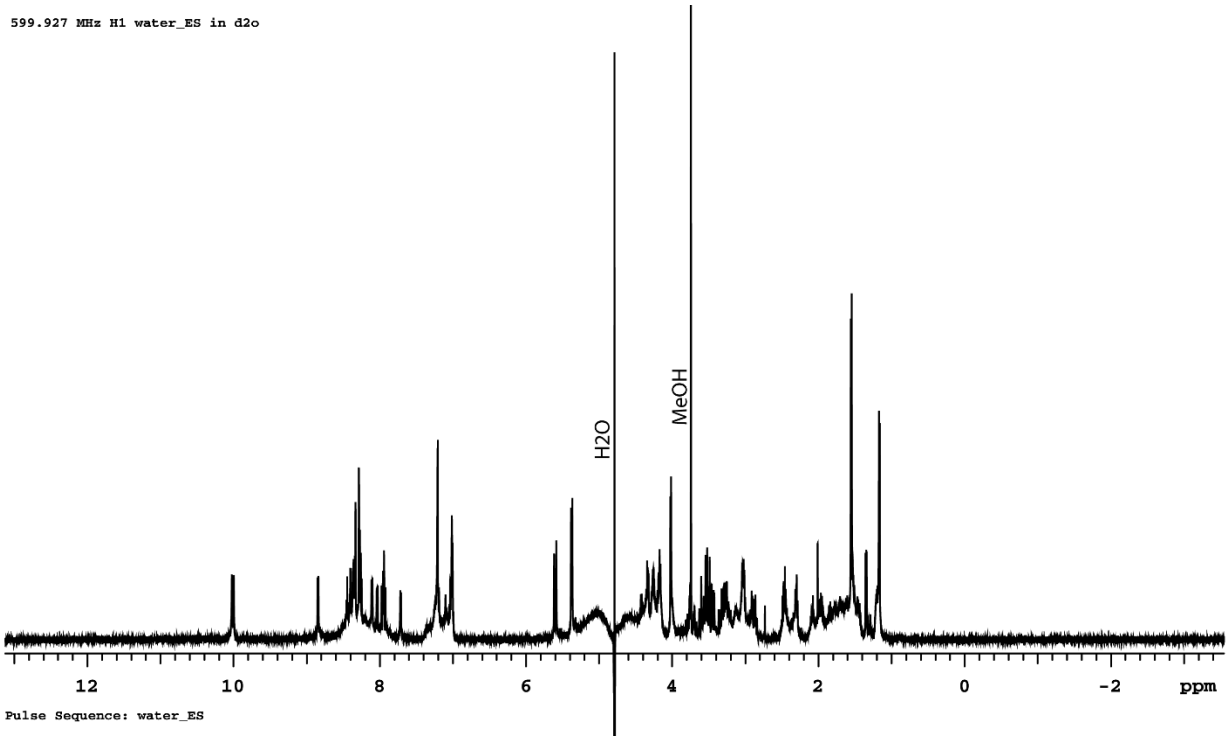
TOCSY-NMR of cyclized peptide ACGFERETCG (**16**)

For numbering of protons, see Table S1 and Scheme 2.

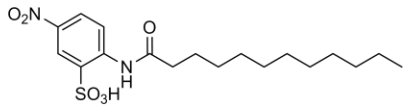


^{1}H- NMR of cyclized peptide ACGFERETCG (**16**)

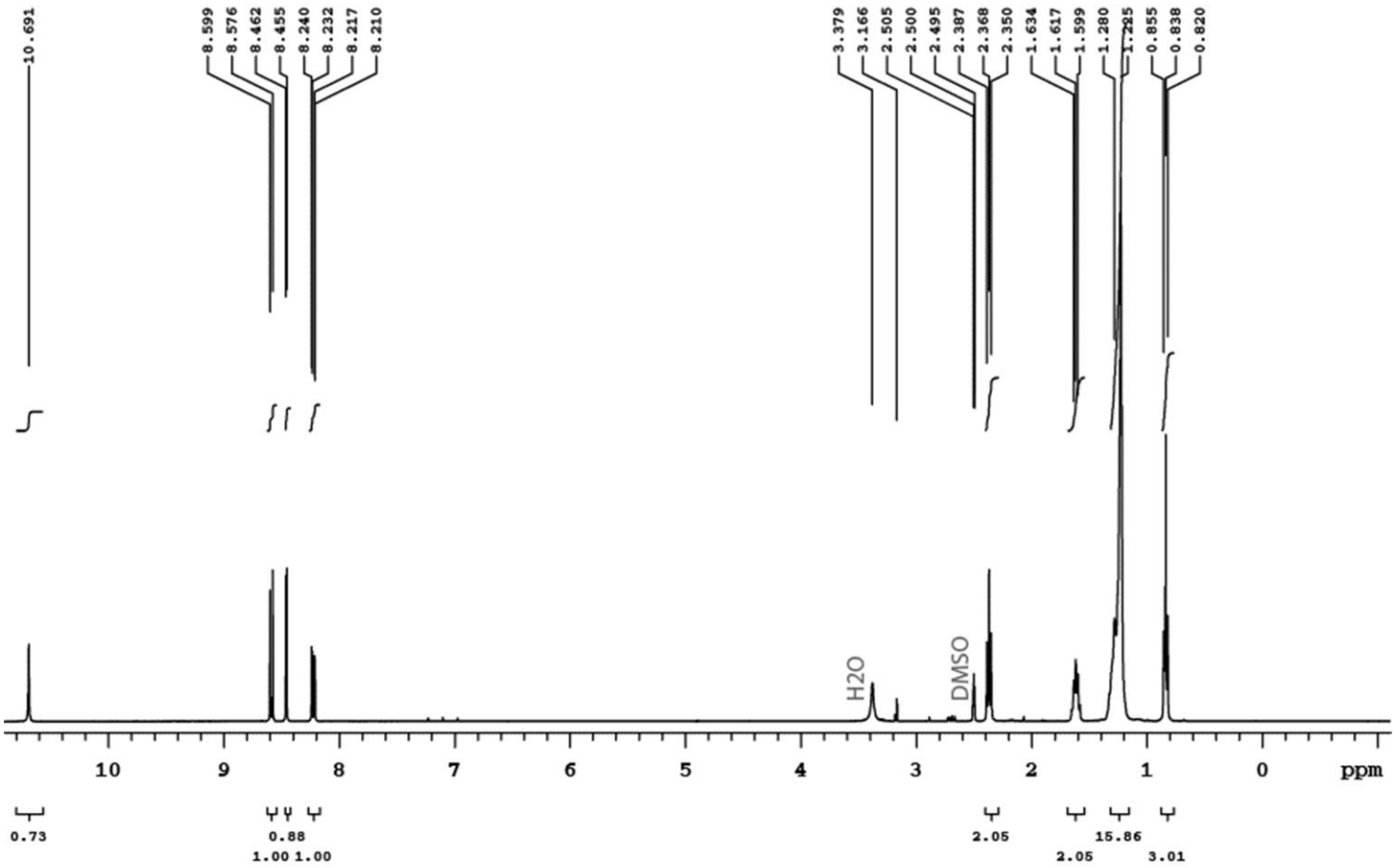




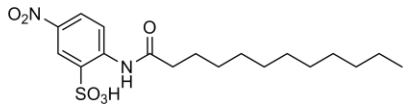
^{1}H-NMR of **2**



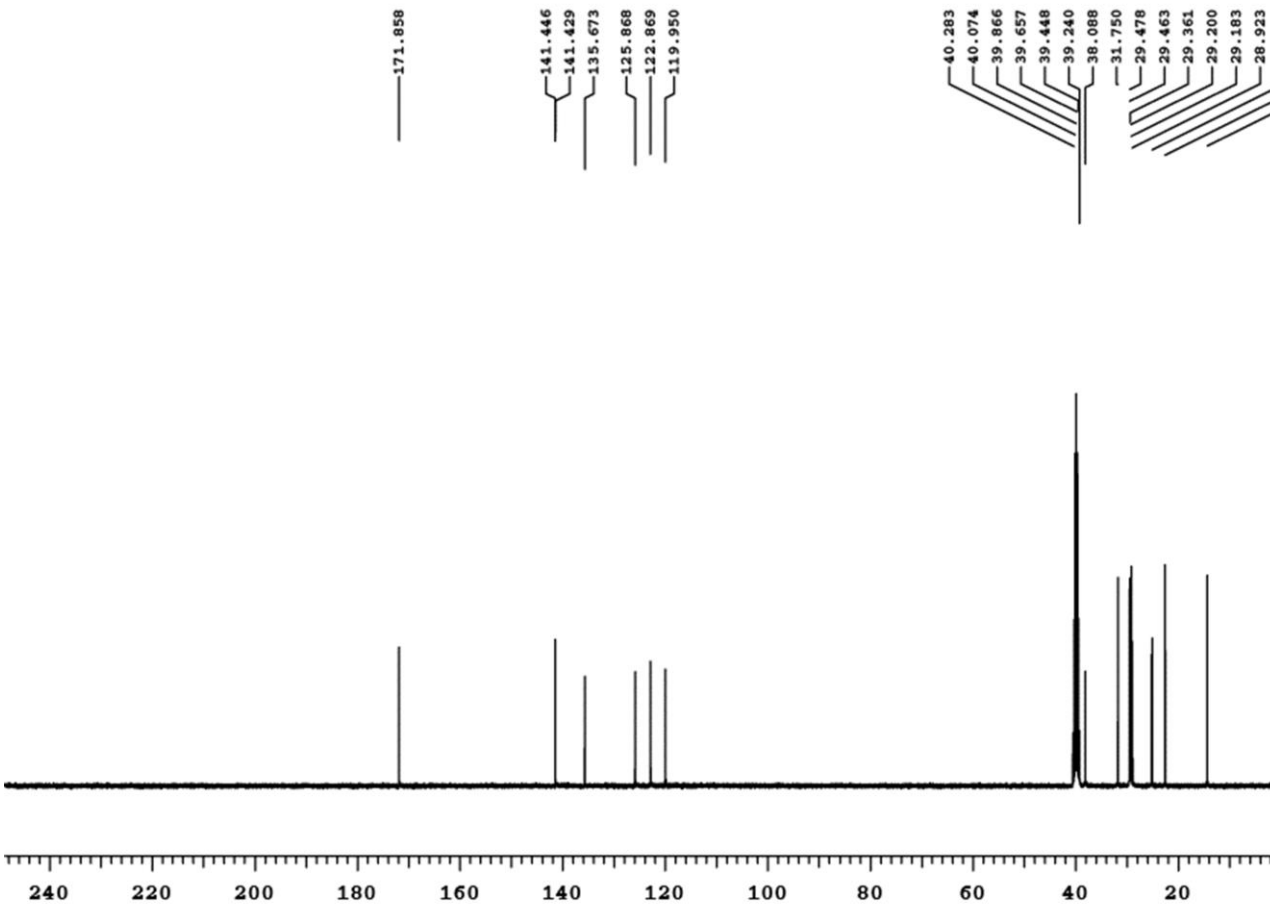
399.796 MHz H1 1D in dmso (ref. to DMSO @ 2.49 ppm), temp 26.5 C -> actual temp = 27.0 C, autoxdb probe



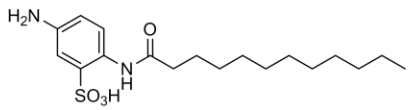
^{13}C-NMR of **2**



100.540 MHz C13[H1] 1D in dmso (ref. to DMSO @ 39.5 ppm), temp 26.5 C -> actual temp = 27.0 C, autoxdb probe

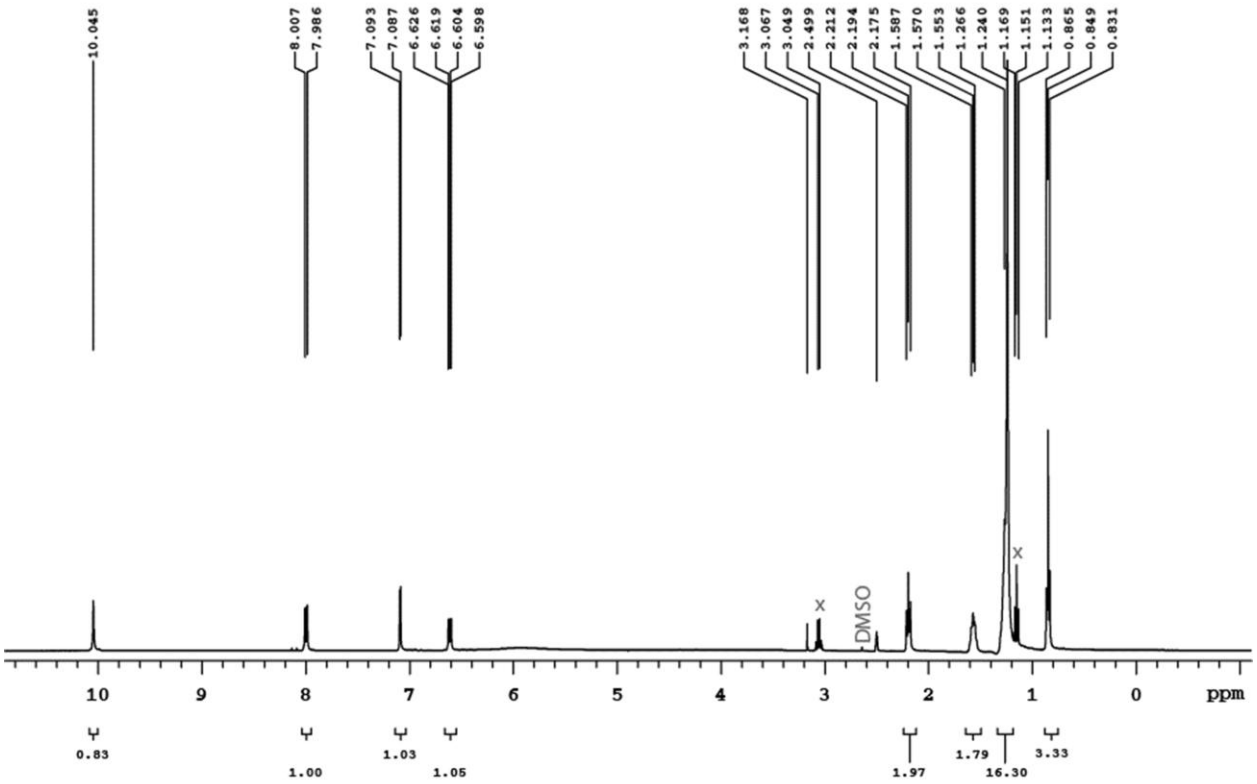


^{1}H-NMR of **3**

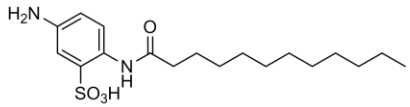


The peaks marked with asterisk (*) show residual ethyl acetate.

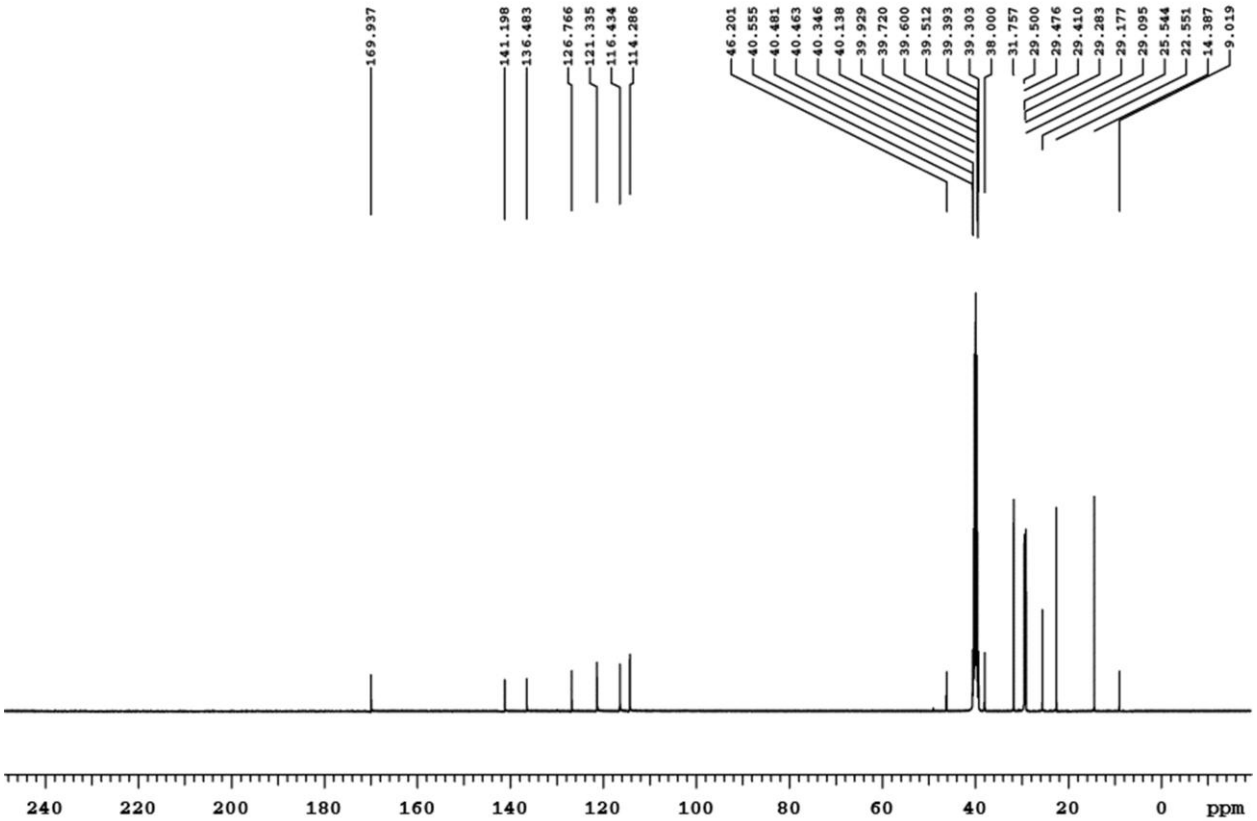
399.796 MHz H1 1D in dmso (ref. to DMSO @ 2.49 ppm), temp 26.5 C -> actual temp = 27.0 C, autoxdb probe



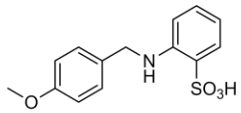
^{13}C-NMR of **3**

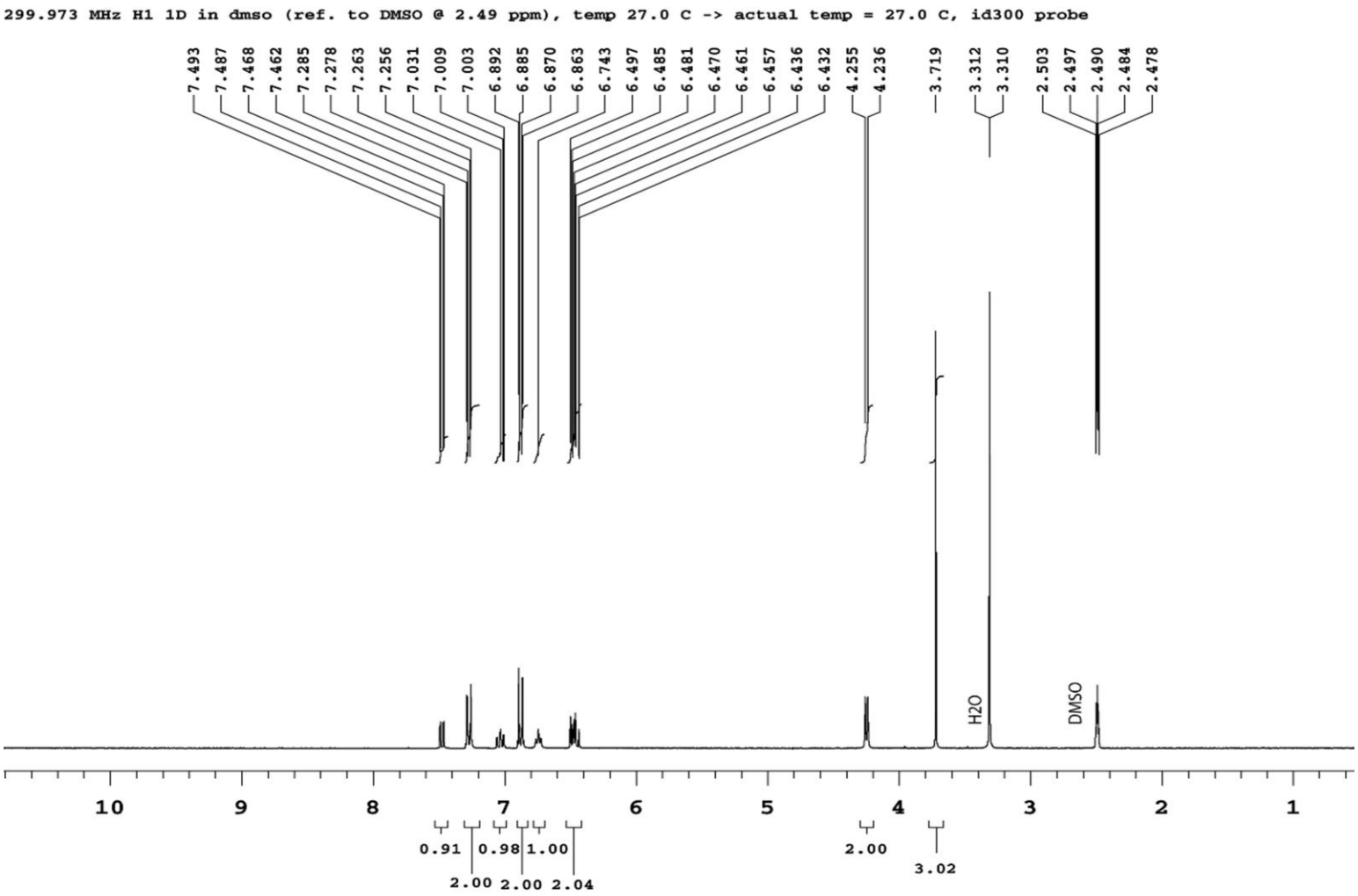


100.540 MHz C13[H1] 1D in dmso (ref. to DMSO @ 39.5 ppm), temp 26.5 C -> actual temp = 27.0 C, autoxdb probe

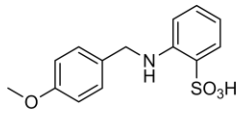


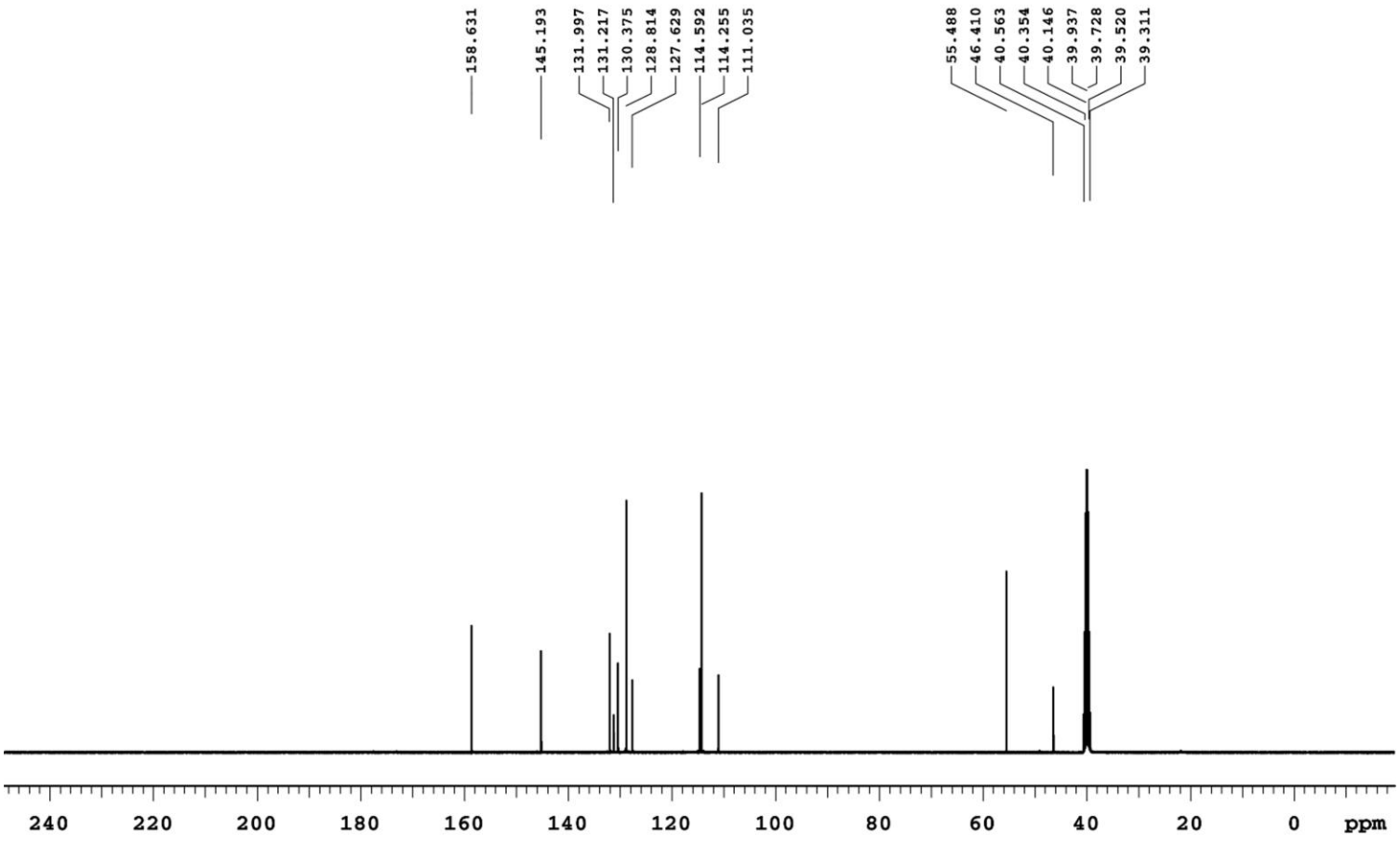
^{1}H-NMR of 2-[(4-methoxybenzyl)amino]benzenesulfonic acid (**4)**



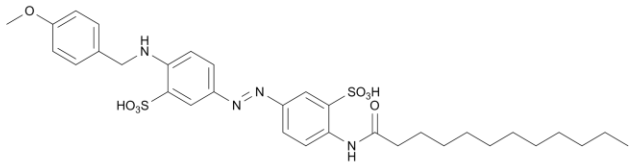


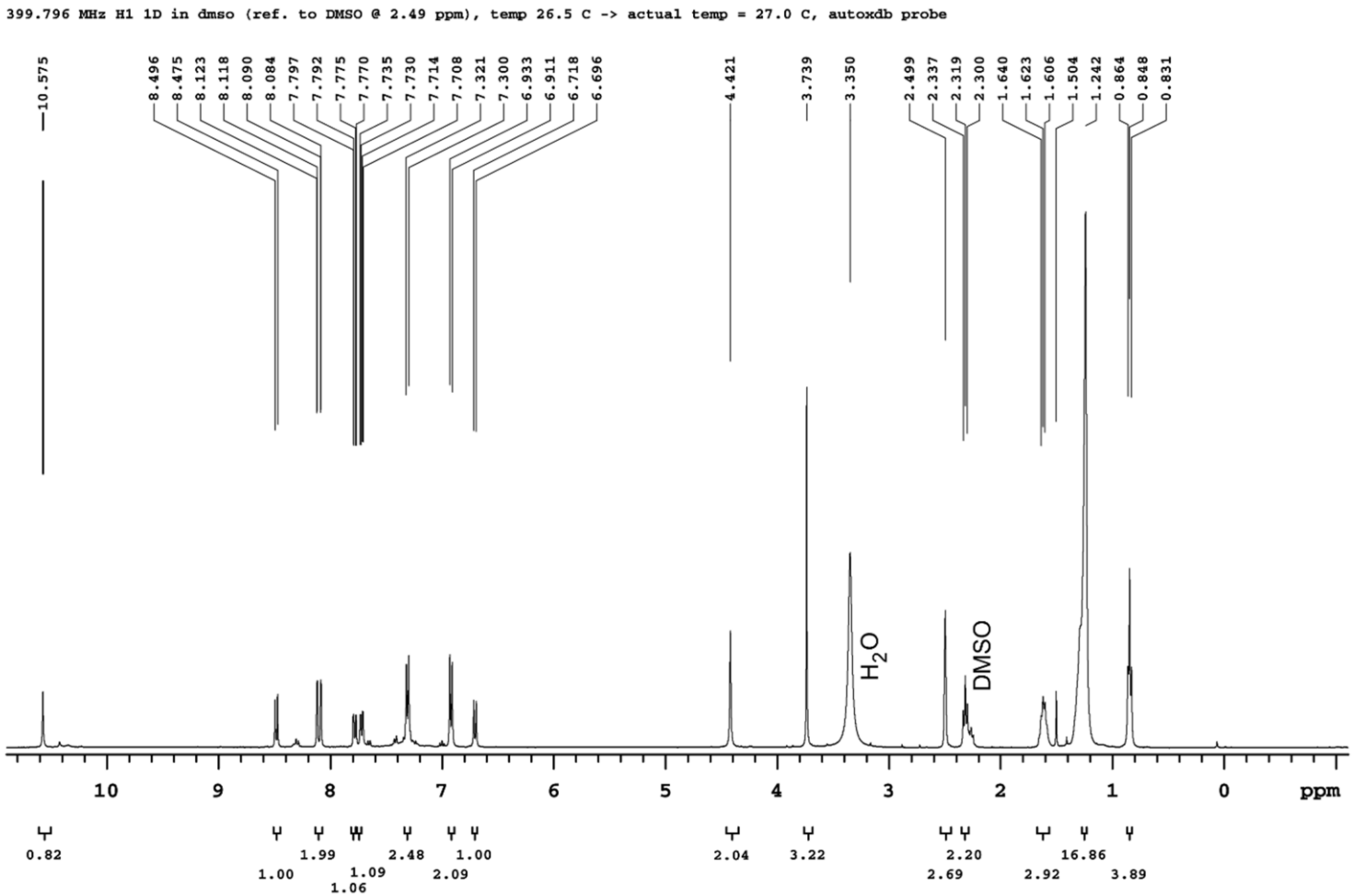
^{13}C-NMR of 2-[(4-methoxybenzyl)amino]benzenesulfonic acid (**4)**



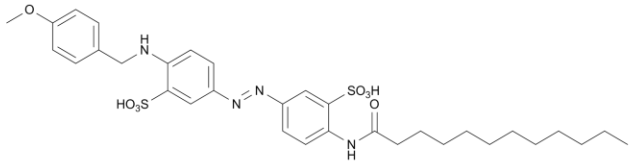


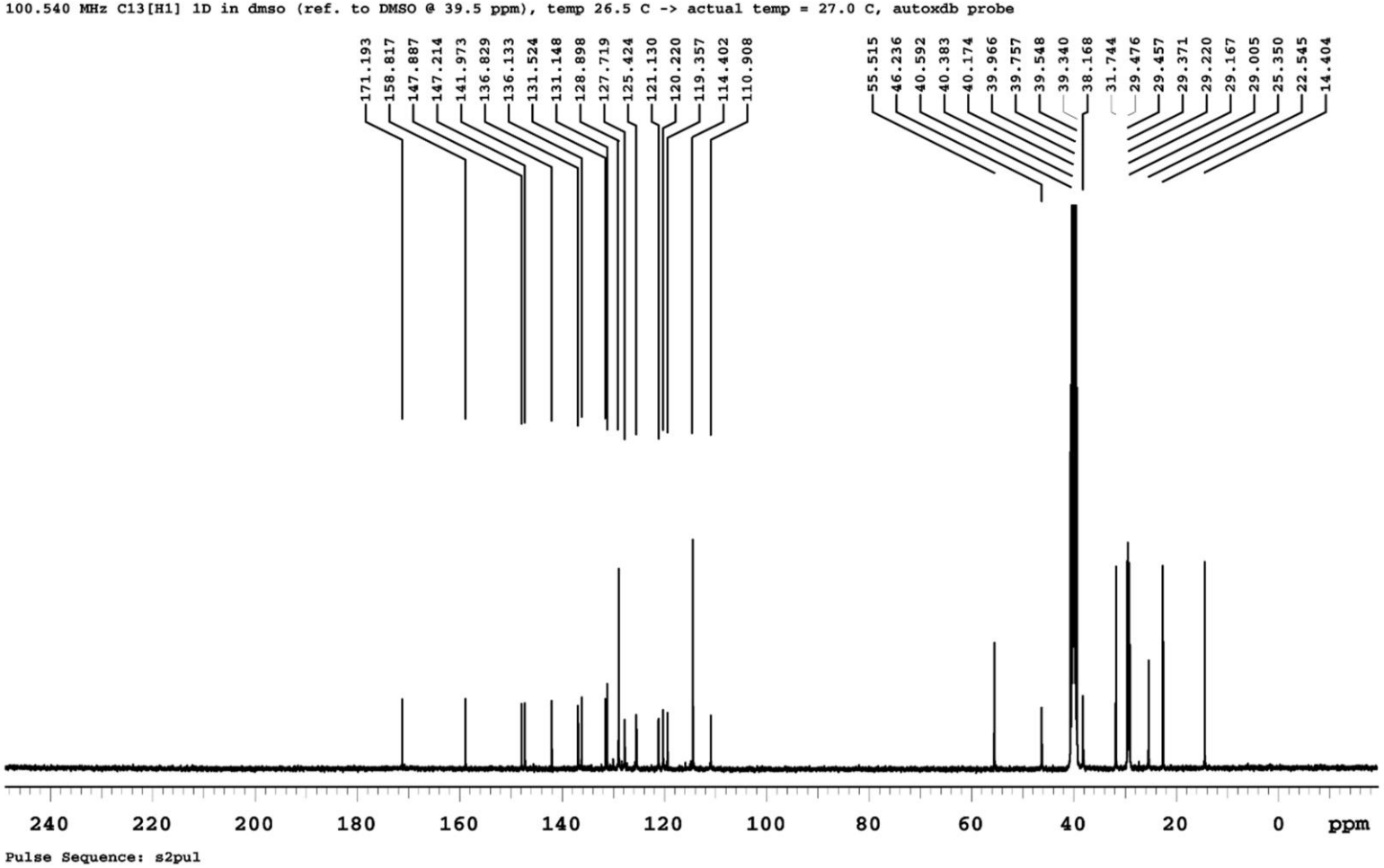
[1]H-NMR of 3,3'-bis(sulfonato)-(4-methoxybenzyl)amido-(4'-dodecanoylamido)-azobenzene (**5**)



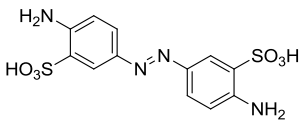


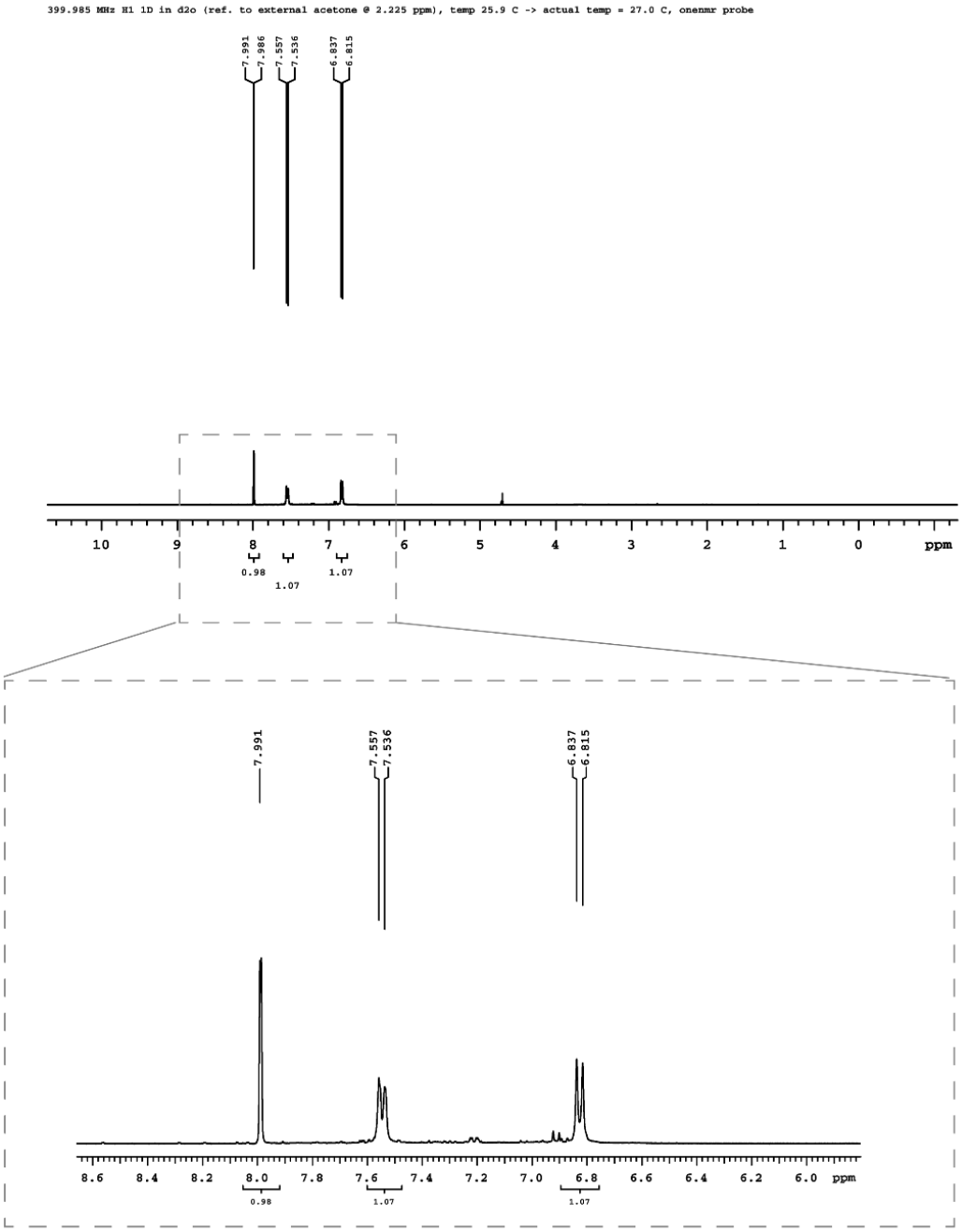
^{13}C-NMR of 3,3'-bis(sulfonato)-(4-methoxybenzyl)amido-(4'-dodecanoylamido)-azobenzene (**5**)





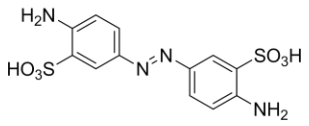
^{1}H-NMR of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6**)

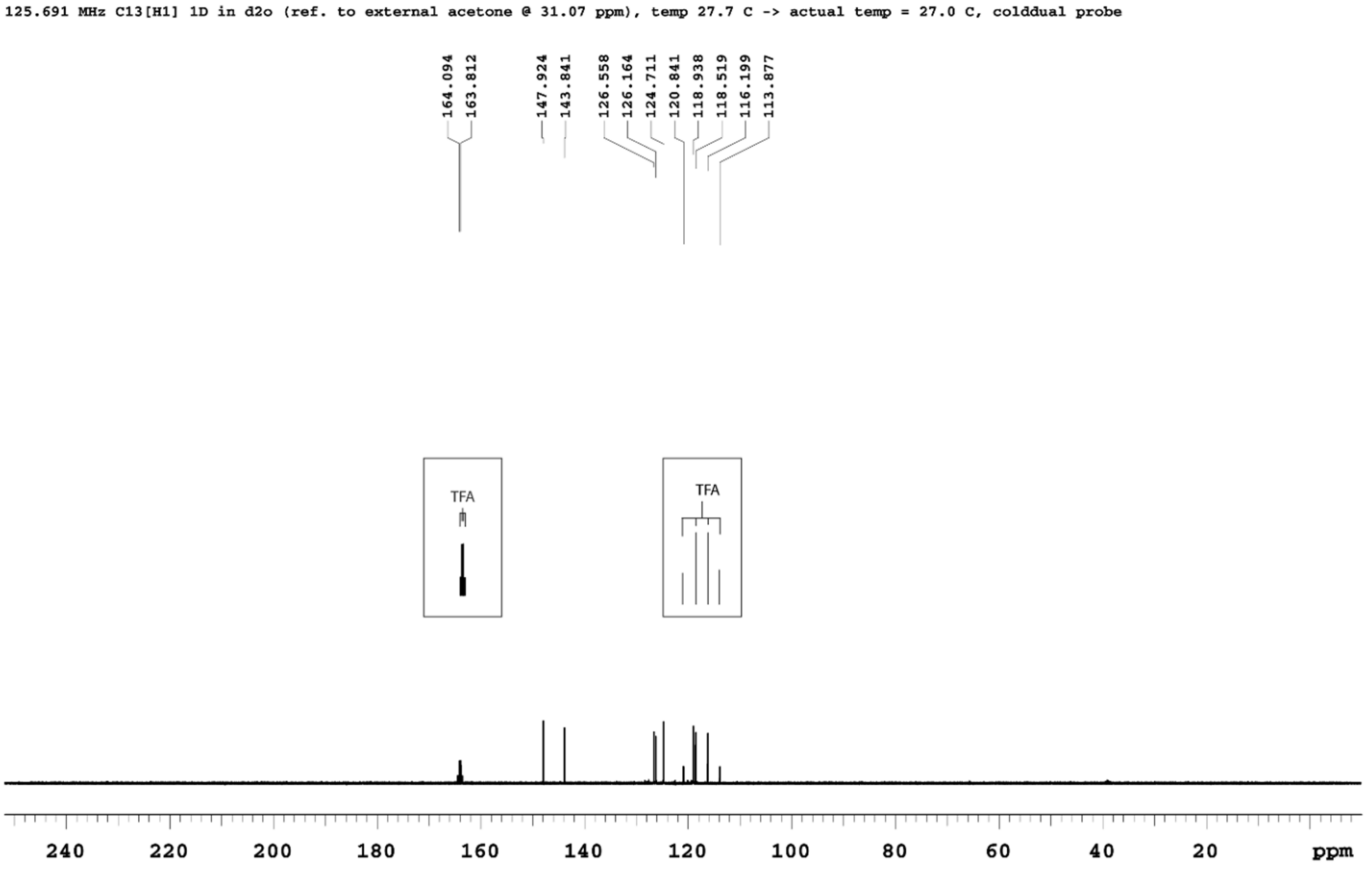




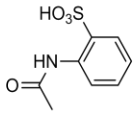
^{13}C-NMR of 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene (**6**)

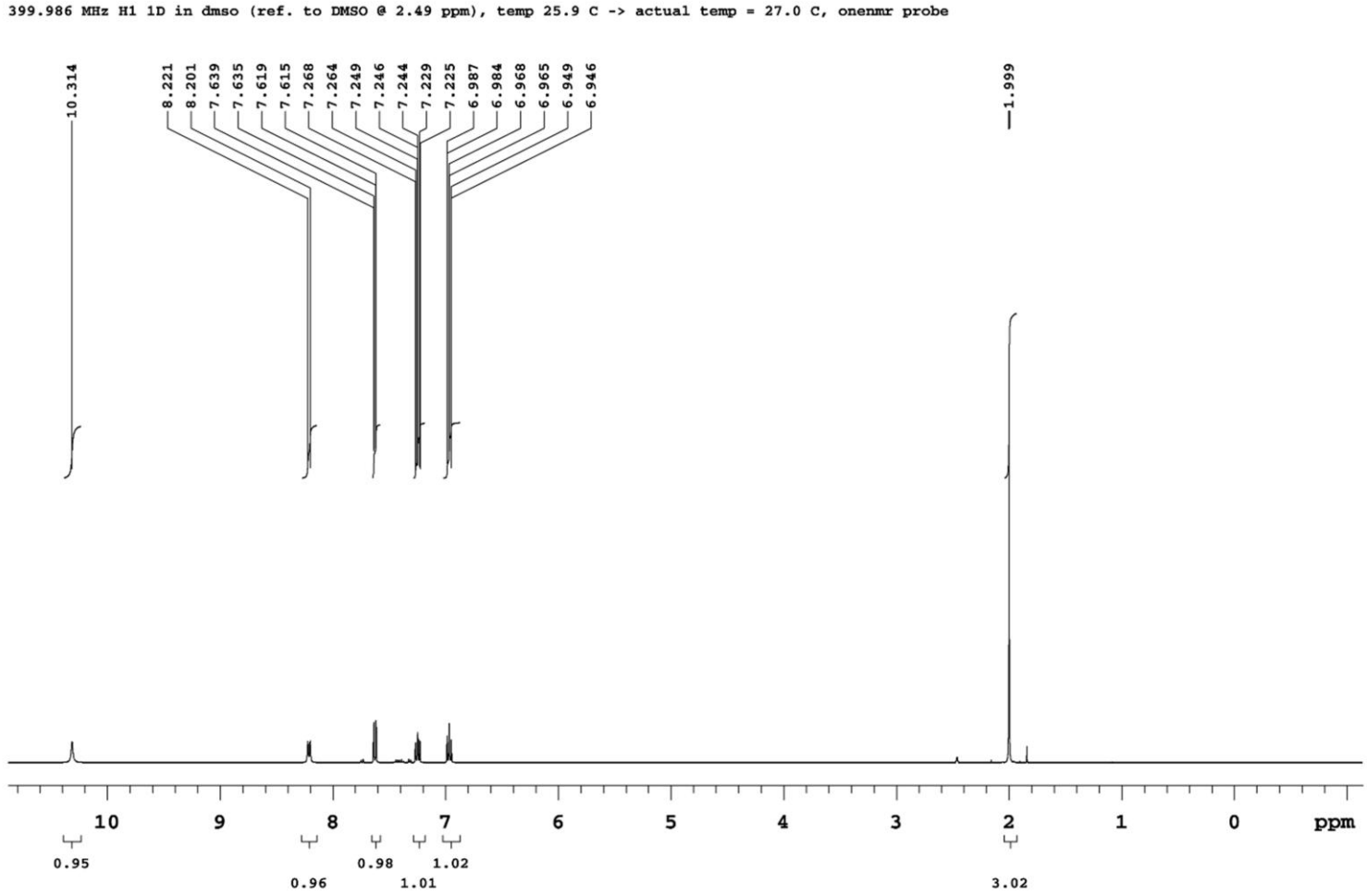
0.1% TFA in D_2O was used as solvent



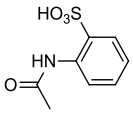


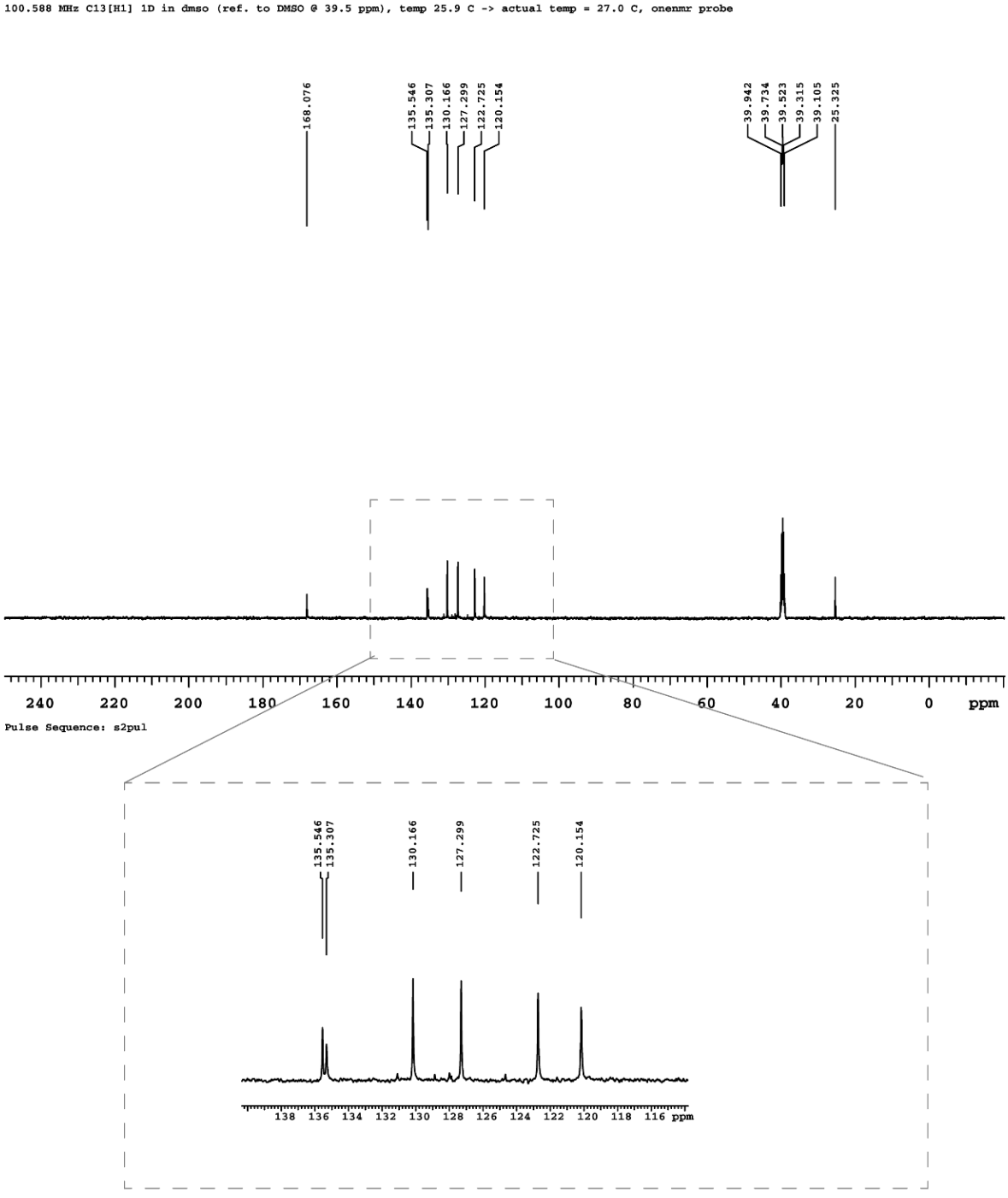
^{1}H-NMR of 2-(acetylamino)benzenesulfonic acid (**7**)



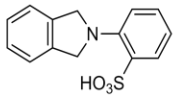


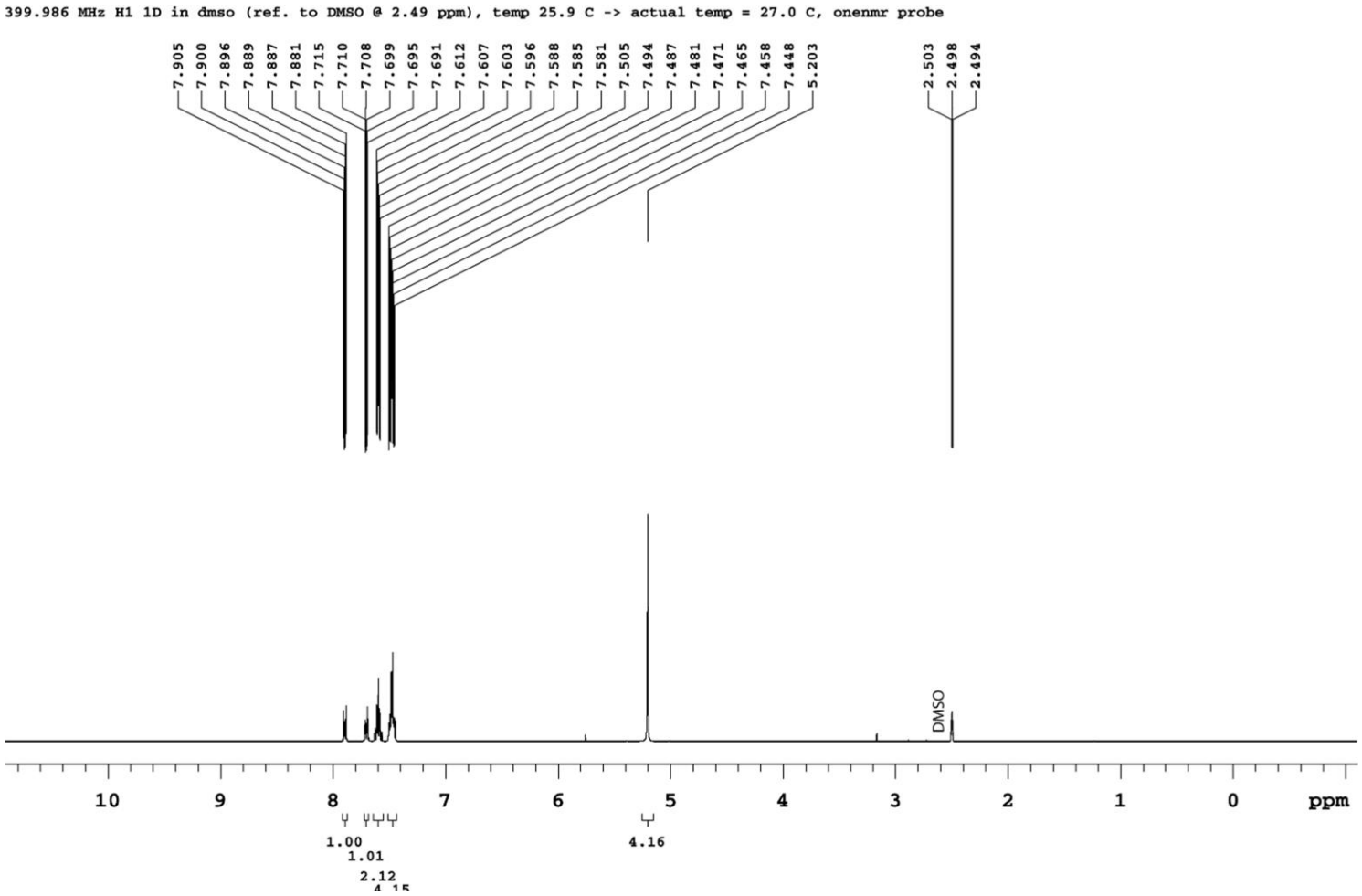
^{1}H-NMR of 2-(acetylamino)benzenesulfonic acid (**7**)



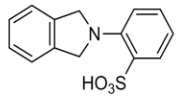


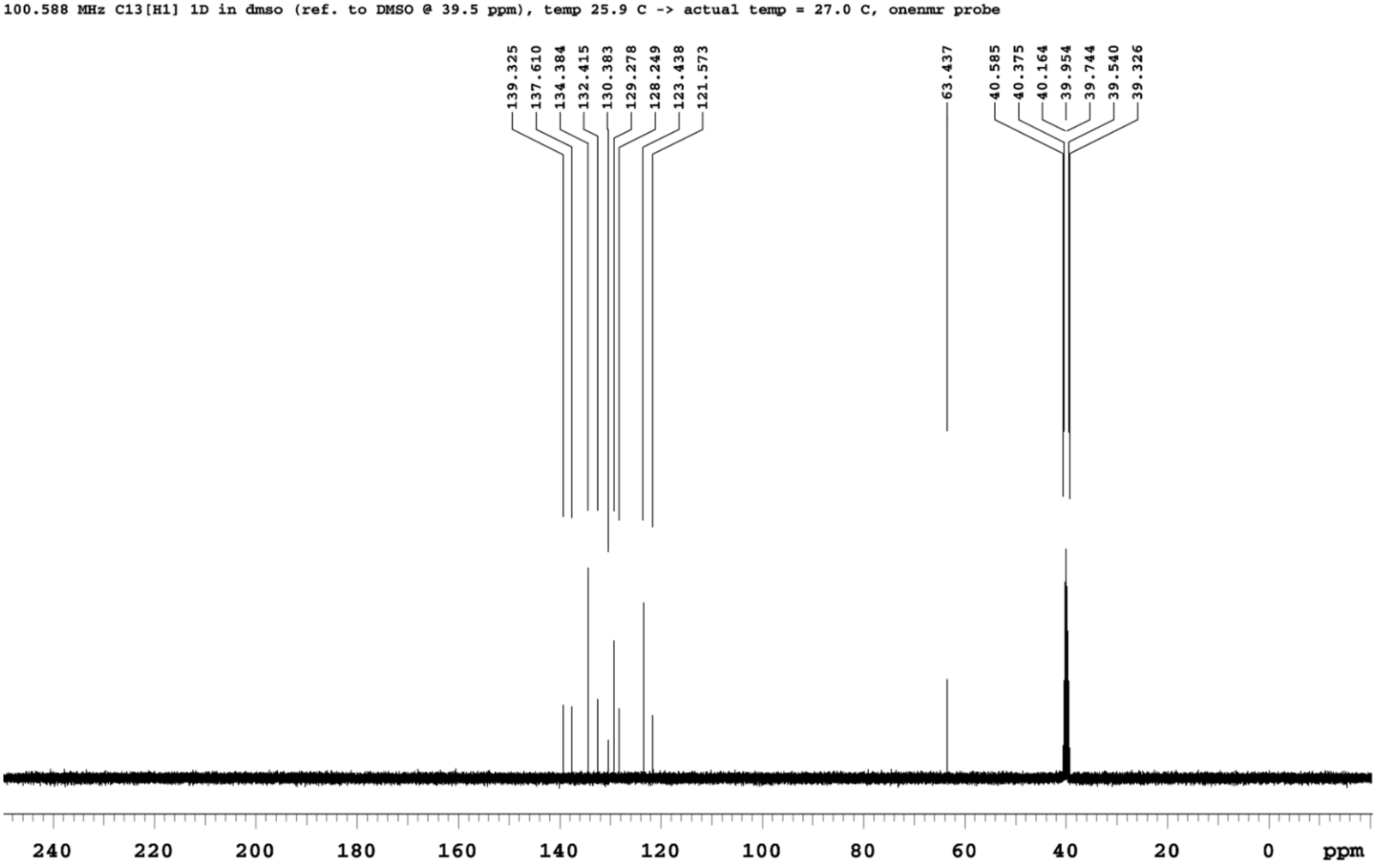
^{1}H-NMR of 2-(1,3-dihydro-2*H*-isoindol-2-yl)benzenesulfonic acid (**9**)



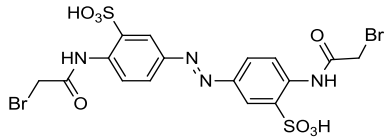


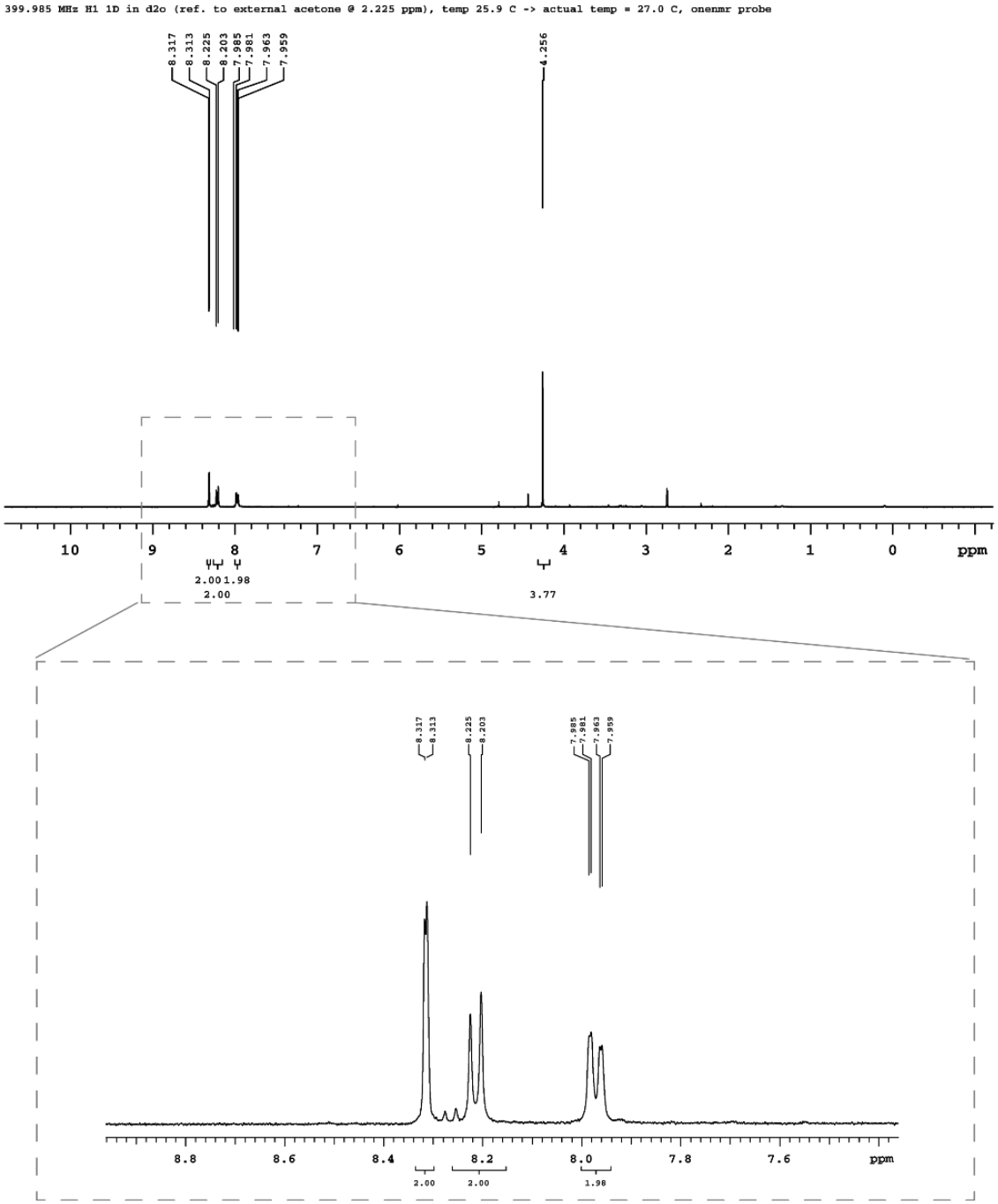
^{13}C-NMR of 2-(1,3-dihydro-2H-isoindol-2-yl)benzenesulfonic acid (**9**)



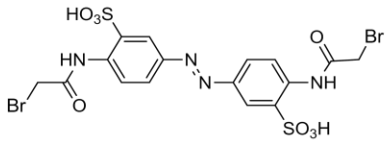


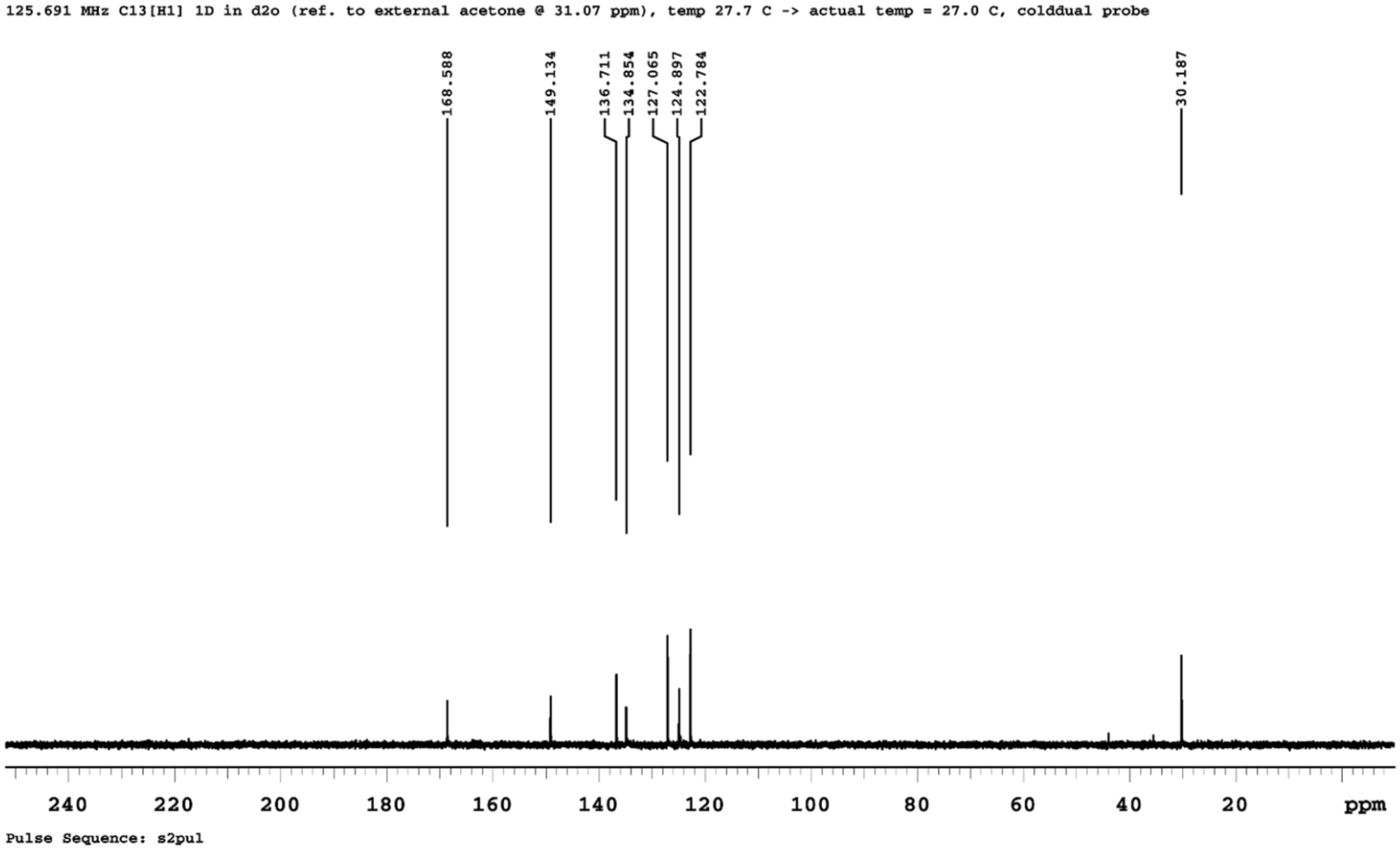
^{1}H-NMR of 3,3'-bis(sulfonato)-4,4'-bis(bromoacetamido)azobenzene (**11**)



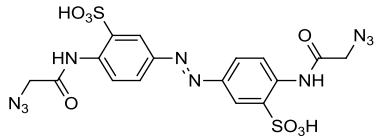


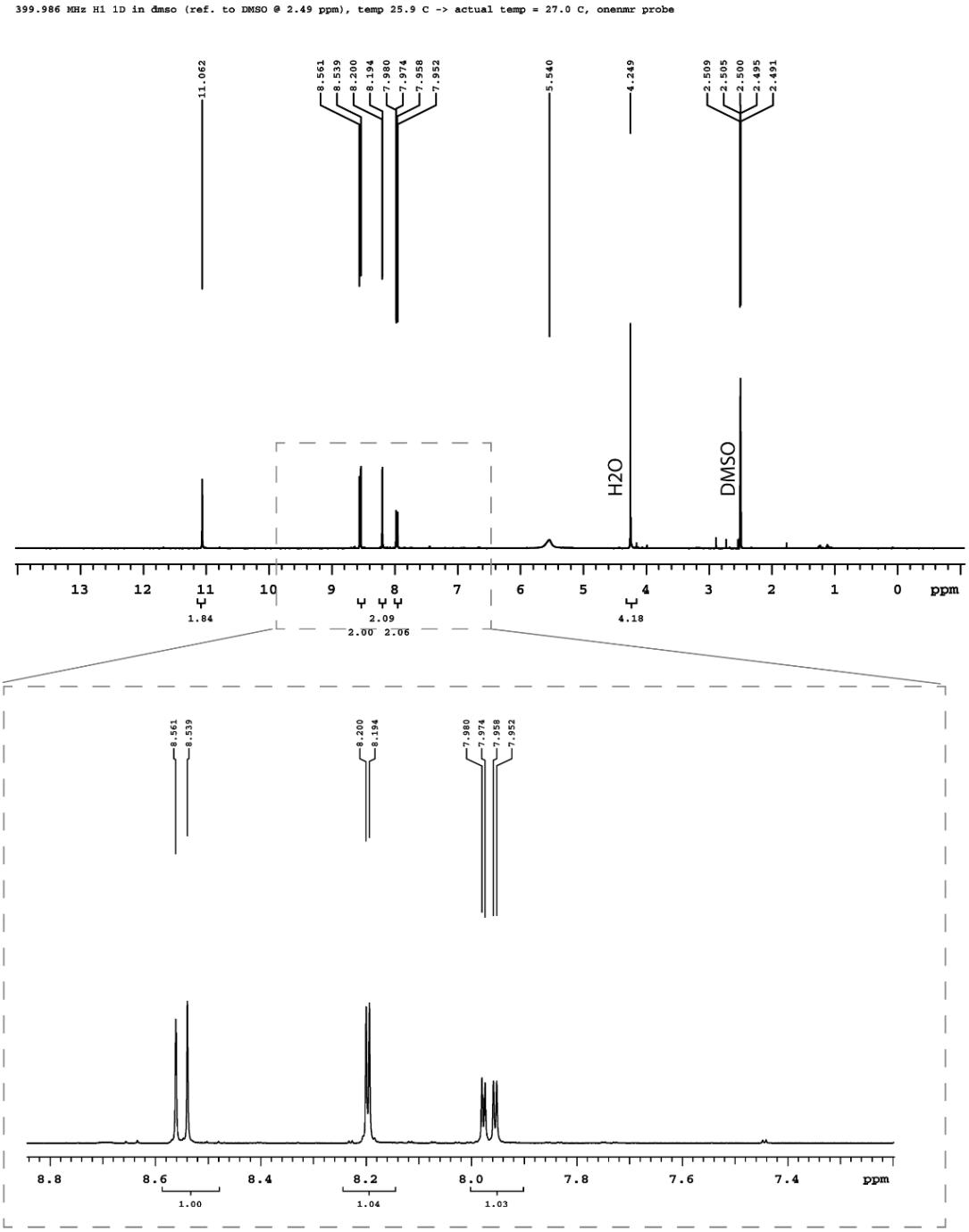
^{13}C-NMR of 3,3'-bis(sulfonato)-4,4'-bis(bromoacetamido)azobenzene (**11**)



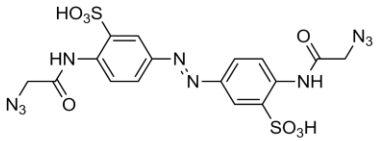


^{1}H-NMR of 3,3'-bis(sulfonato)-4,4'-bis(azidoacetamido)azobenzene (**12**)

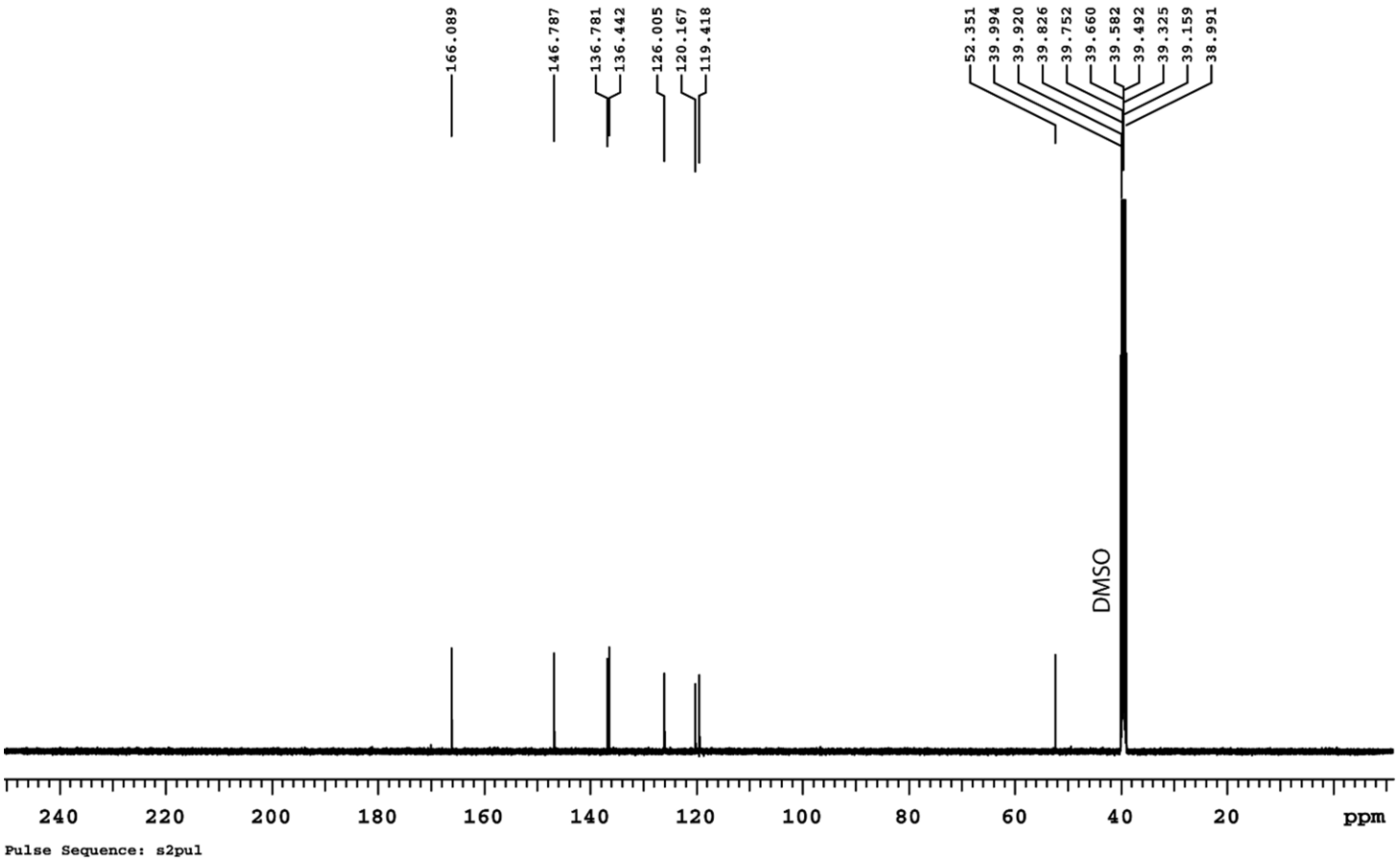




^{13}C-NMR of 3,3'-bis(sulfonato)-4,4'-bis(azidoacetamido)azobenzene (**12**)

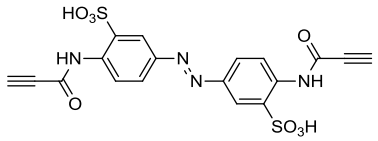


125.691 MHz C13[H1] 1D in dmso (ref. to DMSO @ 39.5 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe

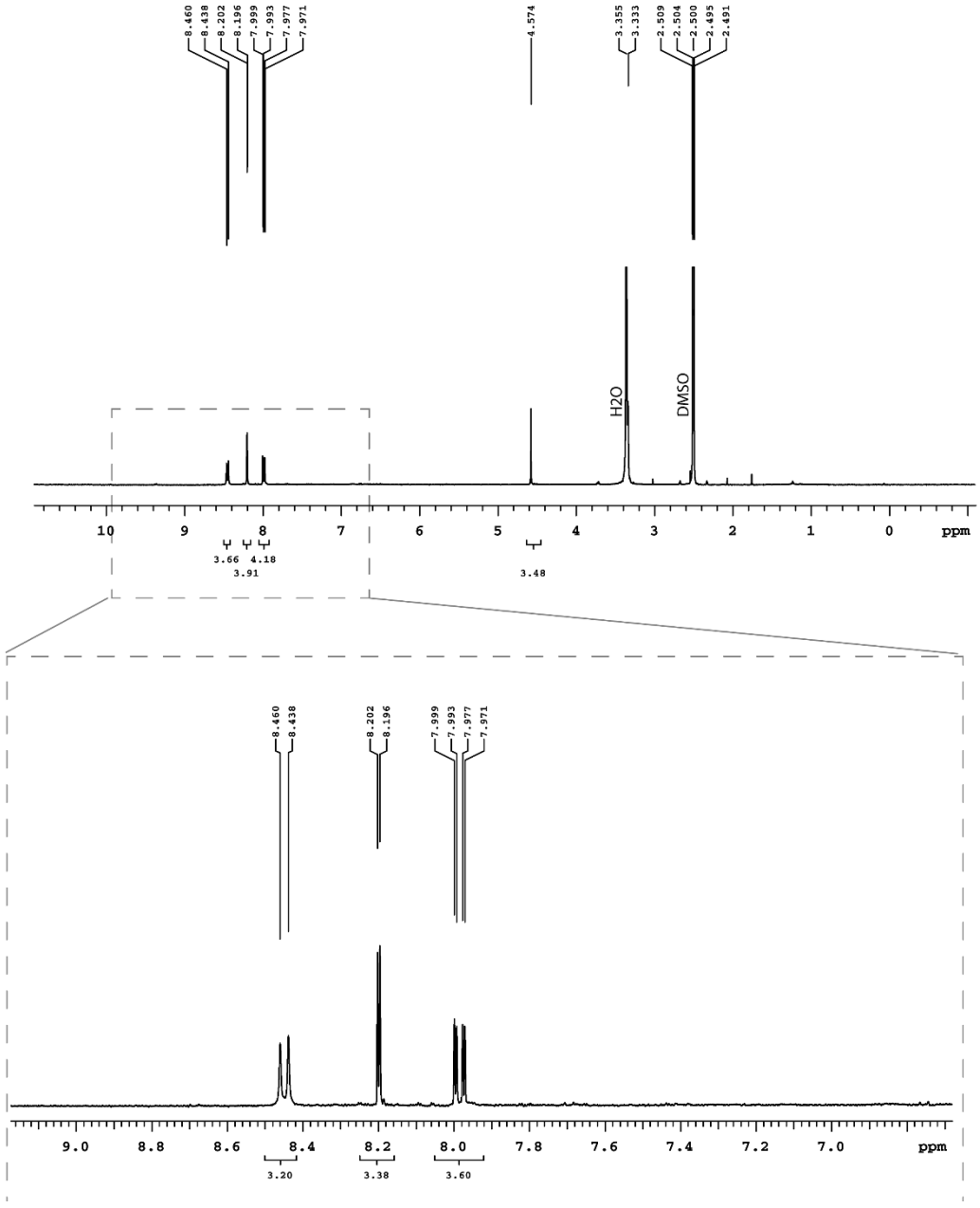


Pulse Sequence: s2pul

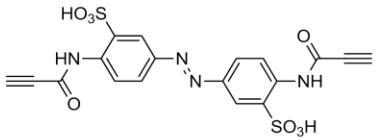
^{1}H-NMR of 3,3'-bis(sulfonato)-4,4'-bis(prop-2-ynoylamido)azobenzene (**13**)

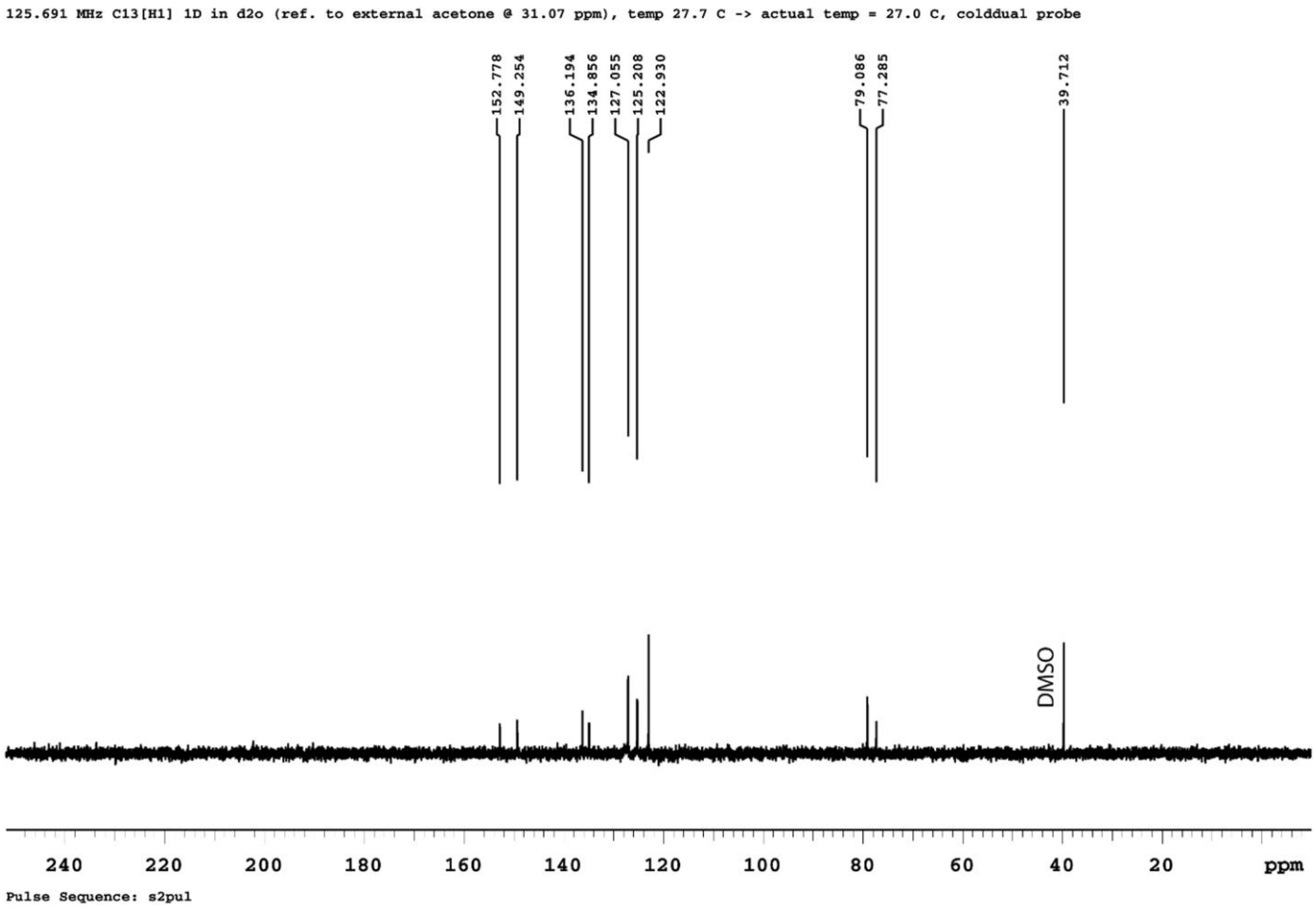


399.986 MHz H1 1D in dmso (ref. to DMSO @ 2.49 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe

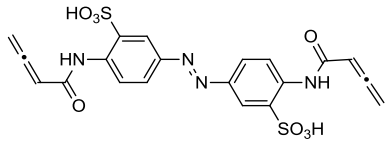


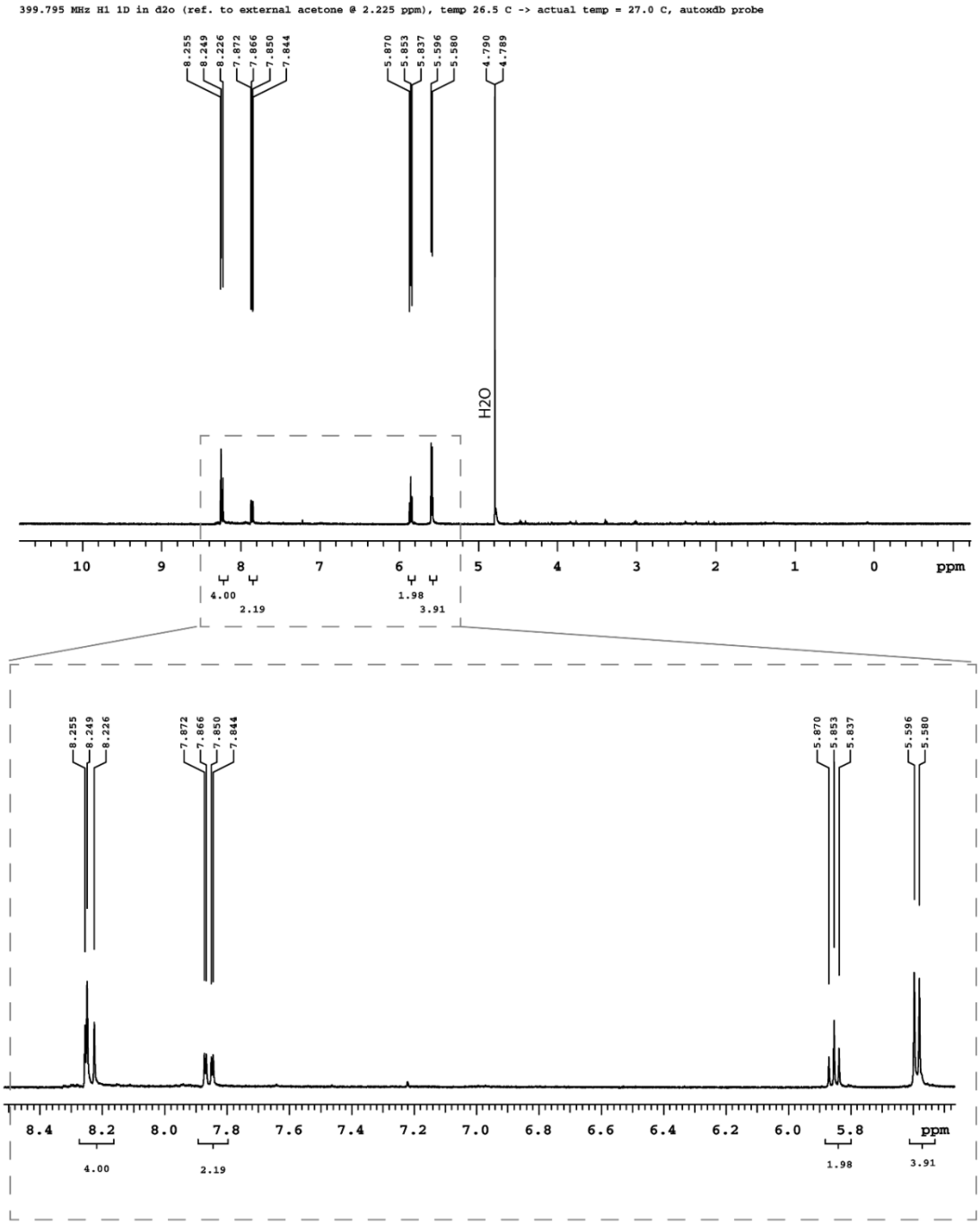
^{13}C-NMR of 3,3'-bis(sulfonato)-4,4'-bis(prop-2-ynoylamido)azobenzene (**13**)



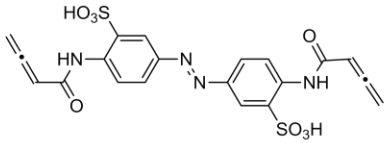


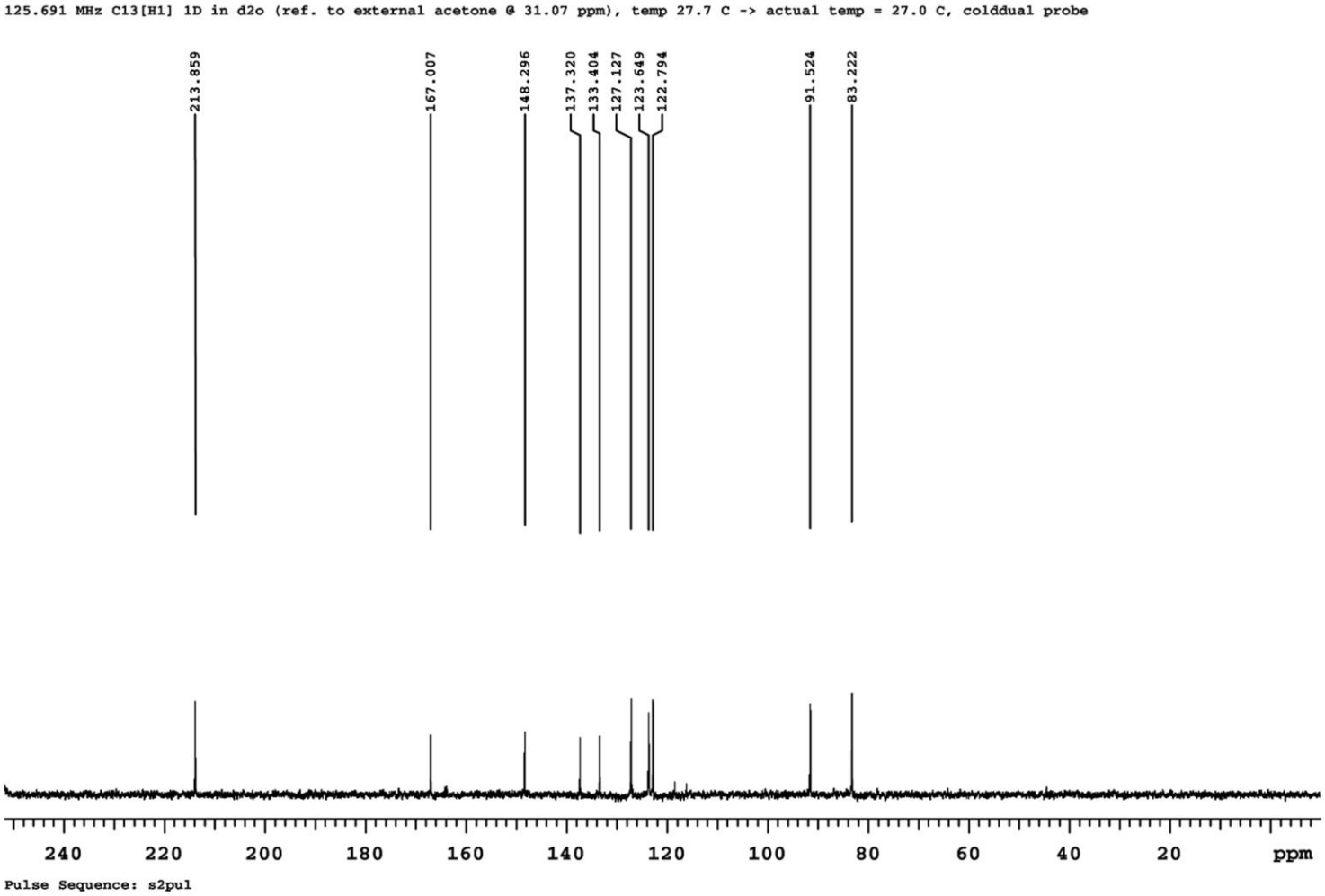
^{1}H-NMR of 3,3'-bis(sulfonato)-4,4'-bis(buta-2,3-dienoylamido)azobenzene (**14**)





C-NMR of 3,3'-bis(sulfonato)-4,4'-bis(buta-2,3-dienoylamido)azobenzene (**14**)





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