Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Design, synthesis, and drug solubilising property of the first folate-calix[4]arene conjugate

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SUPPLEMENTARY INFORMATION

General Considerations

All chemicals and reagents were obtained commercially and used without purification. Reactions were monitored by TLC on Merck silica gel 60 F_{254} plates (0.25 mm) and visualized by UV light and sprying with H_2SO_4-$Ce(SO_4)_2$ mixture. Column chromatography was performed on Merk silica gel 60 (0.040-0.063 mm) and Sigma-Aldrich Sephadex LH-20. ^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker Avance™ 400 spectrometer at 400.13 and 100.61 MHz, respectively. ^{1}H-NMR chemical shifts (δ) are reported in ppm relative to the residual protonated solvent resonance: $CHCl_3$, δ 7.26; MeOH, δ 3.30; DMSO, δ 2.49. Coupling constant (*J*) values are given in Hz. Mass spectra were recorded in ESI mode on a Waters-Micromass ZQ2000 spectrometer (3.5 kV capillary voltage and 20 V cone voltage). Microwave irradiation was performed in a CEM Discover Benchmate apparatus with a single-mode cavity and equipped with a temperature and pressure control devices. HPLC analyses were performed on a Dionex ASI-100 instrument by using a Luna RP C-18 column (5μm, 4.6 x 250 mm, Phenomenex).

Synthesis of 5,11,17,23-Tetra(5-azidopentanoyl)amino-25,26,27,28-tetra[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-calix[4]arene (4)

A mixture of compound **3**[1] (142 mg, 0.13 mmol) and succinimidyl 5-azidopentanoate[2] (254 mg, 1.06 mmol) in dry DMF (5 mL) was stirred at room temperature for 4 days. The solvent was removed under vacuum and the residue was partitioned between CH_2Cl_2 (300 mL) and 0.5 N HCl (150 mL). The organic layer was washed with water (3 x 150 mL), dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. Pure compound **4** was obtained in 81% yield (166 mg) after column chromatography (9:1 AcOEt/MeOH).

^{1}H-NMR (MeOD, 297 K): δ 1.62 (m, J = 7.3 Hz, 8H, 4 × CH_2CH$_2$N$_3$), 1.69 (m, J = 7.3 Hz, 8H, 4 × CH_2CH$_2$CO), 2.28 (t, J = 7.3 Hz, 8H, 4 × CH_2CO), 3.11 and 4.56 (AX system, J = 13.2 Hz, 8H, 4 × ArCH$_2$Ar), 3.28 (overlapped with MeOD, 8H, 4 × CH_2N$_3$), 3.32 (s, 12H, 4 × OCH$_3$), 3.49 (t, J = 5.0 Hz, 8H, 4 × CH_2OCH$_3$), 3.60 (t, J = 5.0 Hz, 8H, 4 × CH_2CH$_2$OCH$_3$), 3.64 and 3.65 (br t, 8H each, 8 × OCH$_2$), 3.93 (br t, 8H, 4 × ArOCH$_2$CH_2O), 4.14 (br t, 8H, 4 × ArOCH_2), 6.91 (s, 8 H, 8 × ArH). ^{13}C-NMR (MeOD, 297 K): 24.0, 29.5 (t, CH$_2$), 32.2 (t, ArCH$_2$Ar), 37.1 (t, CH$_2$), 52.1 (t, CH$_2$N$_3$), 59.1 (q, OCH$_3$), 71.4, 71.5, 71.7, 73.0 (t, OCH$_2$), 74.6 (t, ArOCH$_2$), 121.8 (d, ArC-H), 133.8 (s, Ar*C*-CH$_2$), 136.2 (s, Ar*C*-O), 154.2 (s, Ar*C*-N), 173.3 (s, C=O). ESI-MS *m/z* calcd for $C_{76}H_{113}N_{16}O_{20}^+$ [M+ H]$^+$ 1569.8, found 1569.9; calcd for $C_{76}H_{112}N_{16}O_{20}Na^+$ [M+ Na]$^+$ 1591.8, found 1591.6; calcd for $C_{76}H_{112}N_{16}O_{20}Na_2^{2+}$ [M+ Na]$^{2+}$ 807.4, found 807.2.

[1] E. Galante, C. Geraci, S. Sciuto, V. L. Campo, I. Carvalho, R. Sesti-Costa, P. M. M. Guedes, J. S. Silva, L. Hill, S. A. Nepogodiev, R- A- Field, *Tetrahedron* **2011**, *67*, 5902–5912.
[2] (a) R. P. McGeary, *Tetrahedron Lett.* **1998**, *39*, 3319–3322. (b) T. S Seo, Z. Li, H. Ruparel, J. Ju, *J. Org. Chem.* **2003**, *68*, 609–612.

Synthesis of γ-propargyl folate 6

Tetramethylguanidinium L-methyl folate (γ) **5**[3] (800 mg, 1.40 mmol) was stirred, under inert atmosphere and in the dark, with an excess of propargylamine (10.9 mL, 170 mmol) at 60 °C. The reaction was completed after 4 days, as indicated by analytical HPLC (RP C-18 column, CH_3CN/H_2O containing 0.05% TFA, gradient 5 to 20 % over 30 min; 20 to 30 % over 5 min; flow rate 1 mL/min, λ = 280 nm, retention time 20.8 min). The reaction mixture was transferred to a well-stirred mixture of CH_3CN/Et_2O (1:1, 150 mL). The precipitated solid was collected by centrifugation (3000 rpm, 10 min) and redissolved in water (150 mL). Addition of aqueous 1 N HCl until pH 5 effected reprecipitation of the product. The precipitate was collected by centrifugation, washed with acidic water (pH 5, 75 mL × 2), water (75 mL), CH_3CN (30 mL), and Et_2O (15 mL× 2), and dried under vacuum to give γ-propargyl folate **6** in 66 % yield (440 mg).

^{1}H-NMR (DMSO-d_6, 297 K): δ 1.89 and 2.05 (m, 1H each, glutamyl CHCH_2), 2.19 (m, 2H, glutamyl CH_2CO), 3.04 (s, 1H, propargyl CC*H*), 3.81 (m, 2H, propargyl CH_2CCH), 4.26 (m, 1H, glutamyl CH), 4.47 (d, *J* = 5.9 Hz, 2H, pteroyl CH_2NH), 6.63 (d, *J* = 8.6 Hz, 2H, 2 × pteroyl ArH), 6.89 (t, *J* = 5.9 Hz, 1H, pteroyl CH_2N*H*), 7.63 (d, *J* = 8.5 Hz, 2H, 2 × pteroyl ArH), 8.10 (d, *J* = 7.2 Hz, 1H, glutamyl N*H*CH), 8.26 (t, *J* = 5.9 Hz, 1H, N*H*CH_2CCH), 8.63 (s, 1H, pteroyl NCH). ^{13}C-NMR (DMSO-d6, 297 K): 26.4 (t, glutamyl CH*C*H_2), 27.8 (t, propargyl *C*H_2CCH), 31.7 (t, glutamyl *C*H_2CO), 45.9 (t, pteroyl CH_2NH), 52.2 (d, glutamyl CH), 72.9 (d, propargyl C*C*H), 81.2 (s, propargyl *C*CH), 111.2 (d, pteroyl ArC-H), 121.3 (s, pteroyl Ar*C*-CO), 127.9 (s, pteroyl N*C*CO), 129.0 (d, pteroyl ArC-H), 148.6 (overlapped, pteroyl NCH and CH_2*C*N), 150.8 (s, pteroyl Ar*C*-NH), 153.8 (s, pteroyl NCN), 155.9 (s, pteroyl CNH_2), 161.0 (s, pteroyl NC*C*O), 166.3 (s, pteroyl Ar-CO), 171.4 (s, glutamyl γ-CO), 173.8 (s, glutamyl α-CO). ESI-MS *m/z* calcd. for $C_{22}H_{21}N_8O_5$ $[M-H]^-$ 477.2, found 476.1.

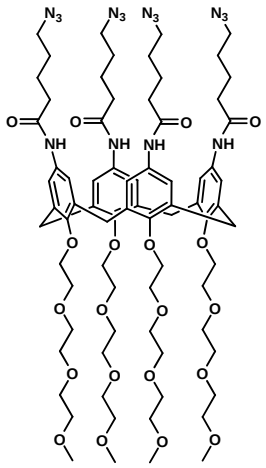
[3] J. Luo, M. D. Smith, D. A. Lantrip, S. Wang, P. L. Fuchs, *J. Am. Chem. Soc.* **1997**, *119*, 10004–10013.

Synthesis of folate-calix[4]arene conjugate 7

To a solution of compound **5** (10 mg, 6.4 µmol) and propargyl folate **6** (24 mg, 50 µmol) in dry DMSO (330 µL), $CuSO_4 \cdot 5H_2O$ (1.2 mg, 4.8 µmol) and sodium ascorbate (9.3 mg, 47 µmol) were added. The mixture was stirred in a 10 mL closed vessel at 70 °C into a microwave apparatus for 30 min. The reaction was stopped by adding cold acetone (2 mL). A precipitate was formed which was collected by centrifugation at 3000 rpm for 5 min. The solid was washed with CH_2Cl_2 (2 mL) and CH_3CN (2 mL), then was suspended in water (2 ml) and 0.1 N HCl was added until to pH 5. The precipitate was collected by centrifugation and washed by CH_3CN (2 mL) and Et_2O (2 mL), and dried in vacuo. The crude product was dissolved in aqueous 0.1 N NaOH/MeOH (9:1) and purified by gel permeation on Sephadex LH-20 (eluent 9:1 H_2O/MeOH). A yellow precipitate was formed by adding 0.1 N HCl until pH 5 to the collected fractions. After centrifugation the solid was washed with CH_3CN (2 mL) and Et_2O (2 × 2 mL) to afford pure compound **7** in 40 % yield (8.9 mg).

^{1}H-NMR (DMSO-d_6, 297 K): δ 1.45 (m, J = 7.8 Hz, 8H, 4 × pentanoyl CH_2CH$_2$CO), 1.75 (m, J = 7.8 Hz, 8H, 4 × pentanoyl CH_2CH$_2$NH), 1.90 and 2.06 (m, 4H each, 4 × glutamyl CHCH_2), 2.13-2.30 (overlapped, 16H, 4 × glutamyl CH$_2$CO and 4 × pentanoyl CH$_2$CO), 3.02 and 4.39 (AX system, J = 12.0 Hz, 8H, 4 × calixarene ArCH$_2$Ar), 3.20 (s, 12H, 4 × OCH$_3$), 3.39 (br t, 8H, 4 × CH_2OCH$_3$), 3.48 (t, J = 5.2 Hz, 8H, 4 × CH_2CH$_2$OCH$_3$), 3.52 and 3.54 (br t, 8H each, 8 × OCH$_2$), 3.82 (br t, 8H, 4 × ArOCH$_2$OCH_2), 4.00 (br t, 8H, 4 × ArOCH$_2$), 4.20-4.36 (overlapped, 20H, 4 × glutamyl CH, 4 × triazole CH$_2$, 4 × pentanoyl CH$_2$N), 4.50 (d, J = 5.3 Hz, 8H, 4 × pteroyl CH_2NH), 6.62 (d, J = 8.5 Hz, 8H, 8 × pteroyl ArH), 6.94-6.97 (overlapped, 12H, 4 × pteroyl CH$_2$NH, and 8 × calixarene ArH), 7.64 (d, J = 8.5 Hz, 8H, 8 × pteroyl ArH), 7.85 (d, 4H, 4 × triazole CH), 8.18 (d, J = 7.2 Hz, 4H, 4 × glutamyl CHNH), 8.32 (t, J = 5.9 Hz, 4H, 4 × CONHCH$_2$), 8.66 (s, 4H, 4 × pteroyl NCH), 9.50 (s, 4H, 4 × calixarene ArNH). ^{13}C-NMR (DMSO-d_6, 297 K): 22.0 (t, pentanoyl CH$_2$CH$_2$CO), 26.4 (t, glutamyl CHCH$_2$), 29.3 (t, pentanoyl CH$_2$CH$_2$NH), 30.8 (t, calixarene ArCH$_2$Ar), 31.8 (t, pentanoyl CH$_2$CO), 34.2 (t, glutamyl CH$_2$CO), 35.3 (t, triazole CH$_2$), 45.8 (t, pteroyl NHCH$_2$), 49.0 (t, pentanoyl CH$_2$N), 52.1 (d, glutamyl CHNH), 58.0 (q, OCH$_3$), 69.6, 69.7 69.8 (t, OCH$_2$), 71.2 (t, Ar-OCH$_2$), 111.2 (d, pteroyl ArC-H), 119.4 (d, calixarene ArC-H), 121.4 (s, calixarene ArC-CO), 122.6 (d, triazole CH), 127.9 (s, pteroyl NCCO), 129.0 (d, pteroyl ArC-H), 133.3 (s, calixarene ArC-CH$_2$), 134.2 (s, calixarene ArC-O), 144.8 (s, triazole C), 148.2 (d, pteroyl NCH), 149.2 (s, pteroyl CH$_2C$N), 150.7 (s, pteroyl ArC-NH), 151.7 (s, calixarene ArC-NH), 153.2 (s, pteroyl NCN), 160.3 (s, pteroyl NCCO), 166.4 (s, pteroyl ArC-CO), 170.0 (s, pentanoyl CO),

171.5 (s, glutamyl γ-*C*O), 173.8 (s, glutamyl α-*C*O). ESI-MS *m/z* calcd for $C_{164}H_{198}N_{48}O_{40}^{2-}$ [M-2H]$^{2-}$ 1739.7, found 1740.2; calcd for $C_{164}H_{197}N_{48}O_{40}^{3-}$ [M-3H]$^{3-}$ 1159.5, found 1159.8; and calcd for $C_{164}H_{196}N_{48}O_{40}^{4-}$ [M-4H]$^{4-}$ 869.4, found 869.2. Additional peaks at mass 1652.6 [M-176-2H]$^{2-}$, 1101.6 [M-176-3H]$^{3-}$, and 825.7 [M-176-4H]$^{4-}$ indicated the occurrence of fragmentation of the folate residues with formation of pteridinyl radicals (176 Da).



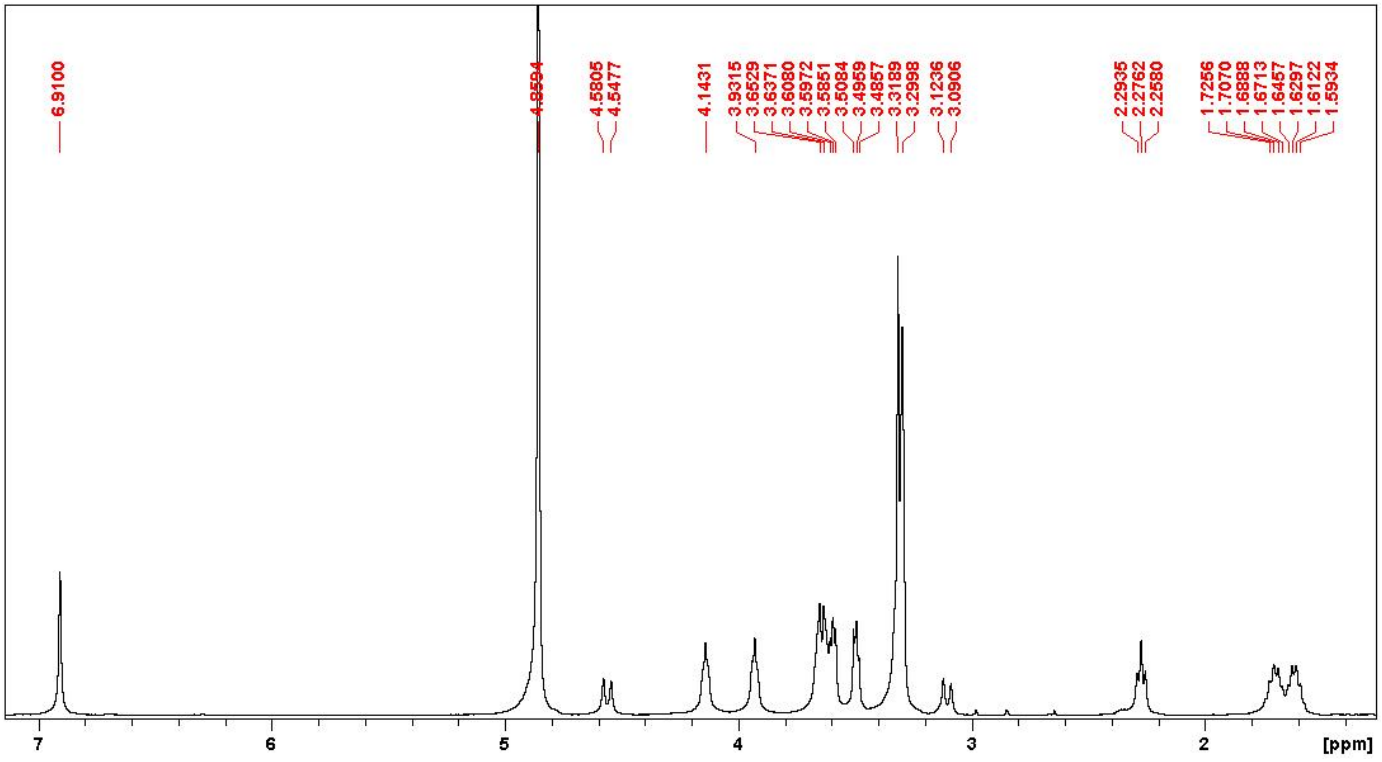


Figure S1: ^{1}NMR spectrum of derivative **4** (MeOD, 400.13 MHz, 297 K)

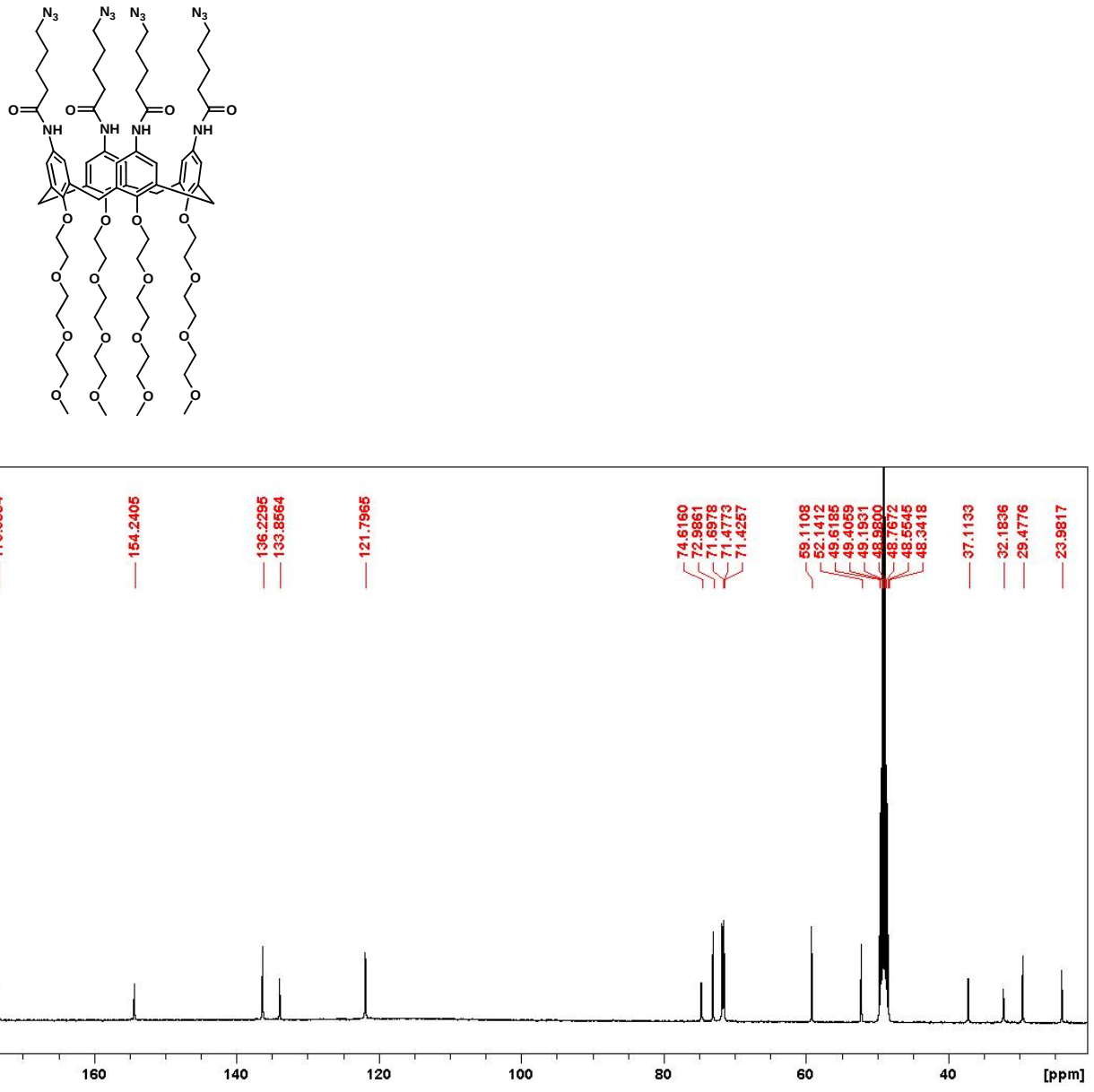
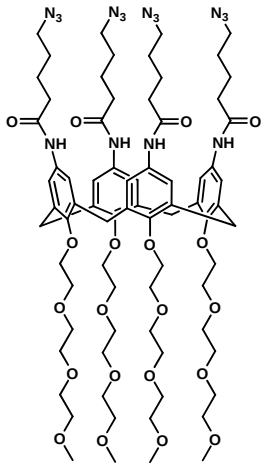


Figure S2: ^{13}C NMR spectrum of derivative **4** (MeOD, 100.61 MHz, 297 K)



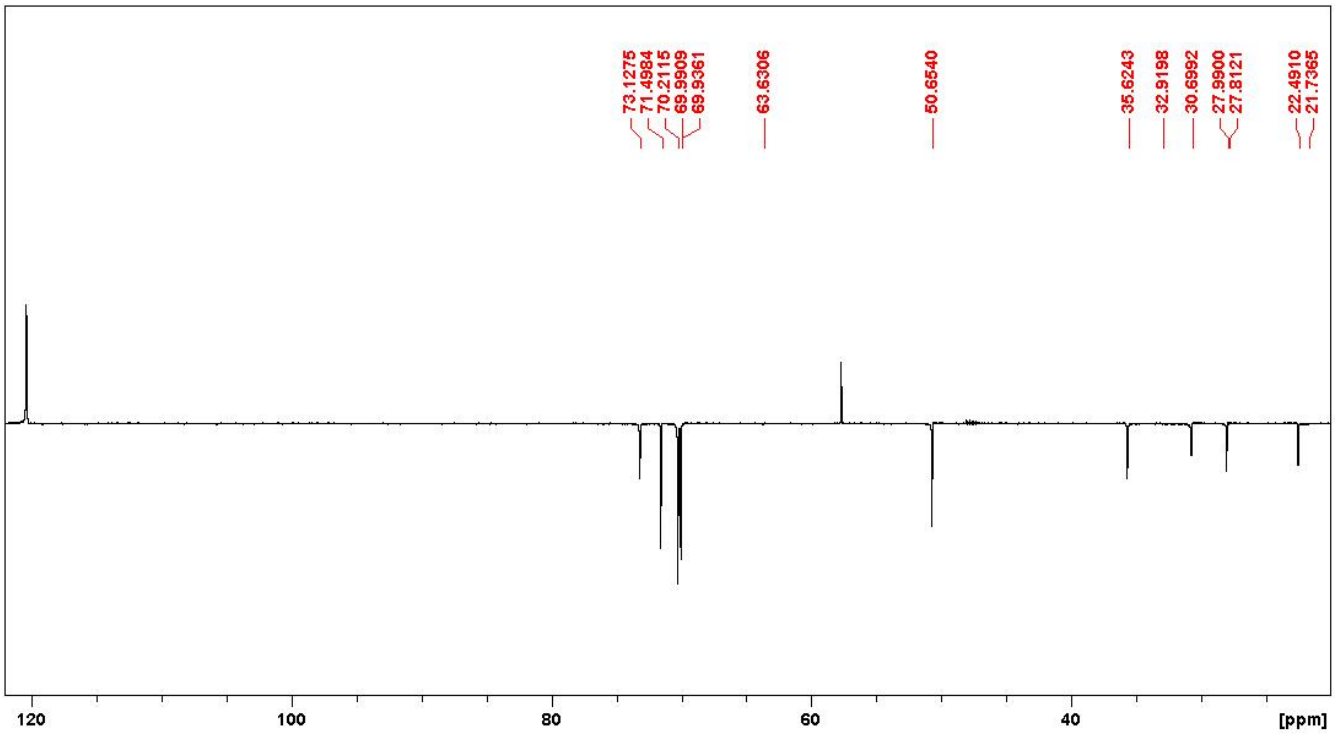


Figure S3: DEPT-135 spectrum of derivative **4** (MeOD, 100.61 MHz, 297 K)

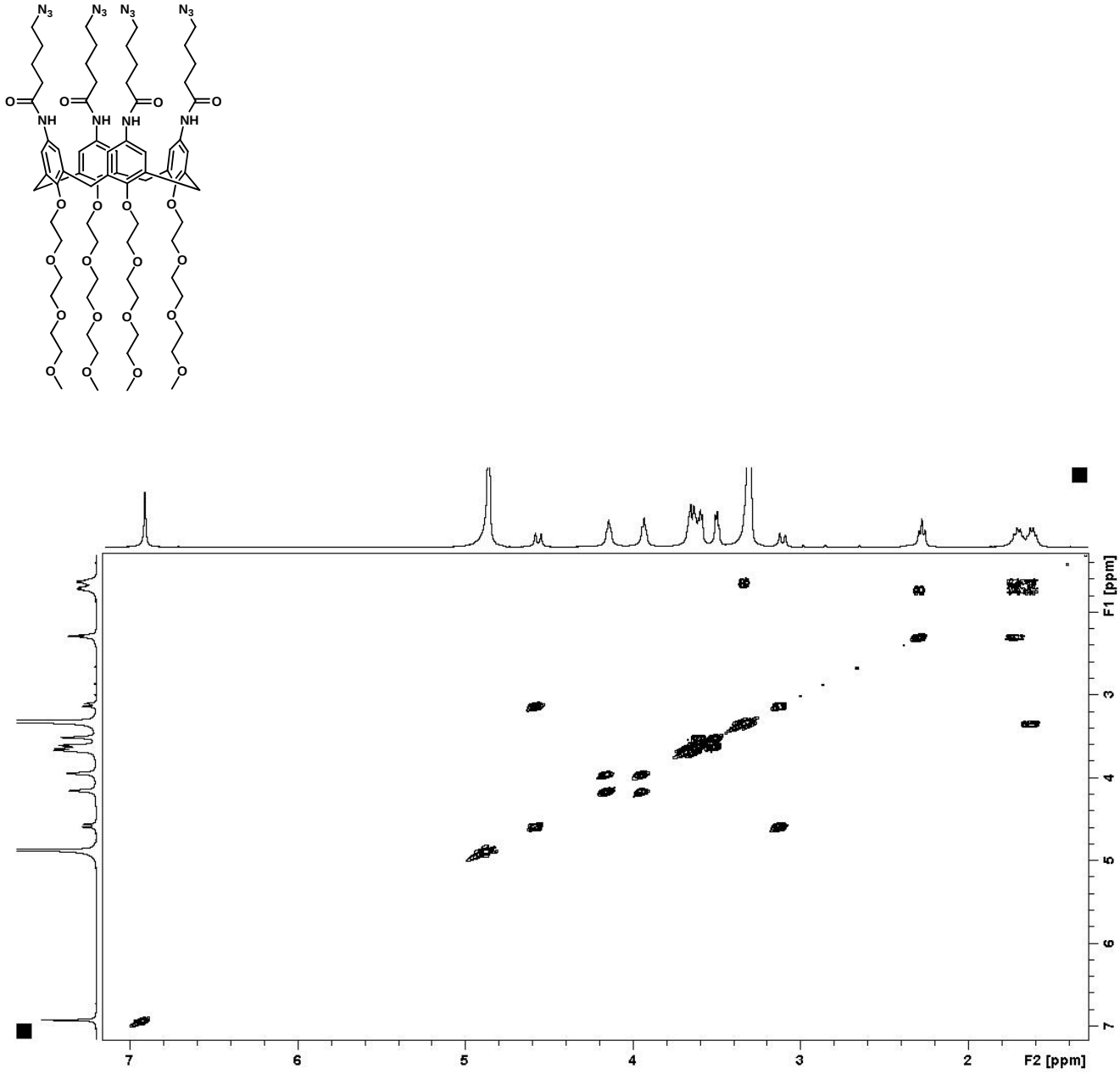
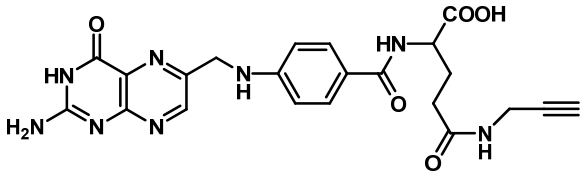


Figure S4: COSY spectrum of derivative **4** (MeOD, 100.61 MHz, 297 K)



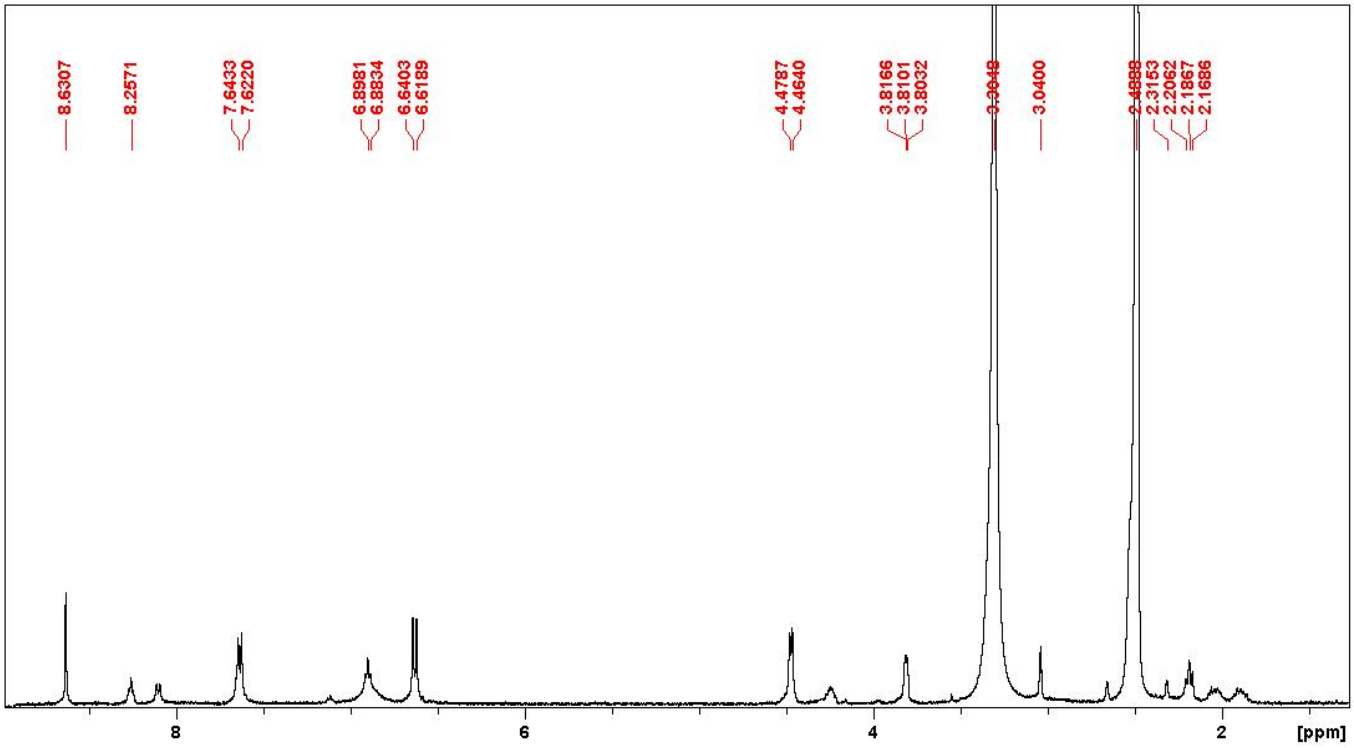
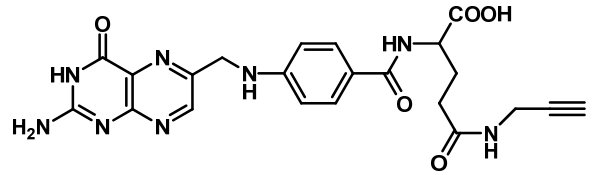


Figure S5: ^{1}NMR spectrum of derivative **6** (DMSO-d_6, 400.13 MHz, 297 K)



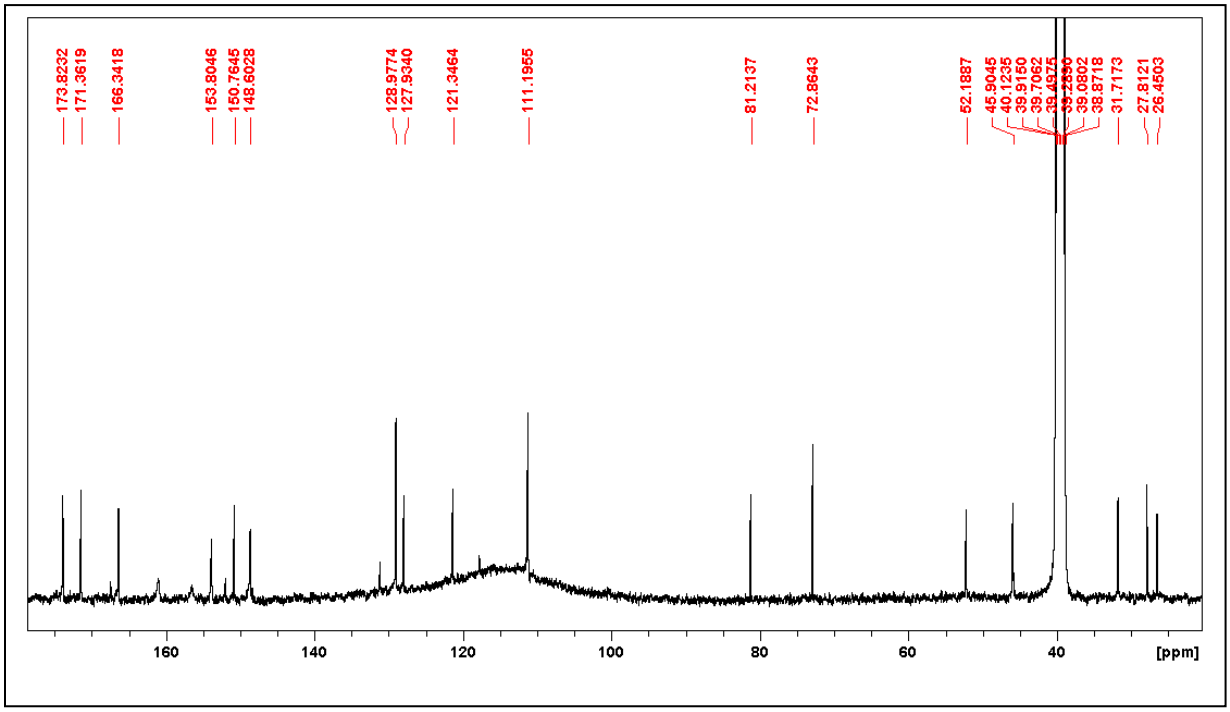


Figure S6: ^{13}C NMR spectrum of derivative **6** (DMSO-d_6, 100.61 MHz, 297 K)

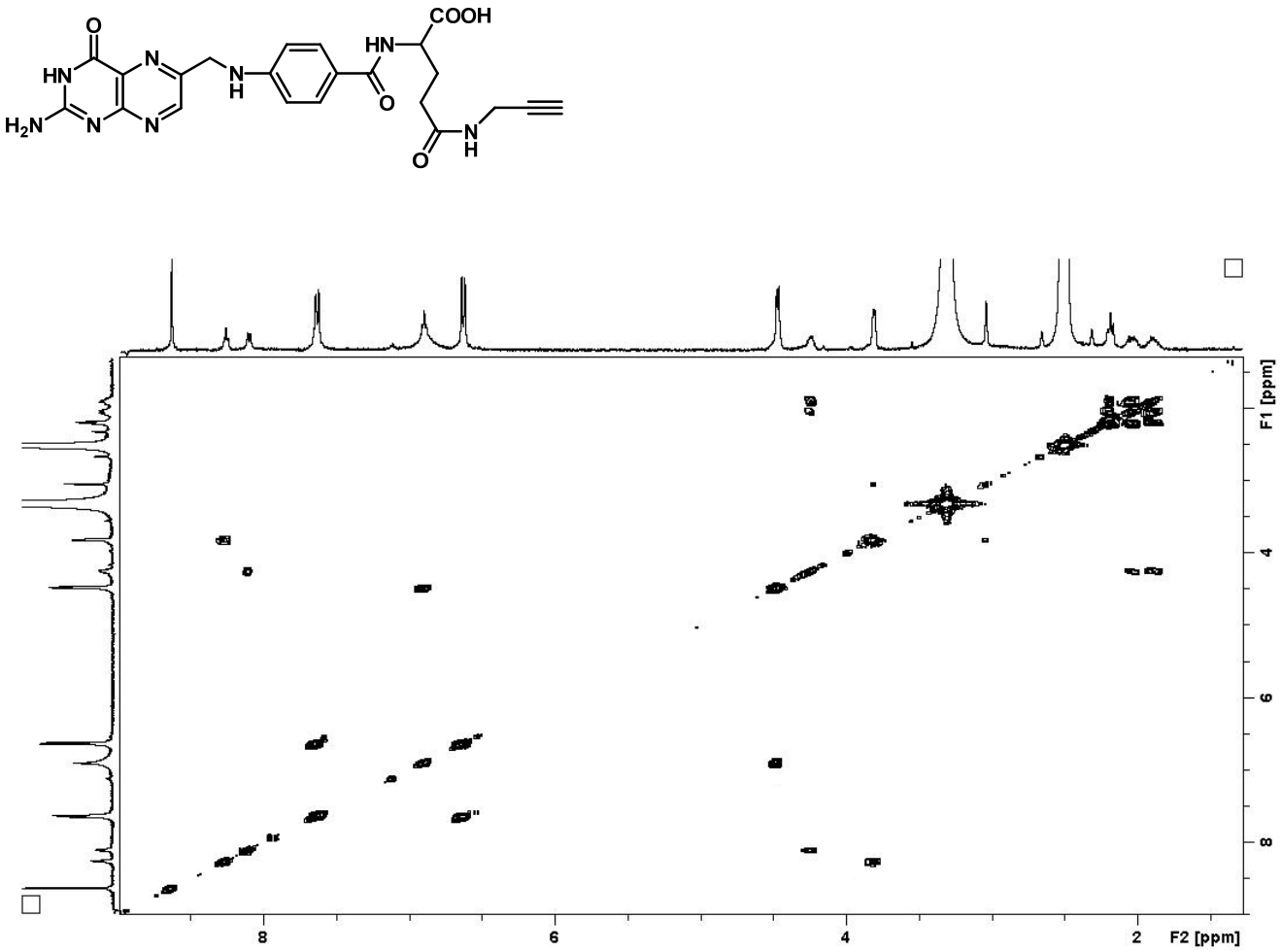


Figure S7: COSY spectrum of derivative **6** (DMSO-d_6, 400.13 MHz, 297 K)

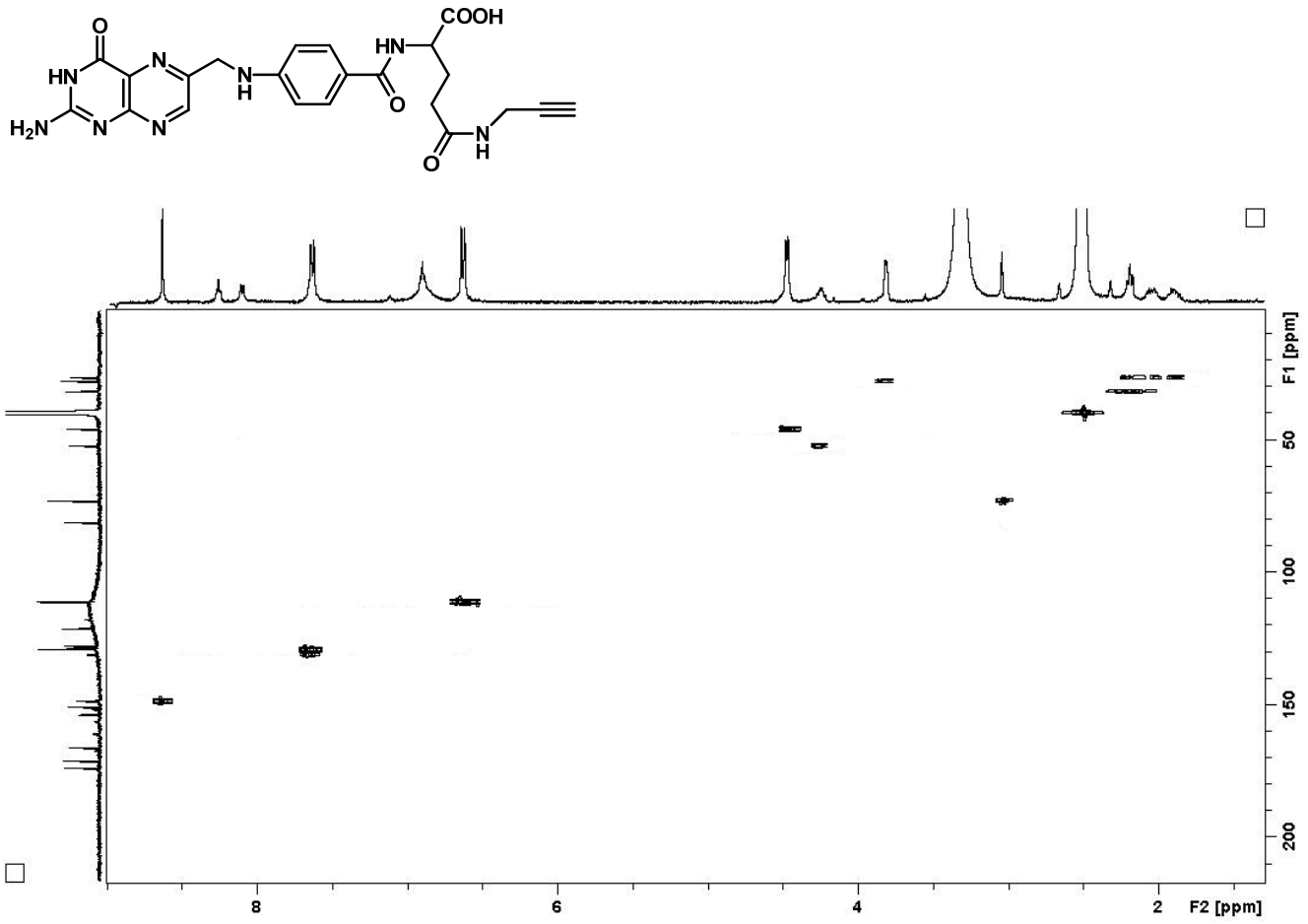
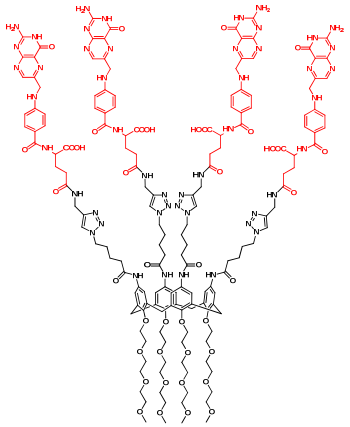


Figure S8: HSQC spectrum of derivative **6** (DMSO-d_6, 400.13 MHz, 297 K)



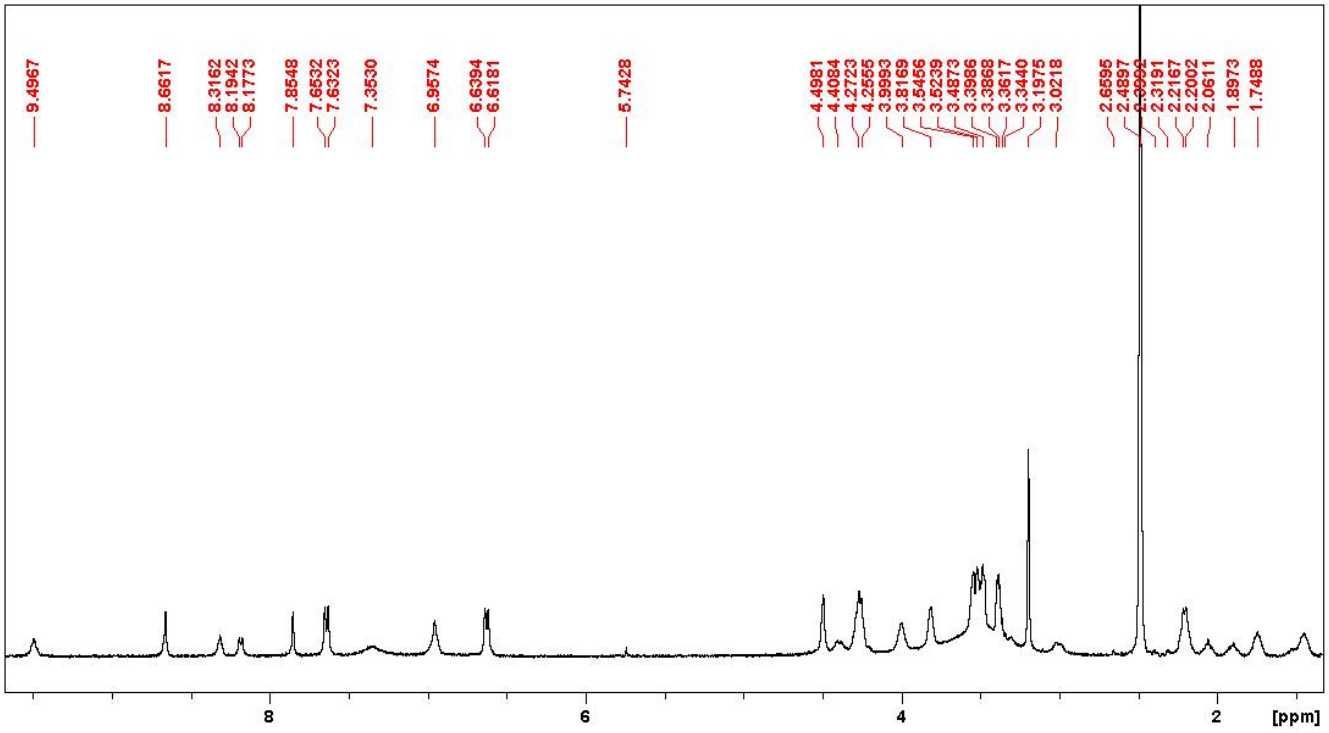
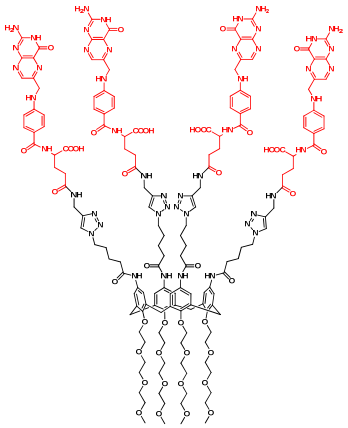


Figure S9: ^{1}NMR spectrum of derivative **7** (DMSO-d_6, 400.13 MHz, 297 K)



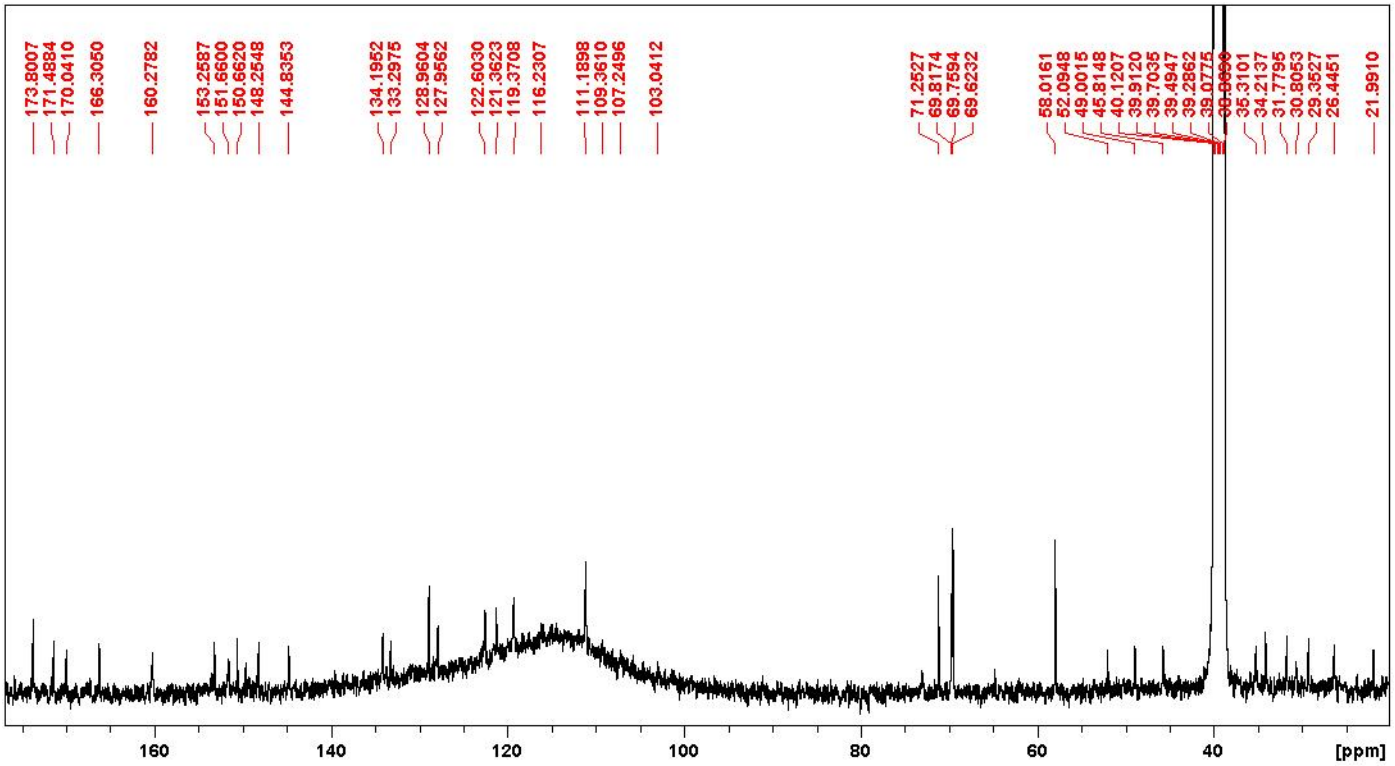


Figure S10: ^{13}C NMR spectrum of derivative **7** (DMSO-d_6, 100.61 MHz, 297 K)

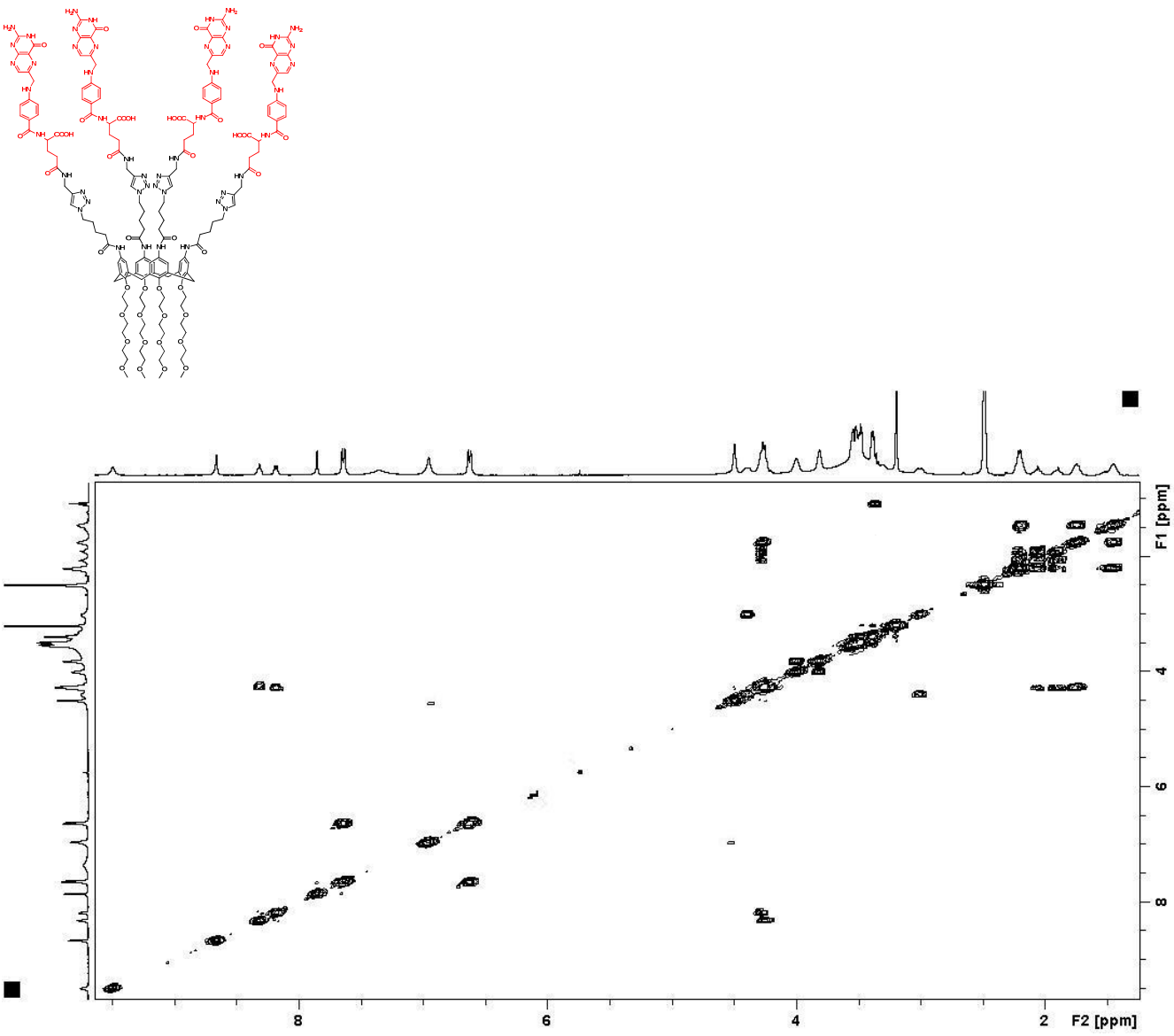
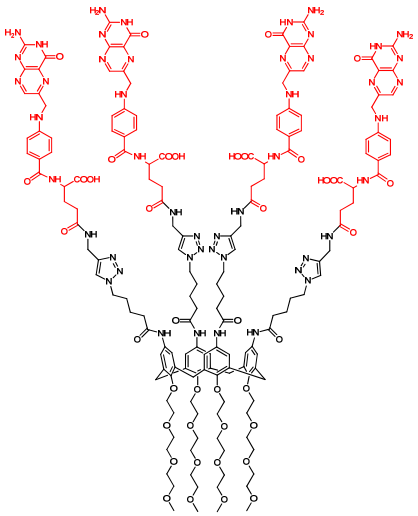


Figure S11: COSY spectrum of derivative **7** (DMSO-d_6, 400.13 MHz, 297 K)



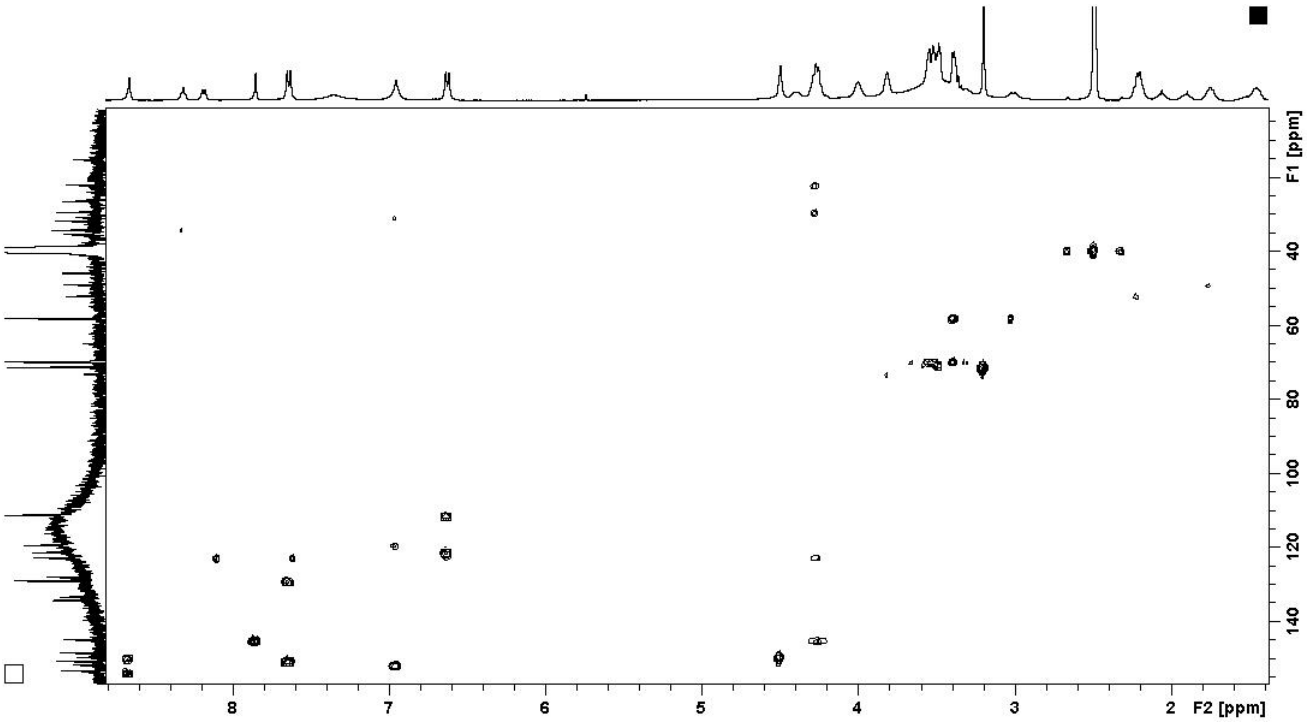


Figure S12: HMBC spectrum of derivative **7** (DMSO-d_6, 297 K)

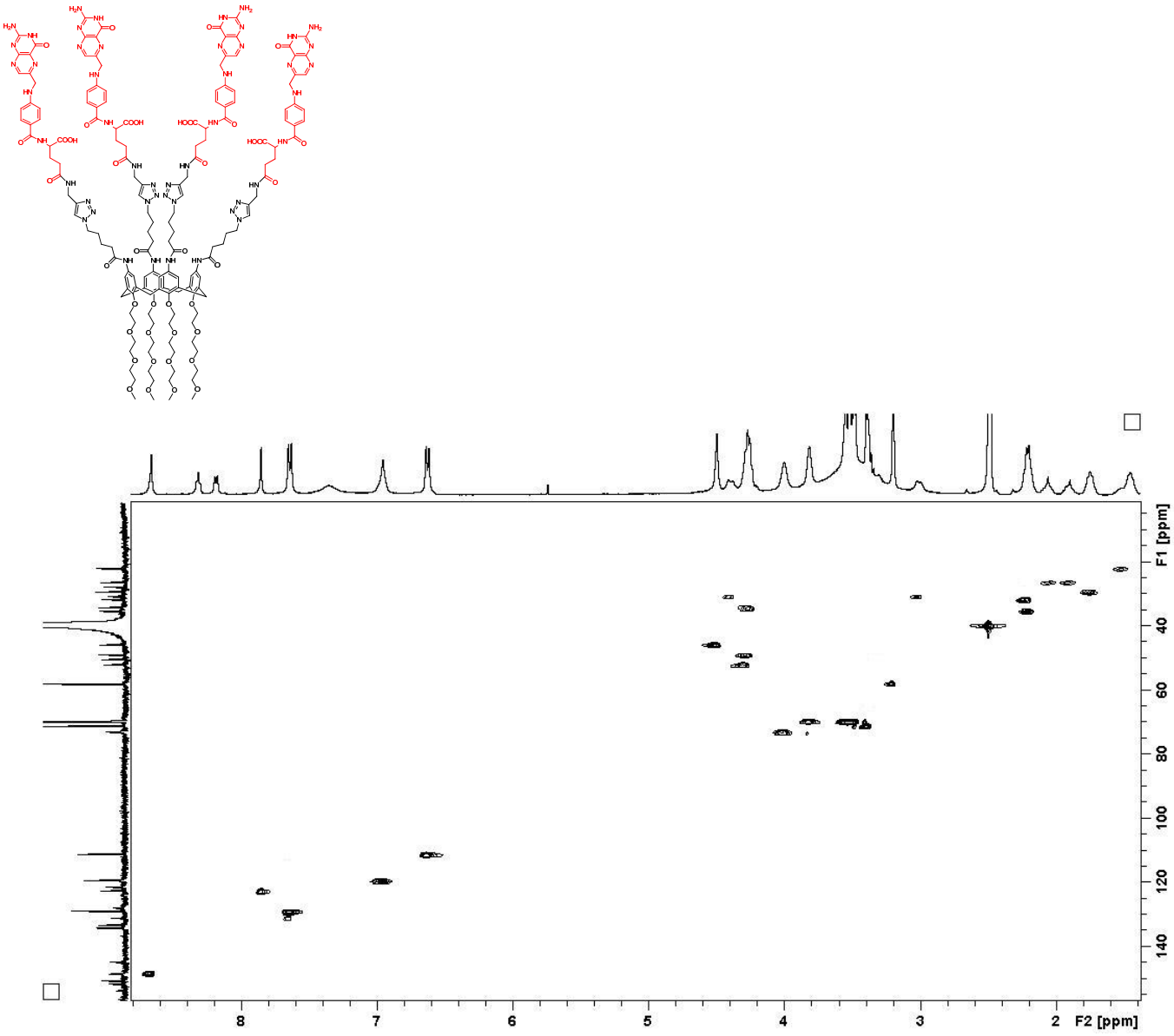


Figure S13 : HSQC spectrum of derivative **7** (DMSO-d_6, 297 K)

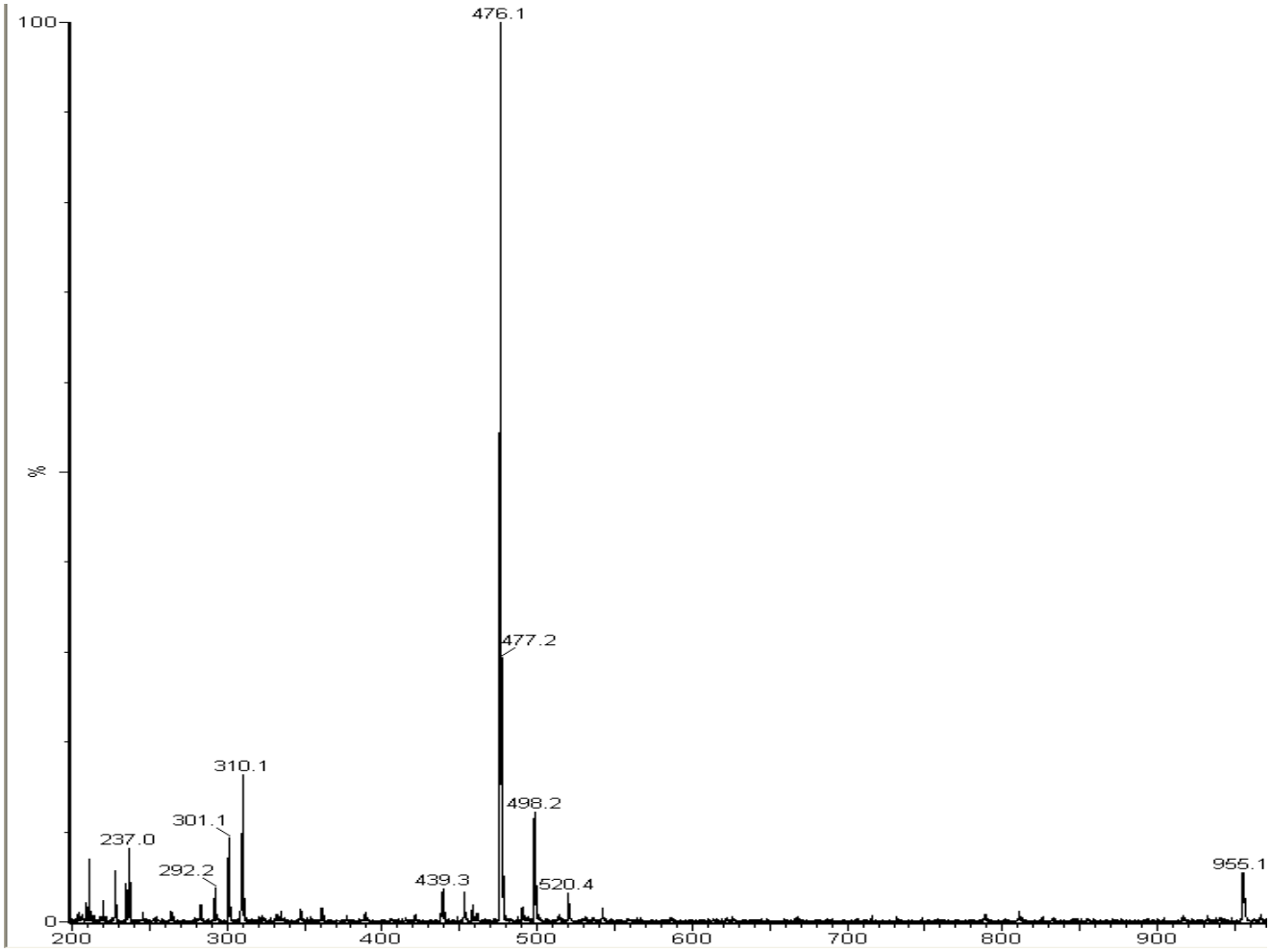


Figure S14: ESI-MS spectrum of derivative **6** in bicarbonate buffer /MeOH, negative mode.

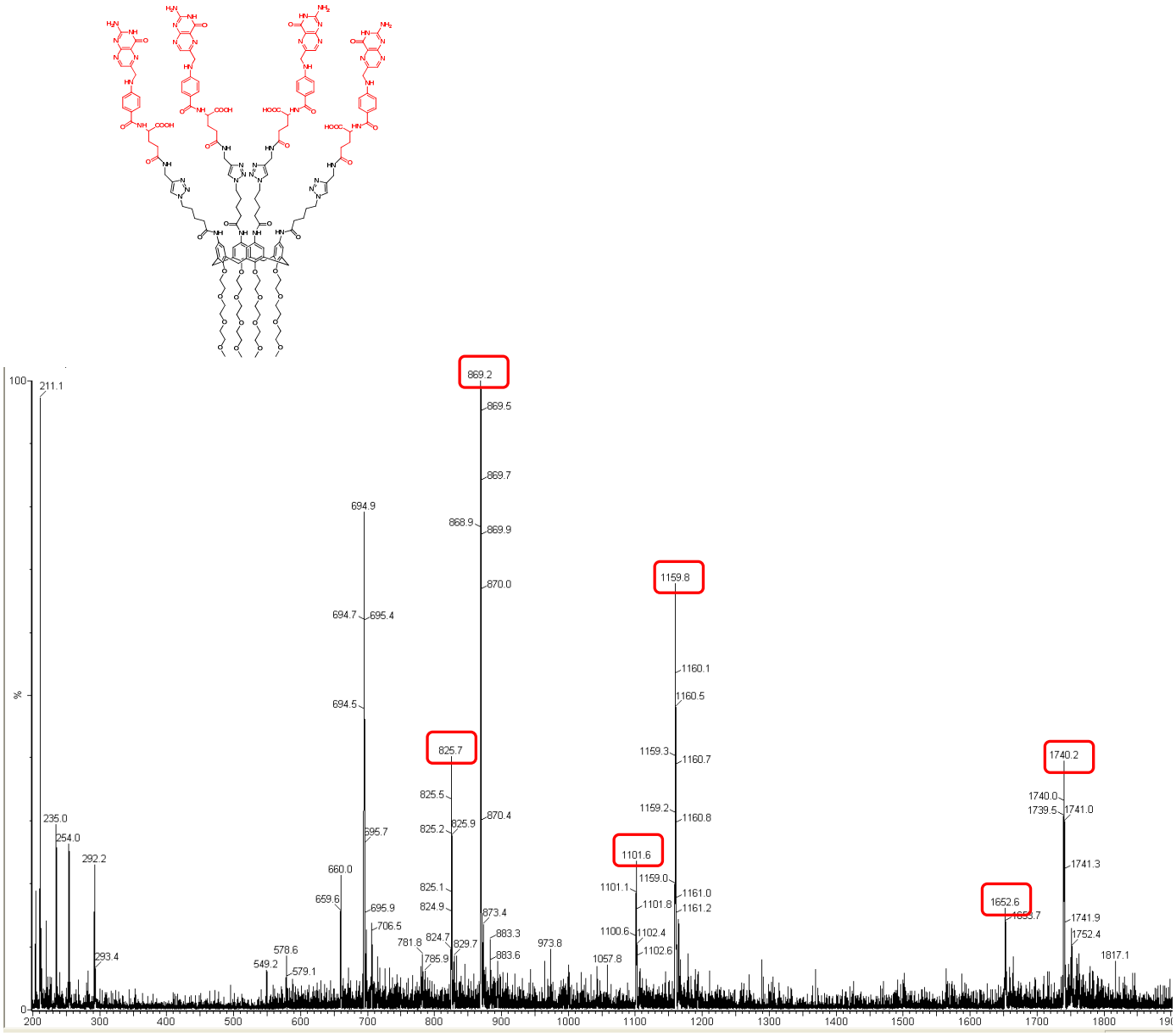


Figure S15: ESI(-)-MS spectrum of derivative **7**.

Diffusion Ordered NMR Spectroscopy (DOSY-NMR)

Diffusion NMR measurements were performed on a Bruker Avance™ 400 spectrometer equipped with a z-gradient system capable of producing magnetic field pulse gradients of about 50 G cm^{-1} and using a 5 mm inverse BBI probe. The measurements were all carried out at 294 ± 0.5 K using LED pulse sequences.[4] The pulsed gradients were incremented from 0.681 to 32.36 G/cm^{-1} in 16 steps.

Gradient duration (δ) and pulse gradient separation (Δ) were 4 ms and 180 ms for sodium salts of **7** (1 mM), indomethacin and their mixture (1:10 molar ratio) in an aqueous NaOD solution (pH 9). The measurements were performed at least three times and diffusion coefficients are reported as the mean ± standard deviation of at least three experiments. All spectra were processed using XWINNMR v3.0 (Bruker) and data analyses were accomplished using t1/t2 routine. The diffusion coefficients were derived by integration of an aromatic peak of indomethacin or compound **7** to a single-exponential decay, using the program Simfit (Bruker). HOD was used as internal standard to calibrate the field gradient.

The diffusion coefficients were used to calculate hydrodynamic radii (r_{exp}) by means of the Einstein-Stokes equation and the obtained values were compared with the calculated average radii (r_{calc}) derived from molecular models.

[4] D. Wu, A. Chen, C. S. Johnson Jr., *J. Magn. Reson.* **1995**, *115*, 260-264.

Solubility Studies

The phase-solubility experiments were performed by the method reported by Higuchi and Connors.[5] Indomethacin (2.5 mg) was added to 0.01 M PBS (200 μL, pH 7.4) containing increasing concentrations of folate─calixarene **7** as sodium salt (0-2 mM). The suspensions were sonicated and shaken at 37 °C, at 300 rpm for 3 days, then centrifugated at 4000 rpm for 10 min. The clear supernatant containing indomethacin─folate-calixarene complex was dried in vacuo. For determining the amount of complexed drug, indomethacin was extracted from the solid by a 1:1 CH_3CN/MeOH mixture (500 μL), filtered (PTFE filter, 0.45 μm), and injected into an HPLC (analytical RP C-18 column, isocratically eluted with a 60:40 CH_3CN/0.1 M aqueous CH_3COOH mixture, flow rate of 1.0 mL/min, 30 °C). The UV detector was set at 250 nm and the retection time was 8.4 min. Samples were prepared in triplicate. The drug content in the solutions was determined according to the peak area, which was referred to the corresponding calibration plot. The phase-solubility diagram was constructed by plotting the dissolved indomethacin concentration against the folate─calix[4]arene (**7**) concentration.

[5] (a) T. Higuchi, K. A. Connors, *Adv. Anal. Chem. Instrum.* **1965**, *4*, 117–212. (b) K. A. Connors, *Binding Constants.* Wiley, New York, 1987, pp. 261-281.

Dynamic Light Scattering

From 2 mM to 0.04 mM solutions were prepared dissolving compound **7** in PBS medium previously filtered by a ChemTeck PTFE 0.45 µm filter to remove dust. The investigated scattering angle was 90°. The scattered light was collected, in a self-beating mode, through an optical fibre matched with a digital Hamamatsu R942 photomultiplier. The signal was sent to a Malvern 4700 submicrometer particle analyzer system in homodyne detection mode to measure the intensity autocorrelation function. Hydrodynamic radius (*r*) of the aggregate molecules was calculated by using CONTIN method see V. Villari, N. Micali, *J. Pharm. Sci.* **2008**, *97*, 1703-1730.

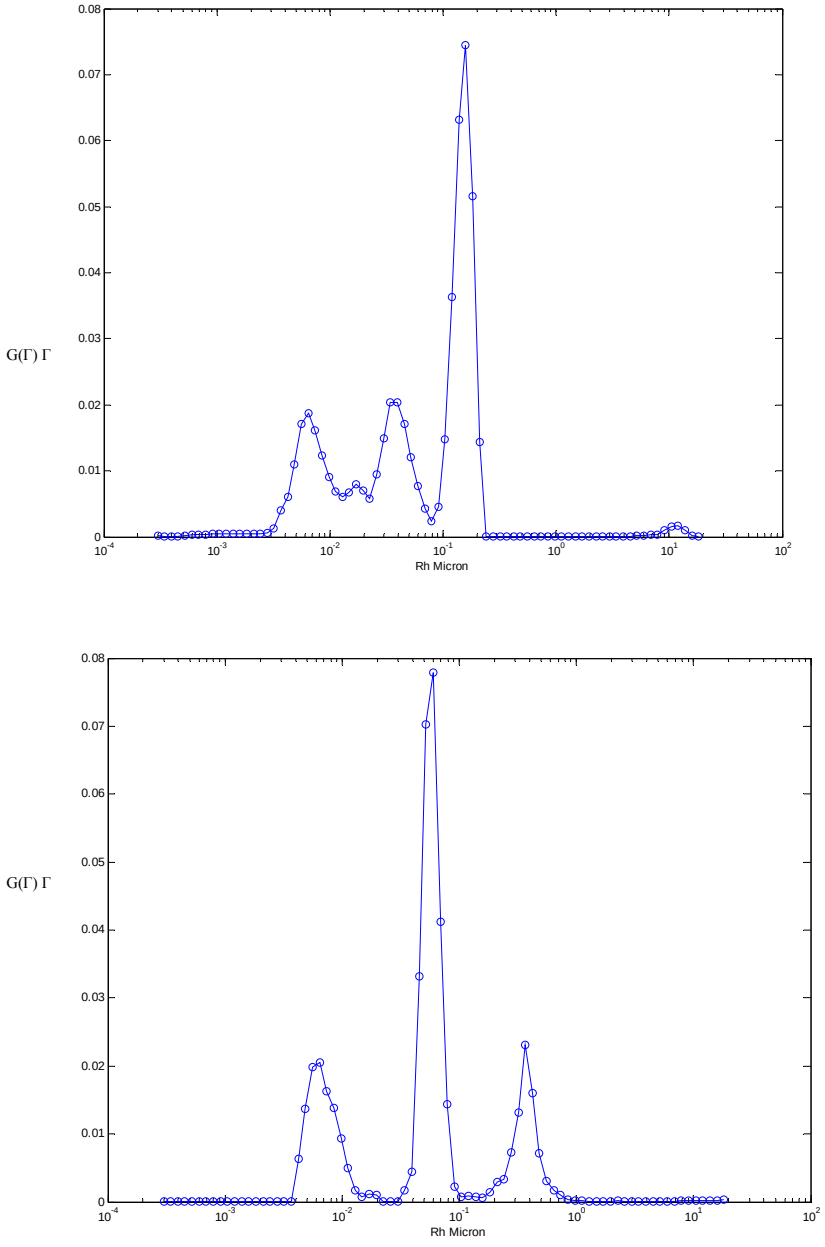


Figure S16. Size distribution of compound 7 in PBS (pH 7.4) at concentration 1mM (top) and 0.06 mM (bottom) obtained by using the algorithm CONTIN as inversion procedure.