Electronic Supplementary Information (ESI) for:

The emergence of halophilic evolutionary patterns from a dynamic combinatorial library of macrocyclic pseudopeptides

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GENERAL CHARACTERISTICS

General: Reagents and solvents were purchased from commercial suppliers (Aldrich, Fluka, or Merck) and were used without further purification. Flash chromatographic purifications and preparative reversed-phase purifications were performed on a Biotage® Isolera Prime™ equipment. TLCs were performed using 6x3 cm SiO_2 pre-coated aluminium plates (ALUGRAM® SIL G/UV_{254}). The compound tritylsulfanyl acetic acid was prepared as previously described.[1]

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) analyses were performed on a Hewlett Packard Series 1100 (UV detector 1315A) modular system using:

i) For the characterization of the intermediates and the final compounds: a reversed-phase X-Terra C_{18} (15 x 0.46 cm, 5 µm) column. (CH_3CN + 0.07% TFA and H_2O + 0.1% TFA) mixtures at 1 mL/min were used as mobile phase and the monitoring wavelengths were set at 220 and 254 nm.

ii) For the analysis of the DCLs: a reversed-phase kromaphase C_{18} (25 x 0.46 cm, 5µm) column. (CH_3CN + 20 mM HCOOH and H_2O + 20 mM HCOOH) mixtures at 1 mL/min were used as mobile phase and the monitoring wavelength was set at 254 nm.

Nuclear Magnetic Resonance (NMR) spectroscopic experiments were carried out on a Varian INOVA 500 spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C) and a Varian Mercury 400 instrument (400 MHz for ^1H and 100 MHz for ^{13}C). The chemical shifts are reported in ppm relative to trimethylsilane (TMS), and coupling constants (J) are reported in Hertz (Hz).

Ultraviolet (UV) and Circular Dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter at room temperature.

pH mesurements were performed on a Crison GLP21 pH-meter with the electrode Crison 50 14T.

High Resolution Mass Spectroscopy (HRMS) analyses were carried out at the IQAC Mass Spectroscopy Facility, using a UPLC-ESI-TOF equipment: [Acquity UPLC® BEH C_{18} 1.7 mm, 2.1x100 mm, LCT Premier Xe, Watters]. (CH_3CN + 20 mM HCOOH and H_2O + 20 mM HCOOH) mixtures at 0.3 mL/min were used as mobile phase.

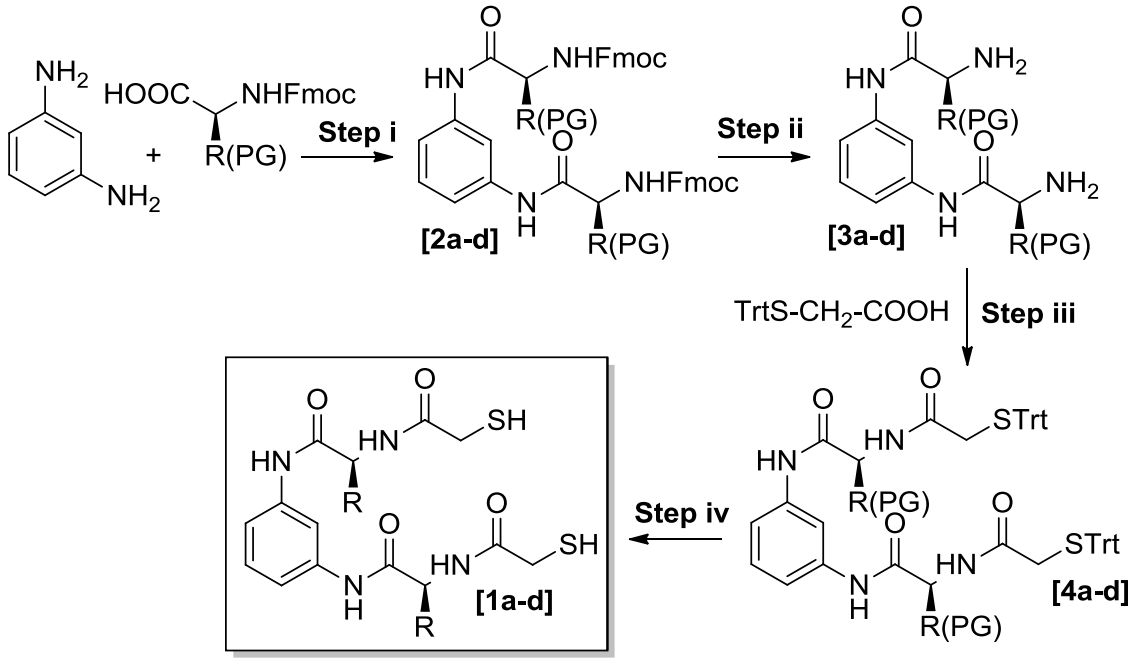
Isothermal Microcalorimetric measurements were carried out at the Scientific and Technologic Center of UB (CCiTUB), using a Microcal VP-ITC Isothermal Titration Calorimeter.

Related references:

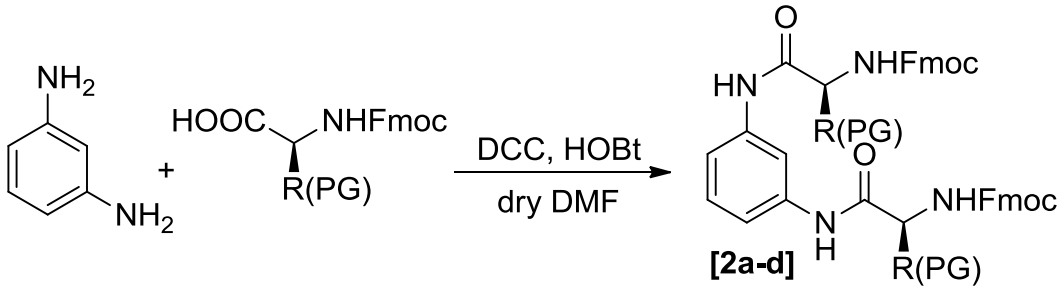
1 A. P. Kozikowski, Y. Chen, A. Gaysin, B. Chen, M. A. D'Annibale, C. M. Suto and B. C. Langley, *J. Med. Chem.*, 2007, **50**, 3054.

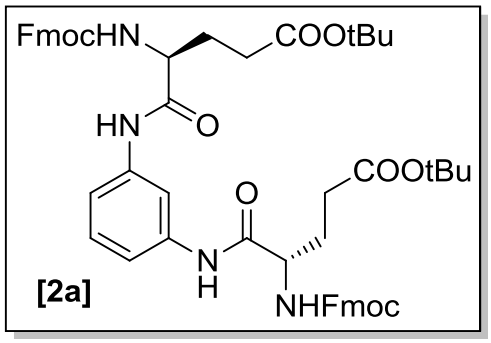
SYNTHESIS OF THE BUILDING BLOCKS **[1a-d]**

General synthetic scheme



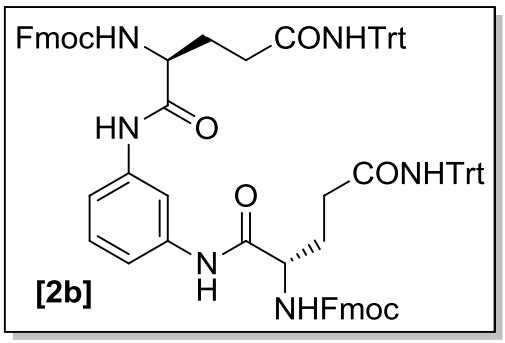
Step i: Experimental procedure for the synthesis of **[2a-d]**



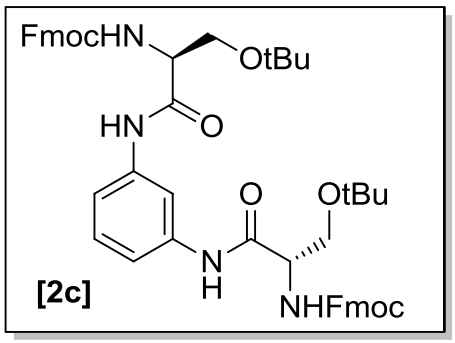


[2a]: Fmoc-Glu(*t*Bu)-OH (2.36 g, 5.55 mmol) was dissolved in dry DMF (20 mL) and both dicyclohexylcarbodiimide (DCC, 1.26 g, 6.10 mmol) and 1-hydroxybenzotriazole (HOBt, 825 mg, 6.10 mmol) were added over the solution. The reaction mixture was cooled to 0ºC. A solution of *m*-phenylenediamine (300 mg, 2.77 mmol) in dry DMF (10 mL) was added over the mixture through a cannula. The solution was stirred at room temperature for 60 hours, after which complete conversion of the starting material was observed by TLC (Rf AcOEt/Hexane, 2:3 (v:v): 0.43). The mixture was filtered, and the filtrate was diluted with DCM, washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 30% to 40% AcOEt) to give 1.79 g of [**2a]** (70% yield) as a white solid. HRMS (ESI+) calcd. for $C_{54}H_{59}N_4O_{10}$ $[M+H]^+$ (m/z): 923.4226, found: 923.4225. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.62 (s, 2H, NH), 7.84 (s, 1H, CH_{Ar}), 7.75 (d, *J* = 7.5 Hz, 4H, CH_{Ar}), 7.59 (t, *J* = 6.8

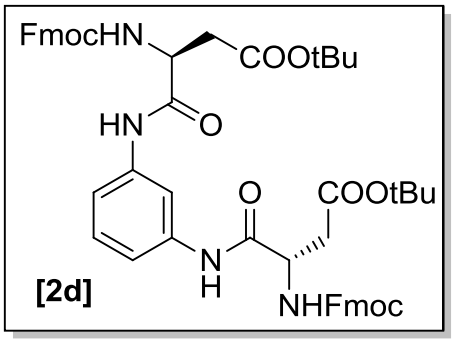
Hz, 4H, CH_{Ar}), 7.38 (t, *J* = 7.4 Hz, 4H, CH_{Ar}), 7.33–7.16 (m, 7H, CH_{Ar}), 5.94 (d, *J* = 7.1 Hz, 2H, NH), 4.47–4.38 (m, 4H, CH_2), 4.37–4.28 (m, 2H, C*H), 4.21 (t, *J* = 7.0 Hz, 2H, CH), 2.59 –2.46 (m, 2H, CH_2COO^tBu), 2.43–2.30 (m, 2H, CH_2COO^tBu), 2.22–2.09 (m, 2H, CH_2C^*H), 2.04–1.93 (m, 2H, CH_2C^*H), 1.46 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.4 (2 x CO), 169.7 (2 x CO), 156.7 (2 x CO), 143.8 (4 x C_{Ar}), 141.4 (4 x C_{Ar}), 138.2 (2 x C_{Ar}), 129.6 (1 x CH_{Ar}), 127.9 (4 x CH_{Ar}), 127.2 (4 x CH_{Ar}), 125.2 (4 x CH_{Ar}), 120.1 (4 x CH_{Ar}), 115.9 (2 x CH_{Ar}), 111.4 (1 x CH_{Ar}), 81.6 (2 x C), 67.4 (2 x CH_2), 55.2 (2 x C*H), 47.2 (2 x CH), 32.1 (2 x CH_2COO^tBu), 28.3 (2 x CH_2C^*H), 28.2 (6 x CH_3).



[2b]: this compound was obtained as described above starting from the Fmoc-Gln(Trt)-OH. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 25% to 40% AcOEt, Rf AcOEt/Hexane, 3:2 (v:v): 0.50) to give 1.11 g of [**2b]** (47% yield) as a white solid. HRMS (ESI+) calcd. for $C_{84}H_{73}N_6O_8$ $[M+H]^+$ (m/z): 1293.5484, found: 1293.5472. ^1H NMR (500 MHz, $CDCl_3$): δ = 8.84 (s, 2H, NH), 7.89 (s, 1H, CH_{Ar}), 7.75 (d, *J* = 7.1 Hz, 4H, CH_{Ar}), 7.61–7.52 (m, 4H, CH_{Ar}), 7.38 (t, *J* = 7.1 Hz, 4H, CH_{Ar}), 7.31–7.18 (m, 34H, CH_{Ar}), 7.10 (t, *J* = 8.0 Hz, 1H, CH_{Ar}), 7.03 (s, 2H, NH), 6.98 (d, *J* = 7.4 Hz, 2H, CH_{Ar}), 6.09 (d, *J* = 4.8 Hz, NH), 4.43–4.30 (m, 4H, CH_2), 4.20 (t, *J* = 7.1 Hz, 2H, CH), 4.17–4.08 (m, 2H, C*H), 2.67–2.55 (m, 2H, $CH_2CONHTrt$), 2.50–2.38 (m, 2H, $CH_2CONHTrt$), 2.19–2.08 (m, 2H, CH_2C^*H), 2.03–1.89 (m, 2H, CH_2C^*H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.5 (2 x CO), 169.5 (2 x CO), 156.5 (2 x CO), 144.5 (6 x C_{Ar}), 144.0 (2 x C_{Ar}), 143.9 (2 x C_{Ar}), 141.4 (2 x C_{Ar}), 141.4 (2 x C_{Ar}), 138.2 (2 x C_{Ar}), 129.2 (1 x CH_{Ar}), 128.8 (12 x CH_{Ar}), 128.2 (12 x CH_{Ar}), 127.8 (6 x CH_{Ar}), 127.2 (4 x CH_{Ar}), 127.2 (4 x CH_{Ar}), 125.3 (4 x CH_{Ar}), 120.1 (4 x CH_{Ar}), 115.9 (2 x CH_{Ar}), 111.6 (1 x CH_{Ar}), 71.0 (2 x C), 67.2 (2 x CH_2), 54.4 (2 x C*H), 47.3 (2 x CH), 34.0 (2 x $CH_2CONHTrt$), 30.4 (2 x CH_2C^*H).

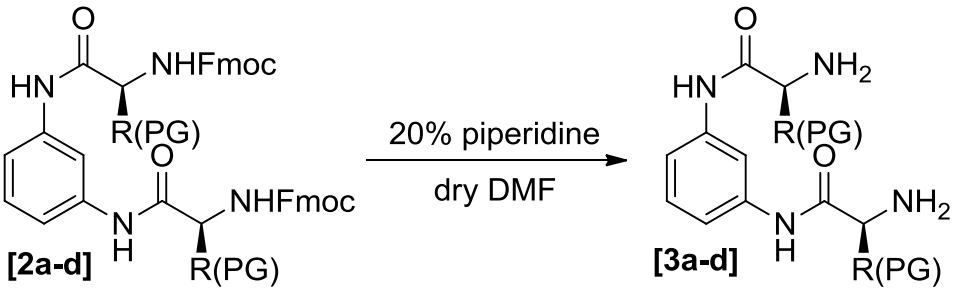


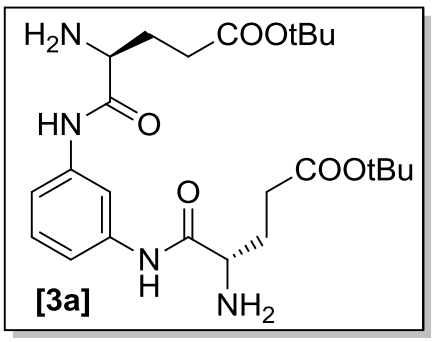
[2c]: this compound was obtained as described above starting from the Fmoc-Ser(tBu)-OH. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 25% to 40% AcOEt, Rf AcOEt/Hexane, 3:2 (v:v): 0.83) to give 1.05 g of [**2c]** (43% yield) as a white solid. HRMS (ESI+) calcd. for $C_{50}H_{55}N_4O_8$ $[M+H]^+$ (m/z): 839.4014, found: 839.4029. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.80 (brs, 2H, NH), 7.96 (s, 1H, CH_{Ar}), 7.77 (d, *J* = 7.6 Hz, 4H, CH_{Ar}), 7.62 (d, *J* = 7.1 Hz, 4H, CH_{Ar}), 7.41 (t, *J* = 7.4 Hz, 4H, CH_{Ar}), 7.32 (t, *J* = 7.8 Hz, 4H, CH_{Ar}), 7.29–7.20 (m, 3H, CH_{Ar}), 5.87 (brs, 2H, NH), 4.44 (d, *J* = 7.0 Hz, 4H, CH_2), 4.35 (brs, 2H, CH_2C^*H), 4.25 (t, *J* = 6.9 Hz, 2H, CH), 3.92 (brs, 2H, CH_2C^*H), 3.45 (t, *J* = 8.7 Hz, 2H: C*H), 1.28 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.5 (2 x CO), 156.2 (2 x CO), 143.9 (4 x C_{Ar}), 141.4 (4 x C_{Ar}), 138.4 (2 x C_{Ar}), 129.9 (1 x CH_{Ar}), 127.9 (4 x CH_{Ar}), 127.2 (4 x CH_{Ar}), 125.2 (4 x CH_{Ar}), 120.2 (4 x CH_{Ar}), 115.5 (2 x CH_{Ar}), 111.0 (1 x CH_{Ar}), 75.1 (2 x C), 67.3 (2 x CH_2), 61.9 (2 x CH_2C^*H), 54.8 (2 x C*H), 47.3 (2 x CH), 27.6 (6 x CH_3).



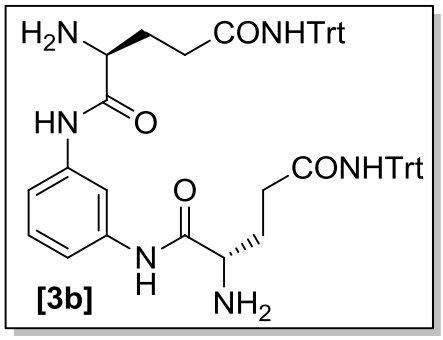
[2d]: this compound was obtained as described above starting from the Fmoc-Asp(tBu)-OH. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 25% to 40% AcOEt, Rf AcOEt/Hexane, 2:3 (v:v): 0.34) to give 978 mg of **[2d]** (42% yield) as a white solid. HRMS (ESI+) calcd. for $C_{52}H_{54}N_4O_{10}Na$ $[M+Na]^+$ (m/z): 917.3732, found: 917.3764. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.57 (s, 2H, NH), 7.80 (s, 1H, CH_{Ar}), 7.76 (d, *J* = 7.4 Hz, 4H, CH_{Ar}), 7.64–7.54 (m, 4H, CH_{Ar}), 7.39 (t, *J* = 7.3 Hz, 4H, CH_{Ar}), 7.34–7.20 (m, 7H, CH_{Ar}), 6.11 (d, *J* = 7.4 Hz, 2H, NH), 4.66 (brs, 2H, C*H), 4.45 (d, *J* = 6.4 Hz, 4H, CH_2), 4.23 (t, *J* = 6.9 Hz, 2H, CH), 2.96 (d, *J* = 16.0 Hz, 2H, CH_2C*H), 2.69 (dd, *J* = 17.0, 6.7 Hz, 2H, CH_2C*H), 1.45 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.5 (2 x CO), 168.7 (2 x CO), 156.4 (2 x CO), 143.8 (4 x C_{Ar}), 141.5 (4 x C_{Ar}), 138.2 (2 x C_{Ar}), 129.7 (1 x CH_{Ar}), 128.0 (4 x CH_{Ar}), 127.3 (4 x CH_{Ar}), 125.2 (4 x CH_{Ar}), 120.2 (4 x CH_{Ar}), 116.1 (2 x CH_{Ar}), 111.5 (1 x CH_{Ar}), 82.5 (2 x C), 67.5 (2 x CH_2), 51.9 (2 x C*H), 47.3 (2 x CH), 34.1 (2 x CH_2COO^{t}Bu), 28.2 (6 x CH_3).

Step ii: Experimental procedure for the synthesis of **[3a-d]**

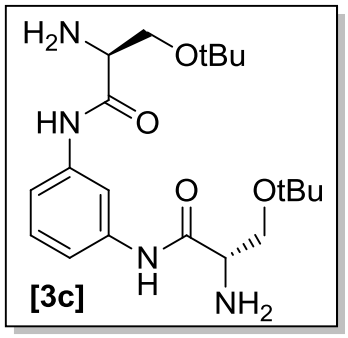




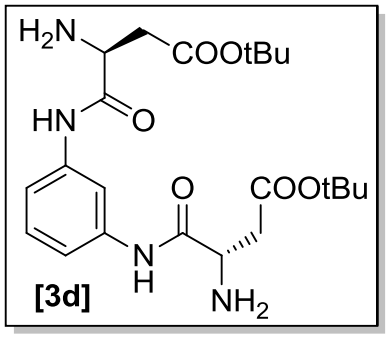
[3a]: [2a] (1.7 g, 1.84 mmol) was dissolved in 4.0 mL of 20% piperidine in dry DMF. After several minutes the product precipitated as a white solid but the mixture was allowed to react for 4 hours until complete conversion of starting material. Diethyl ether was added over the reaction mixture and the product was filtered off and washed with diethyl ether. 1.05 g of **[3a]** were obtained as a white solid (99% yield). HRMS (ESI+) calcd. for $C_{24}H_{39}N_4O_6$ $[M+H]^+$ (m/z): 479.2864, found: 479.2882. ^{1}H NMR (400 MHz, MeOD-*d*$_4$): δ = 7.94 (t, *J* = 2.0 Hz, 1H, CH_{Ar}), 7.37–7.33 (m, 2H, CH_{Ar}), 7.27 (dd, *J* = 9.0, 7.0 Hz, 1H, CH_{Ar}), 3.45 (dd, *J* = 7.2, 6.1 Hz, 2H, C*H), 2.44–2.30 (m, 4H, CH_2COO^{t}Bu), 2.07–1.96 (m, 2H, CH_2C*H), 1.92–1.80 (m, 2H, CH_2C*H), 1.43 (s, 18H, CH_3). ^{13}C NMR (100 MHz, MeOD-*d*$_4$): δ = 175.5 (2 x CO), 174.2 (2 x CO), 140.0 (2 x C_{Ar}), 130.2 (1 x CH_{Ar}), 117.1(2 x CH_{Ar}), 113.1 (1 x CH_{Ar}), 81.7 (2 x C), 56.1 (2 x C*H), 32.7 (2 x CH_2), 31.5 (2 x CH_2), 28.3 (6 x CH_3).



[3b]: 531 mg of [**3b]** (74% yield) were obtained as described above starting from the [**2b]**. HRMS (ESI+) calcd. for $C_{54}H_{53}N_6O_4$ $[M+H]^+$ (m/z): 849.4123, found: 849.4135. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.46 (s, 2H, NH), 7.82 (t, *J* = 1.8 Hz, 1H, CH_{Ar}), 7.35–7.19 (m, 33H, CH_{Ar}), 6.93 (s, 2H, NH), 3.40 (t, *J* = 6.5 Hz, 2H, C*H), 2.53–2.45 (m, 4H, $C\underline{H}_2CO$), 2.13–1.94 (m, 4H, $C\underline{H}_2C$*H), 1.68 (brs, 4H, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.3 (2 x CO), 171.8 (2 x CO), 144.7 (6 x C_{Ar}), 138.4 (2 x C_{Ar}), 129.7 (1 x CH_{Ar}) , 128.8 (12 x CH_{Ar}), 128.1 (12 x CH_{Ar}), 127.2 (6 x CH_{Ar}), 115.2 (2 x CH_{Ar}), 110.5 (1 x CH_{Ar}), 70.7 (2 x C), 54.8 (2 x C*H), 34.1 (2 x $\underline{C}H_2CO$), 31.0 (2 x $\underline{C}H_2C$*H).

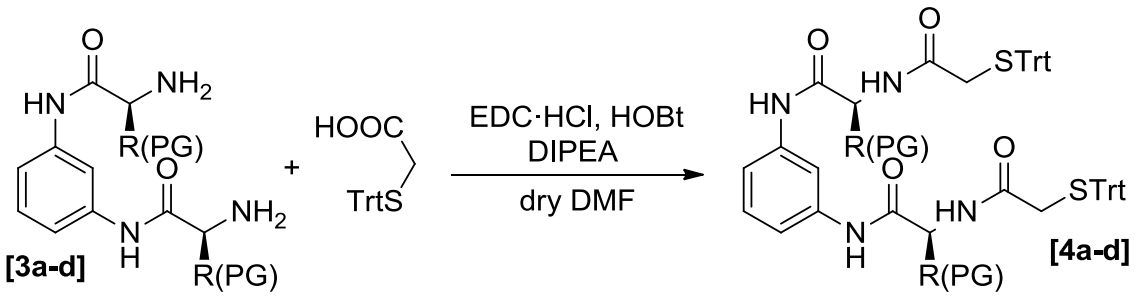


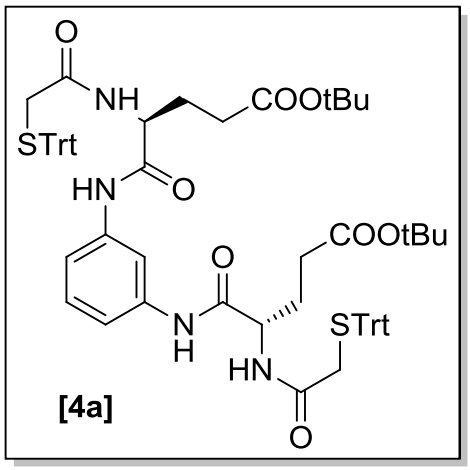
[3c]: 522 mg of [**3c]** (99% yield) were obtained as described above starting from the [**2c]**. HRMS (ESI+) calcd. for $C_{20}H_{35}N_4O_4$ $[M+H]^+$ (m/z): 395.2653, found: 395.2672. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.54 (s, 2H, NH), 7.91 (t, *J* = 2.1 Hz, 1H, CH_{Ar}), 7.39–7.35 (m, 2H, CH_{Ar}), 7.29–7.23 (m, 1H, CH_{Ar}), 3.67 (dd, *J* = 7.2, 3.3 Hz, 2H, CH_2), 3.62–3.55 (m, 4H, 2H x C*H + 2H x CH_2), 2.00 (brs, 4H, NH_2), 1.21 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.6 (2 x CO), 138.6 (2 x C_{Ar}), 129.6 (1 x CH_{Ar}), 115.0 (2x CH_{Ar}), 110.4 (1 x CH_{Ar}), 73.8 (2 x C), 63.8 (2 x CH_2), 56.0 (2 x C*H), 27.7 (6 x CH_3).



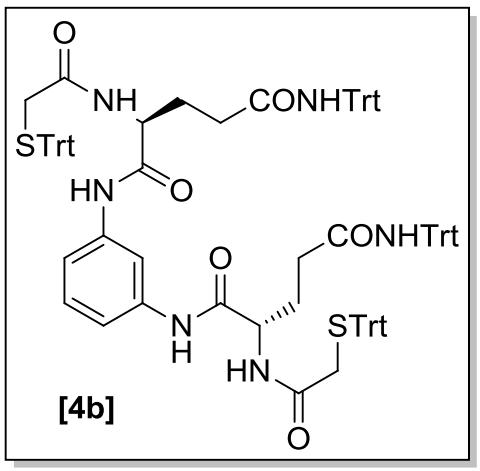
[3d]: 531 mg of [**3d]** (99% yield) were obtained as described above starting from the [**2d]**. HRMS (ESI+) calcd. for $C_{22}H_{35}N_4O_6$ $[M+H]^+$ (m/z): 451.2551, found: 451.2560. ^{1}H NMR (400 MHz, MeOD-*d*$_4$): δ = 7.92 (t, *J* = 2.0 Hz, 1H, CH_{Ar}), 7.38–7.34 (m, 2H, CH_{Ar}), 7.27 (dd, *J* = 8.9, 7.2 Hz, 1H, CH_{Ar}), 3.75 (dd, *J* = 6.7, 6.0 Hz, 2H, C*H), 2.74 (dd, *J* = 16.2, 6.0 Hz, 2H, CH_2), 2.62 (dd, *J* = 16.2, 6.7 Hz, 2H, CH_2), 1.43 (s, 18H, CH_3). ^{13}C NMR (100 MHz, MeOD-*d*$_4$): δ = 174.5 (2 x CO), 172.1 (2 x CO), 140.0 (2 x C_{Ar}), 130.2(1 x CH_{Ar}), 116.9 (2 x CH_{Ar}), 112.8 (1 x CH_{Ar}), 82.3 (2 x C), 53.6 (2 x C*H), 41.5 (2 x CH_2), 28.3 (6 x CH_3).

Step iii: Experimental procedure for the synthesis of **[4a-d]**

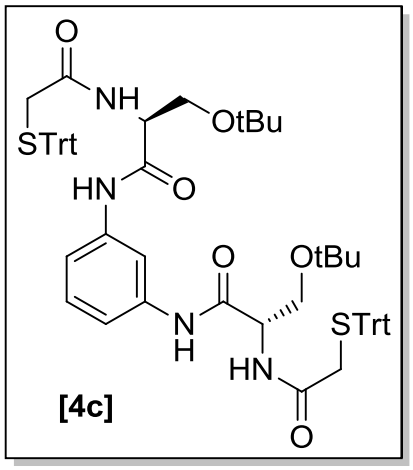




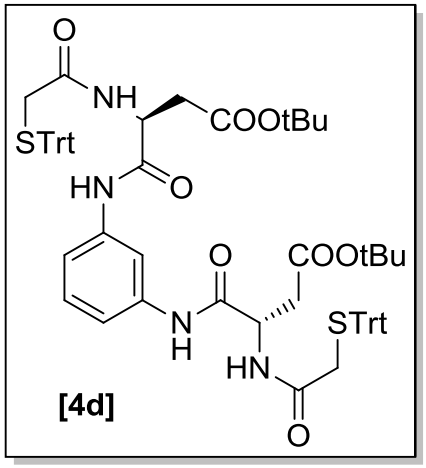
[4a]: tritylsulfanyl acetic acid (243 mg, 0.73 mmol) was dissolved in dry DMF (10 mL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 156 mg, 0.81 mmol), HOBt (110 mg, 0.81 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 255 µL, 1.29 mmol) were added over the solution. The reaction mixture was cooled to 0ºC and then, **[3a]** (166 mg, 0.35 mmol) was added over the mixture. The solution was allowed to stir at room temperature for 60 hours, after which complete conversion of the starting material was observed by TLC (Rf AcOEt/Hexane, 2:3 (v:v): 0.20). The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$ and dried under reduced pressure. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 30% to 40% AcOEt) to give 285 mg of [**4a]** (74% yield) as a white solid. HRMS (ESI+) calcd. for $C_{66}H_{71}N_4O_8S_2$ $[M+H]^+$ (m/z): 1111.4708, found: 1111.4696. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.72 (s, 2H, NH), 7.78 (t, *J* = 1.8 Hz, 1H, CH_{Ar}), 7.44–7.37 (m, 12H, CH_{Ar}), 7.30–7.22 (m, 14H, CH_{Ar}), 7.22–7.16 (m, 7H, CH_{Ar}), 6.80 (d, *J* = 7.2 Hz, 2H, NH), 4.26 (q, *J* = 6.8 Hz, 2H, C*H), 3.14 (ABq, δ_A = 3.12, δ_B = 3.16, *J* = 16.1Hz, 4H, CH_2STrt), 2.45–2.34 (m, 2H, CH_2COO ^{t}Bu), 2.28–2.17 (m, 2H, CH_2COO ^{t}Bu), 2.08–1.96 (m, 2H, CH_2C*H), 1.83–1.71 (m, 2H, CH_2C*H), 1.44 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.1 (2 x CO), 169.0 (2 x CO), 168.8 (2 x CO), 144.0 (6 x C_{Ar}), 138.4 (2 x C_{Ar}), 129.6 (12 x CH_{Ar}), 129.5 (1 x CH_{Ar}), 128.3 (12 x CH_{Ar}), 127.2 (6 x CH_{Ar}), 115.7 (2 x CH_{Ar}), 111.0 (1 x CH_{Ar}), 81.3 (2 x C), 68.1 (2 x C), 53.5 (2 x C*H), 36.1 (2 x CH_2STrt), 32.0 (2 x CH_2COO^{t}Bu), 28.2 (6 x CH_3), 27.8 (2 x CH_2C*H).



[4b]: this compound was obtained as described above starting from [**3b]**. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 30% to 40% AcOEt, Rf AcOEt/Hexane, 1:1 (v:v): 0.23) to give 593 mg of [**4b]** (66% yield) as a white solid. HRMS (ESI+) calcd. for $C_{96}H_{85}N_6O_6S_2$ $[M+H]^+$ (m/z): 1481.5967, found: 1481.5916. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.76 (s, 2H, NH), 7.73 (t, *J* = 1.9 Hz, 1H, CH_{Ar}), 7.44–7.38 (m, 12H, CH_{Ar}), 7.31–6.96 (m, 55H, 43H x CH_{Ar} + 4H x NH + 8H x CH_{Ar}), 4.03 (dd, *J* = 12.6, 6.8 Hz, 2H, C*H), 3.14 (ABq, δ_A = 3.05, δ_B = 3.07, *J* = 15.8Hz, 4H, C$\underline{H}_2$STrt), 2.59–2.49 (m, 2H, C$\underline{H}_2$CONHTrt), 2.39–2.29 (m, 2H, C$\underline{H}_2$CONHTrt), 2.03–1.92 (m, 2H, C$\underline{H}_2$C*H), 1.81–1.70 (m, 2H, C$\underline{H}_2$C*H).^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.5 (2 x CO), 168.8 (2 x CO), 168.6 (2 x CO), 144.5 (3 x C_{Ar}), 144.1 (3 x C_{Ar}), 138.2 (2 x C_{Ar}), 129.7 (6 x CH_{Ar}), 129.2 (1 x CH_{Ar}), 128.8 (6 x CH_{Ar}), 128.3 (6 x CH_{Ar}), 128.1 (6 x CH_{Ar}), 127.2 (3 x CH_{Ar}), 127.1(3 x CH_{Ar}), 115.9 (2 x CH_{Ar}), 111.4 (1 x CH_{Ar}), 70.9 (2 x C), 68.0 (2 x C), 53.1 (2 x C*H), 36.3 (2 x $\underline{C}H_2$STrt), 34.2 (2 x $\underline{C}H_2$CONHTrt), 30.3 (2 x $\underline{C}H_2$C*H).

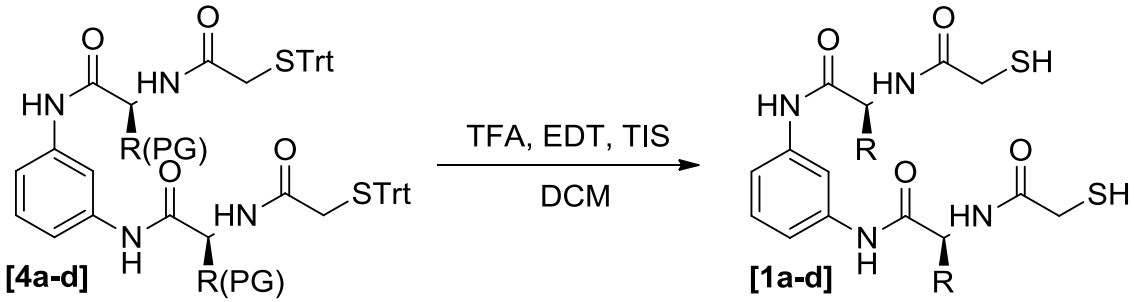


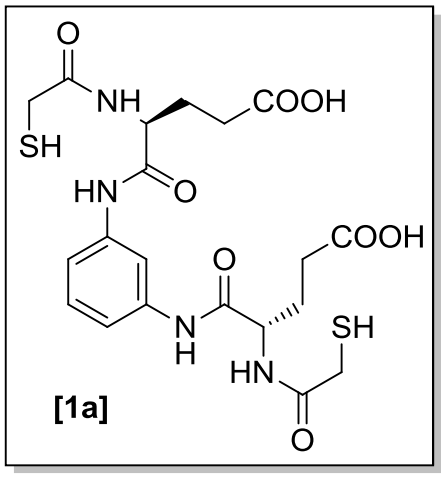
[4c]: this compound was obtained as described above starting from **[3c]**. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 35% to 45% AcOEt, Rf AcOEt/Hexane, 2:3 (v:v): 0.27) to give 612 mg of **[4c]** (51% yield) as a white solid. HRMS (ESI+) calcd. for $C_{62}H_{67}N_4O_6S_2$ $[M+H]^+$ (m/z): 1027.4497, found: 1027.4492. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.68 (s, 2H, NH), 7.79 (t, *J* = 1.8 Hz, 1H, CH_{Ar}), 7.43 (d, *J* = 7.3 Hz, 12H, CH_{Ar}), 7.28 (t, *J* = 7.6 Hz, 12H, CH_{Ar}), 7.25–7.18 (m, 9H, CH_{Ar}), 7.10 (d, *J* = 5.8 Hz, 2H, NH), 4.24–4.18 (m, 2H, C*H), 3.71 (dd, *J* = 8.6, 4.3 Hz, 2H, C$\underline{H}_2$O^{t}Bu), 3.20–3.08 (m, 6H, 2H x C$\underline{H}_2$O^{t}Bu + 4H x C$\underline{H}_2$STrt), 1.22 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.7(2 x CO), 168.2 (2 x CO), 144.1 (6 x C_{Ar}), 138.4 (2 x C_{Ar}), 129.8 (1 x CH_{Ar}), 129.7 (12 x CH_{Ar}), 128.3 (12 x CH_{Ar}), 127.1 (6 x CH_{Ar}), 115.5 (2 x CH_{Ar}), 110.9 (1 x CH_{Ar}), 75.0 (2 x C), 68.0 (2 x C), 61.0 (2 x $\underline{C}H_2$C*H), 53.5 (2 x C*H), 36.2 (2 x CH_2), 27.6 (6 x CH_3).



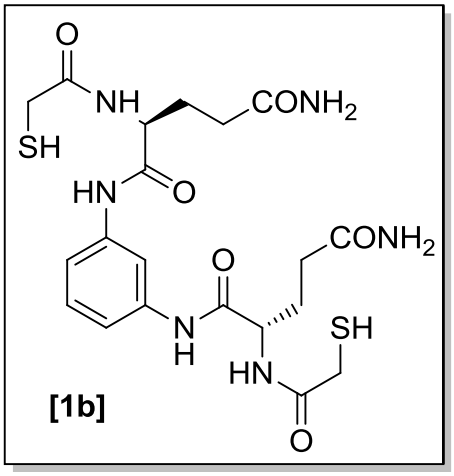
[4d]: this compound was obtained as described above starting from **[3d]**. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 25% to 45% AcOEt, Rf AcOEt/Hexane, 2:3 (v:v): 0.30) to give 644 mg of [**4d]** (69% yield) as a white solid. HRMS (ESI+) calcd. for $C_{64}H_{66}N_4O_8S_2Na$ $[M+Na]^+$ (m/z): 1105.4214, found: 1105.4236. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.54 (s, 2H, NH), 7.63 (s, 1H, CH_{Ar}), 7.40 (d, *J* = 7.9 Hz, 12H, CH_{Ar}), 7.31–7.16 (m, 23H, 21H x CH_{Ar} + 2H x NH), 4.54 (td, *J* = 7.6, 4.0 Hz, 2H, C*H), 3.16 (ABq, δ_A = 3.18, δ_B = 3.14, *J* = 16.3 Hz, 4H, C$\underline{H}_2$STrt), 2.69 (dd, *J* = 17.2, 3.9 Hz, 2H, C$\underline{H}_2$C*H), 2.44 (dd, *J* = 17.1, 7.8 Hz, 2H, C$\underline{H}_2$C*H), 1.43 (s, 18H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.3 (2 x CO), 169.0 (2 x CO), 168.0 (2 x CO), 144.0 (6 x C_{Ar}), 138.3 (2 x C_{Ar}), 129.6 (1 x CH_{Ar}), 129.6 (12 x CH_{Ar}), 128.3 (12 x CH_{Ar}), 127.26 (6 x CH_{Ar}), 116.0 (2 x CH_{Ar}), 111.3 (1 x CH_{Ar}), 82.3 (2 x C), 68.2 (2 x C), 50.4 (2 x C*H), 36.6 (2 x CH_2), 36.0 (2 x CH_2), 28.2 (6 x CH_3).

Step iv: Experimental procedure for the synthesis of **[1a-d]**

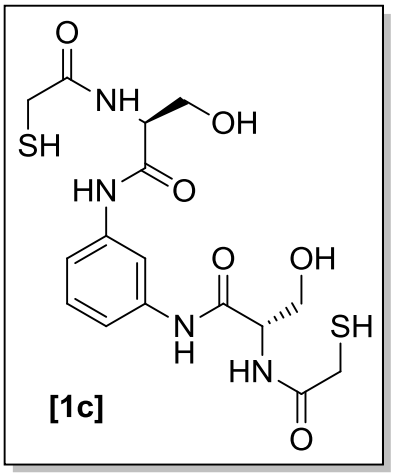




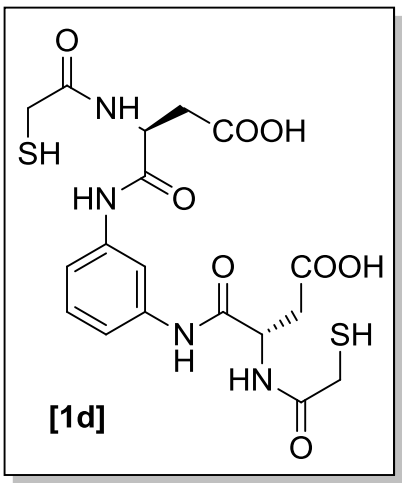
[1a]: [**4a]** was dissolved in DCM (1 mL) and 6.3 mL of trifluoroacetic acid (TFA), 340 µL of triisobutylsilane (TIS) and 170 µL of 1,2-ethanedithiol (EDT) were added rapidly and under stirring. The reaction mixture was allowed to stirr at room temperature for 40 min, after which the solvents were partially evaporated using a N_2 flow. Diethyl ether was added over the reaction mixture and the product was filtered off and washed with diethyl ether. The product was purified using reversed-phase flash chromatography (gradient: from 5% to 30% CH_3CN in H_2O) and 74.6 mg of [**1a]** were obtained as a white solid (89% yield). HRMS (ESI+) calcd. for $C_{20}H_{27}N_4O_8S_2$ $[M+H]^+$ (m/z): 515.1265, found: 515.1271. ^{1}H NMR (400 MHz, MeOD-*d*$_4$): δ = 7.90 (t, *J* = 1.8 Hz, 1H, CH_{Ar}), 7.36–7.30 (m, 2H, CH_{Ar}), 7.29–7.23 (m, 1H, CH_{Ar}), 4.53 (dd, *J* = 8.7, 5.3 Hz, 2H, C*H), 3.24 (s, 4H, C$\underline{H}_2$SH), 2.45 (t, *J* = 7.6 Hz, 4H, C$\underline{H}_2$COOH), 2.24–2.13 (m, 2H, C$\underline{H}_2$C*H), 2.07–1.97 (m, 2H, C$\underline{H}_2$C*H). ^{13}C NMR (100 MHz, MeOD-*d*$_4$): δ = 174.9 (2 x COOH), 172.0 (2 x $\underline{C}OCH_2$), 170.3 (2 x $\underline{C}$OC*H), 138.4 (2 x C_{Ar}), 130.1 (1 x CH_{Ar}), 117.4 (2 x CH_{Ar}), 113.5 (1 x CH_{Ar}), 54.8 (2 x C*H), 31.0 (2 x $\underline{C}H_2$COOH), 28.8 (2 x $\underline{C}H_2$C*H), 28.0 (2 x CH_2SH).



[1b]: 63.6 mg of [**1b]** (92% yield) were obtained as described above starting from the [**4b]**. RP-HPLC (gradient: from 5% to 30% CH_3CN in H_2O). HRMS (ESI+) calcd. for $C_{20}H_{29}N_6O_6S_2$ $[M+H]^+$ (m/z): 513.1585, found: 513.1585. ^1H NMR (400 MHz, DMSO-d_6): δ = 10.08 (s, 2H, NH), 8.29 (d, J = 7.8 Hz, 2H, NH), 7.98–7.93 (m, 1H, CH_{Ar}), 7.34–7.27 (m, 4H, 2H x CH_{Ar} + 2H x NH_2), 7.26–7.18 (m, 1H, CH_{Ar}), 6.76 (s, 2H, NH_2), 4.44–4.34 (m, 2H, C*H), 3.24–3.13 (m, 4H, $C\underline{H}_2SH$), 2.75 (t, J = 8.0 Hz, 2H, $S\underline{H}$), 2.21–2.06 (m, 4H, $C\underline{H}_2CO$), 1.99–1.88 (m, 2H, $C\underline{H}_2C^*H$), 1.88–1.75 (m, 2H, $C\underline{H}_2C^*H$). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 173.3 (2 x $CONH_2$), 169.9 (2 x $\underline{C}OC^*H$), 169.6 (2 x $\underline{C}OCH_2$), 138.9 (2 x C_{Ar}), 138.6 (1 x CH_{Ar}), 124.3 (2 x CH_{Ar}), 120.2 (1 x CH_{Ar}), 62.9 (2 x C*H), 40.9 (2 x $\underline{C}H_2CO$), 37.6 (2 x $\underline{C}H_2C^*H$), 36.5 (2 x $\underline{C}H_2SH$).



[1c]: 76.4 mg of [**1c]** (90% yield) were obtained as described above starting from the [**4c]**. RP-HPLC (gradient: from 5% to 30% CH_3CN in H_2O). HRMS (ESI+) calcd. for $C_{16}H_{23}N_4O_6S_2$ $[M+H]^+$ (m/z): 431.1054, found: 431.1060. ^1H NMR (400 MHz, MeOD-d_4): δ = 7.92 (t, J = 1.8 Hz, 1H, CH_{Ar}), 7.36–7.31 (m, 2H, CH_{Ar}), 7.28–7.23 (m, 1H, CH_{Ar}), 4.56 (t, J = 5.3 Hz, 2H, C*H), 3.92–3.82 (m, 4H, $C\underline{H}_2OH$), 3.28 (s, 4H, $C\underline{H}_2SH$). ^{13}C NMR (100 MHz, MeOD-d_4): δ = 173.0 ($COCH_2$), 170.2 (COCH), 139.6 (2 x C_{Ar}), 129.9 (1 xCH_{Ar}), 117.3 (2 x CH_{Ar}), 113.4 (1 x CH_{Ar}), 62.8 (2 x CH_2OH), 57.2 (2 x C*H), 27.9 (2 x CH_2SH).



[1d]: 75.1 mg of [**1d]** (91% yield) were obtained as described above starting from the [**4d]**. RP-HPLC (gradient: from 5% to 30% CH_3CN in H_2O). HRMS (ESI+) calcd. for $C_{18}H_{23}N_4O_8S_2$ $[M+H]^+$ (m/z): 487.0952, found: 487.0956. ^1H NMR (400 MHz, MeOD-d_4): δ = 7.85 (t, J = 2.0 Hz, 1H, CH_{Ar}), 7.35–7.29 (m, 2H, CH_{Ar}), 7.24 (dd, J = 9.1, 6.9 Hz, 1H, CH_{Ar}), 4.88–4.82 (m, 2H, C^*H+H_2O), 3.24 (s, 4H, $C\underline{H}_2SH$), 2.91 (dd, J = 16.7, 6.4 Hz, 2H, $C\underline{H}_2C^*H$), 2.78 (dd, J = 16.7, 7.0 Hz, 2H, $C\underline{H}_2CO$). ^{13}C NMR (100 MHz, MeOD-d_4): δ = 173.6 (2 x CO), 173.4 (2 x CO), 170.9 (2 x CO), 139.8 (2 xC_{Ar}), 130.1 (1 x CH_{Ar}), 117.6 (2 x CH_{Ar}), 113.8 (1 x CH_{Ar}), 52.3 (2 x C*H), 36.8 (2 x $\underline{C}H_2CO$), 28.1 (2 x CH_2SH).

NMR spectra, HRMS (ESI+) spectra and HPLC traces of **[1a-1d]**

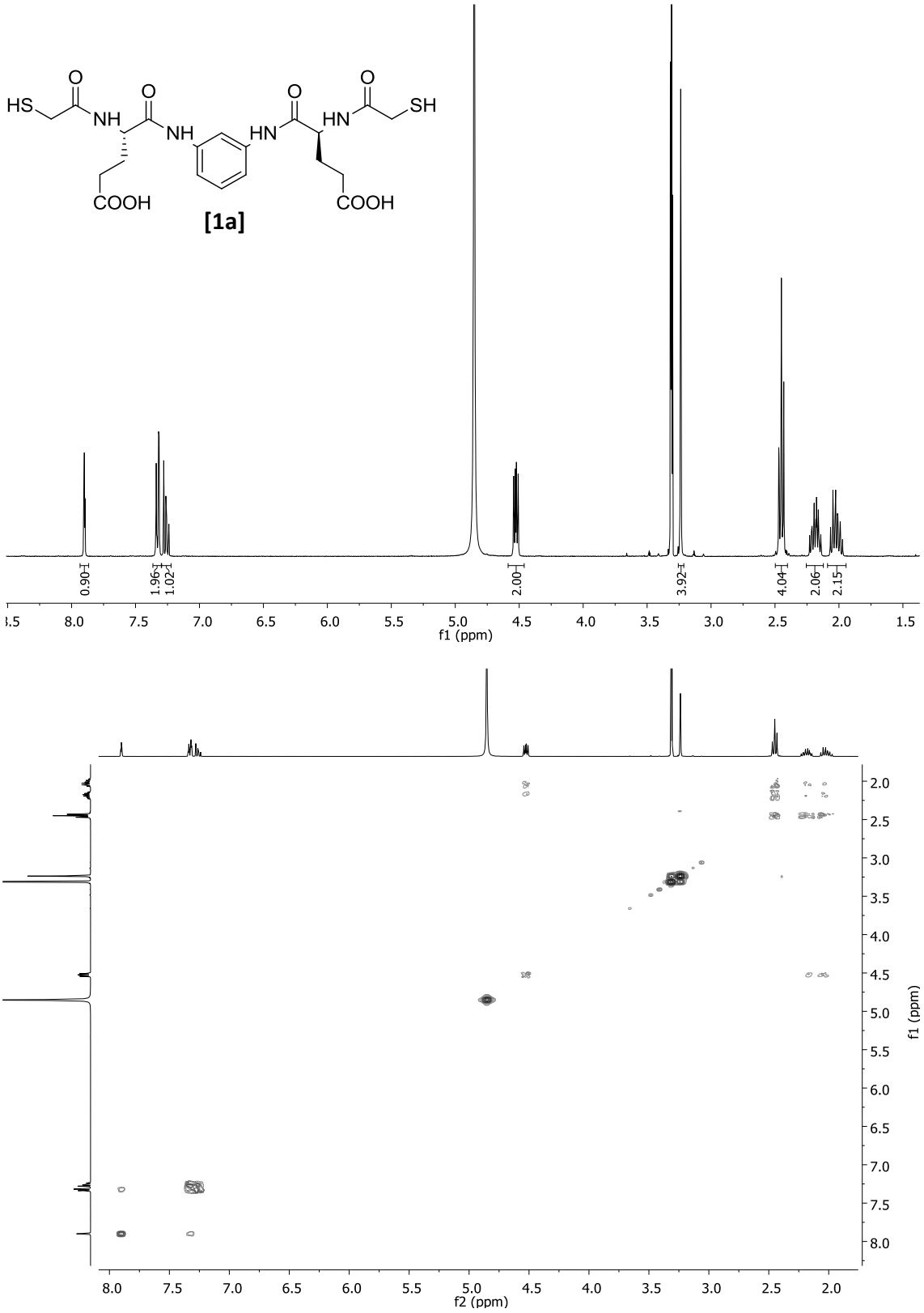


Figure S1: ^{1}H (400 MHz, 298 K in MeOD-d_4) and gCOSY (400 MHz, 298 K in MeOD-d_4) spectra of **[1a]**.

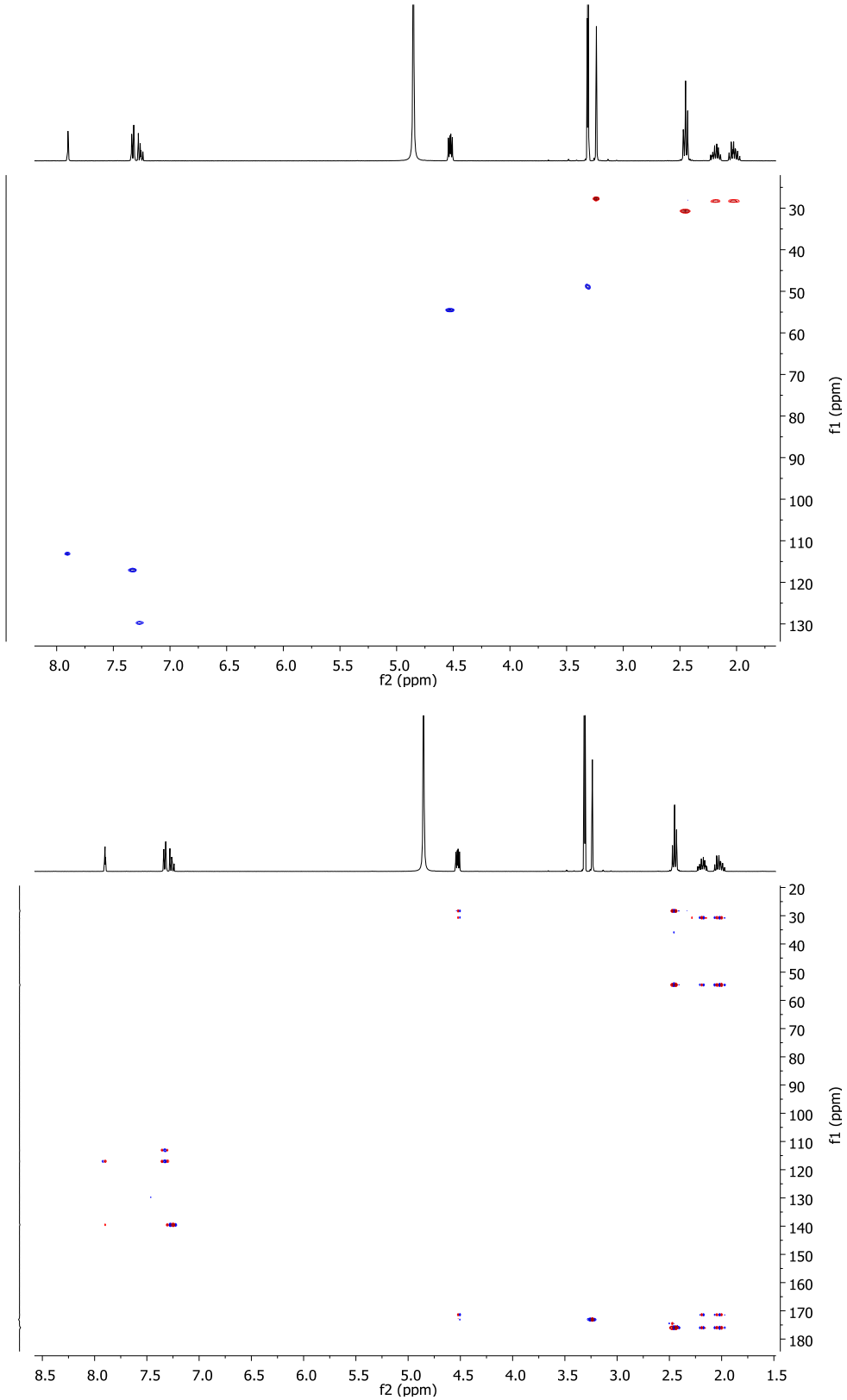


Figure S2: gHSQC (400 MHz, 298 K in MeOD-d_4) and gHMBC (400 MHz, 298 K in MeOD-d_4) spectra of **[1a]**.

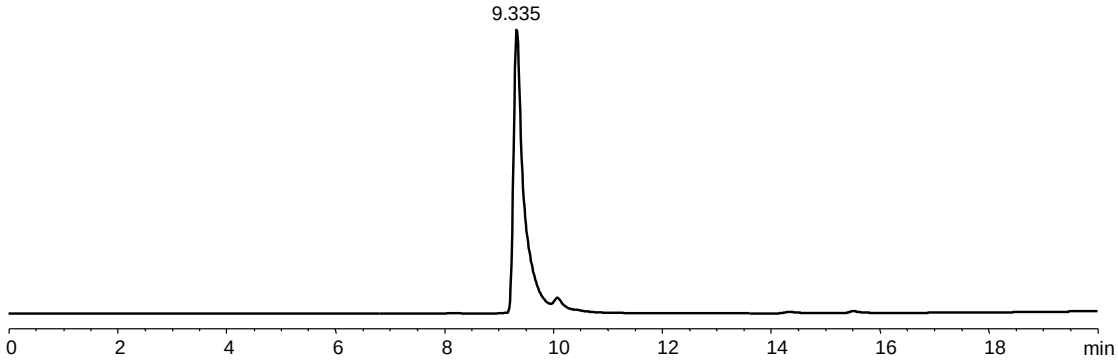


Figure S3: HPLC of **[1a]** (2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 100% CH_3CN over 18 min).

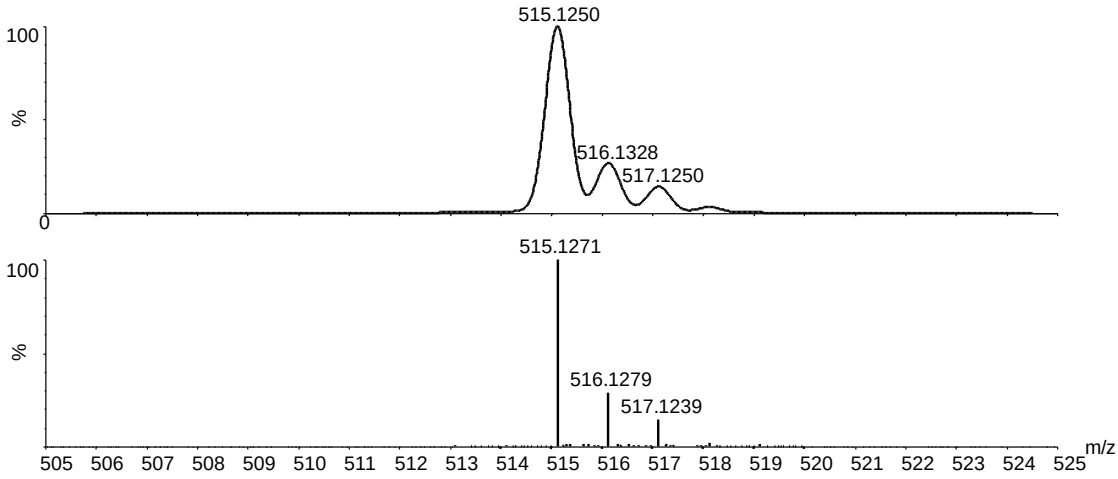


Figure S4: experimental (lower trace) and simulated (upper trace) ESI-TOF mass spectra for $[M+H]^+$ of **[1a]**.

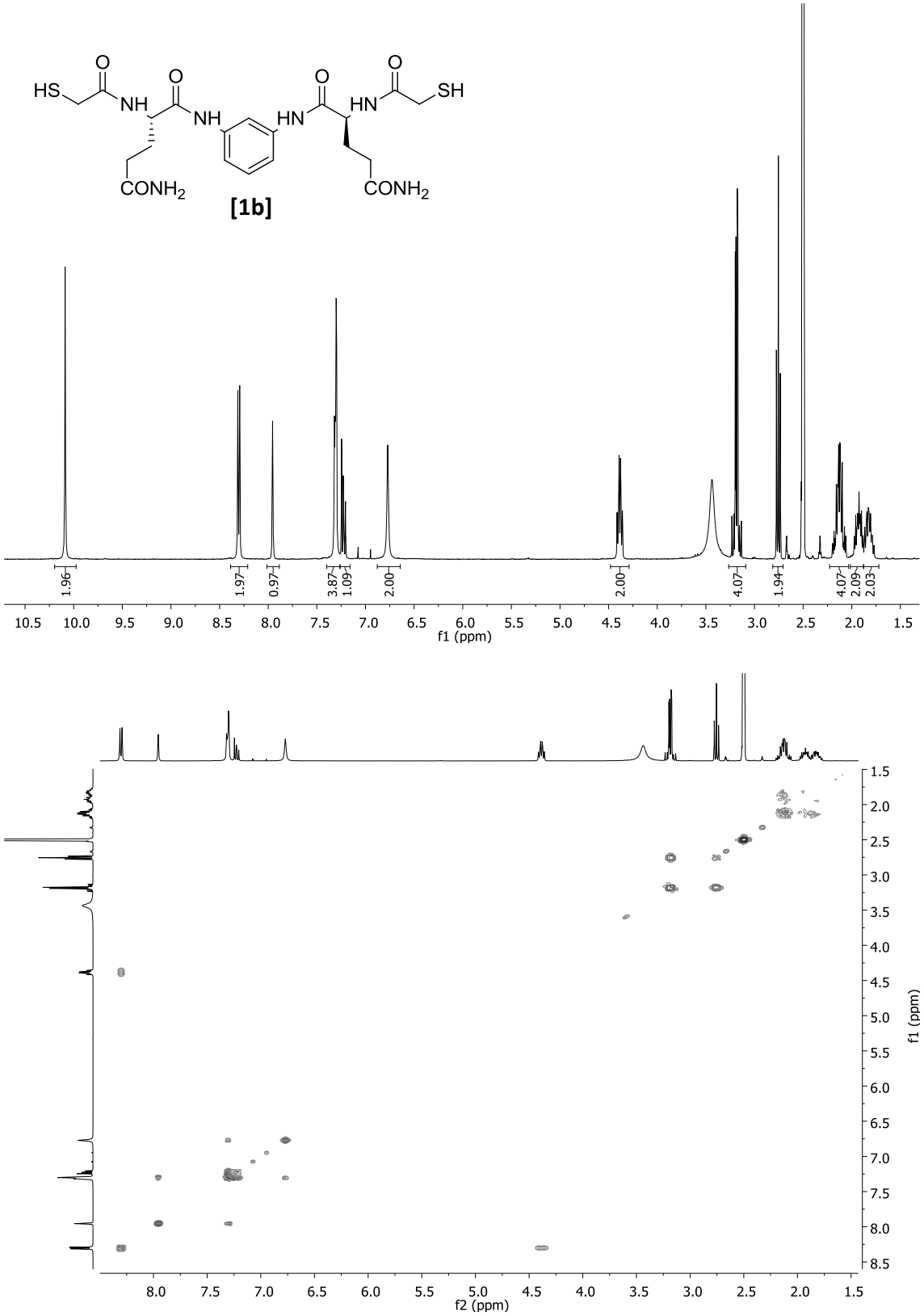


Figure S5: ^{1}H (400 MHz, 298 K in DMSO-d_6) and gCOSY (400 MHz, 298 K in DMSO-d_6) spectra of **[1b]**.

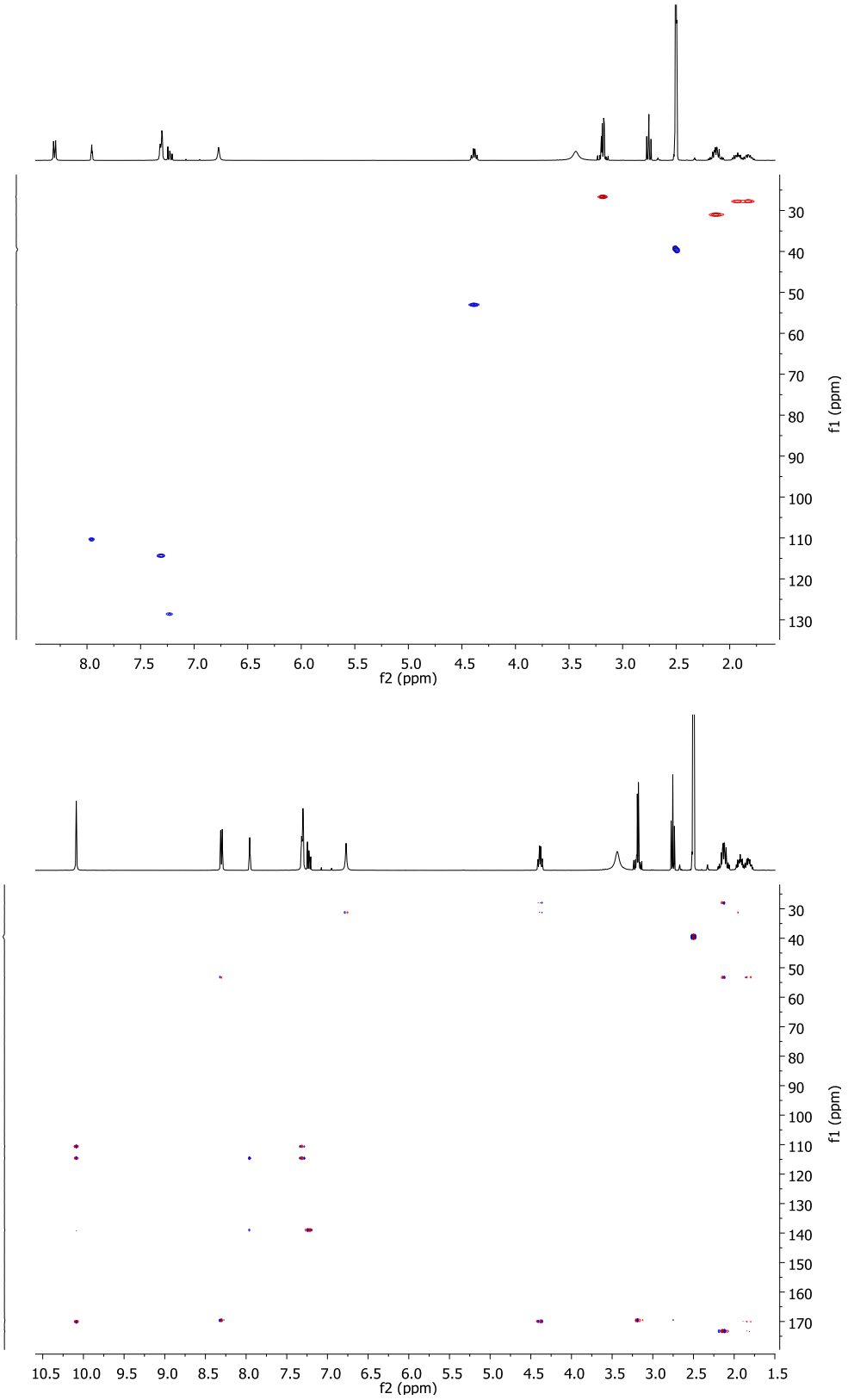


Figure S6: gHSQC (400 MHz, 298 K in DMSO-d_6) and gHMBC (400 MHz, 298 K in DMSO-d_6) spectra of **[1b]**.

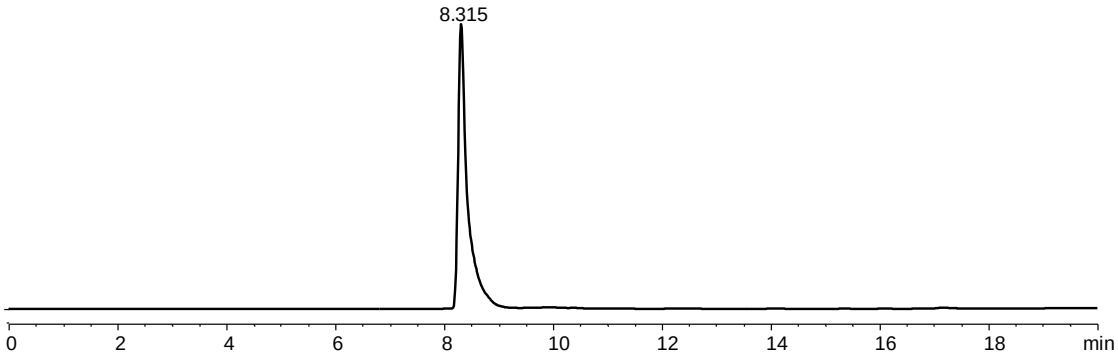


Figure S7: HPLC of **[1b]** (2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 100% CH_3CN over 18 min).

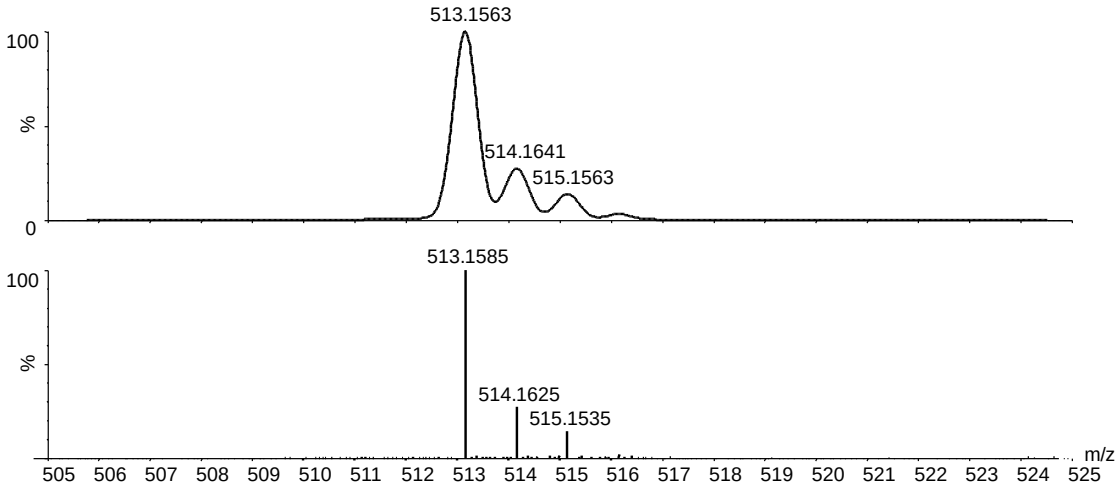


Figure S8: experimental (lower trace) and simulated (upper trace) ESI-TOF mass spectra for $[M+H]^+$ of **[1b]**.

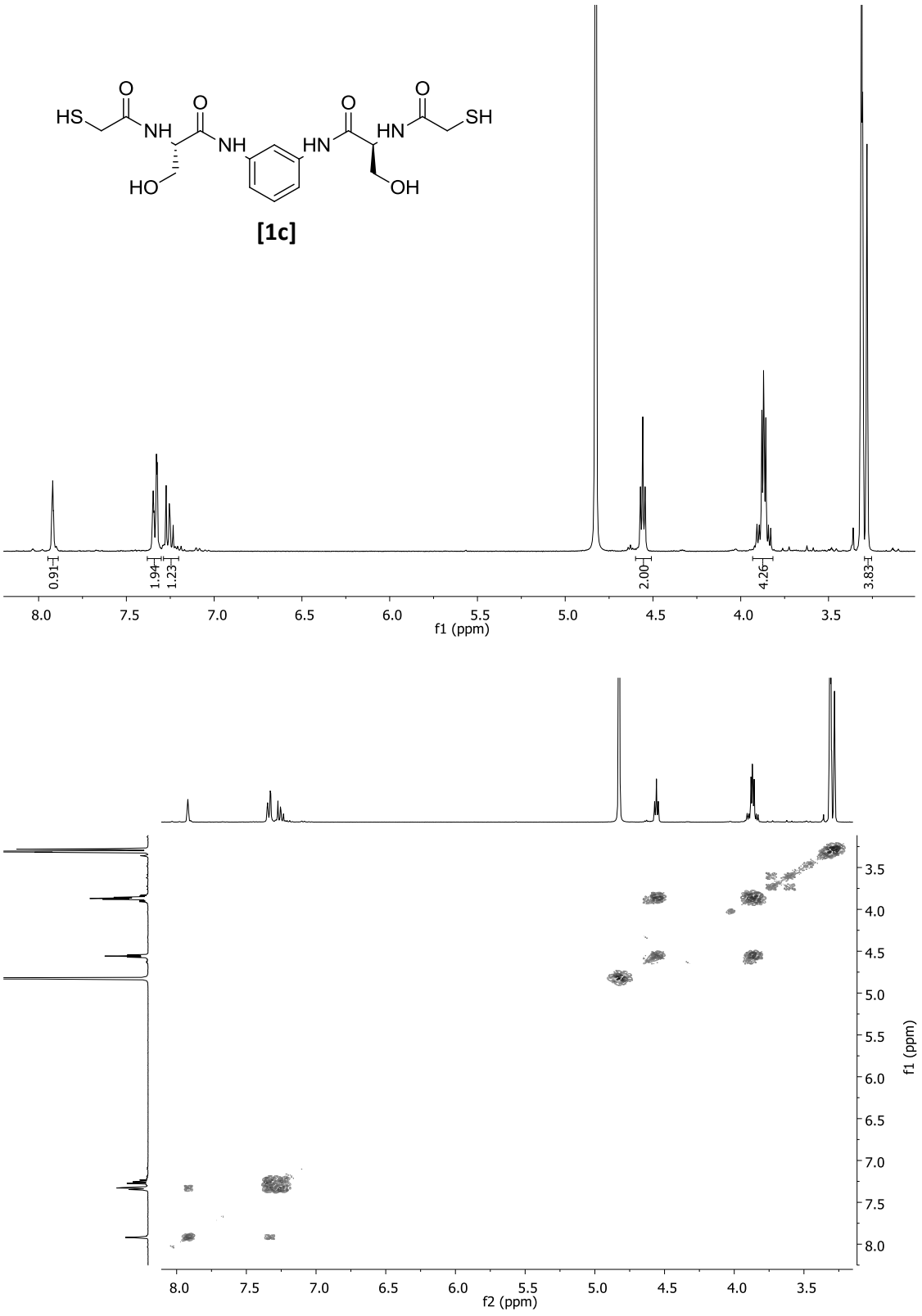


Figure S9: ^{1}H (400 MHz, 298 K in MeOD-d_4) and gCOSY (400 MHz, 298 K in MeOD-d_4) spectra of **[1c]**.

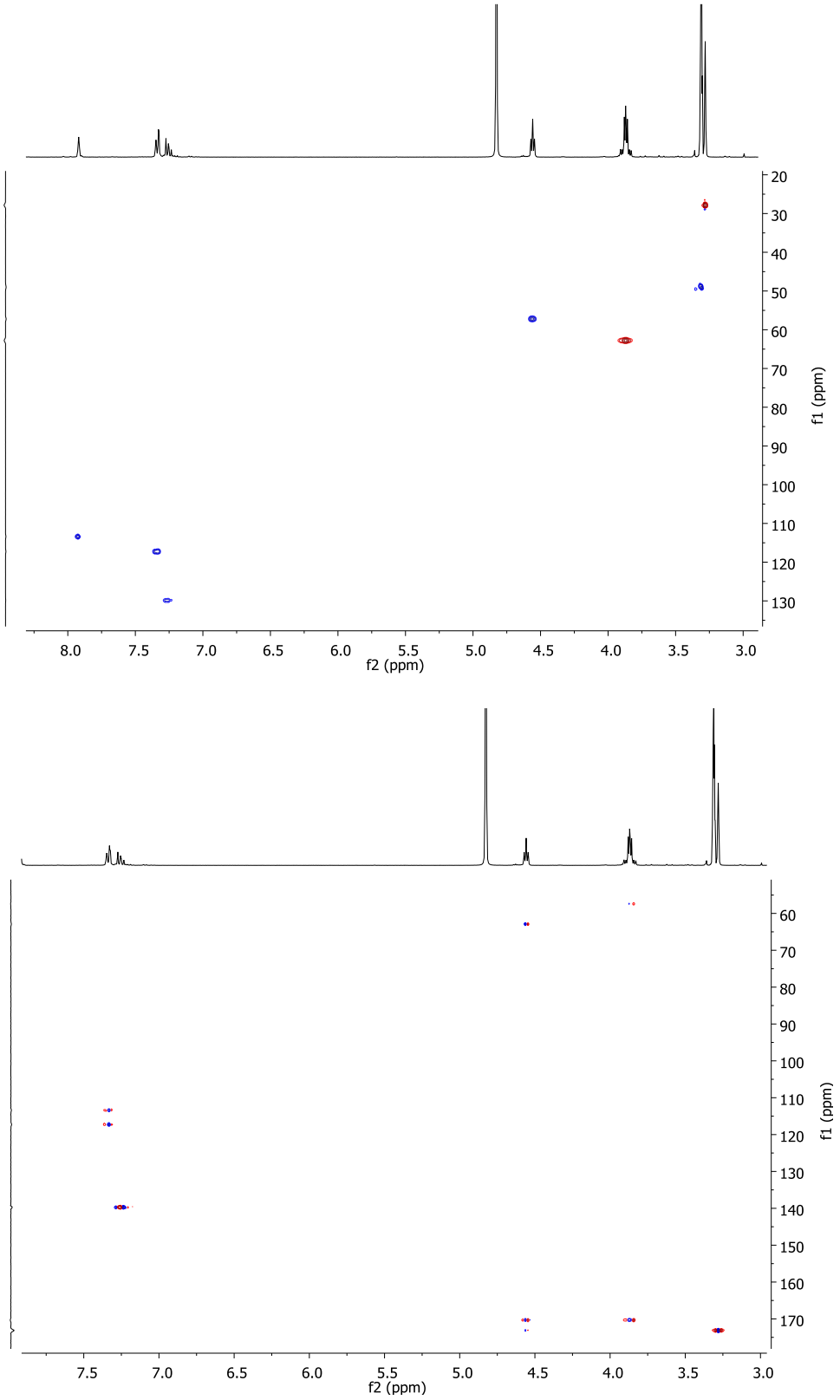


Figure S10: gHSQC (400 MHz, 298 K in MeOD-d_4) and gHMBC (400 MHz, 298 K in MeOD-d_4) spectra of **[1c]**.

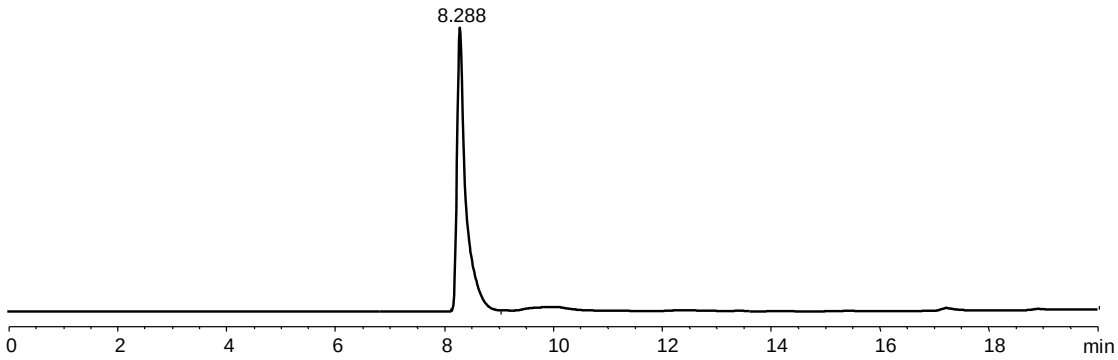


Figure S11: HPLC of **[1c]** (2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 100% CH_3CN over 18 min).

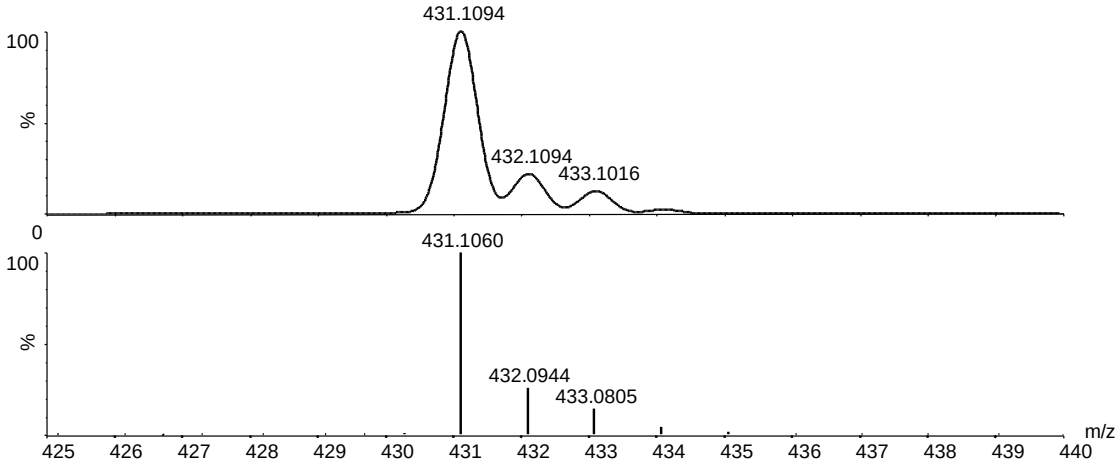


Figure S12: experimental (lower trace) and simulated (upper trace) ESI-TOF mass spectra for $[M+H]^+$ of **[1c]**.

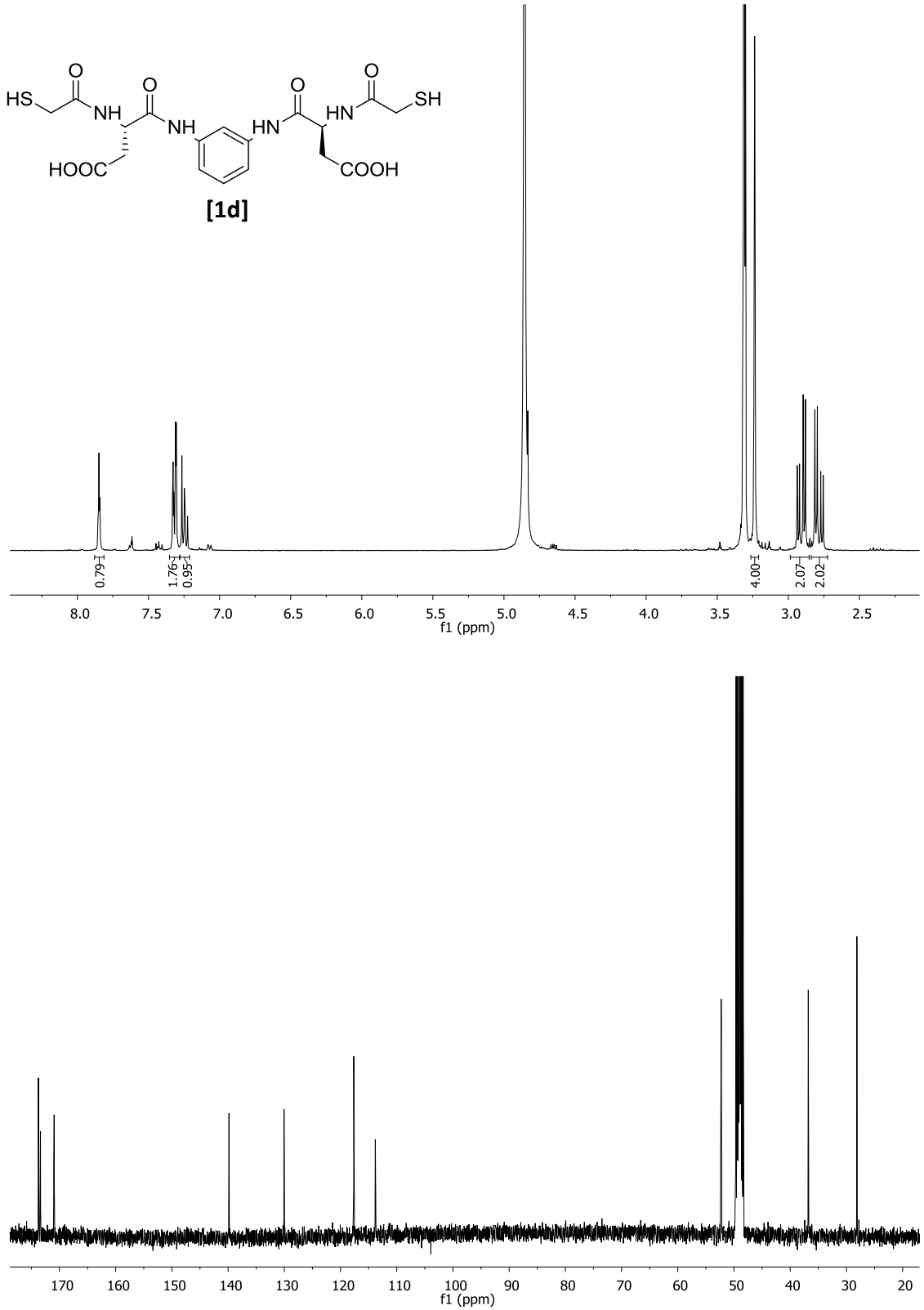


Figure S13: ^{1}H (400 MHz, 298 K in MeOD-d_4) and ^{13}C NMR (100 MHz, 298 K in MeOD-d_4) spectra of **[1d]**.

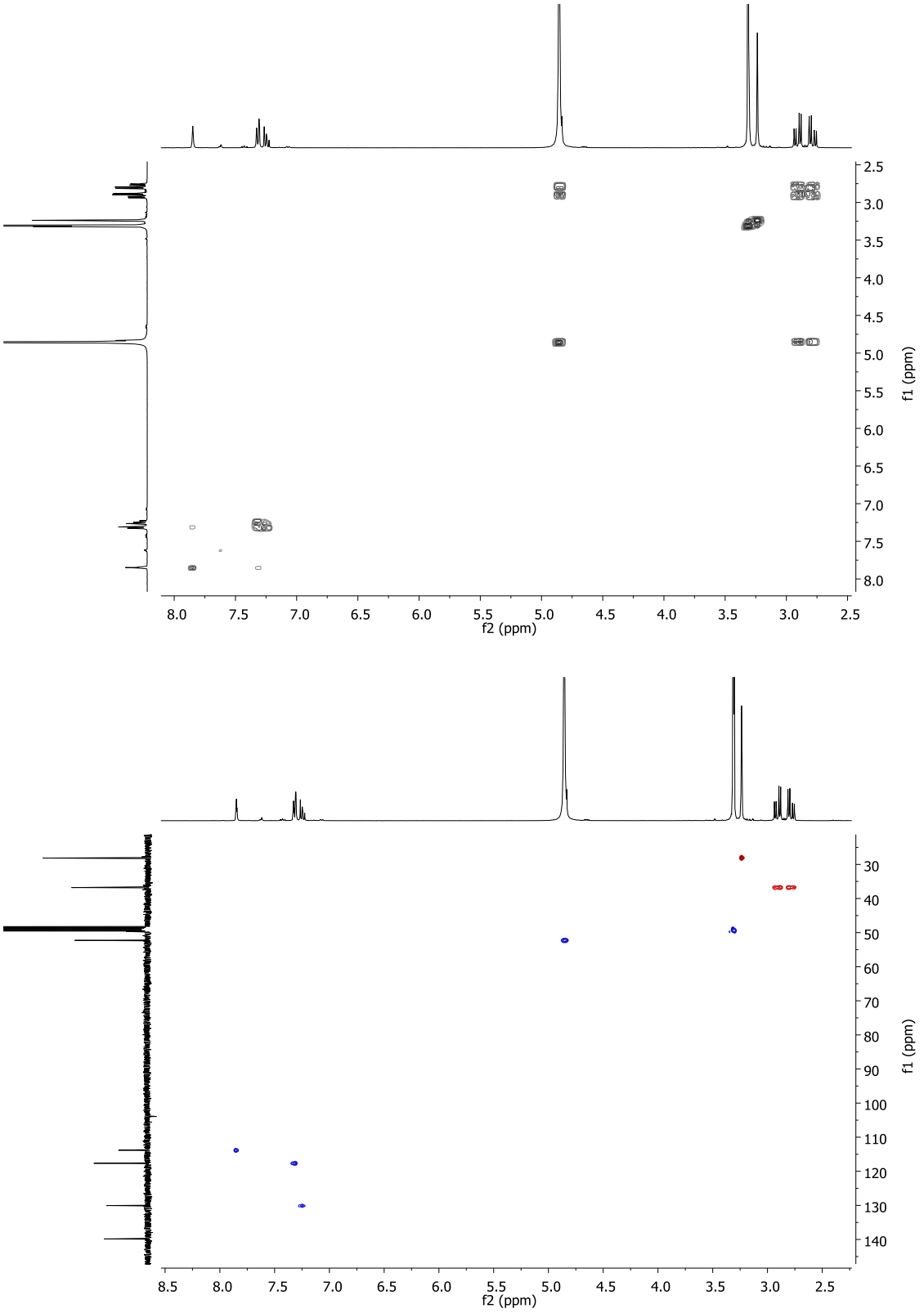


Figure S14: gCOSY (400 MHz, 298 K in MeOD-d_4) and gHSQC (400 MHz, 298 K in MeOD-d_4) spectra of **[1d]**.

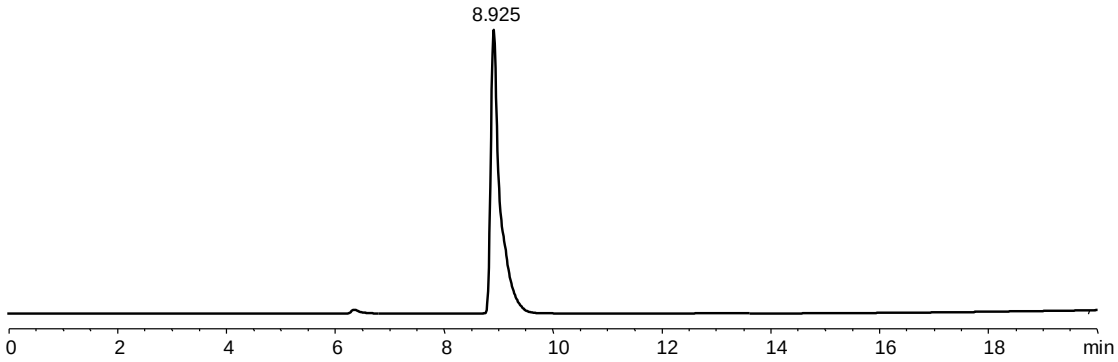


Figure S15: HPLC of **[1d]** (2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 100% CH_3CN over 18 min).

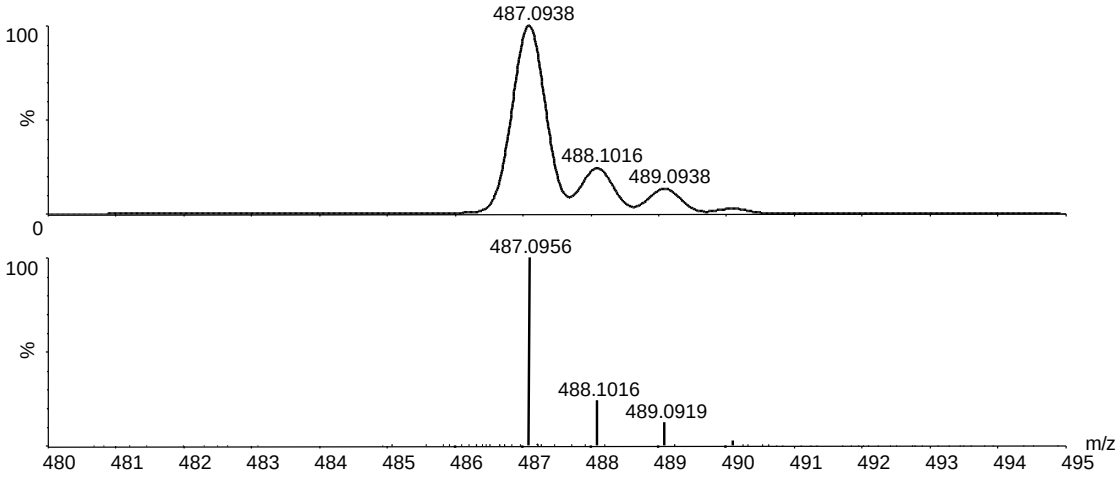
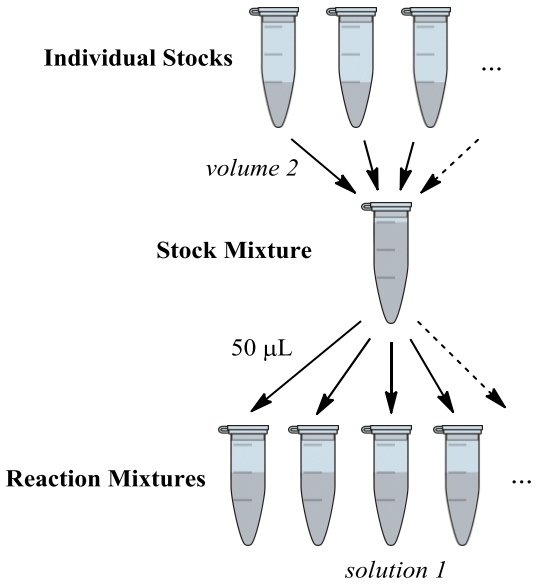


Figure S16: experimental (lower trace) and simulated (upper trace) ESI-TOF mass spectra for $[M+H]^+$ of **[1d]**.

DYNAMIC COMBINATORIAL LIBRARIES (DCLs) EXPERIMENTS

General procedure for the preparation of the DCLs

Individual stocks of each (building block) BB were prepared by dissolving separately *weight 1* of the corresponding BB in *volume 1* of DMSO (see scheme S1). Then a **stock mixture** was prepared by mixing together *volume 2* of each **individual stock**. The **reaction mixtures**, containing 2 mM of each BB in a buffered aqueous solution with 25% of DMSO, were prepared by adding 50 µL of the **stock mixture** to 150 µL of the buffered *solution 1*. After *time 1*, the libraries were quantitatively analyzed by HPLC and the generated compounds identified by UPLC-ESI-TOF.



Scheme S1: preparation of the DCLs.

General procedure for the analysis of the DCLs

The HPLC samples were prepared by adding 15 µL of the corresponding reaction mixture to 140 µL of a solution of 89% H_2O, 10% MeCN and 1% TFA.

The mass spectrometry (MS) samples were prepared by adding 20 µL of the corresponding reaction mixture to 250 µL of a solution of 89% H_2O, 10% MeCN and 1% TFA.

Calculation of the Amplification Factors (AFs)

For a given compound "i" with an area A_i in a mixture "j" with an area summation of all dimers A_T, the **Amplification Factor** (AF) of compound "i" in the mixture "j" was calculated as shown in equation 1:

$$AF_{ij} = \frac{\left(\frac{A_i}{A_T}\right)_j}{\left(\frac{A_i}{A_T}\right)_{No\ salt}} \quad \text{(Equation 1)}$$

DCL 1: dynamic library generated from the mixture of the BBs **1a**, **1b** and **1c** in 20 mM phosphate buffer (pH 7.5) with 25% of DMSO at different salt concentrations

weight 1	volume 1	volume 2	solution 1	time 1
[1a] 2.20 mg **[1b]** 2.21 mg **[1c]** 1.83 mg	170 µL	150 µL	a) 26.7 mM phosphate buffer (pH 7.5) b) 26.7 mM phosphate buffer (pH 7.5) with 0.67 M NaCl / KCl c) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl / KCl d) 26.7 mM phosphate buffer (pH 7.5) with 2.67 M NaCl / KCl	24 h

The composition of the libraries was followed through the time and, at the time of 24 hours, no starting materials were detected by MS (see corresponding section below). Moreover, the composition of the libraries, whether they contain salt of not, proved to reach a stationary situation that remained constant over 13 days. At longer reaction times, overoxidation of disulfides was observed.

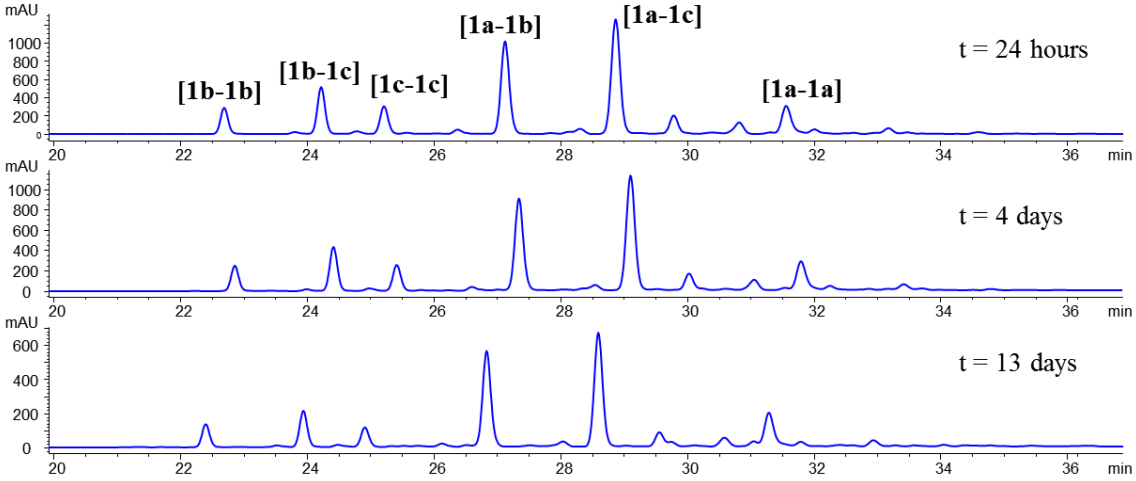


Figure S17: HPLC chromatograms of the DCL 1 in the absence of salt, at three different reaction times.

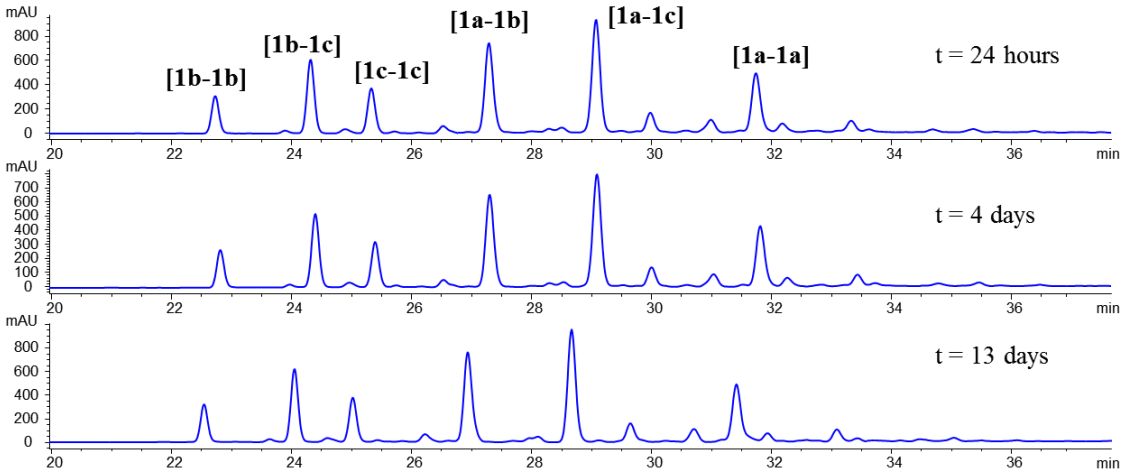
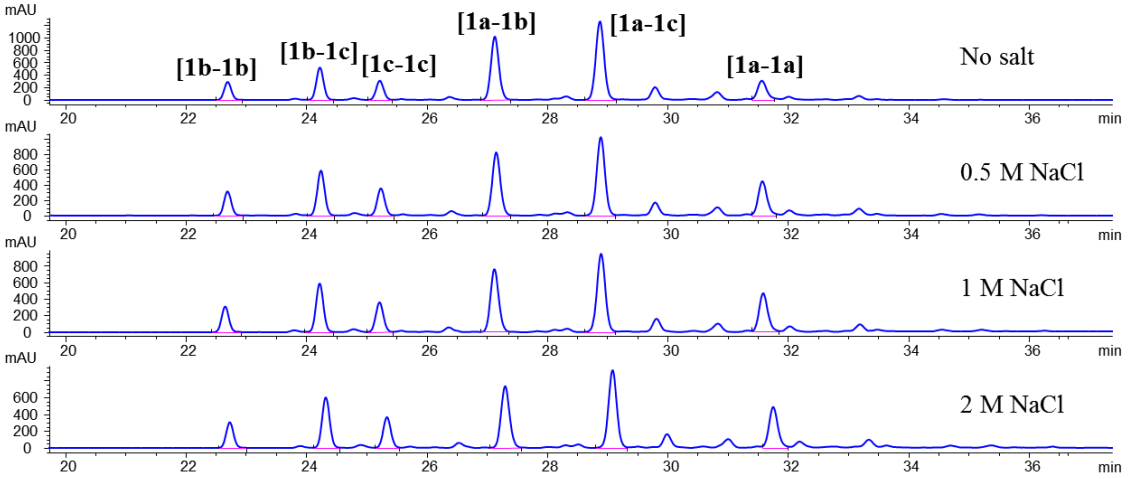


Figure S18: HPLC chromatograms of the DCL 1 with 1M NaCl, at three different reaction times.



	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2471.83	1	2716.98	1.16	2710.29	1.18	2712.62	1.17
[1b-1c]	4502.07	1	5095.20	1.20	5194.51	1.24	5324.44	1.26
[1c-1c]	2755.47	1	3170.87	1.22	3238.47	1.26	3311.48	1.28
[1a-1b]	9567.13	1	7716.81	0.85	7355.68	0.83	7305.47	0.82
[1a-1c]	12537.71	1	9916.41	0.84	9367.20	0.80	9147.65	0.78
[1a-1a]	3271.47	1	4571.02	1.48	4788.85	1.57	5044.30	1.65

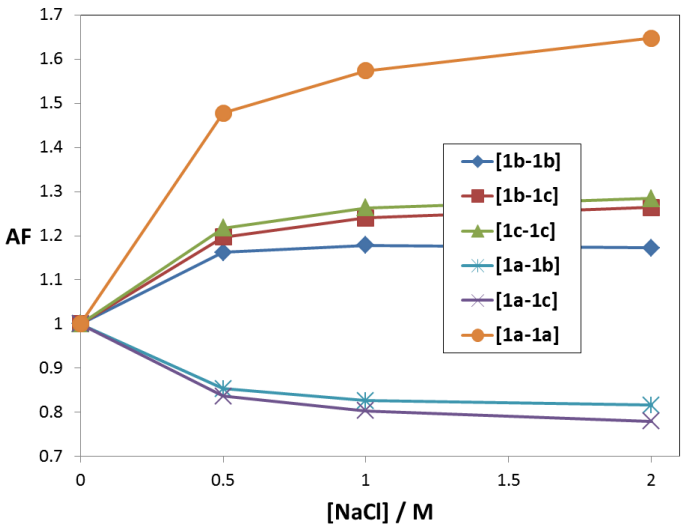
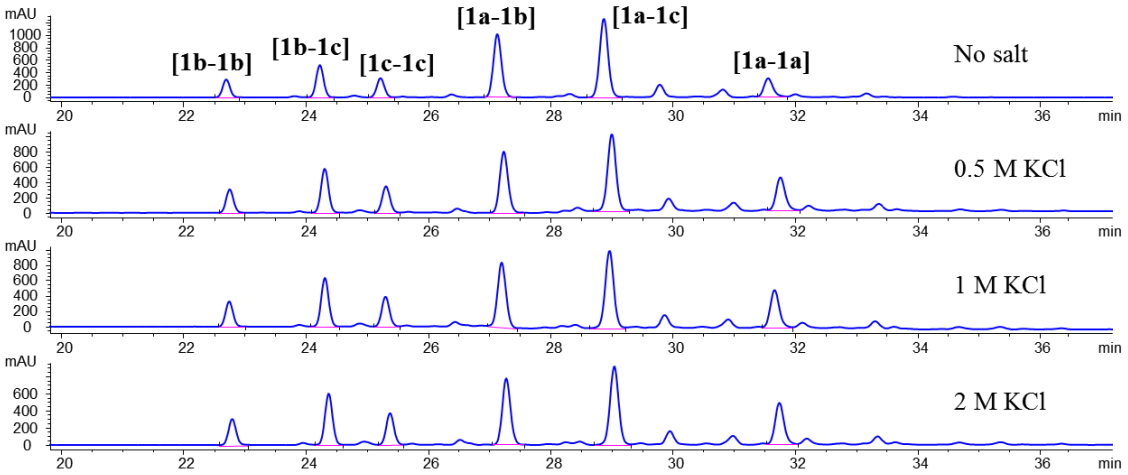


Figure S19. Top: HPLC chromatograms of DCL 1 at four different concentrations of NaCl. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of NaCl. Bottom: Representation of the AFs as a function of the NaCl concentration.



	0 M KCl		0.5 M KCl		1 M KCl		2 M KCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2467.02	1	2708.16	1.16	2920.20	1.17	2707.85	1.17
[1b-1c]	4487.03	1	5041.62	1.19	5503.12	1.22	5288.65	1.26
[1c-1c]	2786.83	1	3142.60	1.19	3478.78	1.24	3303.21	1.27
[1a-1b]	9528.86	1	7743.15	0.86	8203.88	0.85	7325.37	0.82
[1a-1c]	12619.62	1	9972.10	0.84	10206.14	0.80	9243.09	0.78
[1a-1a]	3128.31	1	4480.80	1.52	5026.33	1.59	4889.42	1.67

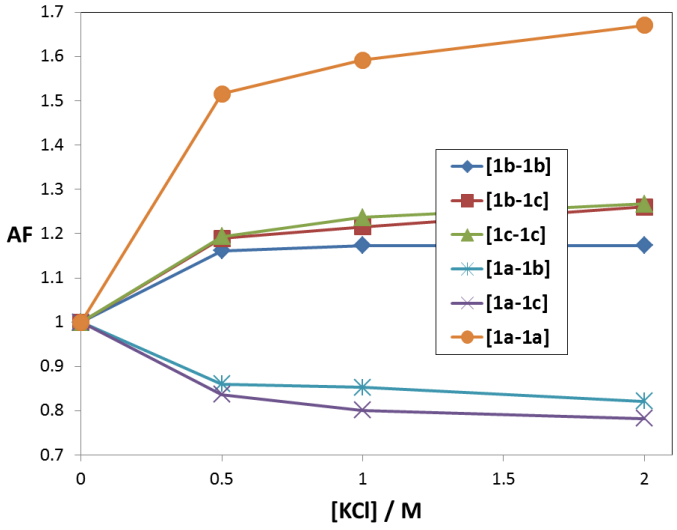


Figure 20. Top: HPLC chromatograms of DCL 1 at four different concentrations of KCl. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of KCl. Bottom: Representation of the AFs as a function of the KCl concentration.

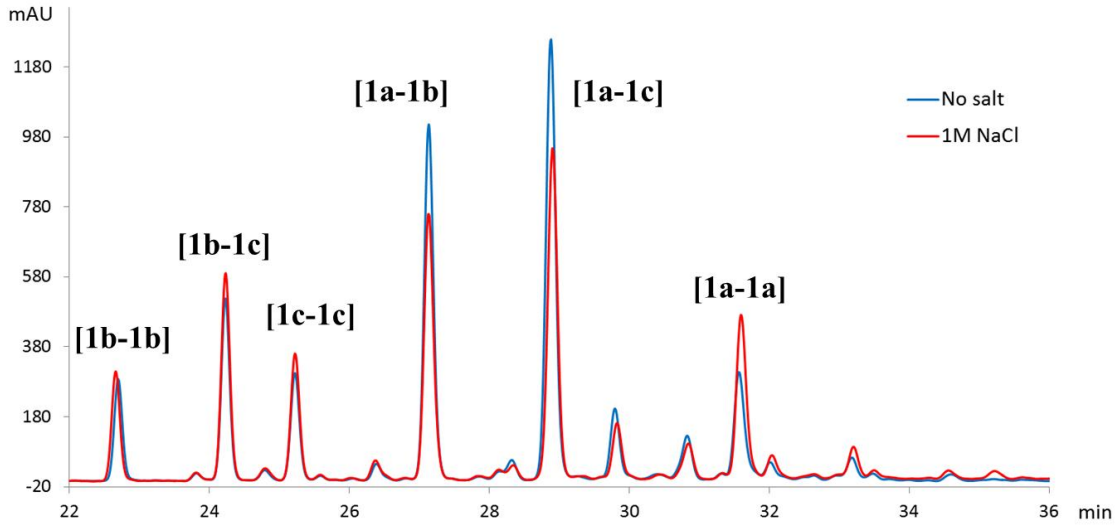
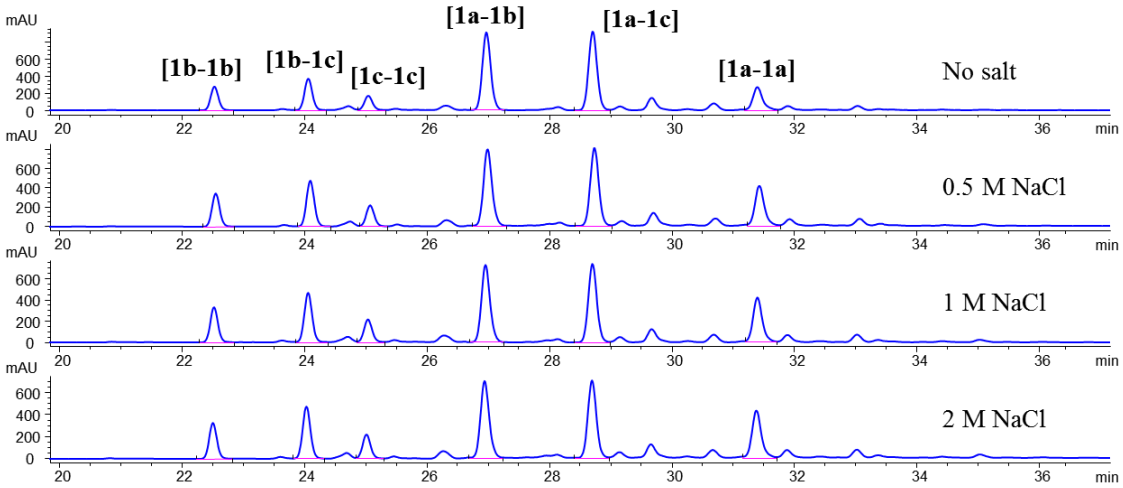


Figure S21: comparison of the HPLC chromatograms of DCL 1 in the absence of salt (**blue**) and in the presence of 1M NaCl (**red**).

DCL 2: replicate of DCL 1

weight 1	volume 1	volume 2	solution 1	time 1
[1a] 2.95 mg **[1b]** 2.83 mg **[1c]** 2.41 mg	239 µL 230 µL 233 µL	220 µL	a) 26.7 mM phosphate buffer (pH 7.5) b) 26.7 mM phosphate buffer (pH 7.5) with 0.67 M NaCl c) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl d) 26.7 mM phosphate buffer (pH 7.5) with 2.67 M NaCl	24 h



	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2515.30	1	3057.57	1.15	2938.16	1.17	2912.74	1.16
[1b-1c]	3345.45	1	4283.40	1.22	4176.24	1.26	4314.33	1.29
[1c-1c]	1561.18	1	1980.67	1.21	1956.31	1.26	2016.09	1.30
[1a-1b]	8745.98	1	7832.40	0.85	7156.92	0.82	7031.37	0.81
[1a-1c]	8962.75	1	7989.16	0.85	7280.86	0.82	7071.72	0.79
[1a-1a]	3023.76	1	4490.09	1.41	4490.30	1.49	4703.73	1.56

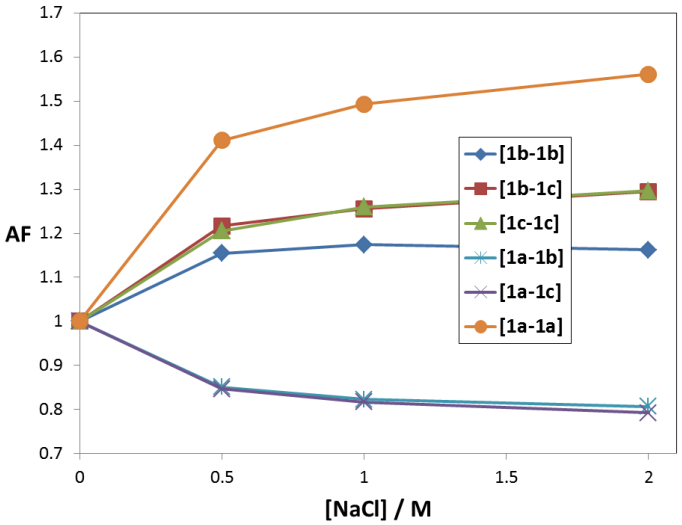
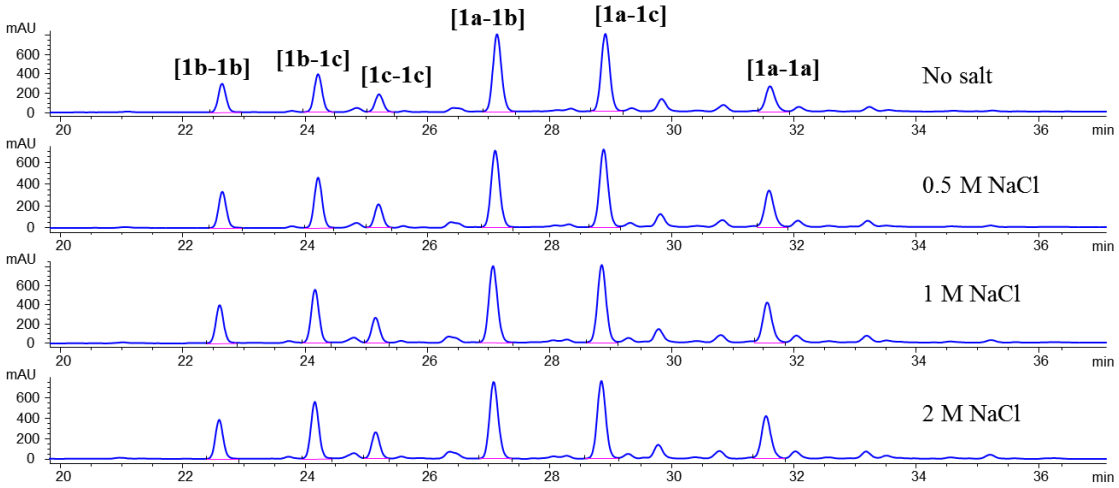


Figure S22. Top: HPLC chromatograms of DCL 2 at four different concentrations of NaCl. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of NaCl. Bottom: Representation of the AFs as a function of the NaCl concentration.

DCL 3: dynamic library generated from the mixture of the BBs **1a**, **1b** and **1c** in 50 mM phosphate buffer (pH 7.5) with 25% of DMSO at different salt concentrations

weight 1	volume 1	volume 2	solution 1	time 1
[1a] 2.72 mg **[1b]** 2.71 mg **[1c]** 2.28 mg	220 µL	200 µL	a) 66.7 mM phosphate buffer (pH 7.5) b) 66.7 mM phosphate buffer (pH 7.5) with 0.67 M NaCl / KCl / $NaNO_3$ c) 66.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl / KCl / $NaNO_3$ d) 66.7 mM phosphate buffer (pH 7.5) with 2.67 M NaCl / KCl / $NaNO_3$	24 h



	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2673.03	1	3015.25	1.13	3620.67	1.14	3493.02	1.14
[1b-1c]	3632.69	1	4158.30	1.14	5154.21	1.19	5099.19	1.22
[1c-1c]	1736.28	1	1943.54	1.12	2448.40	1.18	2379.60	1.19
[1a-1b]	7924.18	1	6974.89	0.88	8059.74	0.85	7610.43	0.83
[1a-1c]	7996.92	1	7082.78	0.89	8161.34	0.86	7634.60	0.83
[1a-1a]	2897.81	1	3683.94	1.27	4606.50	1.33	4701.56	1.41

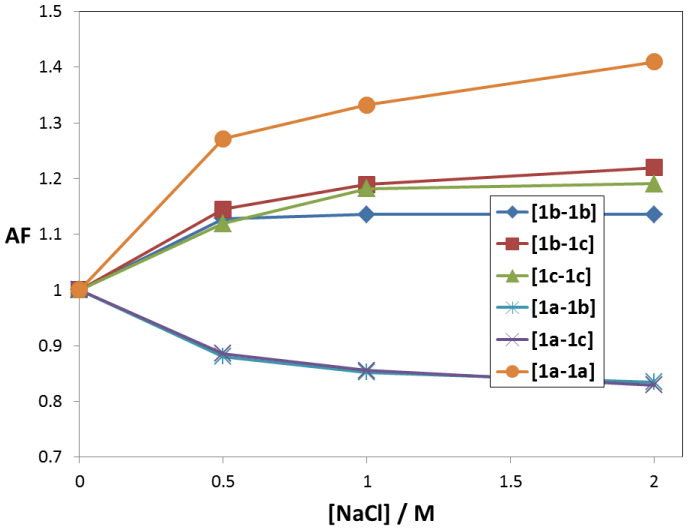
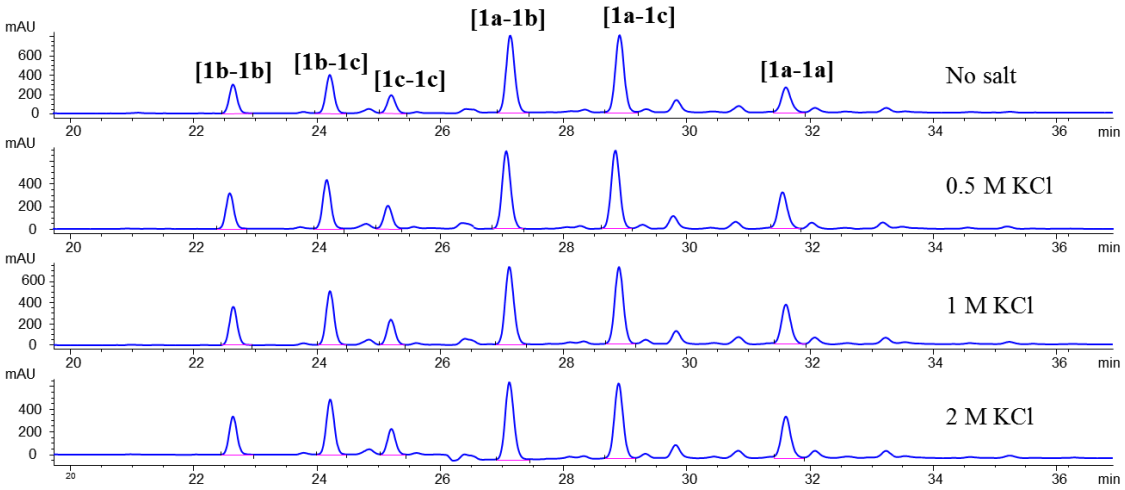


Figure S23. Top: HPLC chromatograms of DCL 3 at four different concentrations of NaCl. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of NaCl. Bottom: Representation of the AFs as a function of the NaCl concentration.



	0 M KCl		0.5 M KCl		1 M KCl		2 M KCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2673.03	1	2851.37	1.11	3242.30	1.13	3062.19	1.13
[1b-1c]	3632.69	1	4021.69	1.16	4654.45	1.20	4506.69	1.22
[1c-1c]	1736.28	1	1863.92	1.12	2174.47	1.17	2123.97	1.20
[1a-1b]	7924.18	1	6759.07	0.89	7326.79	0.87	6854.44	0.85
[1a-1c]	7996.92	1	6772.66	0.88	7261.15	0.85	6670.33	0.82
[1a-1a]	2897.81	1	3469.68	1.25	4047.20	1.31	4078.40	1.38

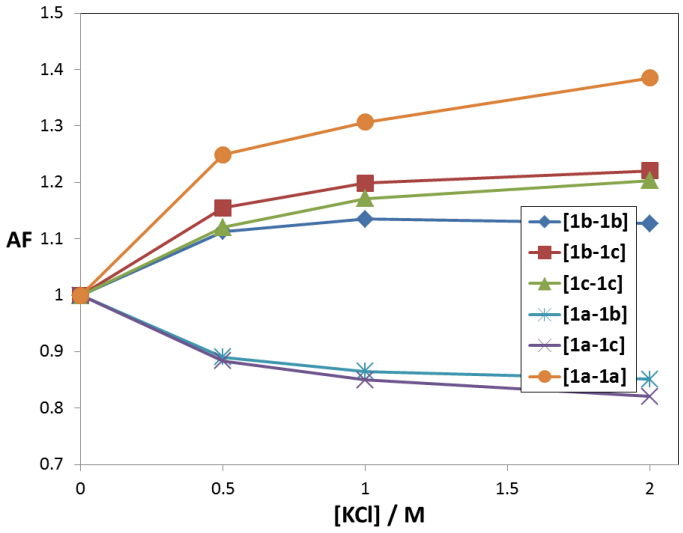
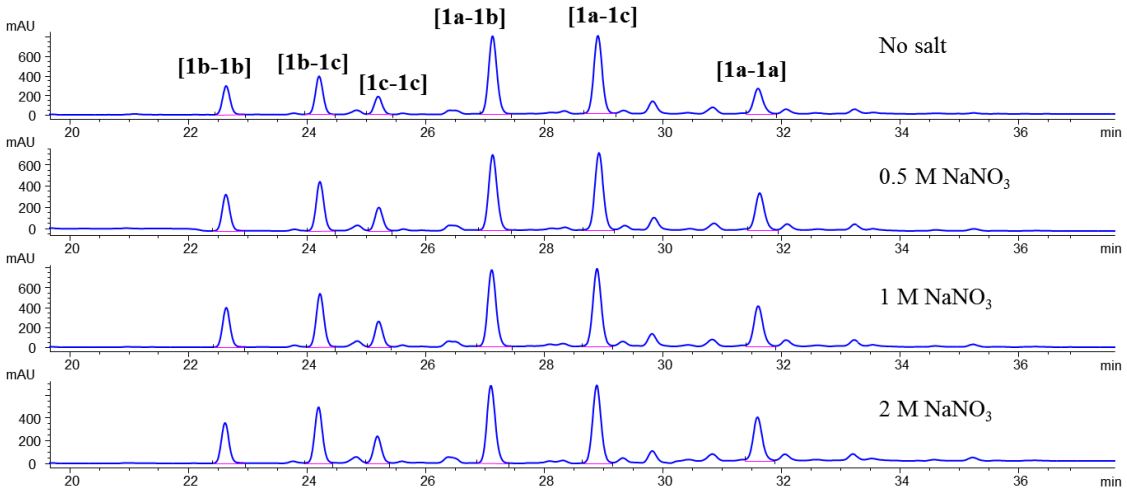


Figure S24. Top: HPLC chromatograms of DCL 3 at four different concentrations of KCl. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of KCl. Bottom: Representation of the AFs as a function of the KCl concentration.



	0 M $NaNO_3$		0.5 M $NaNO_3$		1 M $NaNO_3$		2 M $NaNO_3$	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2673.03	1	3174.09	1.15	3610.87	1.17	3286.71	1.18
[1b-1c]	3632.69	1	4275.28	1.14	4947.67	1.18	4565.10	1.21
[1c-1c]	1736.28	1	2042.21	1.14	2371.06	1.18	2213.08	1.23
[1a-1b]	7924.18	1	7181.93	0.88	7759.35	0.85	6832.24	0.83
[1a-1c]	7996.92	1	7331.80	0.89	7924.91	0.86	6893.72	0.83
[1a-1a]	2897.81	1	3771.32	1.26	4401.53	1.32	4119.31	1.37

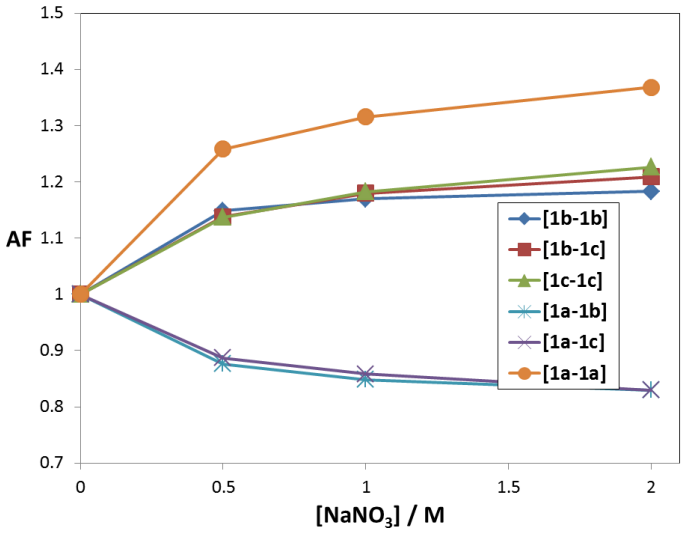
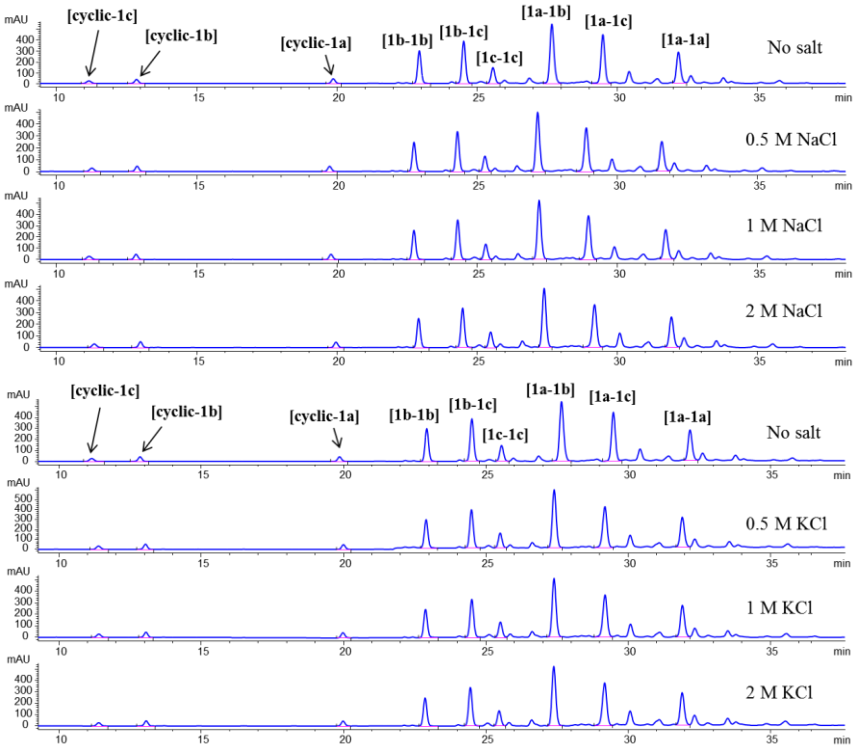


Figure S25. Top: HPLC chromatograms of DCL 3 at four different concentrations of $NaNO_3$. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of $NaNO_3$. Bottom: Representation of the AFs as a function of the $NaNO_3$ concentration.

DCL 4: dynamic library generated from the mixture of the BBs **1a**, **1b** and **1c** in 20 mM phosphate buffer (pH 2.5) with 25% of DMSO at different salt concentrations

weight 1	volume 1	volume 2	solution 1	time 1
[1a] 2.20 mg **[1b]** 2.21 mg **[1c]** 1.84 mg	170 µL	150 µL	a) 26.7 mM phosphate buffer (pH 2.5) b) 26.7 mM phosphate buffer (pH 2.5) with 0.67 M NaCl / KCl c) 26.7 mM phosphate buffer (pH 2.5) with 1.33 M NaCl / KCl d) 26.7 mM phosphate buffer (pH 2.5) with 2.67 M NaCl / KCl	7 days



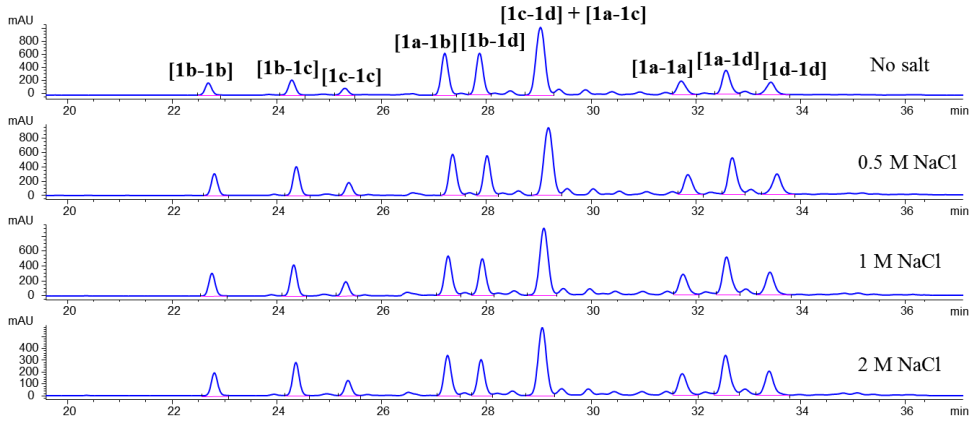
	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2655.21	1	2224.47	0.96	2337.28	0.93	2246.28	0.92
[1b-1c]	3475.24	1	2941.77	0.97	3149.23	0.96	3057.24	0.96
[1c-1c]	1340.58	1	1158.53	0.99	1243.63	0.98	1215.75	0.99
[1a-1b]	5501.16	1	4752.35	0.99	5065.49	0.98	4913.54	0.97
[1a-1c]	4300.03	1	3770.25	1.00	4078.04	1.01	4015.92	1.02
[1a-1a]	2768.16	1	2496.24	1.03	2725.80	1.05	2670.37	1.05

	0 M KCl		0.5 M KCl		1 M KCl		2 M KCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	2642.12	1	2586.97	0.94	2247.45	0.93	2259.46	0.91
[1b-1c]	3470.02	1	3481.45	0.97	3055.41	0.96	3067.02	0.94
[1c-1c]	1342.77	1	1370.80	0.99	1212.66	0.98	1221.73	0.97
[1a-1b]	5503.44	1	5623.42	0.99	4972.99	0.98	5080.71	0.99
[1a-1c]	4312.03	1	4518.85	1.01	3997.04	1.01	4149.00	1.03
[1a-1a]	2756.76	1	3037.33	1.06	2718.70	1.07	2849.10	1.10

Figure S26. Top: HPLC chromatograms of DCL 4 at four different concentrations of NaCl/KCl. Bottom: Tables with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of NaCl/KCl.

DCL 5: dynamic library generated from the mixture of the BBs **1a**, **1b**, **1c** and **1d** in 20 mM phosphate buffer (pH 7.5) with 25% DMSO at different salt concentrations

weight 1	volume 1	volume 2	solution 1	time 1
[1a] 2.30 mg **[1b]** 2.29 mg **[1c]** 1.92 mg **[1d]** 2.18 mg	140 µL	130 µL	a) 26.7 mM phosphate buffer (pH 7.5) b) 26.7 mM phosphate buffer (pH 7.5) with 0.67 M NaCl / KCl / $NaNO_3$ c) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl / KCl / $NaNO_3$ d) 26.7 mM phosphate buffer (pH 7.5) with 2.67 M NaCl / KCl / $NaNO_3$	3 days



	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	1709.68	1	2689.18	1.41	2663.52	1.47	1735.39	1.49
[1b-1c]	2226.91	1	3692.45	1.49	3767.65	1.59	2544.16	1.68
[1c-1c]	973.98	1	1680.52	1.55	1732.87	1.67	1181.81	1.79
[1a-1b]	6218.13	1	5726.78	0.83	5315.76	0.80	3337.54	0.79
[1b-1d]	6210.49	1	5433.87	0.79	4857.46	0.74	2951.24	0.70
[1c-1d] + [1a-1c]	12646.59	1	11530.04	0.82	10481.17	0.78	6490.27	0.76
[1a-1a]	2459.73	1	3239.49	1.18	3137.52	1.20	2043.00	1.22
[1a-1d]	4142.36	1	5867.03	1.27	5865.89	1.33	3880.65	1.38
[1d-1d]	2453.27	1	3573.12	1.31	3661.12	1.40	2373.72	1.42

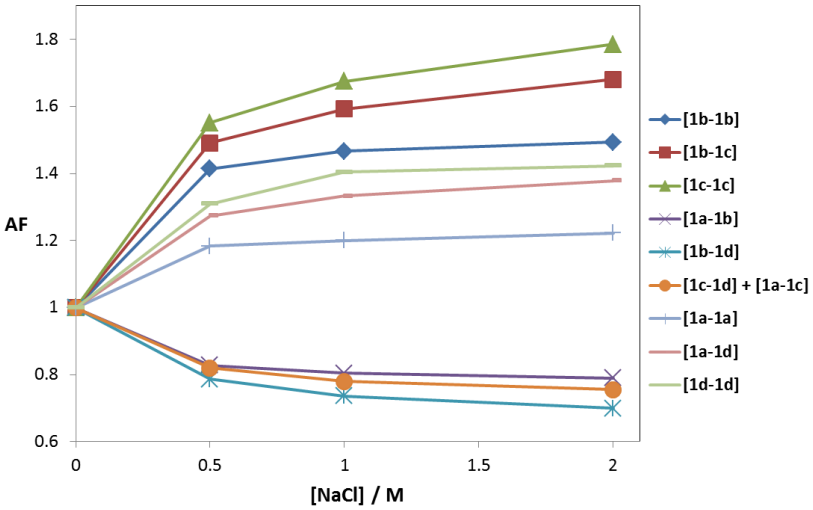
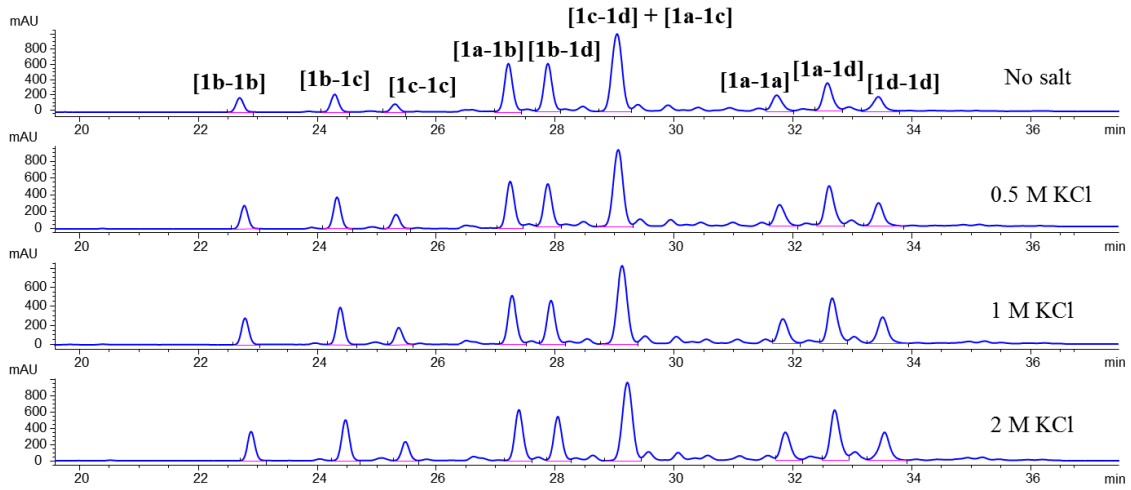


Figure S27. Top: HPLC chromatograms of DCL 5 at four different concentrations of NaCl. Middle: Table with the integrated areas and the calculated AFs of the ten dimers at the four different concentrations of NaCl. Bottom: Representation of the AFs as a function of the NaCl concentration.



	0 M KCl		0.5 M KCl		1 M KCl		2 M KCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	1709.68	1	2432.82	1.37	2435.93	1.44	3145.68	1.49
[1b-1c]	2226.91	1	3378.00	1.46	3484.54	1.59	4603.94	1.67
[1c-1c]	973.98	1	1551.47	1.54	1589.37	1.65	2136.89	1.78
[1a-1b]	6218.13	1	5519.82	0.86	4965.93	0.81	6177.68	0.80
[1b-1d]	6210.49	1	4917.77	0.76	4516.91	0.74	5219.73	0.68
[1c-1d] + [1a-1c]	12646.59	1	10662.28	0.81	9805.95	0.79	11823.63	0.76
[1a-1a]	2459.73	1	3068.93	1.20	2938.57	1.21	3774.74	1.24
[1a-1d]	4142.36	1	5594.42	1.30	5404.10	1.32	6927.48	1.35
[1d-1d]	2453.27	1	3368.97	1.32	3368.96	1.39	4381.33	1.45

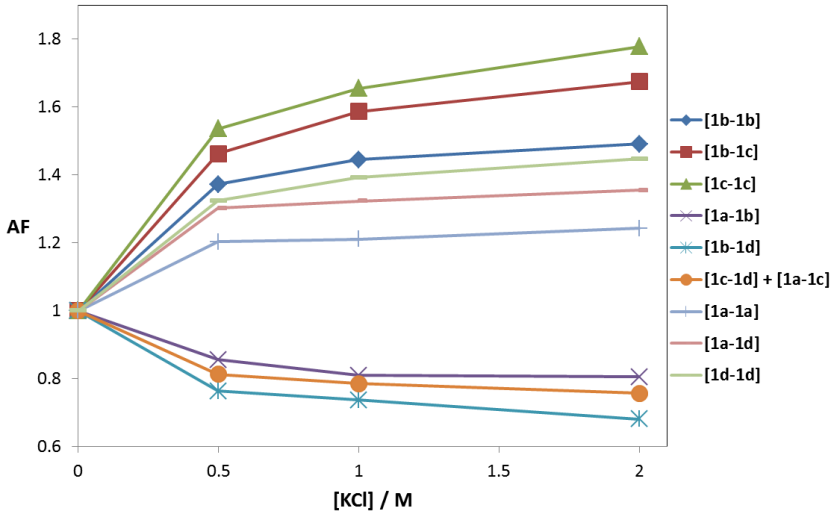
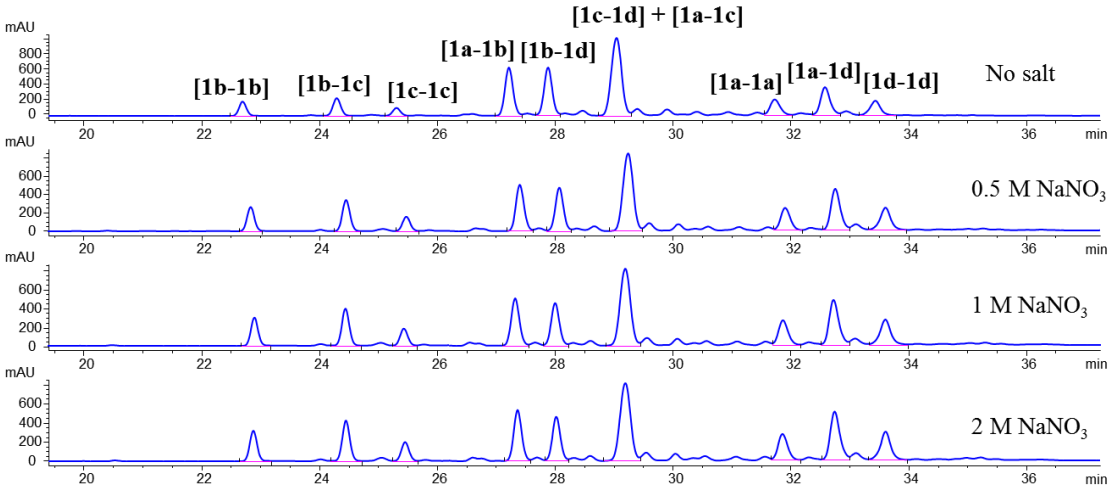


Figure S28. Top: HPLC chromatograms of DCL 5 at four different concentrations of KCl. Middle: Table with the integrated areas and the calculated AFs of the ten dimers at the four different concentrations of KCl. Bottom: Representation of the AFs as a function of the KCl concentration.



	0 M $NaNO_3$		0.5 M $NaNO_3$		1 M $NaNO_3$		2 M $NaNO_3$	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
[1b-1b]	1709.68	1	2405.83	1.45	2587.64	1.51	2876.41	1.59
[1b-1c]	2226.91	1	3252.63	1.50	3499.57	1.57	3876.71	1.64
[1c-1c]	973.98	1	1512.27	1.59	1634.08	1.68	1855.14	1.80
[1a-1b]	6218.13	1	5016.84	0.83	4972.25	0.80	5193.42	0.79
[1b-1d]	6210.49	1	4668.18	0.77	4478.07	0.72	4497.55	0.68
[1c-1d] + [1a-1c]	12646.59	1	10209.61	0.83	10163.57	0.80	10378.37	0.77
[1a-1a]	2459.73	1	2800.64	1.17	2993.63	1.22	3179.63	1.22
[1a-1d]	4142.36	1	5055.01	1.25	5463.48	1.32	5895.54	1.34
[1d-1d]	2453.27	1	3097.55	1.30	3297.75	1.34	3598.16	1.38

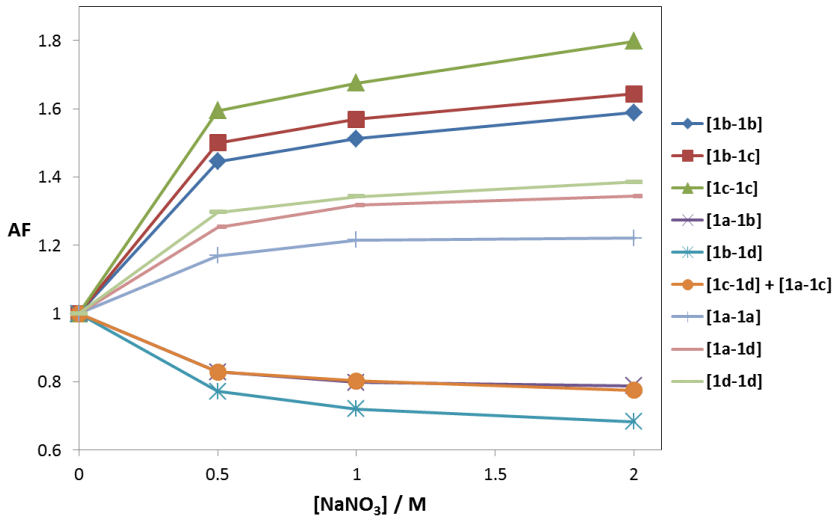
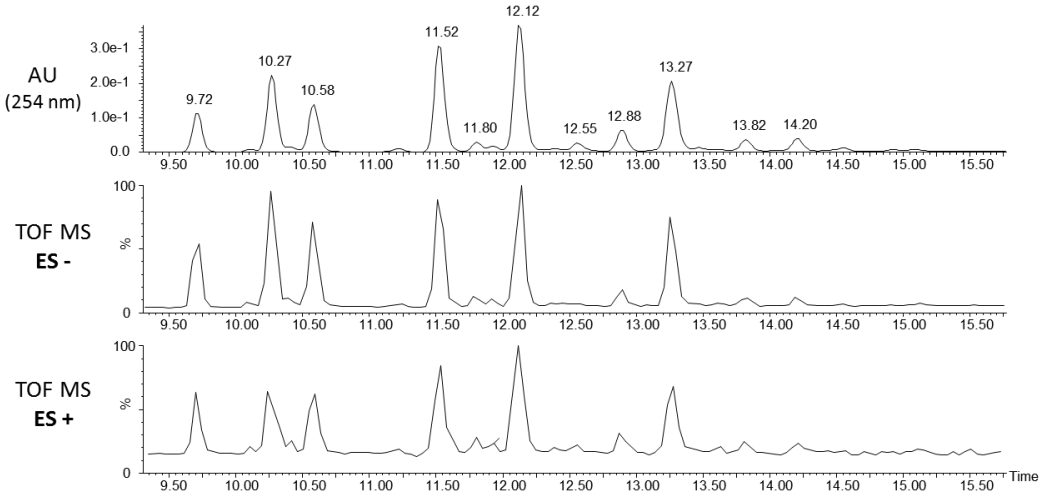


Figure S29. Top: HPLC chromatograms of DCL 5 at four different concentrations of $NaNO_3$. Middle: Table with the integrated areas and the calculated AFs of the six dimers at the four different concentrations of $NaNO^3$. Bottom: Representation of the AFs as a function of the $NaNO_3$ concentration.

MASS SPECTROMETRY (MS)

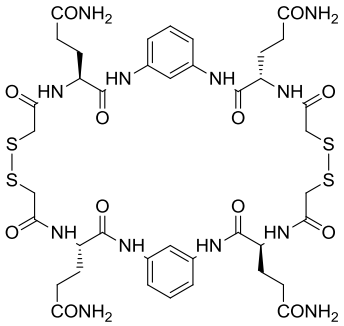
The UPLC-ESI-TOF analyses were performed at both positive and negative detection modes.

Mixture of the three BBs **[1a+1b+1c]** at pH 7.5

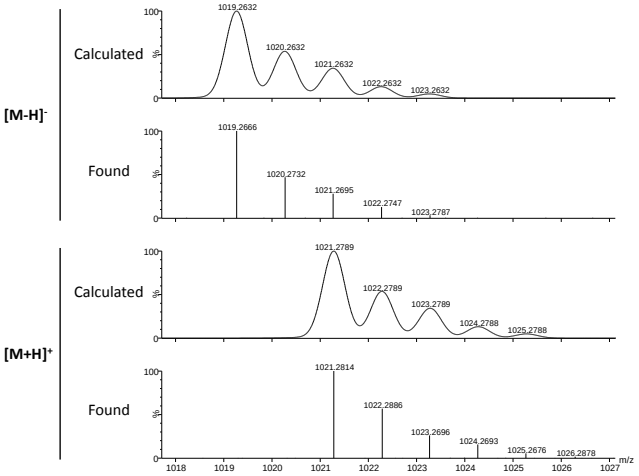


Identification of the dimers:

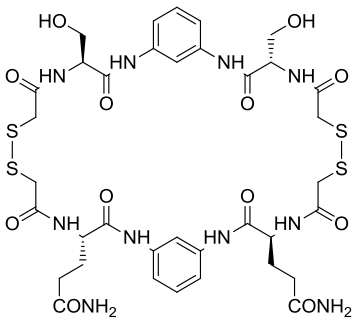
[1b-1b], retention time: 9.72 min



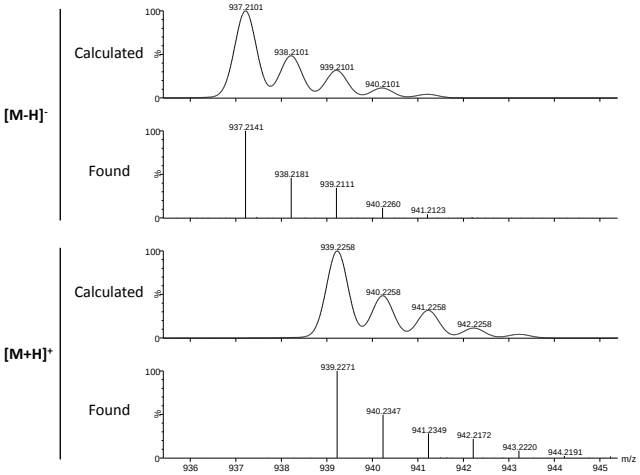
Chemical Formula: $C_{40}H_{52}N_{12}O_{12}S_4$
Exact Mass: 1020.2710



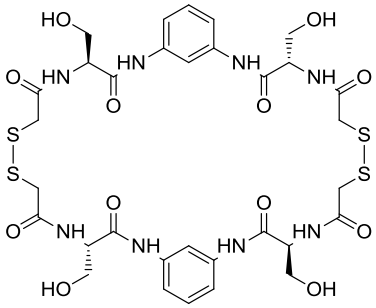
[1b-1c], retention time: 10.27 min



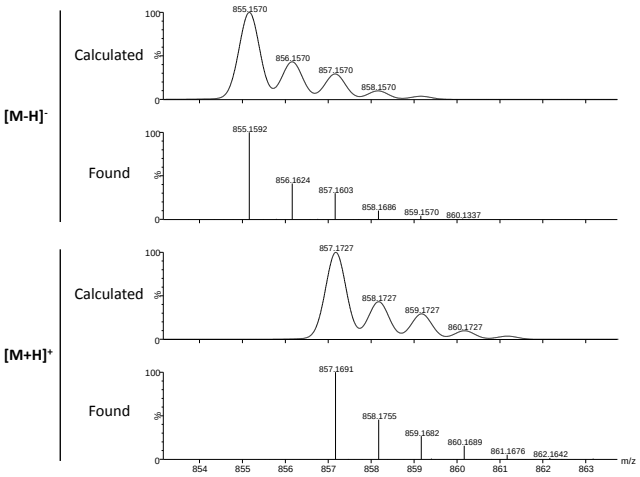
Chemical Formula: $C_{36}H_{46}N_{10}O_{12}S_4$
Exact Mass: 938.2179



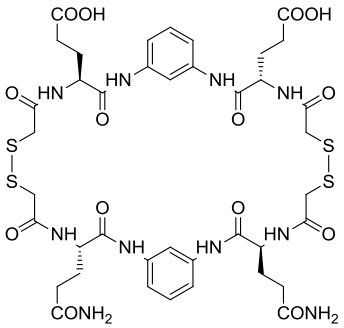
[1c-1c], retention time: 10.58 min



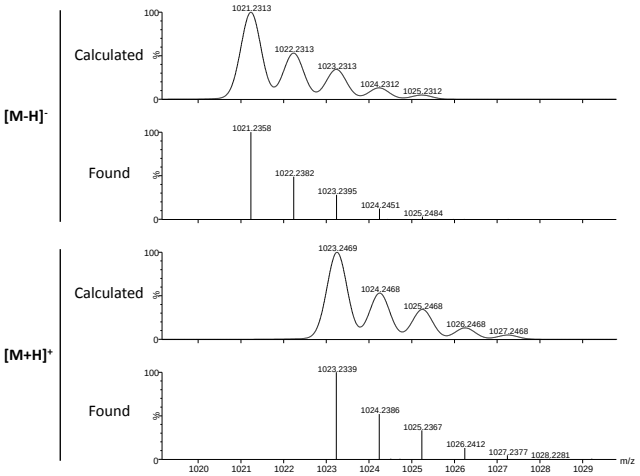
Chemical Formula: $C_{32}H_{40}N_8O_{12}S_4$
Exact Mass: 856.1649



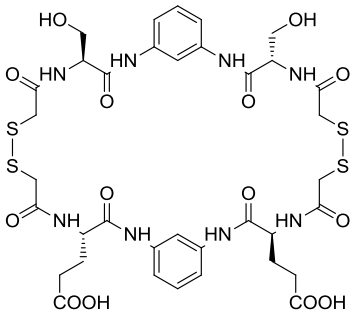
[1a-1b], retention time: 11.52 min



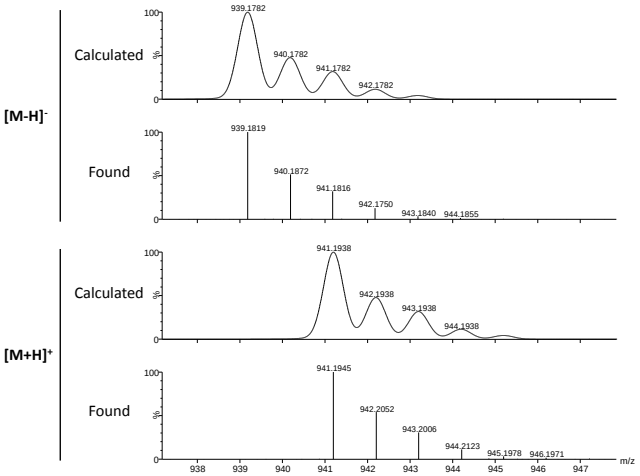
Chemical Formula: $C_{40}H_{50}N_{10}O_{14}S_4$
Exact Mass: 1022.2391



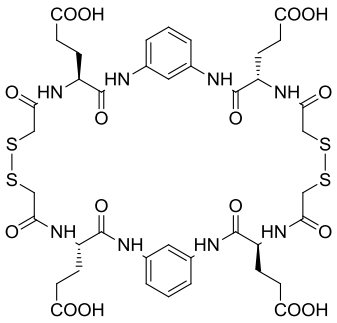
[1a-1c], retention time: 12.12 min



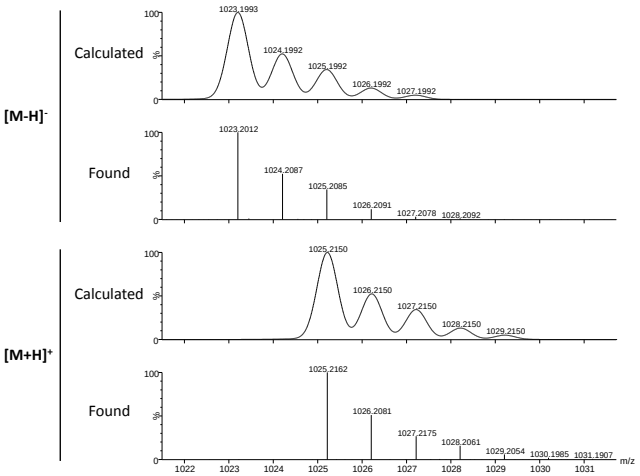
Chemical Formula: $C_{36}H_{44}N_8O_{14}S_4$
Exact Mass: 940.1860



[1a-1a], retention time: 13.27 min

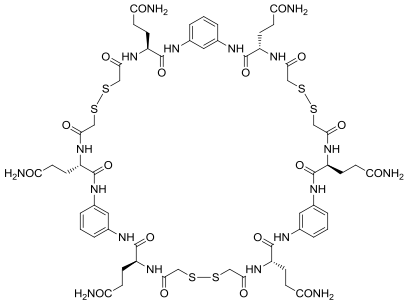


Chemical Formula: $C_{40}H_{48}N_8O_{16}S_4$
Exact Mass: 1024.2071

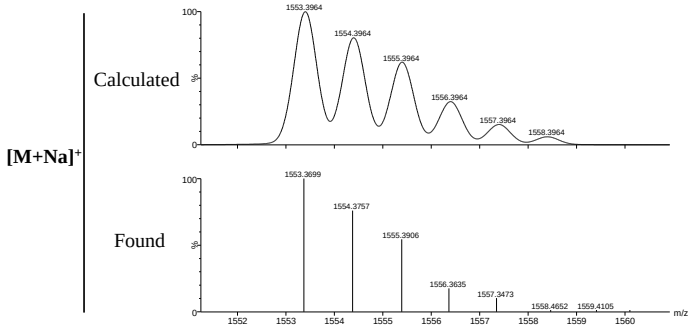


Identification of the trimmers:

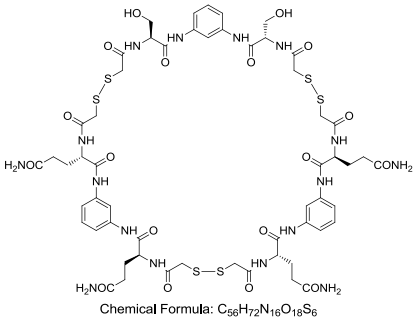
[1b-1b-1b], retention time: 11.22 min



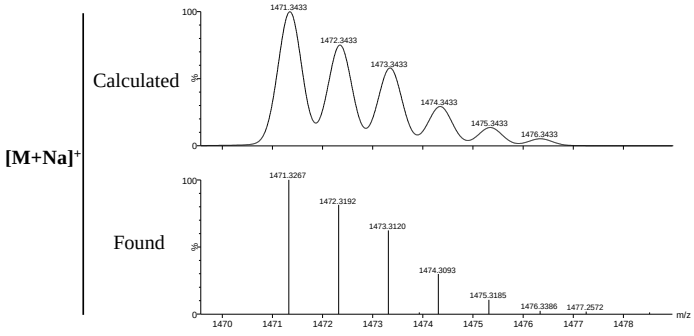
Chemical Formula: $C_{60}H_{78}N_{18}O_{18}S_6$
Exact Mass: 1530.4066



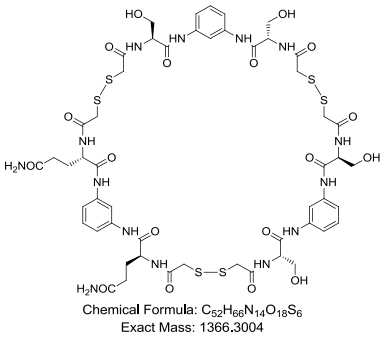
[1b-1b-1c], retention time: 11.52 min



Chemical Formula: $C_{56}H_{72}N_{16}O_{18}S_6$
Exact Mass: 1448.3535

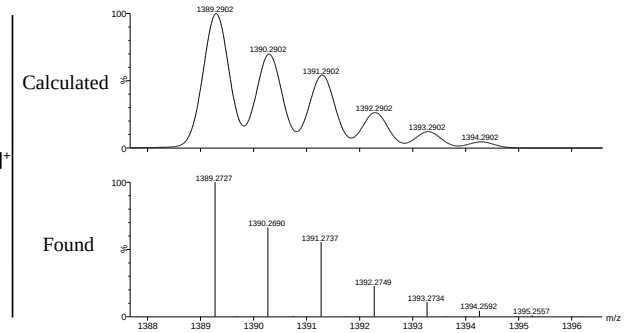


[1b-1c-1c], retention time: 11.80 min

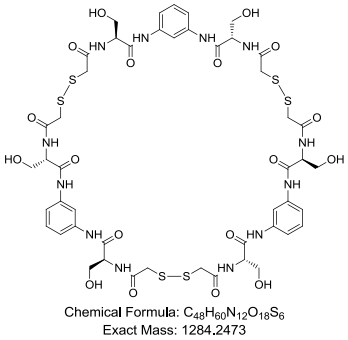


Chemical Formula: $C_{52}H_{66}N_{14}O_{18}S_6$
Exact Mass: 1366.3004

[M+Na]$^+$

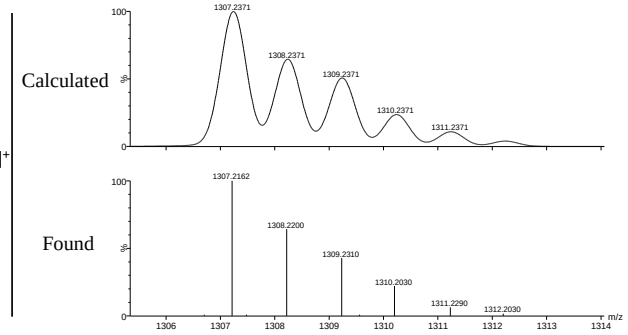


[1c-1c-1c], retention time: 12.12 min

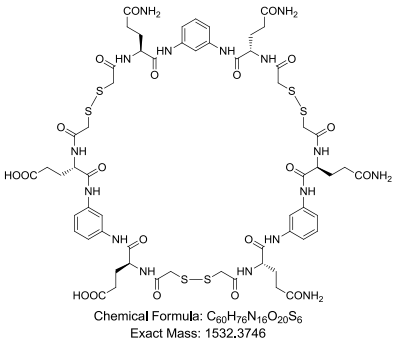


Chemical Formula: $C_{48}H_{60}N_{12}O_{18}S_6$
Exact Mass: 1284.2473

[M+Na]$^+$

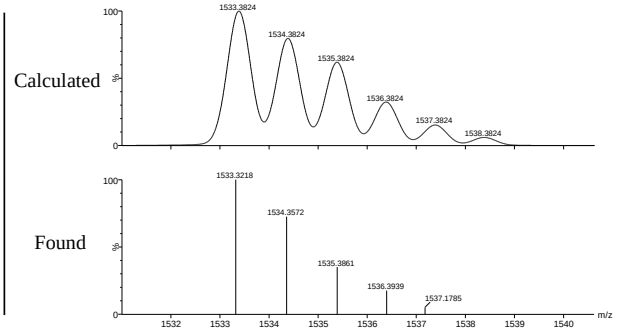


[1a-1b-1b], retention time: 12.55 min

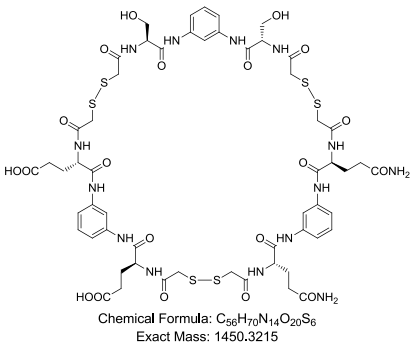


Chemical Formula: $C_{60}H_{76}N_{16}O_{20}S_6$
Exact Mass: 1532.3746

[M+H]$^+$

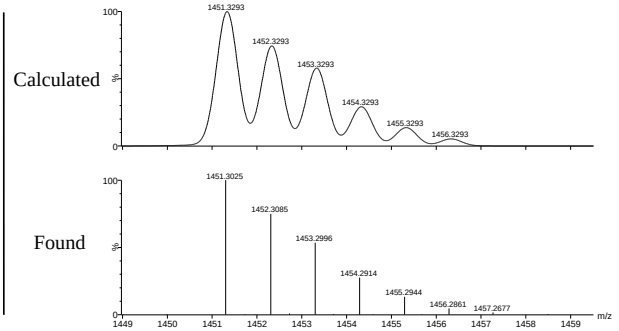


[1a-1b-1c], retention time: 12.88 min

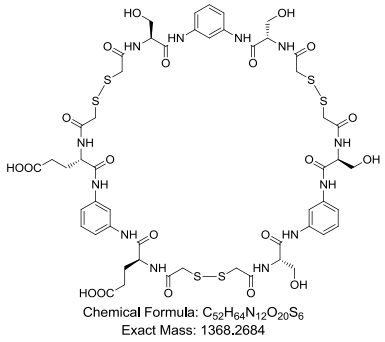


Chemical Formula: $C_{56}H_{70}N_{14}O_{20}S_6$
Exact Mass: 1450.3215

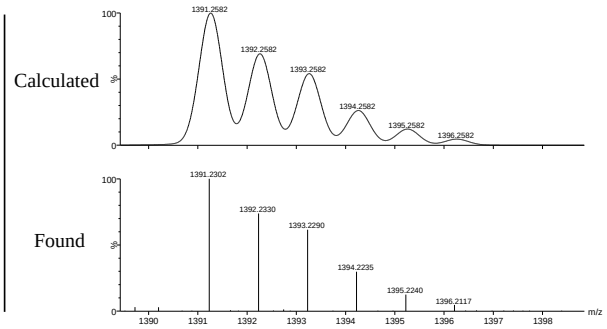
[M+H]$^+$



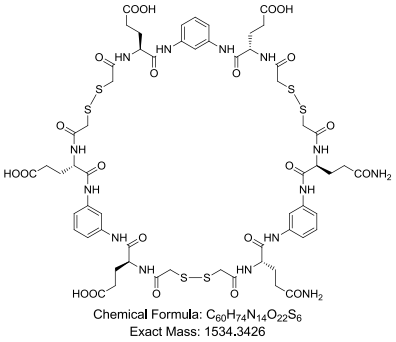
[1a-1c-1c], retention time: 13.27 min



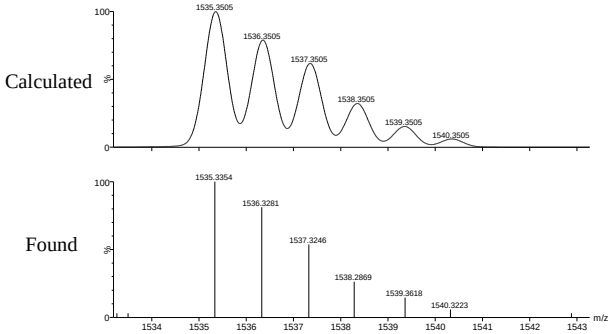
[M+Na]$^+$



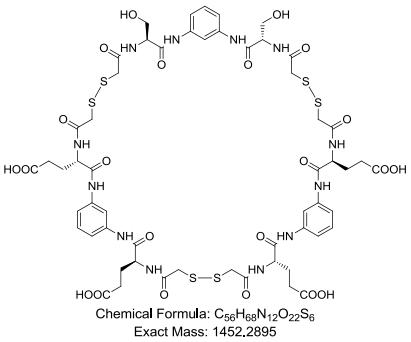
[1a-1a-1b], retention time: 13.82 min



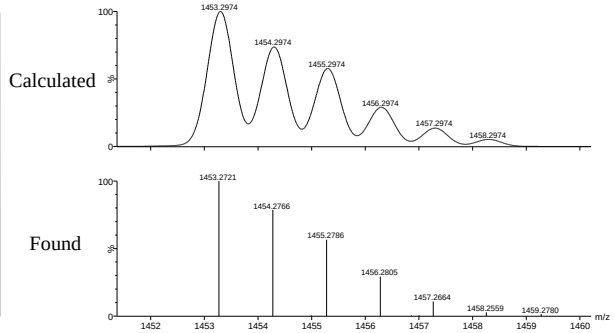
[M+H]$^+$



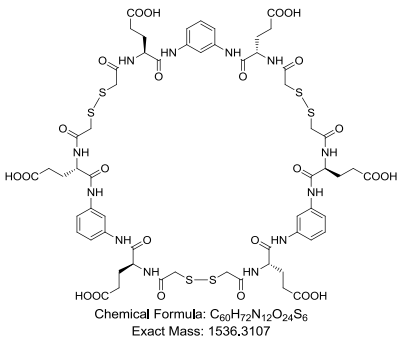
[1a-1a-1c], retention time: 14.20 min



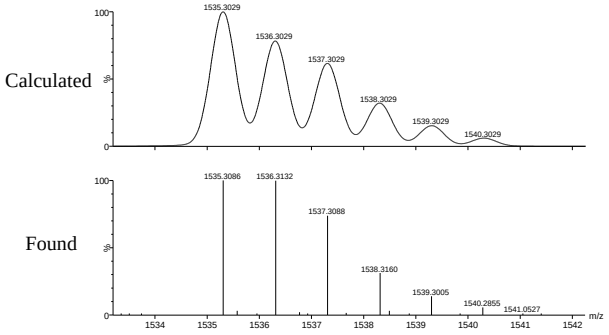
[M+H]$^+$



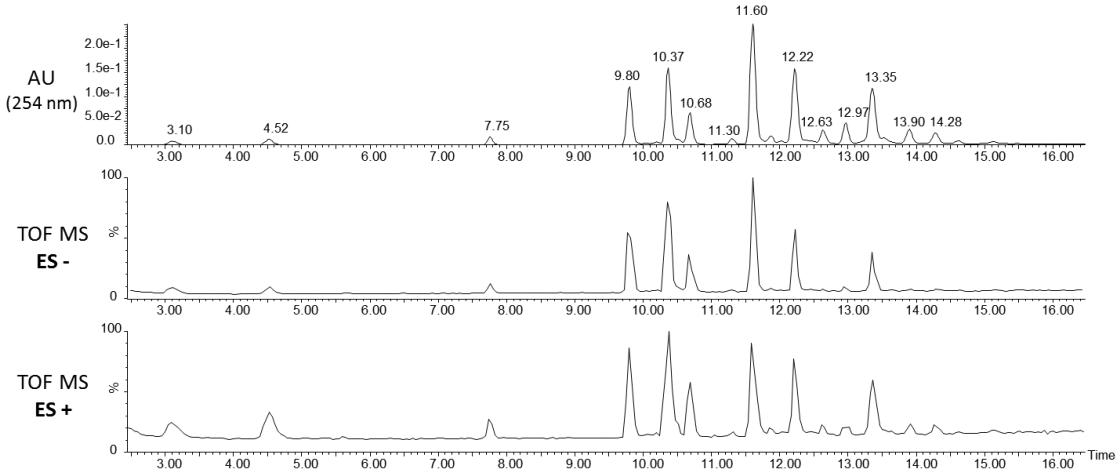
[1a-1a-1a], retention time: 15.12 min



[M-H]$^-$

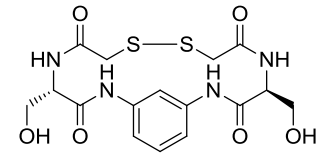


Mixture of the three BBs **[1a+1b+1c]** at pH 2.5

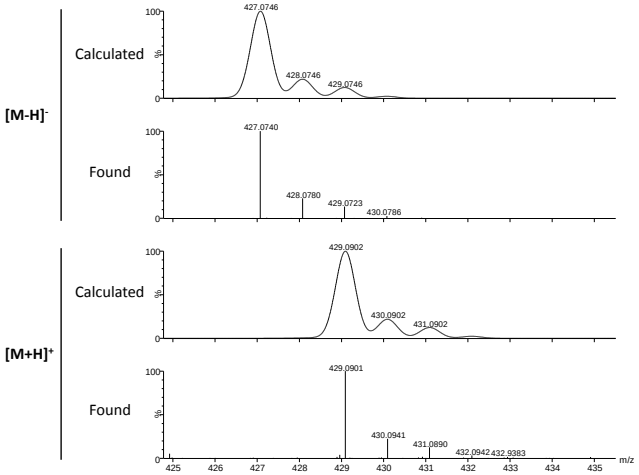


Identification of the cyclic monomers:

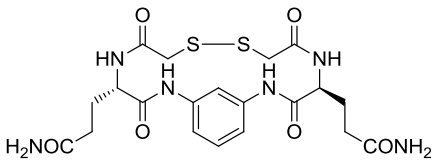
[cyclic-1c], retention time: 3.10 min



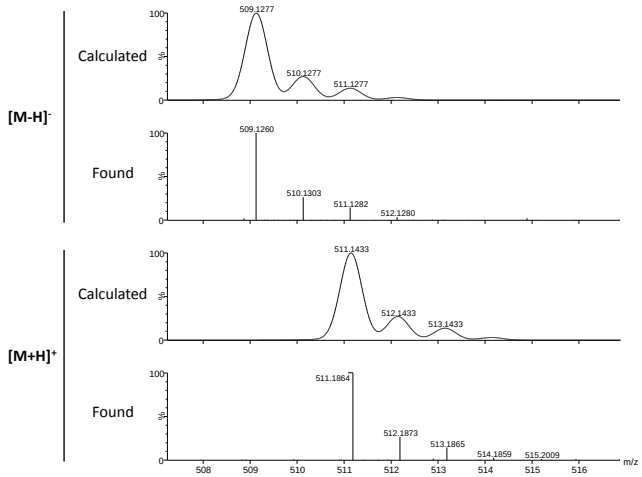
Chemical Formula: $C_{16}H_{20}N_4O_6S_2$
Exact Mass: 428.0824



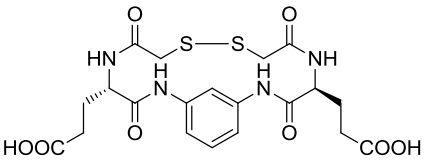
[cyclic-1b], retention time: 4.52 min



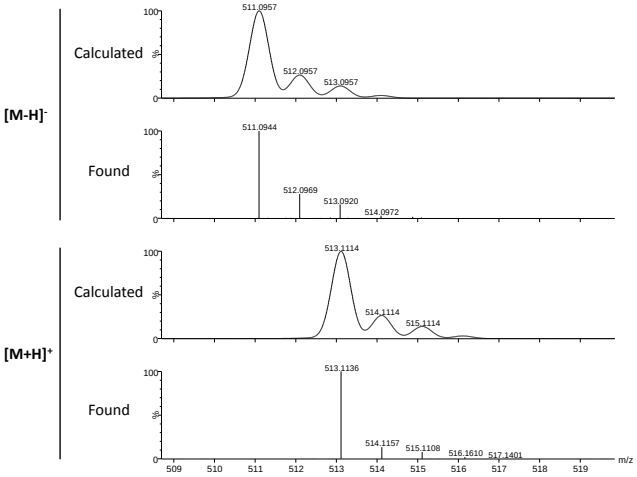
Chemical Formula: $C_{20}H_{26}N_6O_6S_2$
Exact Mass: 510.1355



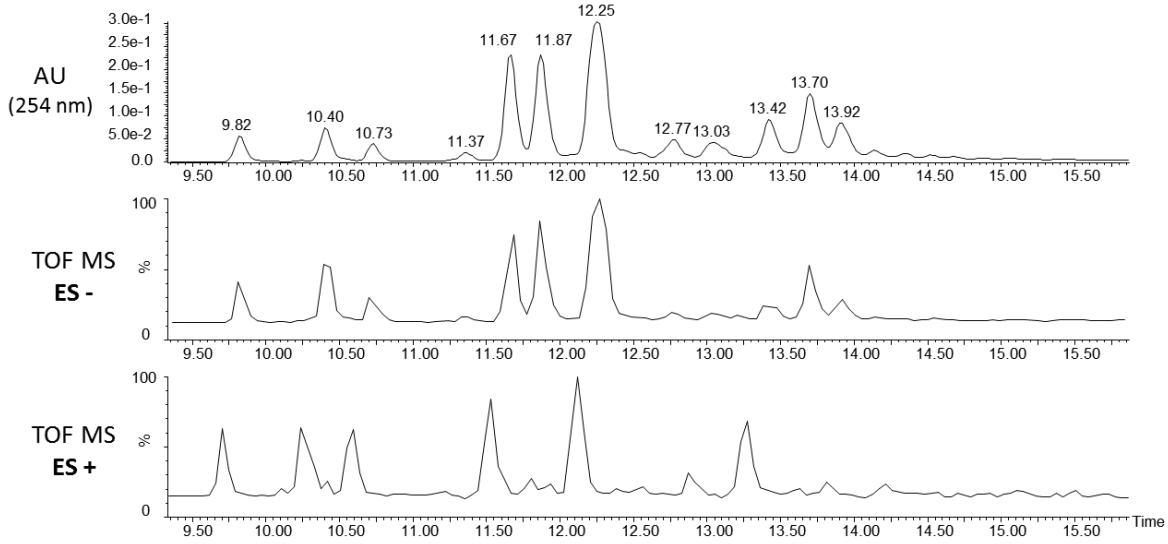
[cyclic-1a], retention time: 7.75 min



Chemical Formula: $C_{20}H_{24}N_4O_8S_2$
Exact Mass: 512.1036

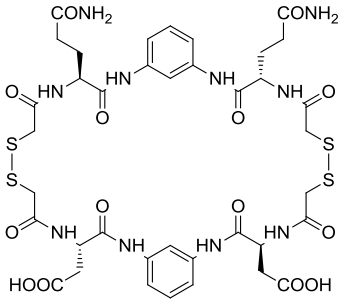


Mixture of the four BBs **[1a+1b+1c+1d]** at pH 7.5

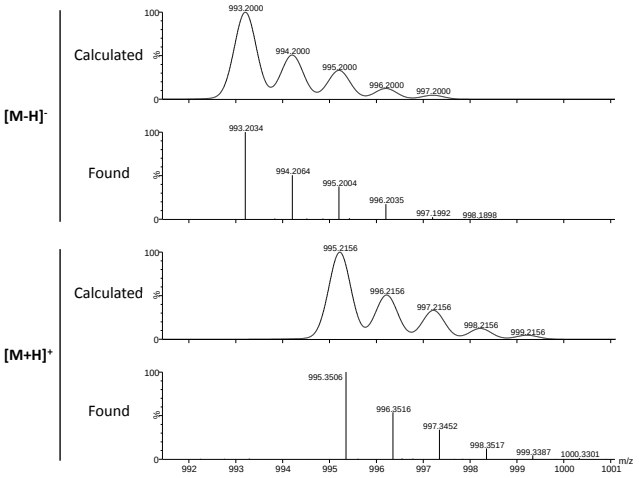


Identification of the dimers containing **[1d]**:

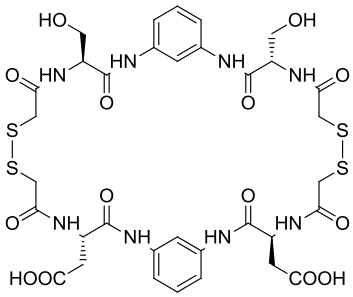
[1b-1d], retention time: 11.87 min



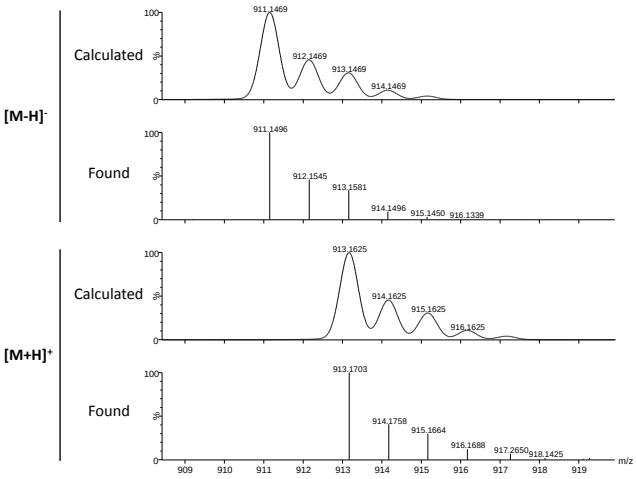
Chemical Formula: $C_{38}H_{46}N_{10}O_{14}S_4$
Exact Mass: 994.2078



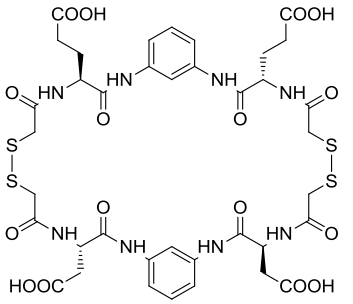
[1c-1d], retention time: 12.25 min



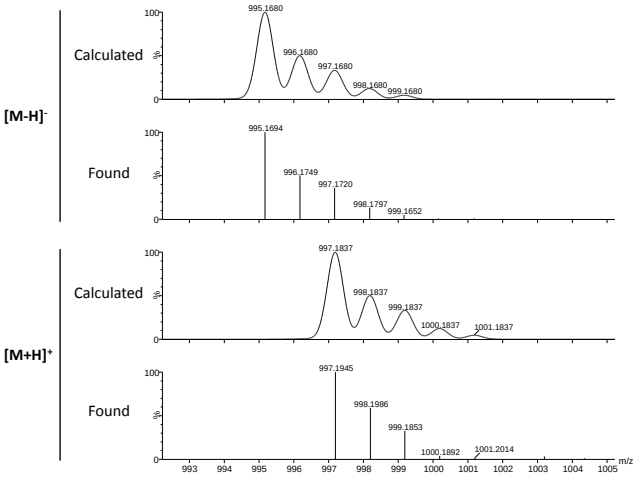
Chemical Formula: $C_{34}H_{40}N_8O_{14}S_4$
Exact Mass: 912.1547



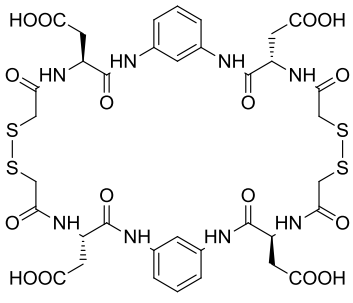
[1a-1d], retention time: 13.70 min



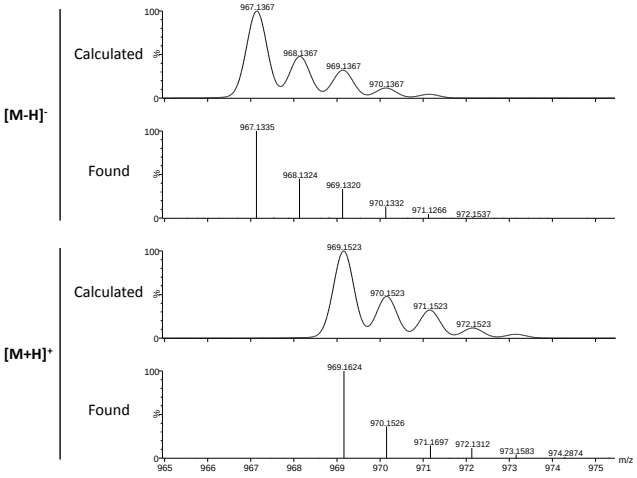
Chemical Formula: $C_{38}H_{44}N_8O_{16}S_4$
Exact Mass: 996.1758



[1d-1d], retention time: 13.92 min



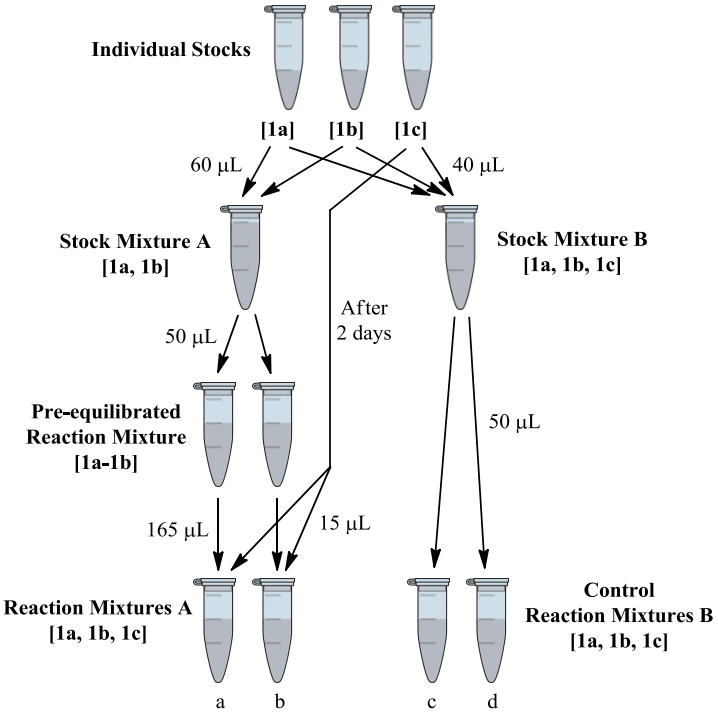
Chemical Formula: $C_{36}H_{40}N_8O_{16}S_4$
Exact Mass: 968.1445



REVERSIBILITY TESTS

Reversibility tests of the DCLs at pH 7.5 and 2.5

Individual stocks (24 mM) of each BB **[1a, 1b, 1c]** were prepared by dissolving separately 1.49 mg of **[1a]**, 1.47 mg of **[1b]** and 1.25 mg of **[1c]**, in 120 μL of DMSO (see scheme S2). From these, a **stock mixture A** with the BBs **[1a]** and **[1b]** was prepared by mixing together 60 μL of their **individual stocks**. Then, two **pre-equilibrated reaction mixtures** were prepared by mixing separately 50 μL of the **stock mixture A** with 225 μL of: a) 26.7 mM phosphate buffer (pH 7.5) and b) 26.7 mM phosphate buffer (pH 2.5). The **individual stock** of **[1c]** was stored at -80 ºC. After 2 days, two **reaction mixtures A** (2 mM of each BB) were prepared by adding 15 μL of the **individual stock** of **[1c]** to 165 μL of each **pre-equilibrated reaction mixture**.



Scheme S2: preparation of the solutions of the reversibility test.

Simultaneously, a stock mixture of the three BBs (**stock mixture B**) was prepared by mixing 40 μL of the three **individual stocks**. Finally, two **control reaction mixtures B** (2 mM of each BB) were prepared by mixing separately 50 μL of the **stock mixture B** with 150 μL of: c) 26.7 mM phosphate buffer (pH 7.5), and d) 26.7 mM phosphate buffer (pH 2.5). After 2 days, the **reaction mixtures A**, the **control reaction mixtures B** and the **pre-equilibrated reaction mixtures** were analyzed by HPLC.

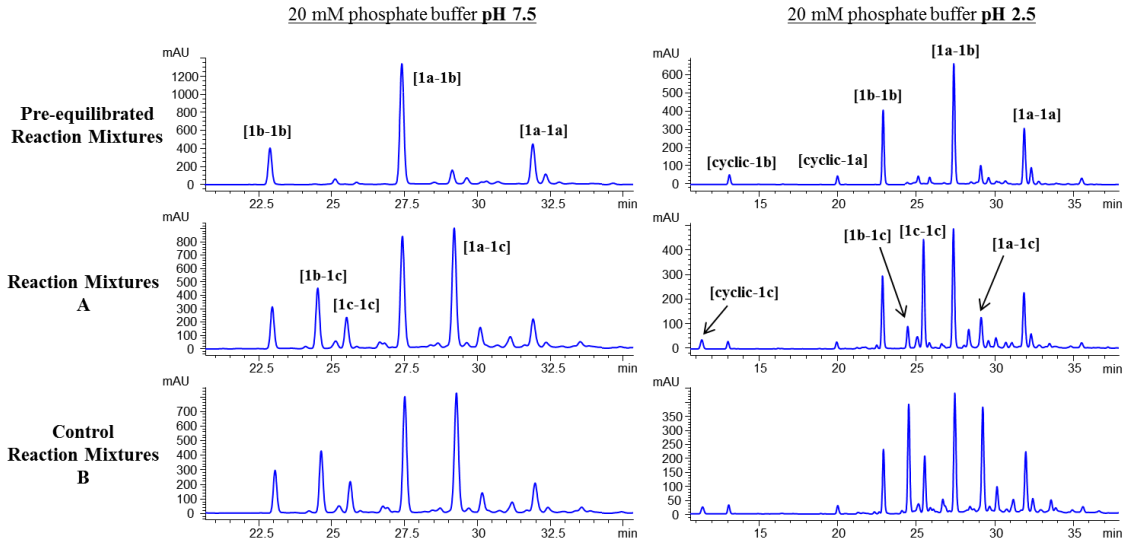


Figure S30: HPLC chromatograms of the pre-equilibrated reaction mixtures and the reaction mixtures A and B, at the pH 7.5 and 2.5.

At pH 7.5, after the addition of **[1c]** to the **pre-equilibrated mixture** of **[1a+1b]**, the mixture evolved to the same final situation as when the three BBs are left to oxidize together. Therefore, at slightly basic pH the mixture proved to reach the thermodynamic equilibrium. However, at pH 2.5 the oxidation rate is faster than the exchange reaction, and thus, **[1c]** is forced to react with itself and only small amounts of the heterodimers containing **[1c]** were formed. Therefore, at acidic pH the mixture is not under equilibrium conditions. The **reaction mixture A** at pH 2.5 was analyzed at longer reaction times but no changes were detected.

Additionally, 0.35 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) were added to both **reaction mixtures A**. After the re-oxidation, their HPLC chromatograms showed that the composition remained unchanged at pH 7.5 but not at pH 2.5, corroborating that the library reaches the equilibrium at pH 7.5 but not at pH 2.5.

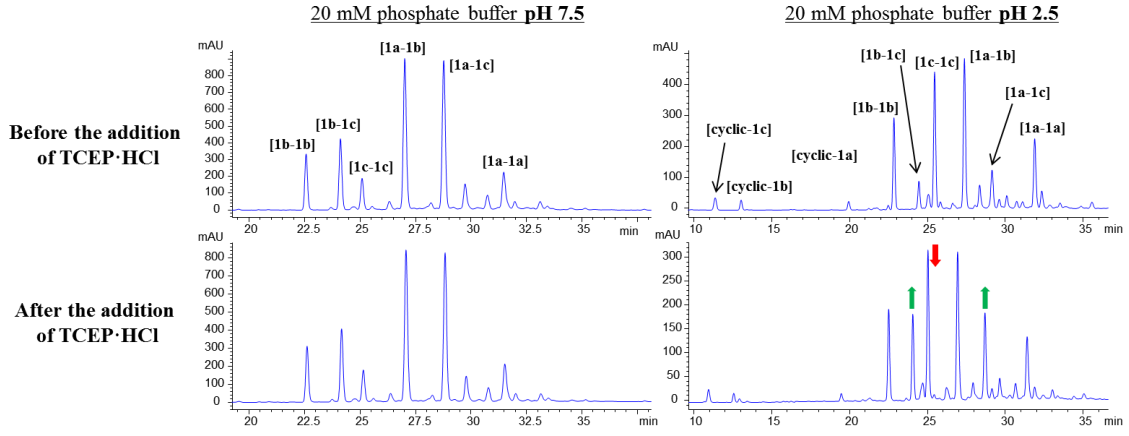


Figure S31: HPLC chromatograms of the reaction mixtures A at the pH values of 7.5 and 2.5, before and after the addition of 0.35 equivalents of TCEP·HCl.

Reversibility test of the DCLs containing high salt concentrations

The three **individual stocks**, the **stock mixtures A** and **B**, the **pre-equilibrated reaction mixture**, the **reaction mixture A** and the **control reaction mixture B** were prepared using the previously explained methodology of the scheme S2. However in this experiment the 26.7 mM phosphate buffer (pH 7.5) used to prepare the samples contained 1.33 M of NaCl.

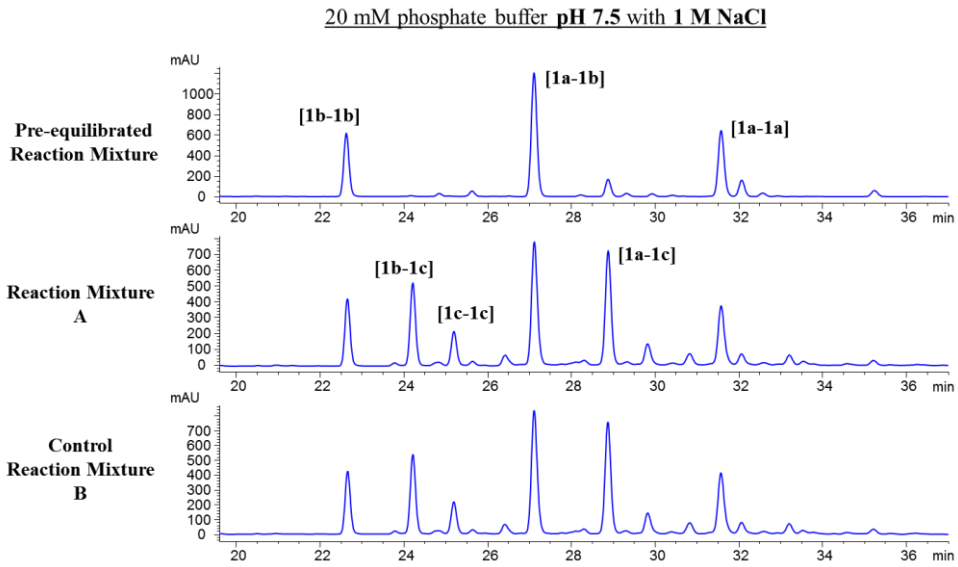


Figure S32: HPLC chromatograms of the pre-equilibrated reaction mixtures and the reaction mixtures A and B, at pH 7.5 with 1M NaCl.

After the addition of **[1c]** to the **pre-equilibrated mixture** of **[1a+1b]**, the mixture evolved to the same final situation as when the three BBs are left to oxidize together. Therefore, at slightly basic pH with a high salt concentration, the mixture proved to reach the thermodynamic equilibrium.

Additionally, 45 µL of the **stock mixture B** were added to 100 µL of a 26.7 mM phosphate buffer (pH 7.5). After 24 hours, 44 µL of a 3.84 M NaCl solution together with 5 µL of the **stock mixture B** were added to 120 µL of the previously oxidized mixture. The resulting solution was let to re-equilibrate and, after 24 hours, its HPLC chromatogram showed that the library had evolved to the same final situation as when the salt was in the library from the beginning (see figure S33).

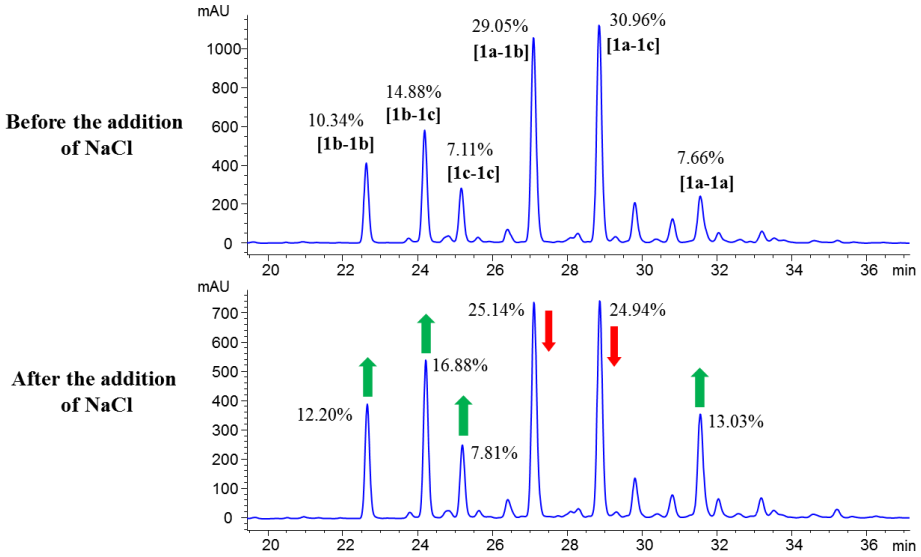


Figure S33: HPLC chromatograms of the DCL generated from **[1a+1b+1c]**, before and after the addition of concentrated NaCl.

Finally, 0.35 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) were added to the **reaction mixture A**. After the re-oxidation, their HPLC chromatograms showed that the composition remained unchanged, corroborating that the library reaches the equilibrium at pH 7.5 with a high salt concentration.

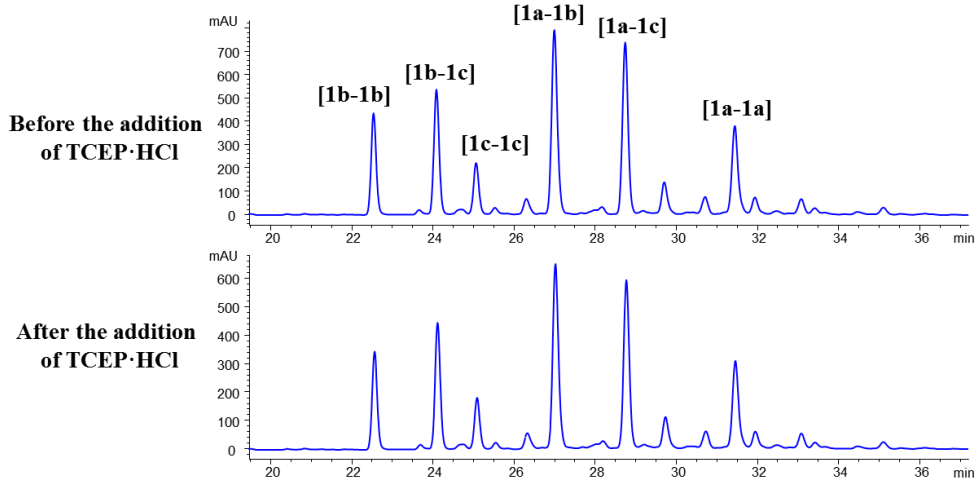
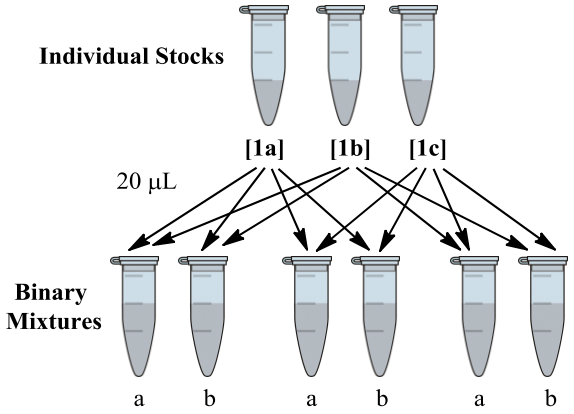


Figure S34: HPLC chromatograms of the reaction mixture A (at pH 7.5 with 1M NaCl), before and after the addition of 0.35 equivalents of TCEP·HCl.

BINARY MIXTURES AND ESTIMATION OF EQUILIBRIUM CONSTANTS

Individual stocks (14 mM) of each BB **[1a, 1b, 1c]** were prepared by dissolving separately 1.23 mg of **[1a]**, 1.18 mg of **[1b]** and 1.36 mg of **[1c]**, in 150 µL, 144 µL and 197 µL of DMSO respectively (see scheme S3). From these, three **binary mixtures** (2 mM of each BB) were prepared by mixing together 20 µL of the two corresponding individual stocks with the solutions: a) 26.7 mM phosphate buffer (pH 7.5), and b) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl. After 24 h, the six mixtures were analyzed by HPLC.



Scheme S3: preparation of the binary mixtures.

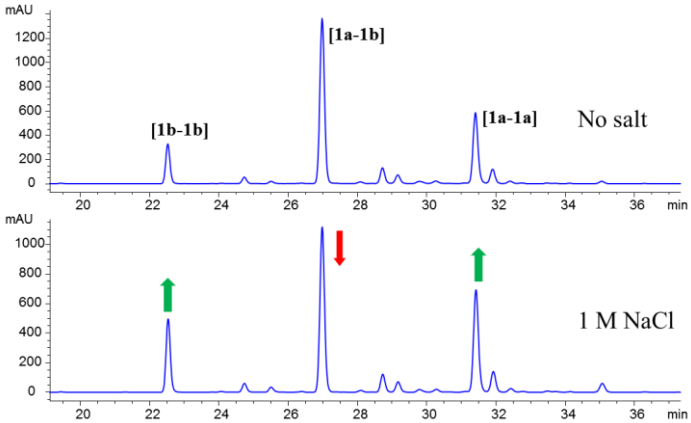


Figure S35: HPLC chromatograms of the binary mixture **[1a+1b]** alone and in the presence of 1M NaCl.

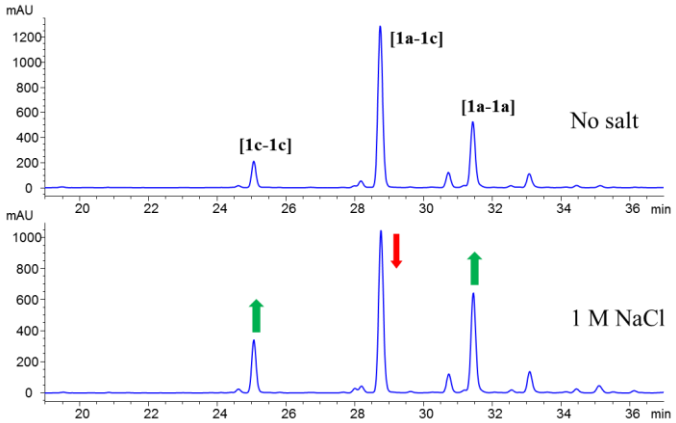


Figure S36: HPLC chromatograms of the binary mixture **[1a+1c]** alone and in the presence of 1M NaCl.

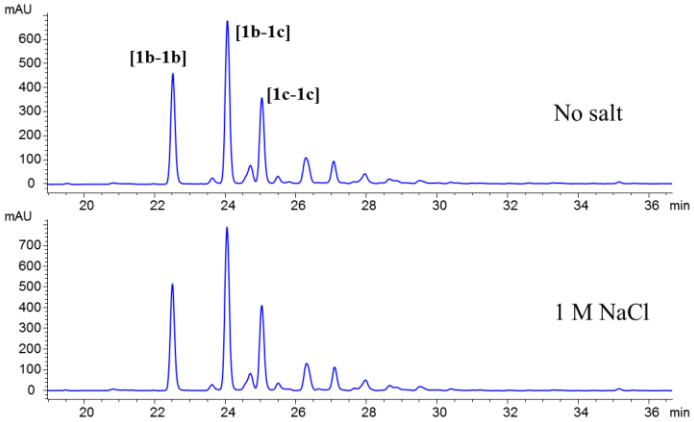


Figure S37: HPLC chromatograms of the binary mixture **[1b+1c]** alone and in the presence of 1M NaCl.

If we consider the DCL consisting of the dimers AA, BB and AB, formed from the connection of the BBs A and B, the formation constant of the three dimers can be expressed as follows:

$$K_{AA} = \frac{[AA]}{[A]^2} \;,\; K_{BB} = \frac{[BB]}{[B]^2} \;,\; K_{AB} = \frac{[AB]}{[A]\cdot[B]} \quad \text{(Equations 2, 3 and 4)}$$

Since in the disulfide-based DCLs, at the equilibrium, the BBs are completely consumed, it is appropriate to combine these three formation constants in order to obtain an expression in which the concentrations [A] and [B] are cancelled:

$$K_{AA} \cdot K_{BB} = K_{AB}{}^2 \cdot C_{[A,B]} \quad \text{(Equation 5)}$$

$$\text{where } C_{[A,B]} = \frac{[AA]\cdot[BB]}{[AB]^2} = \frac{\text{Area}(AA)\cdot\text{Area}(BB)}{\text{Area}(AB)^2} \quad \text{(Equation 6)}$$

The dimensionless $C_{[A,B]}$ magnitude was calculated for the six **binary mixtures**:

	No salt	1 M NaCl
$C_{[1a,1b]}$	0.101	0.278
$C_{[1a,1c]}$	0.065	0.196
$C_{[1b,1c]}$	0.363	0.344

The presence of 1M NaCl did not significantly change the $C_{[1b,1c]}$ value, while $C_{[1a,1b]}$ and $C_{[1a,1c]}$ increased in a factor of 2.8-3.0. This observation is in agreement with the amplification of **[1a-1a]** in the two sublibraries containing the **[1a]** building block. The sublibrary made of only **[1b]** and **[1c]** was insensitive to the salt content.

In order to quantify the salt-induced stabilization of **[1a-1a]**, its formation constant was isolated from equation 5. This was done for the two binary mixtures in which this BB is involved:

$$K_{[1a\text{-}1a]} = \frac{K_{[1a\text{-}1b]}^2 \cdot C_{[1a,1b]}}{K_{[1b\text{-}1b]}} \;,\quad K_{[1a\text{-}1a]} = \frac{K_{[1a\text{-}1c]}^2 \cdot C_{[1a,1c]}}{K_{[1c\text{-}1c]}} \quad \text{(Equations 7 and 8)}$$

The salt-induced stabilization factor of **[1a-1a]** can be calculated by dividing the formation constant in de presence of salt ($K^{NaCl}_{[1a\text{-}1a]}$) by the formation constant in the absence of salt ($K^{No\ salt}_{[1a\text{-}1a]}$). This can be done separately for equations 7 and 8, and similar results should be obtained.

To this purpose, two considerations were taken into account:

1) The formation constants of **[1b-1b]** and **[1c-1c]** are known to be insensitive to the salt content.
2) The formation constants of **[1a-1b]** and **[1a-1c]** can also be considered insensitive to the salt content in order to calculate the stabilization of **[1a-1a]**. Notice that it is equivalent to consider the stabilization of **[1a-1a]** or the destabilization of both **[1a-1b]** and **[1a-1c].** What actually amplifies **[1a-1a]** is the **relative** increase in stability of this dimer in front of the two heterodimers containing **1a**, and thus, the two chemical species that must satisfy the mass balance with **[1a-1a]**.

Accordingly, the ratio of formation constants can be directly calculated by dividing the $C_{[1a,1b]}$ and $C_{[1a,1c]}$ magnitudes respectively:

$$\frac{K^{NaCl}_{[1a\text{-}1a]}}{K^{No\ salt}_{[1a\text{-}1a]}} \simeq \frac{C^{NaCl}_{[1a,1b]}}{C^{No\ salt}_{[1a,1b]}} = 2.8\ , \qquad \frac{K^{NaCl}_{[1a\text{-}1a]}}{K^{No\ salt}_{[1a\text{-}1a]}} \simeq \frac{C^{NaCl}_{[1a,1c]}}{C^{No\ salt}_{[1a,1c]}} = 3.0 \qquad \text{(Equations 9 and 10)}$$

Using 2.9 as the average value of the calculated ratio of $K^{NaCl}_{[1a\text{-}1a]} / K^{No\ salt}_{[1a\text{-}1a]}$, the stabilization of **[1a-1a]** can be easily transformed to a difference of free energy as follows:

$$\Delta\Delta G = -RT \ln\left(\frac{K^{NaCl}_{[1a\text{-}1a]}}{K^{No\ salt}_{[1a\text{-}1a]}}\right) = -2.6\ \text{kJ}\cdot\text{mol}^{-1} \quad \text{(Equation 11)}$$

Finally we checked if the information obtained from the binary mixtures fits the ternary system (DCL 1). With this purpose, the real concentrations of all the six dimers of the ternary mixture were calculated by means of their corresponding areas at the HPLC chromatograms, considering the amount of trimers to be negligible. These calculated values were used as the "experimental data".

For the simulation with the DCLSim 1.1, the temperature was set to 298 K and the three BBs were set as "virtual". The formation constants of the three homodimers were arbitrarily set to the unit, and the formation constants of the three heterodimers were calculated by using the rearranged equation 5 as follows:

$$K_{[1a\text{-}1b]} = \sqrt{\frac{K_{[1a\text{-}1a]} \cdot K_{[1b\text{-}1b]}}{C_{[1a,1b]}}}\ , K_{[1a\text{-}1c]} = \sqrt{\frac{K_{[1a\text{-}1a]} \cdot K_{[1c\text{-}1c]}}{C_{[1a,1c]}}}\ , K_{[1b\text{-}1c]} = \sqrt{\frac{K_{[1b\text{-}1b]} \cdot K_{[1c\text{-}1c]}}{C_{[1b,1c]}}}$$

(Equations 12, 13 and 14)

Notice that it does not matter the attributed value to the formation constants of the homodimers: whatever their value is, the whole system will fit if the formation constants of the heterodimers are calculated by means of the equations 12, 13 and 14.

Finally, the experimental and simulated concentrations of the six dimers were represented together in order to be compared. This was done separately for the ternary mixture in the absence and presence of 1M NaCl (see figure S38).

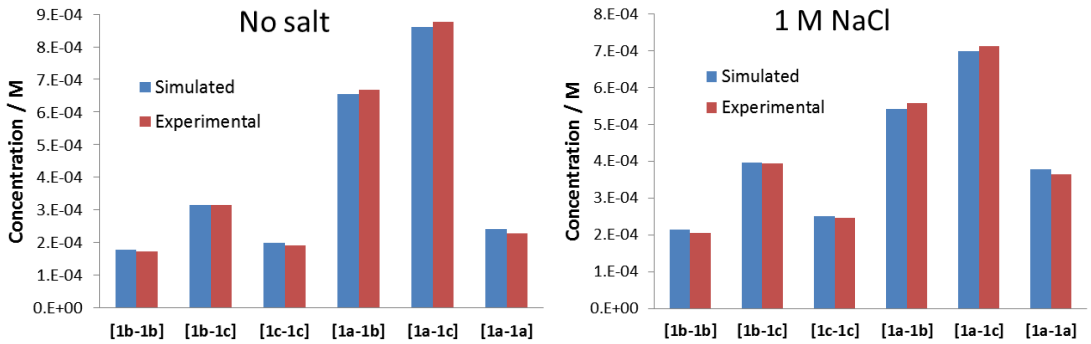
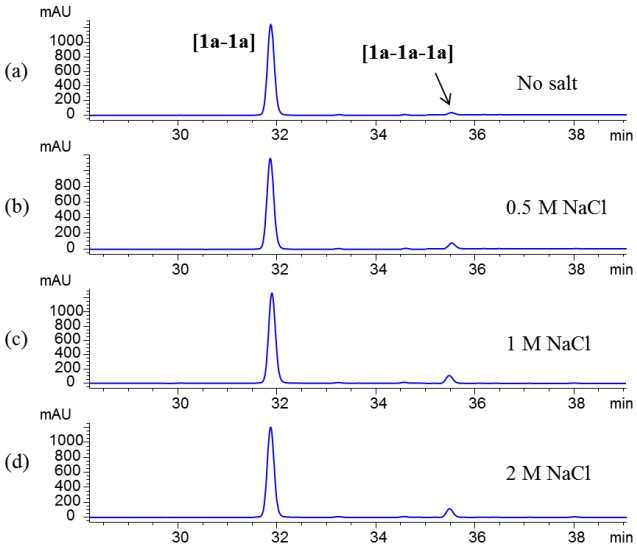


Figure S38: representation of the simulated (**blue**) and experimental (**red**) concentrations of all the six dimers of the ternary mixture, in the presence and absence of 1 M NaCl.

The excellent agreement between the experimental and the calculated values using the estimated equilibrium constants, clearly corroborates that the information obtained from the binary mixtures fits the ternary system. Moreover, the fact that both the binary and ternary libraries can be rationalized by means of the same thermodynamic constants is an extra argument supporting that these chemical systems operate under thermodynamic equilibrium.

STUDY OF THE DIMER/TRIMER RATIO

An 8.54 mM **stock solution** of **[1a]** was prepared by dissolving 2.24 mg in 510 µL of DMSO. Four **reaction mixtures** of 2.13 mM **[1a]** were prepared by mixing 50 µL of the **stock solution** with 150 µL of the following solutions: (a) 26.7 mM phosphate buffer (pH 7.5), (b) 26.7 mM phosphate buffer (pH 7.5) with 0.67 M NaCl, (c) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl, and (d) 26.7 mM phosphate buffer (pH 7.5) with 2.66 M NaCl. After 2 days, the four **reaction mixtures** were analyzed by HPLC.



	0 M NaCl		0.5 M NaCl		1 M NaCl		2 M NaCl	
	Area	**AF**	**Area**	**AF**	**Area**	**AF**	**Area**	**AF**
Dimer	12734.75	1	11859.05	0.96	13075.18	0.95	12463.74	0.94
Trimer	351.92	1	804.93	2.36	1052.78	2.77	1180.95	3.22

The equilibrium constant of the exchange reaction between the dimer and the trimer was calculated as follows:

$$3\ \text{Dimer} \overset{K}{\rightleftharpoons} 2\ \text{Trimer} \qquad K = \frac{[\text{Trimer}]^2}{[\text{Dimer}]^3}$$

$$\frac{\varepsilon_{254}(\text{Dimer})}{2} \simeq \frac{\varepsilon_{254}(\text{Trimer})}{3}$$

$$[\text{Dimer}] = \left(A_{\text{Dimer}} \cdot \frac{2.13 \cdot 10^{-3}\ \text{M}}{A_T} \right) \Big/ 2$$

$$[\text{Trimer}] = \left(A_{\text{Trimer}} \cdot \frac{2.13 \cdot 10^{-3}\ \text{M}}{A_T} \right) \Big/ 3$$

	K / M^{-1}
No salt	0.33
0.5 M NaCl	2.05
1 M NaCl	2.92
2 M NaCl	4.10

The same experiment was performed with the BB **[1b]** and the salt did not induce any modification of the dimer/trimer ratio.

MOLECULAR MODELLING

All the theoretical calculations were performed with Spartan 06 software operating in a Dell workstation. Monte Carlo conformation searches were performed without restrictions by generating 10000-20000 geometries, which were minimized subsequently using the MMFFaq force field. This version of the force field takes into account water solvent as a continuum medium. This force field has proved to be the most suitable for the conformational analysis of pseudopeptide and peptoid molecules.[2] The obtained local minima were ordered following the corresponding MMFFaq energies. The process was repeated several times staring from different initial geometries to ensure mapping all the conformational space. The corresponding conformational searches of the same molecule starting from different geometries rendered identical results. This fact ensures the fidelity and reliability of the results from this conformational analysis. The corresponding Boltzmann distribution and CPK areas were calculated using the same software. The global minima for the tetraanionic and tetraacid forms of **[1a-1a]** are shown in Figure 2 of the manuscript.

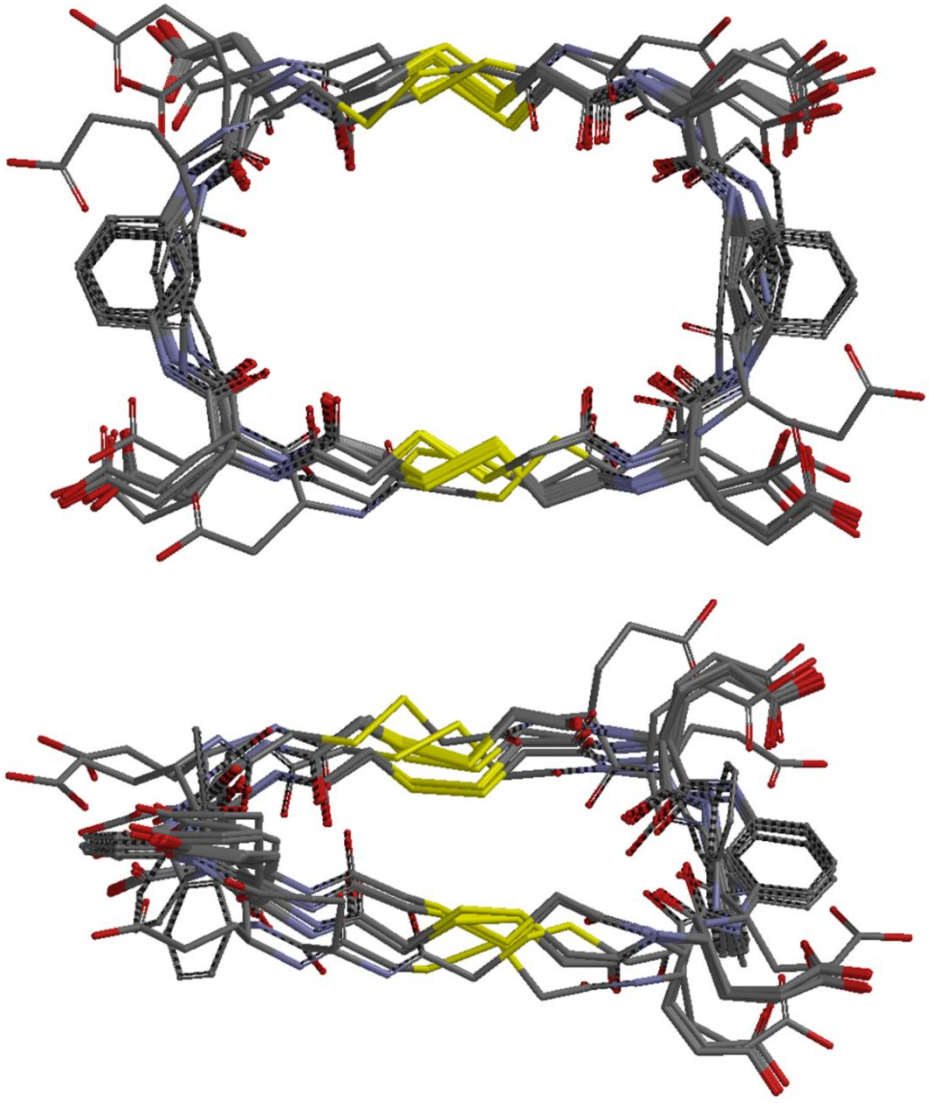


Figure S39: Top and side views of the superposition of the accessible local minima for the tetraacid form of **[1a-1a]**. The figure shows the high flexibility of the macrocyclic framework.

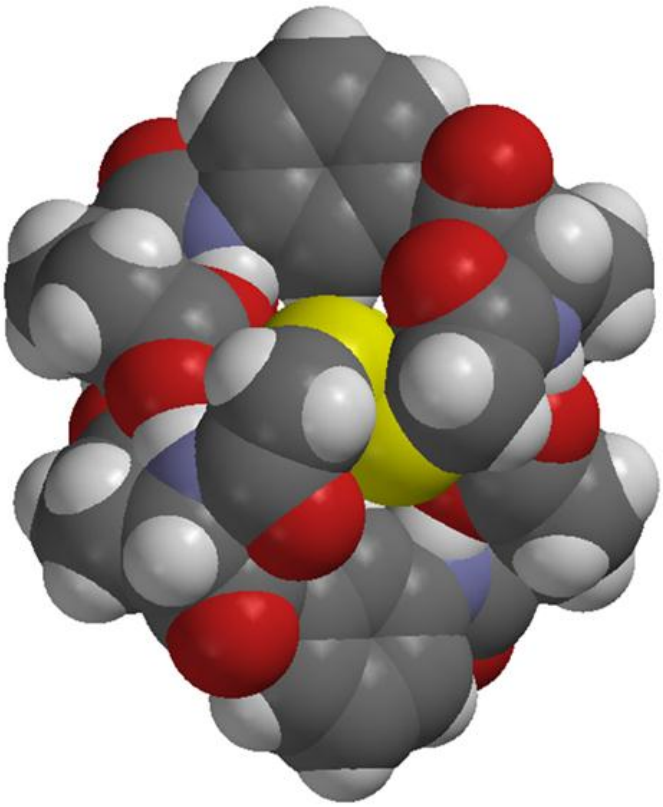


Figure S40: CPK model of the global minimum of the tetraanionic form of **[1a-1a]** macrocycle. The calculated CPK area for this geometry is 871.8 $Å^2$.

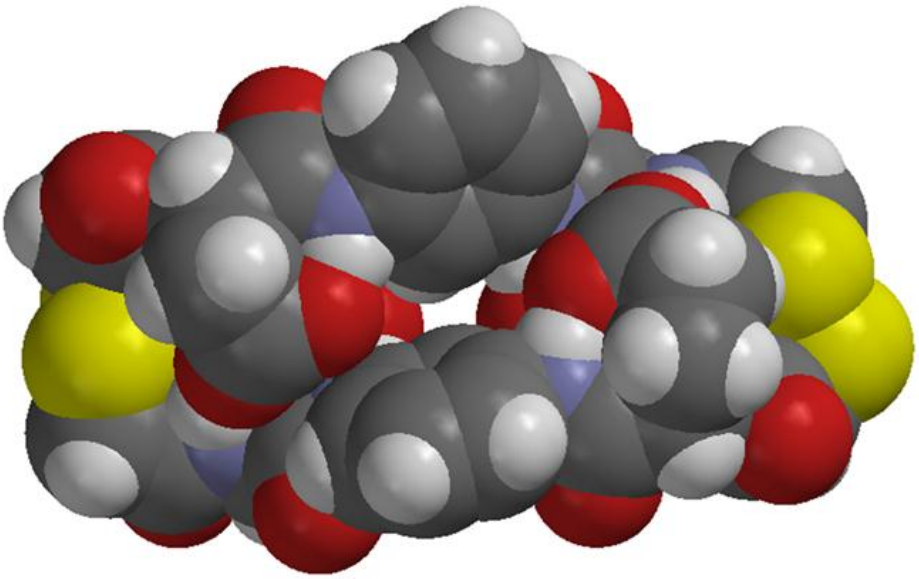


Figure S41: CPK model of the global minimum of the tetraanionic form of **[1a-1d]** macrocycle. The calculated CPK area for this geometry is 839.8 $Å^2$.

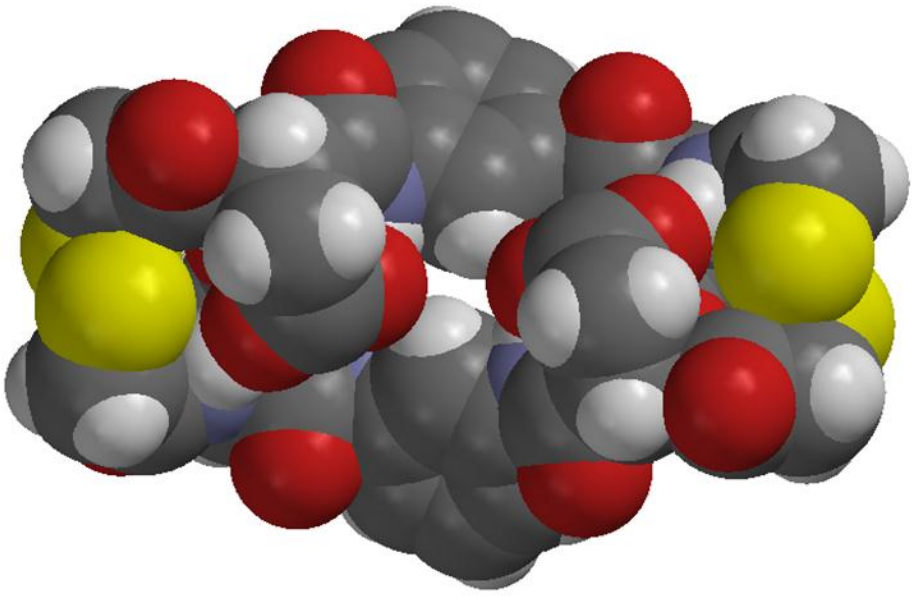


Figure S42: CPK model of the global minimum of the tetraanionic form of **[1d-1d]** macrocycle. The calculated CPK area for this geometry is 801.7 $Å^2$.

Related references:

2 (a) W. Brandt, T. Herberg and L. Wessjohann, *Biopolymers (Protein Science)*, 2011, **96**, 651-667; (b) C. F. Rodriquez, G. Orlova, Y. Guo, X. Li, C.-K. Siu, A. C. Hopkinson and K. W. M.Siu, *J. Phys. Chem. B* 2006**, 110**, 7528-7537; (c) E. F. Strittmatter and E. R. Williams, *J. Phys. Chem. A* 2000**, 104**, 6069-6076; (d) M. D. Beachy, D. Chasman, R. B. Murphy, T. A. Halgren and R. A. Friesner, *J. Am. Chem. Soc.* 1997**, 119**, 5908-5920.

NUCLEAR MAGNETIC RESONANCE (NMR)

An 8.3 mM **stock solution** of **[1a]** was prepared by dissolving 3.22 mg in 750 µL of DMSO-d_6. From this, three NMR samples (2.1 mM) were prepared by mixing 210 µL of the **stock solution** with 630 µL of the following solutions: (a) 26.7 mM phosphate buffer (pH 7.5) with 1.33 M NaCl, (b) 26.7 mM phosphate buffer (pH 7.5), and (c) 26.7 mM phosphate buffer (pH 2.5). Similarly, a 7.1 mM **stock solution** of **[1b]** was prepared by dissolving 1.60 mg in 440 µL of DMSO-d_6. From this, two NMR samples (1.8 mM) were prepared by mixing 210 µL of the **stock solution** with 630 µL of the following solutions: (d) 26.7 mM phosphate buffer (pH 7.5), and (e) 26.7 mM phosphate buffer (pH 2.5). Finally, 5 µL of a 20 mM solution of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in H_2O were added to all the NMR samples. At the time of 24 hours, the five NMR samples were analyzed by HPLC.

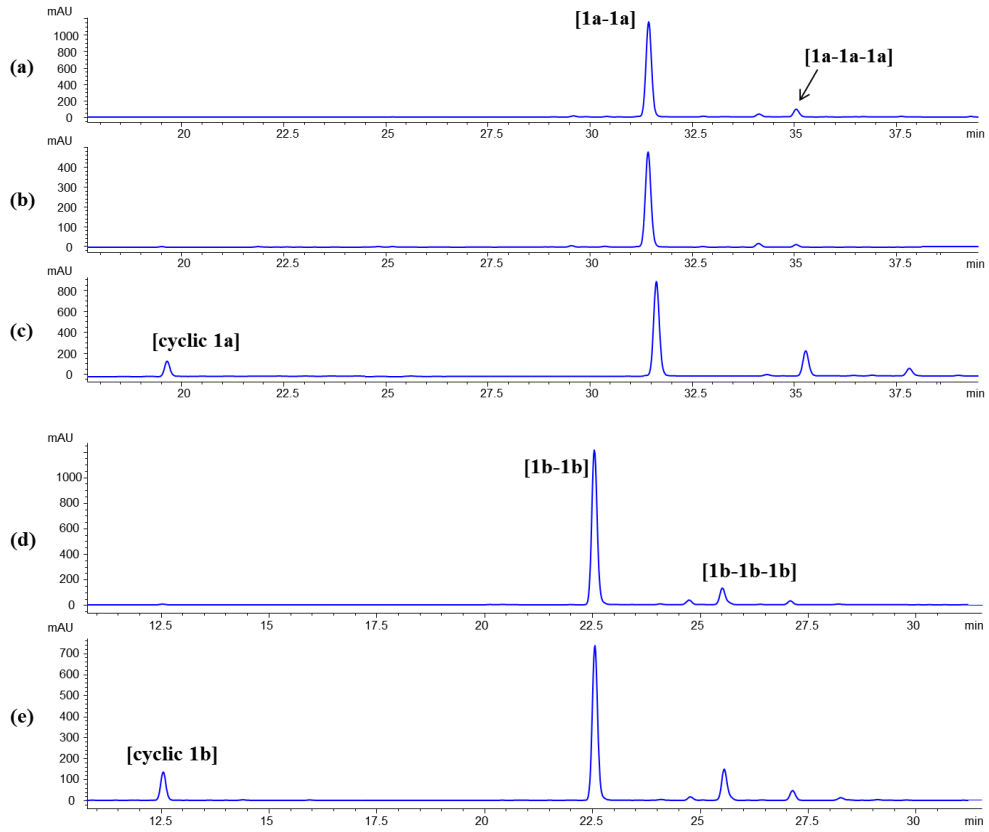
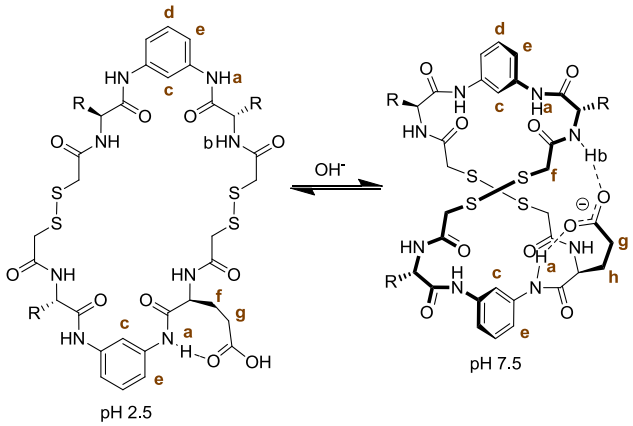


Figure S43: HPLC chromatograms of oxidized **[1a]** in: (a) 20 mM phosphate buffer (pH 7.5) with 1M NaCl and 25% DMSO-d_6, (b) 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6, and (c) 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6. HPLC chromatograms of oxidized **[1b]** in: (d) 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6, and (e) 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6.

The ^{1}H-NMR spectrum of the five samples was recorded on a Varian Mercury 400 instrument (400 MHz for ^{1}H) using the (H)WET water-suppression sequence. The methyl group of DSS was used as a reference at –0.018 ppm. Additionally, a (H)WET-COSY experiment was performed for samples (b), (c), (d) and (e) in order to unambiguously assign the proton signals.



Scheme S4: proposed conformational rearrangement of **[1a-1a]** dimer, produced by a pH change.

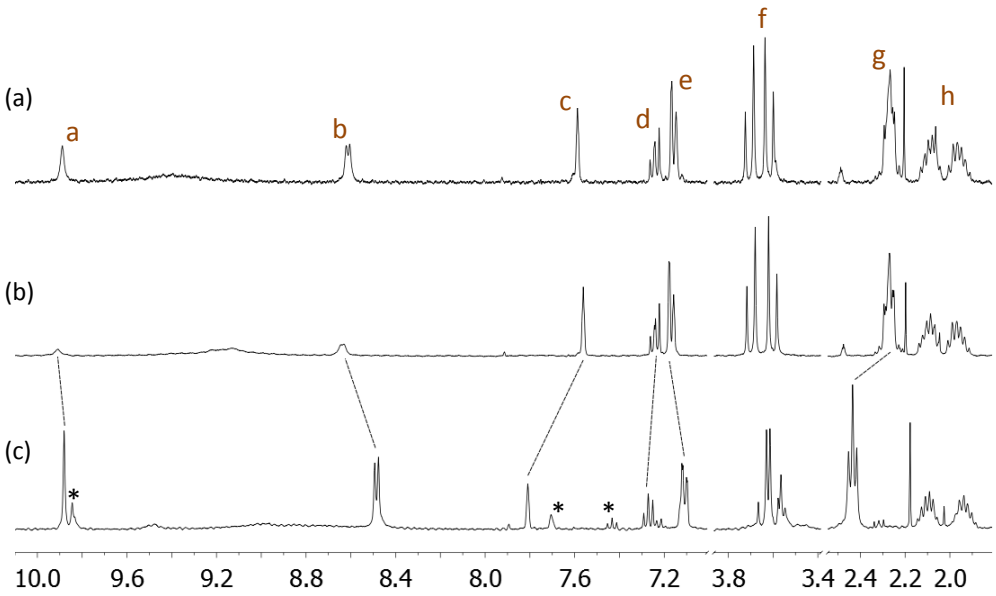


Figure S44: partial (H)WET-^{1}H NMR spectra of oxidized **[1a]** in: (a) 20 mM phosphate buffer (pH 7.5) with 1M NaCl and 25% DMSO-d_6, (b) 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6, and (c) 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6. The symbol (*) identifies signals of **[cyclic-1a]**.

Several signals changed upon increasing the pH from 2.5 to 7.5, due to the deprotonation of the side chains. Thus, the signal **g** (the methylene in α to the carboxylic group) shifts upfield in agreement with the carboxylate formation. Concomitantly, the aliphatic amide NH (**b**) moves downfield as a result of the establishment of an intramolecular H-bond with the carboxylate, which is geometrically favorable in the folded conformation. Interestingly, the aromatic NH (**a**) does not significantly change its chemical shift, which supports that this proton is implicated in similar H-bonding at both acidic and neutral pH, as observed in the modelling. Furthermore, the changes observed for the aromatic protons (**c**, **d** and **e**) are also of note, since **c** and **d** shift upfield and **e** downfield by increasing the pH. This behavior can be explained by considering the rotation of the aromatic amide, which changes the relative disposition of the carbonyl group and the aromatic ring, as observed in the models. Finally, the larger anisochrony of several methylene signals (**g** and, specially, **f**) suggests a more rigid conformation of the cycle in the anionic form.

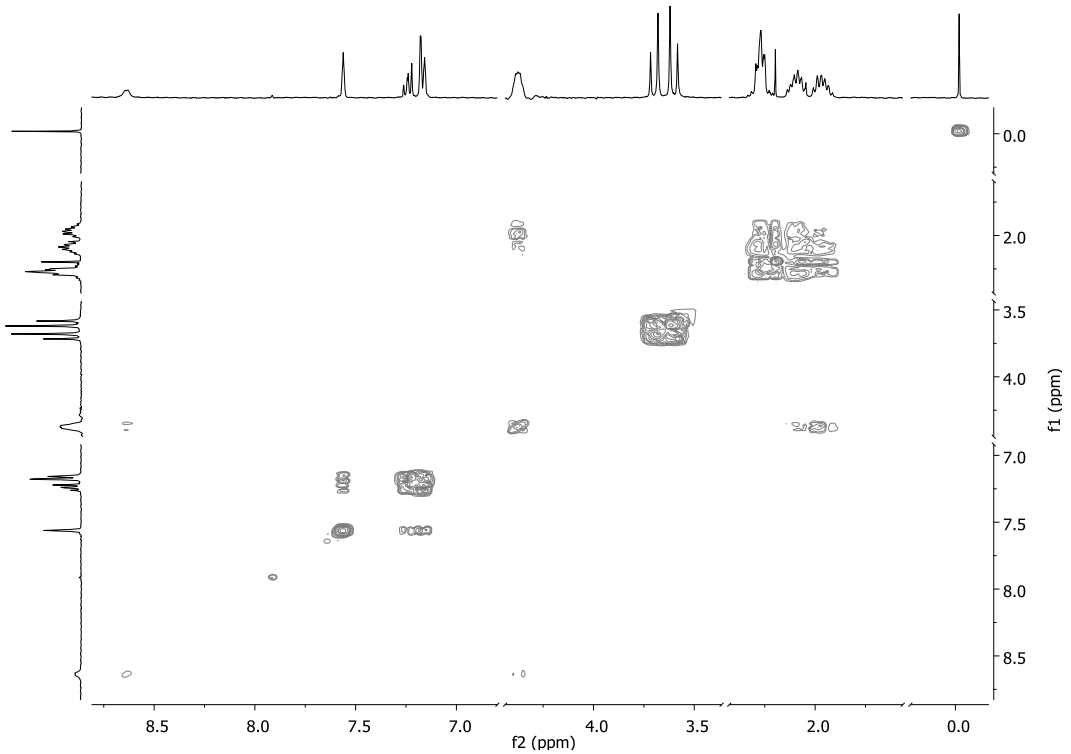


Figure S45: partial (H)WET-COSY spectrum of oxidized **[1a]** in 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6.

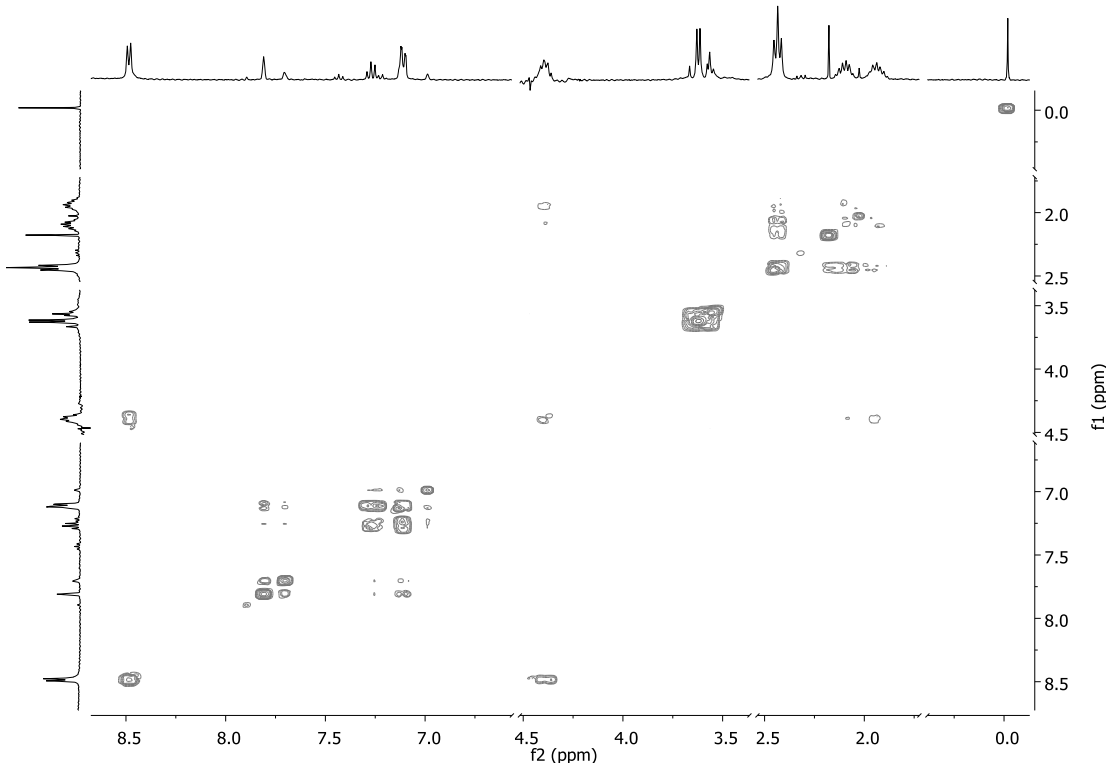


Figure S46: partial (H)WET-COSY spectrum of oxidized **[1a]** in 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6.

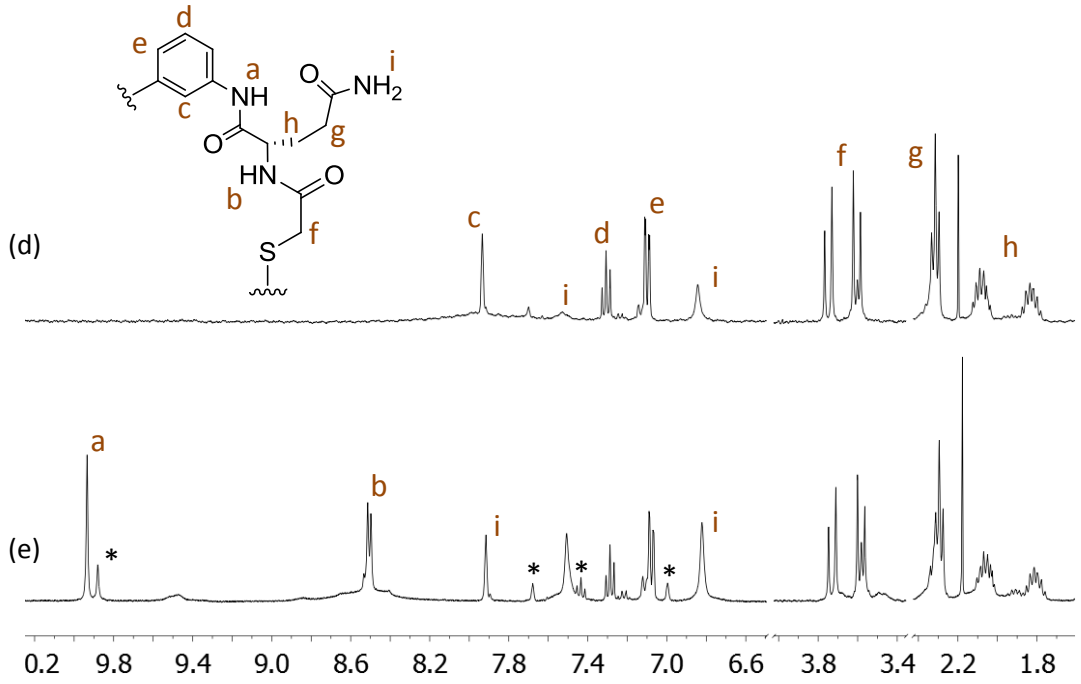


Figure S47: partial (H)WET-^{1}H NMR spectra of oxidized **[1b]** in: (d) 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6, and (e) 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6. The symbol (*) identifies signals of **[cyclic-1b]**.

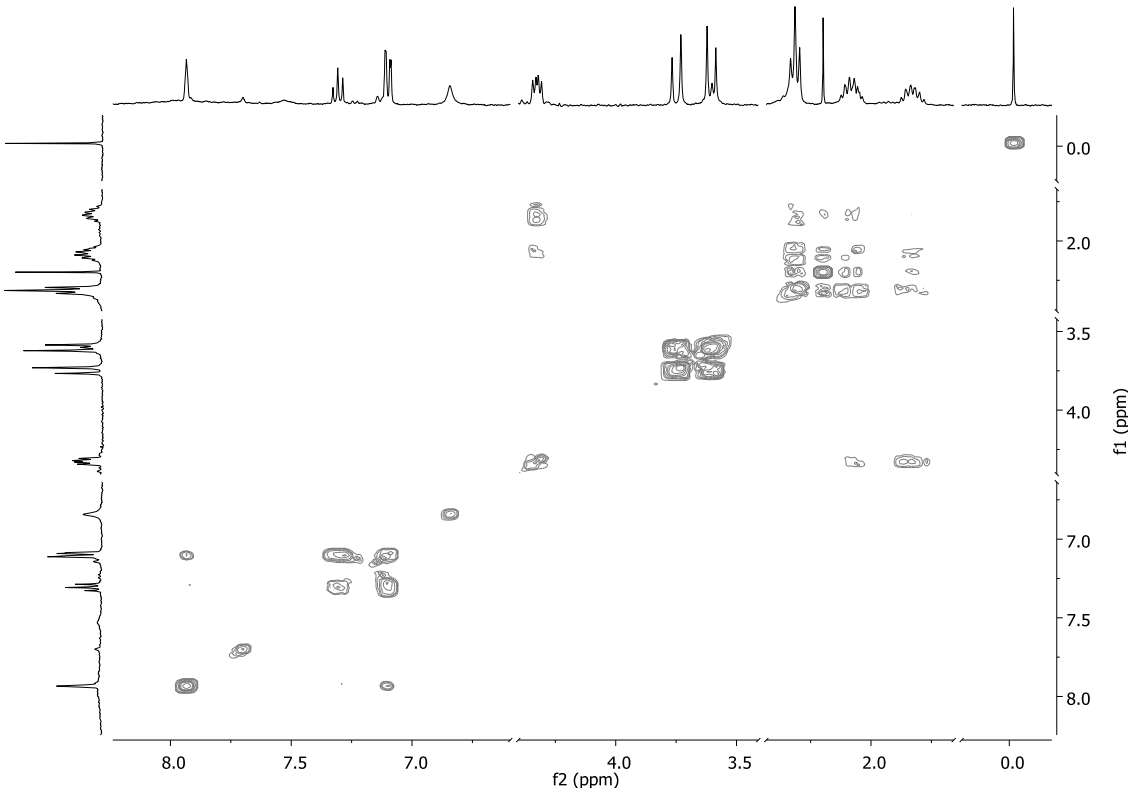


Figure S48: partial (H)WET-COSY spectrum of oxidized **[1b]** in 20 mM phosphate buffer (pH 7.5) with 25% DMSO-d_6.

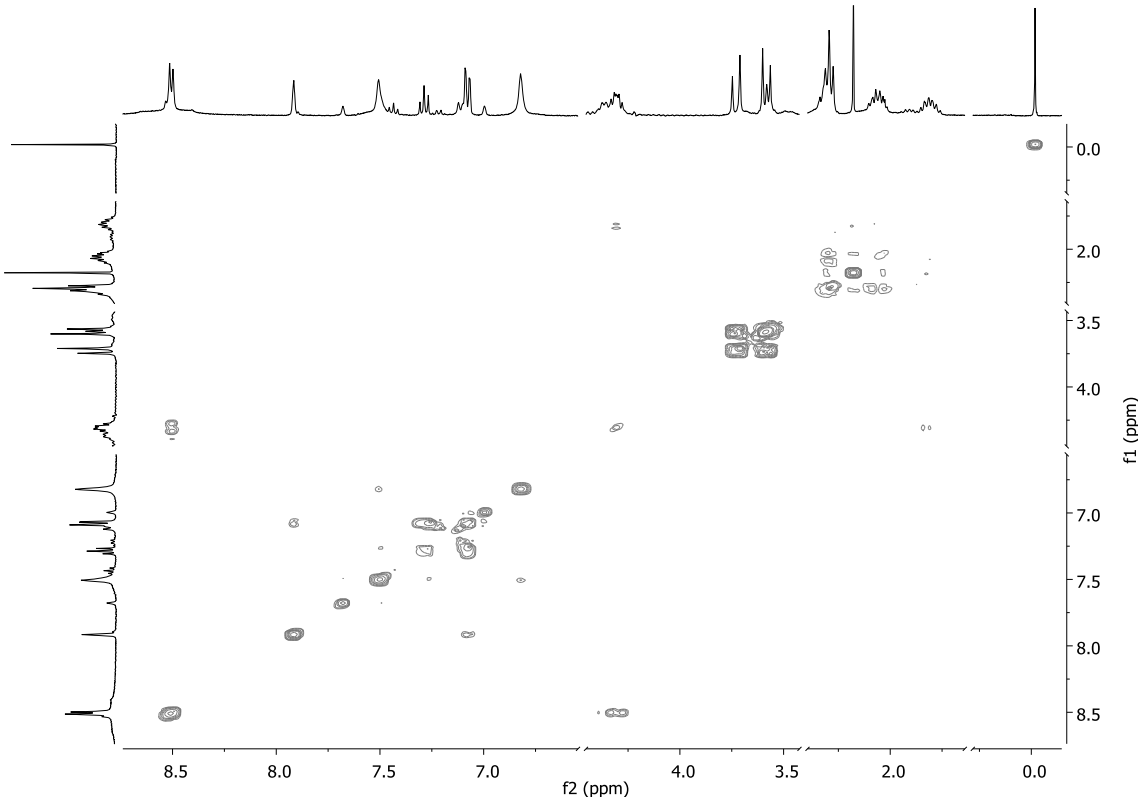


Figure S49: partial (H)WET-COSY spectrum of oxidized **[1b]** in 20 mM phosphate buffer (pH 2.5) with 25% DMSO-d_6.

CIRCULAR DICHROISM (CD)

To a 20 mM phosphate buffer (pH 7.5) with 25% MeOH, 29.5 mg of the BB **[1a]** were added. The oxidation process was monitored until the total consumption of reagent **[1a]**, at the reaction time of 10 days (longer reaction times are required when using MeOH as co-solvent).

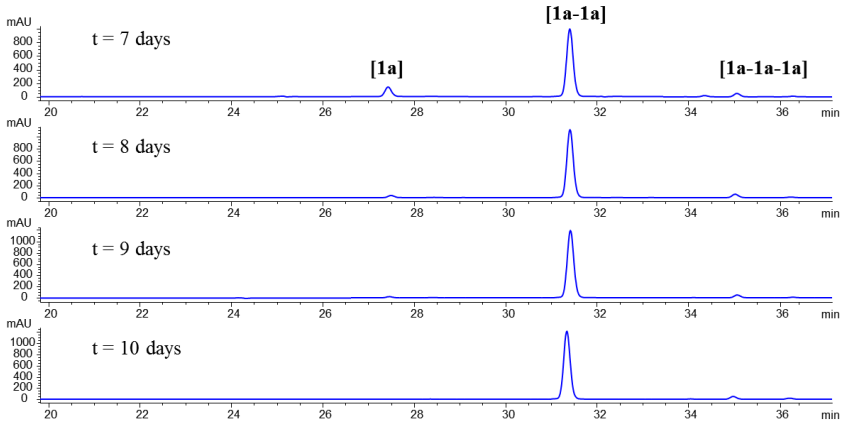


Figure S50: HPLC chromatograms of the oxidation process of the BB **[1a]** in a 20 mM phosphate buffer (pH 7.5) with 25% MeOH, at different reaction times.

The solution was first evaporated under vacuum and then liophilized to remove the H_2O. The resulting solid was purified using preparative reversed-phase chromatography (gradient: from 5% to 30% CH_3CN in H_2O) and 20.1 mg of pure **[1a-1a]** were obtained as a white solid.

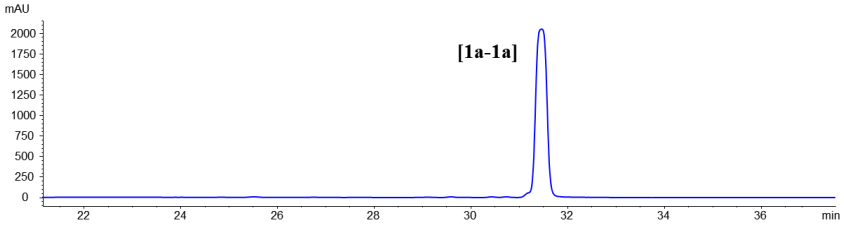


Figure S51: HPLC chromatogram of the dimer **[1a-1a]** after purification.

A 1.07 mM solution of the pure **[1a-1a]** dimer was prepared by dissolving 0.44 mg of the dimer in 400 µL of H_2O with 25% of MeOH. Aliquots of 5 µL of this solution were consecutively added to the following blanks: (a) 1 mL of 20 mM phosphate buffer (pH 7.5) with 25% MeOH, (b) 1 mL of 20 mM phosphate buffer (pH 7.5) with 25% MeOH and 1 M NaCl, and (c) 1 mL of 20 mM phosphate buffer (pH 2.5) with 25% MeOH. The CD and Absorbance (Abs) signals were observed to be proportional to the concentration at least until a final concentration of $2.10 \cdot 10^{-5}$ M of **[1a-1a]**.

The spectra were analyzed with the Spectra Manager software (JASCO Corporation) v. 1.53.01 and a Means-Movement smoothing with a convolution width of 5 was applied to all the CD spectra. For the figure S52, the molar absorption ($\Delta\varepsilon$, $cm^2 \cdot mmol^{-1}$) was calculated as shown in equation 15, where θ is the ellipticity (mdeg), C is the concentration (M) and l is the cell path length (cm). No changes were observed between the normalized spectra at different concentrations.

$$\Delta\varepsilon = \frac{\theta}{32980 \cdot C \cdot l} \quad \text{(Equation 15)}$$

The CD spectra of **[1a-1a]** at different pH values (see figure S52) showed significantly different signatures both at 220 nm and 254 nm, implying a different conformation of the macrocycle in acidic and neutral pH.

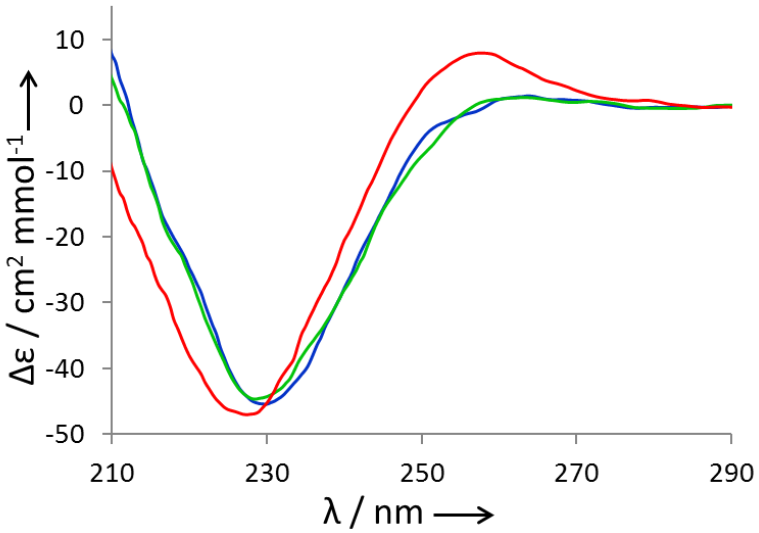


Figure S52: CD spectra of **[1a-1a]** (in 75 : 25, H_2O : MeOH) at pH 2.5 (**red**) and pH 7.5 in the absence (**blue**) and in the presence (**green**) of 1M NaCl.

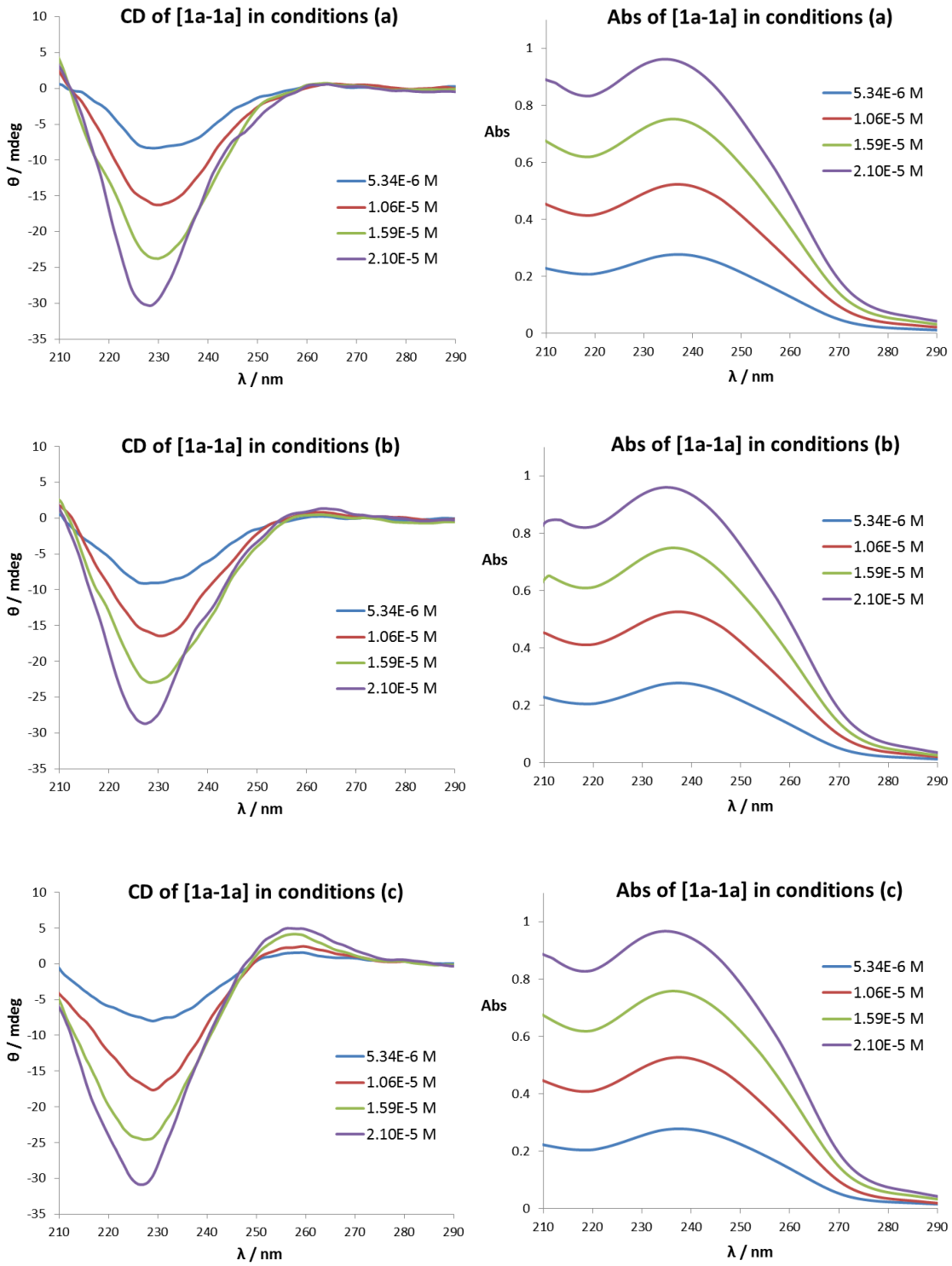


Figure S53: CD and Abs spectra of **[1a-1a]** in: (a) 20 mM phosphate buffer (pH 7.5) with 25% MeOH, (b) 20 mM phosphate buffer (pH 7.5) with 25% MeOH and 1 M NaCl, and (c) 20 mM phosphate buffer (pH 2.5) with 25% MeOH.

ISOTHERMAL MICROCALORIMETRY MEASURMENTS

A 3.59 mM solution of **[1a-1a]** was prepared by dissolving 5.52 mg of the pure dimer in 1500 µL of a blank solution (20 mM phosphate buffer (pH 7.5) with 25% of DMSO). Then, three experiments were performed at 298 K:

A) Successive additions (3 µL each) of the solution of dimer to 1.5 mL of the blank containing 1M NaCl.

B) Successive additions (3 µL each) of the blank solution to 1.5 mL of the blank containing 1 M NaCl.

C) Successive additions (3 µL each) of the solution of dimer to 1.5 mL of the blank.

For each of the three experiments, fourteen of the successive measured enthalpies were represented as a function of the summation of the injected volume (see figure S53). Then the three series were extrapolated to the zero abscises axis in order to obtain the corresponding enthalpies at infinite dilution. The enthalpy values were converted to kJ·mol^{-1} of **[1a-1a]**. Finally, the enthalpy associated to the dilution of **[1a-1a]** in 1M NaCl (ΔH^*) was easily calculated by subtracting the enthalpies associated to the dilution of the salt (ΔH_B) and the dilution of **[1a-1a]** in the blank (ΔH_C).

$$\Delta H_A = 188\ \text{kJ}\cdot\text{mol}^{-1}, \quad \Delta H_B = 166\ \text{kJ}\cdot\text{mol}^{-1}, \quad \Delta H_C \approx 0.0\ \text{kJ}\cdot\text{mol}^{-1}$$

$$\boxed{\Delta H^* = \Delta H_A - \Delta H_B - \Delta H_C = +22\ \text{kJ}\cdot\text{mol}^{-1}}$$

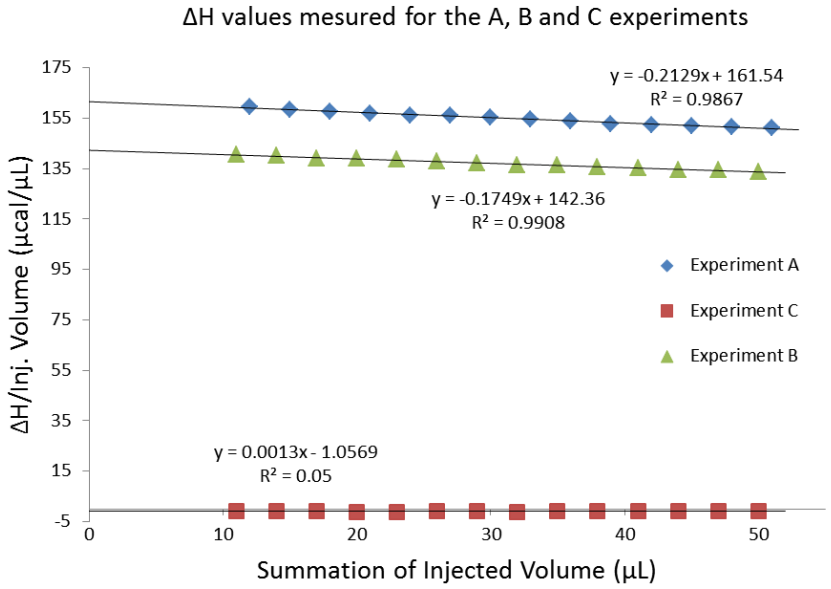


Figure S54: representation of the measured enthalpies of the consecutive additions divided by the volume of each addition, as a function of the summation of the injected volume. Experiments A (**blue**), B (**green**), and C (**red**).

The microcalorimetry measurements rendered that the dilution of **[1a-1a]** in 1 M NaCl at pH 7.5 is endothermic and thus, enthalpically disfavored. Therefore, we excluded the possibility for the salt-induced relative stabilization of **[1a-1a]** to be due to an enthalpically favored binding of the dimer with the salt.