Electronic Supplementary Information (ESI) for:

The effect of DMSO in the aqueous thiol-disulphide dynamic covalent chemistry of model 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}).

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 [**1c**]: 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 Mercury 400 instrument (400 MHz for ^1H and 101 MHz for ^{13}C). The chemical shifts are reported in ppm relative to trimethylsilane (TMS), and coupling constants (J) are reported in Hertz (Hz).

Absorbance measurements were performed on a Molecular Devices SpectraMax M5 microplate reader, at room temperature, and the monitoring wavelength was set at 412 nm. The 96 well microplates, PS, F-bottom, 655101 were used to place the samples when performing the absorbance measurements.

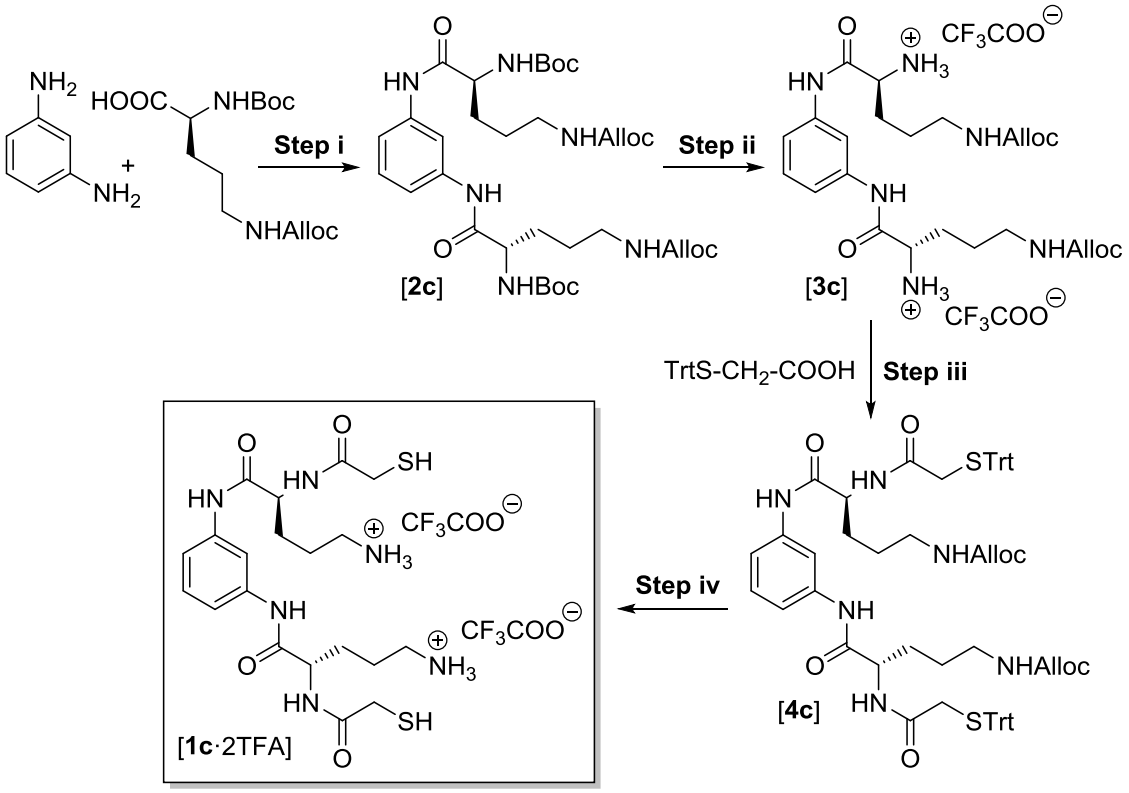
pH measurements were performed at room temperature on a Crison GLP21 pH-meter with the electrode Crison 50 14T.

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

SYNTHESIS OF THE BUILDING BLOCKS [**1c**]

The compound tritylsulfanyl acetic acid was prepared as previously described.[1] Also the compounds [**1a**] and [**1b**] were synthesized as we previously reported.[2]

Synthetic scheme of [**1c**]

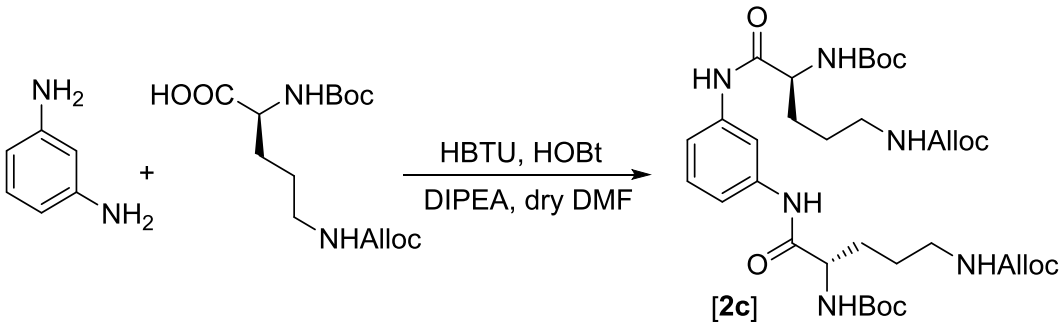


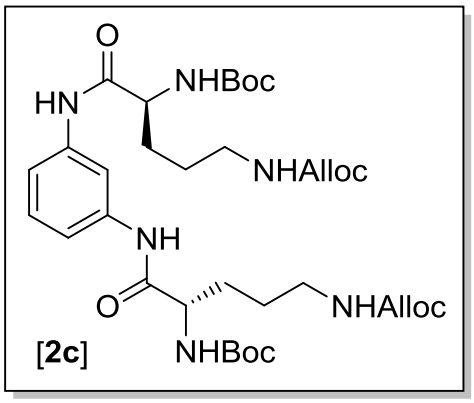
Related references:

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.

2. J. Atcher, A. Moure and I. Alfonso, *Chem. Commun.*, 2013,**49**, 487.

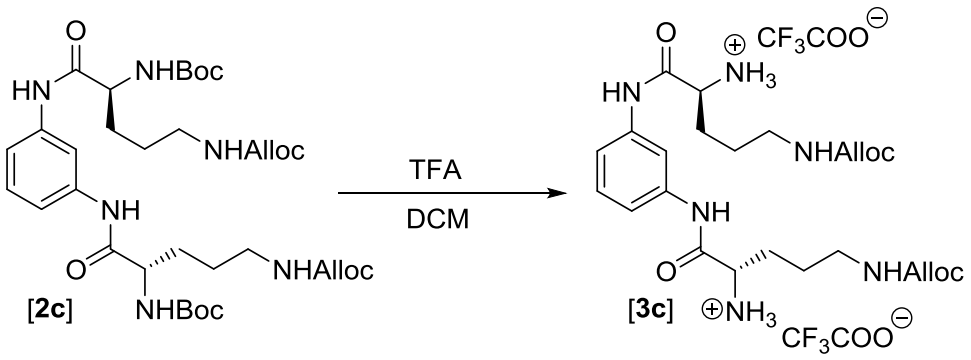
Step i: Experimental procedure for the synthesis of [**2c**]

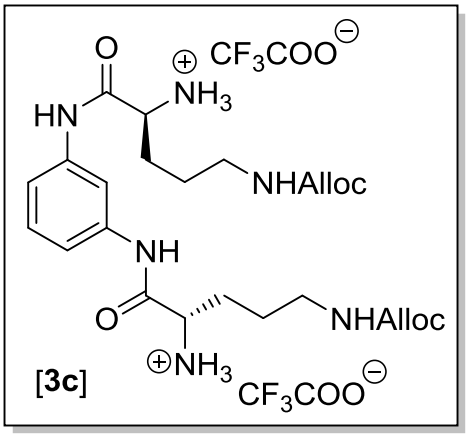




Boc-L-Orn(Alloc)-OH (1.65 g, 5.22 mmol) was dissolved in dry DMF (20 mL) and 1-hydroxybenzotriazole (HOBt, 706 mg, 5.22 mmol), *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU, 2.07 mg, 5.46 mmol) and *N,N*-diisopropylethylamine (DIPEA, 3.50 mL, 20.1 mmol) were added over the solution. The reaction mixture was cooled to 0ºC. A solution of *m*-phenylenediamine (257 mg, 2.37 mmol) in dry DMF (5 mL) was added over the mixture through a cannula. The solution was stirred at room temperature for 5 days, after which complete conversion of the starting material was observed by TLC (Rf AcOEt/Hexane, 3:2 (v:v): 0.23). The mixture 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 45% to 55% AcOEt) to give 1.43 g of [**2c**] (85% yield) as a white solid. HRMS (ESI+) calcd. for $C_{34}H_{52}N_6O_{10}$ $[M+H]^+$ (m/z): 705.3818, found: 705.3813. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.92 (s, 2H, NHCOC*H), 7.77 (s, 1H, CH_{Ar}), 7.36–6.97 (m, 3H, CH_{Ar}), 5.89 (ddt, *J* = 17.2, 10.8, 5.6 Hz, 2H, $NHCOOCH_2CHCH_2$), 5.66 (s, 2H, $NHCOO^tBu$), 5.28 (dq, *J* = 17.2, 1.6 Hz, 2H, $NHCOOCH_2CHCH_2$), 5.22–5.07 (m, 4H: 2H, $NHCOOCH_2CHCH_2$ + 2H, $NHCOOCH_2CHCH_2$), 4.58 (d, *J* = 5.5 Hz, 4H, $NHCOOCH_2CHCH_2$), 4.43 (s, 2H, C*H), 3.40 (s, 2H, $CH_2NHAlloc$), 3.24–3.02 (m, 2H, $CH_2NHAlloc$), 1.96–1.77 (m, 2H, CH_2C*H), 1.76–1.54 (m, 6H: 2H, CH_2C*H + 4H, CH_2CH_2C*H), 1.43 (s, 18H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.3 (2 x CO), 157.2 (2 x CO), 156.4 (2 x CO), 138.5 (2 x C_{Ar}), 133.0 (2 x $NHCOOCH_2CHCH_2$), 129.4 (1 x CH_{Ar}), 117.7 (2 x $NHCOOCH_2CHCH_2$), 115.7 (2 x CH_{Ar}), 111.5 (1 x CH_{Ar}), 80.3 (2 x C), 65.9 (2 x $NHCOOCH_2CHCH_2$), 53.9 (2 x C*H), 38.9 (2 x $CH_2NHAlloc$), 30.3 (2 x CH_2C*H), 28.5 (6 x CH_3), 26.6 (2 x CH_2CH_2C*H).

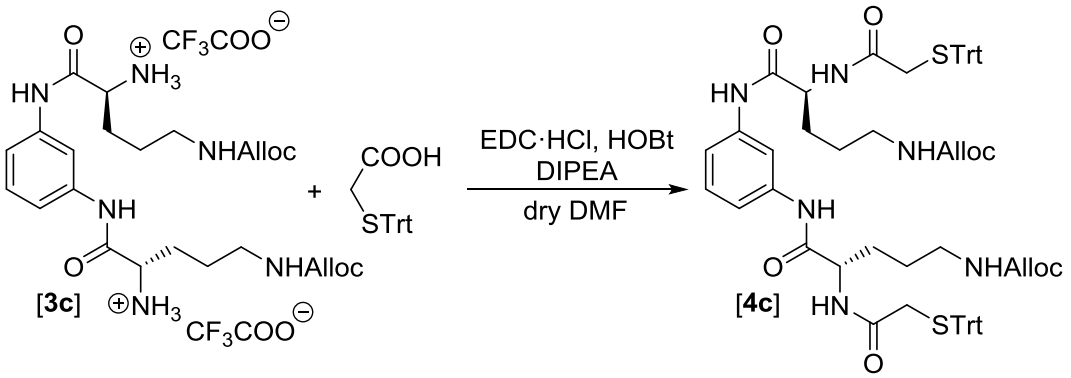
Step ii: Experimental procedure for the synthesis of [**3c**]

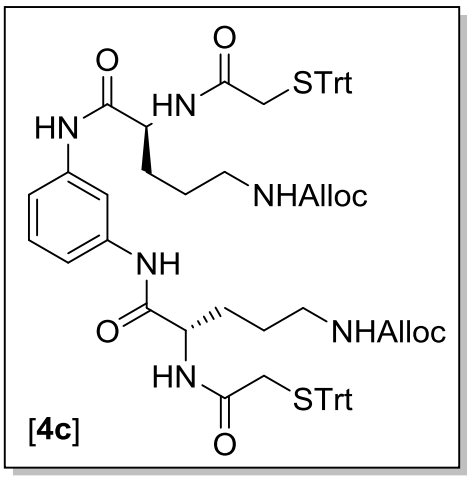




To a solution of [**2c**] (1.30 g, 1.84 mmol) in DCM (30 mL), 5.0 mL of trifluoroacetic acid (TFA) were added. The mixture was allowed to react for 4 hours and then concentrated under reduced pressure. Diethyl ether was added over the residue and the product was filtered and washed with diethyl ether. 1.27 g of [**3c**] were obtained as a white solid (94% yield). HRMS (ESI+) calcd. for $C_{24}H_{36}N_6O_6$ $[M+H]^+$ (m/z): 505.2769, found: 505.2786. ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.08 (t, J = 1.8 Hz, 1H, CH_{Ar}), 7.43–7.26 (m, 3H, CH_{Ar}), 5.90 (ddt, J = 17.3, 10.6, 5.4 Hz, 2H, $NHCOOCH_2C\underline{H}CH_2$), 5.27 (dd, J = 17.3, 1.7 Hz, 2H, $NHCOOCH_2CHC\underline{H}_2$), 5.15 (dd, J = 10.3, 1.0 Hz, 2H, $NHCOOCH_2CHC\underline{H}_2$), 4.52 (dt, J = 5.4, 1.5 Hz, 4H, $NHCOOC\underline{H}_2CHCH_2$), 4.02 (t, J = 6.5 Hz, 2H, C*H), 3.18 (td, J = 6.8, 1.7 Hz, 4H, $C\underline{H}_2NHAlloc$), 2.07–1.83 (m, 4H, $C\underline{H}_2C$*H), 1.74–1.55 (m, 4H, $C\underline{H}_2CH_2C$*H). ^{13}C NMR (101 MHz, MeOD-d_4): δ 168.5 (CO), 159.0 (CO), 139.7 (2 x C_{Ar}), 134.4 (2 x $NHCOOCH_2\underline{C}HCH_2$), 130.5 (1 x CH_{Ar}), 117.5 (2 x $NHCOOCH_2CH\underline{C}H_2$), 117.3 (2 x CH_{Ar}), 112.9 (1 x CH_{Ar}), 66.4 (2 x $NHCOO\underline{C}H_2CHCH_2$), 54.8 (2 x C*H), 40.8 (2 x $\underline{C}H_2NHAlloc$), 30.1 (2 x $\underline{C}H_2C$*H), 26.6 (2 x $\underline{C}H_2CH_2C$*H).

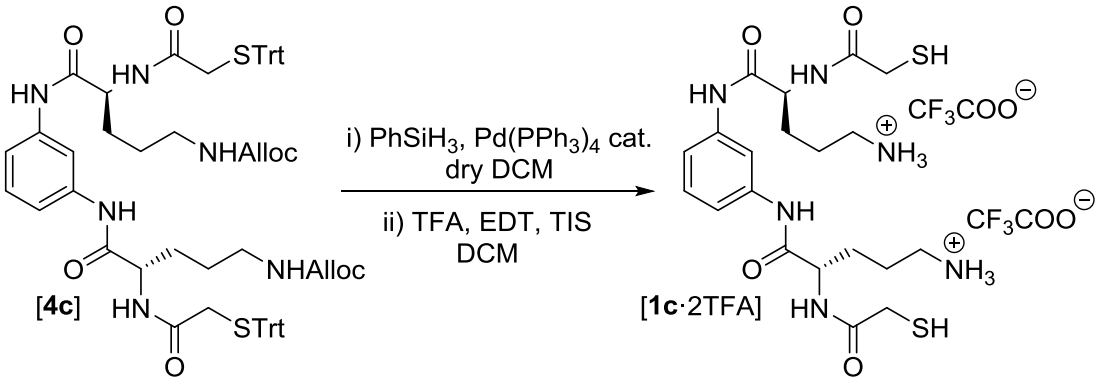
Step iii: Experimental procedure for the synthesis of [**4c**]

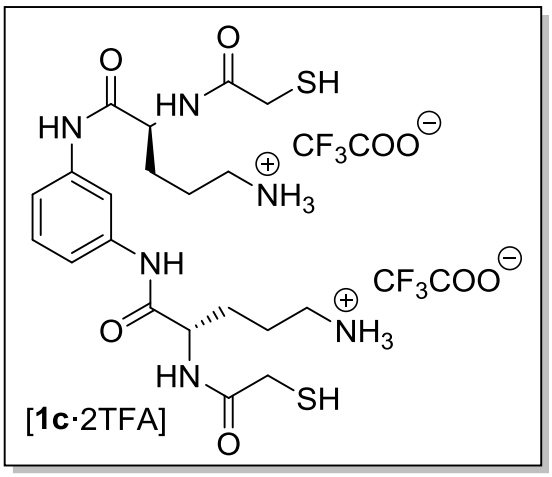




Tritylsulfanyl acetic acid (1.71 g, 5.12 mmol) was dissolved in dry DMF (13 mL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 1.01 mg, 5.29 mmol), HOBt (807 mg, 5.97 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 3.50 mL, 20.1 mmol) were added over the solution. The reaction mixture was cooled to 0ºC and then, [**3c**] (1.25 g, 1.71 mmol) was added over the mixture. The solution was allowed to stir at room temperature for 5 days, and the formation of the product was followed by TLC (Rf AcOEt/Hexane, 4:1 (v:v): 0.51). The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried under reduced pressure. The residue was purified by flash chromatography using hexane: AcOEt as eluent (from 50% to 75% AcOEt) to give 281 mg of [**4c**] (15% yield) as a white solid. HRMS (ESI+) calcd. for $C_{66}H_{68}N_6O_8S_2$ $[M+Na]^+$ (m/z): 1159.4432, found: 1159.4454. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (s, 2H, NHCOC*H), 7.74 (s, 1H, CH_{Ar}), 7.42–7.35 (m, 12H, CH_{Ar}), 7.33–7.23 (m, 15H, CH_{Ar}), 7.22–7.14 (m, 6H, CH_{Ar}), 6.79 (d, *J* = 6.1 Hz, 2H, CONHC*H), 5.86 (ddt, *J* = 16.3, 10.7, 5.5 Hz, 2H, $NHCOOCH_2CHCH_2$), 5.25 (dd, *J* = 17.2, 1.6 Hz, 2H, $NHCOOCH_2CHCH_2$), 5.14 (dd, *J* = 10.6, 1.4 Hz, 2H, $NHCOOCH_2CHCH_2$), 5.02 (s, 2H, $NHCOOCH_2CHCH_2$), 4.56 (d, *J* = 5.2 Hz, 4H, $NHCOOCH_2CHCH_2$), 4.52 (s, 2H, C*H), 3.41 (s, 2H, CH_2NHAlloc), 3.21–2.96 (m, 6H: 2H, CH_2NHAlloc + 4H, CH_2STrt), 1.89–1.66 (m, 2H, CH_2C*H), 1.58–1.35 (m, 6H: 2H, CH_2C*H + 4H, CH_2CH_2C*H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9 (CO), 169.1 (CO), 157.1 (CO), 144.1 (6 x C_{Ar}), 138.5 (2 x C_{Ar}), 133.0 (2 x $NHCOOCH_2CHCH_2$), 129.7 (12 x CH_{Ar}), 129.4 (1 x CH_{Ar}), 128.3 (12 x CH_{Ar}), 127.2 (6 x CH_{Ar}), 117.7 (2 x $NHCOOCH_2CHCH_2$), 115.6 (2 x CH_{Ar}), 111.0 (1 x CH_{Ar}), 68.0 (2 x C), 65.8 (2 x $NHCOOCH_2CHCH_2$), 52.9 (2 x C*H), 39.7 (2 x CH_2NHAlloc), 36.3 (2 x CH_2STrt), 30.0 (2 x CH_2C*H), 26.4 (2 x CH_2CH_2C*H).

Step iv: Experimental procedure for the synthesis of [**1c**·2TFA]





To a solution of [**4c**] (133 mg, 0.117 mmol) in dry DCM (3 mL), $PhSiH_3$ (345 µL, 2.80 mmol) was added under Ar. Then a solution of $Pd(PPh_3)_4$ (18 mg, 0.015 mmol) in dry DCM (2 mL) was also added under Ar. The mixture was allowed to stir at room temperature for 1 hour, after which complete conversion of the starting material was observed by TLC. The crude mixture was filtered through a bed of Celite® and the filtrate was concentrated to dryness under reduced pressure. The resulting residue was redissolved in DCM (1 mL) and 4.5 mL of TFA, 242 µL of triisobutylsilane (TIS, 0.933 mmol) and 117 µL of 1,2-ethanedithiol (EDT, 1.40 mmol) were added rapidly and under stirring. The reaction mixture was allowed to stir at room temperature for 1 hour, 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 and washed with diethyl ether. The product was purified using reversed-phase flash chromatography (gradient: from 5% to 15% CH_3CN in H_2O) and 46.9 mg of [**1c**·2TFA] were obtained as a white solid (56% yield). HRMS (ESI+) calcd. for $C_{20}H_{32}N_6O_4S_2$ $[M+H]^+$ (m/z): 485.1999, found: 485.2007. ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.02–7.97 (m, 1H, CH_{Ar}), 7.34–7.18 (m, 3H, CH_{Ar}), 4.54 (dd, J = 8.0, 5.5 Hz, 2H, C*H), 3.25 (s, 4H, CH_2SH), 3.07–2.90 (m, 4H, $CH_2NH_3^+$), 2.05–1.89 (m, 2H, CH_2C*H), 1.88–1.68 (m, 6H: 2H, CH_2C*H + 4H, CH_2CH_2C*H). ^{13}C NMR (101 MHz, MeOD-d_4): δ 173.51 (2 x $COCH_2$), 171.60 (2 x COC*H), 139.86 (2 x C_{Ar}), 130.20 (1 x CH_{Ar}), 117.45 (2 x CH_{Ar}), 113.53 (1 x CH_{Ar}), 54.78 (C*H), 40.30 ($CH_2NH_3^+$), 30.39 (CH_2C*H), 28.10 (CH_2SH), 24.99 (CH_2CH_2C*H).

NMR spectra, HRMS (ESI+) spectrum and HPLC trace of [**1c**]

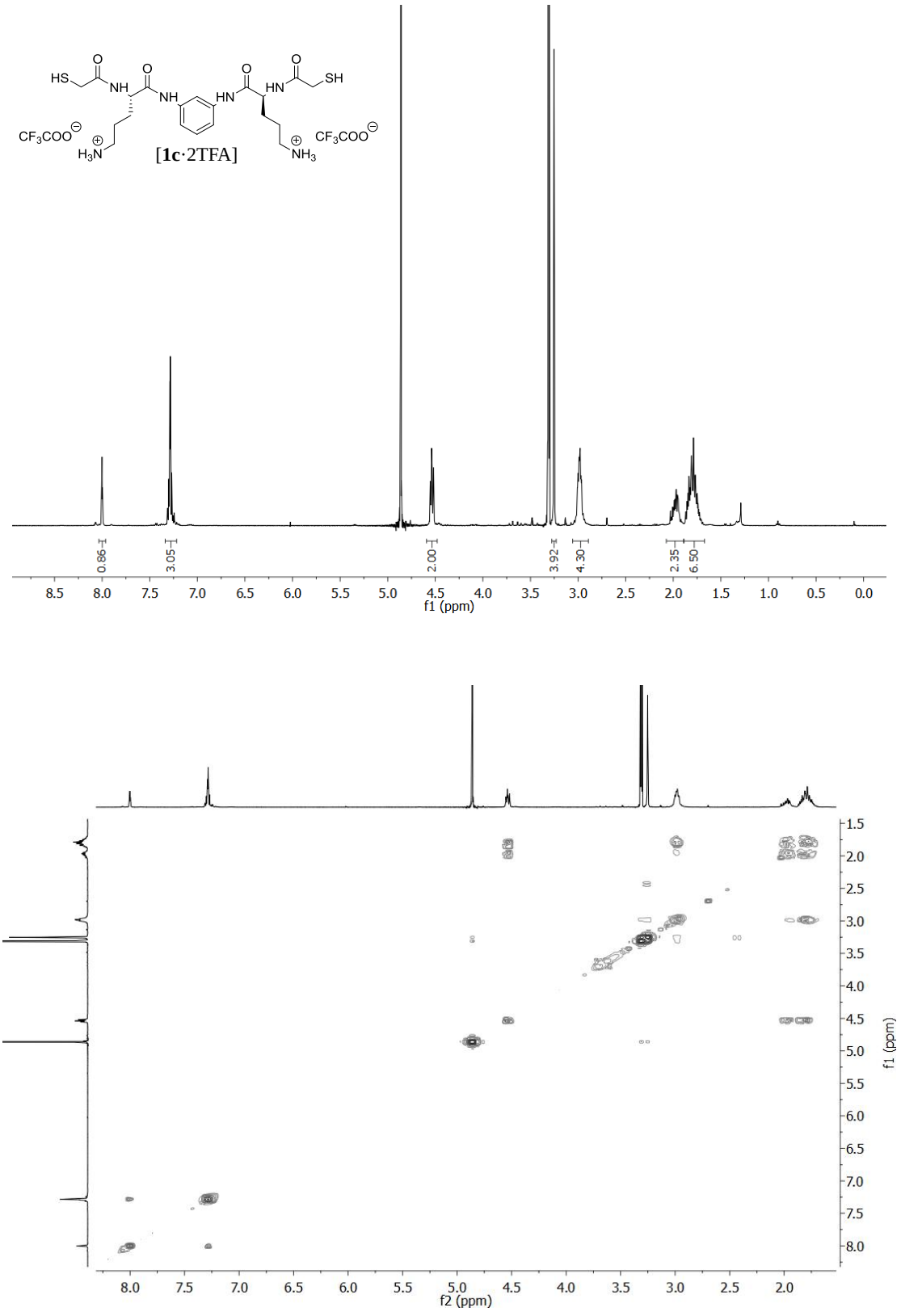


Figure S1: ^{1}H (400 MHz, 298 K in MeOD-d_4) and gCOSY (400 MHz, 298 K in MeOD-d_4) spectra of [**1c**·2TFA].

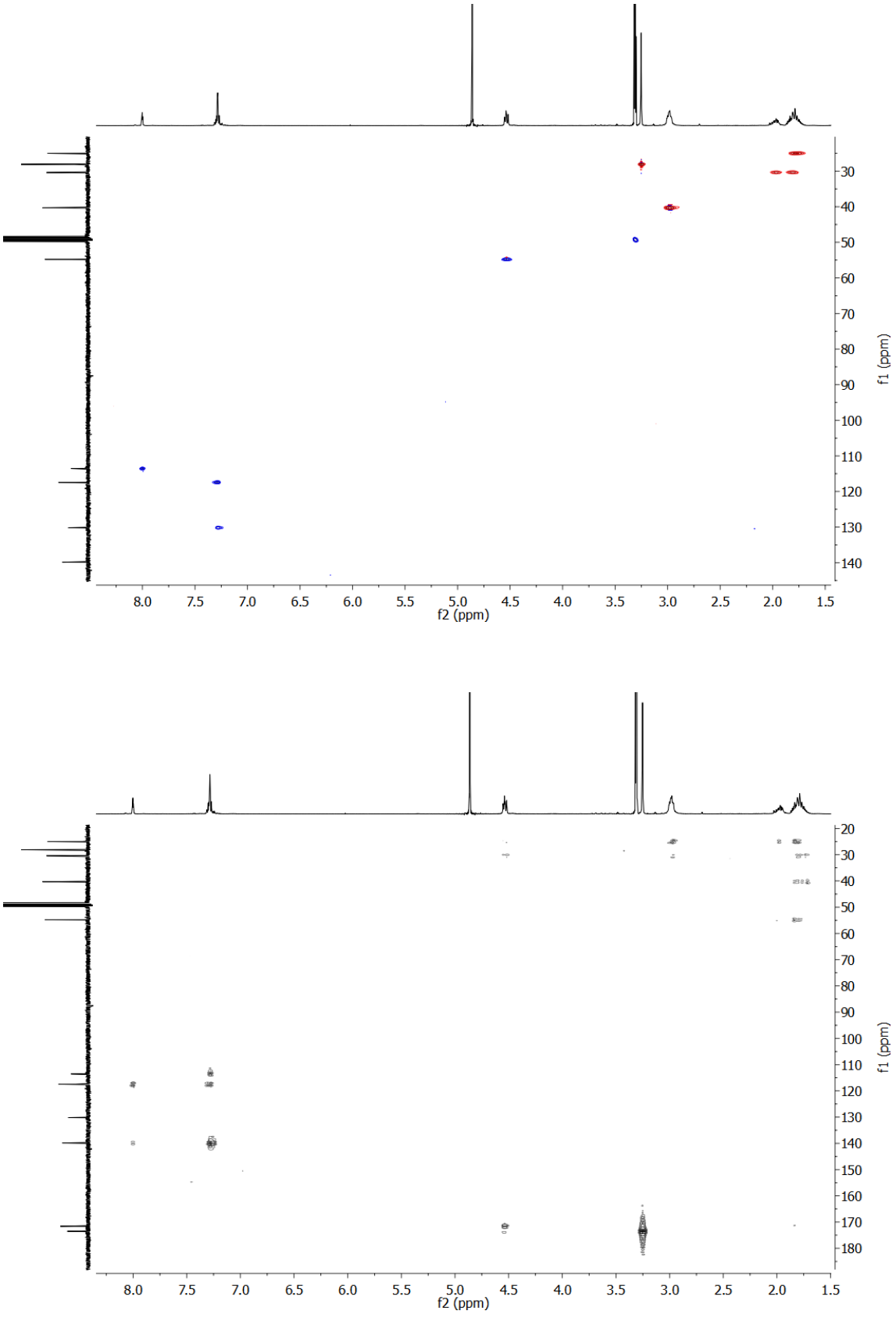


Figure S2: gHSQC (400 MHz, 298 K in MeOD-d_4) and gHMBC (400 MHz, 298 K in MeOD-d_4) spectra of [**1c**·2TFA].

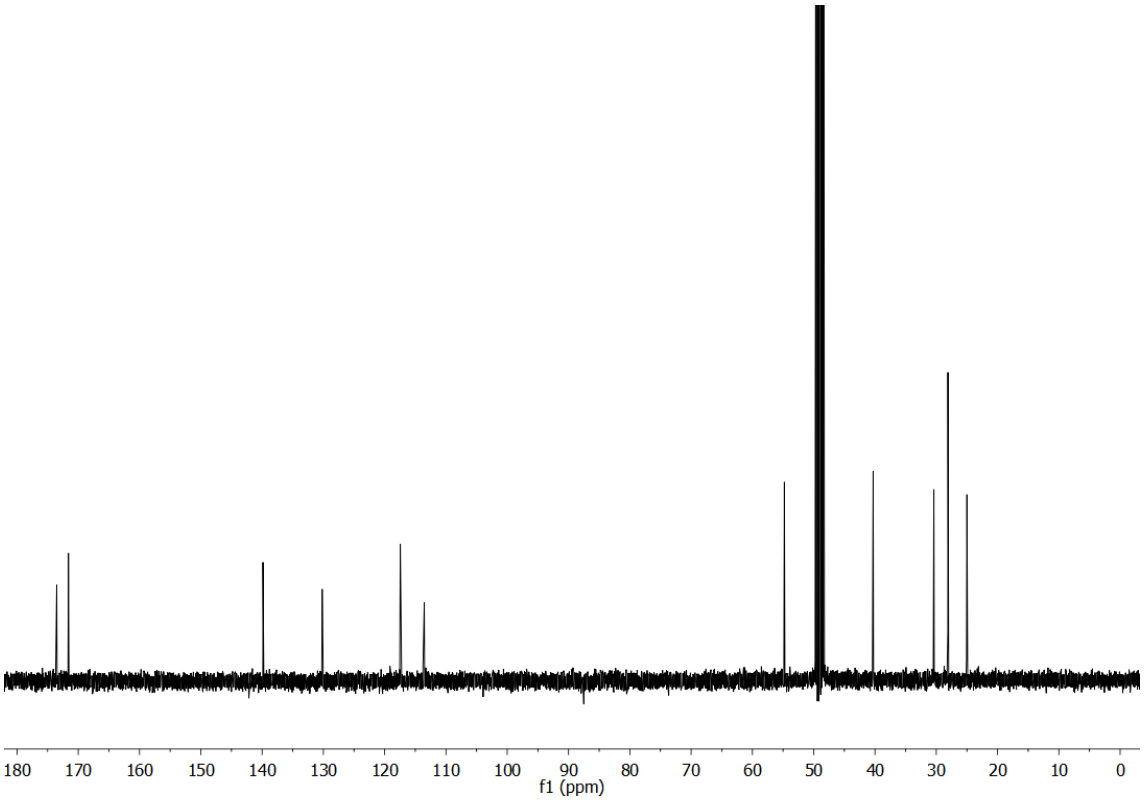


Figure S3: ^{13}C (101 MHz, 298 K in MeOD-d_4) spectra of [**1c**·2TFA].

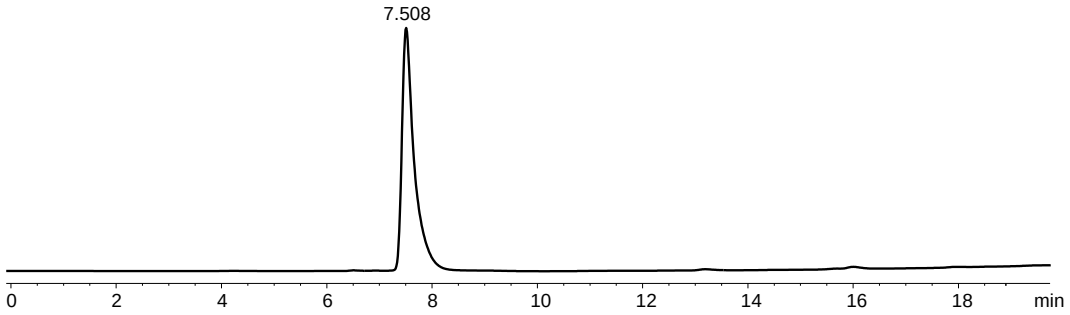


Figure S4: HPLC analysis of [**1c**] (2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 100% CH_3CN over 18 min).

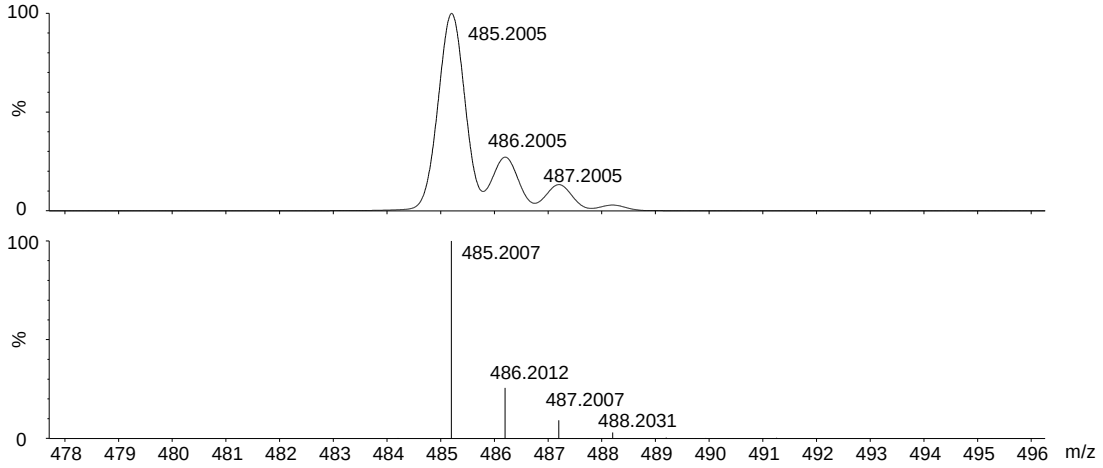


Figure S5: experimental (lower trace) and simulated (upper trace) ESI-TOF mass spectra for $[M+H]^+$ of [**1c**].

PREPARATION OF THE BUFFERS AND pH CONSIDERATIONS

The McIlvaine phosphate-citrate buffer system was used to cover the pH range from 2.5 to 7.5. In order to fix the ionic strength at 0.5 M, different amounts of sodium chloride were added as an inert salt, as previously described.[3] For the pH values of 6.5 and 7.5 no citrate was used in order to avoid precipitation problems. In these two cases the "Buffer Maker" software was used to calculate the needed amounts of the phosphate salts and sodium chloride.

A volume percentages of 10% and 25% DMSO were added to the different buffered solutions and, after the resulting mixtures were left to cool down to room temperature, their pH was measured (see table S1).

Measured pH of the buffer	Measured pH of the buffer with 10% DMSO	Measured pH of the buffer with 25% DMSO
2.50	2.75	3.23
3.49	3.73	4.19
4.53	4.77	5.22
5.53	5.80	6.31
6.52	6.85	7.46
7.66	7.98	8.61

Table S1: pH values of the buffer solutions.

According to a previous study,[4] the "real pH value" of a solution containing a certain percentage of DMSO should be lower than the value measured with a glass electrode system. This difference is a result of the change in the standard potential of the electrode and not the activity of H^+. For the percentages used herein, this difference is small.

Related references:

3. P. J. Elving, J. M. Markowitz and I. Rosenthal, *Anal. Chem.*, 1956, **28**, 1179.
4. P. Mukerjee and J. D. Ostrow, *Tetrahedron Lett.*, 1998, **39**, 423.

KINETIC STUDIES

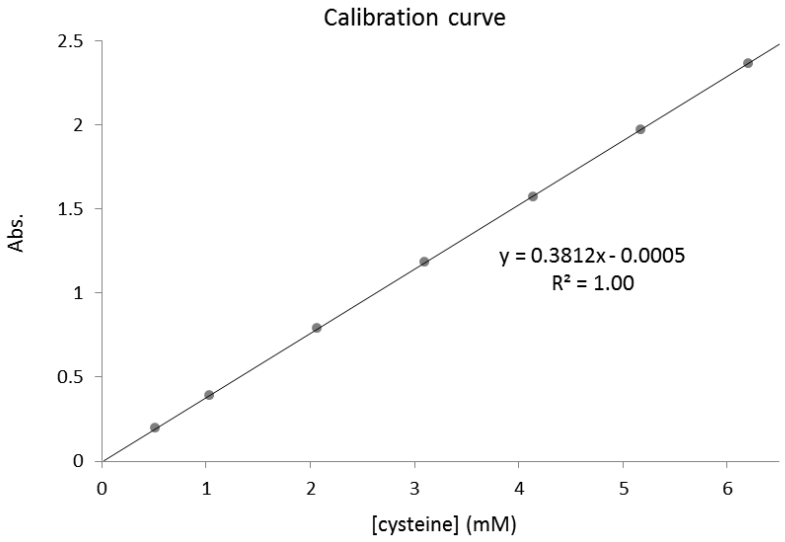
General procedure for the quantification of the free thiols

An 0.80 mM solution of 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) was prepared by dissolving 8.0 mg in 25 mL of a 0.1 M phosphate buffer (pH 8.0). Then 200 µL of this freshly prepared solution were placed in a well of a 96-well plate. Finally, 10 µL of the sample were also added in the well and, after 2 min of incubation at room temperature, the absorbance of the well was measured at 412 nm. The microplate reader was set to shake the samples for 5 seconds before each measurement.

For all the batches and reaction times, the absorbance of a blank was also measured. The blanks were prepared by adding 10 µL of milli-Q water containing the same DMSO percentage as the corresponding sample, to 200 µL of the Ellman's reagent solution. The net absorbance was calculated by subtracting the absorbance of the corresponding blank. All the studied oxidations were carried out at room temperature, in capped vials and without any stirring.

Calibration curve

A 6.205 mM stock solution of cysteine was prepared by dissolving 37.59 mg in 50 mL of milli-Q water. From this, the rest of the stocks (5.171, 4.137, 3.103, 2.068 and 1.034 mM) were prepared by dilution with more milli-Q water. The net absorbance of each of the freshly prepared stocks was represented in front of the concentration and the regression line was obtained by using the linear least square method (see graphic S1). The data showed good linear behaviour within the range of working concentrations demonstrating the reliability of the quantification method.



Graphic S1: linear least square calibration curve.

General procedure for the preparation of the oxidation samples

The following general procedure was used for the preparation of the oxidation samples containing 10% and 25% DMSO.

Individual stocks of the BBs [**1a-c**] (*concentration 1*) were prepared in DMSO. From these, the 2 mM oxidation samples were prepared by adding *volume 1* to *volume 2* of a buffer solution (pH 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 separately). This addition was set as the starting time of the oxidation process.

% DMSO	*Conc. 1*	*Vol. 1*	*Vol. 2*
10	20	30	270
25	8	75	225

Table S2: concentrations (mM) and volumes (μL) of the general procedure for the preparation of the oxidation samples.

For the oxidation samples containing 0% DMSO, 20 μL of a 20 mM **individual stock** prepared in milli-Q water were added to 180 μL of the corresponding buffer solution. For the oxidation samples containing 2% DMSO, 20 μL of a 20 mM **individual stock** prepared in milli-Q water were added to the mixture of 176 μL of the corresponding buffer solution with 4 μL of DMSO. For the oxidation samples containing 100% DMSO, 50 μL of an 8 mM **individual stock** prepared in DMSO were added to 150 μL of DMSO.

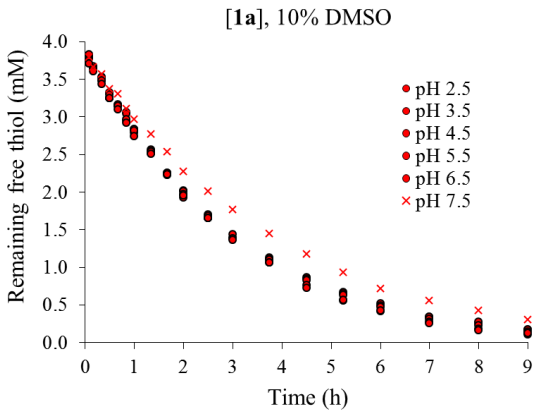
Processing of the kinetic data

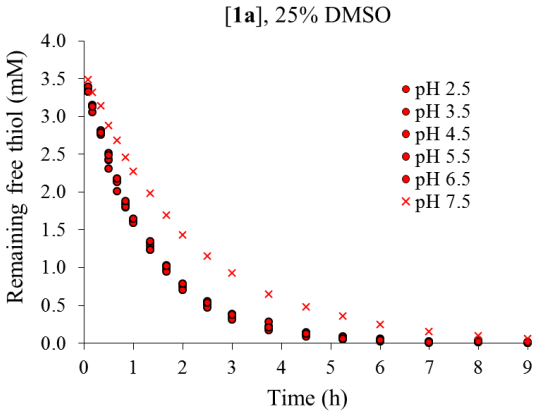
For each oxidation time, the real remaining free thiol concentration was calculated by means of the calibration curve. The points corresponding to the first hour of oxidation (the 2 first hours for the samples with 2% DMSO and the 6 first hours for the samples with 0% and 100% DMSO) were used to adjust a regression line (linear least square method). The slope of the line was taken as minus the initial rate.

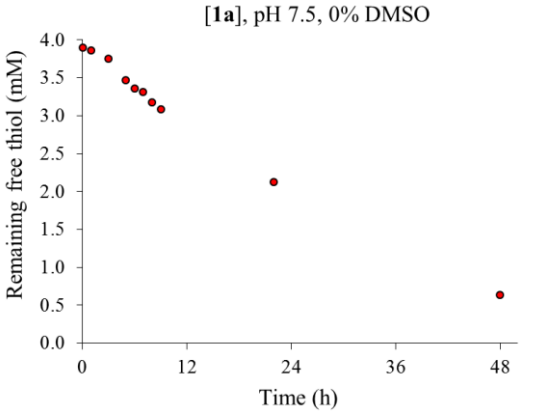
For the calculation of the half-life time, the equation of the straight line containing the two closest points to the 50% concentration (the one just above and the one just below) was used. The time value that fulfilled the equation for the value of 50% was taken as the half-life time.

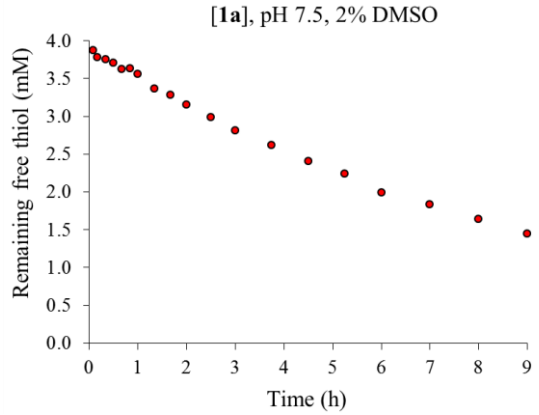
All the experiments were performed at least twice, observing no significant differences within the experimental error.

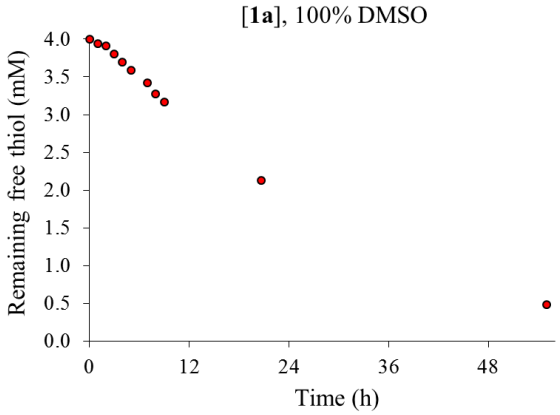
Oxidation process of [**1a**] at different pHs and DMSO percentages



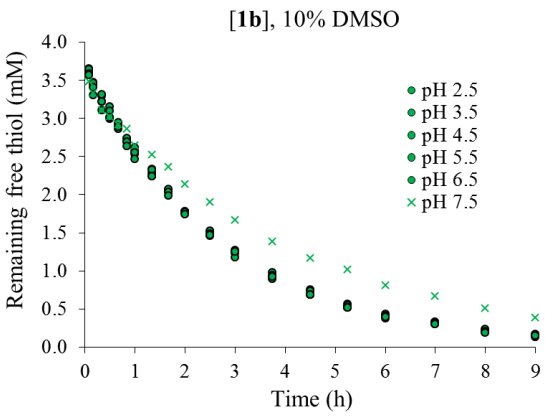


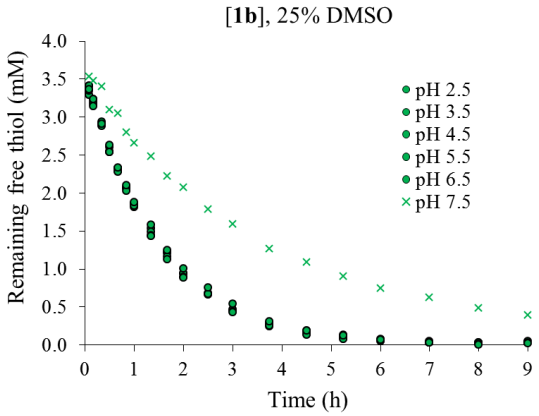


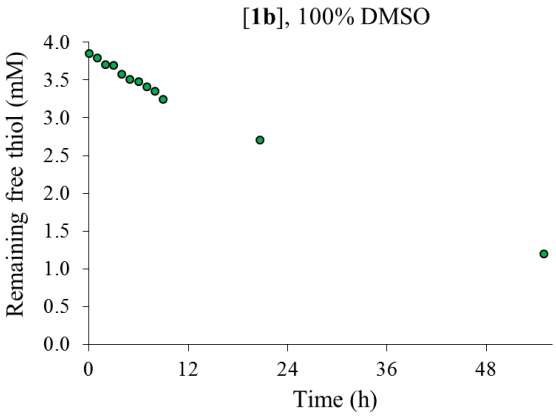




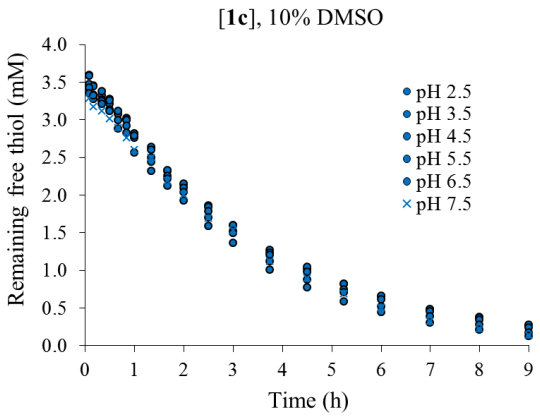
Oxidation process of [**1b**] at different pHs and DMSO percentages

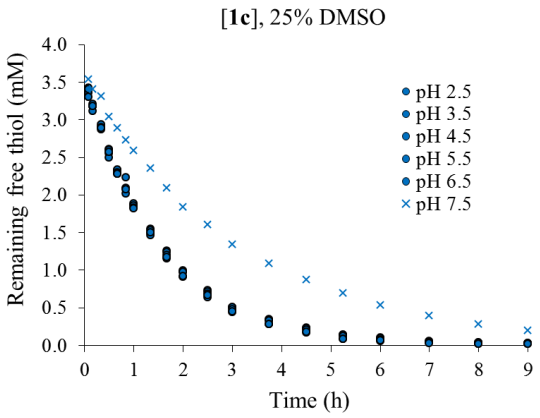


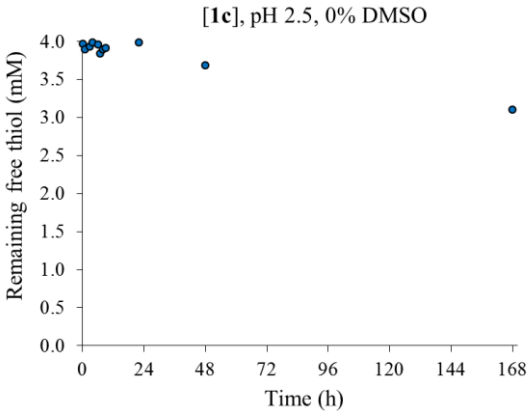


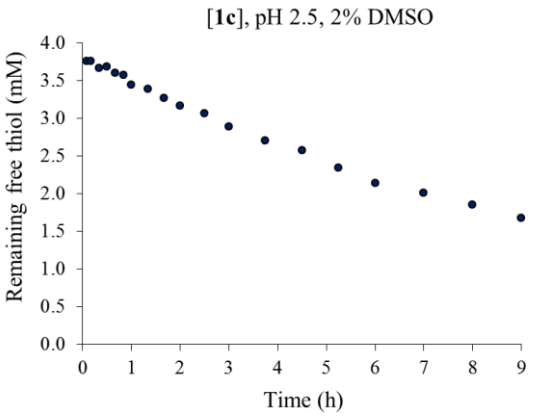


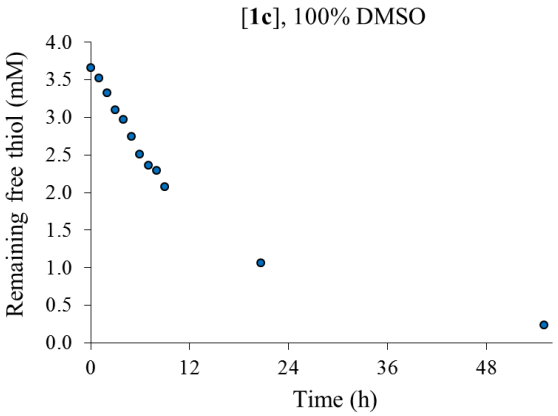
Oxidation process of [**1c**] at different pHs and DMSO percentages



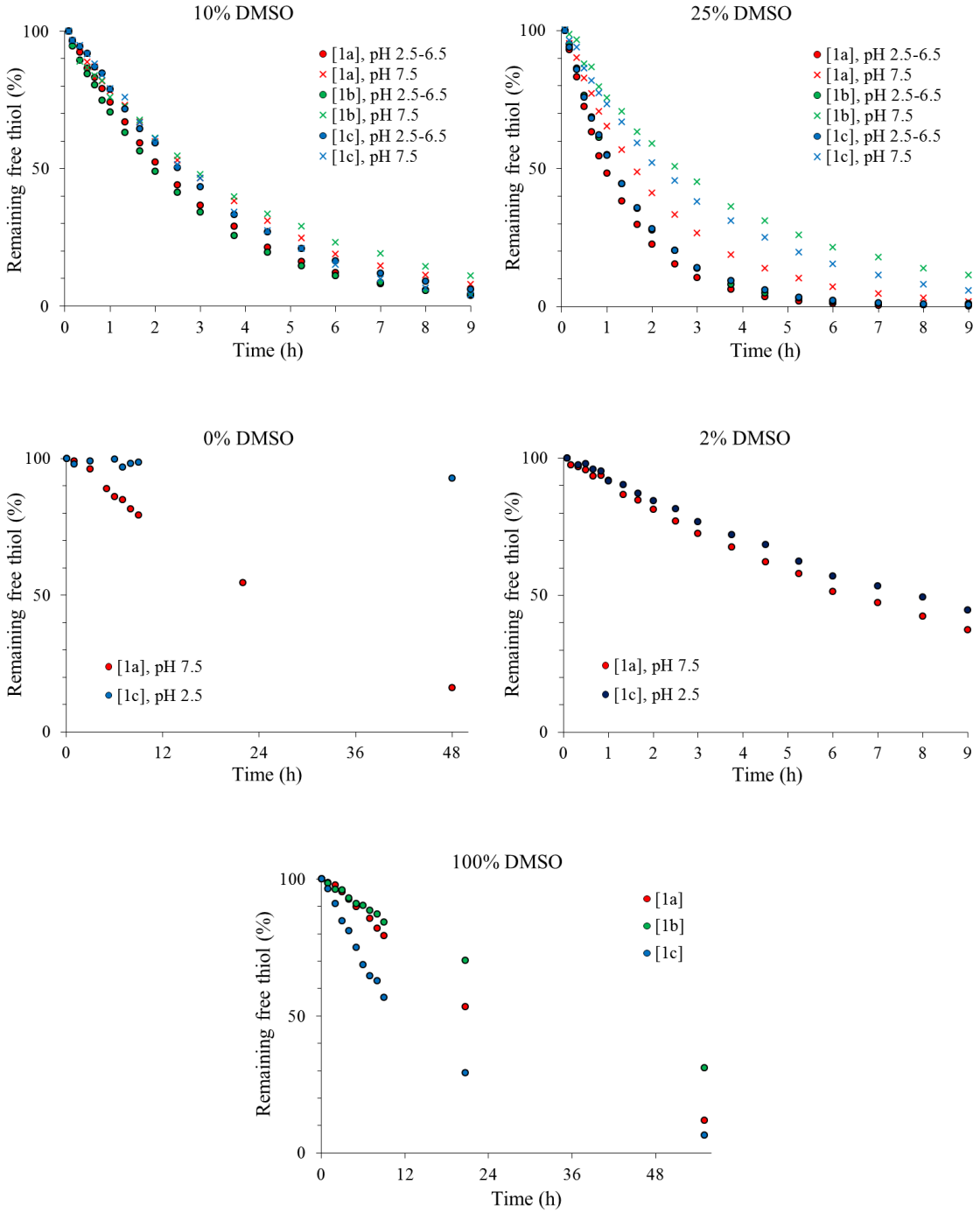








Comparison of the oxidation processes of [**1a-c**]

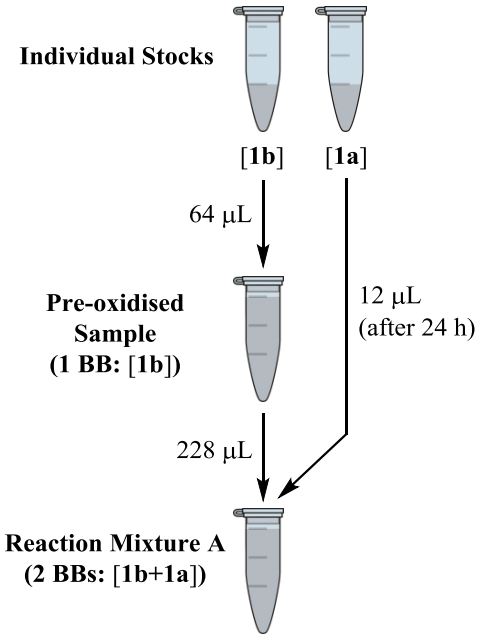


Evaluation of the exchange rates

The following procedure was used for the preparation of the exchange evaluation tests.

A 40 mM **individual stock** of [**1b**] was prepared in DMSO (see scheme S1). From this, a **pre-oxidised sample** was prepared by adding 64 µL of the **individual stock** to 240 µL of a buffer solution (pH 2.5, 4.5 and 6.5 separately).

After 24 hours the **pre-oxidised sample** was analysed by HPLC (eluent: 2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 33% CH_3CN over 28 min) in order to confirm the total oxidation (see figures S6-8). Then a 10 mM **individual stock** of [**1a**] was also prepared in DMSO. From this, a 2 mM of each BB mixture, the **reaction mixture A** (25% DMSO), was prepared by adding 12 µL of the **individual stock** of [**1a**] to 228 µL of the **pre-oxidised sample**. This addition was set as the starting time. At the times of <5 min, 40 min and >24 h the **reaction mixture A** was analysed by HPLC (same eluent as before).



Scheme S1: preparation of the mixtures for the evaluation of the exchange rate.

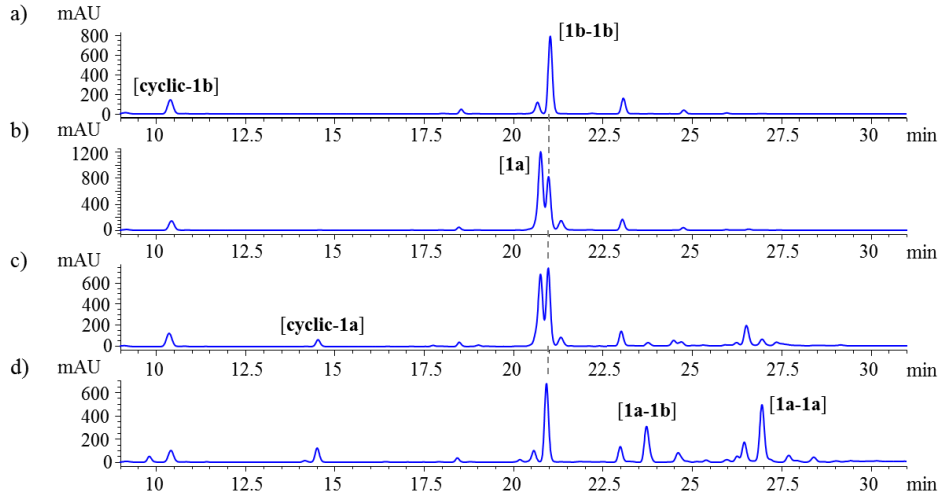


Figure S6: HPLC traces of the exchange evaluation test at pH 2.5, before the addition of [**1a**] (a), after <5 min (b), after 40 min (c), and after > 24 h (d).

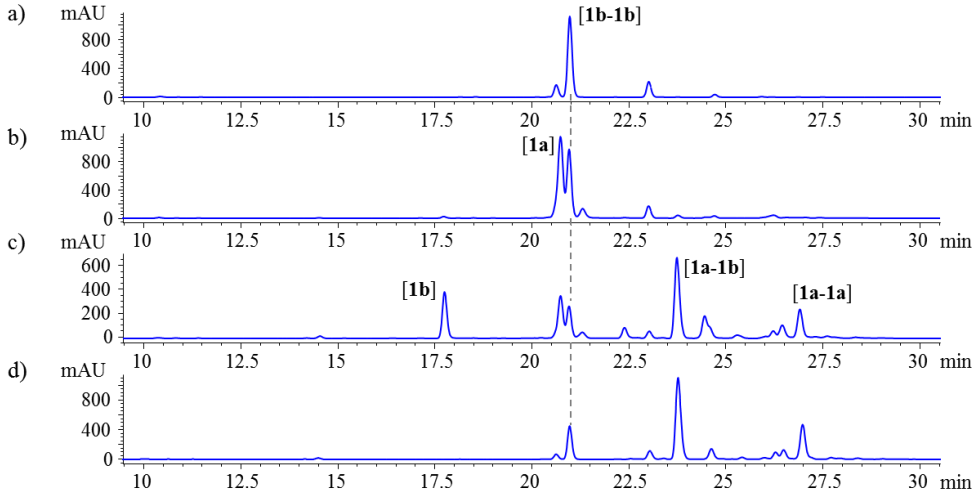


Figure S7: HPLC traces of the exchange evaluation test at pH 4.5, before the addition of [**1a**] (a), after <5 min (b), after 40 min (c), and after > 24 h (d).

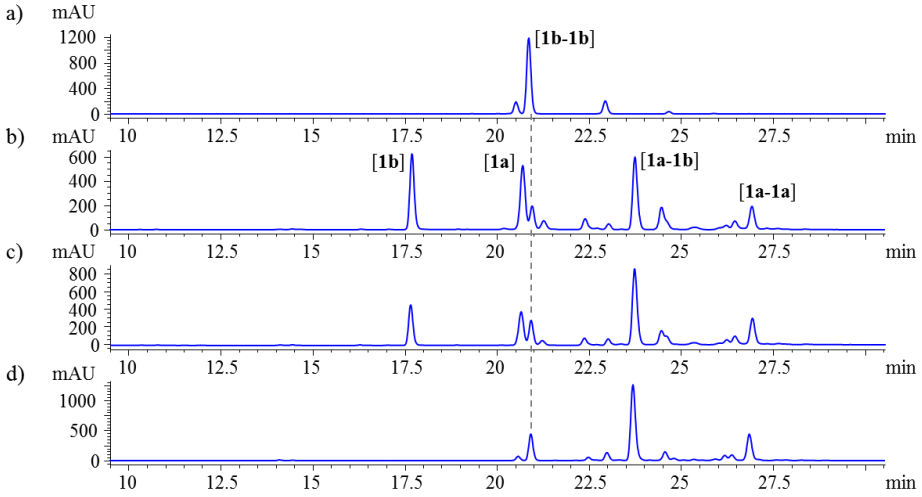


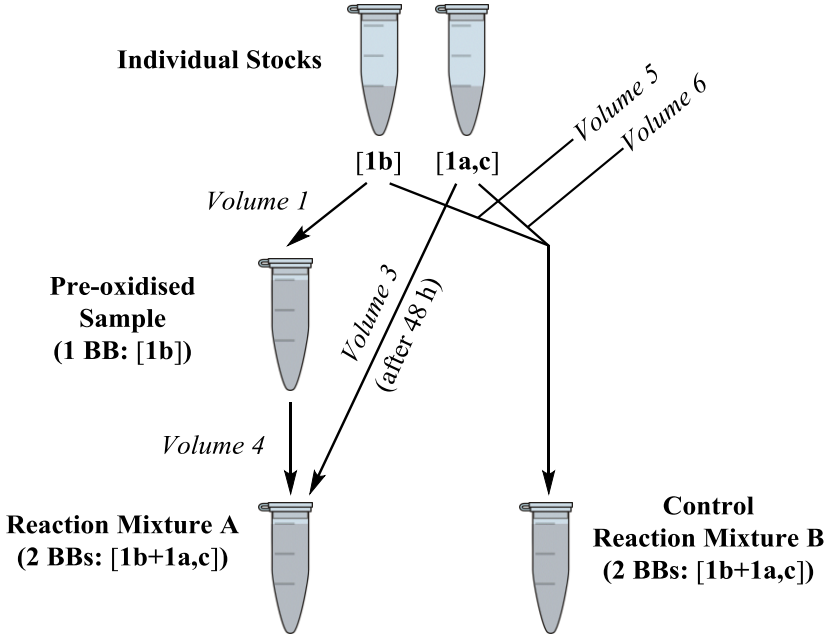
Figure S8: HPLC traces of the exchange evaluation test at pH 6.5, before the addition of [**1a**] (a), after <5 min (b), after 40 min (c), and after > 24 h (d).

REVERSIBILITY TESTS

General procedure for the binary reversibility tests

The following general procedure was used for the preparation of the binary reversibility tests containing 10% and 25% DMSO.

Individual stocks of the two BBs, *concentration 1* of [**1a,c**] and *concentration 2* of [**1b**], were prepared in DMSO (see scheme S1). From these, a **pre-oxidised sample** was prepared by adding *volume 1* of the **individual stock** of [**1b**] to *volume 2* of a buffer solution (pH 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 separately). The **individual stock** of [**1a,c**] was stored at -80 ºC. After 48 hours, a 2 mM of each BB mixture, the **reaction mixture A**, was prepared by adding *volume 3* of the **individual stock** of [**1a,c**] to *volume 4* of the **pre-oxidised sample**. Only for the samples with a final 10% DMSO, 130 µL of the buffer solution (pH 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 separately) were also added to the **mixture A**.



Scheme S2: preparation of the mixtures of the binary reversibility tests.

Simultaneously, a 2 mM of the two BBs mixture, the **control reaction mixtures B**, was prepared by mixing the two **individual stocks**, *volume 5* of [**1b**] and *volume 6* of [**1a,c**], with *volume 7* of a buffer solution (pH 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5 separately). After 48 hours, the **reaction mixture A**, the **control reaction mixture B** and the **pre-oxidised sample** were analysed by HPLC.

% DMSO	*Conc. 1*	*Conc. 2*	*Vol. 1*	*Vol. 2*	*Vol. 3*	*Vol. 4*	*Vol. 5*	*Vol. 6*	*Vol. 7*
10	40	40	15	75	10	60	10	10	180
25	40	10	48	180	10	190	40	10	150

Table S3: concentrations (mM) and volumes (µL) of the general procedure for the binary reversibility tests.

General procedure for the analysis of the mixtures (HPLC and HRMS)

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. Unless otherwise specified, the eluent used for those mixtures not containing the BB [**1c**] was: 2 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 40% CH_3CN over 48 min; and the eluent used for those mixtures containing the BB [**1c**] was: 10 min at 2% CH_3CN in H_2O, then linear gradient from 2% to 40% CH_3CN over 62 min.

The HRMS 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. Eluent used for those mixtures not containing the BB [**1c**]: 2.5 min at 5% CH_3CN in H_2O, then linear gradient from 5% to 50% CH_3CN over 27.5 min. Eluent used for those mixtures containing the BB [**1c**]: 2.5 min at 2% CH_3CN in H_2O, then linear gradient from 2% to 40% CH_3CN over 27.5 min.

Calculation and evaluation of the exchange constants ($K_{[A,B]}$)

For a given mixture of dimers, the exchange constant between two homodimers (AA and BB) and their corresponding heterodimer (AB) (see reaction S1), is defined as shown in equation S1. In the present work, equal extinction coefficients at 254 nm were assumed for all the involved dimers, and thus, the $K_{[A,B]}$ values were directly calculated by means of the corresponding HPLC areas as shown in equation S2.

$$2AB \rightleftharpoons AA + BB \quad \text{(reaction S1)}$$

$$K_{[A,B]} = \frac{[AA]\cdot[BB]}{[AB]^2} \quad \text{(equation S1)} \qquad K_{[A,B]} \simeq \frac{\text{Area}(AA)\cdot\text{Area}(BB)}{\text{Area}(AB)^2} \quad \text{(equation S2)}$$

In order to evaluate if the binary mixtures reached the equilibrium, the difference between the calculated exchange constant for the mixtures A and B (the stepwise and combined additions of the BBs, respectively) was evaluated by means of the relative difference in percentage (Rel. dif. (%), see equation S3), defined as an absolute value. Only for those mixtures reaching the thermodynamic equilibrium, the exchange constant of the mixtures A and B should be the same. Therefore, a zero relative difference (within the experimental error) clearly indicates that the mixture reached the equilibrium, while a higher value indicates that the mixture did not reach the equilibrium.

$$\text{Rel. dif. (\%)} = \left| \frac{\left(K^{A}_{[A,B]} - K^{B}_{[A,B]}\right)}{K^{B}_{[A,B]}} \cdot 100 \right| \quad \text{(equation S3)}$$

Binary mixture of [**1a+1b**] at different pHs and DMSO percentages

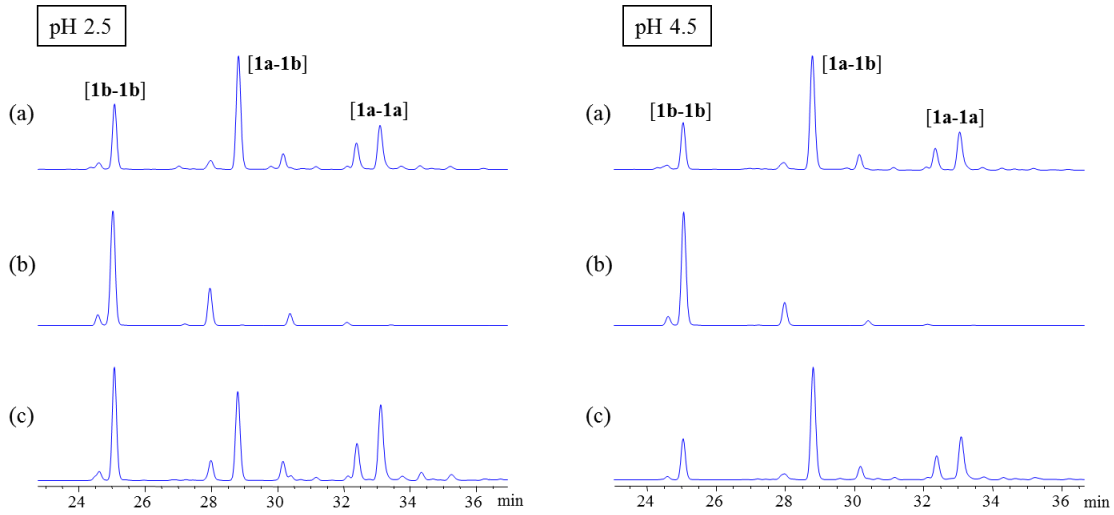


Figure S9: HPLC traces of the control reaction mixture B (a), the pre-oxidised sample (b), and the reaction mixture A (c) of [**1a+1b**] **with 10% DMSO**, for the pHs 2.5 (as an example of non-equilibrium situation) and 4.5 (as an example of equilibrium situation).

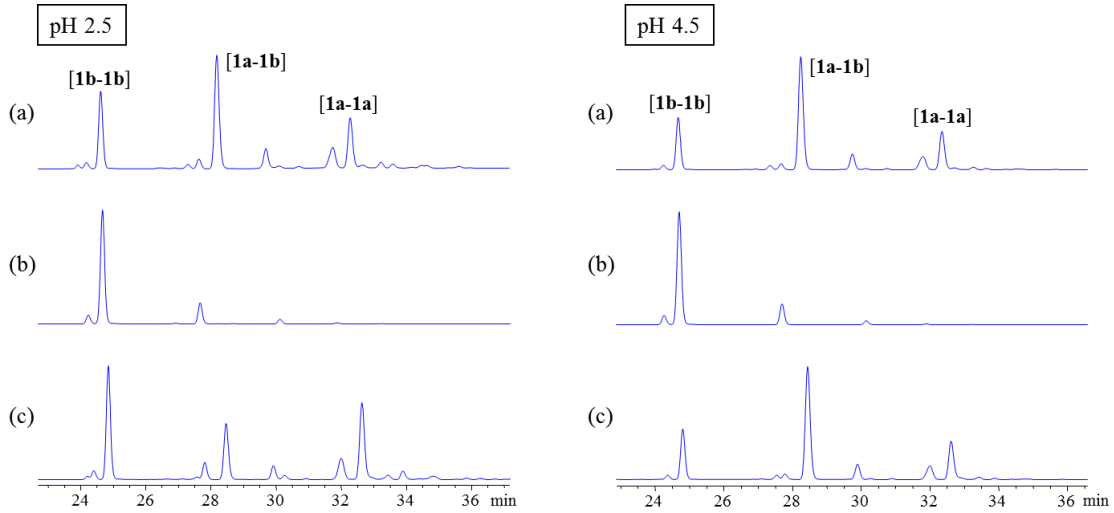


Figure S10: HPLC traces of the control reaction mixture B (a), the pre-oxidised sample (b), and the reaction mixture A (c) of [**1a+1b**] **with 25% DMSO**, for the pHs 2.5 (as an example of non-equilibrium situation) and 4.5 (as an example of equilibrium situation).

Entry	pH	% DMSO	$K^{A}_{[1a,1b]}$	$K^{B}_{[1a,1b]}$	Rel. dif. (%)
1	2.5	10	1.09	0.234	**366**
2	3.5	10	0.213	0.206	**3.4**
3	4.5	10	0.143	0.145	**<2**
4	5.5	10	0.108	0.110	**<2**
5	6.5	10	0.110	0.110	**<2**
6	7.5	10	0.110	0.109	**<2**
7	2.5	25	2.58	0.275	**838**
8	3.5	25	0.288	0.211	**36**
9	4.5	25	0.143	0.141	**<2**
10	5.5	25	0.102	0.101	**<2**
11	6.5	25	0.100	0.100	**<2**
12	7.5	25	0.103	0.103	**<2**

Table S4: values of the exchange constant between [**1a-1a**], [**1b-1b**] and [**1a-1b**] for the reaction mixture A $\left(K^{A}_{[1a,1b]}\right)$ and the control reaction mixture B $\left(K^{B}_{[1a,1b]}\right)$, depending on the pH and the volume percentage of DMSO.

Binary mixture of [**1b+1c**] at different pHs and DMSO percentages

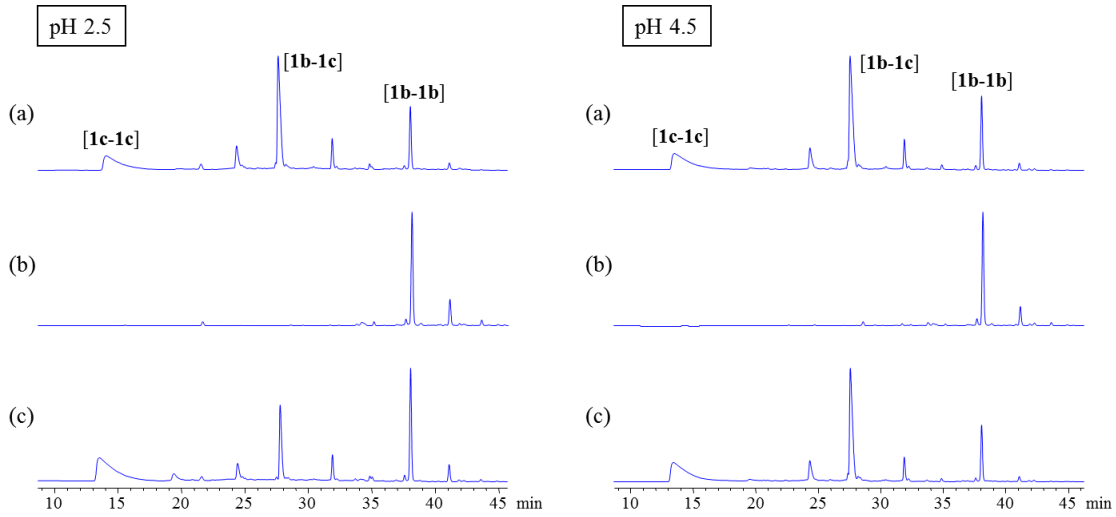


Figure S11: HPLC traces of the control reaction mixture B (a), the pre-oxidised sample (b), and the reaction mixture A (c) of [**1b+1c**] **with 10% DMSO**, for the pHs 2.5 (as an example of non-equilibrium situation) and 4.5 (as an example of equilibrium situation).

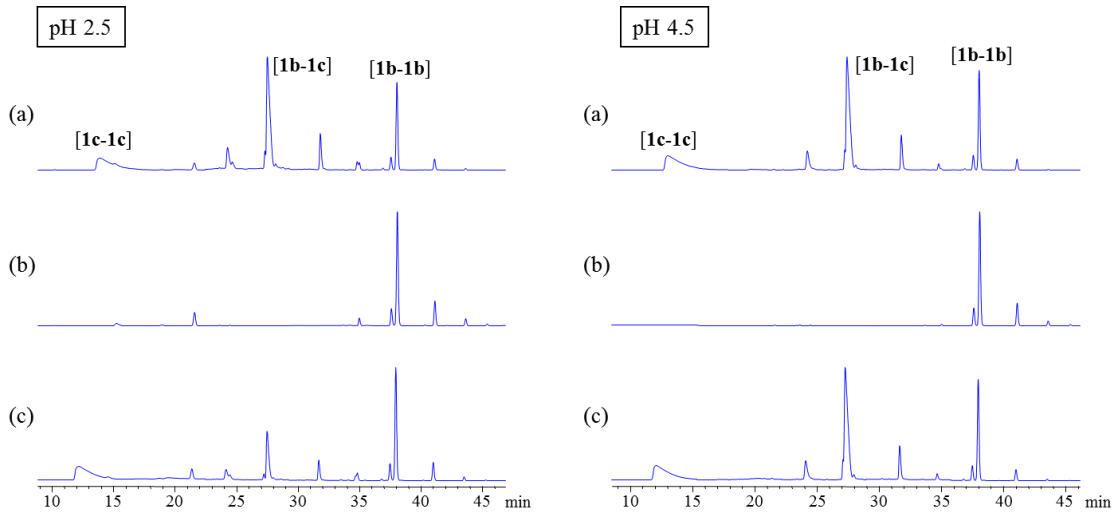


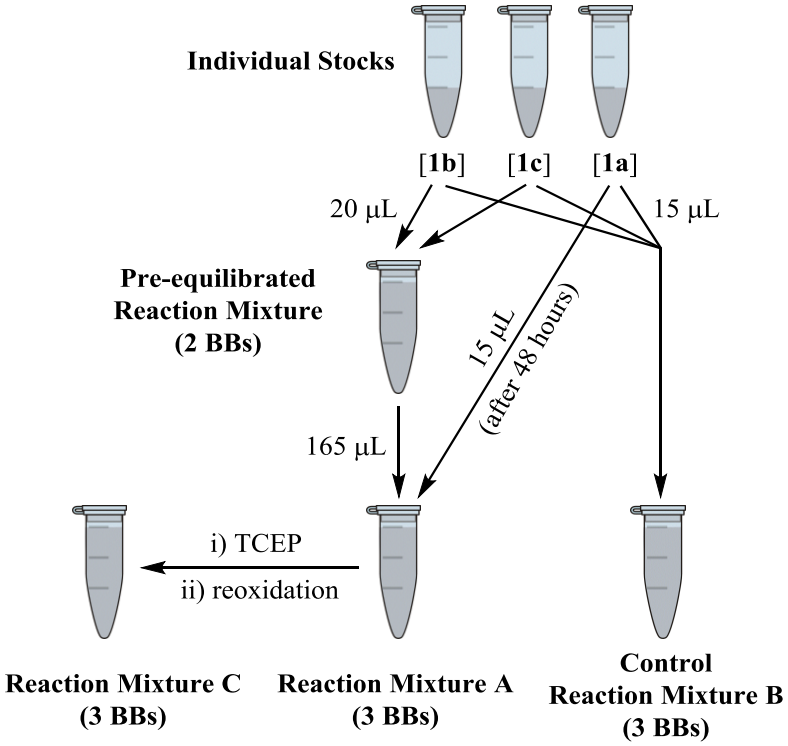
Figure S12: HPLC traces of the control reaction mixture B (a), the pre-oxidised sample (b), and the reaction mixture A (c) of [**1b+1c**] **with 25% DMSO**, for the pHs 2.5 (as an example of non-equilibrium situation) and 4.5 (as an example of equilibrium situation).

Entry	pH	% DMSO	$K^{A}_{[1b,1c]}$	$K^{B}_{[1b,1c]}$	Rel. dif. (%)
1	2.5	10	2.87	0.240	**1096**
2	3.5	10	0.256	0.234	**9.4**
3	4.5	10	0.248	0.248	**<2**
4	5.5	10	0.246	0.247	**<2**
5	6.5	10	0.248	0.250	**<2**
6	7.5	10	0.256	0.253	**<2**
7	2.5	25	2.81	0.234	**1100**
8	3.5	25	0.245	0.235	**4.3**
9	4.5	25	0.237	0.236	**<2**
10	5.5	25	0.256	0.257	**<2**
11	6.5	25	0.264	0.265	**<2**
12	7.5	25	0.269	0.274	**<2**

Table S5: values of the exchange constant between [**1b-1b**], [**1c-1c**] and [**1b-1c**] for the reaction mixture A $\left(K^{A}_{[1b,1c]}\right)$ and the control reaction mixture B $\left(K^{B}_{[1b,1c]}\right)$, depending on the pH and the volume percentage of DMSO.

Ternary mixture of [**1a+1b+1c**] at pH 4.5 with 25% DMSO

24 mM **individual stocks** of each BB [**1a-c**] were prepared in DMSO (see scheme S2). From these, a **pre-equilibrated reaction mixture** was prepared by adding 20 µL of [**1b**] and [**1c**] to 180 µL of a pH 4.5 buffer solution. The **individual stock** of [**1a**] was stored at -80 ºC. After 48 hours, a 2 mM of each BB mixture, the **reaction mixture A**, was prepared by adding 15 µL of the **individual stock** of [**1a**] to 165 µL of the **pre-equilibrated reaction mixture**.



Scheme S3: preparation of the solutions of the ternary reversibility test.

Simultaneously, a 2 mM mixture of each BB, the **control reaction mixture B**, was prepared by mixing 15 µL of each **individual stock** with 135 µL of a pH 4.5 buffer solution. After 48 hours, the **reaction mixture A**, the **control reaction mixture B** and the **pre-equilibrated reaction mixture** were analysed by HPLC (see figure 5 of the manuscript).

Finally, the **reaction mixture C** was prepared by adding 0.35 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl)[5] to the completely oxidised **reaction mixture A**. The substoichiometric amount of TCEP allowed the partial reduction of the disulphides present in the mixture. After the reoxidation of the generated free thiols, 48 hours later, the **reaction mixture C** was analysed by HPLC (see figure S10).

Related references:

5. J. A. Burns, J. C. Butler, J. Moran, and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 2648.

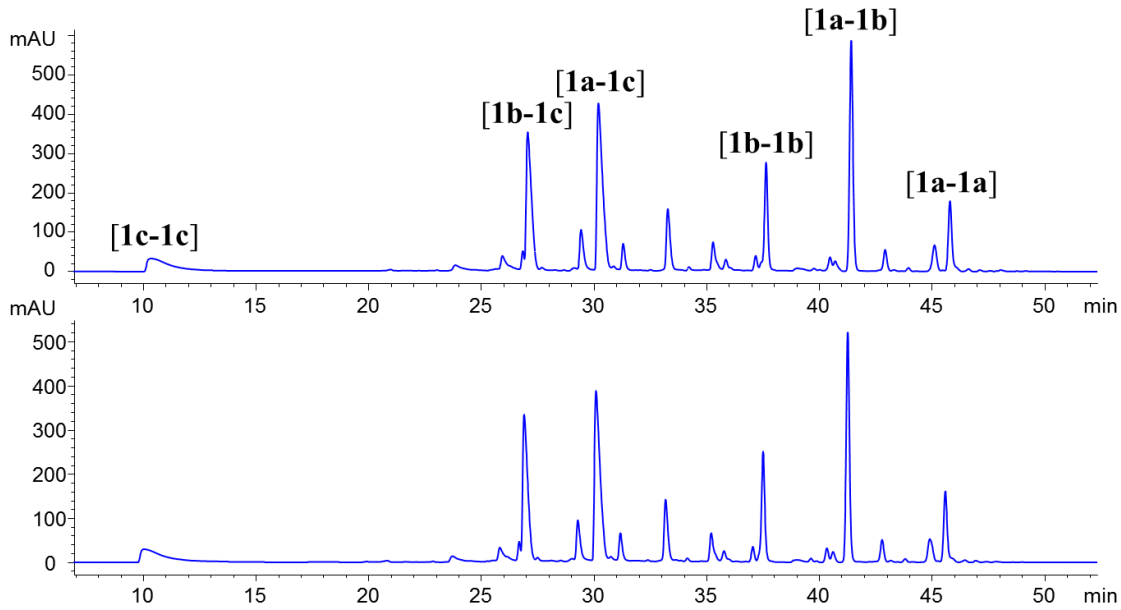


Figure S13: HPLC chromatograms of the reaction mixtures A and C.

After the addition of [**1a**] to the **pre-equilibrated mixture** of [**1b+1c**], the mixture evolved to the same final situation as when the three BBs are left to oxidise together. Moreover, after the partial reduction of the **reaction mixture A** by means of the TCEP, the subsequent reoxidation also led to the same final situation.

These observations were quantitatively verified by means of the $K_{[A,B]}$ constant. No significant changes were observed between the constants calculated for the mixtures A, B and C (see table S4). Therefore, at pH 4.5 and with 25% DMSO, the mixture proved to reach the thermodynamic equilibrium.

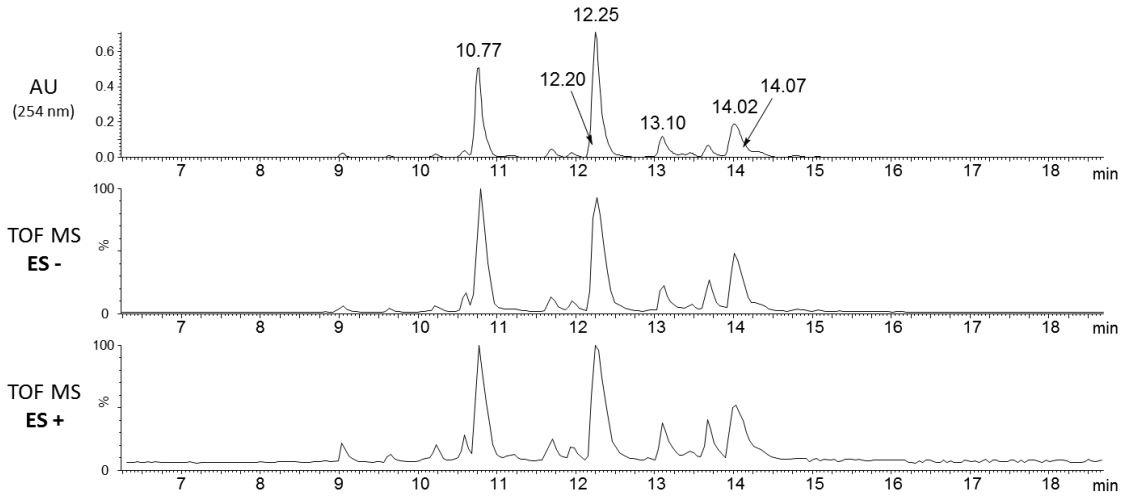
	Mixture A	Mixture B	Mixture C
$K_{[1a,1b]}$	0.141	0.142	0.144
$K_{[1a,1c]}$	0.0911	0.0902	0.0909
$K_{[1b,1c]}$	0.235	0.234	0.232

Table S6: values of the $K_{[A,B]}$ constants calculated for the mixtures A, B and C.

MASS SPECTROMETRY (MS)

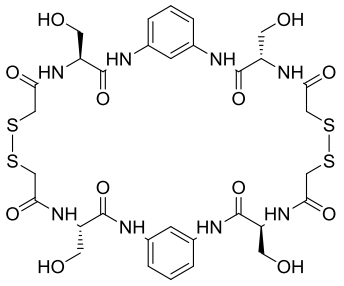
For the different combinations of the BBs [**1a-c**], all the 6 possible dimers, 8 of the ten possible trimers and one tetramer were identified. At acidic pH, the dehydration product was also observed for 3 of the macrocycles bearing the BB [**1a**].

Binary mixture of [**1a+1b**]

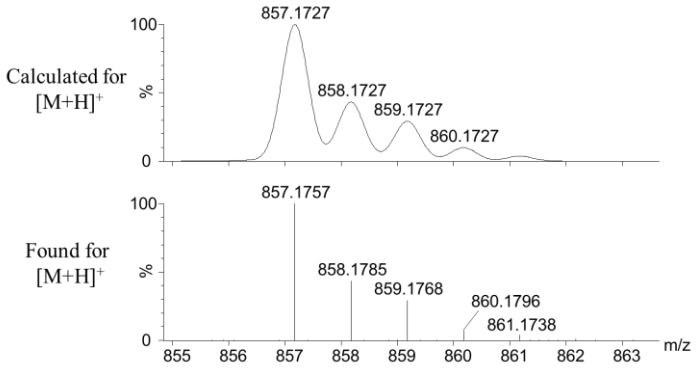


Identification of the dimers:

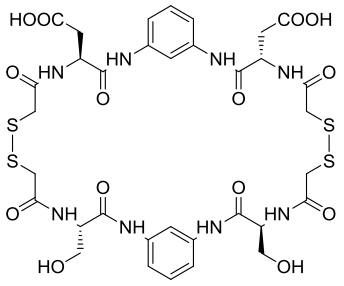
[**1b-1b**], retention time: 10.77 min



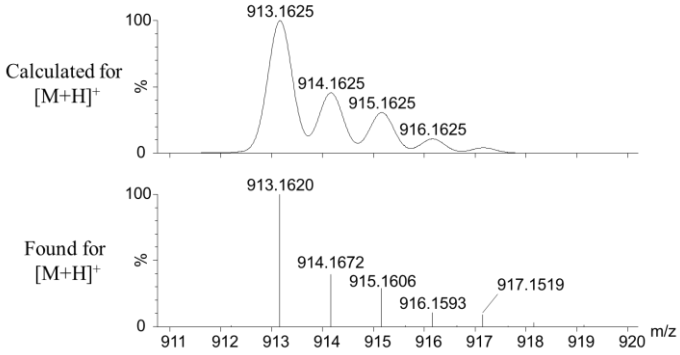
Chemical Formula: $C_{32}H_{40}N_8O_{12}S_4$
Exact Mass: 856.1649



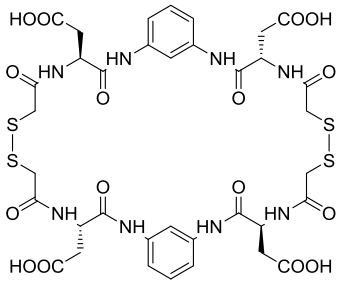
[**1a-1b**], retention time: 12.25 min



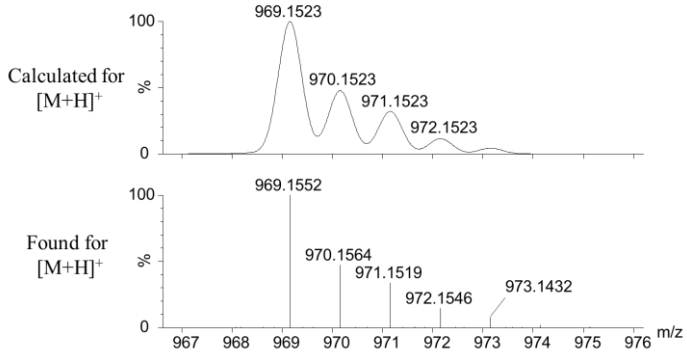
Chemical Formula: $C_{34}H_{40}N_8O_{14}S_4$
Exact Mass: 912.1547



[**1a-1a**], retention time: 14.02 min

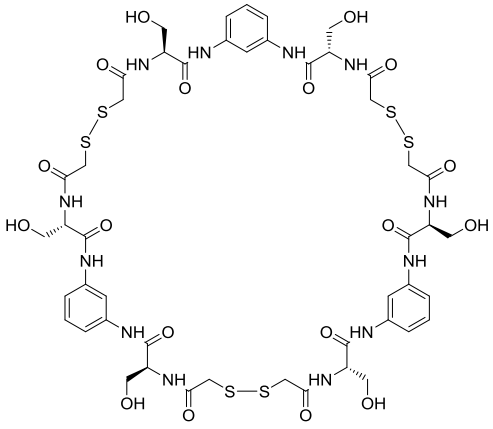


Chemical Formula: $C_{36}H_{40}N_8O_{16}S_4$
Exact Mass: 968.1445

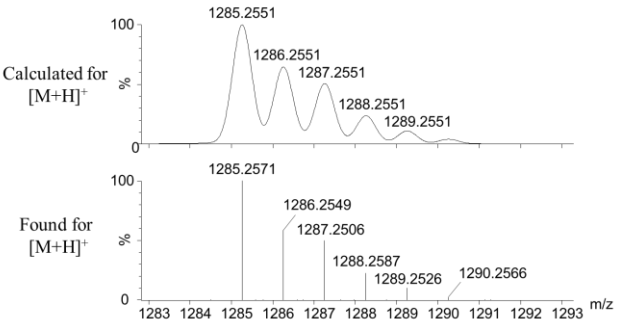


Identification of the trimers:

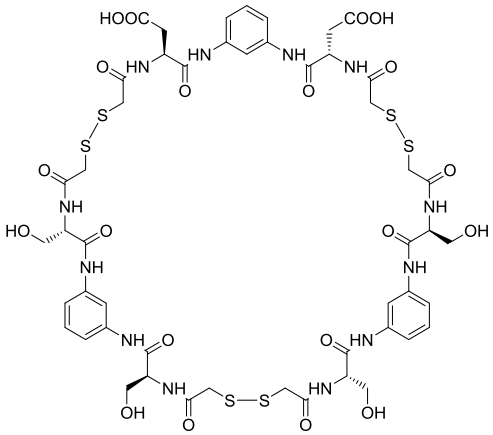
[**1b-1b-1b**], retention time: 12.20 min



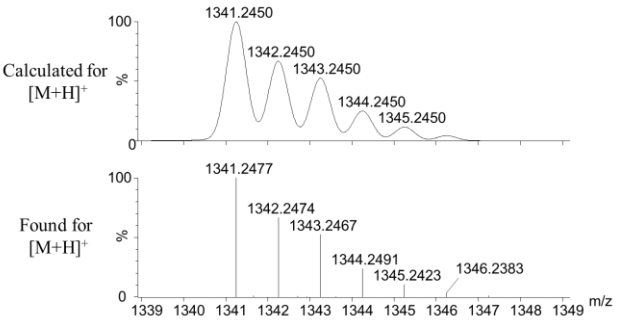
Chemical Formula: $C_{48}H_{60}N_{12}O_{18}S_6$
Exact Mass: 1284.2473



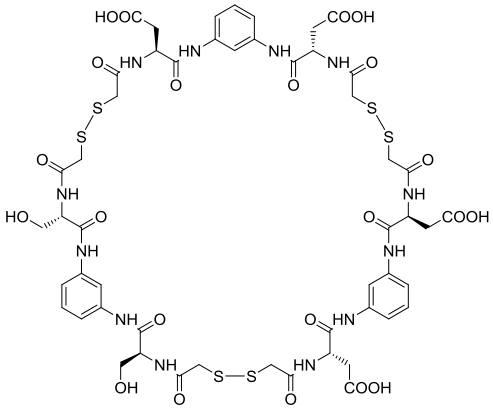
[**1a-1b-1b**], retention time: 13.10 min

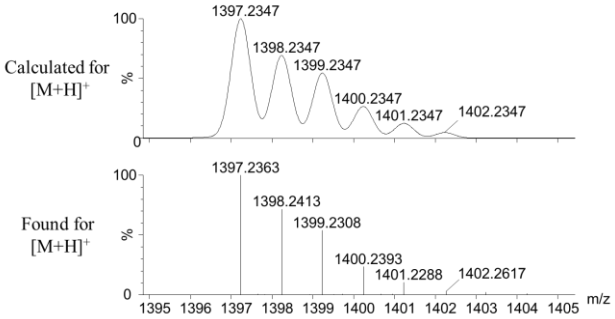


Chemical Formula: $C_{50}H_{60}N_{12}O_{20}S_6$
Exact Mass: 1340.2371

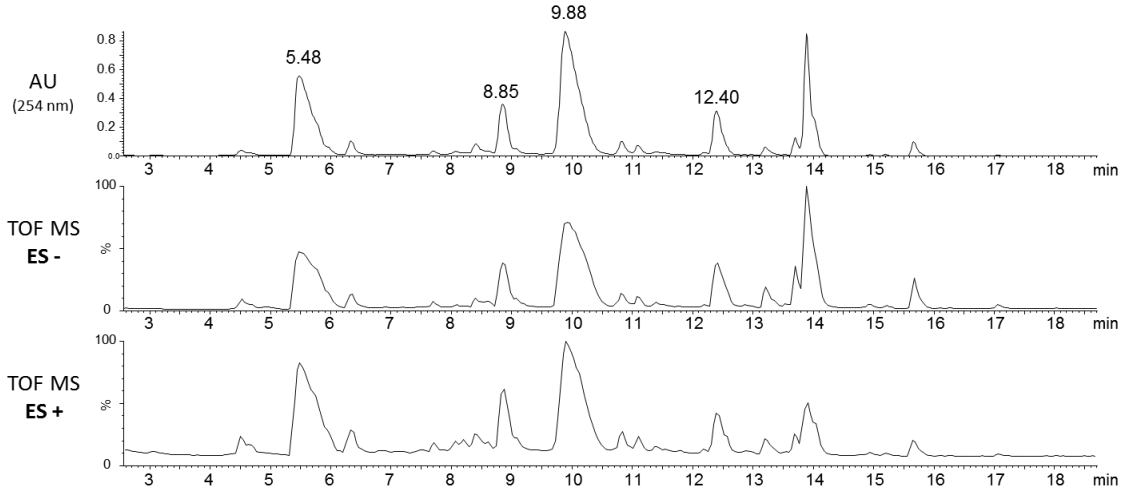


[**1a-1a-1b**], retention time: 14.07 min



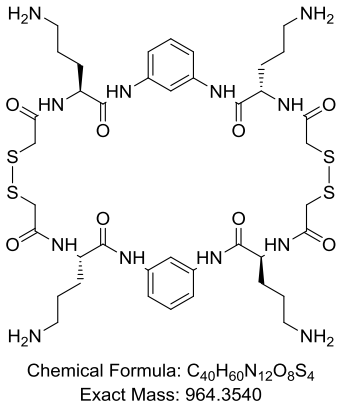


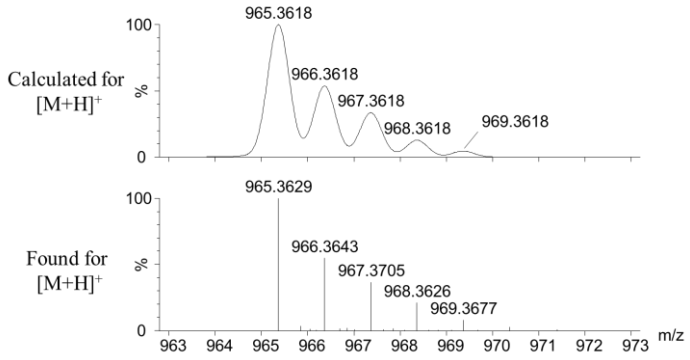
Binary mixture of [**1b+1c**]



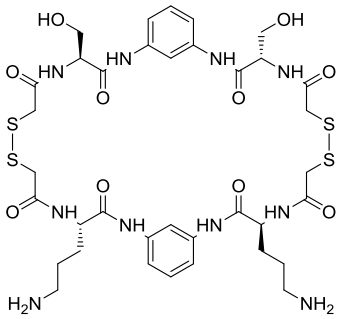
Identification of the dimers (the previously identified dimers are not shown):

[**1c-1c**], retention time: 5.48 min

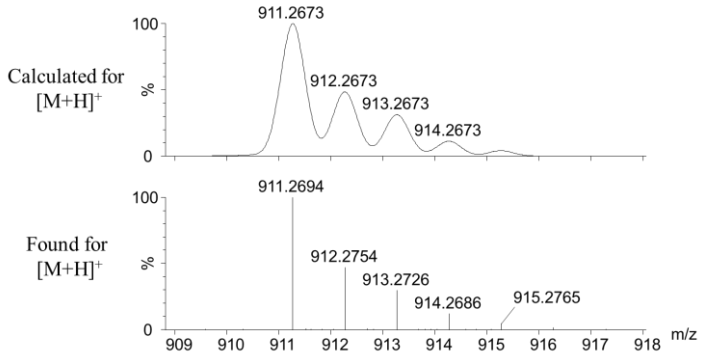




[**1b-1c**], retention time: 9.88 min

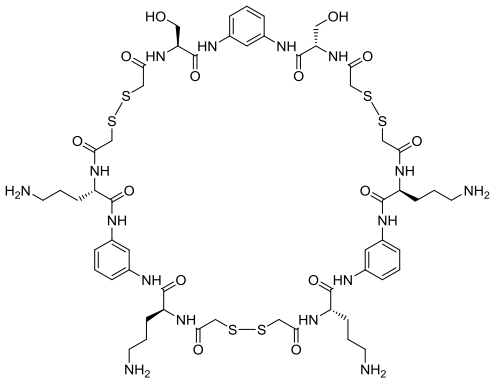


Chemical Formula: $C_{36}H_{50}N_{10}O_{10}S_4$
Exact Mass: 910.2594

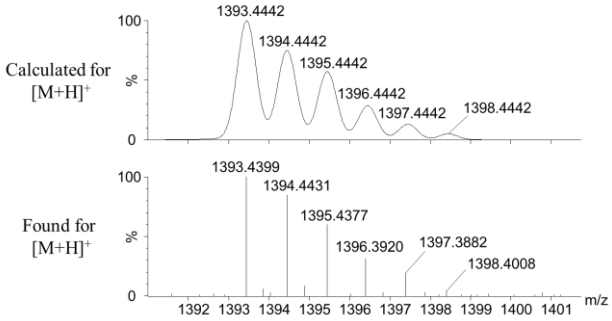


Identification of the trimers (the previously identified trimers are not shown):

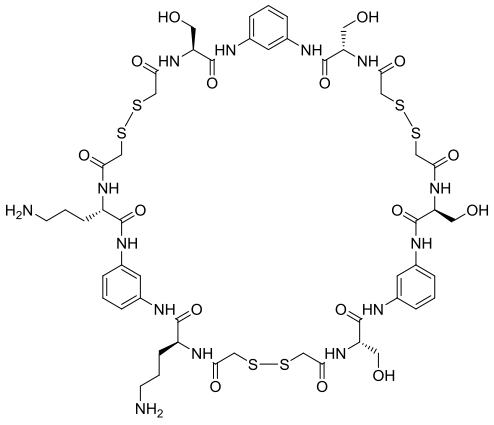
[**1b-1c-1c**], retention time: 8.85 min



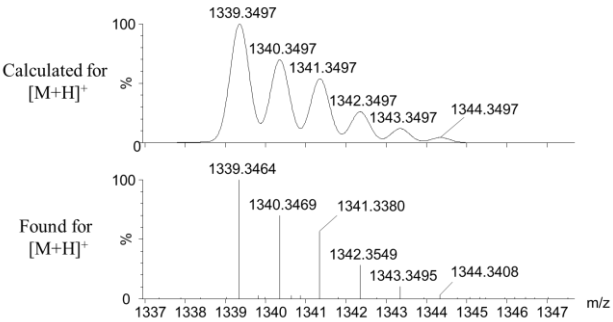
Chemical Formula: $C_{56}H_{80}N_{16}O_{14}S_6$
Exact Mass: 1392.4364



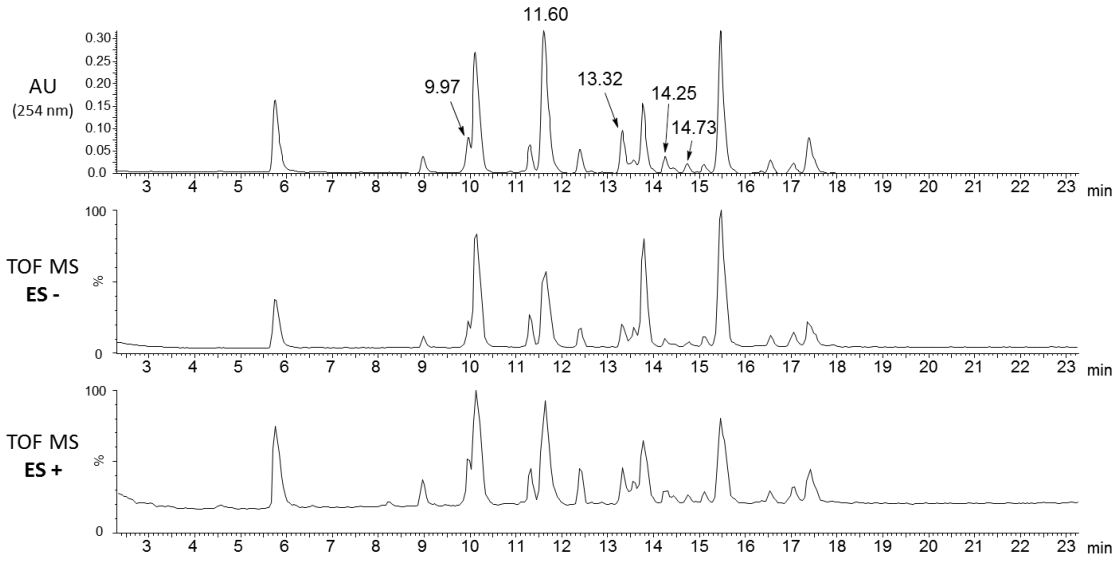
[**1b-1b-1c**], retention time: 12.40 min



Chemical Formula: $C_{52}H_{70}N_{14}O_{16}S_6$
Exact Mass: 1338.3418

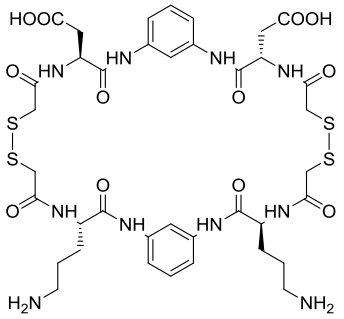


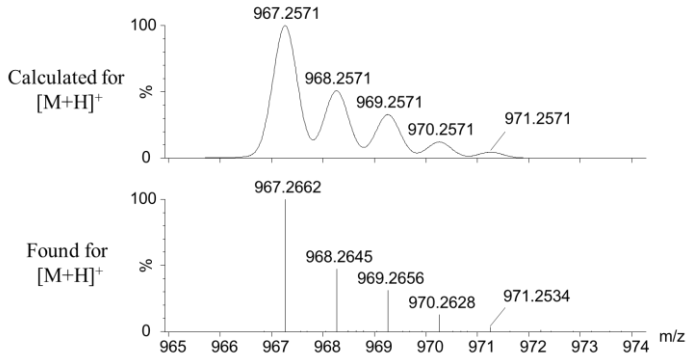
Ternary mixture of [**1a+1b+1c**]



Identification of the dimers (the previously identified dimers are not shown):

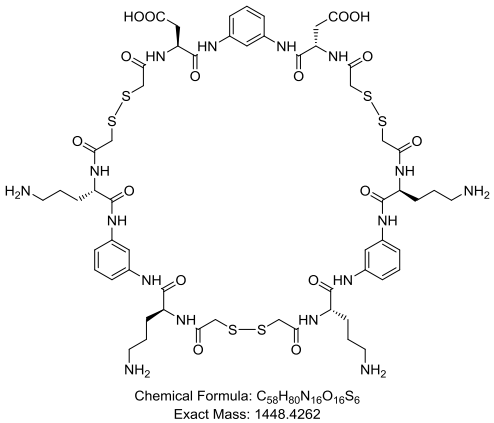
[**1a-1c**], retention time: 11.60

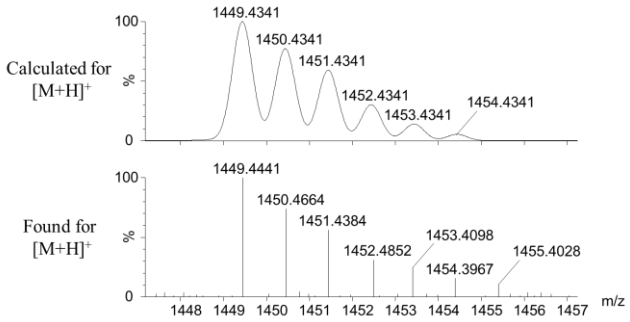




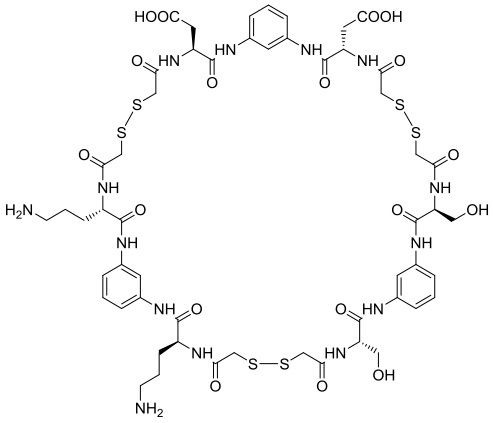
Identification of the trimers (the previously identified trimers are not shown):

[**1a-1c-1c**], retention time: 9.97 min

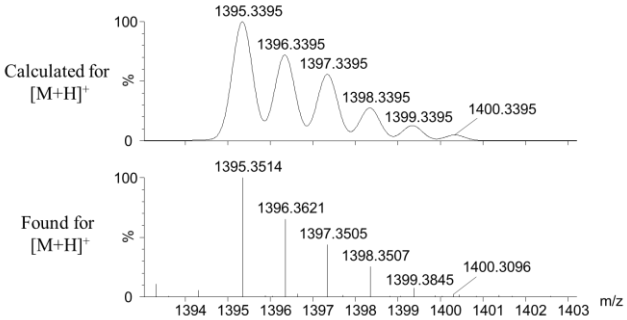




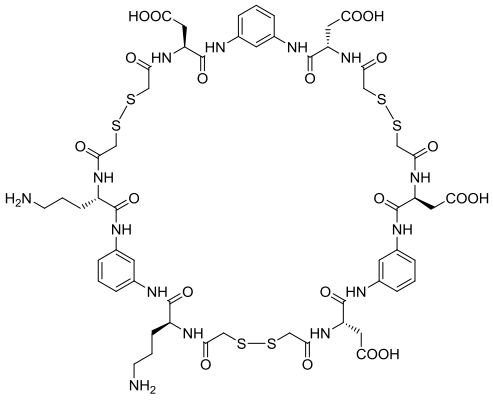
[**1a-1b-1c**], retention time: 13.32 min



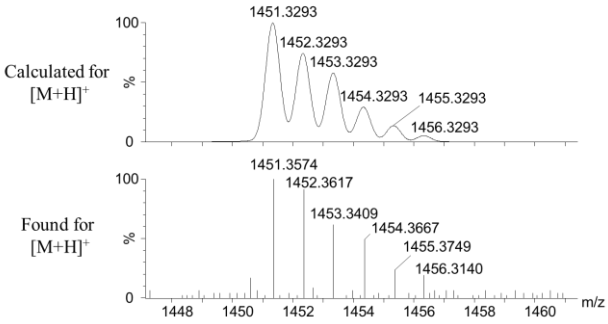
Chemical Formula: $C_{54}H_{70}N_{14}O_{18}S_6$
Exact Mass: 1394.3317



[**1a-1a-1c**], retention time: 14.25 min



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



Identification of the tetramer:

[**1a-1b-1b-1c**], retention time: 14.73 min

In this case the intensity was small and an appropriate isotopic pattern could not be obtained. However, different representative signals were detected:

HRMS (ESI+) calcd. for $[M+2H]^+$ (m/z): 912.2144, found: 912.2171

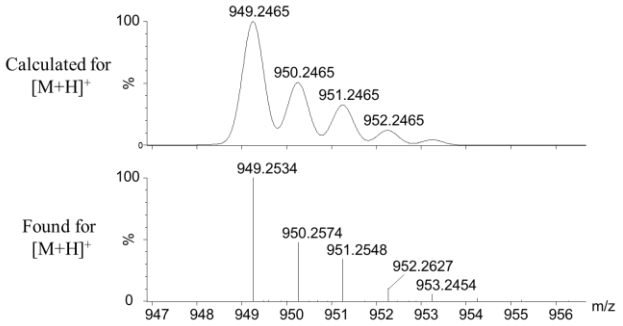
HRMS (ESI-) calcd. for $[M-H]^-$ (m/z): 1821.4068, found: 1821.4384

HRMS (ESI-) calcd. for $[M+Na-2H]^-$ (m/z): 1843.3888, found: 1843.3801

Identification of the dehydration products:

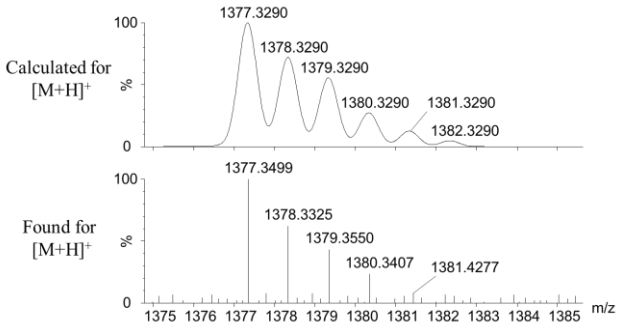
[(**1a-1c**)-H_2O], retention time: 11.30 min

Chemical Formula: $C_{38}H_{48}N_{10}O_{11}S_4$
Exact Mass: 948.2387



[(**1a-1b-1c**)-H_2O], retention time: 13.57 min

Chemical Formula: $C_{54}H_{68}N_{14}O_{17}S_6$
Exact Mass: 1376.3211



[(**1a-1a**)-H_2O], retention time: 17.05 min

Chemical Formula: $C_{36}H_{38}N_8O_{15}S_4$
Exact Mass: 950.1339