Near-Quantitative Aqueous Synthesis of Rotaxanes via Bioconjugation to Oligopeptides and Proteins

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SUPPORTING INFORMATION

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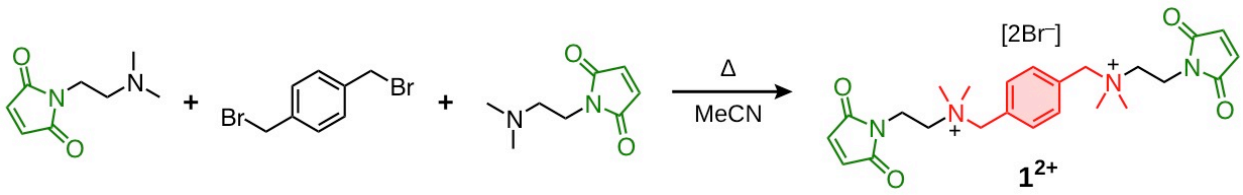
S1. General Methods

The reagents α,α′-dibromo-*para*-xylene (97%), reduced L-glutathione (GSH, ≥98%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, ≥98%), anhydrous Na_2SO_4 (≥99.0%), Cs_2CO_3 (99%), and trifluoroacetic acid (TFA, ≥98%) were purchased from Sigma-Aldrich and used without further purification. Fisher Scientific HPLC-grade MeCN, MeOH, CH_2Cl_2, and ACS-grade *N*,*N*-dimethylformamide (DMF), Me_2SO, and PhMe solvents were used as supplied. Water was filtered and deionized using a Barnstead NANOpure Diamond water purification system. Cucurbit[7]uril[S1] (CB7), *N*-*tert*-butoxycarbonylglutathione (BocGSH),[S2] *N,N*-[(dimethylamino)-ethyl]maleimide,[S3] **S1**,[S4] oxanorbornene imide,[S5] and cyclobis(paraquat-*p*-phenylene)[S6] ($CBPQT^{4+}$) were prepared according to literature procedures. Reactions were performed under ambient air unless otherwise noted. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (E. Merck) and visualized under a UV lamp at 254 nm. Column chromatography was carried out on silica gel 60 (E. Merck, 230–400 mesh). Analytical and semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 Series Capillary Liquid Chromatography (LC) system, eluting with HPLC grade H_2O/MeCN (0.1% v/v TFA) and monitored using an inline diode array UV/Vis detector. A Phenomenex 5 μm C18 Gemini column (150 × 4.6 mm) was used for analytical purifications at a flow rate of 1 ml/min. A Phenomenex 5 μm C18 Gemini column (250 ×10 mm) was used for semi-preparative purifications at a flow rate of 3 ml/min. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance 400 and 600 spectrometers with working frequencies of 400 and 600 MHz for ^{1}H, and 100 and 150 MHz for ^{13}C nuclei, respectively. Chemical shifts are reported in ppm and referenced to the residual non-deuterated solvents for ^{1}H ($CDCl_3$ δ = 7.27 ppm; CD_3CN δ = 1.94 ppm; $(CD_3)_2SO$ δ = 2.50 ppm; D_2O δ = 4.79 ppm) and ^{13}C ($CDCl_3$ δ = 77.0 ppm; CD_3CN δ = 118.26 ppm; $(CD_3)_2SO$ δ = 39.52 ppm) nuclei. High-resolution mass spectrometry (HRMS) was conducted in electrospray ionization (ESI) mode on an Agilent 1260 Series Capillary LC system equipped with an Agilent Poroshell 300SB-C18 analytical column and outfitted with an Agilent 6224 time of flight (TOF) LC-MS system, eluting with HPLC grade H_2O/MeCN (0.1% v/v formic acid) using a gradient of 20–100% MeCN in 7 min at a flow rate of 0.55 ml/min. Data was collected and analyzed using Agilent MassHunter Qualitative Analysis software. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini Gel Tank from Life Technologies on a Bio-Rad PowerPac HC apparatus using pre-

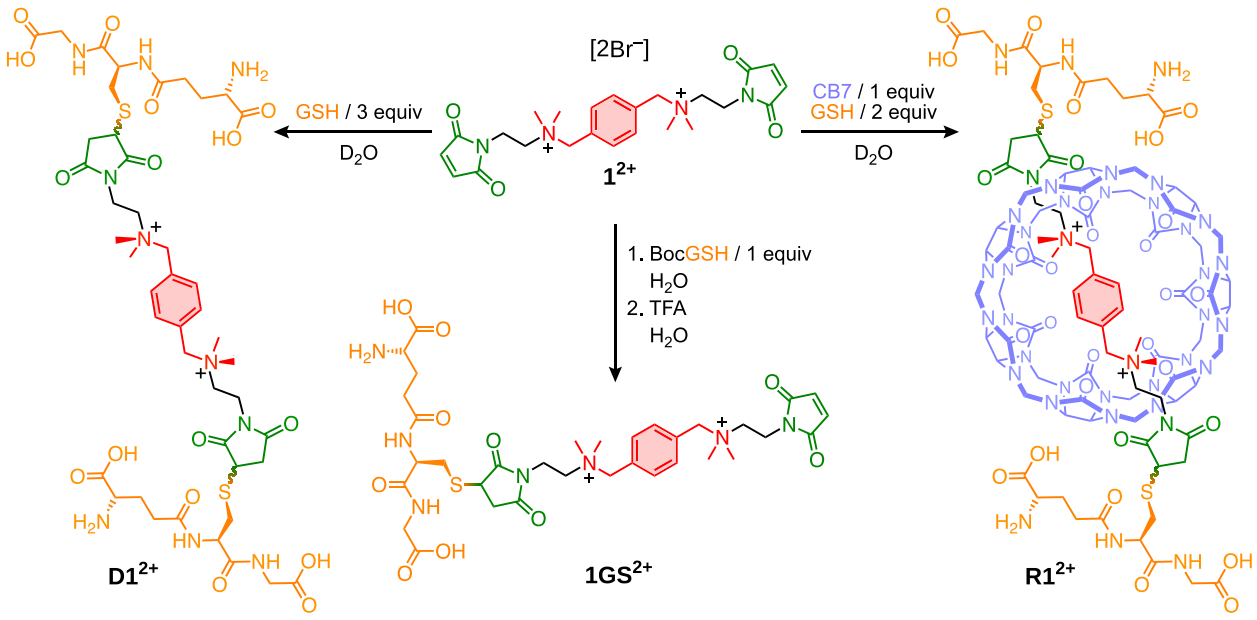
cast 12% Bis-Tris NuPAGE polyacrylamide gels purchased from Invitrogen. Aqueous protein samples were prepared for SDS-PAGE by heating at 100 °C for 10 min in the presence of glycerol (10% v/v), β-mercaptoethanol (10% v/v), and SDS (70 mM). Gels were run for 70 minutes at 150 V, and apparent molecular weights were assigned by comparing bands to a lane containing a protein ladder standard from Bio-Rad. Protein bands were visualized by staining with Coomassie Blue R-250 (Bio-Rad), imaged on a Bio-Rad Gel Doc EZ Imaging system coupled to a PC with Image Lab 5.1 software, and quantified by optical densitometry in ImageJ software. Protein concentrations were determined spectrophotometrically on a Thermo Scientific Nanodrop ND-1000 UV-Vis spectrophotometer.

S2. Synthetic Procedures



Scheme S1. Synthesis of $\mathbf{1}^{2+}$.

Bis-maleimide $\mathbf{1}^{2+}$. A mixture of α,α′-dibromo-*para*-xylene (33 mg, 0.13 mmol) and *N,N*-[(dimethylamino)ethyl]maleimide (52 mg, 0.31 mmol) was heated to reflux in MeCN (3 ml) for 16 h, producing a white precipitate that was collected by vacuum filtration and washed with CH_2Cl_2 (5 ml) to afford $\mathbf{1}^{2+}$ as a white powder (73 mg, 96%). ^{1}H NMR (400 MHz, D_2O, 293 K) δ = 7.73 (s, 4H), 6.90 (s, 4H), 4.65 (s, 4H), 4.09 (t, J = 7.4 Hz, 4H), 3.55 (t, J = 7.4 Hz, 4H), 3.15 (s, 12H). ^{13}C NMR (100 MHz, D_2O, 293 K) δ = 172.6, 135.5, 134.4, 130.0, 68.3, 60.9, 50.8, 31.9. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{24}H_{32}O_4N_4$ [M – 2Br]$^{2+}$ 220.1206, observed 220.1204.



Scheme S2. Synthesis of *p*-xylylene-bisaminium dumbbell $\mathbf{D1}^{2+}$, semidumbbell $\mathbf{1GS}^{2+}$, and the corresponding CB7 [2]rotaxane $\mathbf{R1}^{2+}$, from the bis(maleimide) $\mathbf{1}^{2+}$.

Dumbbell $\mathbf{D1}^{2+}$. GSH (8.2 mg, 27 μmol) and $\mathbf{1}^{2+}$ (5.2 mg, 8.7 μmol) were dissolved in D_2O (120 μl) and the solution was left to stand for 10 min. An analytical sample was obtained by semi-preparative RP-HPLC, eluting in 5:95 MeCN:H_2O with 0.1% TFA over 10 min. ^{1}H NMR (600 MHz, D_2O, 293 K) δ = 7.73 (s, 4H), 4.70–4.63 (m, 5H), 4.13–4.06 (m, 6H), 3.98 (s, 4H), 3.90 (t, J = 6.5 Hz, 2H), 3.53 (t, J = 8.5 Hz, 4H), 3.38–3.15 (m, 4H), 3.15 (s, 12H), 3.03–2.90 (m, 2H), 2.73–2.68 (m, 2H), 2.60–2.50 (m, 4H), 2.22–2.14 (m, 4H). ^{13}C (100 MHz, D_2O, 293 K) δ = 178.4, 177.0, 174.8, 173.6, 173.5, 172.2, 133.8, 129.4, 67.7, 59.4, 53.8, 53.3, 50.3, 41.7, 41.2, 33.2, 32.6, 31.3, 31.2, 26.1. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{44}H_{64}N_{10}O_{16}S_2$ $[M - H]^+$ 1053.4016, observed 1054.3958. *m/z* calcd for $C_{44}H_{64}N_{10}O_{16}S_2$ $[M]^{2+}$ 527.2044, observed 527.7097.

Semidumbbell $\mathbf{1GS}^{2+}$. H_2O (200 μl) was added to a mixture of $\mathbf{1}^{2+}$ (6.6 mg, 11 μmol) and BocGSH (4.8 mg, 12 μmol). After standing for 5 min, the reaction mixture was passed through a 0.22 μm PVDF spin filter in a centrifuge and an aliquot (130 μl) was taken for purification by semi-preparative HPLC, eluting in an aqueous gradient of 0–50% MeCN over 30 min. The fractions containing Boc-protected monoadduct were combined and evaporated to dryness under a flow of N_2. The resulting film was dissolved in H_2O (2 ml) and TFA (0.5 ml), and the solvent

was evaporated gradually under a flow of N_2 overnight, affording a film of **1GS**$^{2+}$, which was used without further purification. ^{1}H NMR (400 MHz, D_2O, 293K) δ = 7.74 (s, 4H), 6.91 (s, 2H), 4.73–4.67 (m, 1H), 4.66 (s, 4H), 4.13–4.06 (m, 6H), 3.97 (s, 2H), 3.85 (t, J = 6.5 Hz, 1H), 3.56 (t, J = 8.5 Hz, 2H), 3.54 (t, J = 8.5 Hz, 2H), 3.39–2.92 (m, 3H), 2.76–2.65 (m, 1H), 2.58–2.50 (m, 2H), 2.21–2.11 (m, 2H). ^{13}C (100 MHz, D_2O, 293 K) δ = 178.1, 176.7, 174.6, 173.3, 173.2, 172.4, 172.1, 135.3, 134.1, 133.5, 129.9, 129.1, 68.1, 67.5, 60.8, 59.3, 53.6, 53.1, 50.6, 50.0, 41.5, 41.0, 32.4, 31.1, 31.0, 30.7, 26.0. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{34}H_{49}N_7O_{10}S_2$ $[M]^{2+}$ 373.6626, found 373.6627.

Rotaxane R1^{2+}. In a glass NMR tube, a 4.0 mM solution of **1**$^{2+}$ (290 µl, 1.2 µmol) was mixed with a 4.0 mM solution of CB7 (290 µl, 1.2 µmol) in D_2O. To this solution was added a 10 mM solution of GSH (240 µl, 2.4 µmol) in portions until ^{1}H NMR spectroscopy indicated the maleimide was completely reacted. ^{1}H NMR (400 MHz, D_2O, 293 K) δ = 6.78 (s, 4H), 5.73 (d, J = 15.5 Hz, 14H), 5.49 (s, 14H), 4.69 (dd, J = 6.0, 6.5 Hz, 2H), 4.26 (t, J = 7.5 Hz, 4H), 4.20 (d, J = 15.5 Hz, 14H), 4.19–4.16 (m, 4H), 4.14 (s, 2H), 4.12 (s, 2H), 3.99 (s, 2H), 3.98 (s, 2H), 3.86 (t, J = 6.0 Hz, 2H), 3.68 (t, J = 8.0, 2H), 3.67 (t, J = 8.0 Hz, 2H), 3.46–3.19 (m, 3H), 3.05–3.00 (m, 1H), 2.83 (s, 6H), 2.81 (s, 6H), 2.76 (d, J = 5.0 Hz, 1H), 2.71 (d, J = 5.0 Hz, 1H), 2.59–2.50 (m, 4H), 2.21–2.14 (m, 4H). ^{13}C (100 MHz, D_2O, 293 K) δ = 178.4, 177.2, 177.1, 174.7, 172.5, 172.3, 156.2, 132.2, 129.7, 71.2, 68.2, 61.2, 55.6, 54.1, 53.3, 52.7, 49.4, 41.7, 41.4, 41.1, 40.2, 33.2, 32.8, 31.3, 31.2, 26.0, 25.4. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{86}H_{108}N_{38}O_{30}S_2$ $[M]^{2+}$ 1108.8801, observed 1109.3793. *m/z* calcd for $C_{86}H_{109}N_{38}O_{30}S_2$ $[M + H]^{3+}$ 739.2532, observed 739.9241.

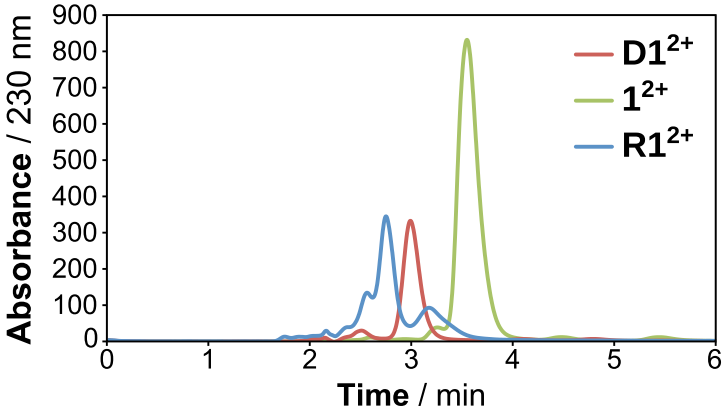
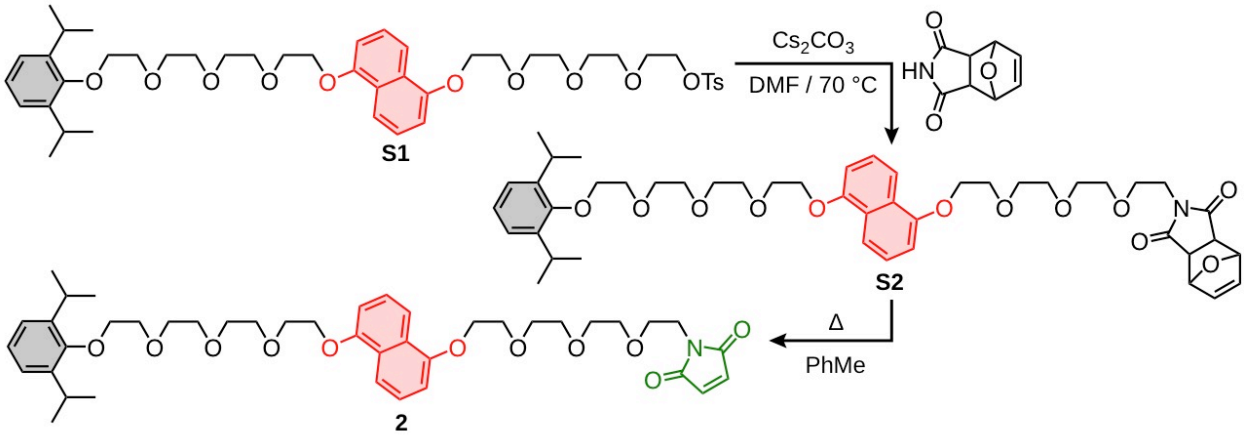


Figure S1. RP-HPLC chromatograms (100% H_2O, 0.1% TFA, 10 min) of **1**$^{2+}$, **D1**$^{2+}$, and **R1**$^{2+}$.

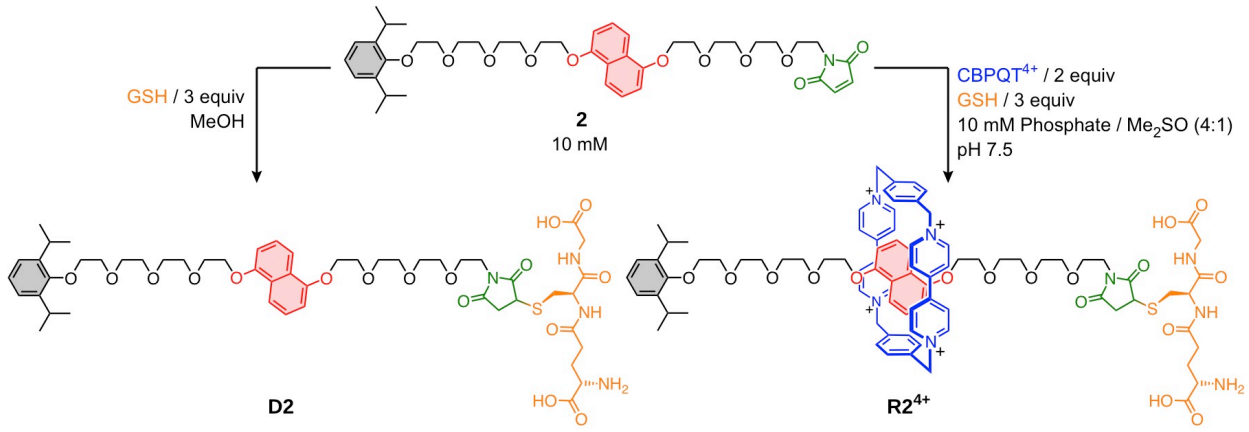


Scheme S3. Synthesis of DNPMI.

Protected Semidumbbell S2. Cs_2CO_3 (260 mg, 0.80 mmol) was added to a solution of **S1** (202 mg, 0.244 mmol) and oxanorbornene imide (111 mg, 0.672 mmol) in DMF (4 ml) and the reaction stirred at 70 °C under an atmosphere of N_2 for 14 h. The reaction mixture was poured into H_2O (30 ml) and extracted with CH_2Cl_2 (3 × 25 ml). The organic extracts were combined, washed with 0.5 M HCl (20 ml), washed with brine (20 ml), dried (Na_2SO_4), concentrated to a volume of approximately 1 ml, and chromatographed on SiO_2, eluting with EtOAc to afford the product, after removal of solvent, as a yellow viscous liquid (193 mg, 97%). ^{1}H NMR (400 MHz, ($CDCl_3$, 293 K) δ = 7.86 (d, J = 8.4 Hz, 2H), 7.34 (t, J = 8.1 Hz, 1H), 7.33 (t, J = 8.1 Hz, 1H), 7.09 (s, 3H), 6.83 (d, J = 7.6 Hz, 2H), 6.37 (s, 2H), 5.20 (s, 2H), 4.28 (q, J = 4.8 Hz, 4H), 4.02-3.95 (m, 4H), 3.92–3.88 (m, 2H), 3.86–3.81 (m, 4H), 3.79–3.72 (m, 8H), 3.69–3.64 (m, 4H), 3.62–3.59 (m, 6H), 3.39 (hept, J = 6.8 Hz, 2H), 2.77 (s, 2H), 1.21 (d, J = 6.9 Hz, 12H). ^{13}C NMR (100 MHz, ($CDCl_3$, 293 K) δ = 175.9, 154.1, 154.1, 152.8, 141.6, 136.3, 126.5, 124.9, 124.9, 124.4, 123.8, 123.8, 114.4, 114.4, 105.5, 105.5, 80.6, 73.7, 70.9, 70.8, 70.8, 70.6, 70.6, 70.5, 70.4, 70.3, 69.9, 69.6, 69.6, 67.7, 67.7, 66.9, 47.2, 38.0, 26.0, 24.0. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{46}H_{61}O_{12}N_1Na$ $[M + Na]^+$ 842.4086, observed 842.4080.

Semidumbbell 2. Compound **S1** (190 mg, 0.23 mmol) was heated to reflux in PhMe (15 ml) for 6 h and the solvent was removed under reduced pressure to afford the product as a yellow viscous liquid (170 mg, 98%). ^{1}H NMR (400 MHz, ($CDCl_3$, 293 K) δ = 7.88 (d, J = 8.1 Hz, 2H), 7.35 (t, J = 8.0 Hz, 2H), 7.11 (s, 3H), 6.84 (d, J = 7.6 Hz, 2H), 6.62 (s, 2H), 4.30 (q, J = 4.3 Hz, 4H), 4.02–3.98 (m, 4H), 3.93–3.91 (m, 2H), 3.88–3.82 (m, 4H), 3.80–3.74 (m, 8H), 3.71–3.66

(m, 4H), 3.64–3.59 (m, 6H), 3.41 (hept, J = 6.8 Hz, 2H), 1.23 (d, J = 6.9 Hz, 12H). ^{13}C NMR (100 MHz, ($CDCl_3$, 293 K) δ = 170.5, 154.1, 154.1, 152.9, 141.6, 133.9, 133.9, 126.6, 124.9, 124.4, 123.8, 123.8, 114.4, 114.4, 105.5, 105.5, 73.7, 70.9, 70.8, 70.8, 70.7, 70.6, 70.6, 70.4, 70.4, 69.9, 69.6, 69.6, 67.7, 67.7, 67.6, 36.9, 26.0, 24.0. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{42}H_{57}O_{11}N_1Na$ $[M + Na]^+$ 774.3824, observed 774.3811.



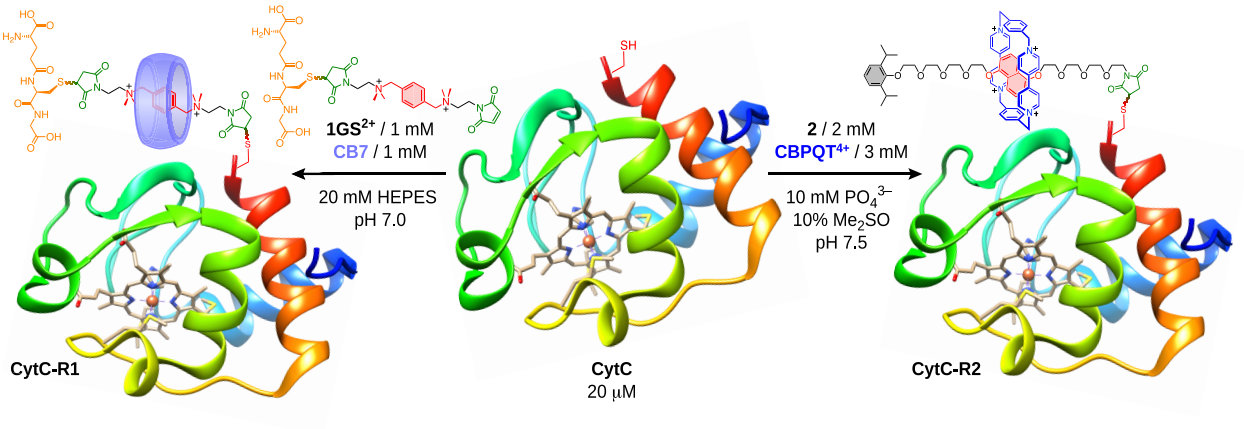
Scheme S4. Synthesis of the DNP dumbbell **D2** and the DNP/CBPQT^{4+} [2]rotaxane **R2**$^{4+}$

Dumbbell D2. GSH (14 mg, 46 μmol) was added to a solution of **2** (17 mg, 23 μmol) in MeOH (2 ml). The reaction was sonicated for 1 min and left to stand for 30 min. The solvent was evaporated under a flow of N_2, affording a pale yellow film. ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 293 K) δ = 8.31 (d, J = 8.5 Hz, 1H), 7.99 (d, J = 8.0 Hz, 1H), 7.87 (t, J = 8.0 Hz, 1H), 7.71 (t, J = 8.0 Hz, 1H), 7.39–7.34 (m, 2H), 7.07 (d, J = 3.5 Hz, 2H), 6.97 (d, J = 7.5 Hz, 1H), 4.55–4.50 (m, 1H), 4.23 (s, 2H), 3.91–3.85 (m, 2H), 3.79–3.75 (m, 2H), 3.70–3.40 (m, 27H), 3.39–3.36 (m, 2H), 3.32 (t, J = 5.5 Hz, 1H), 3.04–2.99 (m, 3H), 2.89–2.87 (m, 1H), 2.73–2.71 (m, 1H), 2.55–2.52 (m, 1H), 2.08–1.94 (m, 4H), 1.13 (d, J = 7.0 Hz, 12H). ^{13}C (150 MHz, $(CD_3)_2SO$, 298 K) δ = 172.6, 172.6, 171.4, 171.3, 171.3, 170.8, 154.2, 153.0, 145.7, 141.7, 131.3, 126.4, 125.8, 125.0, 124.3, 114.2, 106.4, 74.1, 70.5, 70.5, 70.4, 70.3, 70.3, 70.2, 70.1, 70.0, 69.8, 69.4, 69.3, 68.2, 66.5, 60.5, 53.4, 52.4, 41.4, 40.8, 31.8, 26.8, 25.9, 24.3. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{52}H_{75}N_4O_{17}S$ $[M + H]^+$ 1059.4842, observed 1059.4762. *m/z* calcd for $C_{52}H_{76}N_4O_{17}S$ $[M + 2H]^{2+}$ 530.2458, observed 529.7385.

Rotaxane R2^{4+}. Compound **2** (9.8 mg, 13 μmol) and CBPQT·4Cl (14 mg, 21 μmol) were dissolved in Me_2SO (300 μl). The resulting purple-colored solution was diluted with 10 mM phosphate buffer (1.2 ml) at pH 7.5. GSH (16.5 mg, 54 μmol) was added and the solution was sonicated for 1 min. After 1 h, the crude reaction mixture was filtered and purified by preparative HPLC, eluting in an aqueous gradient of 10–100% MeCN (0.1% TFA) over 40 min. The red-colored fractions were combined and the solvent was removed by rotary evaporation to afford **R2^{4+}** as its TFA salt (22.1 mg, 88%). ^{1}H NMR (400 MHz, D_2O, 293 K) δ = 9.10 (d, J = 6.5 Hz, 4H), 8.93 (d, J = 6.5 Hz, 2H), 8.91 (d, J = 6.5 Hz, 2H), 8.13 (s, 4H), 8.01 (s, 2H), 7.99 (s, 2H), 7.58 (d, J = 6.5 Hz, 2H), 7.54 (d, J = 6.5 Hz, 2H), 7.46 (d, J = 6.5 Hz, 2H), 7.44 (d, J = 6.5 Hz, 2H), 7.20–7.15 (m, 3H), 6.42 (d, J = 8.5 Hz, 1H), 6.41 (d, J = 8.5 Hz, 1H), 6.12 (t, J = 8.0 Hz, 2H), 5.92–5.81 (m, 8H), 4.64 (dd, J = 5.5 Hz, 8.0 Hz, 1H), 4.39–4.35 (m, 4H), 4.30–4.26 (m, 4H), 4.16–4.13 (m, 2H), 4.12–4.09 (m, 2H), 4.05–3.93 (m, 8H), 3.89–3.86 (m, 2H), 3.81–3.72 (m, 8H), 3.64–3.61 (m, 2H), 3.57–3.50 (m, 4H), 3.30 (dd, J = 5.0 Hz, 14.0 Hz, 1H), 3.26–3.12 (m, 4H), 2.94 (dd, J = 9.0 Hz, 14.0 Hz, 1H), 2.60–2.54 (m, 2H), 2.54 (d, J = 8.0 Hz, 2H), 2.23–2.18 (m, 2H). ^{13}C NMR (125 MHz, D_2O, 293 K) δ = 184.3, 178.9, 177.7, 174.3, 174.3, 172.79, 162.9, 162.7, 162.4, 151.2, 145.2, 145.0, 143.8, 142.4, 136.4, 136.4, 136.4, 136.4, 131.2, 131.2, 131.2, 131.2, 128.2, 128.2, 128.2, 126.0, 125.9, 125.9, 125.6, 124.7, 124.6, 124.6, 124.5, 124.4, 117.3, 115.4, 108.5, 108.5, 104.2, 104.2, 104.2, 73.4, 70.6, 70.5, 70.3, 70.2, 70.1, 69.8, 69.7, 69.6, 69.5, 69.5, 69.5, 69.3, 68.0, 66.4, 66.4, 65.4, 58.1, 58.1, 53.1, 52.6, 52.4, 52.4, 41.1, 41.1, 40.4, 39.6, 38.2, 38.2, 32.7, 32.5, 30.9, 25.9, 25.5, 23.2, 23.1, 19.1, 12.8. HRMS (ESI-TOF-MS): *m/z* calcd for $C_{88}H_{104}N_8O_{17}S$ $[M - 2H]^{2+}$ 788.3615, observed 788.3626.

S3. Protein Rotaxanation

The *iso*-1-cytochrome *c* (CytC) model protein was purchased from Aldrich (85% purity) and subjected to cation exchange chromatography, eluting in 20 mM HEPES buffer (pH 7) with a step gradient of 0 M, 0.4 M, and 1 M NaCl. Fractions containing ≥95% pure CytC were combined, treated with 2 mM TCEP for 15 min, exchanged into 10 mM phosphate buffer by repeated centrifugal filtration against a 10 kDa molecular weight cutoff (MWCO) membrane, and diluted to a concentration of 100 μM.



Scheme S5. Synthesis of protein-mounted rotaxanes via bioconjugation of semirotaxanes to CytC.

CytC-R1. A 100 μM solution (20 μl) of CytC in 10 mM phosphate buffer, pH 7.5, was added to a 1 mM solution of **1GS**$^{2+}$⊂CB7 (80 μl) in 20 mM HEPES buffer, pH 7.0. The mixture was vortexed for 10 s and left to stand for 30 min. The protein-mounted rotaxane was exchanged into pure water by repeated centrifugal filtration against a 10 kDa MWCO membrane.

CytC-R2. A 10 mM solution (20 μl) of **2**⊂CBPQT^{4+} (15 mM with respect to CBPQT^{4+}) in H_2O:Me_2SO (1:1 v/v) was added to a 25 μM solution of CytC (80 μl) in 10 mM phosphate buffer, pH 7.5. The mixture was vortexed for 10 s and left to stand for 30 min. The protein-mounted rotaxane was exchanged into pure water by repeated centrifugal filtration against a 10 kDa MWCO membrane.

The bioconjugation reactions were immediately analyzed without further purification by ESI-MS (see **Figure 4** and the corresponding discussion in the main text) and SDS-PAGE. The remainder

of the samples were stored under air for at least 24 h at 5 °C and diluted to a concentration of ca. 5 μM, determined spectrophotometrically by the absorbance at 410 nm, using ε_{410} = 101600 $M^{-1}cm^{-1}$ as the value[S7] of the extinction coefficient of CytC. Unedited images of the original gels associated with **Figure 5** of the main text are shown in **Figure S2**.

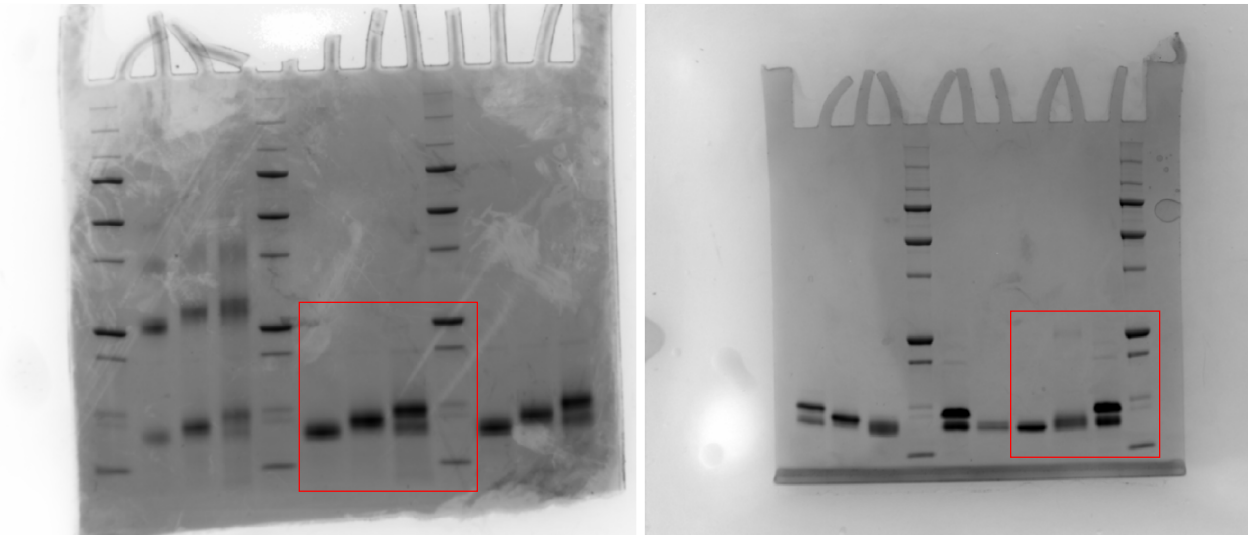


Figure S2. Unedited images of the gels for the rotaxanation of CytC with the xylylene/CB7 (left) and DNP/$CBPQT^{4+}$ (right) semirotaxane maleimides.

S4. ^{1}H NMR Spectra of Dumbbells and Rotaxanes

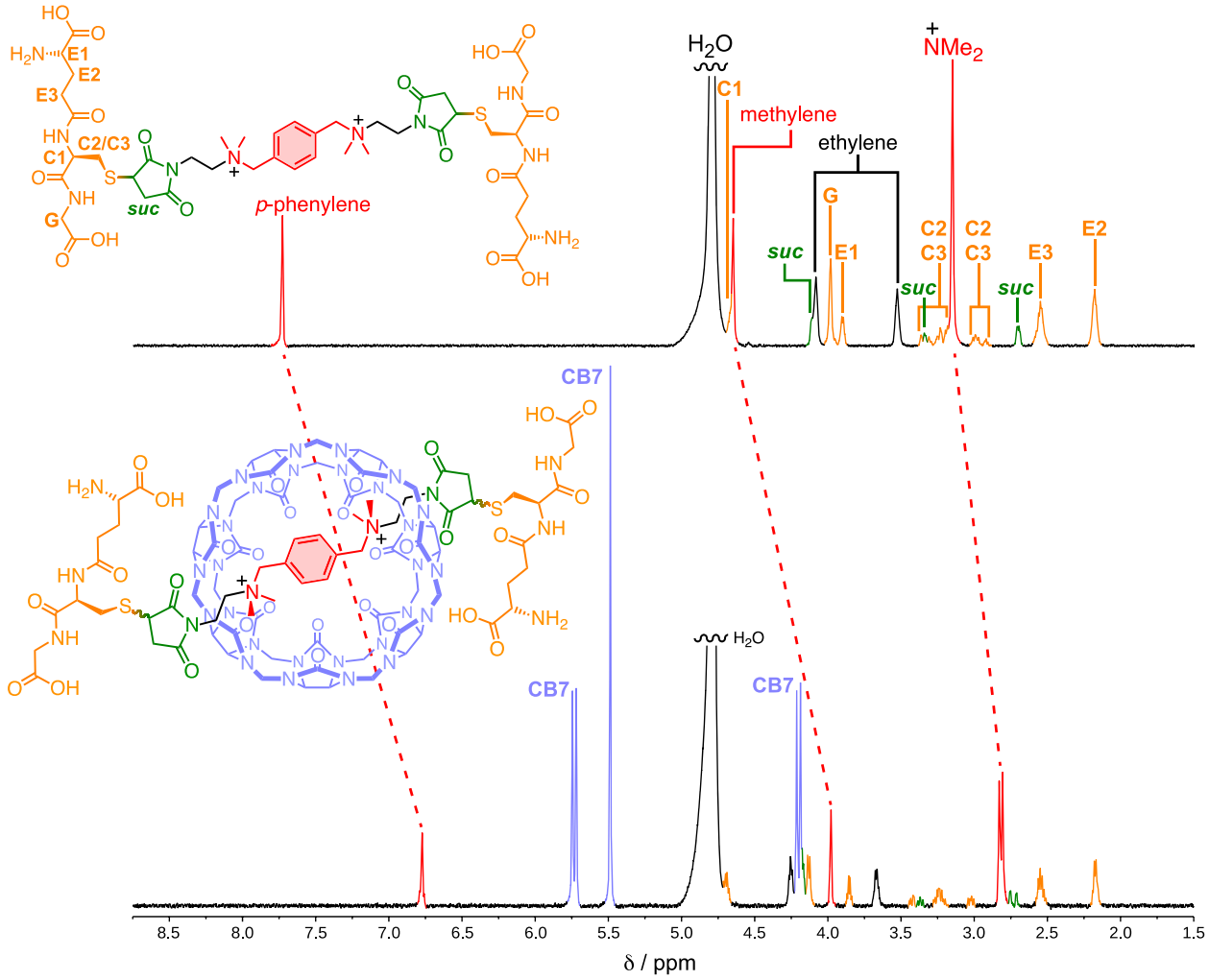


Figure S3. ^{1}H NMR spectra (D_2O, 400 MHz, 293 K) of dumbbell $\mathbf{D1}^{2+}$ and [2]rotaxane $\mathbf{R1}^{2+}$.

The ^{1}H NMR spectra of the free dumbbell $\mathbf{D1}^{2+}$ and the corresponding CB7-threaded [2]rotaxane $\mathbf{R1}^{2+}$ are compared in **Figure S3**. Two *N*-methylammonium proton resonances are observed in the case of $\mathbf{R1}^{2+}$, most likely on account of the stereocenters generated in each of the succinimide units upon bioconjugation to the GSH stoppers.

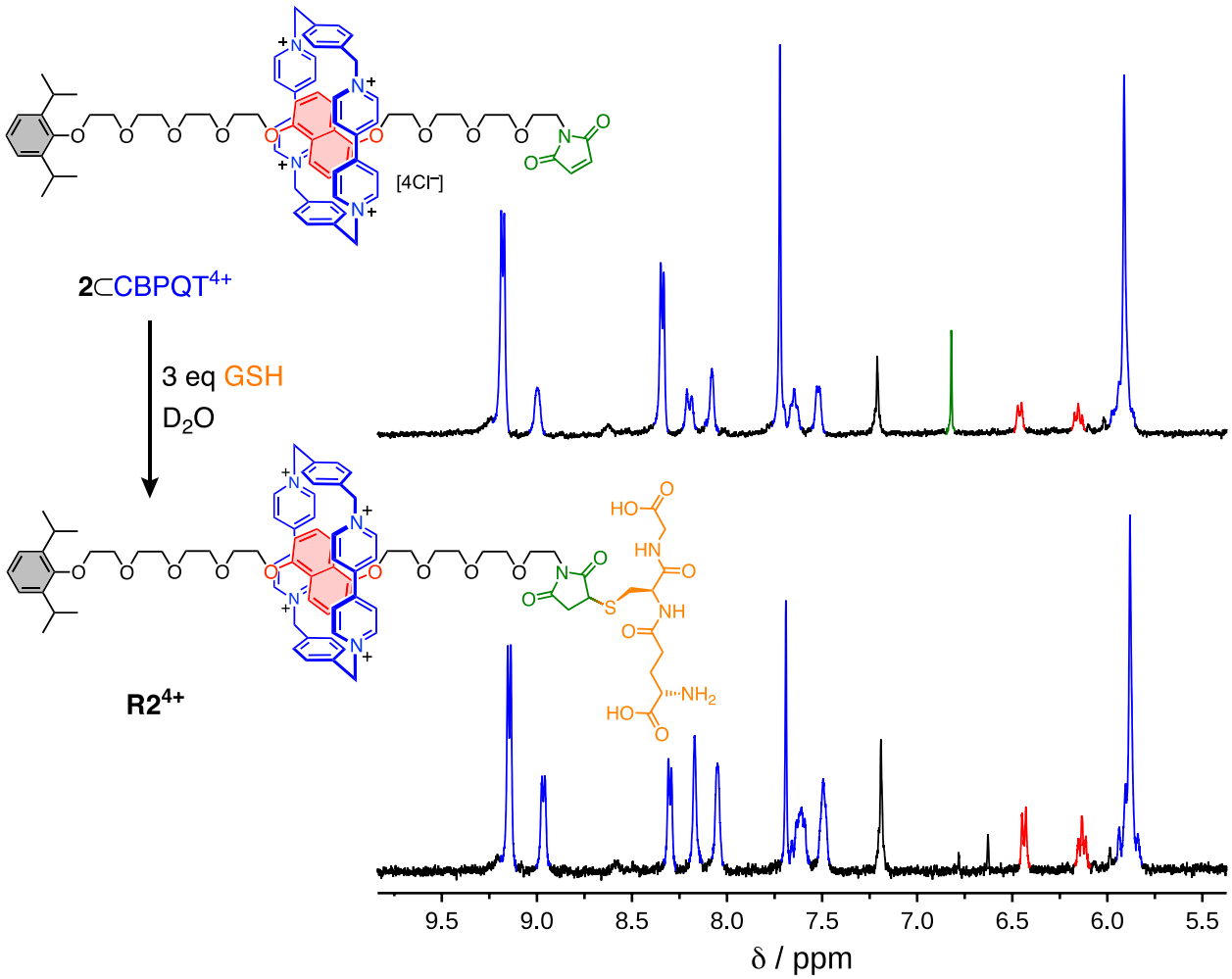


Figure S4. Partial ^{1}H NMR spectra (D_2O, 400 MHz, 293 K) of the semirotaxane **2**⊂CBPQT^{4+} and the corresponding [2]rotaxane obtained approximately 10 min after the addition of 3 eq GSH.

The progress of the rotaxane-stoppering reaction that generates the CBPQT^{4+} [2]rotaxane **R2**$^{4+}$ was monitored (**Figure S4**) *in situ* by ^{1}H NMR spectroscopy. The signal corresponding to the maleimide disappears almost completely within 10 min at 293 K in D_2O upon addition of GSH to the pseudorotaxane **2**⊂CBPQT^{4+}. The fully assigned ^{1}H NMR spectrum of **R2**$^{4+}$ after preparative-scale RP-HPLC purification is illustrated in **Figure S5**.

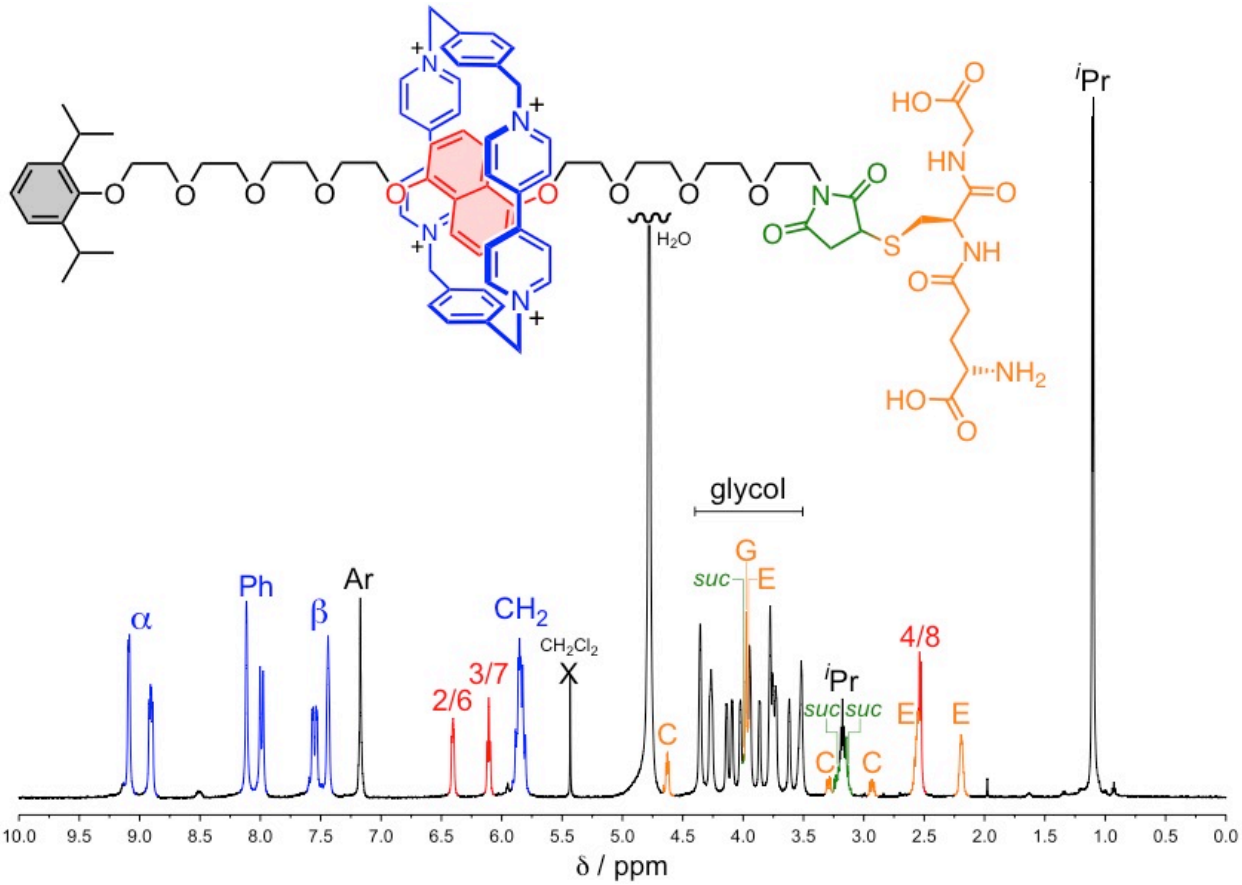


Figure S5. Assigned ^{1}H NMR spectrum (D_2O, 600 MHz, 293 K) of [2]rotaxane **R2**$^{4+}$.

S5. References

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