Electronic Supplementary Information for:

Facile Access to Foldable Redox-Active Flavin Peptide Conjugates

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1. Instrumentation

Mass spectrometry

The mass-spectrometric analyses were performed by the core facilities of Department of Chemistry of the Johannes Gutenberg University Mainz. All test samples were prepared at a concentration of 0.1 g/L using MeOH as solvent. The electrospray ionization measurements (ESI) were recorded on a *Waters Micromass QTof Ultima 3* or *Agilent 6545 QToF* instrument.

MALDI-TOF MS

Matrix assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry measurements were performed by the core facilities of Department of Chemistry of the Johannes Gutenberg University Mainz, on a *Shimadzu Axima CFR MALDI-ToF* or *Bruker autoflex maX MALDI-ToF/ToF*.

NMR-spectroscopy

NMR spectra were recorded on a *BRUKER ARX 300 spectrometer*, a *BRUKER Avance II 400* or a *BRUKER Avance III 600* spectrometer. All measurements were carried out using DMSO-d_6, $CDCl_3$, HFIP-d_2 or D_2O as deuterated solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the chemical shifts of the residual protons in the deuterated solvent. The spin multiplicities of the signals are assigned as follows: s (br) (singlet (broad)), s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). All measured coupling constants are stated in Hertz (Hz). All NMR spectra were analyzed using the software MestReNova.

Circular dichroism (CD) spectroscopy

All spectra were recorded using a peptide concentration of 50 µM in 10 mM phosphate buffer using a quartz cell with a path length of 2 mm or 1 mm. CD-spectra were recorded on a JASCO J-815 CD spectrometer using the software Spectra Manager 2.08.04. An average of three scans was reported. All spectra were corrected by the subtraction of the buffer (background). All data was processed using OriginPro 7.5.

Circular dichroism melting curves were performed using a Peltier thermostated cell holder. The CD spectra were recorded at various temperatures from 60 °C to 5 °C, while equilibrating the sample at each temperature for 5 min. The intensity was plotted at λ = 228 nm for **2** and **3** as function of temperature. To obtained the melting temperatures T_m, the resulting normalized melting curves were fitted according to a two-state equilibrium folding model, as published previously.[1]

Cyclic voltammetry (CV)

Cyclic voltammetry was performed in an undivided 3-electrode cell under argon atmosphere with a Metrohm 663 VA stand equipped with a µ-Autolab type III potentiostat (Metrohm AG, Herisau, Switzerland). WE: electrode tip (glassy carbon), 2 mm in diameter; CE: glassy carbon rod, RE: Ag/AgCl (silver wire in 3 M KCl in Milli-Q H_2O) for measurements in H_2O; v = 50 mV/s. Prior to the measurement sample solutions were degassed with argon for at least 1-2 min.

High Performance Liquid Chromatography (HPLC)

Analytical and semipreparative reversed phased high performance liquid chromatography (HPLC) was performed on a LC-4000 system from JASCO (Tokyo, Japan). The set-up consisted of a binary pump system, an in-line degasser, dynamic mixer and a UV/VIS-detector. Signal detection was achieved between 200–450 nm. The system was operated with the JASCO ChromNAV (V 2.00.02) software. For analytical runs the samples were provided via an autosampler using a Merck Chromolith® RP-C18e, 100×4.6, 150 Å column with suitable gradients of acetonitrile (ACN) and water with auxiliary 0.1 v% TFA. Typical gradient conditions were 5 to 100% organics, if not stated otherwise. Samples for semipreparative purification were injected manually via Rheodyne® valve with a 2000 µL sample loop on a Luna C18-2, 250×30, 10 µm, 100 Å, Phenomenex (Torrance, USA) column. An automatic fraction collector CHF122SC from Advantec MFC Inc. (Dublin, USA) was used to collect the purified products. All separations were performed at room temperature if not stated otherwise. All solvents were used in HPLC quality: Ultrapure water was generated by a PURELAB flex 4 system from Veolia (Paris, France). Acetonitrile was purchased from Fisher Scientific (Hampton, USA) or Sigma Aldrich (St. Louis, USA).

2. Materials and Methods

All reactions involving air- and moisture-sensitive compounds or intermediates were performed under argon atmosphere using standard Schlenk techniques. The glassware was dried in an oven at 120 °C or heat gun dried under high vacuum prior to use. All reagents and solvents were added using disposable syringes and needles through septa. Solids were added using an argon or nitrogen counter flow. Degassing of solvents was achieved by performing multiple freeze-pump-thaw cycles until no more formation of gas bubbles could be observed. The vacuum level used for the removal of organic solvents was about 1 mbar and 0.1 mbar for the removal of water (Christ Alpha 1-2 LD plus freeze dryer).

Solvents and reagents

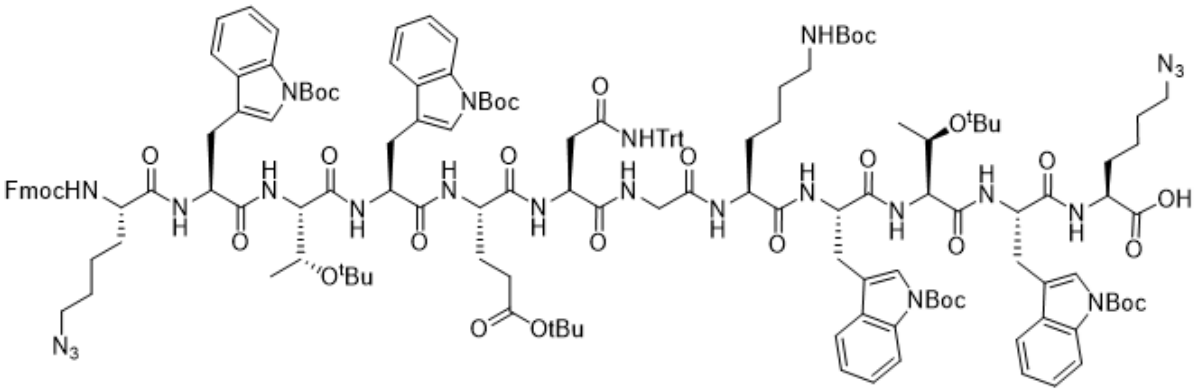
Unless stated otherwise, all solvents and reagents were obtained from commercial sources in the highest purity available and used without further purification. The list of suppliers includes SIGMA-ALDRICH (Sigma-Aldrich Chemie GmbH, Taufkirchen) ACROS ORGANICS (Thermo Scientific GmbH, Nidderau), MERCK (Merck KGaA, Darmstadt), ALFA AESAR (Alfa Aesar GmbH & Co. KG, Karlsruhe), CARBOLUTION Chemicals (Carbolution Chemicals GmbH, Saarbrücken), BACHEM (Bachem, Bubendorf) and IRIS BIOTECH (Iris Biotech GmbH, Marktredwitz). Water was demineralized prior to use. DMF and piperidine were purchased in peptide grade quality.

Chromatography

Qualitative thin layer chromatography was carried out on silica-coated aluminium sheets (60, F254) with a fluorescence indicator from MERCK. The indication of the analytes was achieved by irradiation of the TLC plates with UV light (λ = 254 nm). Alternatively, the plates were dipped into a $KMnO_4$, cerium molybdate or ninhydrin solution followed by heating. Size exclusion chromatography was performed using a Sephadex® LH-20 column with methanol as the eluent.

3. Synthesis

Fmoc-Lys(Az)-Trp(Boc)-Thr(^{t}Bu)-Trp(Boc)-Glu(^{t}Bu)-Asn(Trt)-Gly-Lys(Boc)-Trp(Boc)-Thr(^{t}Bu)-Trp(Boc)-Lys(Az)-OH (1)



The 2-chlorotrityl chloride resin (0.6 g, loading capacity 1.6 mmol/g) was put into a Merrifield flask under argon atmosphere and a solution of Fmoc-Lys(N_3)-OH (synthesized as previously reported)[2] (0.75 g, 1.9 mmol, 2.0 eq. relative to the resin loading capacity) in DCM (6 mL) and DMF (1 mL, all DMF in this procedure was used in SPPS grade) was added. DIPEA (0.33 mL, 1.9 mmol, 2.0 eq. relative to the resin loading capacity) was added and the reaction vessel was shaken for 5 min at room temperature (r.t.). Additional DIPEA (0.5 mL, 2.9 mmol, 3.0 eq. relative to the resin loading capacity) was added subsequently and the reaction vessel was shaken for 1 h at r.t. This was followed by the addition of MeOH (0.6 mL, 1 mL/g resin) and shaking of the reaction vessel for a further 15 min. Afterwards, the vessel was drained and the resin was washed with DCM, DMF, MeOH, DCM and dried under high vacuum overnight.

The automated step-wise chain elongation was performed using a CS136XT peptide synthesizer, which is an automated batch peptide synthesizer. The beads were swollen in DCM and DMF while shaking the reaction vessel. After draining a piperidine solution (20 % in DMF) was added and the vessel was shaken for 20 min. The piperidine solution was disposed and the resin was washed four times with DMF and twice with DCM. After the addition of the Fmoc-protected amino acid (4.0 eq. of resin loading capacity), HBTU (4.0 eq.), HOBt (4.0 eq.) and DIPEA (6.0 eq.) each in DMF were added to the reaction vessel. After shaking for one hour the solution was removed and the beads washed five times with DMF. A complete conversion of the primary amine to the corresponding amide was controlled via Kaiser test after each coupling step. This procedure was repeated for the following 11 amino acids. For the coupling of the Fmoc-Lys(N_3)-OH, only 3.0 eq. (6.0 eq. for DIPEA) were employed, and the reaction time was extended to 2 h. After the final step, the resin was washed with DCM.

The peptide was cleaved from the resin by shaking the beads in a 4:1 DCM (8 mL) and TFE (2 mL) mixture. The solution was drained and the beads were washed with DCM (2x 10 mL). The solution was concentrated under reduced pressure and the product precipitated in cold Et_2O (30 mL). The mixture was centrifuged, decanted and the remaining solvent was removed under high vacuum to isolate the product **1a** as a colorless solid (1.84 g, 0.65 mmol).

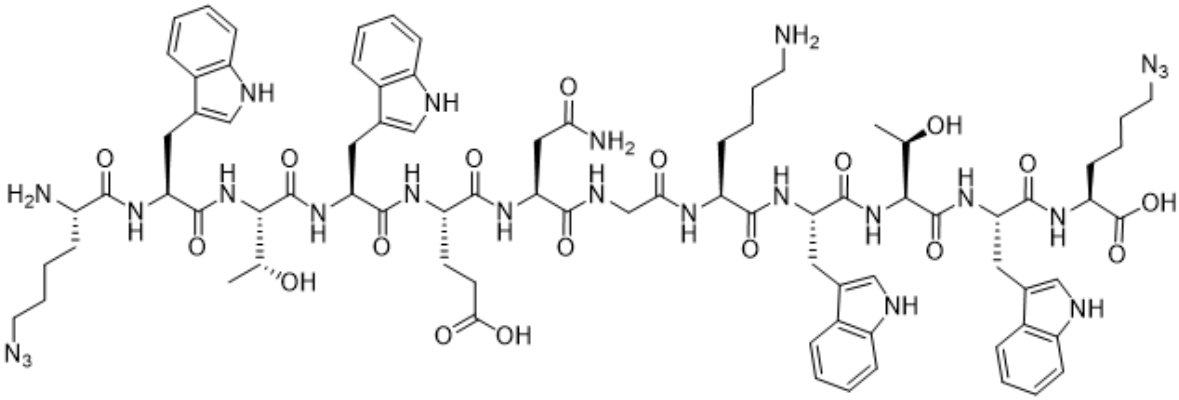
Molecular formula: $C_{152}H_{192}N_{24}O_{30}$.

^{1}H NMR (400 MHz, DMSO-d_6, 293 K): δ/ppm: 12.63 (brs, 1H, COO*H*), 8.66 (s, 1H, N*H*^{Asn}Trt), 8.33–6.91 (m, 55H, *CH*^{Ar}, α-N*H*), 6.72–6.63 (m, 1H, N*H*^{Boc}), 4.92–4.65 (m, 4H, α-C*H*, C*H*^{Fmoc},

CH_2^{Fmoc}), 4.52–3.64 (m, 12H, α-CH_2^{Gly}, α-C*H*), 3.25–3.18 (m, 4H, CH_2N_3) 3.17–2.62 (m, 14H, β-CH_2^{Trp}, β-CH^{Thr}, β-CH_2^{Asn}, ε-CH_2^{Lys}), 2.15 (t, *J* = 8.3 Hz, 2H, γ-CH_2^{Glu}), 1.75–0.78 (m, 100H, CH_3^{Boc}, CH_3^{t}Bu, CH_3^{Thr}, β-CH_2^{Glu}, CH_2^{Lys}, CH_2^{AzLys}).

ESI-HRMS (MeOH) (*m/z*): Calculated for $[C_{152}H_{192}N_{24}O_{30}Na_2]^{2+}$: 1439.7016, found: 1439.7028.

H-Lys(Az)-Trp-Thr-Trp-Glu-Asn-Gly-Lys-Trp-Thr-Trp-Lys(Az)-OH (2)



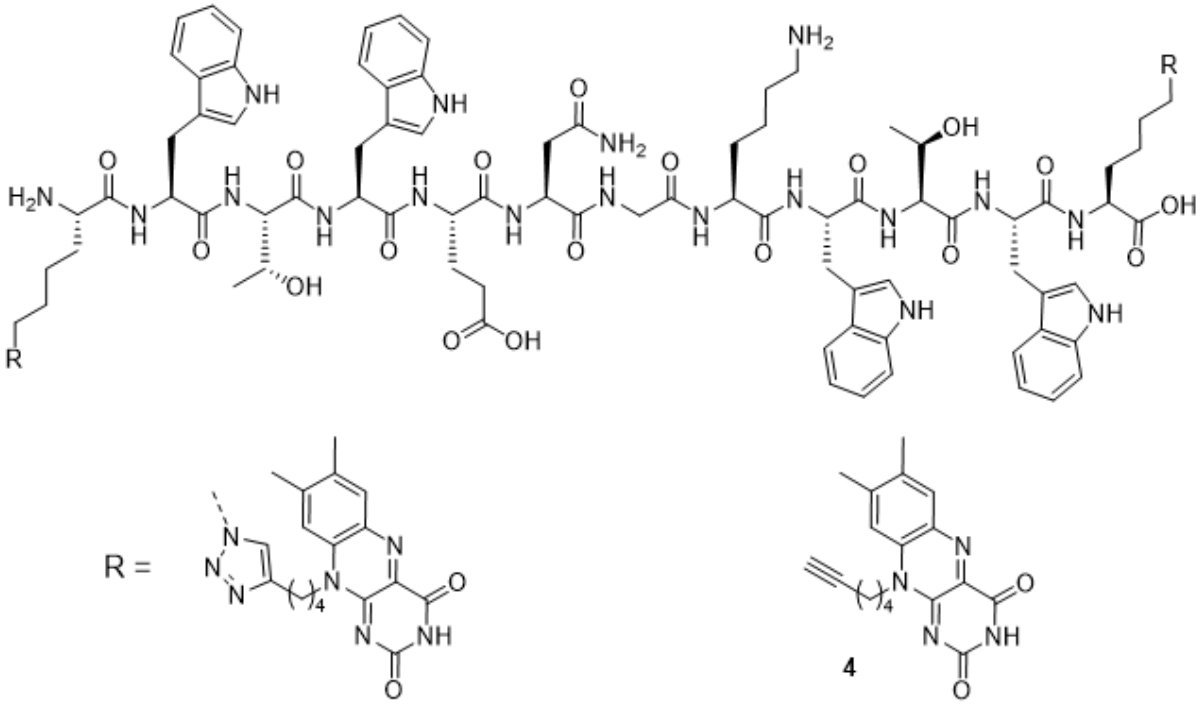
1 (200.0 mg, 70.5 µmol, 1.0 eq.) was dissolved in 5 mL of a 95:5 mixture of TFA (4.75 mL) and EDT (0.25 mL) and the solution was stirred for 30 min at room temperature. The solution was concentrated under reduced pressure and the product was precipitated in cold Et_2O (10 mL). The mixture was centrifuged, decanted and the remaining solvent was removed under high vacuum. The solid was taken up in DMF (4 mL) and piperidine (1 mL) was added. The solution was stirred for 30 min at room temperature. The solution was concentrated under reduced pressure and the product was precipitated in cold Et_2O (10 mL). The mixture was centrifuged, decanted and the remaining residue was purified by semipreparative RP-HPLC and subsequently lyophilized yielding in a colorless solid (50.0 mg, 29.4 µmol, 42%).

Molecular formula: $C_{81}H_{104}N_{24}O_{18}$.

^{1}H-NMR (600 MHz, DMSO-d_6, 293 K): 12.31 (s, 1H, COO*H*), 10.91–10.59 (m, 4H, NH^{Indol}), 8.61 (d, *J* = 7.9 Hz, 1H, NH^{Trp}), 8.26 – 8.13 (m, 3H, NH^{Trp} , NH^{AzLys}, NH^{Glu}), 8.12 – 7.98 (m, 4H, NH^{Thr}, NH^{Gly}, NH_2^{AzLys}), 7.94 (d, *J* = 8.2 Hz, 1H, NH^{Lys}), 7.92 – 7.88 (m, 2H, NH^{Trp}), 7.83 – 7.76 (m, 1H, NH^{Thr}), 7.70 – 7.53 (m, 7H, NH^{Asn}, CH^{Indol}, NH_2^{Lys}), 7.47 (s, 1H, CONH_2^{Asn}), 7.33 – 7.27 (m, 4H, CH^{Indol}), 7.18 – 7.10 (m, 4H, CH^{Indol}), 7.08 – 6.99 (m, 5H, CH^{Indol}, CONH_2^{Asn}), 6.98 –6.87 (m, 4H, CH^{Indol}), 5.10 – 4.94 (m, 2H, OH^{Thr}), 4.74 (td, J = 8.3, 4.5 Hz, 1H, α-CH^{Trp}), 4.66 – 4.52 (m, 4H, α-CH^{Trp}, α-CH^{Asn}), 4.38 – 4.20 (m, 4H, α-CH^{Thr}, α-CH^{Glu}, α-CH^{Lys}), 4.18 – 4.10 (m, 1H, α-CH^{AzLys}), 4.01 – 3.90 (m, 2H, β-CH^{Thr}), 3.82 (dd, J = 16.8 Hz, 6.1 Hz, 1H, CH_2^{Gly}), 3.74 – 3.68 (m, 1H, α-CH^{Lys}), 3.57 (dd, J = 16.8 Hz, 5.1 Hz, 1H, CH_2^{Gly}), 3.31 – 3.23 (m, 4H, CH_2N_3), 3.22 – 2.91 (m, 8H, β-CH_2^{Trp}), 2.73 – 2.66 (m, 2H, $CH_2NH_2^{Lys}$), 2.27 – 2.15 (m, 2H, γ-CH_2^{Glu}), 1.95 – 1.82 (m, 1H, β-CH_2^{Glu}), 1.77 – 1.53 (m, 7H, β-CH_2^{Glu}, β-CH_2^{Lys}, β-CH_2^{AzLys}), 1.52 – 1.41 (m, 6H, δ-CH_2^{Lys}, δ-CH_2^{AzLys}), 1.36 – 1.16 (m, 6H, γ-CH_2^{Lys}, γ-CH_2^{AzLys}), 1.03 – 0.92 (m, 6H, CH_3^{Thr}).

MALDI-ToF-MS (MeOH) (*m/z*): Calculated for $[C_{81}H_{104}N_{24}O_{18}Na]^+$: 1724.863, found: 1724.500.

H-Lys(Fla)-Trp-Thr-Trp-Glu-Asn-Gly-Lys-Trp-Thr-Trp-Lys(Fla)-OH (3)



2 (39.1 mg, 23.0 µmol, 1.0 eq.) and **4** (synthesized as previously reported)[3] (18.0 mg, 56.0 µmol, 2.5 eq.) were dissolved in DMSO (1.0 mL) in a dry Schlenk tube. The solution was degassed twice and CuI (26.0 mg, 138.0 µmol, 6.0 eq.) and TBTA (73.0 mg, 138.0 µmol, 6.0 eq.) were added under argon cross-flow. The mixture was stirred at 40 °C overnight, precipitated from Et_2O (10.0 mL), centrifuged and decanted. The remaining solid was purified by semi-preparative RP-HPLC and subsequently lyophilized yielding in a yellow solid (8.5 mg, 3.6 µmol, 16 %).

Molecular formula: $C_{117}H_{140}N_{32}O_{22}$.

^{1}H-NMR (400 MHz, DMSO-d_6, 293 K): δ/ppm: 11.38 – 11.23 (m, 2H, NH^{Fla}), 10.87 – 10.67 (m, 4H, NH^{Indol}), 8.57 – 6.72 (m, 43H, NH^{Trp},CH^{Ar}, α-NH, NH_2^{Asn}, NH_2^{Lys}, NH_2^{AzLys}), 4.78 – 3.64 (m, 23H, OH^{Thr}, α-CH_2^{Gly}, α-CH, β-CH^{Thr}, N^{Fla}CH_2, N^{Tria}-CH_2), 3.17 – 2.89 (m, 8H, β-CH_2^{Trp}), 2.71 – 2.64 (m, 2H, CH_2NH$_2^{Lys}$), 2.39 (s, 6H, CH_3^{Fla}), 2.23 – 2.16 (s, 2H, β-CH_2^{Asn}), 1.94 – 0.90 (m, 36H, CH_3^{Thr}, β-CH_2^{Glu}, CH_2^{Lys}, CH_2^{AzLys}, CH_2^{Fla}).

MALDI-ToF-MS (MeOH) (*m/z*): Calculated for $[C_{117}H_{140}N_{32}O_{22}Na]^+$: 2369.599, found: 2369.031.

4. Spectra and chromatograms

Figure S1: ^{1}H NMR spectrum of **2** in d_6-DMSO at 293 K. Asterisk denotes residual solvent resonance.

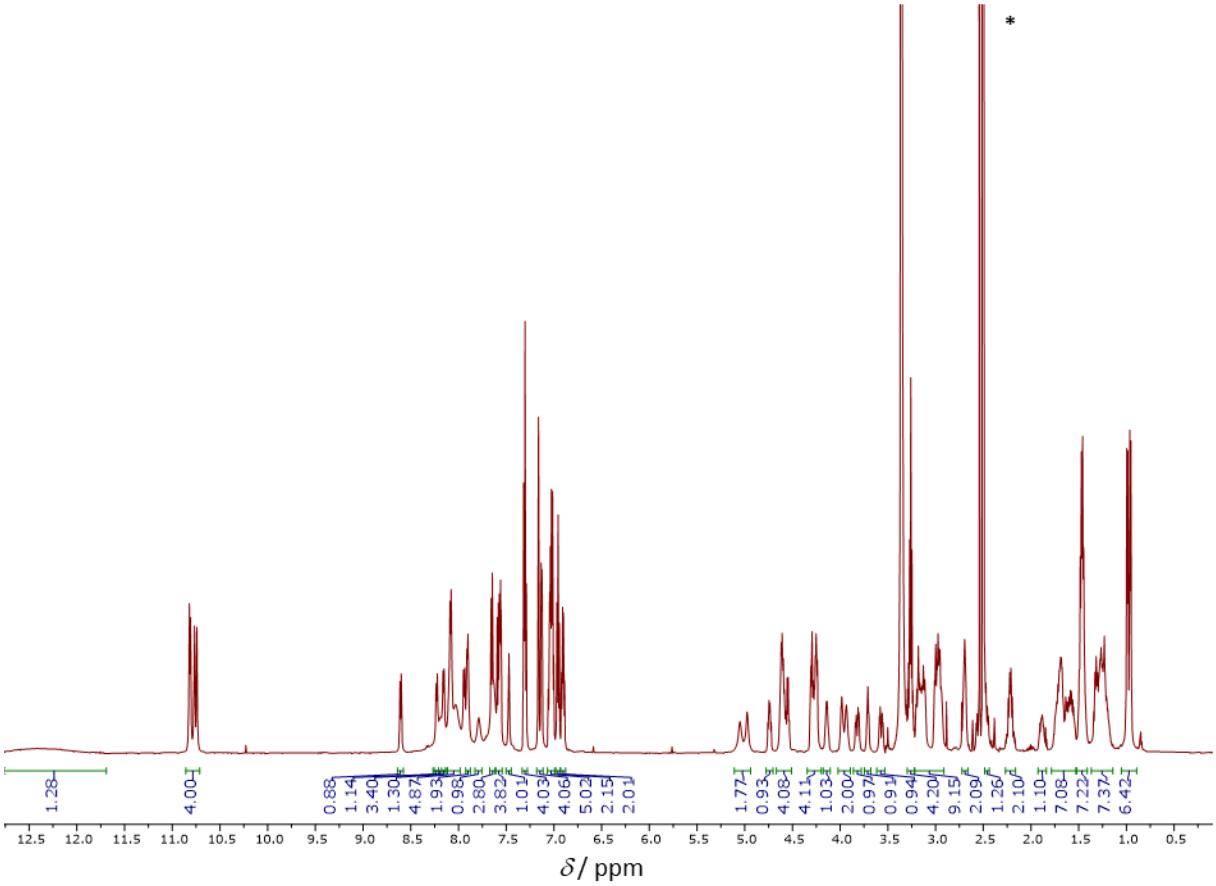


Figure S2: NOESY NMR spectrum of **2** in a d_2-HFIP / 10 mM phosphate buffer (in D_2O) mixture (35:65) at 293 K measured with water supression.

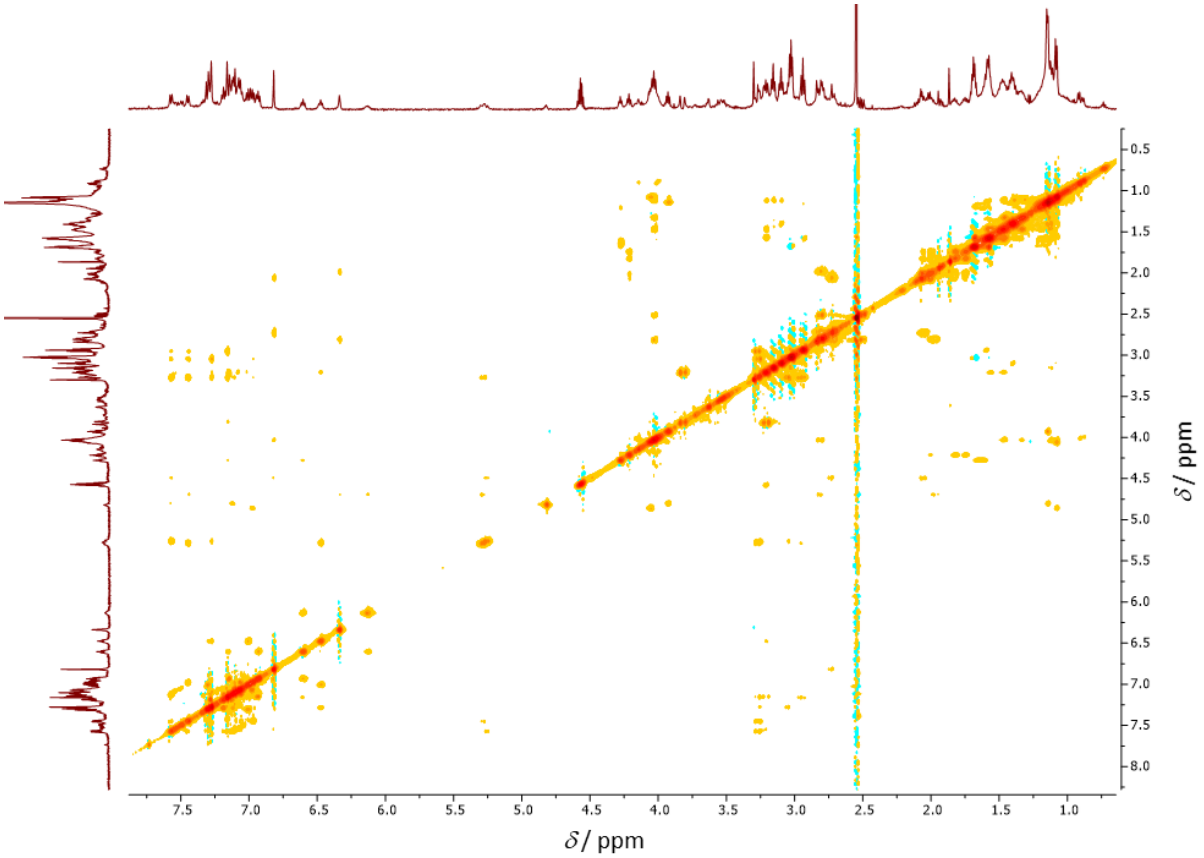


Figure S3: ^{1}H NMR spectrum of **3** in d_6-DMSO at 293 K. Asterisk denotes residual solvent resonance.

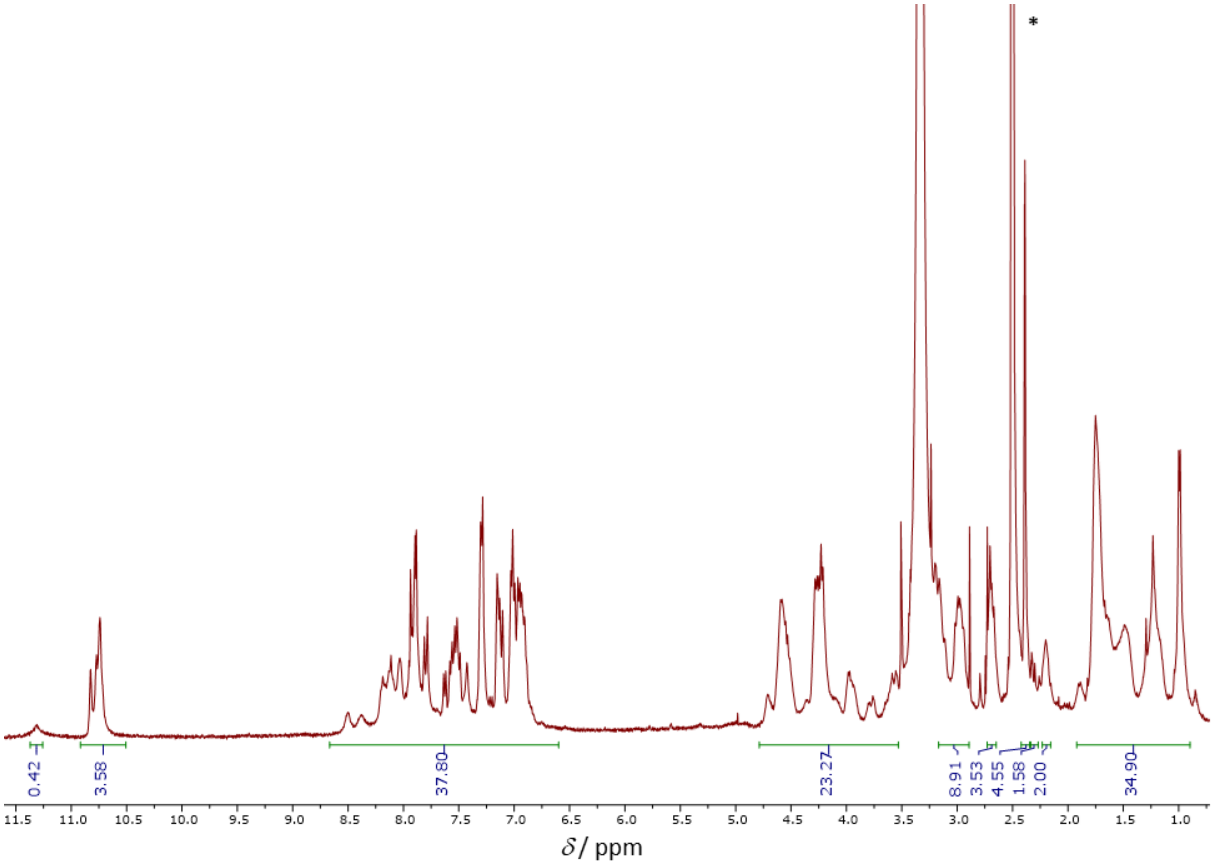


Figure S4: MALDI-TOF-MS of **2** embedded in a CHCA matrix. Top full spectrum; bottom section 1660–1740.

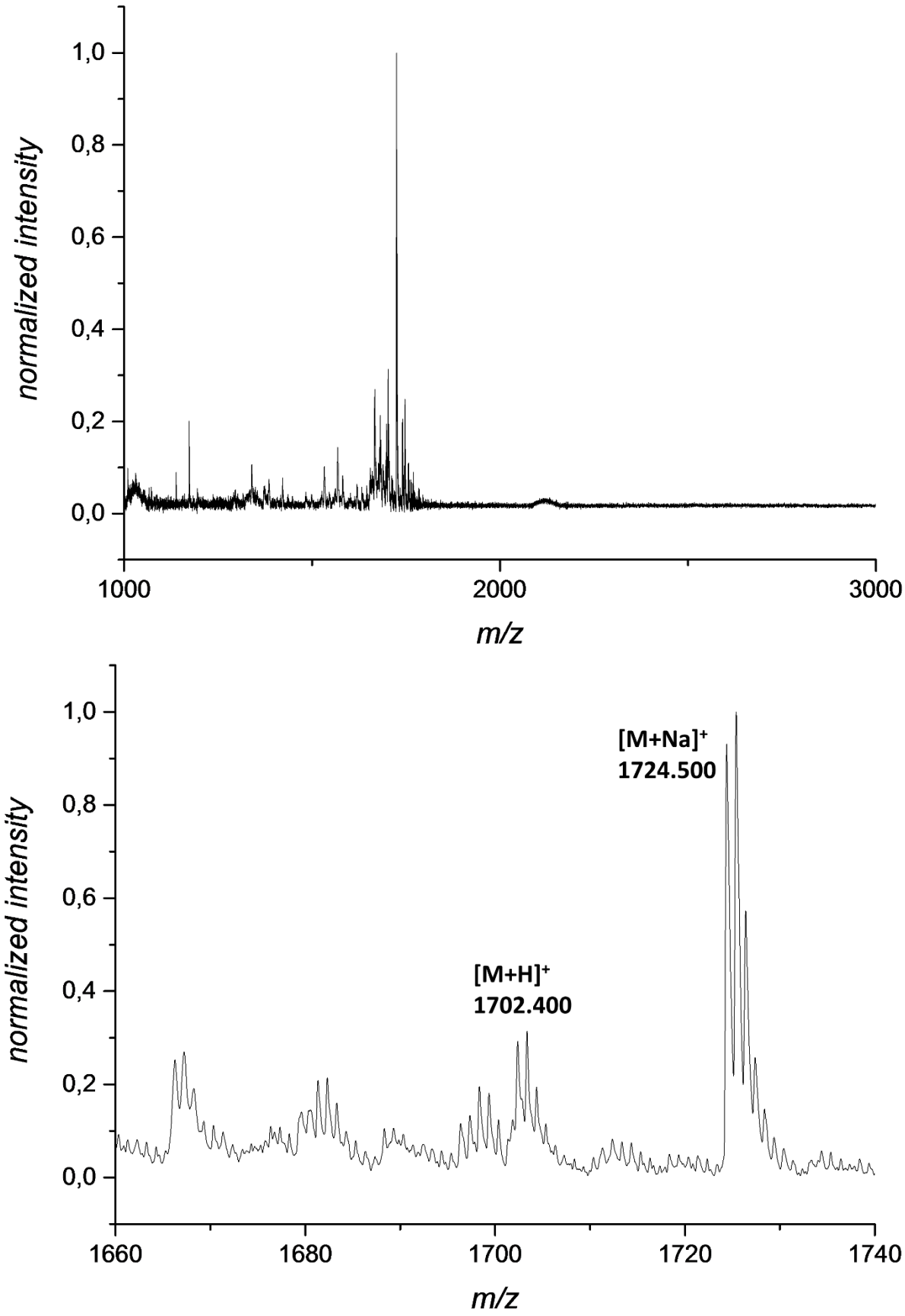


Figure S5: MALDI-TOF-MS of **3** embedded in a CHCA matrix. Top full spectrum; bottom section 1660–1740.

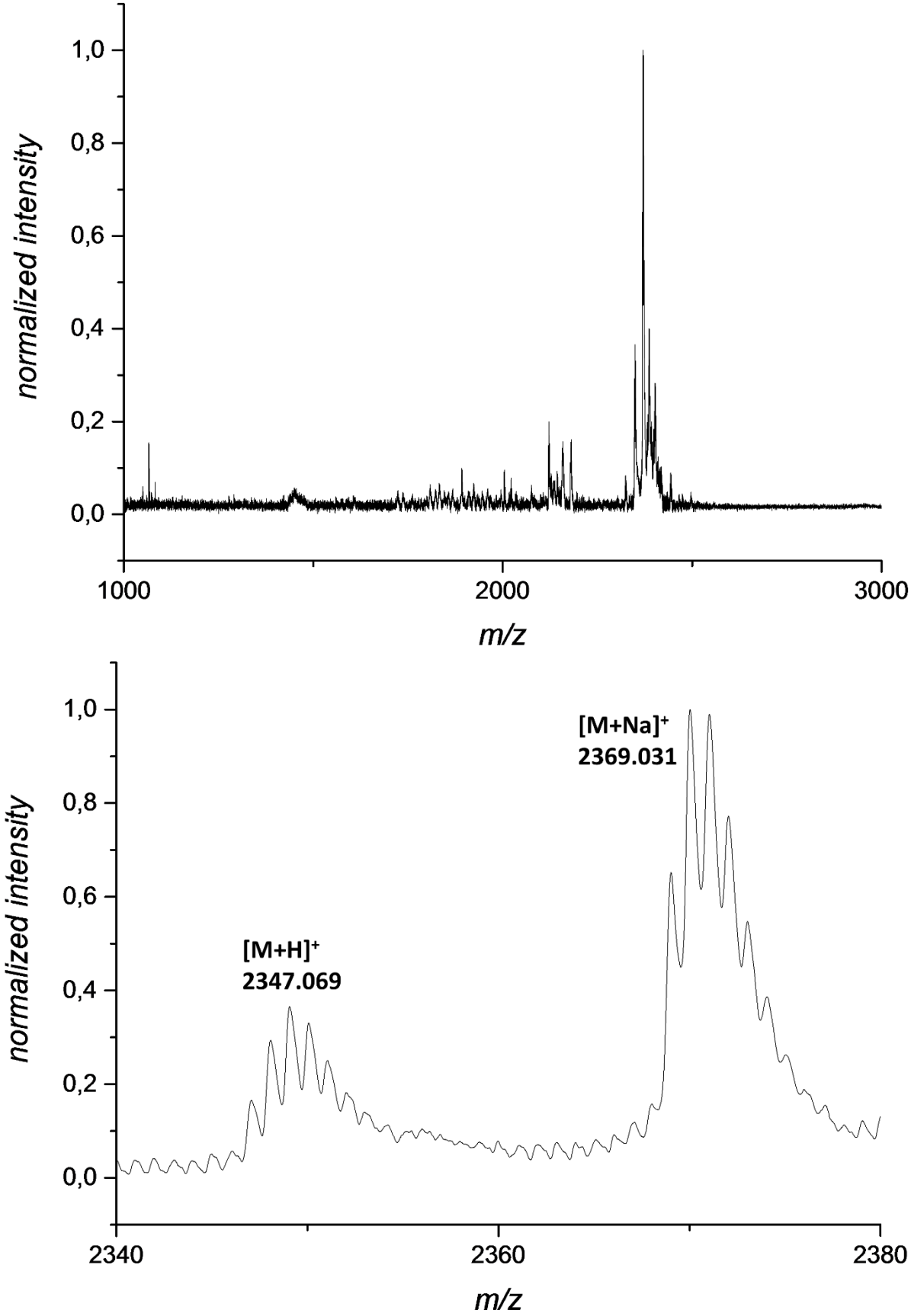


Figure S6: Analytic RP-HPLC chromatogram of **2**.

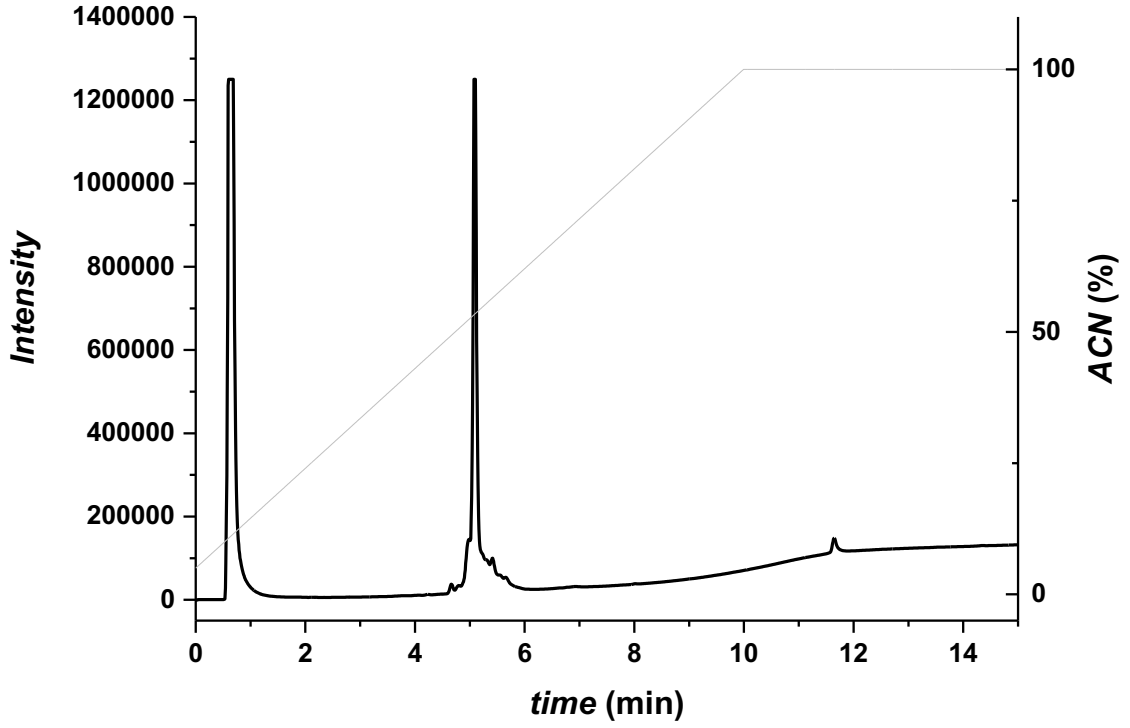


Figure S7: Analytic RP-HPLC chromatogram of **3**.

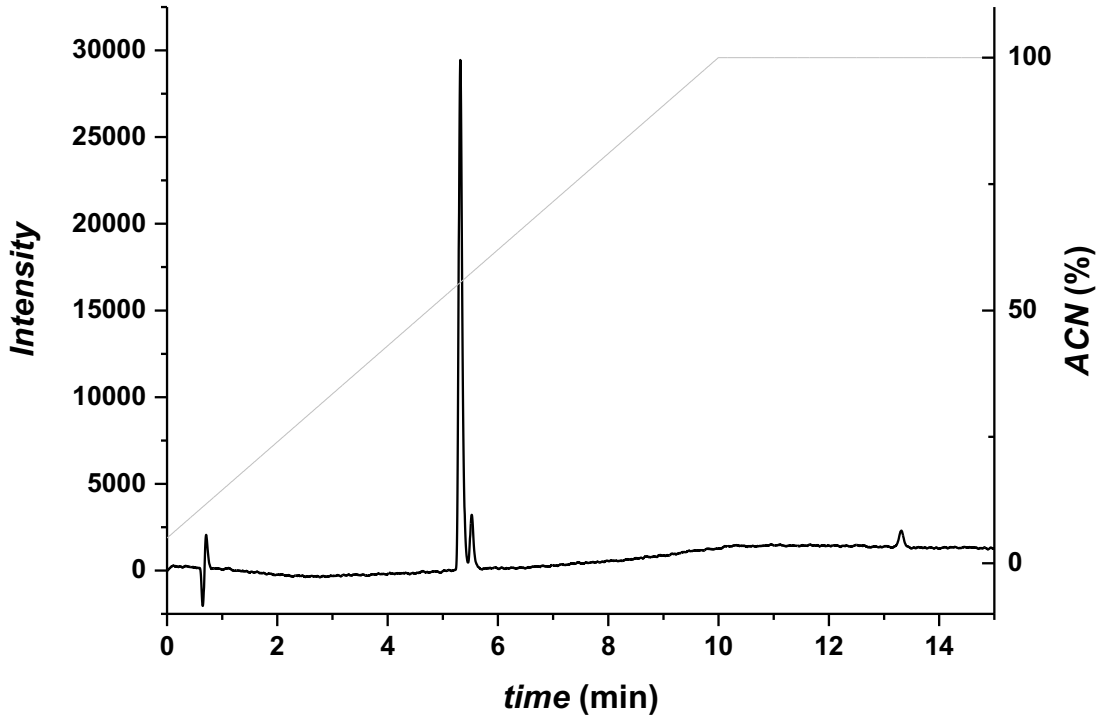


Figure S8: Temperature dependent CD spectra of **2**; all CD measurements were performed using a 50 µM solution of **2** in 10 mM phosphate buffer (pH 7.4)/HFIP (35 % v/v).

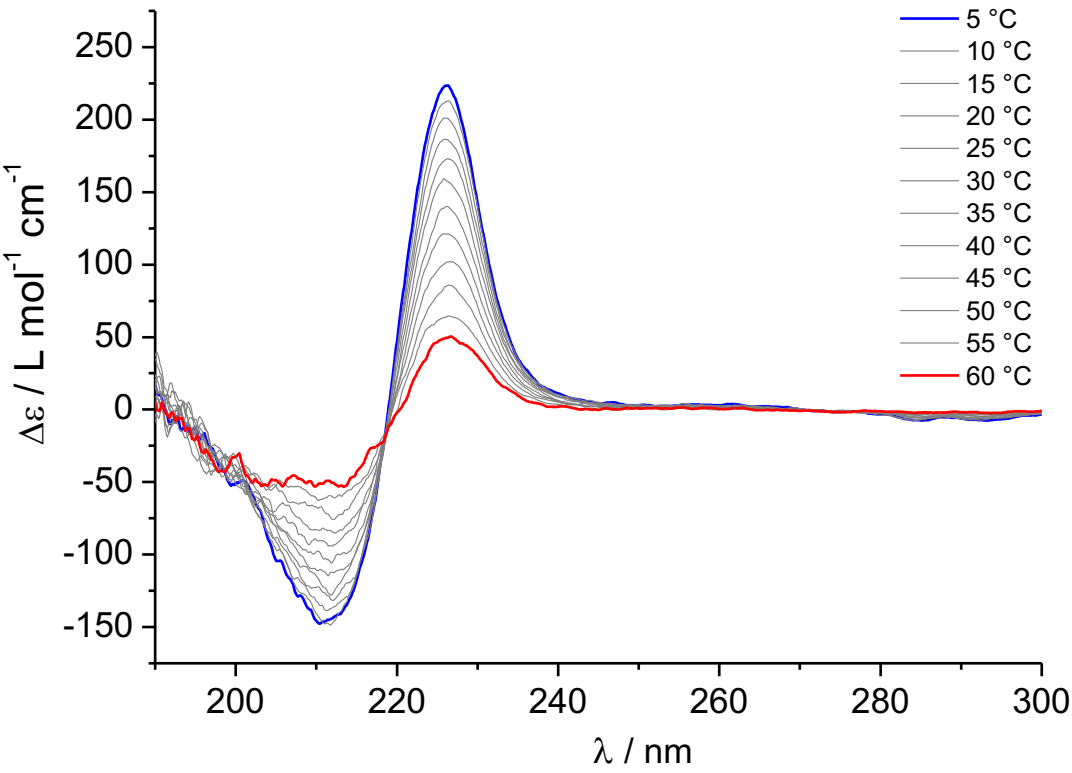
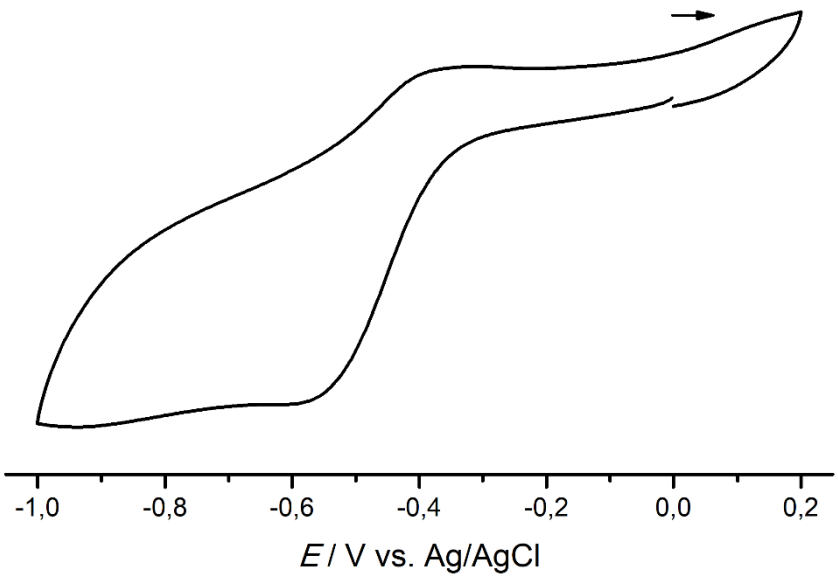


Figure S9: Cyclic voltammogram of **2** in 10 mM phosphate buffer / HFIP (20% v/v), using a sweep rate of 50 mV/s.



6. References

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2 C. Byrne, P. A. McEwan, J. Emsley, P. M. Fischer and W. C. Chan, *Chem. Commun.*, 2011, **47**, 2589–2591.

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