Supplementary Material (ESI) for New Journal of Chemistry
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Denaturation and Accelerated Proteolysis of Sizeable Heme Proteins by Synthetic Metalloporphyrins

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Supporting Information

General

Horse heart cytochrome c, horse skeletal myoglobin, bovine hemoglobin, azurin and α-lactalbumin were purchased from Sigma and used without further purification, unless otherwise stated. Trypsin, modified sequencing grade, was purchased from Roche. meso-Tetra-(4-carboxyphenyl)porphine was purchased from Strem. All other reagents were purchased from Aldrich or Novabiochem and used without further purification. All solvents were distilled immediately prior to use. NMR spectra were obtained on a Brucker 400 or 500 DPX spectrometer. UV-vis spectra were recorded on an Agilent A453 spectrometer.

2-(R)-[4’-(Benzyloxycarbonylaminoethyl)-benzoylaminol]-pentanedioic acid dimethyl ester (4).

To a suspension of 4-(benzyloxycarbonylamino-methyl)-benzoic acid (285 mg, 1 mmol) in freshly distilled CH₂Cl₂ (20 mL) was added N-methyl morpholine (220 μL, 2 mmol) and isobutyl chloroformate (130 μl, 1 mmol) at 0 °C. After 5 minutes, the solution had become homogeneous. H-Glu(OMe)-OMe.HCl (212 mg, 1 mmol) was added in one portion, followed by further N-methyl morpholine (100 μL, 1 mmol). The reaction was allowed to warm gradually to RT overnight. After 16 h, the reaction was diluted with CH₂Cl₂ (60 mL), washed with 0.1 M HCl (20 mL), sat. NaHCO₃ (20 mL), brine (20 mL), dried (Na₂SO₄), filtered and concentrated. The residue was dry-loaded onto silica gel, and purified by flash column chromatography, eluting with hexanes:EtOAc, 2:3 to afford the title compound as a white powder (429 mg, 97%).

\[ \delta^1H (400 MHz, CDCl₃) 2.11 – 2.21 (m, 1 H, CH₂CH₂CO₂CH₃), 2.29 – 2.37 (m, 1 H, CH₂CH₂CO₂CH₃), 2.41 – 2.57 (m, 2 H, CH₂CO₂CH₃), 3.67 (s, 3 H, CO₂CH₃), 3.79 (s, 3 H, CO₂CH₃), 4.45 (d, J = 6.0 Hz, 2 H, CH₃NHCbz), 4.79 – 4.84 (m, 1 H, NHCHCO), 5.12 – 5.20 (m, 3 H, NHCbz, CH₂ (Cbz)), 7.03 (d, J = 7.6 Hz, 1 H, CONH), 7.31 – 7.42 (m, 7 H, Ph (Cbz), Ph), 7.79 (d, J = 8.0 Hz, 1 H, CONH), 7.89 (d, J = 8.0 Hz, 1 H, CONH), 7.93 (d, J = 8.0 Hz, 1 H, CONH), 8.07 (d, J = 8.0 Hz, 1 H, CONH), 8.11 (d, J = 8.0 Hz, 1 H, CONH).]}

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2 H, Ph); δ_C (100 MHz, CDCl3) 27.1, 30.2 (CH2CH2CO2CH3), 44.7 (NHCHCO), 51.9, 52.3, 52.6 (2 CO2CH3, CH2NHCbz), 67.0 (CH3 (Cbz)), 127.5, 128.1, 128.2, 128.5, 133.7, 136.4, 142.6 (9 CH & 3 C Ph (Cbz), Ph)), 156.4 (CH2CONH), 166.7 (CONH), 172.5 (CO2CH3), 173.6 (CO2CH3); m/z (ESI) 443 (M + H).

2-(R)-(4’-Aminomethyl-benzoylamino)-pentanedioic acid dimethyl ester (11).

H2 was bubbled through a solution of 4 (300 mg, 0.68 mmol) and 10% Pd/C (30 mg) in absolute MeOH (12 mL) for 15 mins, then the reaction mixture was stirred under a balloon of H2 for 2 h. After this time, the reaction was filtered through Celite, washing with MeOH. The filtrate was concentrated, then purified by silica gel flash column chromatography (eluent: Solvent A:CH2Cl2, 1:1), to afford 11 as a sticky oil (199 mg, 95%): δ_H (400 MHz, d4-MeOD) 2.05 – 2.15 (m, 1 H, CH2CH2CO2CH3), 2.25 – 2.34 (m, 1 H, CH2CH2CO2CH3), 2.50 (t, J = 7.2 Hz, 2 H, CH2CO2CH3), 3.65 (s, 3 H, CO2CH3), 3.75 (s, 3 H, CO2CH3), 4.63 – 4.67 (m, 1 H, NHCHCO), 4.85 (s, 2 H, CH2NH2) 7.45 (d, J = 8.0 Hz, 2 H, CH (Ph)), 7.83 (d, J = 8.4 Hz, 2 H, CH (Ph)); δ_C (100 MHz, d4-MeOD) 27.4, 31.3 (CH2CH2CO2CH3), 46.3 (NHCHCO), 52.3, 52.9, 53.7 (2 CO2CH3, CH2NH2), 128.5, 128.8, 133.6 (4 CH & 2 C (Ph)), 170.2 (CONH), 173.7 (CO2CH3), 174.9 (CO2CH3); m/z (ESI) 309 (M + H).

_meso-Tetrakis-[4-carboxyphenyl-2’-(R)-(4”-aminomethyl-benzoylamino)-pentanedioic acid dimethyl ester] Porphyrin (1).

To a stirring suspension of _meso-tetrakis-4-(carboxyphenyl)porphine_ (18; 138 mg, 0.174 mmol) in freshly distilled CH2Cl2 (30 mL) was added oxalyl chloride (542 μL, 6.10 mmol) and catalytic DMF (1 drop). The reaction mixture was allowed to stir for 16 h at RT, then concentrated under vacuum, followed by drying under high vacuum for a further 8 h. The residue was re-dissolved in anhydrous THF (12 mL) to which a solution of 11
(220 mg, 0.714 mmol) and DIPEA (246 μL, 1.43 mmol) in anhydrous CH_2Cl_2 (20 mL) was added via cannular at 0 °C. After 15 min., the reaction was complete. The reaction mixture was dry-loaded onto silica gel and purified twice by flash column chromatography, the first time eluting with 10% MeOH / CH_2Cl_2, then the second time eluting with CH_2Cl_2:MeOH:NH_4OH, 96:3.5:0.5, to give the title compound as a purple solid (160 mg, 47%): \( \delta^H \) (400 MHz, \( d_6 \)-DMSO) -2.91 (br s, 2 H, Ar), 1.99 – 2.23 (m, 8 H, CH_2CH_2CO_2CH_3), 2.42 – 2.52 (m, 8 H, CH_2CO_2CH_3), 3.61 (s, 12 H, CO_2CH_3), 3.68 (s, 12 H, CO_2CH_3), 4.46 – 4.55 (m, 4 H, NHCH), 4.69 – 4.79 (m, 8 H, CONHCH), 7.59 (d, \( J = 8.0 \) Hz, 8 H, CH (Ph)), 7.95 (d, \( J = 8.0 \) Hz, 8 H, CH (Ph)), 8.34 (app s, 16 H, Ar), 8.80 (d, \( J = 7.6 \) Hz, 4 H, CONHCH), 8.90 (s, 8 H, Ar), 9.49 – 9.58 (m, 4 H, CONHCH); MALDI-TOF MS \( m/z \) 1953 [M + H]^+; \( \text{UV-Vis (H}_2\text{O, 5 mM Na}_2\text{HPO}_4, pH 7.4) } \lambda 416, 524, 563, 594, 648 \text{ nm.} \)

**meso-Tetakis-[4-carboxyphenyl-2’-(R)-(4”-aminomethyl-benzoylamino)-pentanedioic acid] Porphyrin Dihydrochloride Salt (1a).**

LiOH.H_2O (12.0 mg, 287 μmol) was added to a stirring solution of 1 (40 mg, 20.5 μmol) in THF-MeOH-H_2O (3:2:1; 1.6 mL) at 0 °C. After 5 h, the reaction was neutralized with 1 N HCl, then all solvents were removed \textit{in vacuo}, and the residue was re-dissolved in H_2O (ca. 10 mL). The crude product was precipitated by the addition of 1 N HCl, then centrifuged and the supernatant was removed. The pellet was re-suspended in 1 N HCl (10 mL), centrifuged and decanted, and this procedure was repeated twice. The pellet was dissolved in CH_3CN:H_2O, 1:1 and lyophilized to give the title compound as a fluffy, green powder: \( \delta^H \) (400 MHz, \( d_6 \)-DMSO) 1.94 – 2.06 (m, 4 H, CH_2CH_2CO_2H), 2.08 – 2.19 (m, 4 H, CH_2CH_2CO_2H), 2.33 – 2.44 (m, 8 H, CH_2CO_2H), 4.37 – 4.50 (m, 4 H, NHCHCO), 4.68 – 4.79 (m, 8 H, CONHCH), 7.59 (d, \( J = 8.4 \) Hz, 8 H, CH (Ph)), 7.95 (d, \( J = 8.4 \) Hz, 8 H, CH (Ph)), 8.34 (s, 16 H, Ar), 8.64 (d, \( J = 8.0 \) Hz, 4 H, CONHCH), 8.90 (s, 8 H, Ar), 9.51 – 9.58 (m, 4 H, CONHCH); MALDI-TOF MS \( m/z \) 1841 [M + H]^+; \( \text{UV-Vis (H}_2\text{O, 5 mM Na}_2\text{HPO}_4, pH 7.4) } \lambda 416, 524, 563, 594, 648 \text{ nm.} \)
Copper (II) meso-Tetrakis-[4-carboxyphenyl-2'-(R)-(4”-aminomethyl-benzoylamino)-pentanedioic acid] Porphyrin (1b).

Porphyрин 1a (9.7 mg, 4.69 μmol) was dissolved in spectrophotometric grade MeOH (50 mL). Copper (II) chloride (97 mg, 723 μmol) was added. The reaction mixture was stirred for 3 h under reflux, after which time a red precipitate had formed and all green colour had vanished from the solution. The solvent was reduced to around 5 mL under vacuum, then an equal volume of H2O was added. The reaction mixture was centrifuged and decanted. The pellet was re-suspended in 0.01 N HCl (10 mL), centrifuged and decanted, and this process was repeated a further two times. The pellet was lyophilized from CH3CN:H2O, 1:1 to furnish the title compound as a brick red, fluffy powder (50%): MALDI-TOF MS m/z 1902 [M + H]⁺; UV-Vis (H2O, 5 mM NaH2PO4, pH 7.4) λ 416, 547, 586 nm.

Porphyрин 19a.

δH (400 MHz, d6-DMSO) –2.91 (br s, 2 H), 3.94 – 3.97 (m, 8 H), 4.68 – 4.74 (m, 8 H), 7.58 (d, J = 8.4 Hz, 8 H), 7.93 (d, J = 8.4 Hz, 8 H), 8.37 (br s, 16 H), 8.62 – 8.72 (m, 12 H), 9.48 – 9.54 (m, 4 H); MALDI-TOF m/z 1551.5 [M + H]⁺.
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Porphyrin 20a.

\[
\delta_1 (500 \text{ MHz}, d_6\text{-DMSO}) -2.93 \text{ (br s, 2 H), 3.79 – 3.85 (m, 8 H), 4.48 - 4.52 (m, 4 H), 4.68 – 4.76 (m, 8 H), 7.58 (d, } J = 8.3 \text{ Hz, 8 H), 7.95 (d, } J = 8.3 \text{ Hz, 8 H), 8.32 – 8.38 (m, 16 H), 8.42 (d, } J = 8.0 \text{ Hz, 4 H), 8.88 (s, 8 H), 9.52 (t, } J = 5.5 \text{ Hz, 4 H); MALDI-TOF } m/z 1672.5 \text{ [M + H]}^+.
\]

Porphyrin 21a.

\[
\delta_1 (400 \text{ MHz}, d_6\text{-DMSO}) -2.93 \text{ (br s, 2 H), 2.97 (dd, } J = 13.8, 11.3 \text{ Hz, 4 H), 3.08 (dd, } J = 13.8, 4.4 \text{ Hz, 4 H), 4.50 – 4.52 (m, 4 H), 4.53 – 4.55 (m, 8 H), 6.66 (d, } J = 8.4 \text{ Hz, 8 H), 7.12 (d, } J = 8.4 \text{ Hz, 8 H), 7.55 (d, } J = 8.0 \text{ Hz, 8 H), 7.86 (d, } J = 8.0 \text{ Hz, 8 H), 8.37 \text{ (br s, 16 H), 8.64 (d, } J = 8.0 \text{ Hz, 4 H), 8.89 (s, 8 H), 9.51 (t, } J = 5.6 \text{ Hz, 4 H); MALDI-TOF } m/z 1976.7 \text{ [M + H]}^+.
\]
Porphyrin 22a.

\[ \delta_H (400 \text{ MHz, } d_6-\text{DMSO}) -2.92 \text{ (br s, 2 H), 3.19 (dd, } J = 14.5, 9.8 \text{ Hz, 4 H), 3.30 (dd, } J = 14.5, 5.0 \text{ Hz, 4 H), 4.66 - 4.74 \text{ (m, 8 H), 4.76 - 4.82 (m, 4 H), 7.43 (s, 4 H), 7.56 (d, } J = 8.2 \text{ Hz, 8 H), 7.87 (d, } J = 8.2 \text{ Hz, 8 H), 8.34 (br s, 16 H), 8.82 (d, } J = 8.0 \text{ Hz, 4 H), 8.88 (s, 8 H), 8.99 (s, 4 H), 9.49 - 9.55 \text{ (m, 4 H); MALDI-TOF } m/z 1872.7 [M + H]^+ . \]

Porphyrin 23a.

\[ \delta_H (400 \text{ MHz, } d_6-\text{DMSO}) -2.90 \text{ (br s, 2 H), 3.16 - 3.36 (m, 8 H), 4.66 - 4.72 (m, 12 H), 7.00 (t, } J = 7.7 \text{ Hz, 4 H), 7.07 (t, } J = 7.7 \text{ Hz, 4 H), 7.23 (s, 4 H), 7.33 (d, } J = 7.7 \text{ Hz, 4 H), 7.46 (d, } J = 7.6 \text{ Hz, 8 H), 7.62 (d, } J = 7.7 \text{ Hz, 4 H), 7.88 (d, } J = 7.6 \text{ Hz, 8 H), 8.29 - 8.40 \text{ (m, 16 H), 8.63 (d, } J = 7.6 \text{ Hz, 4 H), 8.88 (s, 8 H), 9.45 - 9.53 \text{ (m, 4 H), 10.82 (m, 4 H); MALDI-TOF } m/z 2068.7 [M + H]^+ . \]

Porphyran 24a.

\[
\begin{align*}
\delta_H (400 \text{ MHz}, d_6-\text{DMSO}) & \quad -2.90 \text{ (br s, 2 H)}, 1.48 - 1.50 \text{ (m, 8 H)}, 1.51 - 1.53 \text{ (m, 8 H)}, \\
& \quad 1.56 - 1.58 \text{ (m, 8 H)}, 2.75 - 2.85 \text{ (m, 8 H)}, 4.38 - 4.45 \text{ (m, 4 H)}, 4.70 - 4.75 \text{ (m, 8 H)}, \\
& \quad 7.58 \text{ (d, } J = 8.0 \text{ Hz, 8 H)}, 7.66 \text{ (br s)}, 7.95 \text{ (d, } J = 8.0 \text{ Hz, 8 H}), 8.37 \text{ (s, 16 H)}, 8.61 \text{ (d, } J = 7.6 \text{ Hz, 4 H)}, 8.88 \text{ (s, 8 H)}, 9.50 - 9.55 \text{ (m, 4 H)}; \text{MALDI-TOF } m/z \quad 1836.8 \quad [\text{M + H}]^+.
\end{align*}
\]

Standardisation of Protein Solutions. Stock solutions of proteins and porphyrins were prepared in 5 mM NaH2PO4 buffer, pH 7.4 and the pH was re-adjusted to 7.4 with 1 N solutions of HCl and NaOH as required. The concentration of proteins was determined from the absorbance at the following wavelengths (nm): cytochrome c, \( \varepsilon_{550} = 29500 \text{ M}^{-1} \text{ cm}^{-1} \) (after reduction to Fe(II) with excess sodium dithionite); myoglobin \( \varepsilon_{422} = 116000 \text{ M}^{-1} \text{ cm}^{-1} \) (as ferric cyanide derivative\(^2\)) and hemoglobin \( \varepsilon_{540} = 12500 \text{ M}^{-1} \text{ cm}^{-1} \) (as ferric cyanide derivative). Trypsin solutions were prepared by dissolving the enzyme in 0.1 N HCl at a concentration of 1 mg/mL and used fresh.

Screening Procedure. The free base porphyrins (1a, 19a – 22a, 24a) were dissolved in 5 mM NaH2PO4, pH 7.4 to a concentration of 0.25 mM. The pH was re-adjusted back to 7.4, if necessary, with 1 N solutions of HCl and NaOH. Porphyrin 23a was insoluble and was not studied further. The heme proteins cyt c, myoglobin and hemoglobin were prepared as described above such that the final concentration in the CD cuvette would be 20 \( \mu \text{M} \). Porphyrins 1a, 19a – 22a, 24a were then screened for their abilities to denature these proteins at a concentration of 40 \( \mu \text{M} \) by conducting thermal denaturation experiments as described below. Of the free base porphyrins, 1a was the most potent denaturant in that it caused the greatest reduction in protein melting temperature of cyt c and myoglobin (>15 °C) at 222 nm, and caused a reduction of around 20 mdeg in the CD signal of hemoglobin at 222 nm and at 25 °C. Then, the copper derivative (1b) of 1a was prepared, and as reported previously was found to be more potent than the free base 1a, therefore 1b was selected as the focus of this study.

\(^2\) The protein stock solution was titrated into an aqueous solutions of NaHCO3 (0.1%), K3[Fe(CN)6] (0.02%) and KCN (0.005%); Drabkin, D. L. and Austin, J. H., J. Biol. Chem. 1935, 112, 51 - 65.
Thermal Denaturation Experiments. For CD thermal denaturation studies, 400 μL of 20 μM protein in 5 mM NaH₂PO₄ buffer at pH 7.4 and various equivalents of porphyrins were briefly vortexed, then placed in 1 mm quartz CD cells. The CD signal at 222 nm was recorded every 1 °C with an averaging time of 15 s, from 25 °C to 100 °C. The heating rate used was 10 °C/min, with the constraint that the temperature had to remain within 0.5 °C of the target temperature for one minute before data was recorded, to ensure a smooth heating rate.

For CD wavelength studies, 400 μL of 20 μM protein in 5 mM NaH₂PO₄ buffer at pH 7.4 and various equivalents of porphyrins were briefly vortexed, then placed in 1 mm quartz CD cells. The CD signal at 222 nm was recorded every 1 nm at 25 °C from 250 nm to 190 nm, with an averaging time of 15 s.

Protein Controls that show little or no change in protein melting temperatures in the presence of 1b.

Proteolysis Experiments. For CD kinetic studies, 400 μL of 20 μM protein in phosphate buffer (5 mM NaH₂PO₄, 50 mM NaCl and pH 7.4) and various equivalents of porphyrins were equilibrated to 37 °C for 30 min. Next, 8 μL of a trypsin solution (1 mg/mL in 0.1 N HCl) was added and the samples were briefly vortexed, then placed in 1 mm quartz CD cells pre-equilibrated to 37 °C inside the CD sample holder. The CD signal at 222 nm was recorded every minute with an averaging time of 15 s for 20 h.

For SDS-PAGE analyses, 100 μL of protein (20 μM) and porphyrin (80 μM) were incubated for 30 min at 37 °C (5 mM NaH₂PO₄, 50 mM NaCl and pH 7.4). Trypsin (2 μL of a 1 mg/ml in 0.1 N HCl) was added to the reaction mixture. At appropriate intervals, 10 μL of the solution was removed from the reaction mixture and quenched by the addition to 20 μL tris sample buffer (Biorad 2%), followed by heating to 95 °C for 5 min. At the end of the reaction, the samples were loaded onto 16.5% tris/tricine gels. The sizes of the markers used for gels 5a and 5b were 26.6 kDa, 17.0 kDa, 14.4 kDa, 6.5 kDa, 3.6 kDa, 1.4 kDa (Biorad, catalog no.: 161-0326, used according to the manufacturer’s directions). The sizes of the markers used for gel 5c were 201.1 kDa, 115.7 kDa, 93.6
kDa, 50.4 kDa, 37.4 kDa, 29.0 kDa, 19.4 kDa, 6.9 kDa (Biorad, catalog no.: 161-0318, used according to the manufacturer’s directions). The gels were run with a constant voltage of 100 V for 2 h (running buffer 10x SDS tris/tricine). The gels were washed with water (5 min), GelCode Blue stain reagent (Pierce) (2 h), then finally water again (2 h). Finally, the gels were dried in the standard manner, then imaged using a digital camera.

Protein Controls that show little or no change in proteolysis rates in the presence of 1b.