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Supporting Information

Selective Protein-Surface Sensing Using Ruthenium (II) Tris-(Bipyridine) Complexes

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General Considerations

All reagents were obtained from Aldrich, Alfa Aesar, Acros or Fluka and used without further purification. All solvents used were HPLC grade. Dry solvents were distilled from sodium/benzophenone (THF, Et_2O) or calcium hydride (CH_2Cl_2) immediately prior to use. Analytical TLC was performed using 0.2 mm silica gel 60 F_{254} pre-coated aluminium sheets (Merck) and visualised using UV irradiation or, in the case of amine intermediates, by staining with a ninhydrin solution. Flash column chromatography was carried out on silica gel 60 (35 to 70 micron particles, FluoroChem). Solvent ratios are described where appropriate. Solvents were removed under reduced pressure using a Buchi rotary evaporator at diaphragm pump pressure. Samples were freed of remaining traces of solvents under high vacuum. ^{1}H and ^{13}C NMR spectra were measured on a Bruker DPX300 or a Bruker Avance 500 spectrometer using an internal deuterium lock. Chemical shifts are reported in parts per million (ppm) downfield from TMS in δ units and coupling constants are given in hertz (Hz). Coupling constants are reported to the nearest 0.1 Hz. TMS is defined as 0 ppm for ^{1}H NMR spectra and the centre line of the triplet of $CDCl_3$ was defined as 77.10 ppm for ^{13}C NMR spectra. When describing ^{1}H NMR data the following abbreviations are be used; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, app = apparent and br = broad. Melting points were determined using a Griffin D5 variable temperature apparatus and are uncorrected. Microanalyses were obtained on a Carlo Erba Elemental Analyser MOD 1106 instrument, found composition is reported to the nearest 0.05%. Infrared spectra were recorded on a Perkin-Elmer FTIR spectrometer and samples analysed as solids (unless stated). Mass spectra (HRMS) were recorded in house using a Micromass GCT Premier, using electron impact ionisation (EI) or a Bruker Daltonics micrOTOF, using electro-spray ionisation (ESI). LC-MS experiments were run on a Waters Micromass ZQ spectrometer, samples ionised by ESI and analysed by a time-of-flight mass spectrometer, or a Bruker Daltronics esquire^{TM} series spectrometer, samples ionised by ESI and analysed by a quadrupole ion trap mass spectrometer. All experiments were run through a C18 column on an acetonitrile/water gradient (typically 0-100% acetonitrile over 3 minutes).

Stock solutions of receptors were prepared to a concentration of 1 mM in 5 mM phosphate buffer. Once the receptors had been dissolved, the pH was adjusted to 7.4

via the addition of 1*N* sodium hydroxide or 1*N* HCl. Likewise, stock solutions of hen egg white lysozyme, horse skeletal myoglobin, horse heart cyt *c*, spinach ferredoxin and horse radish peroxidase (all obtained from Sigma and used without further purification) were prepared to a concentration of ~1 mM in 5 mM sodium phosphate buffer and the pH adjusted to 7.4 *via* the addition of 1*N* sodium hydroxide or 1*N* HCl and diluted appropriately. The concentration of protein in the stock solution was verified from the absorbance at 280nm in 6 M GdmHCl (extinction coefficients and pI's were calculated using the program 'ProtParam' available at www.expasy.org). The concentrations of horse heart cyt *c* and acetylated cyt *c* were determined using the molar extinction coefficient at 550 nM of 2.95×10^4 after reduction using dithionite.

Synthetic Procedures

General procedure for isophthalic acid coupling to an amine

5-Nitroisophthaloyl dichloride (1 eq.) was dissolved in dry dichloromethane and added dropwise to a stirred solution of amine (2.2 eq.) and triethylamine (2.2 eq.) in dry dichloromethane at 0^oC under N_2. The reaction mixture was allowed to warm to room temperature and stirring continued for between 2.5 - 24 hrs, where upon TLC showed the reaction to be complete. The reaction mixture was washed with 1*N* hydrochloric acid, saturated sodium bicarbonate and saturated sodium chloride. The organic layer was dried over sodium sulphate, concentrated and dried thoroughly on a vacuum line to yield the product.

General Procedure for conversion of 2,2'-bipyridine-4,4'-dicarboxylic acid to 2,2'-bipyridine-4,4'-dicarbonyl dichloride

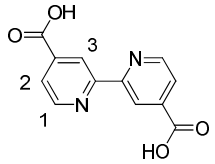
The carboxylic acid was refluxed with one drop of triethylamine in an excess of thionyl chloride overnight. The reaction mixture was concentrated and Dried thoroughly under vacuum. Once dried the acid chloride was used immediately.

General procedure for hydrogenation

Palladium on carbon (10 % w/v) was suspended in a suitable solvent, usually methanol, and added carefully to a solution of nitro-ligand dissolved in the same solvent under an atmosphere of N_2. The flask was twice evacuated and H_2 was passed

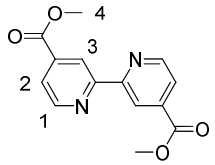
over the reaction for between 12 - 16 hrs. The reaction mixture was filtered through celite, concentrated and dried thoroughly on a vacuum line to yield the NH_2 product.

2,2'-Bipyridine-4,4'-dicarboxylic acid[1, 2]



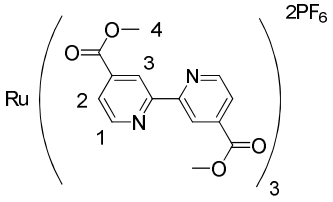
4,4'-Dimethyl-2,2'-bipyridine (5.68 g, 30.8 mmol) in conc. sulphuric acid (142 mL) at 70-80ºC was oxidized by the addition of potassium dichromate (26 g, 87.8 mmol) slowly over 2 hrs, maintaining the temperature between 70-80ºC. The deep green mixture was then poured over 800 mL of ice/water affording a light green precipitate that was isolated by vacuum filtration. The solid was washed in water and refluxed in 50% conc. nitric acid (150 mL) for 2 hrs. The cooled reaction mixture was poured onto ice water (1 L). A white precipitate was isolated and washed with water. The resultant white solid product (7.0 g, 93%) was insoluble in all known solvents except strong acids; m.p. > 250ºC; δ_H (300 MHz, TFA-d): 8.07 (2H, d, *J* = 5.6 Hz, H1), 8.65 (2H, d, *J* = 5.6 Hz, H2) and 8.75 (2H, s, H3); ESI-MS found *m/z* 245 $[M+H]^+$.

4,4′-Dimethylester-2,2′-bipyridine[3]

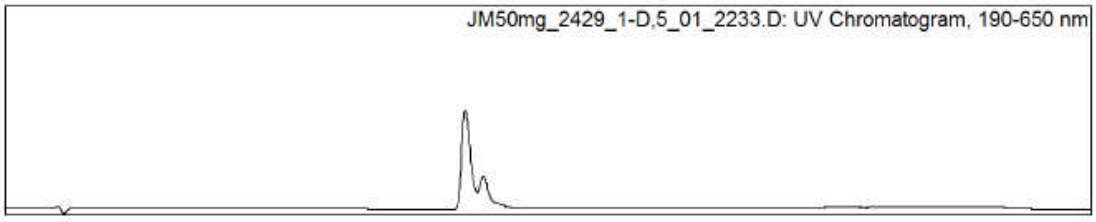


Minor modifications were made to procedure taken used by Azzoumanain and Bakhtchadjian[3] as follows: 2,2'-bipyridine-4,4'-dicarboxylic acid (3.0 g, 12.29 mmol) was suspended in methanol (200 mL) to which thionyl chloride (20 mL) was added dropwise over 5 mins. The mixture was refluxed for 20 hrs and quenched with saturated aqueous sodium hydrogen carbonate solution. The reaction mixture was extracted (chloroform 3 × 200 mL) and the organic layers combined and dried over magnesium sulphate. The organic layers were evaporated to dryness and the product recrystallised from chloroform and acetone to yield (2.80 g, 84%) the product as a white crystalline product; m.p. 221.1-221.5ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.10 (6H, s, H4), 7.92 (2H, d, *J* = 6.0 Hz, H1), 8.87 (2H, d, *J* = 6.0 Hz, H2), 8.98 (2H, s, H3).

Tris (4,4′-Dimethylester-2,2′-bipyridine) ruthenium (II) dichloride[4]

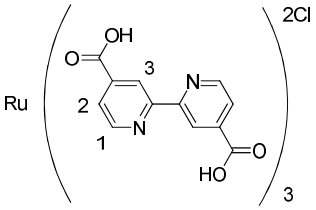


4,4′-Dimethylester-2,2′-bipyridine (1.17 g, 7.44 mmol) was refluxed with (dimethylsulfoxide)dichlororuthenium (II) (416 mg, 1.64 mmol) and silver nitrate (57 mg, 3.28 mmol) in ethanol (35 mL) for 7 days. The hot solution was then filtered and concentrated. The solid was dissolved in water (10 mL) and the ruthenium product precipitated upon the addition of ammonium hexaflorophosphate (excess). The precipitate was recrystallised overnight from acetone and water to yield (100 mg, 10%) as red crystals; m.p. 217.8-218.5ºC; ^{1}H NMR (300 MHz, Acetone-d_6): δ = 2.79 (18H, s, H4), 6.72 (6H, d, *J* = 6.0 Hz, H2), 6.95 (6H, d, *J* = 6.0 Hz, H1), 8.06 (6H, s, H3); ESI-MS: *m/z* = 459 $[M]^{2+}$ (note: some ethyl ester exchange products are observed in the mass spectrum)



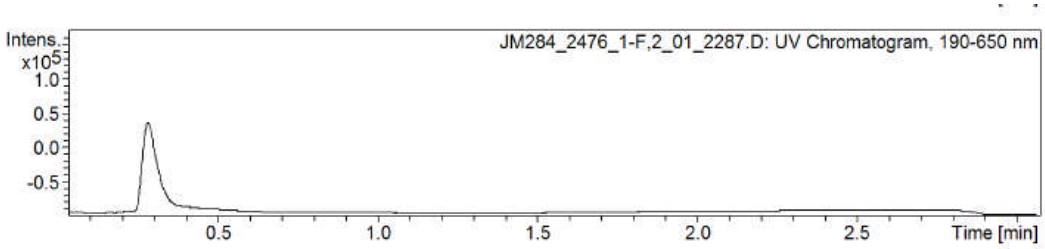
LC of Tris(4,4′-Dimethylester-2,2′-bipyridine) ruthenium (II) dichloride

Tris (2,2'-Bipyridine-4,4'-dicarboxylic acid) ruthenium(II) dichloride 1



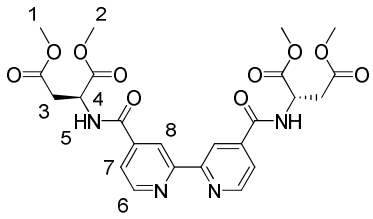
To a strirred solution of tris(4,4′-dimethylester-2,2′-bipyridine) ruthenium (II) dichloride (50 mg, 0.0409 mmol) in tetrahydrofuran: water (1:1, v:v, 10 mL) was added lithium hydroxide (25 mg, 0.6138 mmol) in small portions. After an hour the solution was neutralised using 1*N* hydrochloric acid to pH 7.0 and concentrated. The residual salt was removed by dialysis against pure water using 1,000 Da MW cut-off disposable dialyser (Aldrich) and the product crystallised from methanol and acetone

to yield (49 mg, quantitative) the product as a deep solid; m.p. > 250°C; ^{1}H NMR (500 MHz, D_2O): δ = 7.54 (2H, dd, *J* = 6.0 and 1.6 Hz, H2), 7.74 (2H, d, *J* = 6.0 Hz, H1), 8.74 (2H, d, *J* = 1.6 Hz, H3); ESI-MS: *m/z* = 417 $[M]^{2+}$; λ_{max} (5 mM phosphate, pH 7.4): 300 nm (ε/dm^{-3} mol^{-1} cm^{-1} 64272)



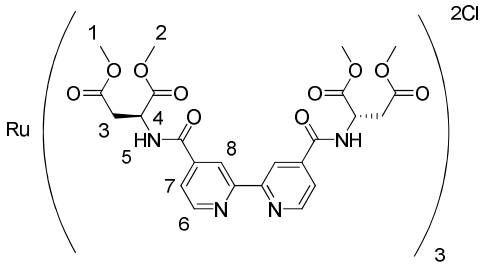
*LC of compound **1***

(2S,2'R)-tetramethyl-2,2'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl)) disuccinate

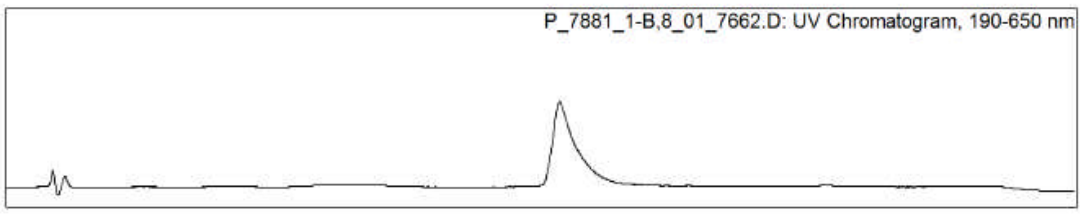


4,4'-Carboxy-2,2'-bipyridine (100 mg, 2.048 mmol) was converted to the acid chloride by treatment with thionyl chloride in the standard manner. Once thoroughly dried the acid chloride was dissolved in dry chloroform (20 mL) and added dropwise to a stirred solution of dimethyl L-aspartic acid hydrochloride (178 mg, 4.505 mmol) and triethylamine (1.27 mL, 9.010 mmol) in dry chloroform (25 mL) under nitrogen at 0ºC. Once addition was complete the flask was allowed to warm to room temperature and the reaction mixture refluxed for 48 hrs. The reaction mixture was concentrated and purified using flash silica chromatography (gradient of 1-8% methanol in chloroform of 0.5% intervals, 250 mL of each increment) to yield (210 mg, 75%) a cream solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.02 (2H, dd, *J* = 17.1 and 4.3 Hz, H3a), 3.17 (2H, dd, *J* = 17.1 and 4.3 Hz, H3b), 3.74 (6H, s, H1), 3.83 (6H, s, H2), 5.10 (2H, dt, *J* = 7.7 and 4.3 Hz, H4), 7.45 (2H, d, *J* = 7.7 Hz, H5), 7.75 (2H, dd, *J* = 5.1 and 1.7 Hz, H7), 8.75 (2H, app s, H8), 8.85 (2H, d, *J* = 5.1 Hz, H6); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 35.9, 49.1, 52.2, 53.0, 118.1, 121.9, 141.9, 150.2, 156.3, 159.9, 165.1, 170.8; IR (solid state): υ_{max} = 3285, 3059, 2940, 1717, 1690, 1436, 1200, 1019, 666 cm^{-1}; ESI-HRMS: found *m/z* 531.1720 $[M+H]^+$, $C_{24}H_{27}N_4O_{10}$ requires 531.1722.

Tris ((2S,2'R)-tetramethyl-2,2'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl)) disuccinate) ruthenium(II) dichloride

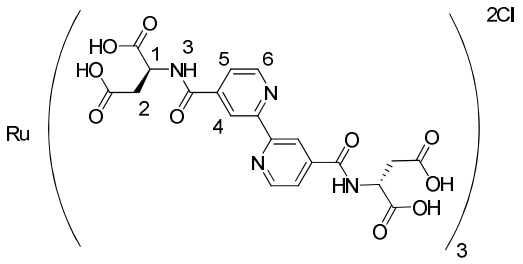


(2S,2'R)-Tetramethyl-2,2'- (([2,2'-bipyridine]-4,4'-dicarbonyl) bis(azanediyl)) disuccinate (250 mg, 0.471 mmol) was refluxed with (dimethylsulfoxide)dichlororuthenium (II) (71.3 mg, 0.147 mmol) and silver nitrate (50 mg, 0.294 mmol) in ethanol (20 mL) for 7 days. After reflux the solution was filtered hot and concentrated. The solid was redissolved in the minimum amount of ethanol and loaded on to a SP Sephadex cation exchange column. The ruthenium(II) complex was eluted off the column with an aqueous 0.1 M sodium chloride solution. The red fractions were concentrated, redissolved in acetone and filtered to remove the sodium chloride. This process was repeated until no white salt could be seen in the concentrated product. Mass spectrometry analysis showed ester exchange had taken place during the complexation. As the next step of the reaction was hydrolysis, the bright red material (150 mg, 60%) was taken forward with no further purification; ^{1}H NMR (300 MHz; D_2O): δ = 2.90 (12H, m, H3), 3.55 (18H, s, H1), 3.60 (18H, s, H2), 4.8 (6H, m, H4), 7.59 (6H, br s, H6), 7.85 (6H, br s, H7), 8.85 (6H, br s, H8); IR (solid state): υ_{max} = 3265, 3052, 2956, 1717, 1652, 1473, 1436, 1160, 1021, 665 cm^{-1}; ESI-MS: *m/z* 846 = $[M]^{2+}$.

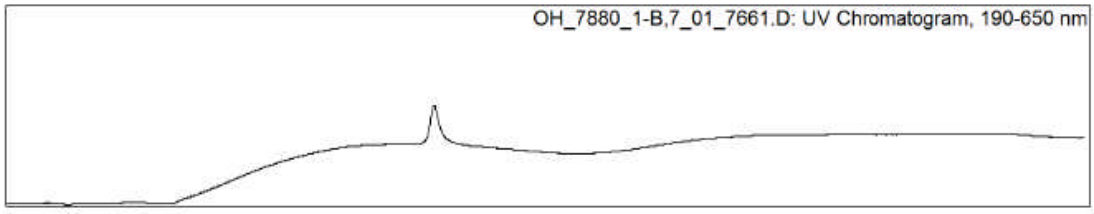


LC of product

Tris (2S,2'R)-2,2'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl))disuccinic acid) ruthenium(II) dichloride 2

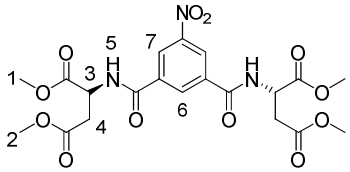


Tris ((2S,2'R)-tetramethyl-2,2'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl)) disuccinate) ruthenium(II) dichloride (100 mg, 0.0567 mmol) was dissolved in a 1:1 mixture of ethanol and water (30 mL) and treated with two additions (an hour apart) of 1M sodium hydroxide solution (2.04 mL, 2.041 mmol. After stirring for 2 hrs the solution was neutralised with 1*N* hydrochloric acid solution to pH 7.0 and the reaction concentrated. The residual salt was removed by dialysis against pure water using 1,000 Da mw cut-off disposable dialyser (Aldrich) and the product crystallised from methanol and acetone to yield (98 mg, quantitative) the product as a deep red solid; m.p. > 250^{o}C; ^{1}H NMR (500 MHz, D_2O): δ = 2.72 (6H, app m, H2a), 2.87 (6H, dd, *J* = 16.2 and 3.4 Hz, H2b), 4.70 (6H, app dd, *J* = 9.4 and 3.4 Hz, H1), 7.78 (6H, m, H6), 7.75 (6H, d, *J* = 5.1 Hz, H5), 8.75 (6H, app s, H4); IR (solid state): υ_{max} = 3082, 2554, 2351, 1717, 1643, 1541, 1473, 1026, 763 cm^{-1}; ESI-MS: *m/z* = 761 $[M]^{2+}$; λ_{max} (5 mM phosphate, pH 7.4): 301 nm (ε/dm^{-3} mol^{-1} cm^{-1} 64578).



*LC of compound **2***

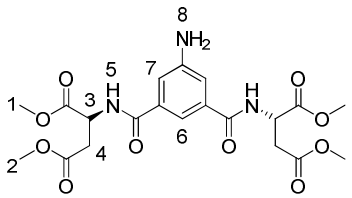
(2S,2'S)-Tetramethyl 2,2'-((5-nitroisophthaloyl)bis(azanediyl))disuccinate



5'-Nitroisophthaloyl dichloride (2.00 g, 8.06 mmol), dimethyl L-aspartic acid hydrochloride (3.50 g, 17.71 mmol), triethylamine (3.59 g, 35.45 mmol) in dry

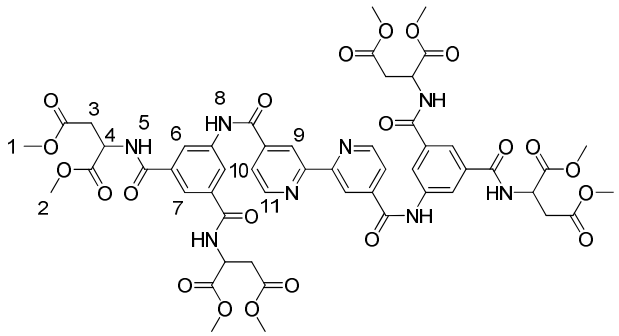
dichloromethane (50 mL) was stirred for 18 hrs. The reaction mixture was washed with aqueous hydrochloric acid (1*N*, 50 mL), saturated sodium hydrogen carbonate solution (50 ml) and saturated sodium chloride solution (50 mL). The crude product was purified by flash silica chromatography (3:7 ethyl acetate: dichloromethane) to yield (3.34 g, 82.5 %) a white product; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.01 (2H, dd, *J* = 18.0 and 4.3 Hz, H4a), 3.19 (2H, dd, *J* = 18.0 and 4.3 Hz, H4b), 3.73 (6H, s, H2), 3.82 (6H, s, H1), 5.10 (2H, dt, *J* = 4.3 and 8.0 Hz, H3), 7.54 (2H, br, *J* = 7.8 Hz, H5), 8.58 (1H, t, *J* = 1.3 Hz, H6), 8.81 (2H, d, *J* = 1.3 Hz, H7); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.3, 49.3, 52.8, 53.6, 125.7, 131.7, 136.2, 148.9, 164.3, 171.2, 172.0; IR (solid state): υ_{max} = 3603, 3354, 2846, 2328, 2019, 1895, 1732, 1657, 1535, 1465 1351, 1091, 919, 863 cm^{-1}; ESI-HRMS: found *m/z* 498.1359 $[M+H]^+$, $C_{20}H_{23}N_3O_{12}$ requires 498.1354.

(2S,2'S)-Tetramethyl 2,2'-((5-aminoisophthaloyl)bis(azanediyl))disuccinate



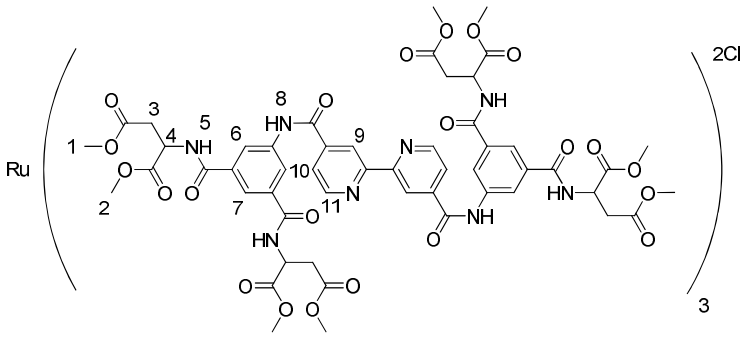
(2S,2'S)-Tetramethyl 2,2'-((5-nitroisophthaloyl)bis(azanediyl))disuccinate (3.2 g, 6.44 mmol) and 10% palladium on charcoal (300 mg) dissolved in methanol (30 ml) were stirred under a hydrogen atmosphere for 24 hrs. The reaction mixture was filtered through a celite pad and concentrated to yield (2.97 g, 98%) a fluffy white product; m.p. 81.5-82.5ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 2.95 (2H, dd, *J* = 18.0 and 4.3 Hz, H4a), 3.12 (2H, dd, *J* = 18.0 and 4.3 Hz, H4b), 3.73 (6H, s, H2), 3.83 (6H, s, H1), 5.10 (2H, dt, *J* = 7.9 and 4.4 Hz, H3), 7.60 (2H, br, *J* = 8.0 Hz, H8), 8.62 (1H, t, *J* = 1.5 Hz, H6), 8.81 (2H, d, *J* = 1.5 Hz, H7); ^{13}C NMR (75 MHz; $CDCl_3$): δ = 36.5, 49.4, 52.6, 53.4, 115.6, 117.2, 135.6, 147.5, 166.9, 171.6, 172.0; IR (solid state): υ_{max} 3364, 3006, 2956, 2850, 2609, 1736, 1650, 1600, 1528, 1439, 1369, 1052, 999, 877 cm^{-1}; ESI-HRMS: found *m/z* 468.1614 $[M+H]^+$, $C_{20}H_{26}N_3O_{10}$ requires 468.1613.

(2S,2'S,2''S,2'''S)-octa-tetramethyl 2,2',2'',2'''-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl))bis(isophthaloyl))tetrakis(azanediyl))tetrasuccinate

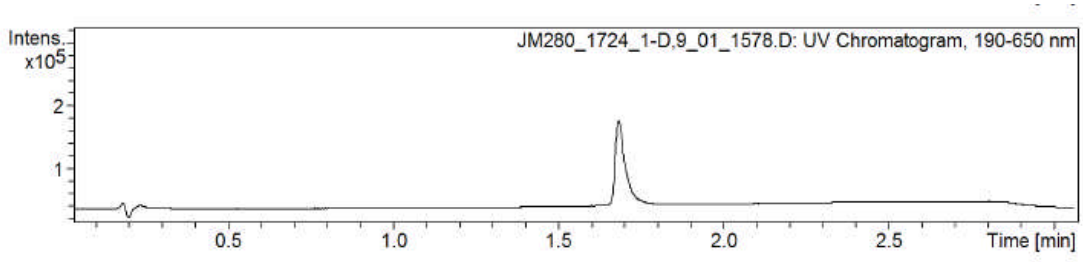


4,4'-Carboxy-2,2'-bipyridine (250 mg, 1.025 mmol) was converted to the acid chloride by treatment with thionyl chloride in the standard manner. Once thoroughly dried it was dissolved in dry chloroform (20 mL) and added dropwise to a stirred solution of (2S,2'S)-tetramethyl 2,2'-((5-aminoisophthaloyl)bis(azanediyl))disuccinate (921 mg, 1.970 mmol) and triethylamine (199 mg, 1.970 mmol) in dry chloroform (25 mL) under nitrogen at 0ºC. Once addition was complete the flask was allowed to warm to room temperature and the reaction mixture refluxed for 48 hrs. The reaction mixture was concentrated and purified using flash silica chromatography (gradient of 1-8% methanol in chloroform of 0.5% intervals, 250 mL of each increment) to yield a yellow product (530 mg, 51%); m.p.: 118.1-119.0^{o}C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 2.90 (4H, dd, *J* = 16.2 and 7.7 Hz, H3a), 3.00 (4H, dd, *J* = 16.2 and 6.0 Hz, H3b), 3.66 (12H, s, H1), 3.69 (12H, s, H2), 4.91 (4H, dt, *J* = 7.7 and 6.0 Hz, H4), 8.06 (2H, d, *J* = 5.1 Hz, H11) 8.11 (2H, s, H7), 8.45 (4H, s, H6), 9.01 (2H, d, *J* = 5.1 Hz, H10), 9.02 (2H, s, H9), 9.16 (4H, d, *J* = 7.7 Hz, H5), 11.09 (2H, s, H8); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 35.6, 49.7, 52.1, 52.7, 118.8, 122.5, 122.8, 123.2, 135.0, 139.2, 143.2, 150.7, 155.9, 164.5, 166.1, 170.9, 171.5; IR (solid state): υ_{max} = 3378, 3071, 2958, 1720, 1642, 1599, 1539, 1436, 1174, 1055, 751 cm^{-1}; ESI-HRMS: found *m/z* 1165.3289 $[M+Na]^+$, $C_{52}H_{54}N_8O_{22}Na$ requires 1165.3245.

Tris ((2S,2'S,2''S,2'''S)-octa-tetramethyl 2,2',2'',2'''-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl))bis(isophthaloyl))tetrakis(azanediyl))tetrasuccinate) ruthenium(II) dichloride

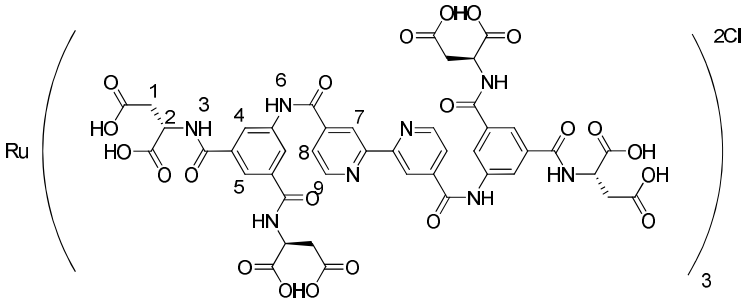


(2S, 2'S, 2''S, 2'''S)-Octa-tetramethyl 2,2',2'',2'''-((5,5'-(([2,2'-bipyridine] -4,4'-dicarbonyl) bis(azanediyl)) bis(isophthaloyl)) tetrakis(azanediyl)) tetrasuccinate (200 mg, 0.175 mmol) was refluxed with (dimethylsulfoxide)dichlororuthenium (II) (26.5 mg, 0.0547 mmol) and silver nitrate (18.9 mg, 0.109 mmol) in ethanol (20 mL) for 7 days. The solution was then filtered and concentrated. The solid was redissolved in the minimum amount of ethanol and loaded on to a SP Sephadex cation exchange column. The ruthenium(II) complex was eluted off the column with an aqueous 0.2 M sodium chloride solution. The red fractions were concentrated, redissolved in acetone and filtered to remove the sodium chloride. This process was repeated until no white salt could be seen in the concentrated product. Mass spectrometry analysis showed a large amount of ester exchange had taken place during the complexation. As the next step of the reaction was ester hydrolysis, the bright red material (100 mg, 58.8%) was taken forward with no further purification; ^{1}H NMR (500 MHz, MeOD): δ = 2.87 (12H, br m, H3a), 2.93 (12H, br m, H3b), 3.60 (36H, br s, H1), 3.65 (36H, br s, H2), 4.91 (12H, br s, H4), 7.79 (6H, br s, H11) 7.87 (6H, br s, H7), 8.21 (12H, br s, H6), 8.25 (6H, br s, H10) and 8.70 (6H, s, H9); IR (solid state): υ_{max} = 2955, 2342, 1728, 1432, 1172, 993, 858, 756 and 683 cm^{-1}; ESI-MS: *m/z* = 1765.2 $[M]^{2+}$, 1190.2 $[M+H]^{3+}$, 893.6 $[M+2H]^{4+}$ (ethyl trans-esterification products observed)

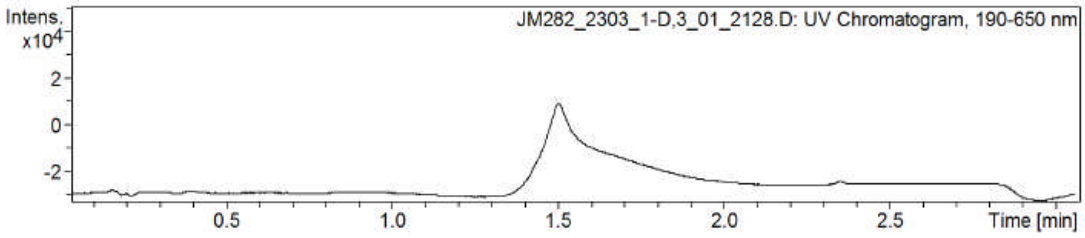


LC of product

Tris ((2S,2'S,2''S,2'''S)-2,2',2'',2'''-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis (azanediyl))bis(isophthaloyl))tetrakis(azanediyl))tetrasuccinic acid) ruthenium (II) dichloride 3

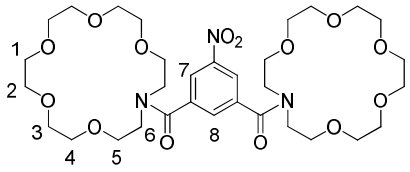


Tris ((2S,2'S,2''S,2'''S)-octa-tetramethyl 2,2',2'',2'''-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl) bis(azanediyl)) bis(isophthaloyl)) tetrakis (azanediyl)) tetrasuccinate) ruthenium(II) dichloride (50 mg, 0.016 mmol, 1 eq.) was dissolved in 1:1 mixture of ethanol and water (1:1 5 mL total,) and treated with two additions (three hours apart) of 1M sodium hydroxide solution (1.2 mL, 1.021 mmol). After stirring for 6 hrs the solution was neutralised with 1*N* hydrochloric acid solution to pH 7.0 and the reaction concentrated. The residual salt was removed by dialysis against pure water using 1,000 Da MW cut-off disposable dialyser (Aldrich) and the product crystallised from methanol and acetone to yield (49 mg, quantitative) a deep red product; m.p.: > 250^{o}C; ^{1}H NMR (500 MHz, D_2O): δ = 2.61 (12H, dd, *J* = 15.4 and 9.4 Hz, H1a), 2.76 (4H, dd, *J* = 15.4 and 3.4 Hz, H1b), 4.59 (12H, app dd, *J* = 9.4 and 3.4 Hz, H2), 7.94 (6H, d, *J* = 6.0 Hz, H8), 8.04 (6H, s, H5), 8.08 (2H, d, *J* = 6.0 Hz, H9), 8.15 (12H, s, H4), 9.17 (6H, s, H7); ^{13}C NMR (75 MHz, D_2O) δ = 30.5, 39.8, 49.1, 54.2, 124.6, 135.4, 137.7, 157.8, 165.4, 168.9, 178.8, 179.3; IR (solid state): υ_{max} = 3283, 3060, 1637, 1593, 1541, 1330, 1297 1108, 897 cm^{-1}; ESI-MS *m/z* = 1596.6 $[M]^{2+}$; λ_{max} (5 mM phosphate, pH 7.4) 305 nm (ε/dm^{-3} mol^{-1} cm^{-1} 82883).



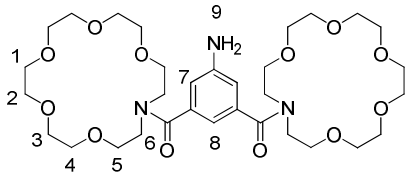
*LC of compound **3***

(5-Nitro-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)methanone)



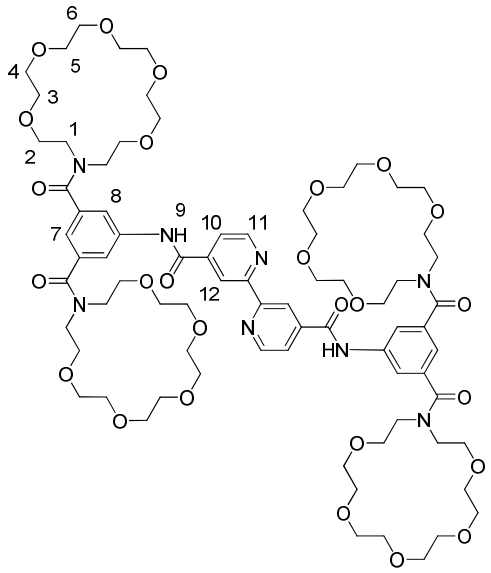
5'-Nitroisophthaloyl dichloride (1.00 g, 4.03 mmol), 1-aza-18-crown-6 (2.33 g, 8.87 mmol), triethylamine (0.9 g, 8.87 mmol) and dry dichloromethane (50 mL) was stirred for 15 hrs. The reaction mixture was concentrated and purified by flash silica chromatography (100:40:8 $CHCl_3$:Acetone:EtOH) to yield an orange oil (2.0 g 70%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.48-3.82 (48H, m, H1-6), 7.87 (1H, s, H8) 8.41 (2H, s, H7); ^{13}C NMR (100 MHz; $CDCl_3$) δ = 27.5, 45.8, 50.4, 68.7, 69.4, 70.4, 70.5, 70.6, 71.1, 71.9, 122.9, 131.9, 138.8, 147.6, 169.4; IR (NaCl disk): υ_{max} = 3436, 2915, 2083, 1626, 1540, 1479, 1354, 1109, 943 cm^{-1}; ESI-HRMS: found *m/z* 724.3267 $[M+Na]^+$, $C_{32}H_{51}N_3O_{14}Na$ requires 724.3263.

(5-Amino-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)methanone)



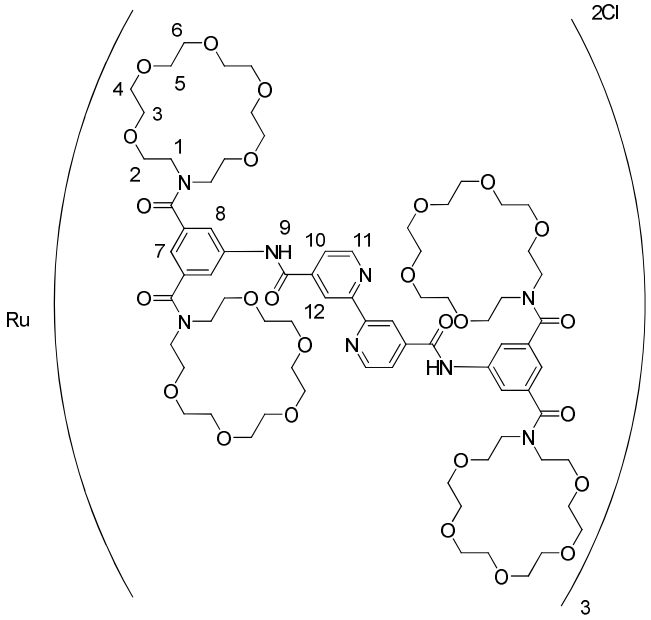
(5-Nitro-1,3-phenylene) bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) methanone) (1.9 g, 2.71 mmol) and 10% palladium on charcoal (200 mg) were stirred under an atmosphere of hydrogen in methanol (30 mL) for 24 hrs. The reaction mixture was filtered through a celite pad and concentrated to yield (1.75 g, 96%) a viscous orange oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.55-3.83 (48H, m, H1-6), 4.00 (2H, s, H9), 6.75 (1H, s, H7), 7.27 (2H, s, H8); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 46.1, 50.1, 69.4, 69.7, 70.6, 70.7, 113.9, 114.6, 138.1, 146.8 and 171.6; IR (NaCl disk): υ_{max} = 3436, 2917, 2873, 1621, 1597, 1471, 1439, 1384, 1112, 947 cm^{-1}; ESI-HRMS: found *m/z* 672.7838 $[M+H]^+$, $C_{32}H_{53}N_3O_{12}$ requires 672.7841.

N4,N4'-bis(3,5-di(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-16-arbonyl) phenyl)-[2,2'-bipyridine]-4,4'-dicarboxamide

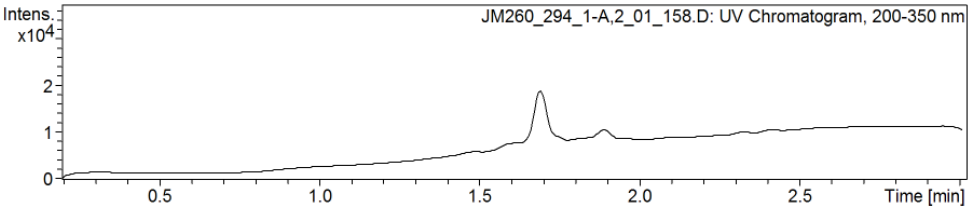


4,4’-carboxy-2,2’-bipyridine (139 mg, 0.496 mmol) was converted to the acid chloride by treatment with thionyl chloride in the standard manner. Once thoroughly dried it was dissolved in dry chloroform (20 mL) and added dropwise to a stirred solution of (5-Amino-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)methanone) (700 mg, 1.042 mmol) and triethylamine (104 mg, 1.042 mmol) in dry chloroform (20 mL) under nitrogen at 0ºC. Once addition was complete the flask was allowed to warm to room temperature and the reaction mixture refluxed for 48 hrs. The reaction mixture was concentrated and purified using flash alumina chromatography (gradient of 1-8% methanol in chloroform of 0.5% intervals, 250 mL of each increment) to yield an orange oil (530 mg, 51%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.65 (80H, br s, H2-6), 3.75 (16H, br s, H1), 7.32 (2H, s, H7), 7.90 (2H, d, *J* = 3.4 Hz, H11), 8.03 (4H, s, H8), 8.85 (2H, d, *J* = 3.4 Hz, H10), 8.93 (2H, s, H12); IR (solid state): υ_{max} = 3380, 3060, 2911, 1950, 1630, 1450, 845, 757 cm^{-1}; ESI-HRMS: found *m/z* 1551.7604 $[M+H]^+$, $C_{76}H_{111}N_8O_{26}$ requires 1551.7613.

Tris (N4,N4'-bis(3,5-di(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-16-arbonyl) phenyl)-[2,2'-bipyridine]-4,4'-dicarboxamide) ruthenium(II) dichloride 4

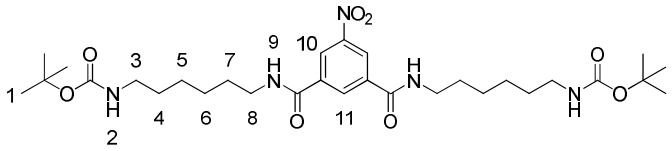


N4,N4'-bis(3,5-di(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-16-arbonyl) phenyl)-[2,2'-bipyridine]-4,4'-dicarboxamide (300 mg, 0.193 mmol) was refluxed with (dimethylsulfoxide)dichlororuthenium (II) (31.2 mg, 0.064 mmol) and silver nitrate (22 mg, 0.128 mmol) in ethanol (20 mL) for 7 days. After reflux the hot solution was filtered and concentrated. The solid was redissolved in the minimum amount of ethanol and loaded on to a SP Sephadex cation exchange column. The ruthenium(II) complex was eluted off the column with an aqueous 0.4 M sodium chloride solution. The red fractions were concentrated, redissolved in acetone and filtered to remove the sodium chloride. This process was repeated until no white salt could be seen in the concentrated product. This afforded (75 mg, 25%) of the product as a red solid; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.5-3.9 (288H, br, H1-6), 6.8-8.8 (36H, br m, H7-11); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 31.2, 46.4, 50.4, 69.9, 70.8, 71.0, 77.1, 120.3, 122.4, 138.1 and 171.0; IR (solid state): υ_{max} = 3390, 3054, 2961, 2626, 1974, 1654, 1440, 960 and 757 cm^{-1}; ESI-MS *m/z* = 1189.7 $[M+2H]^{4+}$, 1195.0 $[M+H+Na]^{4+}$, 1200.4 $[M+2Na]^{4+}$, 973.4 $[M+3H]^{5+}$, 815.2 $[M+4H]^{6+}$; λ_{max} (5 mM phosphate, pH 7.4) 300 nm (ε/dm^{-3} mol^{-1} cm^{-1} 76945)



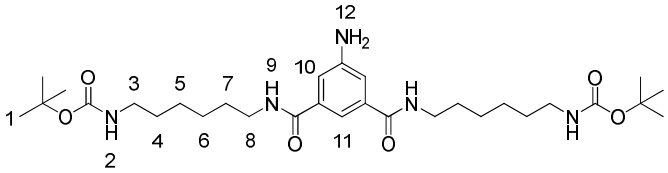
*LC Chromatogram of Compound **4***

Di-tert-butyl(((5-nitroisophthaloyl)bis(azanediyl))bis(hexane-6,1-diyl)) dicarbamate



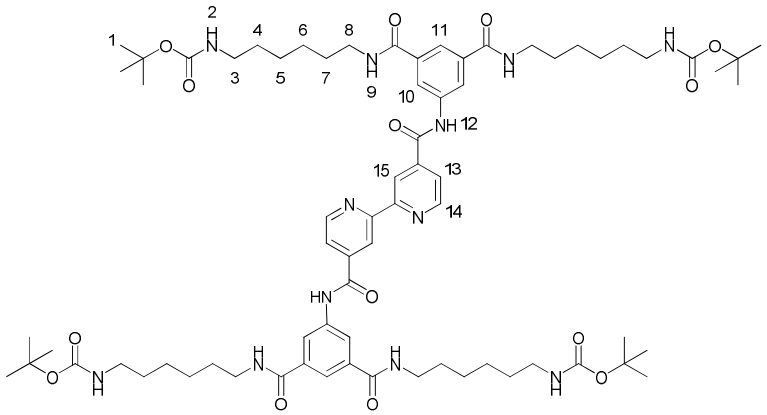
5'-Nitroisophthaloyl dichloride (1.00 g, 4.03 mmol), *N*-boc-1,6-diaminohexane (1.92 g, 8.87 mmol) and triethylamine (0.9 g, 8.87 mmol) in dry dichloromethane (60 mL) were stirred for 15 hrs. The reaction mixture was washed with 1*N* hydrochloric acid solution (50 mL), saturated sodium hydrogen carbonate solution (50 mL) and saturated sodium chloride solution (50 mL). The organic layers were dried with sodium sulphate and concentrated. The crude product was purified by flash silica chromatography (1:4 ethyl acetate: dichloromethane) followed by recrystallisation from hot ethyl acetate to yield (2.45 g, 98%) a white product; m.p. 78.7-79.2^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.29 (4H, m, H5), 1.32 (18H, s, H1), 1.36 (4H, m, H6), 1.43 (3H, t, *J* = 6.8 Hz, H4), 1.57 (4H, m, H7), 3.10 (4H, dt, *J* = 6.8 and 6.0 Hz, H3), 3.39 (4H, dt, *J* = 6.8 and 6.0 Hz, H8), 4.73 (2H, br s, H2), 7.22 (2H, br s, H9), 8.60 (1H, s, H11), 8.81 (2H, s, H10); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 23.8, 25.2, 27.4, 27.8, 29.0, 38.3, 38.4, 78.5, 124.2, 128.1, 135.5, 147.7, 155.8, 163.3; IR (solid state): υ_{max} = 3365, 3296, 3089, 2929, 1683, 1640, 1515, 1443, 1390, 1364, 1276, 1170, 917, 725 cm^{-1}; ESI-HRMS: found *m/z* 630.3486 $[M+Na]^+$, $C_{30}H_{49}N_5O_8Na$ requires 630.3479.

Di-tert-butyl(((5-aminoisophthaloyl)bis(azanediyl))bis(hexane-6,1-diyl)) dicarbamate



Di-tert-butyl (((5-nitroisophthaloyl) bis(azanediyl)) bis (hexane-6,1-diyl)) dicarbamate (2.3 g, 3.79 mmol) and 10% palladium on charcoal (200 mg) were stirred under a hydrogen atmosphere in methanol (40 ml) for 24 hrs. The reaction mixture was filtered through a celite pad and concentrated to yield (2.18 g, quantitative) a white product; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.35 (4H, m, H5), 1.41 (18H, s, H1), 1.42 (4H, m, H6), 1.49 (4H, m, H4), 1.58 (4H, m, H7), 3.15 (4H, dt, *J* = 6.8 and 6.0 Hz, H3), 3.41 (4H, dt, *J* = 6.0 and 6.8 Hz, H8), 4.70 (2H, br s, H2), 6.82 (2H, broad s, H9), 7.32 (2H, s, H10), 7.58 (1H, s, H11); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 167.5, 156.9, 147.5, 142.5, 136.2, 117.2, 79.7, 40.3, 39.9, 30.4, 29.6, 28.9, 26.3, 26.1; IR (solid state): υ_{max} = 3361, 2936, 1684, 1620, 1593, 1516, 1389, 1275, 1172, 1044, 868, 760 cm^{-1}; ESI-HRMS: found *m/z* 600.3732 $[M+Na]^+$, $C_{30}H_{51}N_5O_6Na$ requires 600.3737.

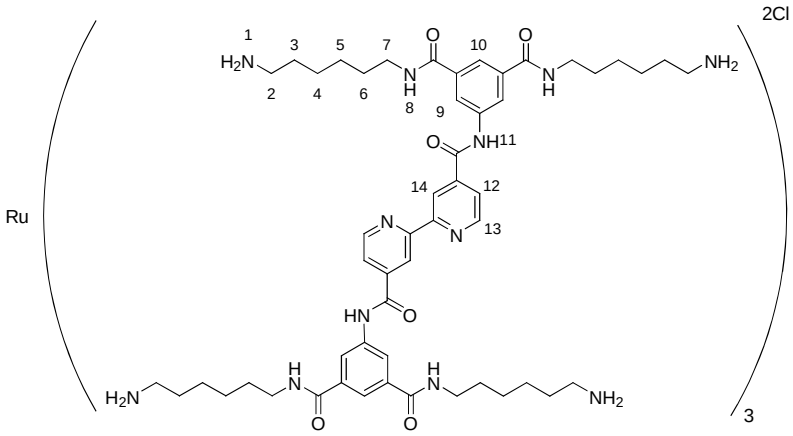
tetra-tert-butyl(((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl)) bis(isophthaloyl))tetrakis(azanediyl))tetrakis(hexane-6,1-diyl)) tetracarbamate



4,4'-Carboxy-2,2'-bipyridine (109.7 mg, 0.393 mmol) was converted to the acid chloride by treatment with thionyl chloride in the standard manner. Once thoroughly dried it was dissolved in dry chloroform (30 mL) and added dropwise to a stirred solution of di-tert-butyl(((5-amino isophthaloyl) bis(azanediyl)) bis(hexane-6,1-diyl)) dicarbamate (500 mg, 0.865 mmol, 2.2 eq.) and triethylamine (350 µL, 2.43 mmol,

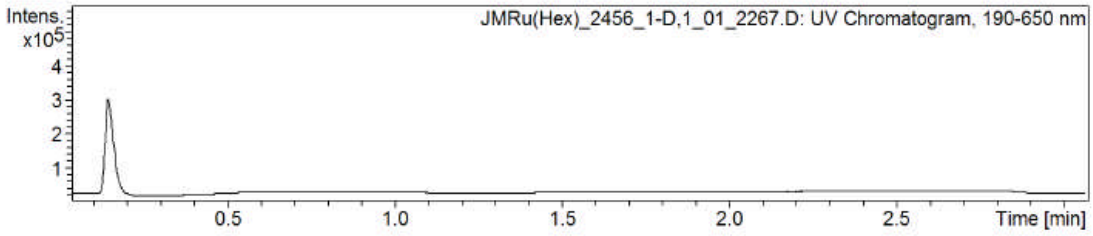
6.2 eq.) in dry chloroform (25 mL) under nitrogen at 0ºC. Once addition was complete the flask was allowed to warm to room temperature and the reaction mixture refluxed for 48 hrs. The reaction mixture was concentrated and purified using flash silica chromatography (gradient of 1-8% methanol in chloroform of 0.5% intervals, 250 mL of each increment) to yield (300 mg, 56%) a cream solid; ^{1}H NMR (400 MHz; DMSO-d_6): δ = 1.32 (8H, t, *J* = 6.4 Hz, H5), 1.38 (36H, s, H1), 1.40 (8H, t, *J* = 6.4 Hz, H7), 1.56 (8H, t, *J* = 6.4 Hz, H4), 2.92 (8H, app q, *J* = 6.4 Hz, H8), 3.29 (8H, app q, *J* = 6.4 Hz, H3), 6.74 (4H, t, *J* = 6.4 Hz, H9), 8.04 (2H, s, H11), 8.06 (2H, d, *J* = 1.5 Hz, H14), 8.40 (4H, s, H10), 8.52 (4H, t, *J* = 5.3 Hz, H2), 8.99 (2H, s, H15), 9.00 (2H, d, *J* = 1.2 Hz, H13), 10.97 (2H, s, H12); ^{13}C NMR (100 MHz; DMSO-d_6): δ = 26.0, 26.2, 28.2, 29.0, 29.1, 29.5, 38.9, 77.2, 118.6, 121.4, 122.1, 122.3, 135.7, 138.6, 142.9, 150.2, 155.6, 164.0, 165.8; IR (solid state) υ_{max} = 3356, 2926, 1682, 1632, 1525, 1364, 1172 and 706 cm^{-1}; ESI-HRMS: found *m/z* 1364.8136 $[M+H]^+$, $C_{72}H_{107}N_{12}O_{14}$ requires 1364.8102.

Tris (5,5'-(([2,2'-bipyridine] -4,4'-dicarbonyl) bis(azanediyl)) bis(N1,N3-bis(6-aminohexyl)isophthalamide)ruthenium(II) dichloride 5



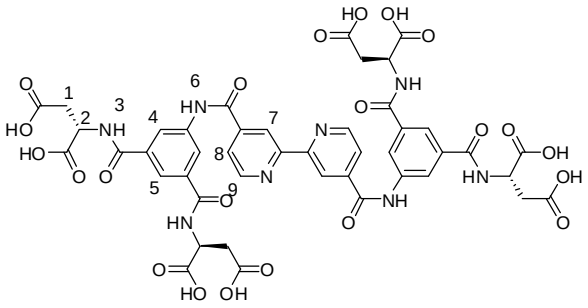
Tetra-tert-butyl (((5,5'-(([2,2'-bipyridine] -4,4'-dicarbonyl) bis(azanediyl)) bis(isophthaloyl)) tetrakis(azanediyl)) tetrakis(hexane-6,1-diyl)) tetracarbamate (90 mg, 0.066 mmol) was refluxed with (dimethylsulfoxide)dichlororuthenium (II) (10.3 mg, 0.021 mmol) and silver nitrate (7.1 mg, 0.042 mmol) in ethanol (10 mL) for 7 days. After reflux the hot solution was filtered and concentrated. The solid was redissolved in the minimum amount of ethanol and loaded on to a SP Sephadex cation exchange column. The ruthenium(II) complex was eluted off the column with an aqueous 0.4 M sodium chloride solution. The red fractions were concentrated,

redissolved in acetone and filtered to remove the sodium chloride. This process was repeated until no white salt could be seen in the concentrated product. The crude intermediate was stirred in 1M HCl in dioxane for 4 hrs. The reaction mixture was concentrated, dissolved in water and the pH adjusted carefully to 7. The neutralised product was crystallised from methanol and acetone to give the product (45 mg, 50%) as a red powder; m.p. 227.0 - 227.8.0ºC; ^{1}H NMR (300 MHz; D_2O): δ = 1.51 (96H, br s, H3-6), 2.86 (48H, br s, H2+7), 7.2-8.7 (36H br s, H9-13); IR (solid state): υ_{max} = 2861, 2062, 1249, 1159, 970, 897 cm^{-1}; ESI-HRMS: found *m/z* 1364.8136 $[M]^+$, $C_{72}H_{108}N_{12}O_{14}$ requires 1364.8102; λ_{max} (5 mM phosphate, pH 7.4): 308 nm (ε/dm^{-3} mol^{-1} cm^{-1} 85729)



*LC of compound **5***

(2S,2'S,2''S,2'''S)-2,2',2'',2'''-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanedi-yl))bis(isophthaloyl))tetrakis(azanediyl))tetrasuccinic acid 6



2S,2'S,2"S,2"'S)-octa-tetramethyl 2,2',2",2"'-((5,5'-(([2,2'-bipyridine]-4,4'-dicarbonyl) bis (azanediyl)) bis(isophthaloyl))tetrakis(azanediyl))tetrasuccinate (20 mg, 0.0175 mmol) was suspended in a stirred mixture of ethanol and water (1:1, 8 mL). Lithium hydroxide solution (9 mg, 0.210 mmol in water, 200 µL) was added in 50 µL portions at 30 min intervals. Mass spectrometry analysis showed an incomplete reaction so a second portion of lithium hydroxide was added to the reaction mixture (2 mg, 0.047 mmol) and stirring continued for a further hour. The reaction mixture was concentrated, dissolved in water and neutralised by the addition of 1N hydrochloric acid. The reaction mixture was concentrated and dissolved in a minimum amount of

water with acetone added to precipitate the product (12 mg, 67% isolate yield) as a white solid; m.p.: > 250°C; ^{1}H NMR (500 MHz, D_2O): δ = 2.60 (4H, dd, *J* = 15.4 and 9.4 Hz, H1a), 2.75 (4H, dd, *J* = 15.4 and 4.3 Hz, H1b), 4.58, (4H, dd, *J* = 9.4 and 4.3 Hz, H2), 7.92 (2H, *J* = 5.1 Hz, H8), 8.02 (2H, s, H5), 8.13 (4H, s, H4), 8.59 (2H, s, H7) and 8.84 (2H, d, *J* = 5.1 Hz, H9); ^{13}C NMR (75 MHz, D_2O): δ = 40.0, 54.4, 120.5, 122.8, 124.5, 135.7, 137.9, 144.0, 150.7, 152.5, 156.2, 169.1, 178.9, 179.4; IR (solid state): υ_{max} = 3347, 2217, 1557, 1415, 1309, 1259, 1107, 894 cm^{-1}; ESI-MS *m/z* = 1031 $[M+H]^+$ and 516 $[M+H]^{2+}$.

Fluorescence titration assay

During the course of all titrations the guest solution contained a concentration of the host equivalent to that in the host solution. The data from three runs were averaged and used for curve fitting with the curve fitting error quoted. Fitting of each run separately and averaging then taking the standard deviation gave similar errors.

Equation (1) operating in Origin7 was used to derive the dissociation constant for a 1:1 binding isotherm:

$$f = m[(nc + x + K) - \sqrt{\{(nc + x + K)^2 - 4ncx\}}]/2nc \qquad (1)$$

Where f = change in relative fluorescence (given by $[F/F_0]$-1), m = maximum value of f, n = stoichiometry, c = concentration of receptor, K = dissociation constant, x = concentration of protein added.

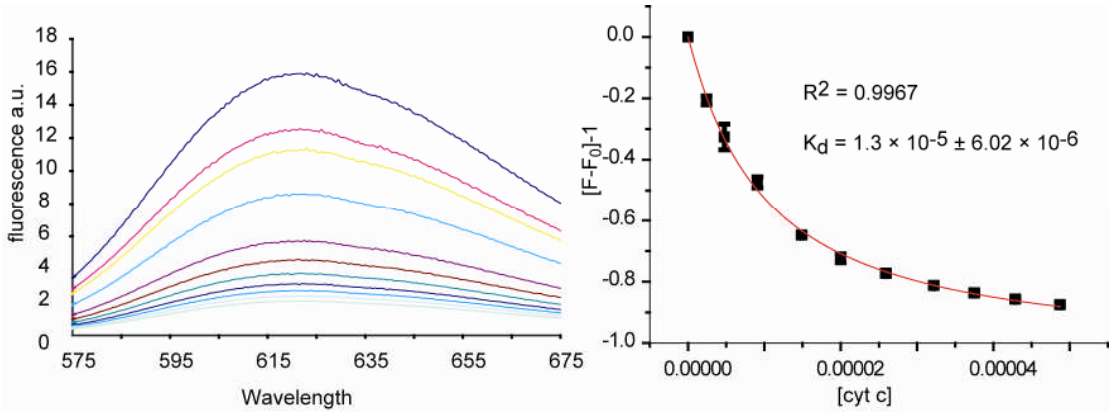


Figure ESI-1 Data for binding of receptor **1** to cyt *c*. Emission response of receptor **1** (1000 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c* (a) emission spectrum with various concentrations of protein (b) change in t emission maximum of 625 nm and fit by non-linear regression

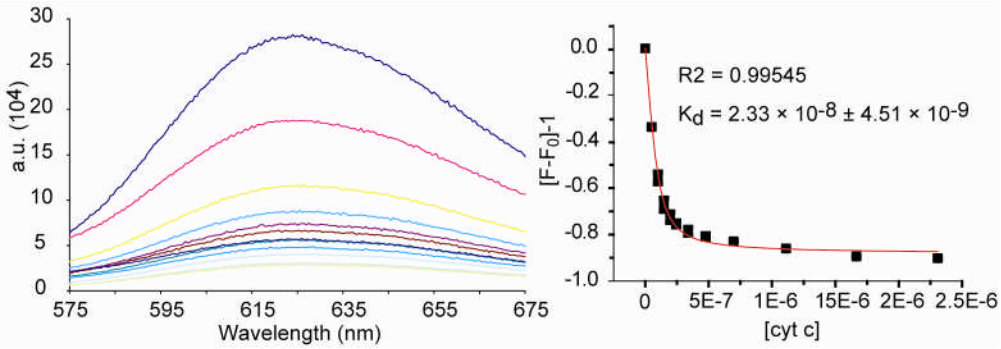


Figure ESI-2 Data for binding of receptor **2** to cyt *c*. Emission response of receptor **2** (100 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c*: emission spectrum with various concentrations of protein and change in emission maximum of 625 nm and fit by non-linear regression

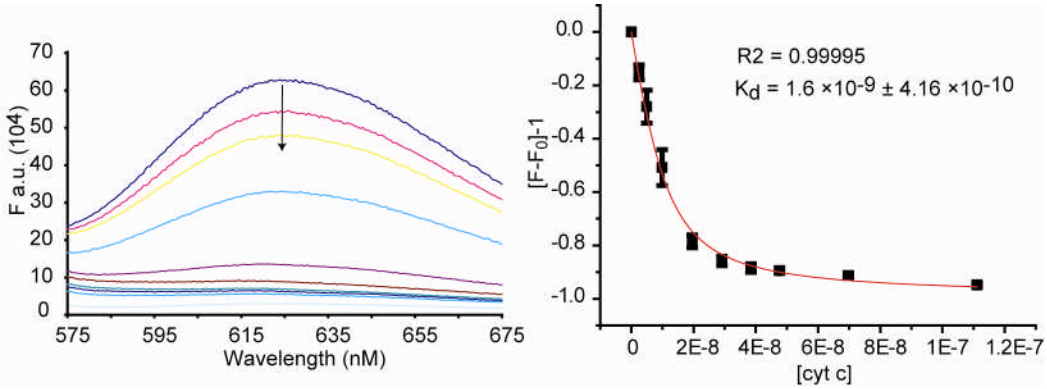


Figure ESI-3 Data for binding of receptor **3** to cyt *c*. Emission response of receptor **3** (10 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c*: emission spectrum with various concentrations of protein and change in emission maximum of 625 nm and fit by non-linear regression

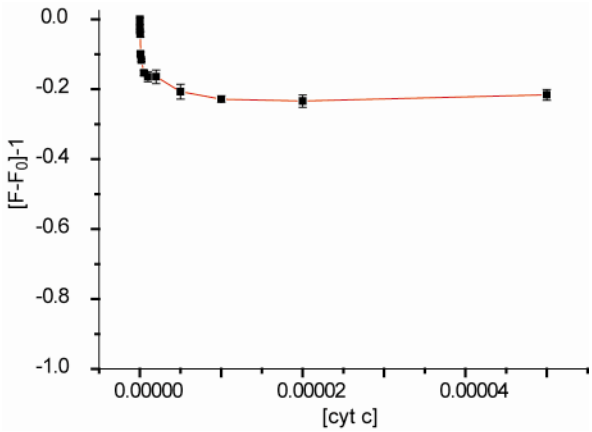


Figure ESI-4 Data for binding of receptor **4** to cyt *c*. Emission response of receptor **4** (1000 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c*: emission spectrum with various concentrations of protein and change in emission maximum of 625 nm and fit by non-linear regression. For **4** is was necessary to run the experiment in a plate reader as we observed in fluorescence reduction of **4** on its own with successive excitations presumably due to photoinduced degradation of the Ru(II) complex induced by the mildly basic nitrogen atom of the aza crown moiety.

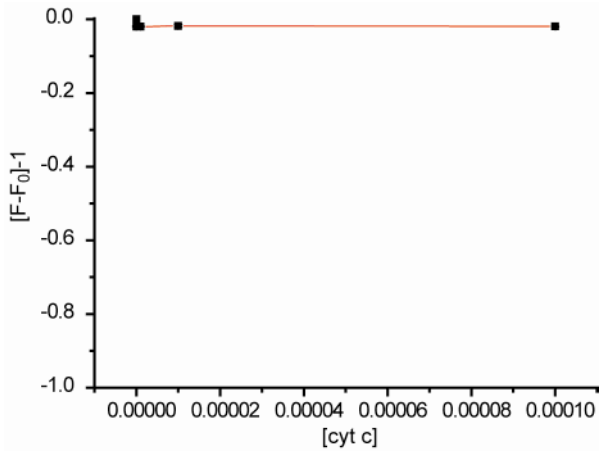


Figure ESI-5 Data for binding of receptor **5** to cyt *c*. Emission response of receptor **5** (1000 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c*: emission spectrum with various concentrations of protein and change in emission maximum of 625 nm and fit by non-linear regression. For **5** is was necessary to run the experiment in a plate reader as we observed in fluorescence reduction of **5** on its own with successive excitations presumably due to photoinduced degradation of the Ru(II) complex induced by the mildly basic nitrogen atom of the aza crown moiety.

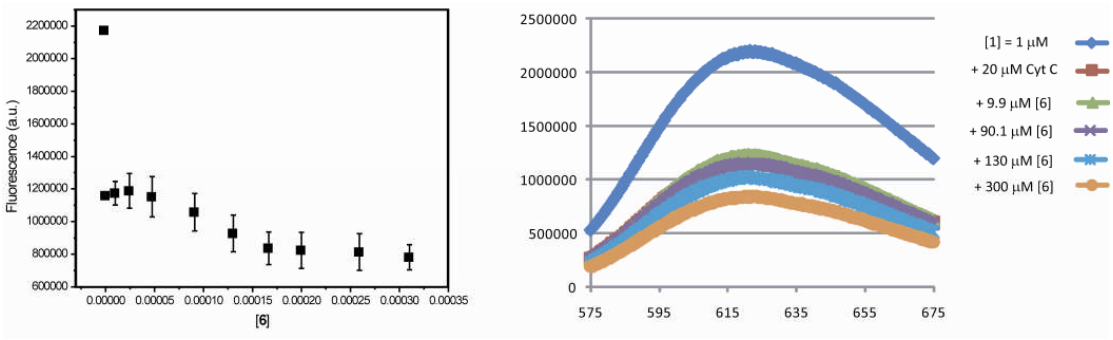


Figure ESI-6 Data for competition experiment with receptor **6** to cyt *c*-**1** complex. Emission response of receptor **1** (1000 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of cyt *c*: emission spectrum with various concentrations of **6** and change in emission maximum of 625 nm.

UV-Vis-monitored ascorbate reduction assay

A solution containing cyt *c* was incubated with or without synthetic receptors in a 4 mL quartz cuvette with a path length of 10 mm, and the volume adjusted to 2.85 mL by addition of 5 mM phosphate buffer (pH 7.4). After 10 minutes of incubation at room temperature, the spectrophotometer was started and 150 µL of a 20 mM stock solution of ascorbate in 5 mM phosphate buffer (pH 7.4) was added to the cuvette. The final concentration of the receptors and cyt *c* was 16 µM. the cuvette was immediately agitated with a glass rod. Absorbance at 550 nm was monitored every 0.5 seconds for at least two minutes. Initial rates were normalised and plotted against time.

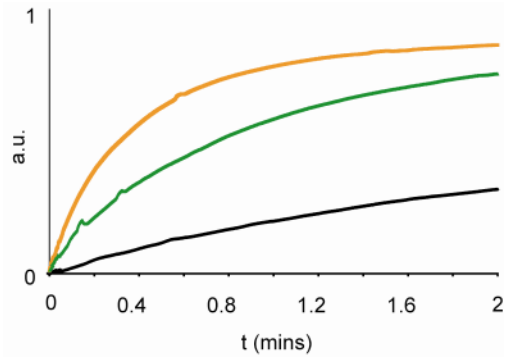


Figure ESI-7 Reduction of cyt *c* (10 µM) by 20 mM ascorbate (5 mM phosphate, pH 7.4) (orange) and in the presence of receptors **2** (green) and **3** (black).

Mass Spectrometry

A method to rapidly screen this class of receptors against a panel of proteins would be highly desirable. The mass spectrum of a constant concentration of cyt *c* (2 µM, 10

mM ammonium acetate, pH 7.0) was analysed in the presence an increasing concentration of receptor **3** (0.5 μM, 2.0 μM and 6.0 μM respectively). As the concentration of ruthenium increases the native cyt *c* peaks decrease until all detectable cyt *c* is bound in a complex with the receptor. ESI-MS is known to increase electrostatic interactions between molecules, shown here by the presence of 2:1 and 3:1 stoichiometry. In contrast no non-covalent adducts were observed when the same experiment was repeated with myoglobin.

Samples were analyzed by positive ionisation nanoelectrospray using an LCT Premier mass spectrometer (Waters Corp., Manchester, UK) equipped with a NanoMate (Advion, Inc., Ithaca, NY, USA) temperature-controlled automated sample handling and ionisation interface. A capillary voltage of 1.95 kV was set with a nitrogen gas flow of 0.5 psi for sample introduction and ionization. The sampling cone voltage was optimized at 70 V, ion guide 1 at 100 V, and aperture 1 at 70 V. Data were acquired over the range m/z 500–5,000 and data processing was performed using the MassLynx software supplied with the mass spectrometer. An external calibration using horse heart myoglobin was applied to ensure mass accuracy. Multiple different charge states are observed for both the protein and the protein- ruthenium complex adducts. The charge state distribution of the protein changes upon addition of the ruthenium complex. punctuation

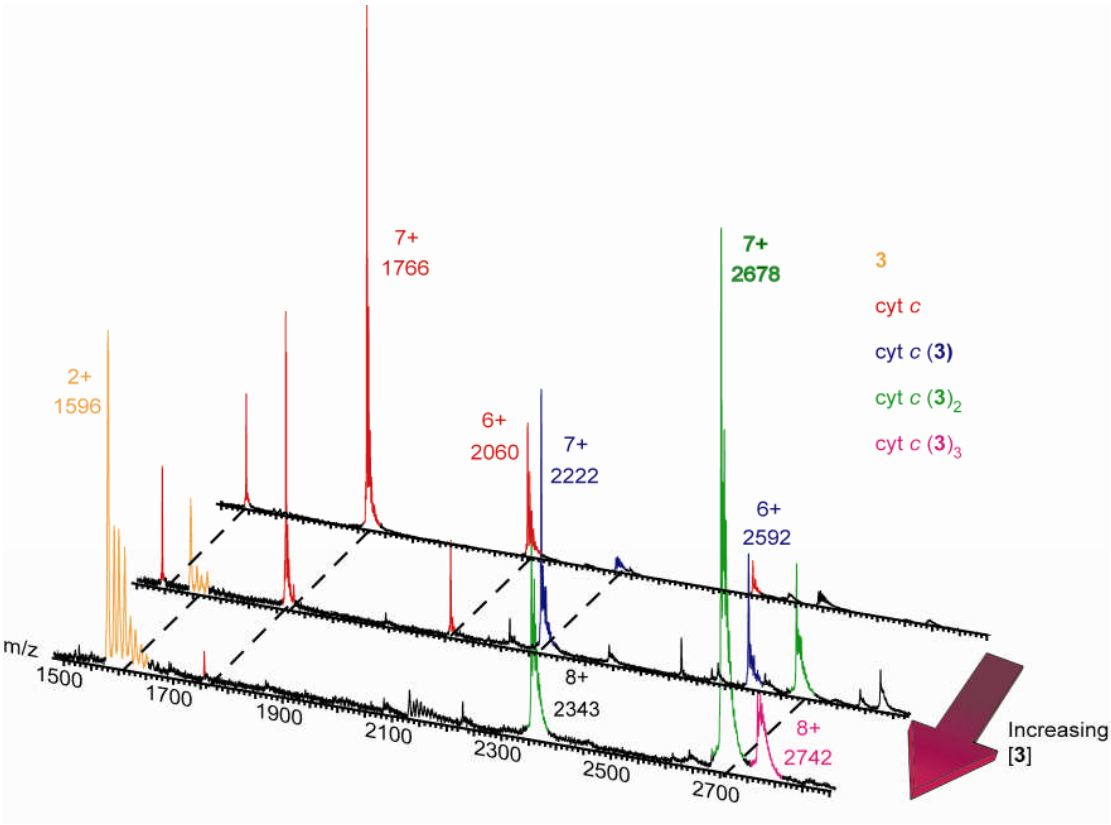


Figure ESI-8 Stacked mass spectra of cyt *c* with different concentrations of receptor **3**. Confirms binding to cyt *c* and potential of MS to be used a screening tool for protein surface recognition.

Fluorescence testing against other proteins

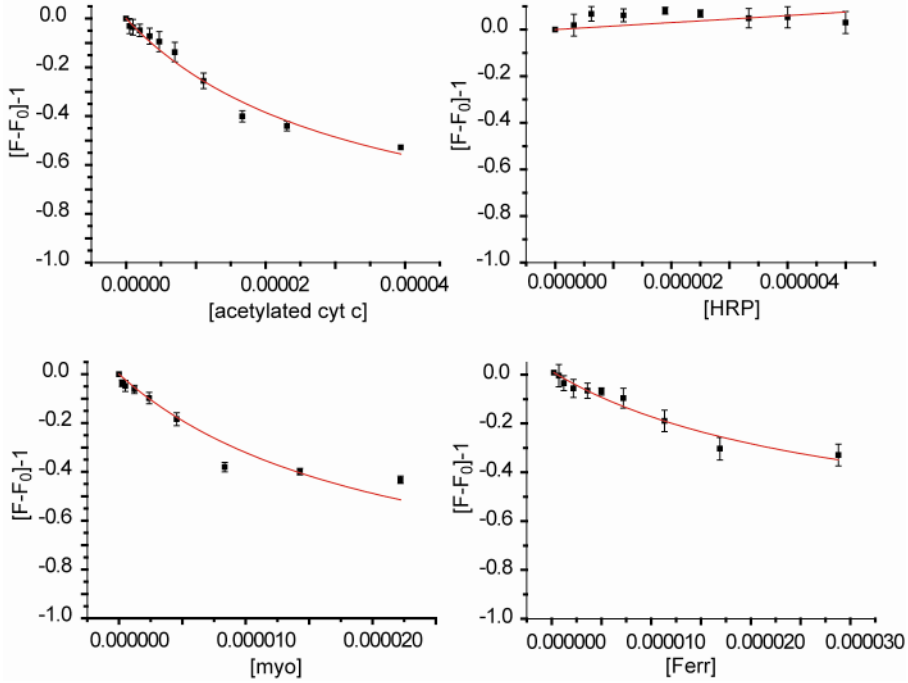


Figure ESI-9 Emission response of receptor **3** (1000 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of acetylcated cyt *c*, horse radish peroxidise, myoglobin and ferredoxin: change in emission maximum of 625 nm and fit by non-linear regression.

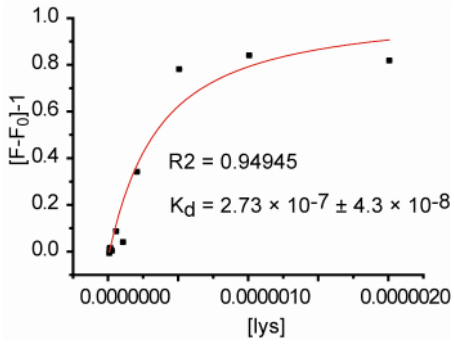


Figure ESI-10 anisotropy response of receptor **3** (100 nM, 5 mM sodium phosphate buffer, pH 7.4, ex 467 nm) on addition of α-chymotrypsin and lysozyme: change in anisotropy at 625 nm and fit by non-linear regression.

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