Protein Surface Recognition by Dendritic Ruthenium(II) Tris(bipyridine) Complexes

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Electronic supplementary information

**General Methods.** Buffer reagents were purchased from Wako, and α-chymotrypsin (ChT), soybean trypsin inhibitor (STI), and N-benzoyltyrosine-p-nitroanilide (BTNA) were purchased from Sigma and used without purification. Reagents and solvents were obtained from commercial sources without further purification unless otherwise noted. Melting points were determined with an Electrothermal capillary melting point apparatus and are uncorrected. $^1$H and $^{13}$C NMR spectra were recorded on a JEOL JNM-LA 400 spectrometer. Chemical shifts were reported in δ (ppm) relative to tetramethylsilane. All coupling constants were described in Hz. Elemental analyses were performed by Perkin Elmer-2400CHN. Flash column chromatography was performed on silica gel (40-63 μm) under a pressure of about 4 psi. Synthesized final compounds were checked for purity by analytical HPLC, which was performed using a JASCO PU-2086 and a JASCO UV-2075 detector with a GL Science Inertsil 150 mm x 4.6 mm, 5 μm C-18 column or TOSO TSKgel Amide-80 eluted with gradient 10% to 90 % of acetonitrile in 0.1 % TFA in water in 30 min. High-resolution mass data (HRMS) and low-resolution mass data (LRMS) of the compounds except for 3a,b were analyzed by Nagasaki University Instrument Center under the guidance of Profs. T. Maki and N. Yamaguchi. Mass spectra of 1 and 2 were taken by Prof. K. Yamaguchi from Tokushima Bunri University. MALDI-TOF mass spectra of 3a,b were measured by Shimadzu KRATOX AXIMA using a mixture of 2',4',6'-trihydroxyacetophenone and dihydrogen ammonium citrate as a matrix. The enzyme inhibition assays were performed with a JASCO V-550 UV/Vis spectrophotometer equipped with a Temp Controller (JASCO). For the binding titrations, RF-5300PC equipped with SA-100 temperature controller (Sansyo) was used.

**General procedure for ligand synthesis and preparation of Ru(bpy)$_3$ Complexes (Scheme S1).** **2,2'-Bipyridine-4,4'-dicarboxylic acid (5).** In a 100 mL of round-bottomed flask 4,4'-dimethyl-2,2'-bipyridine (1.72 g, 9.3 mmol) was dissolved in conc. sulfuric acid (22 mL), and the solution was cooled at 0 °C. To the solution was added CrO$_3$ (5.6 g, 56 mmol) in portion under vigorously stirring, and the
resulting dark blue mixture was stirred at 80 °C for 8h, and at rt overnight. The mixture was poured into crashed ice (ca. 200 mL), and the resulting pale yellow precipitate was collected by filtration. This solid was dissolved in KOH aqueous solution (1 g / 20 mL), and the insoluble pale blue materials were removed by filtration. The pale yellow filtrate was neutralized with 5M HCl, and the resulting white precipitates were washed with water, methanol, and Et2O, and dried (1.90 g, 83 %).

This white solid (1.86 g, 7.6 mmol) was suspended in dry methanol (250 mL), and conc. sulfuric acid (2.5 mL) was slowly added in to the suspension. The mixture was refluxed overnight. After evaporation of methanol, the product was extracted with 10% methanol in CHCl3 (350 mL), and the organic layer was washed with water several times until pH turned to ~7, brine, and dried (Na2SO4). The crude solid was recrystallized from chloroform and methanol to give the 2,2'-bipyridine-4,4'-dicarboxylic acid dimethyl ester as a colorless solid (1.50 g, 72 %): mp 214 °C; 1H-NMR (400 MHz, DMSO-d6) δ 8.98 (s, 2H), 8.87 (d, J = 5.8 Hz, 2H), 7.91 (dd, J = 5.8 and 1.6 Hz, 2H), 4.04 (s, 6H).
A solution of 2,2'-bipyridine-4,4'-dicarboxylic acid dimethyl ester (1.0 g, 3.68 mmol) and 1M NaOH (71 mL) in methanol (200 mL) was refluxed for overnight. The insoluble material was filtered off, and the filtrate was then concentrated, and adjusted to pH ~6 with 5 M HCl at 0 °C. The resulting white solid was collected by filtration, washed with water, methanol, and Et2O, and dried to give 7 (863 mg, 96 %).

2-{4'-(1,3-Bis-methoxycarbonyl-propylcarbamoyl)-[2,2']bipyridinyl-4-carbonyl]-amino}-pentanedioic acid dimethyl ester (6). Compound 5 (500 mg, 1.78 mmol) was treated with SOCl2 (11 mL) under reflux condition overnight to give the corresponding acid chloride as a white solid. To a solution of L-glutamic acid dimethyl ester hydrochloride (917 mg, 4.33 mmol) and Et3N (0.5 mL, 3.61 mmol) in CH2Cl2 (20 mL) was added a suspension of the acid chloride (406 mg, 1.44 mmol) in toluene (30 mL), and the mixture was stirred at rt overnight. The product was extracted with AcOEt, and the organic layer was washed with water, sat. NaHCO3, and brine, dried (MgSO4), and evaporated. The crude material was purified by SiO2 column chromatography (chloroform : acetone: EtOH = 100:40:8) to give a white solid (457 mg, 56 %): mp 214 °C; 1H-NMR (400 MHz, CDCl3) δ 8.84 (d, J = 5.0 Hz, 2H), 8.77 (s, 2H), 7.38 (d, J = 7.4 Hz, 2H, NH), 4.85 (m, 2H, α-H), 3.81 (s, 6H, OCH3), 3.71 (m, 6H, OCH3), 2.53 (m, 4H, CH2), 2.37 (m, 2H), 2.20 (m, 2H): 13C-NMR (100 MHz, CDCl3) δ 26.9, 30.2, 52.0, 52.5, 52.7, 117.9, 121.9, 141.8, 150.1, 156.1, 165.3, 172.0, 173.6: HR FAB-MS calcd for C26H31N4O10 [M+H]+ 559.2040, found 559.2073.

Tris[4,4'-bis((S)-1,3-dimethoxycarbonyl-propylaminocarbonyl)-2,2'-bipyridine]ruthenium( II ) Bis(hexafluorophosphate) (7), Δ/Λ mixture. This complex was prepared by a similar method to that previously reported.[1] A solution of 8 (391 mg, 0.67 mmol) and anhydrous RuCl3 (45 mg, 0.21 mmol) in dry EtOH (60 mL) was refluxed under Ar atmosphere for 27 h. After evaporation of solvent, the residue was dissolved in distilled water (30 mL), and insoluble materials were filtered off. To the filtrate was added a solution of NH4PF6 in distilled water (15 mL). The resulting red precipitates were collected by filtration to give the product as a red crystal (247 mg, 55 %): 1H NMR (CD3OD, 400 MHz) δ 9.21 (s, 6H), 8.04 (d, J = 5.9 Hz, 6H), 7.89 (d, J = 5.9 Hz, 6H), 4.71 (m, 6H, α-H), 3.75 (s, 18H, CH3), 3.65 (s, 18H, CH3), 2.52 (m, 12H, CH2CO), 2.27 (m, 6H, CHHCH2), 2.13 (m, 6H, CHHCH2): UV-Vis (MeCN), [9] = 48.7 μM, λmax nm (log ε): 250 (4.72), 302 (4.80), 348 (4.28), 467 (4.41): LR ESI-MS calcd for C78H90N12O30Ru [M+] 1776, found 1776: Anal. calcd for C 78H90F12N12O3P2Ru·5EtOH: C, 46.01; H, 5.27; N, 7.32. found: C, 45.33; H, 4.39; N, 8.13.

Tris[4,4'-bis((S)-1,3-dicarbonyl-propylaminocarbonyl)-2,2'-bipyridine]ruthenium( II ) Bis(hydroxylate), (1), Δ/Λ mixture. Complex 7 (50 mg, 0.023 mmol) was hydrolyzed by treatment with 1M NaOH (414 μL) in MeOH (2.5 mL). After stirring the mixture at rt overnight, the excess NaOH was neutralized with 1M HCl, and the solvent was evaporated. The residual solid was dissolved in aqueous MeOH, and insoluble materials were filtered off. The filtrate was concentrated to dryness, and
the red solid was suspended in dry EtOH (10 mL). The resulting red powder was collected by centrifugation to give the product as a dark red solid (20 mg, 49 %): $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 9.21 (s, 6H), 8.04 ($J = 5.9$ Hz, 6H), 7.90 ($J = 5.9$ Hz, 6H), 4.72 (m, 6H, $\alpha$-H), 2.51 (m, 12H, CH$_2$CO), 2.30 (m, 6H, CHHCH$_2$), 2.05 (m, 6H, CHHCH$_2$): UV-Vis (5 mM phosphate buffer, pH 7.4), $[\lambda]_{max}$ = 469 (4.07). Emission (5 mM phosphate buffer, pH 7.4), $\lambda_{ex} = 459$ nm, $\lambda_{em} = 626$ nm: LR TOF-MS calcd for C$_{66}$H$_{66}$N$_{12}$O$_{30}$Ru [M]$^+$ 1608, found 1608.

General procedure for ligand synthesis and preparation of Ru(bpy)$_3$ complexes containing dipeptide side chains (Scheme S2).

H-Glu(OMe)-Phe-OMe trifluoroacetate. A coupling reaction of Boc-Glu(OMe)-OH (3.0 g, 11.5 mmol) and HCl-H-Phe-OMe (2.47 g, 11.5 mmol) was carried out with EDCI-HCl (2.20 g, 11.5 mmol), HOBt (1.55 g, 11.5 mmol), and Et$_3$N (1.6 mL, 11.5 mmol) in DMF (50 mL) to give Boc-Glu(OMe)-Phe-
OMe. This compound was recrystallized from AcOEt-hexanes to give the pure product as colorless needles (4.29 g, 88 %): $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 7.31-7.22 (m, 5H, Aryl H), 6.66 (br s, 1H, NH), 5.17 (br s, 1H, NH), 4.83 (m, 1H, $\alpha$-CH), 4.13 (m, 1H, $\alpha$-CH), 3.72 (s, 3H, OCH$_3$), 3.68 (s, 3H, OCH$_3$), 3.12 (m, 2H, CH$_2$Ph), 2.43-2.39 (m, 2H, CH$_2$CO), 2.07 (m, 1H, CHHCH$_2$), 1.88 (m, 1H, CHHCH$_2$), 1.43 (s, 9H, tBu).

This compound was deprotected by treatment with 50% TFA in dichloromethane to give the desired product as a white solid (100 %). This compound was used for the next step without further purification.

4-({4'-[3-Methoxycarbonyl-1-(1-methoxycarbonyl-2-phenyl-ethylcarbamoyl)-propylcarbamoyl]-[2,2']bipyridinyl-4-carbonyl}-amino)-4-(1-methoxycarbonyl-2-phenyl-ethylcarbamoyl)-butyric acid methyl ester (8). This compound was prepared by a similar method described for 6 using 5 (232 mg, 0.95 mmol) and TFA·H-Glu(OMe)-Phe -OMe (1.01 g, 2.37 mmol). The crude product was purified by SiO$_2$ column chromatography (CH$_2$Cl$_2$: hexane = 4:1) to give desired product as a yellow solid (332 mg, 41 %): $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.80 (d, $J$ = 5.0 Hz, 2H, bpy), 8.68 (s, 2H, bpy), 7.72 (dd, $J$ = 6.6 and 1.6 Hz, 2H, bpy), 7.68 (d, $J$ = 7.2 Hz, 2H, NH), 7.26-7.11 (m, 10H, Aryl H), 7.06 (d, $J$ = 7.8 Hz, 2H, NH), 3.75 (s, 6H), 3.69 (s, 6H), 3.18 (dd, $J$ = 13.9 and 5.5 Hz, 2H), 3.07 (dd, $J$ = 13.9 and 7.0 Hz, 2H), 2.66-2.47 (m, 4H), 2.26-2.09 (m, 4H): HR FAB-MS calcd for C$_{44}$H$_{49}$N$_6$O$_{12}$ [M+H]$^+$ 853.3409, found 853.3426.

Tris[4,4'-bis(methoxycarbonyl-2-(S)-benzyl-ethylcarbamoyl-(S)-3-methoxycarbonyl-propylaminocarbonyl)-2,2'-bipyridine]ruthenium (II) Bis(hexafluorophosphate)s, (9), $\Delta/\Lambda$ mixture. This compound was prepared by a similar method to that described for 7. The crude complex was purified by SiO$_2$ column chromatography (CH$_2$Cl$_2$:acetone:EtOH = 100:80:16 to CH$_2$Cl$_2$:MeOH = 5:1) to give the product as a red solid (82 mg, 49 %). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 9.46 and 9.39 (s each, 1.5H each), 9.21 and 9.19 (s each, 1.5H each), 8.64 and 8.60 (d each, $J$ = 7.4 and 7.6 Hz, 1.5H each), 8.40 (d, $J$ = 7.4 Hz, 0.75H), 8.22 (s, 0.75H), 8.08 (s, 0.75H), 7.99 (m, 6H), 7.87 (m, 6H), 7.76 (s, 0.75H), 7.30-7.10 (m, 36H), 4.55 and 4.46 (m, 10.8 H), 4.20 and 3.99 (m, 1.2H), 3.62, 3.58, 3.57, 3.53, 3.51 (s each, 36H), 3.05-2.95 (m, 12H), 2.42-2.37 (m, 6H), 2.04 (m, 12H): LR ESI-MS calcd for C$_{132}$H$_{144}$N$_{18}$O$_{36}$Ru [M+H]$^+$ 2658, found [M]$^+$2658.

Tris[4,4'-bis(methoxycarbonyl-2-(S)-benzyl-ethylcarbamoyl-(S)-3-methoxycarbonyl-propylaminocarbonyl)-2,2'-bipyridine]ruthenium (II) Bis(hydroxylate)s, (2), $\Delta/\Lambda$ mixture. This compound was prepared by a similar method to that described for 1. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 9.26-9.18 (m, 6H), 7.96 (m, 12H), 7.25-7.01 (m, 30H), 4.54 and 4.45 (m, 12H), 3.17 (m, 6H), 3.00 (m, 6H), 2.30 (m, 12H), 2.09 (m, 12H): UV-Vis (5 mM phosphate buffer, pH 7.4), $[\epsilon]$ = 50.0 $\mu$M, $\lambda_{\text{max}}$ nm (log $\varepsilon$): 439 (4.14), 467 (4.23): LR ESI-MS calcd for C$_{120}$H$_{120}$N$_{18}$O$_{36}$Ru [M+H]$^+$ 2491, found [M+H]$^+$2491.
Procedure for ligand synthesis and preparation of Ru(bpy)$_3$ complexes containing isophtalamide spacers (Scheme S3).

$N_1,N_3$-di($(S)$-benzyl-methoxycarbonylmethyl)-5-nitro-1,3-benzene dicarboxyamide (10). A solution of 5-nitroisophthalic acid (2.11 g, 10 mmol) and thionyl chloride (20 mL) was refluxed for 15 h, and the excess thionyl chloride was evaporated to give the diacid chloride as a colorless solid. The acid chloride was reacted with HCl•H-Phe-OMe (6.47 g, 30 mmol) in a mixture of dichloromethane (50 mL) and Et$_3$N (6.96 mL, 50 mmol) at rt overnight. The product was extracted with chloroform and H$_2$O, and the combined organic layer was washed with 10 % citric acid, sat. NaHCO$_3$, and dried (anhydrous MgSO$_4$). After concentration, the residual solid was recrystallized from chloroform and Et$_2$O to afford the desired product as a colorless solid (4.65 g, 87 %). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.64 (s, 2H, Aryl H), 8.36 (s, 1H, Aryl H), 7.34-7.14 (m, 10H, Aryl H), 6.88 (d, $J = 7.6$ Hz, 2H, NH), 5.10 (m, 2H, $\alpha$CH), 3.81 (s, 6H, CH$_3$ x 2), 3.27 (dd, $J = 13.9$ and 6.2 Hz, 2H, CH$_2$ x 2): HR FAB-MS calcd for C$_{28}$H$_{28}$N$_3$O$_8$ $[M+H]^+$ 534.1877, found 534.1915.

$N_1,N_3$-Di($(S)$-1-(butoxycarbamino)ethyl-methoxycarbonylmethyl)-5-nitro-1,3-benzene dicarboxyamide (11). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 8.75 (s, 2H, Aryl H), 8.53 (s, 1H, Aryl H), 7.73 (br s, 2H, NH), 4.80 (br s, 2H), 4.73 (br s, 2H), 3.32 (s, 6H, CH$_3$ x 2), 3.13 (br s, 4H, CH$_2$N x 2), 2.09-1.80 (m, 4H), 1.53-1.26 (m, 8H), 1.37 (s, 18H, $t$Bu): HR FAB-MS calcd for C$_{32}$H$_{50}$N$_5$O$_{12}$ $[M+H]^+$ 696.3456, found 696.3433.

$N_1,N_3$-di($(S)$-benzyl-methoxycarbonylmethyl)-5-amino-1,3-benzene dicarboxyamide (12). Compound 10 (600 mg, 1.12 mmol) was dissolved in THF (30 mL). Palladium on Carbon (10 % w/w) was added to the solution which was then hydrogenated for 24 h at rt and 1 atm of hydrogen. The mixture was filtered through Celite and concentrated. The crude material was purified by chromatography (chloroform:acetone:EtOH = 100:10:2) to obtain a pale yellow solid (411 mg, 73 %). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 7.31-7.09 (m, 13H), 6.70 (d, $J = 7.7$ Hz, 2H, NH), 5.05 (m, 2H, $\alpha$CH x 2), 3.76 (s, 3H, CH$_3$ x 2), 3.28 (dd, $J = 13.9$ and 5.8 Hz, 2H), 3.20 (dd, $J = 13.9$ and 6.0 Hz, 2H): HR FAB-MS calcd for C$_{28}$H$_{30}$N$_3$O$_6$ $[M+H]^+$ 504.2135, found 504.2161.

$N_1,N_3$-Di($(S)$-butoxycarbamino)ethyl-methoxycarbonylmethyl)-5-amino-1,3-benzene dicarboxyamide (13). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 7.55 (s, 1H, Aryl H), 7.26 (s, 2H, Aryl H), 7.00 (br s, 2H, NH x 2), 4.77 (m, 2H, $\alpha$CH x 2), 4.66 (br s, 2H, NH$_2$ x 2), 3.99 (br s, 2H, NH$_2$), 3.78 (s, 6H, CH$_3$ x 2), 3.11 (m, 4H), 1.97-1.82 (m, 4H), 1.56-1.49 (m, 4H), 1.40 (s, 18H): HR FAB-MS calcd for C$_{32}$H$_{52}$N$_5$O$_{10}$ $[M+H]^+$ 666.3756, found 666.3735.

4,4'-Bis{$(N_1,N_3$-di($(S)$-benzyl-methoxycarbonylmethyl)-1,3-benzene dicarboxyamide-5-aminocarbonyl)-2,2'-bipyridine (14). Compound 7 (150 mg, 0.45 mmol) was converted to the
Scheme S3. Synthesis of Ru(bpy)$_3$ (3a-b)

Reagents and conditions: (a) (i) SOCl$_2$, 95 °C, overnight (ii) HCl•H-AA-OMe, CH$_2$Cl$_2$, rt, overnight; (b) H$_2$, 10% Pd-C, THF, 24h; (c) 2,2'-bipyridine-4,4'-dicarboxylic acid dichloride, triethylamine, CH$_2$Cl$_2$, overnight; (d) RuCl$_3$, 50% DMF in EtOH, 60 °C, 5 days; (e) LiOH, THF; (f) 50% TFA in CH$_2$Cl$_2$, rt.

corresponding diacid chloride by refluxing in thionyl chloride (5 mL) overnight. The resulting acid chloride was treated with compound 12 (500 mg, 0.99 mmol) and triethylamine (138 μL, 0.99 mmol) in dichloromethane (4 mL). The mixture was stirred at rt for 30 min, and refluxed overnight. The crude material was purified by column chromatography (chloroform:acetone:EtOH = 100:10:2 to 100:20:4) to afford the desired product as a white solid (512 mg, 94%). $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 11.05 (s, 2H, NH x 2), 9.04 (d, J = 7.7 Hz, 4H, NH x 4), 8.98 (d, 2H, bpy-3H), 8.98 (s, 2H, bpy-6H), 8.35 (s, 4H,
Aryl H), 8.00 (m, 4H, bpy-4H x 2 and Aryl H x 2), 7.30-7.20 (m, 20H, Aryl H), 4.70 (m, 4H, αCH x 4), 3.64 (s, 12H, CH3 x 4), 3.15 (m, 8H, CH2 x 4): HR FAB-MS calcd for C68H63N8O14 [M+H]+ 1215.4461, found 1215.4447.

4,4’-Bis{(N1,N3-Di((S)-1-(butoxycarbonylamino)ethyl-methoxycarbonylmethyl)-1,3-benzene dicarboxyamide-5-aminocarbonyl)}-2,2’-bipyridine (15). 1H NMR (DMSO-d6, 400 MHz) δ 11.05 (s, 2H, NH x 2), 9.01 (s, 4H), 8.92 (d, J = 6.9 Hz, 4H, NH x 4), 8.44 (s, 4H, Aryl H), 8.14 (s, 2H), 8.04 (d, J = 4.6 Hz, 2H), 6.80 (m, 4H, NH x 4), 4.43 (m, 4H, αCH x 4), 3.67 (s, 12H, CH3 x 4), 2.91 (m, 8H, CH2N x 4), 1.82 (m, 8H, CH2 x 4), 1.40 (m, 16H, CH2CH2 x 4), 1.35 (s, 36H, tBu): HR FAB-MS calcd for C76H107N12O22 [M+H]+ 1539.7623, found 1539.7593.

4,4’-Bis{(N1,N3-Di((S)-benzyl-carboxyllmethyl)-1,3-benzene dicarboxyamide-5-aminocarbonyl)}-2,2’-bipyridine (4). Compound 14 (11 mg) was treated with 1M LiOH (71 μL, 71 μmol) in THF at rt overnight. No starting material was observed on TLC. After evaporation of THF, the mixture was neutralized by adding 1M HCl (71 μL, 71 μmol). The resulting white precipitate was further purified by size exclusion column (Sephadex LH-20; MeOH: dichloromethane = 1:1) to obtain the desired compound as a colorless amorphous solid (10 mg, 100 %). 1H NMR (CD3OD, 400 MHz) δ 8.87 (s, 2H), 8.83 (d, J = 4.5 Hz, 2H), 8.25 (s, 4H), 7.87 (br s, 4H), 7.29-7.17 (m, 20H), 4.85 (m, 4H), 3.35 and 3.13 (dd, J = 2.0 and 9.6 Hz, 2H each): HR ESI-MS calcd for C64H55N8O14 [M+H]+ 1159.3838, found 1159.3816.

Tris[4,4’-Bis{(N1,N3-di((S)-benzyl-methoxycarbonylmethyl)-1,3-benzene dicarboxyamide-5-aminocarbonyl)}-2,2’-bipyridine]ruthenium (II) Bischlorides (16), Δ/Λ mixture. This complex was prepared by a similar method to that previously reported. A solution of 14 (84 mg, 67 μmol) and anhydrous RuCl3 (4.2 mg, 20.3 μmol) in EtOH (2 mL) and DMF (2 mL) was placed in a glass tube, which was degassed and sealed. The mixture was heated at 60 °C for 5 days, and the resulting dark red solution was concentrated. The residual dark red solid was subjected to SiO2 column chromatography (chloroform alone, chloroform:acetone:EtOH = 100:20:4 to 100:40:8), and the orange fluorescent fractions were collected. The resulting solid was further purified by size exclusion chromatography (Sephadex LH-20; MeOH: dichloromethane = 1:1) to afford the desired product as a dark red solid (43 mg, 56 %). 1H NMR (DMSO-d6, 400 MHz) δ 11.14 (s, 3H), 11.02 (s, 3H), 9.53 (br s, 3H), 9.06-9.04 (m, 9H), 8.99 (br s, 6H), 8.37 and 8.35 (s, 6H each), 8.15-8.02 (m, 18H), 7.31-7.16 (m, 60H), 4.74 (m, 12H), 3.66 and 3.63 (s, 18H each), 3.18 (m, 24H): LR ESI-MS calcd for C204H187N24O42Ru [M+H]+ 3746, found 3746.
24H), 6.78 (m, 12H, BocNH), 4.42 (m, 12H, αCH), 3.67 and 3.64 (s each, 36H), 2.91 (m, 24H, CH₂NH), 1.82 (m, 24H, CH₂CH₂NH), 1.35, 1.33, 1.24 (s each, 156H, tBu, CH₂CH₂): LR FAB-MS calcd for C₂₂₃H₃₁₀N₃₆O₆₄Ru [M-H]⁺ 4617, found 4617.

Tris[4,4’-Bis{(N1,N3-di((S)-benzyl-carboxy carbonylmethyl)-1,3-benzene dicarboxyamide-5-aminocarboxyl)le}]-2,2’-bipyridine[ruthenium (II) Bis(hydroxylate)s (3a), Δ/Λ mixture. Compound 16 (11 mg, 2.78 μmol) was treated with 1M LiOH (133 μL, 133 μmol) in THF (1 mL). The solution was neutralized with 1M HCl and evaporated to dryness. The residual solid was purified by size exclusion column chromatography (Sephadex LH-20; MeOH: dichloromethane = 1:1), and the luminous fractions identified by TLC analysis were collected to afford a dark red solid (8.1 mg, 81 %). ¹H NMR (DMSO-d₆, 400 MHz) δ 11.14 (s, 3H), 11.02 (s, 3H), 9.53 (br s, 3H), 9.06-9.04 (m, 9H), 8.99 (br s, 6H), 8.37 and 8.35 (s, 6H each), 8.15-8.02 (m, 18H), 7.31-7.16 (m, 60H), 4.74 (m, 12H), 3.66 and 3.63 (s, 18H each), 3.18 (m, 24H): UV-Vis (acetonitrile), [3a] = 25 μM, λmax nm (log ε): 305 (4.71), 469 (4.04): Anal. calcd for C₁₉₂H₁₅₂Li₁₂N₂₄O₄₄Ru·14H₂O: C,58.59; H,4.61; N, 8.54. found: C, 58.66; H, 4.74; N, 8.62: MALDI-MS calcd for C₁₉₂H₁₆₂N₂₄O₄₂Ru M⁺ 3579, found 3579.

Tris[4,4’-Bis{(N1,N3-Di((S)-1-aminoethyl-methoxycarbonylmethyl)-1,3-benzene dicarboxyamide-5-aminocarboxyl)le}]-2,2’-bipyridine[ruthenium (II) Bis(trifluoroacetate)s (3b), Δ/Λ mixture. This compound was derived from compound 17 (5.3 mg) by treatment with 50 % TFA in dichloromethane for 30 min. at rt. The mixture was evaporated to dryness, and the red solid was passed through Sephadex LH-20 column (dichloromethane:MeOH = 1:1) to give the product as a dark red solid (6 mg, quant.). ¹H NMR (CD₃OD, 400 MHz) δ 9.46 (s, 3.3H), 9.00 and 8.03 (s and d, J = 4.4 Hz, 0.9H), 8.58-8.40 (m, 15H), 8.17-7.97 (m, 15H), 7.31 (br s, 1.8H), 4.64 (m, 12H), 3.78 and 3.75 (s each, 36H), 2.94 (m, 24H), 2.01 (m, 12H), 1.92 (m, 12H), 1.83 (m, 24H), 1.56 (m, 24H): Anal. calcd for C₁₇₂H₂₂₂F₆N₃₆O₄₆Ru·14CF₃CO₂H·14H₂O: C,58.59; H,4.61; N, 9.38. found: C, 44.80; H, 4.73; N, 9.06: MALDI-MS calcd for C₁₄₄H₁₃₈N₂₄O₆₆Ru [M+H]⁺ 3519, found 3519.

Fluorescence Titrations. Fluorescence spectra were obtained on a Shimadzu RF-5300PC equipped with SA-100 temperature controller (Sansyo) with 3 nm excitation and emission slit widths. Stock solutions of compounds were prepared in DMSO (~ 5 mM) and diluted in 2 mL of sodium phosphate buffer (5 mM, pH 7.4) in a 10 mm cuvette. The final concentration of compound was adjusted to 10 μM, and the final content of DMSO was 0.2%. Titration was performed by adding 0 to 50 μM of ChT with the sodium phosphate stock solution (1 mM, pH 7.4). Before spectral measurement after each titration, the solution was incubated for 10 min at 25 °C. The emission spectrum was measured at 620 nm through excitation of the MLCT bands (~ 470 nm). Three scans were averaged, and recorded. The changes in volume at the end of the titration were typically around 5%, and thus the data were uncorrected for dilution. The
obtained data set in case of 3a was analyzed using Hyperquad2006 or WinEQNMR based on the following multi equilibrium model (Figure S1):

\[
\begin{align*}
M + L & \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ML \\
ML + L & \underset{K_3}{\overset{K_2}{\rightleftharpoons}} ML_2 
\end{align*}
\]

where M and L are Ru(bpy)₃ and ChT, respectively.

\[
\log \beta_1 = \log K_1 \\
\log \beta_2 = \log K_1 K_2
\]

**Figure S1.** The data set for 3a was fitted to the multiple-equilibrium model involving 1:1 and 1:2 complexes of 3a with ChT.

**General Conditions for Enzyme Assay.** α-Chymotrypsin inhibition assay was carried out by a similar method to that previously reported.³ To a solution of α-chymotrypsin (1.5 μM, 443 μL) in sodium phosphate buffer (5 mM, pH 7.4) was mixed with a stock solution of each complex in DMSO (5 mM, 6.75 μL). For the control the same amount of DMSO was added. To the solution was added 2 μL of a 10 mM stock solution of BTNA (100 μM), and hydrolysis was followed by monitoring product formation at 405 nm for 5 min at 30 °C. The data was analyzed by Sigma Plot 10. Incubation of BTNA with 3a alone
did not cause any background hydrolysis under the experimental condition.

**Typical Example of Absorbance and Emission Spectrum.**

![Figure S2. Absorbance (left) and emission spectra (right; excitation at 470 nm) of compound 3a (10 μM in 5 mM phosphate buffer, pH 7.4). λ\text{max} nm (log ε): 470 (4.34). Emission, λ\text{ex} = 470 nm, λ\text{em} = 620 nm.](image)

**Figure S3.** A plausible model for ternary complex of ChT bound to 3a (CPK) and \(N\)-benzoyltyrosine-\(p\)-nitroanilide (BTNA, CPK in pink). Docking BTNA into ChT active pocket was performed by Glide in Maestro Ver. 8.0)
MALDI-TOF data of 3a.

![MALDI-TOF data graph](image)

References:

