Supporting Information

Lanthanide-based Protease Activity Sensors for Time-resolved Fluorescence Measurements

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1. Materials and instruments

Z-L-Tyr(*t*Bu)-OH·DCHA was purchased from Watanabe Chemical Industries, Ltd. HBTU was purchased from Peptide Institute, Inc. Calpain I was purchased from CALBIOCHEM. All other reagents were purchased from Tokyo Chemical Industries, Wako Pure Chemical, or Aldrich Chemical Co. They were of highest grade available, and used as received without further purification. Silica gel column chromatography was performed using BW-300, or Chromatorex NH (Fuji Silysia Chemical Ltd.).

NMR spectra were recorded on a JEOL JNM-EX270 instrument at 270 MHz for ^{1}H NMR and at 64.5 MHz for ^{13}C NMR, and a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H, at 100.4 MHz for ^{13}C NMR, using tetramethylsilane (in $CDCl_3$, CD_3OD) as an internal standard. Mass spectra (CI, FAB) were taken on a JEOL JMS-700. ESI-TOF MS was taken on a Waters LCT-Premier XE. IR spectra were measured using a JASCO FT/IR-460 Plus. UV-visible spectra were measured using a Shimadzu UV-1650PC. Fluorescence spectra were measured using a Hitachi F4500 spectrometer. The slit width was 5.0 nm for both excitation and emission. The photomultiplier voltage was 700 V. Time-resolved luminescence spectra and lanthanide luminescence lifetimes were measured using a HORIBA JOBIN YVON S.A.S. SPEX spectrometer. The slit width was 5.0 nm for both excitation and emission. The photomultiplier voltage was 950 V.

2. Synthesis

DO3A *tert*-butyl ester hydrobromide salt (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid *tert*-butyl ester hydrobromide salt) (5·HBr)

Cyclen (2.42 g, 14.1 mmol, 1.0 eq.) was dissolved in acetonitrile (80 mL) under argon. An excess of $NaHCO_3$ (5.91 g, 70.0 mmol, 5.0 eq.) was added to the solution and the mixture was stirred at 0 °C for 30min. *tert*-Butyl bromoacetate (9.05 g, 46.4 mmol, 3.3 eq.) was dropped to the mixture for 60 min. The mixture was cooled and allowed to be stirred at RT for 24 h. The mixture was then filtered and the solvent was evaporated. The residue was recrystallized from toluene to give **5**·HBr as a colorless solid (3.88 g, y. 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 27H), 2.88–2.93 (m, 12H), 3.11 (s, 4H), 3.29 (s, 2H), 3.38 (s, 4H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.74, 27.81, 28.12, 28.16, 47.41, 49.18, 51.30, 58.08, 81.61, 81.77, 169.59, 170.47; IR (KBr) ν 1226, 1252, 1368, 1732, 2861, 2979, 3115, 3462; HRMS (ESI^+) *m/z*: 515.3797 (Calcd for $[M+H]^+$: 515.3809).

4-Nitrobenzyl DO3A *tert*-butyl ester (6)

Compound **5**·HBr (3.60 g, 7.00 mmol 1.0 eq.) and 4–nitrobenzyl bromide (1.51 g, 7.00 mmol, 1.0 eq.) were dissolved in acetonitrile (70 mL) under argon. An excess of $NaHCO_3$ (2.94 g, 35.0 mmol, 5.0 eq) was added to the solution. The mixture was refluxed at 100°C for 8 h. The mixture was then filtered and the solvent was evaporated. The residue was purified using silica gel column chromatography eluting with dichloromethane/methanol (95/5) to give **6** as a pale yellow solid (4.25 g, y. 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 27H), 2.19–3.50 (m, 24H), 7.72 (d, J = 8.0 Hz, 2H), 8.18 (d, J = 8.0 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.74, 27.84, 27.97, 28.02, 50.02, 55.69, 56.01, 58.85, 81.86, 82.39, 82.97, 123.55, 130.98, 145.30, 147.20, 172.57, 173.60; IR (KBr) ν 1164, 1230, 1344, 1522, 1718, 2848, 2981, 3462; HRMS (ESI$^+$) m/z: 650.4142 (Calcd for $[M+H]^+$: 650.4129).

4-Aminobenzyl DO3A *tert*-butyl ester (7)

Compound **6** (1.40 g, 2.16 mmol) was dissolved in methanol (10 mL). The mixture was stirred with 10% Pd/C (40 mg) under H_2 for 12 h. The mixture was then filtered and the solvent was evaporated to give **7** as a colorless solid (1.26 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 27H), 2.21-3.67 (m, 26H), 6.62 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.4 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.74, 27.84, 28.05, 28.08, 50.34, 55.58, 55.79, 58.78, 81.82, 82.19, 82.64, 114.98, 126.38, 130.83, 146.01, 172.29, 173.24; IR (KBr) ν 1162, 1225, 1368, 1718, 2830, 2976, 3202, 3410; HRMS (ESI$^+$) m/z: 620.4350 (Calcd for $[M+H]^+$: 620.4387).

4-Aminobenzyl DO3A (Abd) (1)

Compound **7** (962 mg, 1.55 mmol) was dissolved in TFA (15 mL) and was stirred for 9 h. The mixture was evaporated and crystallized from diethyl ether to give **1** as a pale yellow solid (645 mg, y. 92%). ^{1}H NMR (400 MHz, D_2O) δ 2.79–3.78 (m, 24H), 7.01 (d, J = 8.0 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 43.70, 49.92, 50.30, 52.42, 54.14, 56.55, 59.25, 116.67, 119.58, 133.69, 162.60, 162.95; IR (KBr) ν 1203, 1700, 3110, 3421; HRMS (ESI$^+$) m/z: 452.2462 (Calcd for $[M+H]^+$: 452.2509).

4-Acetylaminobenzyl DO3A *tert*-butyl ester (8)

Compound **6** (594 mg, 0.914 mmol) was dissolved in Ac_2O/AcOH (*v*/*v* = 2/1, 6 mL). The mixture was stirred with 10% Pd/C (26 mg) under H_2 for 40 h. The mixture was then filtered and the solvent was removed under vacuum. The residue was purified using silica gel column chromatography eluting with dichloromethane/methanol (95/5) to give **8** as a yellow solid (268 mg, y. 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 3H), 1.51 (s, 30H), 2.28–3.47(m, 24H), 7.45 (d, J = 7.8 Hz, 2H), 7.64 (d, J = 7.8 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 24.66, 27.83, 27.95, 28.03, 28.11, 50.47, 55.55, 55.89, 59.11, 82.08, 82.56, 83.19, 119.93, 130.05, 131.03, 139.28, 170.04, 172.14, 173.11; IR (KBr) ν 1160, 1228, 1312, 1718, 2834, 2979, 3229, HRMS (ESI$^+$) *m/z*: 662.4456 (Calcd for $[M+H]^+$: 662.4493).

4-Acetylaminobenzyl DO3A (Ac-Abd) (2)

Compound **8** (962 mg, 1.55 mmol) was dissolved in TFA (15 mL) and was stirred for 9 h. The mixture was evaporated and crystallized from diethyl ether to give **2** as a pale yellow solid (645 mg, 92%). ^{1}H NMR (400 MHz, D_2O) δ 2.03 (s, 3H), 2.89–3.80 (m, 24H), 7.33 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H); ^{13}C-NMR (100 MHz, CD_3OD) δ 23.95, 30.71, 50.58, 51.47, 52.18, 55.68, 57.09, 58.50, 121.74, 132.14, 139.96, 171.15, 171.45, 209.85; IR (KBr) ν 1399, 1635, 3432; HRMS (ESI$^+$) *m/z*: 494.2570 (Calcd for $[M+H]^+$: 494.2615).

General synthesis of lanthanide complexes ([1-Ln^{3+}], [2-Ln^{3+}])

Ligand compound (**1**, **2**) was dissolved in 100m M HEPES buffer (pH 7.4) and $Ln(OTf)_3$ (Ln = Tb or Eu) was added. The mixture was stirred for 1 h. The obtained product was purified with reversed-phase HPLC, eluted with 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile to yield the lanthanide complexes (Figure S1-S4). HRMS (FAB$^+$) *m/z*: [**1**-Tb^{3+}]: 608.1537 (Calcd for $[M+H]^+$: 608.1528), [**2**-Tb^{3+}]: 650.1630 (Calcd for $[M+H]^+$: 650.1633), [**1**-Eu^{3+}]: 602.1475 (Calcd for $[M+H]^+$: 602.1487), [**2**-Eu^{3+}]: 644.1588 (Calcd for $[M+H]^+$: 644.1592).

4-Nitroacetophenyl DO3A *tert*-butyl ester (9)

Compound **5**·HBr (1.00 g, 1.95 mmol 1.0 eq.) and 2-bromo-4-nitroacetophenone (0.470 g, 0.195 mmol, 1.0 eq.) were dissolved in acetonitrile (100 mL) under argon. An excess of $NaHCO_3$ (0.660 g, 7.93 mmol, 4.0 eq) was added to the solution. The mixture was refluxed at 100°C for 24 h. The mixture was then filtered and the solvent was evaporated. The residue was purified using silica gel column chromatography eluting with dichloromethane/methanol (95/5) to give **9** as a pale yellow solid

(0.815 g, y. 64%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.46 (s, 27H), 2.16-3.46 (br, 22H), 4.30 (s, 2H), 8.23 (d, J = 8.7 Hz, 2H), 8.31 (d, J = 8.7 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.79, 27.87, 28.16, 28.19, 51.33, 55.57, 55.86, 61.19, 81.61, 81.77, 82.06, 123.84, 129.11, 140.02, 150.58, 172.76, 173.17, 199.36; IR (KBr) ν 1161, 1229, 1368, 1533, 1730, 2832, 2977, 3447; HRMS (ESI$^+$) m/z: 678.4078 (Calcd for $[M+H]^+$: 678.4078).

4-Aminoacetophenyl DO3A *tert*-butyl ester (10)

Compound **9** (150 mg, 0.222 mmol) was dissolved in methanol (50 mL). The mixture was stirred with 10% Pd/C (15 mg) under H_2 for 12 h. The mixture was then filtered and the solvent was evaporated. The residue was purified using silica gel column chromatography eluting with dichloromethane/methanol (98/2) to give **10** as a pale yellow solid (90.7 mg, y. 64%). ^{1}H NMR (270 MHz, CD_3OD) δ 1.43 (s, 27H), 2.86 (s, 4H), 2.86-3.01 (m, 16H), 3.43 (s, 6H), 3.57 (s, 2H), 6.62 (d, J = 8.5 Hz, 2H), 7.74 (d, J = 8.5 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.78, 27.85, 28.12, 28.16, 51.26, 55.57, 55.73, 59.18, 81.57, 81.91, 82.05, 113.68, 125.12, 129.82, 152.68, 169.62, 172.57, 196.40; IR (KBr) ν 1163, 1229, 1368, 1734, 2827, 2975, 3197, 3420; HRMS (ESI$^+$) m/z: 648.4336 (Calcd for $[M+H]^+$: 648.4294).

4-Aminoacetophenyl DO3A (Aad) (3)

Compound **10** (84.2 mg, 0.130 mmol) was dissolved in TFA (10 mL) and was stirred for 12 h. The mixture was evaporated and purified with reversed-phase HPLC, eluted with H_2O/acetonitrile containing 0.1% TFA to yield **3** as a colorless solid (52.7 mg, y. 85%). ^{1}H NMR (270 MHz, CD_3OD) δ3.16-3.62 (m, 16H), 3.81 (s, 6H), 4.12 (s, 2H), 6.64 (d, J = 8.6 Hz, 2H), 7.73 (d, J = 8.6 Hz, 2H); ^{13}C-NMR (100 MHz, CD_3OD) δ 52.79, 54.21, 55.39, 60.31, 76.82, 80.19, 81.50, 114.27, 116.30, 119.18, 132.04, 156.21, 162.10, 174.21; IR (KBr) ν 1197, 1696, 3097, 3446; HRMS (ESI$^+$) m/z: 480.2412 (Calcd for $[M+H]^+$: 480.2458).

4-Acetylaminoacetophenyl DO3A *tert*-butyl ester (11)

Compound **9** (120 mg, 0.233 mmol) was dissolved in Ac_2O/AcOH (v/v = 2/1, 50 mL). The mixture was stirred with 10% Pd/C (12 mg) under H_2 for 24 h. The mixture was then filtered and the solvent was removed under vacuum. The residue was purified using silica gel column chromatography eluting with dichloromethane/methanol (95/5) to give **11** as a yellow solid (37.5 mg, y. 44%). ^{1}H NMR (270

MHz, $CDCl_3$) δ 1.43 (s, 27H), 2.86–3.01 (m, 16H), 3.43 (s, 6H), 3.57 (s, 2H), 6.62 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 24.88, 27.74, 27.80, 28.04, 28.07, 48.78, 55.50, 55.64, 59.52, 82.00, 82.13, 82.39, 119.17, 128.36, 129.68, 145.03, 170.82, 172.52, 172.66, 197.63; IR (KBr) ν 1164, 1229, 1368, 1730, 2831, 2978, 3245, 3447; HRMS (ESI^+) m/z: 690.4463 (Calcd for $[M+H]^+$: 690.4442).

4-Acetylaminoacetophenyl DO3A (Ac-Aad) (4)

Compound **11** (37.0 mg, 0.0537 mmol) was dissolved in TFA (10 mL) and was stirred for 12 h. The mixture was evaporated and purified with reversed-phase HPLC, eluted with H_2O/acetonitrile containing 0.1% TFA to yield **4** as a colorless solid (7.70 mg, y. 27%). ^{1}H NMR (270 MHz, CD_3OD) δ 1.92 (s, 3H), 3.16-3.67 (m, 22H), 4.41 (s, 2H), 7.70 (d, J = 8.6 Hz, 2H), 7.97 (d, J = 8.6 Hz, 2H); ^{13}C-NMR (100 MHz, CD_3OD) δ 22.47, 48.78, 54.29, 55.31, 57.11, 80.92, 81.11, 81.96, 115.08, 118.44, 129.06, 144.12, 144.50, 160.97, 161.31, 170.50; IR (KBr) ν 1201, 1696, 3447; HRMS (ESI^+) m/z: 522.2575 (Calcd for $[M+H]^+$: 522.2564).

Z-Tyr(*t*Bu)-Abd *tert*-butyl ester (12)

Compound **7** (718 mg, 1.16 mmol, 1.0 eq) was dissolved in DMF (25 mL) and then was added with Z-Tyr(*t*Bu)-OH·DCHA (706 mg, 1.28 mmol, 1.1 eq), HOBt·H_2O (355 mg, 2.32 mmol, 2.0 eq), HBTU·PF_6 (879 mg, 2.32 mmol, 2.0 eq.), and DIEA. The mixture was stirred overnight at RT. The mixture was diluted with sat. $NaHCO_3$ aq. and was extracted by ethyl acetate. The organic layer was washed by sat. $NaHCO_3$ aq., water and brine. The organic layer was dried over $MgSO_4$ and was evaporated to give the crude product. The crude product was purified using silica gel column chromatography eluting with dichloromethane/methanol (98/2) to give **12** as a pale yellow solid (892 mg, y. 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.48 (s, 27H,), 2.53–3.69 (m, 28H), 4.47 (m, 1H), 6.60 (d, J = 8.0 Hz, 2H), 6.90 (d, J = 8.0 Hz, 2H), 7.13 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 8.0 Hz, 1H), 7.31–7.35 (m, 5H), 7.43 (d, J = 8.0 Hz, 2H), 7.86 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.53, 27.85, 27.94, 28.07, 28.80, 29.02, 38.59, 55.61, 55.97, 58.95, 67.18, 78.49, 82.40, 82.69, 82.92, 82.94, 115.09, 119.99, 124.39, 127.90, 128.22, 128.55, 129.74, 130.61, 131.06, 133.21, 135.99, 136.92, 154.50, 169.39, 172.50, 173.44; IR (KBr) ν 843, 1162, 1233, 1368, 1718, 2838, 2979, 3447; HRMS (ESI^+) m/z: 973.6016 (Calcd for $[M+H]^+$: 973.6014).

Tyr(*t*Bu)-Abd *tert*-butyl ester (13)

Compound **12** (832 mg, 0.855 mmol) was dissolved in methanol (50 mL). The mixture was stirred with 10% Pd/C (27 mg) under H_2 for 12 h. The mixture was then filtered and the solvent was evaporated to give **13** as a pale yellow solid (695 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.46 (s, 27H), 2.21–3.73 (m, 31H), 6.94 (d, J = 8.4 Hz, 2H), 7.17 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 9.47 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.47, 22.86, 24.67, 27.83, 28.08, 38.56, 49.62, 55.60, 55.97, 59.01, 67.36, 81.87, 82.41, 83.02, 119.98, 124.27, 128.08, 128.51, 129.62, 130.42, 131.37, 154.39, 171.01, 172.43, 173.37; IR (KBr) ν 842, 1161, 1256, 1368, 1743, 2867, 2939, 3095, 3447; HRMS (ESI$^+$) *m/z*: 839.5655 (Calcd for $[M+H]^+$: 839.5646).

Z-LY(*t*Bu)-Abd *tert*-butyl ester (14)

Compound **13** (647 mg, 0.772 mmol, 1.0 eq.) was dissolved in DMF (15 mL) and then was added with Z-Leu-OH (246 mg, 0.946 mmol, 1.2 eq), HOBt·H_2O (236 mg, 1.54 mmol, 2.0 eq) HBTU·PF_6 (584 mg, 1.54 mmol, 2 eq) and DIEA. The mixture was stirred for 2.5 h. The mixture was diluted with sat. $NaHCO_3$ aq. and was extracted by ethyl acetate. The organic layer was washed by sat. $NaHCO_3$ aq., water and brine. The organic layer was dried over $MgSO_4$ and evaporated to give the crude product. The crude product was purified using silica gel column chromatography eluting with dichloromethane/methanol (98/2) to give **14** as a pale yellow solid (388 mg, y. 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87–0.90 (m, 9H), 1.30 (s, 9H), 1.46 (s, 27H), 2.21-3.49 (m, 28H), 4.06 (m, 1H); 4.68 (m, 1H); 6.53 (d, J = 8.0 Hz, 1H); 6.90 (d, J = 8.4 Hz, 2H); 6.92 (d, J = 8.4 Hz, 2H), 7.08 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 7.34 (m, 5H), 7.59 (d, J = 8.0 Hz, 1H), 8.86 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 21.31, 22.35, 22.44, 25.56, 25.78, 26.61, 26.89, 27.01, 27.39, 35.00, 42.92, 45.07, 48.60, 49.91, 53.59, 54.21, 57.85, 80.71, 81.93, 82.95, 115.06, 120.16, 121.70, 124.15, 126.88, 127.97, 128.25, 129.37, 129.85, 130.57, 132.72, 135.89, 137.58, 156.86, 169.24, 170.95, 172.88; IR (KBr) ν 841, 1162, 1233, 1368, 1718, 2835, 2978, 3397; HRMS (ESI$^+$) *m/z*: 1086.6824 (Calcd for $[M+H]^+$: 1086.6855).

LY(*t*Bu)-Abd *tert*-butyl ester (15)

Compound **14** (355 mg, 0.327 mmol) was dissolved in methanol (40 mL). The mixture was stirred with 10% Pd/C (13.0 mg) under H_2 for 28 h. The mixture was then filtered and the solvent was evaporated to give **15** as a pale yellow solid (295 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86–0.99 (m, 9H), 1.31 (s, 9H), 1.46 (s, 27H), 2.09-3.49 (m, 28H), 4.06 (m, 1H), 4.68 (m, 1H), 6.92 (d, J = 8.4

Hz, 2H), 7.15 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 7.98 (d, J = 8.0 Hz, 1H), 8.61 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 20.31, 21.59, 21.64, 23.76, 23.87, 26.41, 26.51, 26.54, 27.28, 36.96, 42.19, 48.98, 52.35, 54.85, 55.10, 55.73, 77.63, 81.46, 82.01, 119.28, 123.33, 129.01, 129.02, 129.91, 131.21, 132.58, 136.98, 153.56, 172.42, 173.22 IR (KBr) v 843, 1160, 1233, 1368, 1723, 2869, 2978, 3392; HRMS (ESI^+) m/z: 952.6531 (Calcd for $[M+H]^+$: 952.6487).

Suc-LY(*t*Bu)-Abd *tert*-butyl ester (16)

Compound **15** (275 mg, 0.289 mmol, 1.0 eq.) was dissolved in DMF (10 mL) and then was added with succinic anhydride (31.8 mg, 0.318 mmol, 1.1 eq.) and DIEA. The mixture was allowed to be stirred for 7 h. The solvent was evaporated and then the mixture was purified using silica gel column chromatography eluting with dichloromethane/methanol (90/10) to give **16** as a colorless solid (176 mg, y. 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83–0.96 (m, 9H), 1.30 (s, 9H), 1.47 (s, 27H), 1.62-3.90 (m, 28H), 4.13 (m, 1H), 4.75 (m, 1H), 6.86 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.62 (d, 1H), 7.86(d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.4 Hz, 2H), 8.55 (s, 1H), 9.17 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 21.63, 23.33, 25.86, 28.45, 29.24, 30.23, 30.72, 31.50, 31.63, 37.45, 41.14, 52.06, 53.68, 54.51, 55.88, 56.77, 57.04, 57.97, 79.54, 80.21, 83.12, 121.65, 125.27, 127.21, 130.76, 133.09, 133.91, 140.93, 155.34, 171.69, 172.00, 175.03, 176.08, 177.45; IR (KBr) v 846, 1162, 1255, 1368, 1729, 2873, 2980, 3093, 3414; HRMS (ESI^+) m/z: 1052.6617 (Calcd for $[M+H]^+$: 1052.6647).

Suc-LY-Abd (17)

Compound **16** (25.0 mg, 0.0238 mmol) was dissolved in TFA (2 mL) and was stirred for 24 h. The mixture was evaporated and purified with reversed-phase HPLC, eluted with H_2O/acetonitrile containing 0.1% TFA to yield **17** as a colorless solid (14.0 mg, 0.0169 mmol, y. 71 %). ^{1}H NMR (400 MHz, CD_3OD) δ 0.85 (d, J = 6.6 Hz, 3H), 0.90 (d, J = 6.6 Hz, 3H), 1.39 (m, 2H), 1.43 (m, 1H), 1.94 (s, 3H), 2.92–3.53 (m, 30H), 4.17 (m, 1H), 4.58 (m, 1H), 6.69 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.6 Hz, 2H), 7.70 (d, J = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 21.88, 22.51, 23.37, 25.82, 32.17, 32.22, 37.58, 41.22, 50.35, 51.79, 52.94, 54.35, 57.14, 57.55, 58.59, 116.16, 116.44, 121.70, 129.28, 131.19, 132.37, 139.79, 157.12, 171.85, 172.77, 174.81, 176.42, 177.52, 178.41; IR (KBr) v 1201, 1638, 3447; HRMS (ESI^-) m/z: 826.4012 (Calcd for $[M-H]^-$: 826.3987).

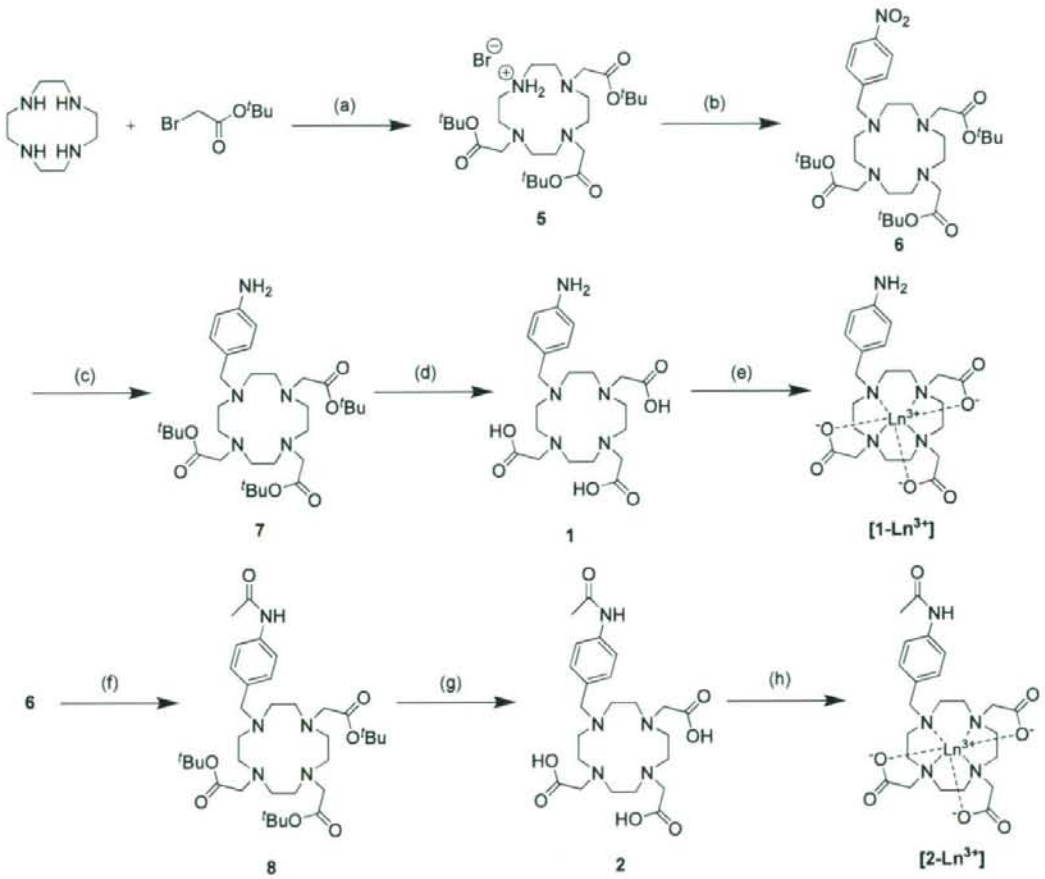
Suc-LY-Abd-Tb

Compound **17** was dissolved in methanol, and then $Tb(NO_3)_3 \cdot 6H_2O$ and Na_2CO_3 were added. The mixture was stirred for 1 h. The solution was evaporated and was purified using Chelex 100, eluting with water to yield Suc-LY-Abd-Tb. The purity was confirmed with reversed-phase HPLC (Figure S5). HRMS (FAB^+) *m/z*: 984.3154 (Calcd for $[M+H]^+$: 984.3162).

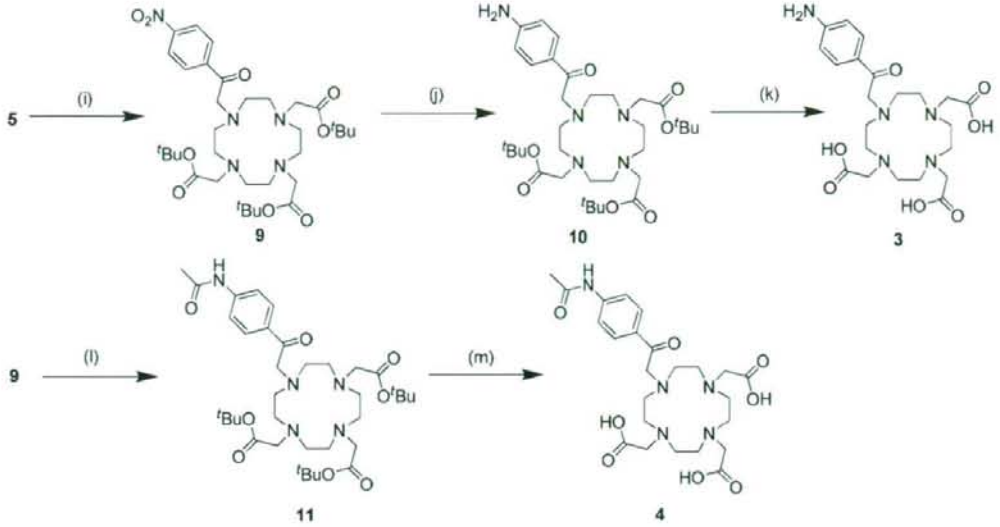
Leu-Abd

Compound **7** (50.0 mg, 0.0806 mmol, 1.0 eq) was dissolved in DMF(1 mL) and then was added with Boc-Leu-OH (25.5 mg, 0.110 mmol, 1.4 eq), $HOBt \cdot H_2O$ (20.8 mg, 0.136 mmol, 1.7 eq), $HBTU \cdot PF_6$ (58.4 mg, 0.154 mmol, 1.9 eq) and DIEA. The mixture was stirred for 3 h. The mixture was evaporated. After dryness the crude product was dissolved in TFA (10 mL) and was stirred for 12 h. The mixture was evaporated and purified with reversed-phase HPLC, eluted with H_2O/acetonitrile containing 0.1% TFA to yield Leu-Abd as a colorless solid (20.0 mg, 0.0354 mmol, y. 44 %). ^1H NMR (400 MHz, CD_3OD) δ 0.93 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 5.8 Hz, 6H), 1.65 (m, 3H), 2.89–3.333 (m, 25H), 7.50 (d, J = 8.3 Hz, 2H), 7.67 (d, J = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 21.96, 23.21, 41.66, 53.75, 58.74, 116.69, 119.61, 121.77, 133.40, 163.02, 169.38; IR (KBr) ν 1137, 1648, 3447; HRMS (FAB^+) *m/z*: 565.3353 (Calcd for $[M-H]^+$: 565.3271).

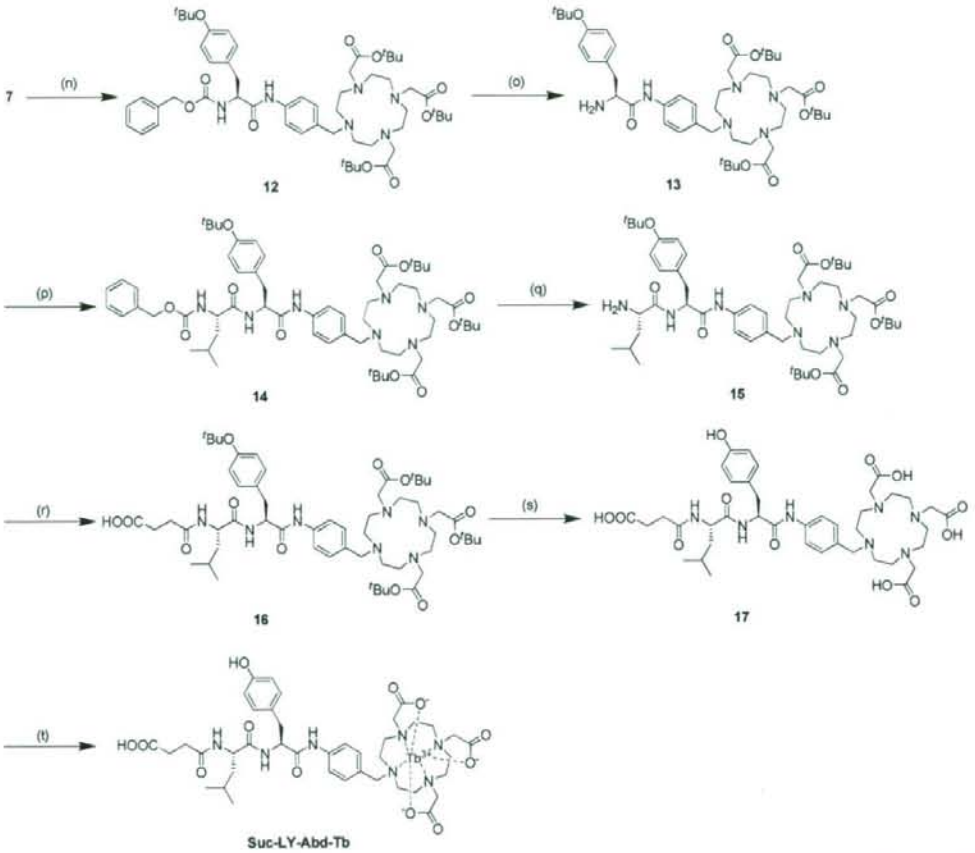
Synthetic schemes



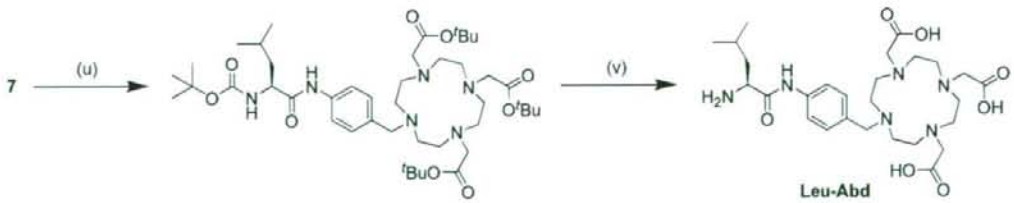
Scheme S1. Synthetic route to [**1**-Ln^{3+}] and [**2**-Ln^{3+}]. (a) $NaHCO_3$, CH_3CN, (b) 4-nitrobenzyl bromide, $NaHCO_3$, CH_3CN, (c) H_2/Pd, MeOH, (d) TFA, (e) $Ln(OTf)_3$ (f) H_2/Pd, Ac_2O/AcOH, (g) TFA, (h) $Ln(OTf)_3$.



Scheme S2. Synthetic route to **3** and **4**. (i) 2-Bromo-4-nitroacetophenone, $NaHCO_3$, CH_3CN, (j) H_2/Pd, MeOH, (k) TFA, (l) H_2/Pd, Ac_2O/AcOH, (m) TFA.



Scheme S3. Synthetic route to Suc-LY-Abd-Tb. (n) Z-Tyr(*t*Bu)-OH·DCHA, HOBt·H_2O, HBTU·PF_6, DMF, DIEA, (o) H_2/Pd, MeOH, (p) Z-Leu-OH, HOBt·H_2O, HBTU·PF_6, DMF, DIEA, (q) H_2/Pd, MeOH, (r) succinic anhydride, DMF, DIEA, (s) TFA, (t) $Tb(NO_3)_3 \cdot 6H_2O$, Na_2CO_3, MeOH.



Scheme S4. Synthetic route to Leu-Abd. (u) Boc-Leu-OH, HOBt·H_2O, HBTU·PF_6, DMF, DIEA, (v) TFA.

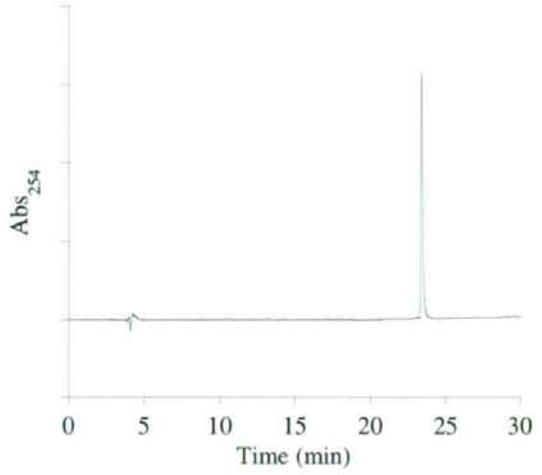


Figure S1. Reversed-phase HPLC diagram of [**1**-Tb^{3+}]. Eluent: 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile: 100/0 (*v*/*v*) (0 min) → 20/80 (*v*/*v*) (30 min).

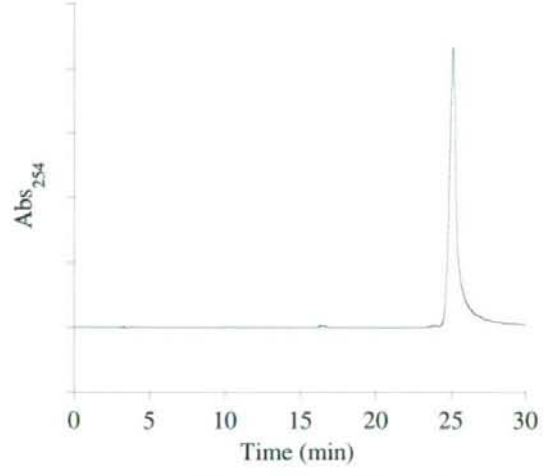


Figure S2. Reversed-phase HPLC diagram of [**2**-Tb^{3+}]. Eluent: 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile: 95/5 (*v*/*v*) (0 min) → 70/30 (*v*/*v*) (30 min).

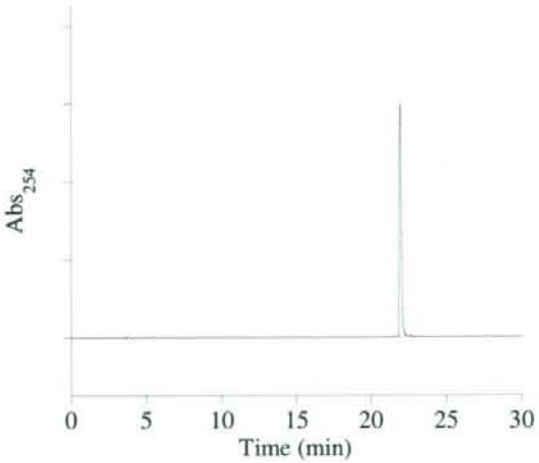


Figure S3. Reversed-phase HPLC diagram of [**1**-Eu^{3+}]. Eluent: 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile: 100/0 (*v/v*) (0 min) → 20/80 (*v/v*) (30 min).

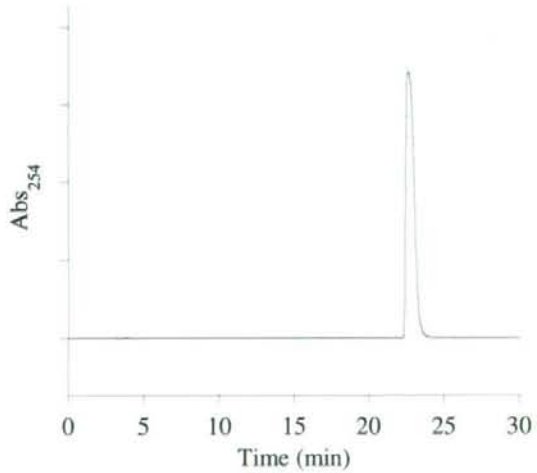


Figure S4. Reversed-phase HPLC diagram of [**2**-Eu^{3+}]. Eluent: 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile: 95/5 (*v/v*) (0 min) → 70/30 (*v/v*) (30 min).

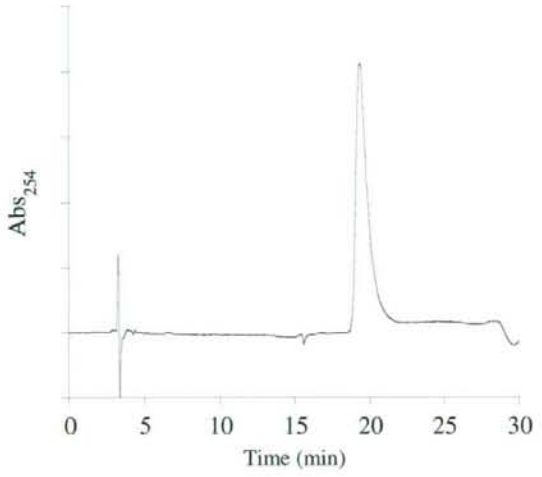


Figure S5. Reversed-phase HPLC with absorbance detection of Suc-LY-Abd-Tb. Eluent: 100 mM triethylammonium-acetate buffer (pH 6.8)/acetonitrile: 63/37 (*v*/*v*) (0 min) → 58/42 (*v*/*v*) (25 min).

3. Physical properties

I. Absorption spectra

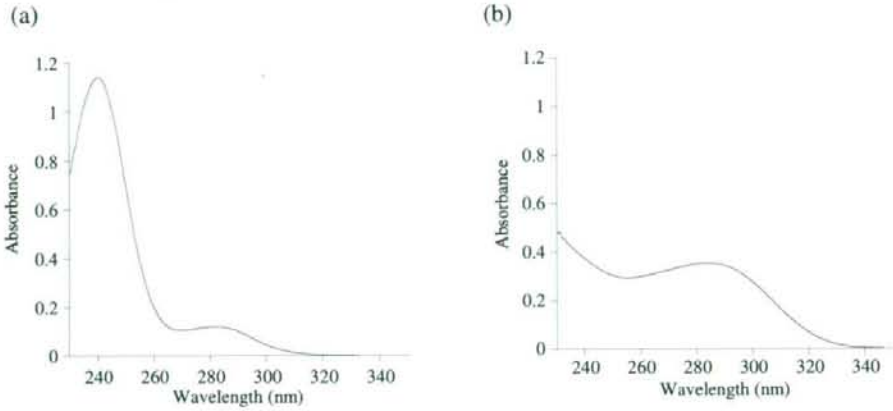


Figure S6. Absorption spectra of 100 μM (a) [**1**-Tb^{3+}] and (b) [**2**-Tb^{3+}] in 100 mM HEPES buffer (pH 7.4) at 25 °C.

II. Emission spectra

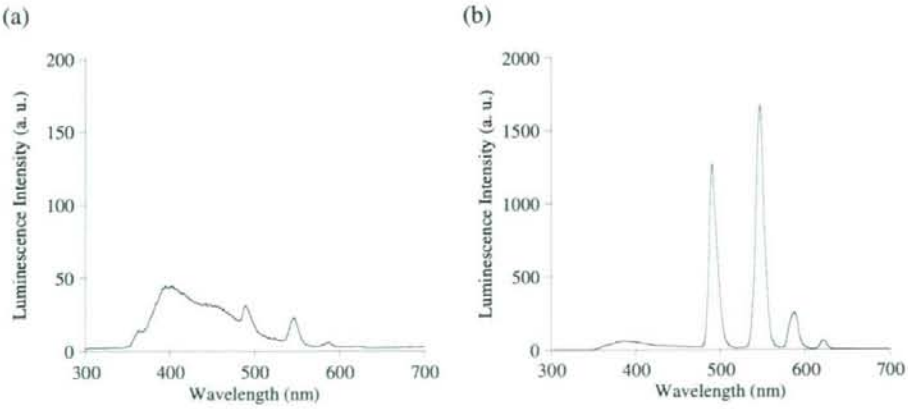


Figure S7. Emission spectra (λ_{ex} = 320 nm) of (a) 10 μM **3** with 10 μM $Tb(NO_3)_3$, (b) 10 μM **4** with 10 μM $Tb(NO_3)_3$ in 100 mM HEPES buffer (pH 7.4) at 25 °C. The spectra were measured after enough time for the complexation.

III. Excitation spectra

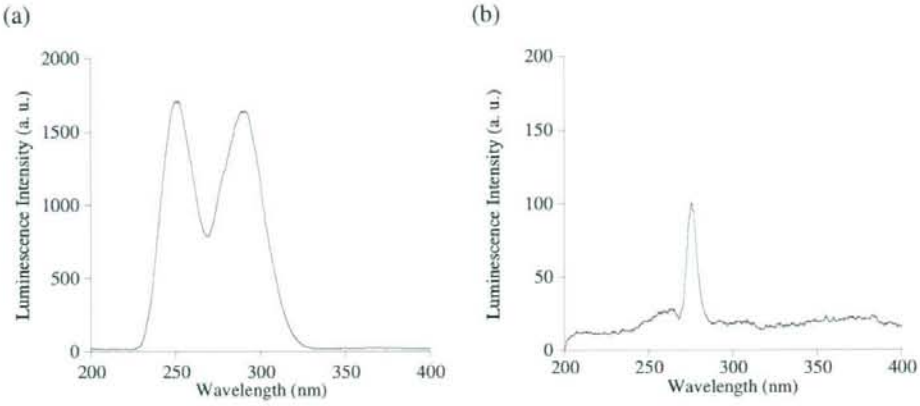


Figure S8. Excitation spectra (λ_{em} = 545 nm) of 10 μM (a) [**1**-Tb^{3+}], (b) [**2**-Tb^{3+}] in 100 mM HEPES buffer (pH 7.4) at 25 °C.

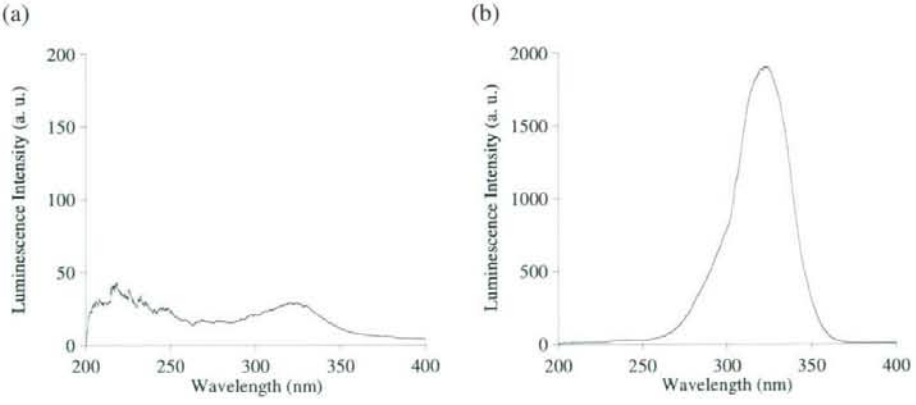


Figure S9. Excitation spectra (λ_{em} = 545 nm) of (a) 10 μM **3** with 10 μM $Tb(NO_3)_3$, (b) 10 μM **4** with 10 μM $Tb(NO_3)_3$ in 100 mM HEPES buffer (pH 7.4) at 25 °C. The spectra were measured after enough time for the complexation.

IV. Time-resolved luminescence spectra

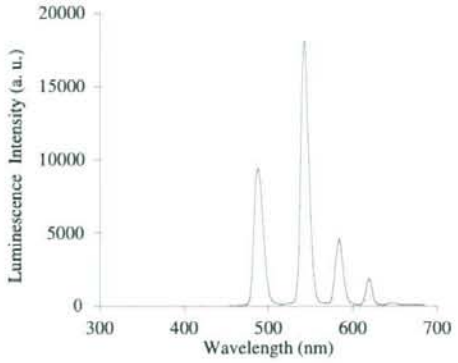


Figure S10. Time-resolved luminescence spectra (λ_{ex} = 250 nm, delay time: 60 μs, gate time: 2.0 ms) of 10 μM [**1**-Tb^{3+}] in 100 mM HEPES buffer (pH 7.4) at 25 °C.

V. Luminescence lifetime

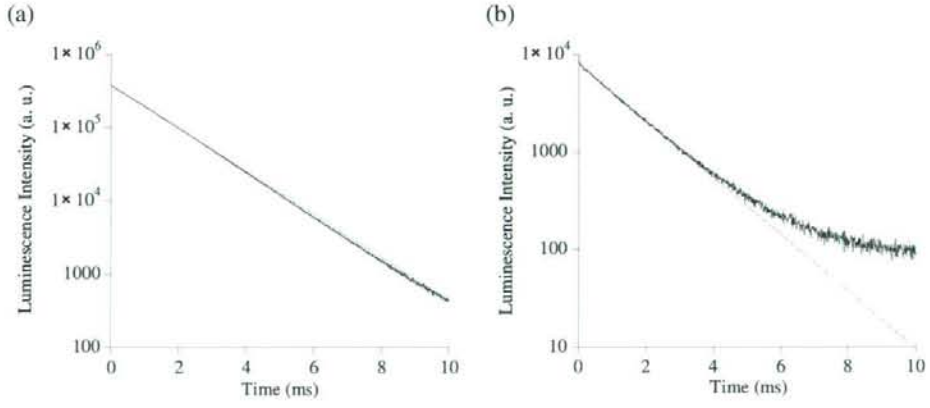


Figure S11. Luminescence lifetime (λ_{ex} = 250 nm, λ_{em} = 545 nm) of 10 μM (a) [**1**-Tb^{3+}], (b) [**2**-Tb^{3+}] in 100 mM HEPES buffer (pH 7.4) at 25 °C. Luminescence lifetime was calculated by the following formula: $I = I_0 \exp(-t/\tau)$.

VI. Quantum yields

Relative quantum yields of luminescence of the synthesized compounds in 100 mM HEPES buffer (pH 7.4) were calculated as described elsewhere[S1] using phenol as the reference ($\Phi_{phenol} = 0.14$).[S2]

VII. Photostability of 3-Tb^{3+} and 4-Tb^{3+} complexes

It has been reported that *p*-aminophenacyl group undergoes photodecomposition by UV irradiation.[S3] Therefore, the photostability of [**3**-Tb^{3+}] and [**4**-Tb^{3+}] was investigated. Figures S12 and S13 show the excitation spectral and ^{1}H NMR spectral changes of (a) [**3**-Tb^{3+}] and (b) [**4**-Tb^{3+}] under UV irradiation by the fluorometer. As a result, no spectral change under UV irradiation was observed in the all cases. This result means that both [**3**-Tb^{3+}] and [**4**-Tb^{3+}] are photostable under the experimental condition.

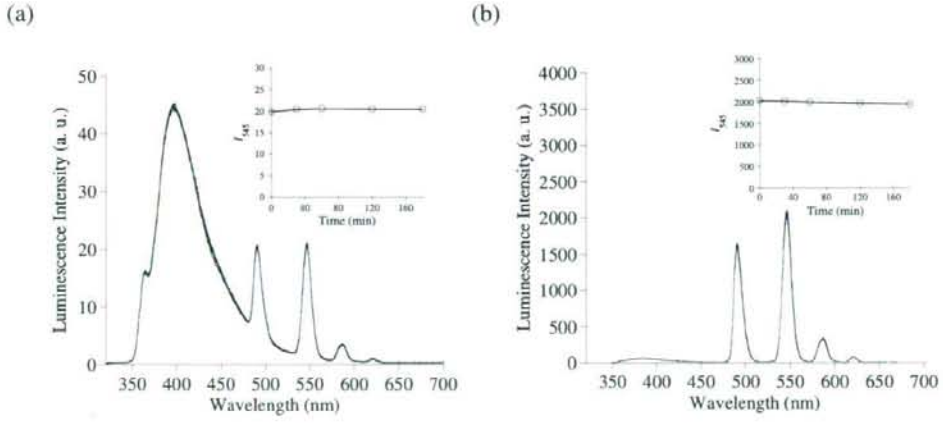


Figure S12. Emission spectra (λ_{ex} = 320 nm) of (a) 10 μM **3** with 10 μM $Tb(NO_3)_3$ and (b) 10 μM **4** with 10 μM $Tb(NO_3)_3$ under UV irradiation by the fluorometer. The spectra were measured in 100 mM HEPES buffer (pH 7.4) at 25 °C. Exposure time was 0, 30, 60, 120, 180 min, respectively. (Inset) Plots of the luminescence intensity (λ_{ex} =320 nm, λ_{em} = 545 nm) vs. time.

4. Enzyme assays

Calpain I assay

Each of 1 μM Suc-LY-Abd-Tb and LY-MCA was dissolved in the reaction buffer (100 mM HEPES (pH 7.4), 10 mM $CaCl_2$, 2.5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA). The samples were incubated with 45 μg/mL calpain I at 30 °C.

Calpain I assays in the presence of fluorescent compound were performed using 1 μM probes with 1 μM umbelliferone in the reaction buffer at 30 °C.

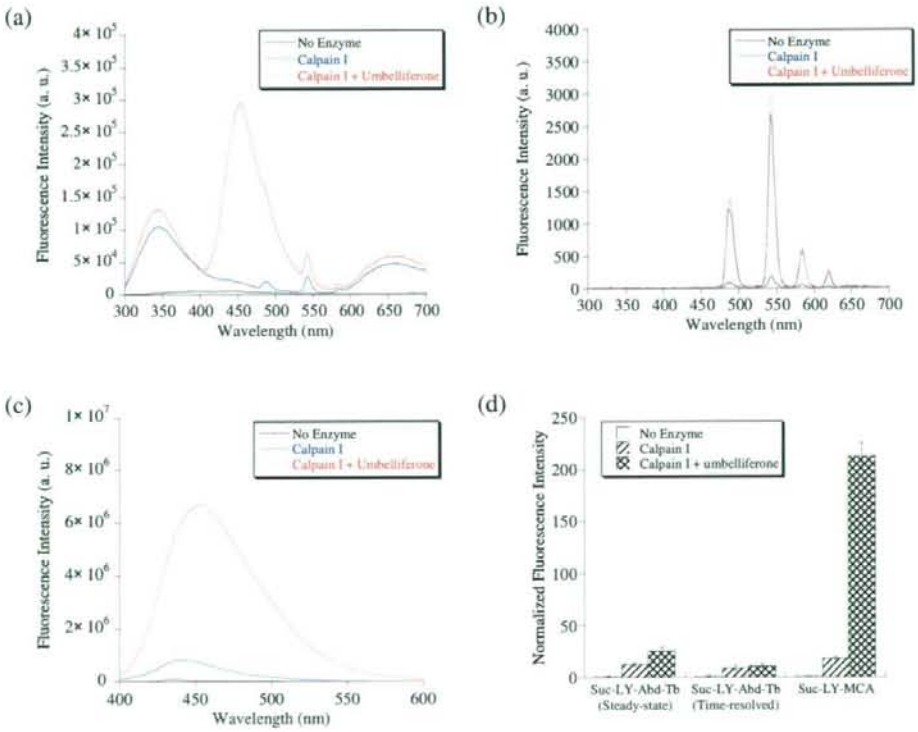


Figure S13. Fluorescence spectra of (a, b) 1 μM Suc-LY-Abd-Tb (λ_{ex} = 250 nm): (a) steady-state measurement), (b) time-resolved measurement, and (c) 1 μM Suc-LY-MCA (λ_{ex} = 380 nm) with (red line) or without (blue line) 1 μM umbelliferones in calpain I assay. Panel (d) shows a comparison of the normalized fluorescence intensities between the fluorescence assays. The error bar denotes the standard deviation (n = 3).