

Electronic Supplementary Information

Detection of NAD(P)H-dependent enzyme activity with dynamic luminescence quenching of terbium complexes

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Experimental Methods

Materials and Instruments.

L-Lactate dehydrogenase (from pig heart) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Alcohol dehydrogenase (from *S. cerevisiae*) and malate dehydrogenase (from porcine heart) were purchased from Sigma (MO, USA). Unit definitions of these enzymes were in accordance with the manufacturers' usage. All the other reagents were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or Sigma-Aldrich Corporation (MO, USA); they were of the highest grade available, and were used as received. UV-visible spectra were obtained on a Shimadzu UV-1800 (Tokyo, Japan). Time-resolved luminescence spectra and luminescence lifetimes were obtained using a Horiba Jobin-Yvon Fluorolog-3 (Kyoto, Japan). For assays using microplates, a PerkinElmer EnVision multifunctional plate reader was used.

UV-Visible Absorption Spectral Measurements.

Unless otherwise stated, absorption spectra of the complexes were measured at 4 μM in 20 mM Tris-HCl buffer (pH 7.4). Each sample contained less than 0.5% DMSO as a cosolvent. All measurements were performed in 1 cm quartz cells at ambient temperature (approx. 25 $^{\circ}\text{C}$).

Luminescence Spectral Measurements.

Unless otherwise stated, luminescence spectra of the complexes were measured at 4 μM in 20 mM Tris-HCl buffer (pH 7.4), with excitation at 340 nm. Each sample contained less than 0.5% DMSO as a cosolvent. All measurements were performed in 1 cm quartz cells at ambient temperature. For time-resolved measurements, delay time and gate time were set at 100 μs and 2.0 ms respectively.

Luminescence Lifetime Measurements.

Unless otherwise stated, luminescence lifetimes of the complexes were measured at 4 μM in 20 mM Tris-HCl buffer (pH 7.4), with an excitation wavelength of 340 nm. The luminescence intensity at 545 nm for Tb^{3+} complexes was measured at every 50 μs after pulse excitation, while the gate time was set at 3.5 ms for Tb^{3+} complexes. Data were fitted to a single exponential decay curve (see below), where I and I_0 are the luminescence intensity at time t and time 0 respectively. τ represents the luminescence lifetime.

$$I = I_0 \exp(-t/\tau) + C$$

Quantum Yield Measurements.

Quantum yields of the complexes were estimated by a relative method using Tb-DTPA-cs124 ($\Phi = 0.32$ in water) as a standard.^{S1} Quantum yields were calculated according to the following equation.

$$\Phi_x/\Phi_{\text{st}} = [A_{\text{st}}/A_x] [n_x^2/n_{\text{st}}^2] [D_x/D_{\text{st}}]$$

Where Φ is the quantum yield (subscript “st” stands for the reference and “x” for the sample), A is the absorbance at the excitation wavelength, n is the refractive index (if both spectra are obtained in aqueous solution, this term is cancelled out), and D is the area under the luminescence spectra on an energy scale. The sample and the reference were excited at the same wavelength, at which the absorbance was kept lower than 0.05. All spectra were obtained with a Horiba Jobin-Yvon Fluorolog-3 (Kyoto, Japan).

LDH Assay Using A1Tb in a Quartz Cell.

0.8 mM A1Tb stock solution in DMSO was diluted to a final concentration of 4 μM in 20 mM Tris-HCl buffer (pH 7.4) containing 100 μM NADH and 50 mU/mL LDH. The solution was incubated at room temperature for 5 min. Sodium pyruvate (0.125 mg/mL) was subsequently added to the solution.

Absorbance at 340 nm, luminescence intensity (excitation wavelength: 340 nm, emission wavelength: 545 nm, delay time: 100 μ s, gate time: 2.0 ms), and luminescence lifetime (excitation wavelength: 340 nm, emission wavelength: 545 nm, gate time: 3.5 ms) were measured in a 1 cm quartz cell containing a total volume of 3 mL of the mixture. LDH was excluded from the negative control.

LDH Assay Using A1Tb on a 96-Well Plate.

LDH assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, purchased from Corning) with a total volume of 100 μ L. The reaction was performed in 20 mM Tris-HCl buffer (pH 7.4) containing A1Tb (1 μ M), NADH (100 μ M), sodium pyruvate (0.125 mg/mL) and the indicated units of LDH. The plate was incubated at ambient temperature, and the luminescence intensity was measured every 5 min with an EnVision reader. Measurement conditions were as follows: excitation filter: 340/60 nm, dichroic mirror: 400 nm, emission filter: 545/7 nm, delay time: 100 μ s, gate time: 400 μ s, and cycle time: 5 ms.

Colorimetric Assay on a 96-Well Plate Format.

LDH assay was performed on a 96-well microplate (flat-bottomed, non-treated clear plate, purchased from Nunc) with a total volume of 100 μ L. The reaction was carried out in 20 mM Tris-HCl buffer (pH 7.4) containing various units of LDH, 0.125 mg/mL sodium pyruvate, and 100 μ M NADH. The plate was incubated at ambient temperature, and measurement was performed every 5 min with an EnVision reader. For colorimetric assay, increase of NADH absorbance was monitored at 340/60 nm via a filter.

ADH Assay Using A1Tb on a 96-Well Plate.

ADH assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, purchased from Corning) with a total volume of 100 μ L. The reaction was performed in 20 mM Tris-HCl buffer (pH 7.4) containing A1Tb (1 μ M), NADH (100 μ M), acetone (2 μ L) and 0.2 unit of ADH. The plate was incubated at ambient temperature, and the luminescence intensity was measured every 3 min with an EnVision reader. Measurement conditions were as follows: excitation filter: 340/60 nm, dichroic mirror: 400 nm, emission filter: 545/7 nm, delay time 100 μ s, gate time 400 μ s, and cycle time: 5 ms.

MDH Assay Using A1Tb on a 96-Well Plate.

MDH assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, purchased from Corning) with a total volume of 100 μ L. The reaction was performed in 20 mM Tris-HCl buffer (pH 7.4) containing A1Tb (1 μ M), NADH (100 μ M), oxaloacetic acid (0.2 mM) and 0.02 unit of MDH. The plate was incubated at ambient temperature, and the luminescence intensity

was measured every 3 min with an EnVision reader. Measurement conditions were as follows: excitation filter: 340/60 nm, dichroic mirror: 400 nm, emission filter: 545/7 nm, delay time 100 μ s, gate time 400 μ s, and cycle time: 5 ms.

Monitoring Reversible Enzyme Reaction of ADH

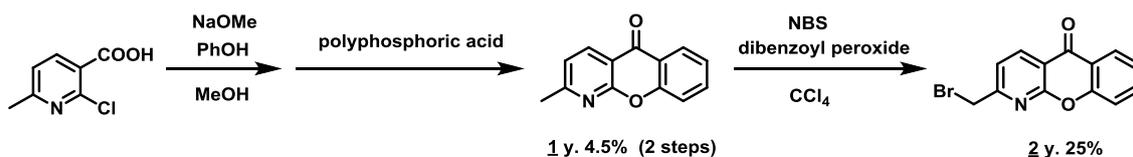
1 mM A1Tb stock solution in DMSO was diluted to a final concentration of 1 μ M in 20 mM Tris-HCl buffer (pH 9.0, 3.0 mL) containing 100 μ M NAD⁺ and 1-propanol (6 μ L). The solution was incubated at room temperature for 5 min, and the time-resolved luminescence spectrum (excitation wavelength: 340 nm, delay time: 100 μ s, gate time: 2.0 ms) was measured (= 0 min). ADH (6 U) was then added to the solution, and the spectra were measured after incubation of 5 and 20 min. Then, propionaldehyde (12 μ L) was added to the cuvette, and the spectrum was measured at 30 min after initial addition of ADH.

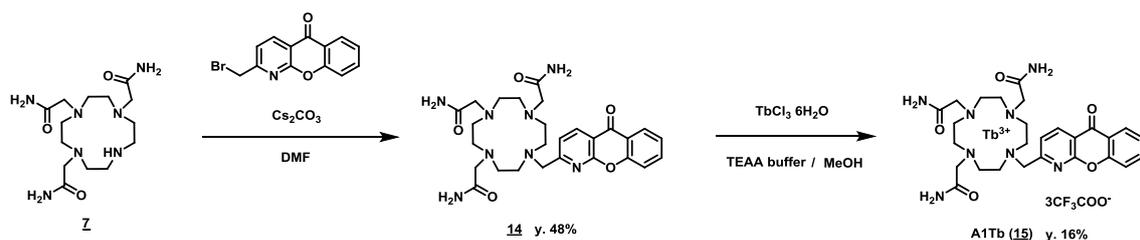
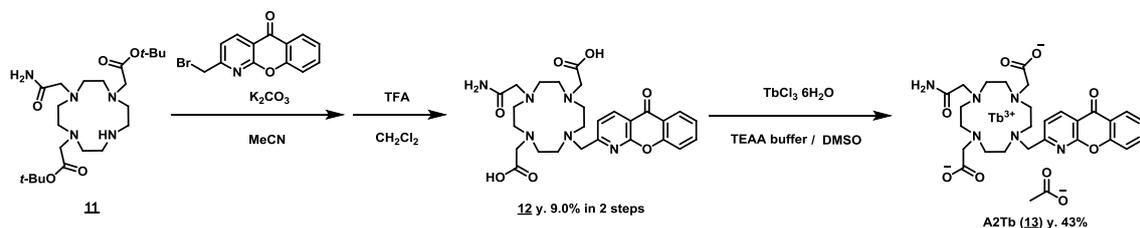
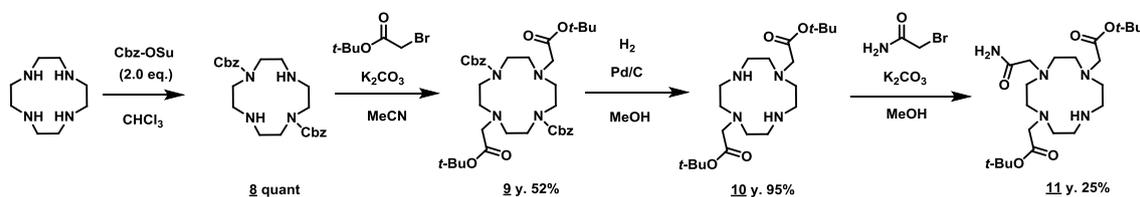
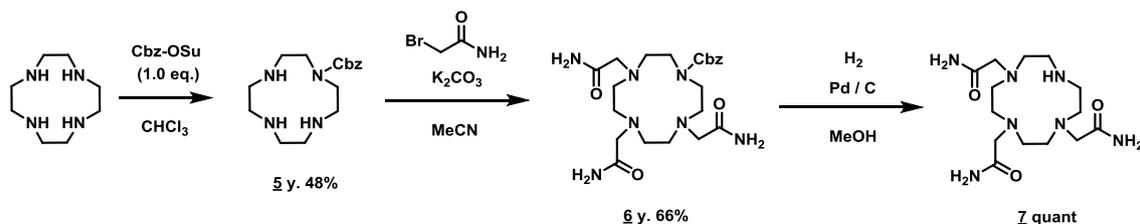
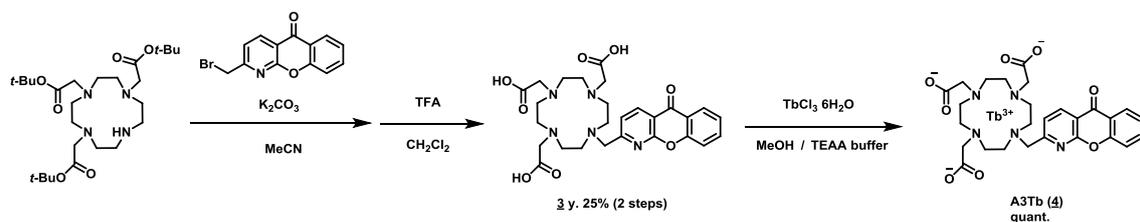
Synthetic Procedure and Characterization Data for Synthesized Compounds

General Information.

Dibenzoyl peroxide was purchased from Acros Organics (NJ, USA). Cyclen was purchased from Strem Chemicals (MA, USA). Tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7-triacetate (DO3A-tri-*tert*-butyl ester) was synthesized according to a previous report.^{S2} 2.0 M triethylamine acetate (TEAA, pH 7) was purchased from Glen Research (VA, USA). C-18 Sep-Pak cartridge was purchased from Waters (Ireland). All the other reagents were purchased from Tokyo Chemical Industry Co., Ltd. (Japan), or Wako Pure Chemical Industries, Ltd. (Japan); they were of the highest grade available, and were used without further purification. ¹H-NMR and ¹³C-NMR spectra were recorded on a JEOL JNM-LA300 or JNM-LA400; δ values are given in ppm relative to tetramethylsilane. Mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer (EI⁺) or a JEOL JMS-T100LC (ESI⁺, ESI⁻). Lyophilization of compounds dissolved in aqueous solution was performed by VD-800R (TAITEC, Japan). Silica gel column chromatography was performed using either silica gel 60N (Kanto Kagaku Co., Ltd), silica gel 60 (Kanto Kagaku Co., Ltd), or NH silica gel (Fuji Silysia Chemical Ltd). Column sizes (diameter \times length) were as follows: 50 mm \times 200 mm (for reactions starting from more than 5.0 mmol of materials), 35 mm \times 150 mm (for reactions starting from 0.5 to 5.0 mmol of materials), and 20 mm \times 100 mm (for reactions starting from less than 0.5 mmol of materials). Preparative HPLC was performed on a reverse-phase ODS column (GL Sciences, Inertsil ODS-3 30 mm \times 250 mm) fitted on a JASCO PU-1587 HPLC system, with a flow rate of 25 mL/min or a reverse-phase ODS column (GL Sciences, Inertsil ODS-3 10 mm \times 250 mm) fitted on a JASCO PU-2086 Plus HPLC system, with a flow rate of 5 mL/min. HPLC analyses were performed on a reverse-phase ODS column (GL Sciences, Inertsil ODS-3 4.6 mm \times 250 mm) using eluent A and eluent B specified below, fitted on a JASCO PU-2080 Plus system, with a flow rate of 1.0 mL/min.

Synthesis of A1Tb, A2Tb, and A3Tb.





Compound **1**

Compound **1** was synthesized according to the reported procedure with slight modifications.^{S3} Sodium methoxide (680 mg, 12.6 mmol) was dissolved in MeOH (6 mL), followed by addition of 6-methyl-2-chloronicotinic acid (1.0 g, 6.0 mmol) and phenol (2.8 g, 30 mmol). The solvent was evaporated and the residue was heated for 9 hours at 180 °C under an Ar atmosphere with stirring. After cooling, the

product was taken up in water (40 mL) and the solution was washed with CH₂Cl₂ (30 mL × 3). The aqueous layer was acidified with acetic acid to pH 4, and extracted with CH₂Cl₂ (40 mL × 2). The organic layer was dried over Na₂SO₄, followed by evaporation to afford a yellow solid. The solid was dissolved in liquid polyphosphoric acid (approximately 50 mL) and the reaction mixture was heated at 120 °C for 2 hours under an Ar atmosphere. After cooling, the resulting brown liquid was slowly poured into ice-cooled water. The solution was adjusted to pH 12 by carefully adding NaOH pellets and extracted with CH₂Cl₂ (30 mL × 3). Then, the organic layer was washed with water (30 mL × 1), dried over Na₂SO₄, filtered, and evaporated under reduced pressure to give compound 1 (58 mg, 4.5%). ¹H-NMR (300 MHz, CDCl₃) δ 2.72 (3H, s), 7.31 (1H, d, *J* = 8.1 Hz), 7.41-7.46 (1H, m), 7.61 (1H, d, *J* = 7.3 Hz), 7.77-7.79 (1H, m), 8.32 (1H, dd, *J* = 8.1 Hz, 2.2 Hz), 8.60 (1H, d, *J* = 7.3 Hz).

Compound 2

Compound 2 was synthesized according to the reported procedure with slight modifications.^{S3} Compound 1 (50 mg, 0.24 mmol) was dissolved in CCl₄ (10 mL) and the solution was heated to 80 °C under an Ar atmosphere. *N*-Bromosuccinimide (21 mg, 0.12 mmol) and dibenzoyl peroxide (4 mg) were added and the reaction course was monitored using TLC (SiO₂, CH₂Cl₂). After 12 hours and the addition of 2.0 equivalents of *N*-bromosuccinimide, the reaction mixture was allowed to cool to room temperature and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (CH₂Cl₂ as eluent) to yield compound 2 as a white solid. (18 mg, 25%). ¹H-NMR (300 MHz, CDCl₃) δ 4.61 (2H, s), 7.44-7.48 (1H, m), 7.58 (1H, d, *J* = 8.1 Hz), 7.63 (1H, d, *J* = 8.1 Hz), 7.79-7.82 (1H, m), 8.33 (1H, dd, *J* = 8.1 Hz, 1.5 Hz), 8.73 (1H, d, *J* = 8.1 Hz). TLC (SiO₂, CH₂Cl₂, R_f = 0.35).

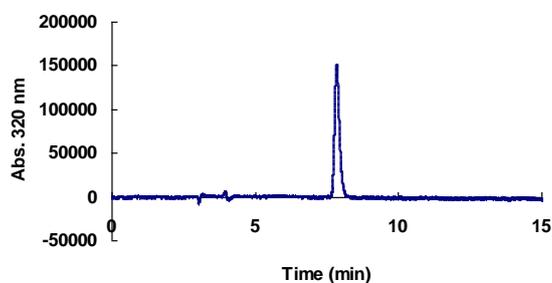
Compound 3

Compound 3 was synthesized according to the reported procedure with slight modifications.^{S3} Compound 2 (14 mg, 0.048 mmol), DO3A-tri-*tert*-butyl ester (25 mg, 0.049 mmol),^{S2} and potassium carbonate (16 mg, 0.049 mmol) were dissolved in MeCN (10 mL) and the mixture was stirred overnight at 70 °C under an Ar atmosphere. After evaporation, the residue was dissolved in CH₂Cl₂ (20 mL). The solution was washed with brine (10 mL × 2), dried over Na₂SO₄, and evaporated under reduced pressure. The residue was dissolved in CH₂Cl₂ (2 mL)/TFA (2 mL) and the solution was stirred overnight at room temperature. After evaporation of the solvent, the crude product was purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 70:30 to 40:60 in 30 minutes; eluent A: 0.1% TFA, H₂O; eluent B: 0.1% TFA, 80% acetonitrile, 20% H₂O). Lyophilization of the eluted fraction afforded compound 3 (7 mg, 25%) as a white powder. ¹H-NMR (300 MHz, CD₃CN) δ 3.07-3.24 (18H, brm), 3.72 (4H, s), 4.48 (2H, s), 7.46 (1H, t, *J* = 7.7 Hz), 7.51 (1H, s), 7.67 (1H, d, *J* = 8.1 Hz), 7.84 (1H, t, *J* = 7.7 Hz), 8.16 (1H, d, *J* = 8.1 Hz), 8.58 (1H, s); HRMS (ESI⁺):

calcd for $[M + H]^+$, 556.2407 ; found, 556.2371 (-3.7 mmu).

A3Tb (**4**)

Compound **3** (4 mg, 0.0072 mmol) and $TbCl_3 \cdot 6H_2O$ (69 mg, 0.18 mmol) were dissolved in MeOH (2 mL) and 0.1 M TEAA buffer (2 mL). The reaction mixture was stirred overnight at 70 °C under an Ar atmosphere and then purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 70:30 to 40:60 in 30 minutes; eluent A: H_2O ; eluent B: 80 % acetonitrile, 20% H_2O). Lyophilization of the eluted fraction afforded A3Tb (6 mg, quant). HRMS (ESI⁺): calcd for $[M + H]^+$, 712.1426; found, 712.1378 (-4.8 mmu). HPLC analysis: gradient elution with eluent A (0.1% TFA, H_2O) and eluent B (0.1% TFA, 80% acetonitrile, 20% H_2O), A/B = 90/10→50/50 (30 min), detection by absorbance measurement at 320 nm. R_t : 7.9 min.



Compound **5**

1.0 g (5.8 mmol) of cyclen was dissolved in $CHCl_3$ (20 mL), and 1.5 g (6.0 mmol) of *N*-(benzyloxycarbonyloxy)succinimide (Cbz-OSu) was slowly added to the solution. The reaction mixture was stirred at room temperature for 3 hours under an Ar atmosphere and then 2 N NaOH aq. (50 mL) was added. The mixture was extracted with CH_2Cl_2 (20 mL \times 3), and the organic layer was washed with brine (20 mL \times 1), dried over Na_2SO_4 , and evaporated. The residue was purified by preparative reverse-phase HPLC (linear gradient from eluent A: eluent B = 80:20 to 0:100 in 30 minutes; eluent A: 0.1% TFA, H_2O ; eluent B: 0.1% TFA, 80% acetonitrile, 20% H_2O). Lyophilization of the eluted fraction afforded compound **5** (890 mg, 48%) as a white powder. 1H -NMR (300 MHz, $CDCl_3$) δ 3.13 (4H, s), 3.23 (4H, s), 3.35 (4H, s), 3.57 (4H, s), 5.08 (2H, s), 7.28-7.36 (5H, m), 8.96 (3H, br); ^{13}C -NMR (75 MHz, CD_3OD) δ 44.4, 46.4, 47.0, 47.9, 69.3, 120.1, 129.4, 129.6, 137.5, 158.6; HRMS (ESI⁺): calcd for $[M + H]^+$, 307.2134 ; found, 307.2139 (0.5 mmu).

Compound **6**

Compound **5** (437 mg, 1.4 mmol) and potassium carbonate (1.38 g, 14 mmol) were dissolved in MeCN (20 mL), followed by addition of 2-bromoacetamide (966 mg, 7.0 mmol). The reaction mixture was

stirred at room temperature overnight under an Ar atmosphere, then evaporated, and the residue was purified by preparative reverse-phase HPLC (linear gradient from eluent A: eluent B = 90:10 to 50:50 in 30 minutes; eluent A: 0.1% TFA, H₂O; eluent B: 0.1 % TFA, 80 % acetonitrile, 20% H₂O). Lyophilization of the eluted fraction afforded compound 6 (441 mg, 66%). ¹H-NMR (300 MHz, D₂O) δ 2.88-3.91 (18H, brm), 4.13 (2H, s), 4.46 (2H, s), 5.17 (2H, s), 7.26 (5H, m); ¹³C-NMR (100 MHz, CD₃OD) δ 46.6, 52.2, 52.5, 54.0, 54.5, 54.7, 70.4, 114.5, 129.5, 129.7, 137.1, 158.9, 167.9, 177.8; HRMS (ESI⁺): calcd for [M + H]⁺, 478.2778 ; found, 478.2735 (-4.3 mmu).

Compound 7

A mixture of compound 6 (410 mg, 0.86 mmol) and a portion of 10% Pd/C in MeOH (10 mL) was stirred under a hydrogen atmosphere at room temperature for 6 hours, and then filtered through a pad of Celite. Evaporation of the filtrate afforded compound 7 (339 mg, quant). ¹H-NMR (300 MHz, D₂O) δ 2.48 (4H, s), 2.57 (4H, s), 2.71 (4H, s), 3.03 (4H, s), 3.06 (2H, s), 3.18 (4H, s); ¹³C-NMR (75 MHz, CD₃OD) δ 43.6, 49.9, 51.0, 53.4, 55.5, 56.4, 168.0, 175.4; HRMS (ESI⁺): calcd for [M + H]⁺, 344.2410 ; found, 344.2398 (-1.2 mmu).

Compound 8

Compound 8 was synthesized according to the reported procedure with slight modifications.^{S4} In brief, 1.0 g (5.8 mmol) of cyclen and 2.9 g (11.6 mmol) of *N*-(benzyloxycarbonyloxy)succinimide (Cbz-OSu) were dissolved in CHCl₃ (20 mL), and the solution was stirred at room temperature for 30 minutes under an Ar atmosphere. After evaporation, the residue was dissolved in CH₂Cl₂ (60 mL). The organic layer was washed with 2 N NaOH aq. and brine (40 mL each), dried over Na₂SO₄, and evaporated to afford compound 8 (2.97 g, quant). The obtained compound was used without further purification. ¹H-NMR (300 MHz, CDCl₃) δ 2.76-2.94 (8H, brm), 3.34-3.51 (8H, m), 5.15 (4H, s), 7.31-7.35 (10H, m); HRMS (ESI⁺): calcd for [M + H]⁺, 441.2502 ; found, 441.2495 (-0.7 mmu).

Compound 9

Compound 9 was synthesized according to the reported procedure with slight modifications.^{S4} To a mixture of compound 8 (2.97 g, 6.8 mmol) and potassium carbonate (2.4 g, 17 mmol) in MeCN (25 mL) was added *tert*-butyl bromoacetate (2.0 mL, 13.5 mmol). After refluxing overnight, the solvent was evaporated. The residue was purified by silica gel column chromatography (MeOH/CH₂Cl₂ = 2/98) to afford compound 9 (2.36 g, 52%). ¹H-NMR (400 MHz, CDCl₃) δ 1.42 (18H, s), 2.89 (8H, br), 3.31-3.42 (12H, brm), 5.12 (4H, s), 7.27-7.34 (10H, m); HRMS (ESI⁺): calcd for [M + H]⁺, 669.3863 ; found, 669.3890 (2.7 mmu).

Compound 10

Compound 10 was synthesized according to the reported procedure.^{S4} A mixture of compound 9 (6.29 g, 9.4 mmol) and a portion of 10% Pd/C in MeOH (90 mL) was stirred under a hydrogen atmosphere at room temperature overnight, and then filtered through a pad of Celite. Evaporation of the filtrate afforded compound 10 (3.58 g, 95%). ¹H-NMR (300 MHz, CDCl₃) δ 1.46 (18H, s), 2.61 (8H, m), 2.80 (8H, m), 3.31 (4H, s); HRMS (ESI⁺): calcd for [M + H]⁺, 401.3128 ; found, 401.3102 (-2.6 mmu).

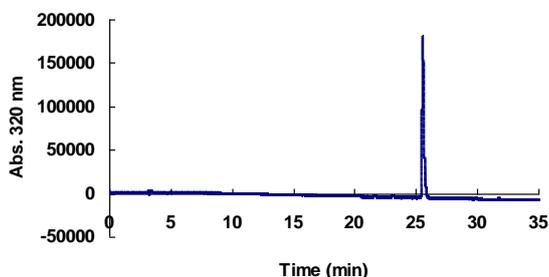
Compound 11

Compound 10 (1.79 g, 4.5 mmol) was dissolved in MeCN (20 mL), followed by addition of potassium carbonate (1.23 g, 9.0 mmol). A solution of 2-bromoacetamide (478 mg, 3.5 mmol) dissolved in MeCN (10 mL) was added dropwise to the mixture at 0 °C, and the reaction mixture was stirred at room temperature overnight under an Ar atmosphere. The solvent was removed under reduced pressure and the residue was diluted with AcOEt (50 mL). The solution was washed with saturated aqueous sodium hydrogen carbonate and brine (30 mL each), dried over Na₂SO₄, and evaporated. The residue was purified by NH silica column chromatography (MeOH 0% to 10% in CH₂Cl₂) to afford compound 11 (506 mg, 25%) as a brown powder. ¹H-NMR (300 MHz, CDCl₃) δ 1.46 (18H, m), 2.61 (8H, m), 2.80 (8H, m), 3.06 (2H, s), 3.28 (2H, s), 3.32 (2H, s); ¹³C-NMR (75 MHz, CDCl₃) δ 28.1, 45.7, 47.7, 52.0, 52.3, 57.2, 57.3, 80.9, 170.8, 170.9; HRMS (ESI⁺): calcd for [M + H]⁺, 458.3342; found, 458.3364 (2.2 mmu).

Compound 12

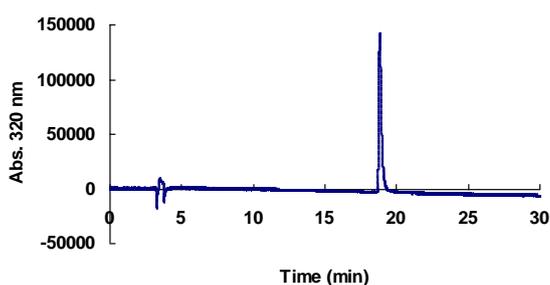
Compound 11 (50 mg, 0.11 mmol), compound 2 (30 mg, 0.10 mmol), and potassium carbonate (36 mg, 0.26 mmol) were dissolved in MeCN (5 mL) and the solution was stirred for 3 hours at 70 °C under an Ar atmosphere. After evaporation, the residue was dissolved in a mixture of water and DMF (1 mL/1 mL). The solution was filtered through a Celite pad and roughly purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 80:20 to 0:100 in 20 minutes; eluent A 0.1% TFA, H₂O; eluent B 0.1% TFA, 80% acetonitrile, 20% H₂O). Lyophilization of the eluted fraction afforded a solid, which was dissolved in a mixture of CH₂Cl₂ (2 mL) and TFA (2 mL). The solution was stirred for 6 hours at room temperature under an Ar atmosphere and then evaporated. The residue was purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 99:1 to 60:40 in 30 minutes; eluent A 0.1% TFA, H₂O; eluent B 0.1% TFA, 80% acetonitrile, 20% H₂O). Lyophilization of the eluted fraction afforded the title compound 12 (6 mg, 9.0%). ¹H-NMR (300 MHz, D₂O) δ 3.02-3.87 (22H, brm), 4.05 (2H, s), 7.37 (1H, t, *J* = 7.3 Hz), 7.52 (1H, d, *J* = 8.1 Hz), 7.64 (1H, d, *J* = 8.1 Hz), 7.76 (1H, t, *J* = 7.3 Hz), 8.05 (1H, d, *J* = 8.1 Hz), 8.46 (1H, d, *J* = 8.1 Hz); ¹³C-NMR (100 MHz, DMSO-*d*₆) δ 45.7, 48.3, 50.5, 50.9, 52.8, 54.8, 56.7, 115.6, 118.3, 118.7, 121.1, 121.6, 126.1, 136.3, 138.2, 155.1, 157.8, 158.1, 159.2, 171.9, 176.6; HRMS (ESI⁺): calcd for

[M + H]⁺, 555.2567 ; found, 555.2608 (4.1 mmu). HPLC analysis: gradient elution with eluent A (0.1% TFA, H₂O) and eluent B (0.1% TFA, 80% acetonitrile, 20% H₂O), A/B = 99/1 to 60/40 (30 min); detection by absorbance measurement at 320 nm. *R*_t : 25.6 min.



A2Tb (**13**)

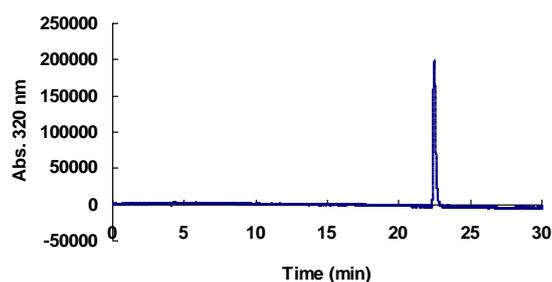
Compound **12** (6 mg, 0.0090 mmol) and TbCl₃·6H₂O (60 mg, 0.32 mmol) were dissolved in DMSO (1 mL) and 0.1 M TEAA buffer (3 mL). The reaction mixture was stirred overnight at 70 °C under an Ar atmosphere, and then purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 99:1 to 60:40 in 30 minutes; eluent A: 0.1 M TEAA buffer; eluent B: 20% 0.1 M TEAA buffer, 80% acetonitrile). The obtained fractions were loaded on a C-18 Sep-Pak cartridge, which was washed thoroughly with water to remove excess TEAA salt, then eluted with MeOH. The eluted fraction was evaporated, and the residue was lyophilized to afford A2Tb (3 mg, as acetate, 43%). HRMS (ESI⁺): calcd for [M]⁺, 711.1586; found, 711.1540 (-4.6 mmu). HPLC analysis: gradient elution with eluent A (0.1 M TEAA buffer) and eluent B (20% 0.1 M TEAA buffer, 80 % acetonitrile), A/B = 99/1 to 60/40 (30 min); detection by absorbance measurement at 320 nm. *R*_t : 18.9 min.



Compound **14**

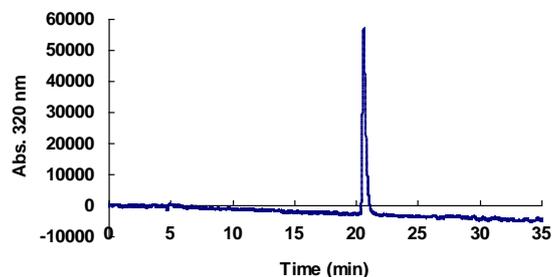
Compound **2** (15 mg, 0.05 mmol), compound **7** (20 mg, 0.05 mmol), and cesium carbonate (34 mg, 0.01 mmol) were dissolved in DMF (3 mL) and the reaction mixture was stirred overnight at 50 °C under an Ar atmosphere, then diluted in 9 mL of 0.1% TFA and purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 70:30 to 40:60 in 50 minutes; eluent A: 0.1% TFA, H₂O; eluent B: 0.1% TFA, 80% acetonitrile, 20% H₂O). Lyophilization of the eluted fraction afforded

compound **14** (13 mg, 48%) as a yellow solid. $^1\text{H-NMR}$ (300 MHz, D_2O) δ 3.33-3.61 (24H, brm), 7.37 (1H, t, $J = 7.3$ Hz), 7.48-7.52 (2H, brm), 7.76 (1H, t, $J = 7.3$ Hz), 8.04 (1H, d, $J = 8.1$ Hz), 8.48 (1H, d, $J = 8.1$ Hz); $^{13}\text{C-NMR}$ (75 MHz, CD_3OD) δ 31.7, 36.9, 51.4, 55.5, 56.3, 58.6, 69.1, 115.7, 117.2, 119.5, 119.8, 122.7, 126.2, 127.4, 137.3, 139.7, 157.1, 161.1, 161.5, 162.0, 164.9; HRMS (ESI $^+$): calcd for $[\text{M} + \text{H}]^+$, 553.2887 ; found, 553.2853 (-3.4 mmu). HPLC analysis: gradient elution with eluent A (0.1% TFA, H_2O) and eluent B (0.1% TFA, 80% acetonitrile, 20% H_2O), A/B = 99/1 to 60/40 (30 min); detection by absorbance measurement at 320 nm. R_t : 22.5 min.



A1Tb (15)

Compound **14** (13 mg, 0.024 mmol) and $\text{TbCl}_3 \cdot 6\text{H}_2\text{O}$ (25 mg, 0.068 mmol) were dissolved in 0.1 M TEAA buffer/MeOH = 1/1 (4 mL). The solution was stirred at 70 $^\circ\text{C}$ for 12 hours under an Ar atmosphere, and then purified by preparative reversed-phase HPLC (linear gradient from eluent A: eluent B = 99:1 to 80:20 in 30 minutes; eluent A: 0.1% TFA, H_2O ; eluent B: 0.1% TFA, 80% acetonitrile, 20% H_2O). Lyophilization of the eluted fraction afforded A1Tb (4 mg, as trifluoroacetate, 16 %) as a white powder. HRMS (ESI $^+$): calcd for $[\text{M} - 2\text{H}]^+$, 709.1906 ; found, 709.1866 (-4.0 mmu). HPLC analysis: gradient elution with eluent A (0.1% TFA H_2O) and eluent B (0.1% TFA, 80% acetonitrile, 20% H_2O), A/B = 99/1 to 80/20 (30 min); detected in terms of absorbance at 320 nm. R_t was 20.6 min.



Supplementary Figures and Tables

Table S1. Oxidation potentials of representative biological reductants and k_q values of A1Tb upon titration of biological reductants.

Biological reductants	E_0' (V)	k_q ($M^{-1}s^{-1}$) ^a
ascorbate ^{S5}	0.30	7.0×10^6
GSH ^{S6}	-0.24	n.d. ^b
NADH ^{S7}	-0.32	1.2×10^8
NADPH ^{S8}	-0.32	2.6×10^8

^a k_q was determined from Stern-Volmer analysis of the luminescence lifetime at 545 nm.

^b No statistically significant change in the luminescence lifetime of A1Tb was observed upon titration up to 100 μ M of GSH.

Table S2. Photophysical properties of Tb³⁺ complexes.^a

Probe	Abs. _{max} (nm)	Em. _{max} (nm)	Quantum yield (%) ^b
A1Tb	335	545	17
A2Tb	335	546	27
A3Tb	336	546	28

^a Measurement was performed with Tb³⁺ complex (4 μ M) in 20 mM Tris-HCl buffer (pH 7.4), with excitation at 340 nm. Each sample contained less than 0.5% DMSO as a cosolvent.

^b Quantum yields were determined using Tb-DTPA-cs124 (0.32) in H₂O as a reference.^{S1}

Table S3. k_q values for Tb^{3+} complexes upon titration of NADPH measured at different excitation wavelengths.

Probe	Net charge	k_q ($M^{-1}s^{-1}$) ^a	k_q ($M^{-1}s^{-1}$) ^a
		Ex. 340 nm	Ex. 488 nm
A1Tb	+3	2.6×10^8	2.4×10^8
A2Tb	+1	2.3×10^7	2.2×10^7
A3Tb	0	4.1×10^6	3.7×10^6

^a k_q was determined from Stern-Volmer analysis of the luminescence lifetime at 545 nm.

Table S4. k_q values for Tb^{3+} complexes upon titration of NADH measured at different excitation wavelengths.

Probe	Net charge	k_q ($M^{-1}s^{-1}$) ^a	k_q ($M^{-1}s^{-1}$) ^a
		Ex. 340 nm	Ex. 488 nm
A1Tb	+3	1.2×10^8	1.1×10^8
A2Tb	+1	2.1×10^7	1.8×10^7
A3Tb	0	9.1×10^6	8.6×10^6

^a k_q was determined from Stern-Volmer analysis of the luminescence lifetime at 545 nm.

Table S5. Effect of NAD⁺, ATP, and other compounds on luminescence intensity and lifetime of A1Tb.

Added compound	Luminescence intensity	Luminescence lifetime
	k_q (M ⁻¹ s ⁻¹)	k_q (M ⁻¹ s ⁻¹)
NADH	1.2×10^8 ^a	1.2×10^8
NAD ⁺	7.8×10^5	n. d. ^b
ATP	1.7×10^8	n. d. ^b
ADP	6.8×10^7	n. d. ^b
AMP	1.1×10^7	n. d. ^b
cAMP	n. d. ^b	n. d. ^b

k_q values were calculated from the linear regression of Stern-Volmer plot on luminescence intensity and lifetime upon addition of each compound (0, 10, 30, 100 μ M). Luminescence intensity and lifetime of 4 μ M A1Tb were measured in 20 mM Tris-HCl buffer (pH 7.4) containing less than 0.5% DMSO as a cosolvent. Ex. 340 nm (slit 10 nm). Em. 545 nm (slit 2 nm). Data are shown as mean of 3 measurements.

^a Data between 0 to 10 μ M of NADH were used for the determination of k_q value (see discussion on Figure S1).

^b Quenching was not observed (k_q values were lower than 1.0×10^5 M⁻¹s⁻¹).

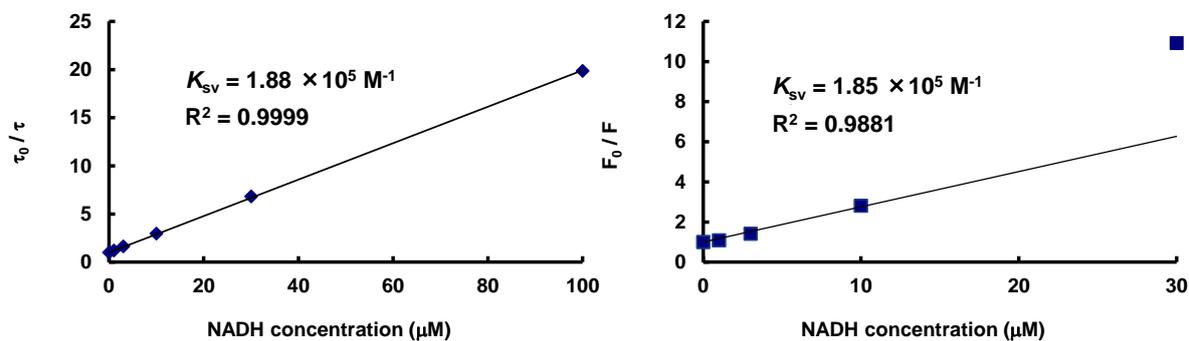


Figure S1. Stern-Volmer plot of luminescence lifetime (left) and intensity (right) of A1Tb upon titration of NADH. The K_{sv} values obtained from the linear regression are shown in the inset. Data are shown as mean \pm S.D. ($n = 3$), but error bars are smaller than data points. The linear regression and K_{sv} value were determined from data between 0 to 10 μM of NADH (see Note below).

Note: At concentrations higher than 10 μM , intensity-based data deviate from the line due to the inner filter effect of NADH. Therefore, we cannot completely exclude the possibility of static quenching in this concentration range.

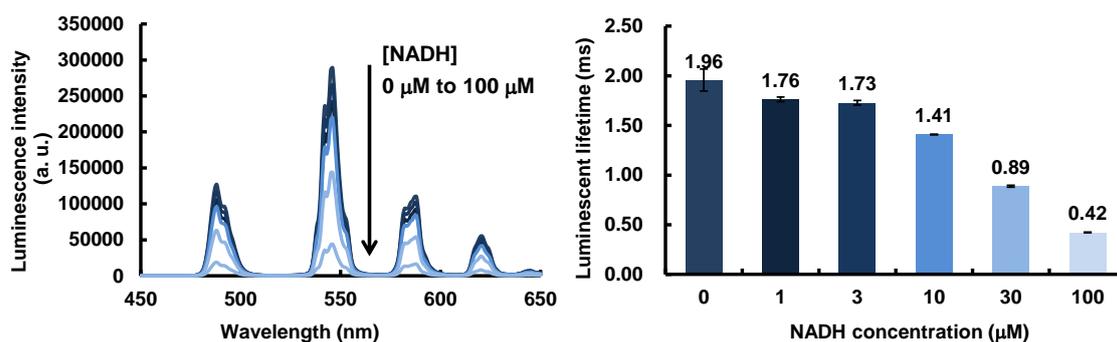


Figure S2. Luminescence spectra (left) and lifetime (right) of A2Tb upon titration of NADH from 0 μM (dark blue) to 100 μM (aqua). Error bars represent S. D. ($n = 3$).

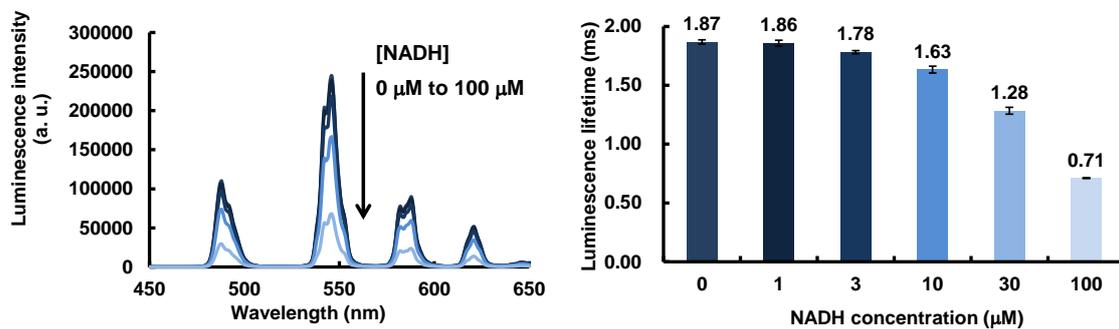


Figure S3. Luminescence spectra (left) and lifetime (right) of A3Tb upon titration of NADH from 0 μM (dark blue) to 100 μM (aqua). Error bars represent S. D. (n = 3).

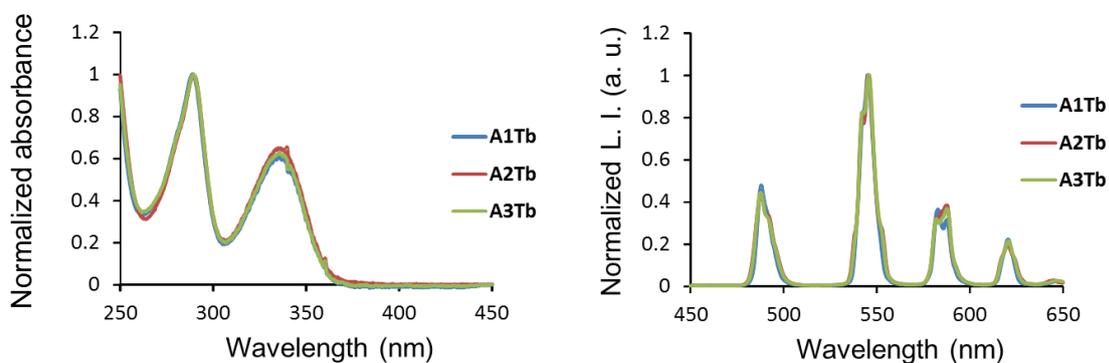


Figure S4. Absorption (left) and emission (right) spectra of Tb³⁺ complexes. Measurements were performed in 20 mM Tris-HCl buffer (pH 7.4).

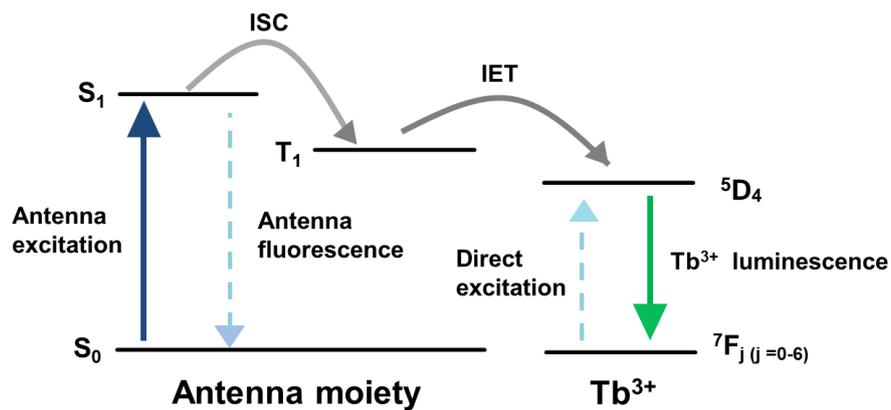


Figure S5. Energy diagram of Tb³⁺ complexes. ISC: intersystem crossing. IET: intramolecular energy transfer.

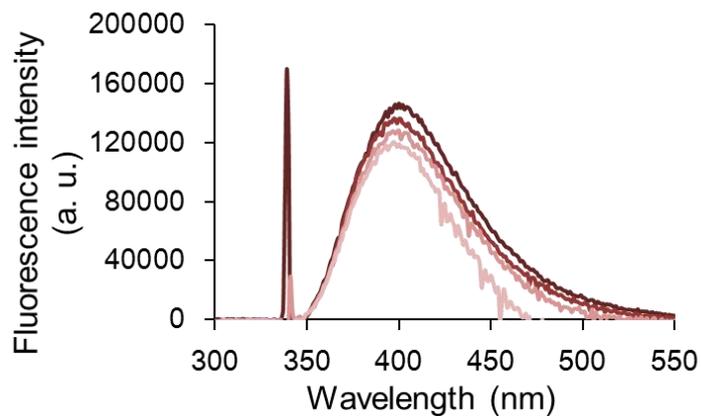


Figure S6. Fluorescence spectra of 10 μM 2-Me azaxanthone (**1**) upon titration of NADH from 0 μM (dark brown), 1 μM (red), 3 μM (pink), and up to 10 μM (light pink). Measurements were performed in 20 mM Tris-HCl buffer (pH 7.4) and 0.1% DMSO as a cosolvent. Spectra were obtained by subtracting fluorescence from the corresponding concentration of NADH by itself.

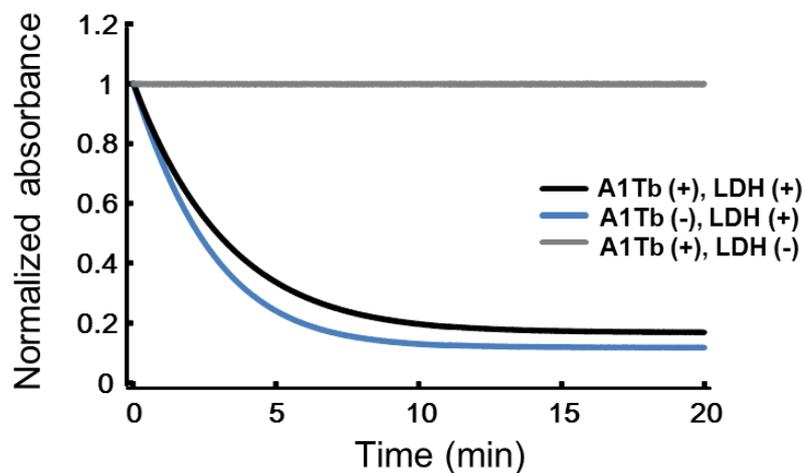


Figure S7. Monitoring LDH activity by detecting decrease in NADH absorption at 340 nm in the presence of 4 μM A1Tb (purple line). 100 μM NADH, 125 $\mu\text{g/mL}$ sodium pyruvate, and 50 mU/mL LDH were incubated at room temperature. A1Tb was omitted from A1Tb (-) (light blue line), while LDH was omitted from LDH (-) (gray line). The apparent difference of residual absorbance at the end point between A1Tb (+) and (-) is due to the absorption of A1Tb itself.

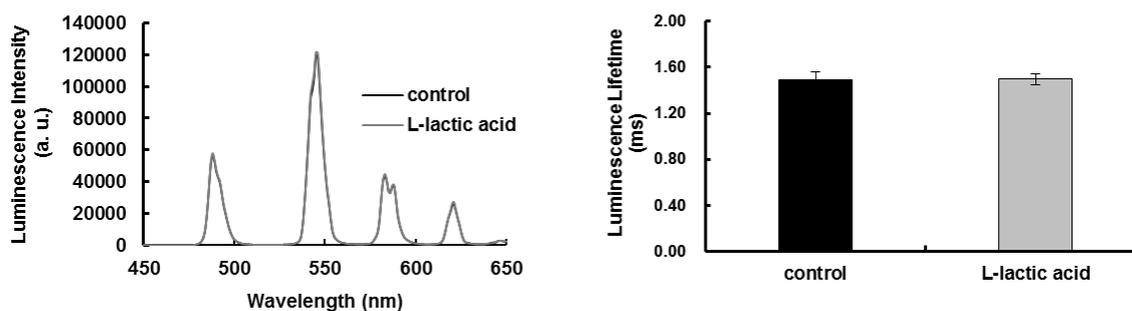


Figure S8. Luminescence spectra (left) and lifetime (right) of 4 μM A1Tb in the absence (black line/bar) or presence (gray line/bar) of 100 μM L-lactic acid. Ex. 340 nm (slit 10 nm). Em. 545 nm (slit 2 nm). Error bars represent S. D. ($n = 3$).

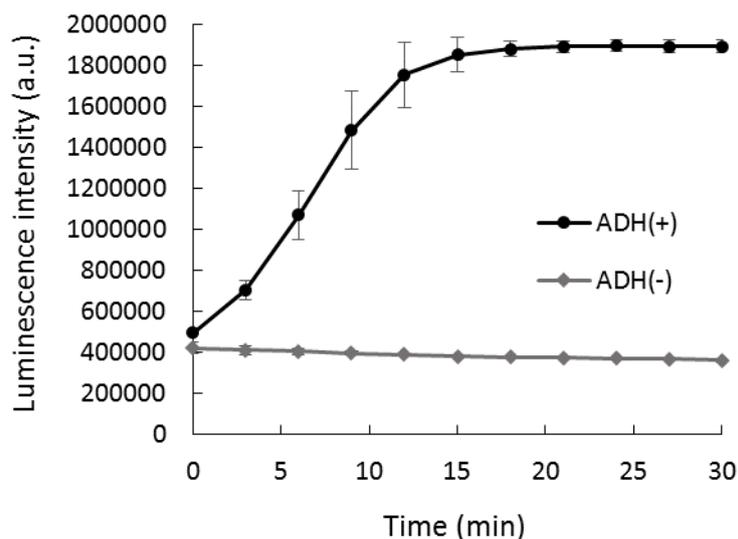


Figure S9. Time course of luminescence intensity of 1 μ M A1Tb in the presence (black circle) and absence (gray diamond) of ADH (2 U/mL). Initial concentrations of acetone and NADH were 2% (v/v) and 50 μ M, respectively. Error bars represent S. D. (n = 4).

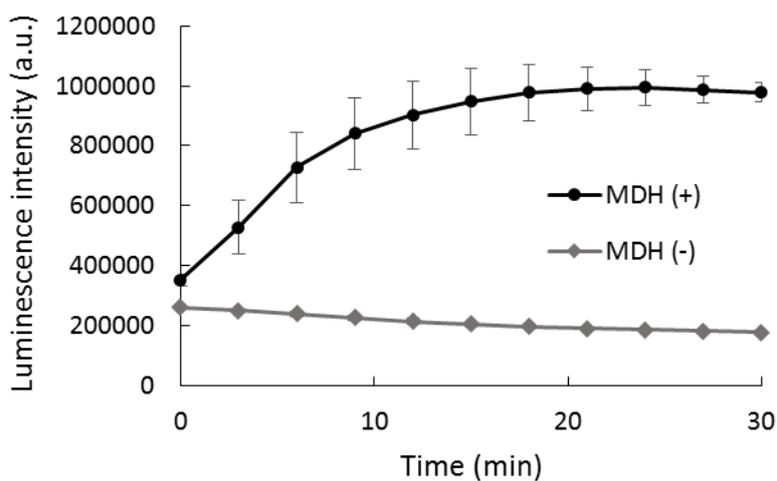


Figure S10. Time course of luminescence intensity of 1 μ M A1Tb in the presence (black circle) and absence (gray diamond) of MDH (0.2 U/mL). Initial concentrations of oxaloacetic acid and NADH were 0.2 mM and 50 μ M, respectively. Error bars represent S. D. (n = 4).

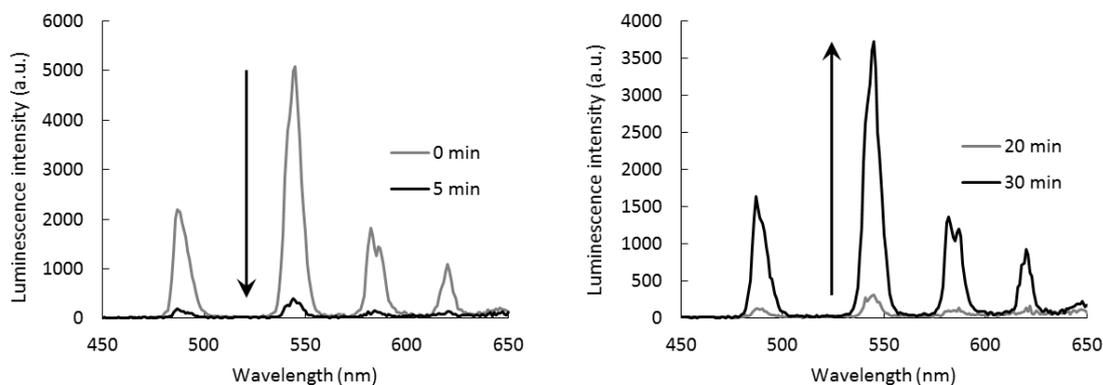


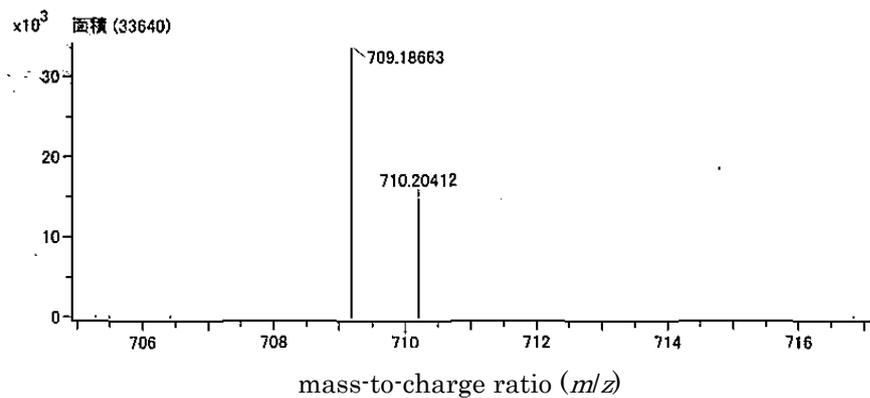
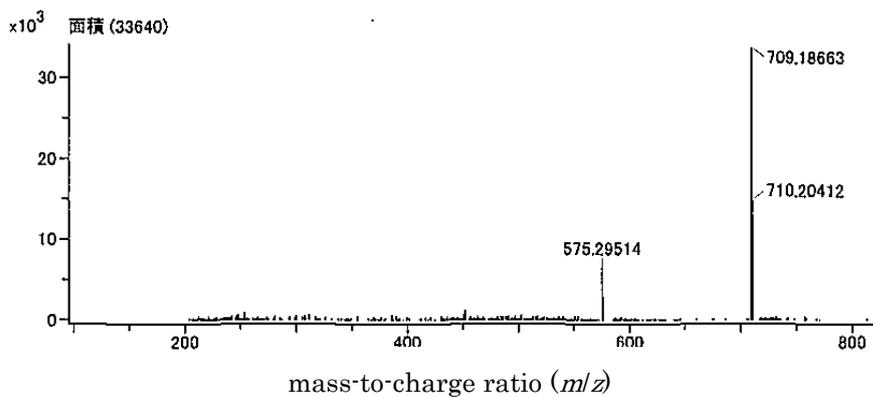
Figure S11. Luminescence spectra of 1 μM A1Tb in reversible ADH assay. The production of NADH and propionaldehyde from NAD^+ and 1-propanol was initiated at 0 min by addition of ADH, and the reverse reaction was accelerated by addition of excess propionaldehyde to the same cuvette at 20 min. Time-resolved emission spectra of A1Tb at 0 and 5 min (left) correspond to the increase of NADH, and those at 20 and 30 min (right) correspond to the decrease.

Supplementary References

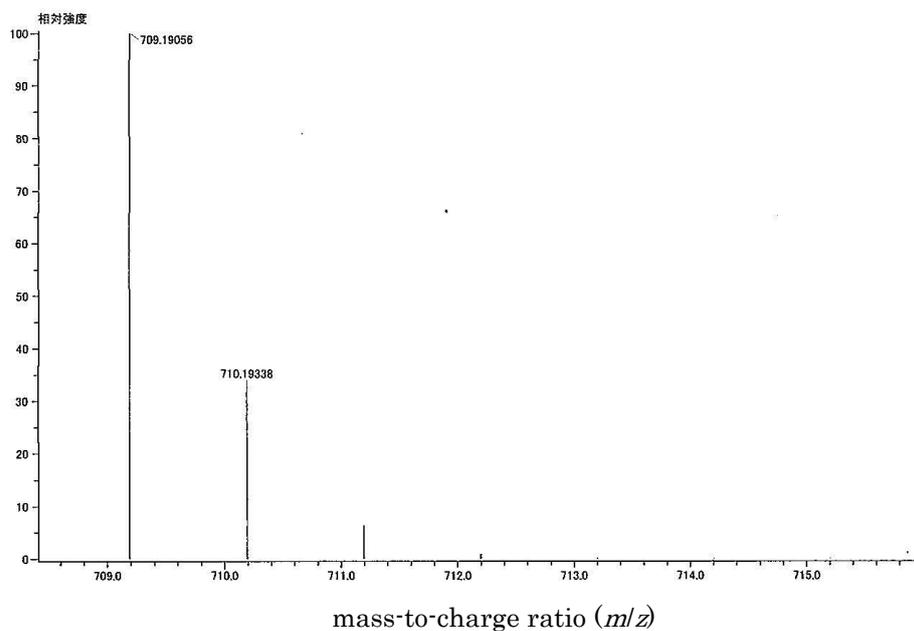
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ESI(+)-MS data of A1Tb, A2Tb, and A3Tb (calibrated chart and simulated spectra)

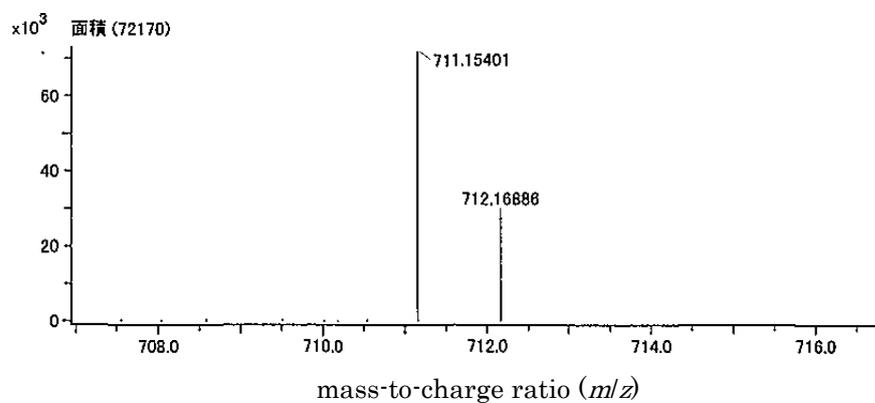
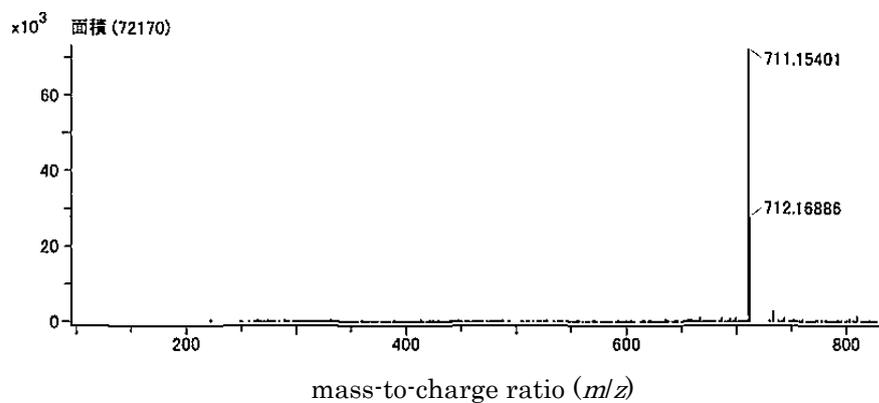
Spectra of A1Tb (lower panel: magnified view)



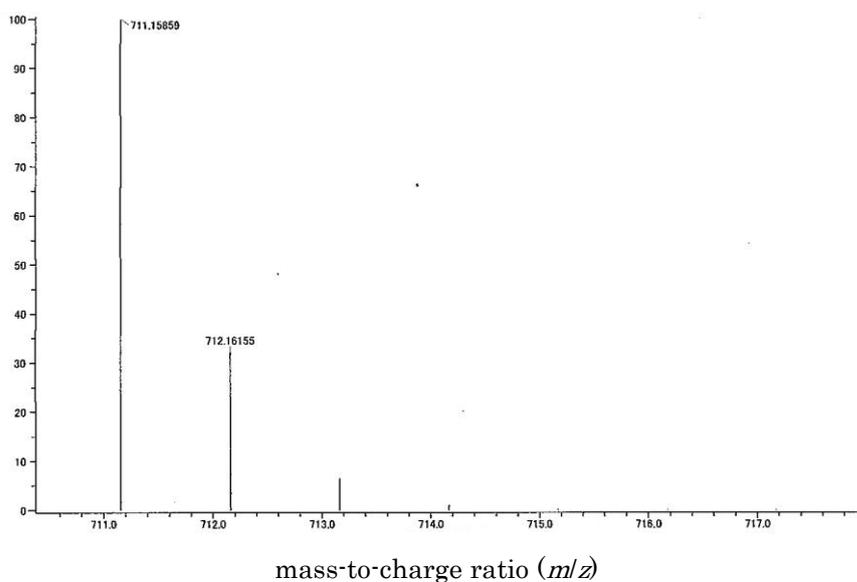
Simulated spectra of A1Tb ($C_{27}H_{34}N_8O_5Tb$, $[M - 2H]^+$)



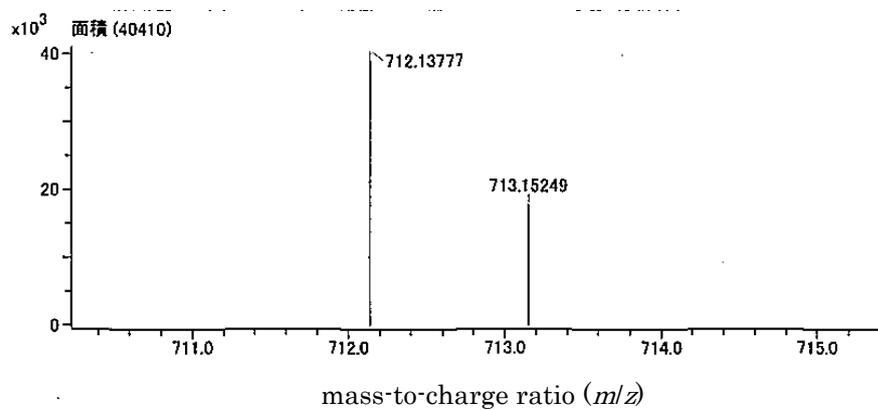
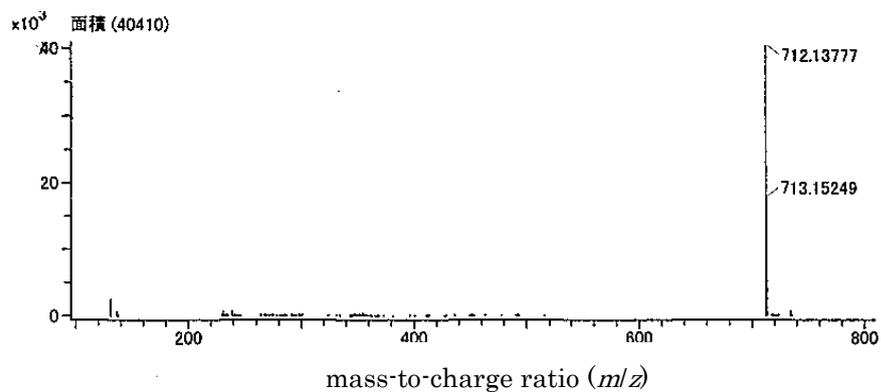
Spectra of A2Tb (lower panel: magnified view)



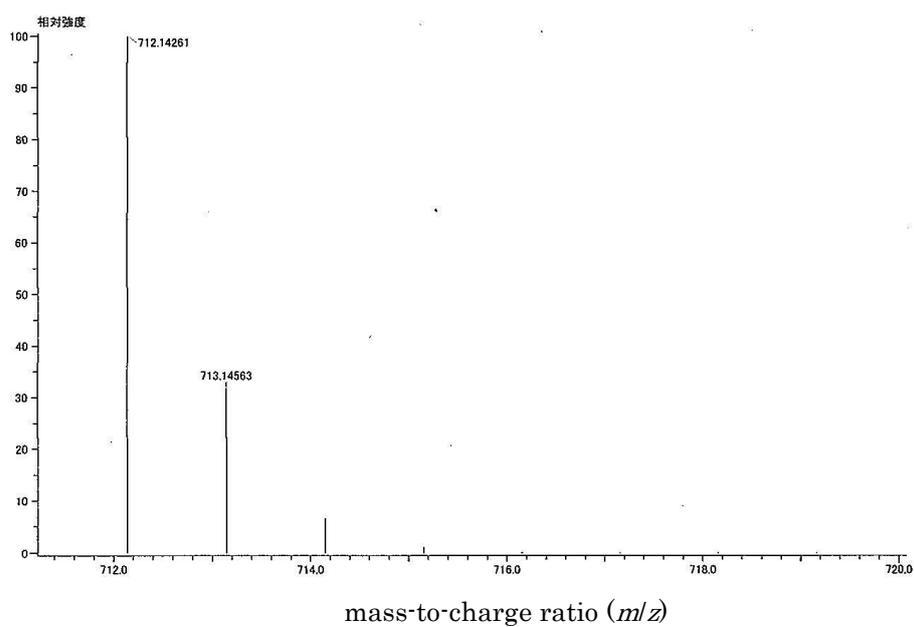
Simulated spectra of A2Tb ($C_{27}H_{32}N_6O_7Tb$, $[M]^+$)



Spectra of A3Tb (lower panel: magnified view)



Simulated spectra of A3Tb ($C_{27}H_{31}N_5O_8Tb$, $[M + H]^+$)



Note: NMR was not measured for Tb^{3+} complexes due to their paramagnetic nature.