

Supplementary Information

A Long-Lived Luminescent Probe to Sensitive Detect Arylamine *N*-Acetyltransferase (NAT) Activity of Cells

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Table of Contents

1. Materials and methods
2. Supplementary figures and tables
3. Supplementary references

1. Materials and methods

Materials

General chemicals were of the best grade available, supplied by Aldrich Chemical Co., Ltd, Tokyo Chemical Industries, and Wako Pure Chemical Industries. They were used without further purification. Dimethyl sulfoxide and *N,N*-dimethylformamide (from Dojindo) used in spectroscopic analysis were of fluorometric grade. Biochemical reagents including carnitine acetyltransferase and NAT from pigeon liver were purchased from SIGMA, and recombinant human NAT1 and NAT2 (insect cell cytosol) were purchased from BD Biosciences. The activity of the enzymes was confirmed by using standard substrates (PABA for NAT1 and SMZ for NAT2). Protein concentration and the definition of activity unit were in accordance with the distributor's instructions. The complexes **1** and **2** were synthesized and fully characterized as described in the literature.¹³

Instruments

UV-Visible spectra were obtained on a Shimadzu UV-1800 spectrophotometer. Steady-state fluorescence studies were performed on a Hitachi F4500 spectrofluorimeter and time-resolved luminescence spectra were obtained on a Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter. Luminescence lifetime data were determined on a PerkinElmer LS55 spectrofluorimeter, and fitted to a single exponential decay curve. For assays using microplates, a PerkinElmer EnVision multifunctional plate reader was used. HPLC analyses were performed on an Inertsil ODS-3 (4.6 × 250 mm) column (GL Sciences Inc., Japan) using an HPLC system composed of a pump (PU-2080, JASCO, Japan) and a detector (MD-2015 and FP-2020, JASCO, Japan), using eluent A (0.1 M triethylammonium acetate) and eluent B (CH₃CN with 20% H₂O containing 0.1 M triethylammonium acetate).

Spectroscopic measurements

Measurements were performed at 298 K unless otherwise indicated. Samples were prepared in a quartz cuvette (*l* = 1 cm). Throughout the work, Tb³⁺ complexes were prepared at 10 mM in DMSO, and the stock solution was diluted with an appropriate aqueous buffer to the indicated concentration. Each solution contained up to 0.1% (v/v) DMSO as a cosolvent. Luminescence spectra were corrected according to the manufacturer's instructions.

Quantum yields of luminescence

For determination of the quantum efficiency of luminescence (Φ_{lum}), quinine sulfate ($\Phi = 0.546$ in 1 N H₂SO₄) was used as a standard.^{S1} Excitation wavelength was 330 nm. Efficiency was calculated with the following equation (*L* denotes the area under the luminescence band ($L = \sum I_{\text{lum}}(\lambda)$), where

$I_{lum}(\lambda)$ is the luminescence intensity at each emission wavelength), Abs denotes the absorbance at the excitation wavelength, and n denotes the refractive index of the solvent).

$$\Phi_{lum}^{sample} = \Phi_{lum}^{standard} \times (L^{sample} / L^{standard}) \times (n^{sample} / n^{standard})^2 \times (Abs^{standard} / Abs^{sample})$$

Estimation of water coordination number (q)

Time-dependent luminescence profiles of the Tb^{3+} complexes in H_2O and D_2O containing 100 mM HEPES (pH or pD 7.4) were obtained, and the data were fitted to single exponential decay curves to calculate luminescence lifetimes (τ). The coordination number of water molecules (q) was estimated from τ (μs) in H_2O and D_2O by use of the following equation, which was proposed by Parker and coworkers.^{S2}

$$q_{Tb} = 5.0 \times \left(\frac{1}{\tau_{H_2O}} - \frac{1}{\tau_{D_2O}} - 0.06 \right)$$

NAT assay monitored in a 1-cm cuvette (for Fig. 3 and Fig. S3)

NAT assay was performed in an Eppendorf tube at 37 °C in 0.1 M Tris-HCl buffer (pH 7.4), containing 0.5 mM DTT, 0.5 mM EDTA, 0.1 mM acetyl CoA, 2 mM acetyl carnitine, 1 U carnitine acetyltransferase, 0.2 U NAT from pigeon liver, and 0.1 mM complex 1. Total volume was 0.3 mL. At the indicated times, an aliquot (30 μL) was taken, diluted a hundred times with the Tris-HCl buffer, and used for luminescence measurements. Excitation wavelength was 340 nm. For time-resolved measurement, the delay time was set to 0.1 ms and the gate time was set to 2.0 ms. In either measurement, no subtraction of background signal was performed.

Comparison with other probes (for Fig. 4)

NAT assay was performed on a 96-well microplate (non-treated black plate from IWAKI) in 0.1 M Tris-HCl buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, 0.5 mM acetyl CoA, 0.01 U/mL NAT from pigeon liver, and 20 μM substrate (total volume 200 μL). The plate was incubated at ambient temperature, and the emission intensity was measured every 5 min with an LS55 spectrofluorimeter. Excitation/emission wavelengths were 360/425 nm, 475/575 nm, and 330/545 nm for 2-aminoanthracene, cresyl violet, and complex 1, respectively. For the Tb^{3+} complex, time-resolved measurement with a delay time of 50 μs and gate time of 2.0 ms was performed.

NAT assay using a 96-well microplate (for Fig. S1 and S4)

NAT assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, obtained from Corning) with a total volume of 50 μ L. The reactions were carried out in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA, 0.5 mM acetyl CoA, 0.04 U/mL NAT from pigeon liver, and 2 μ M complex **1**. The plate was incubated at ambient temperature, and the luminescence intensity was measured every 10 min with an EnVision reader. As a control, wells containing an inhibitor (20 μ M quercetin or 1 mM PABA) and wells without acetyl CoA were also prepared. Measurement conditions were as follows. Filter set: LANCE/DELFLIA, delay time 100 μ s, gate time 400 μ s, cycle time 5 ms.

NAT assay using recombinant human enzyme (for Fig. S5)

NAT assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, obtained from Corning) with a total volume of 100 μ L. The assay was performed in 0.1 M Tris-HCl buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, 0.05 mM acetyl CoA, 5 mM acetyl carnitine, 10 U/mL carnitine acetyltransferase, 5 μ g/mL recombinant NAT1 or NAT2 insect cell cytosol, and 1 μ M complex **1**. The plate was incubated at ambient temperature, and the luminescence intensity was measured every 5 min with an EnVision reader. As a control, control cytosol (BD Biosciences) was added in place of NAT1 or NAT2 cytosol. Measurement conditions were as follows. Filter set: LANCE/DELFLIA, delay time 100 μ s, gate time 400 μ s, cycle time 5 ms.

NAT assay using cell cytosol (for Fig. 5, Fig. S6)

Cell cytosol was obtained by means of a procedure slightly modified from that described in the literature.²⁵ At approximately 60-80% confluence, HeLa, A549, or HepG2 cell monolayers (cultured in 100 mm dish) were washed with PBS (5 mL). The cells were scraped off and suspended in PBS (5 mL), then centrifuged for 2 min at 1000 rpm. The supernatant was removed and the cells were gently resuspended in CelLytic M (SIGMA, 1 mL) containing 1 mM DTT, 1 mM EDTA, and protease inhibitor cocktail (SIGMA, 20 μ L). Then the lysate was centrifuged for 15 min at 13000 rpm at 4 °C, and the supernatant was immediately used for further analysis. For comparison with other lysates, total protein concentration was measured by Bradford's method and appropriate corrections were made. The assay was performed on a 96-well half area microplate (Costar, flat-bottomed, non-treated black plate, obtained from Corning) with a total volume of 50 μ L. The reactions were carried out in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5 mM acetyl CoA, cell cytosol (25 μ L), and 10 μ M complex **1**. The plate was incubated at ambient temperature, and the luminescence intensity was measured every 5 min with an EnVision reader. As a control, wells containing 10 μ M quercetin were also prepared.

2. Supplementary figures and tables

Table S1 Photophysical properties of complexes **1** and **2**.

	λ_{Ex} /nm	ϵ (cm ⁻¹ M ⁻¹)	Φ_{Lum} (%)	τ (H ₂ O) /ms	τ (D ₂ O) /ms	q
complex 1	330	1.3×10^4	0.01	n.d. ^a	n.d. ^a	n.d. ^a
complex 2	330	1.3×10^4	4.8	1.15	1.80	1.27

^aNot determined due to the very weak luminescence.

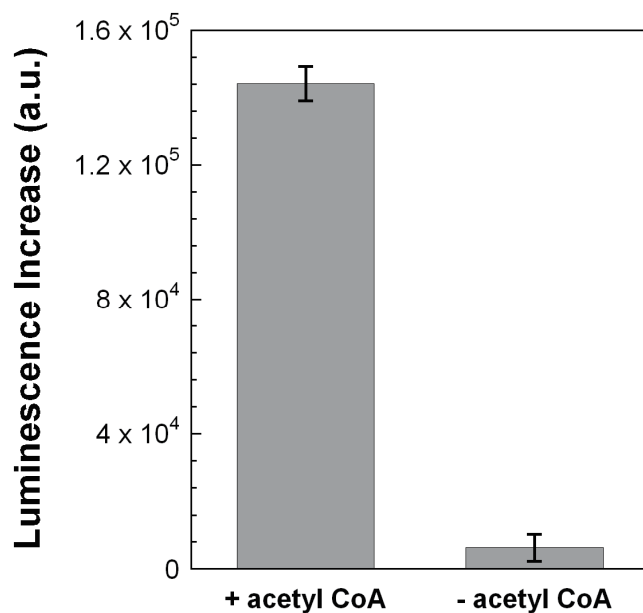


Fig. S1 Dependence of the enzyme activity on acetyl CoA. The assay was performed on a 96-well microplate and the luminescence increase over 60 min was plotted as mean \pm SEM ($n = 3$).

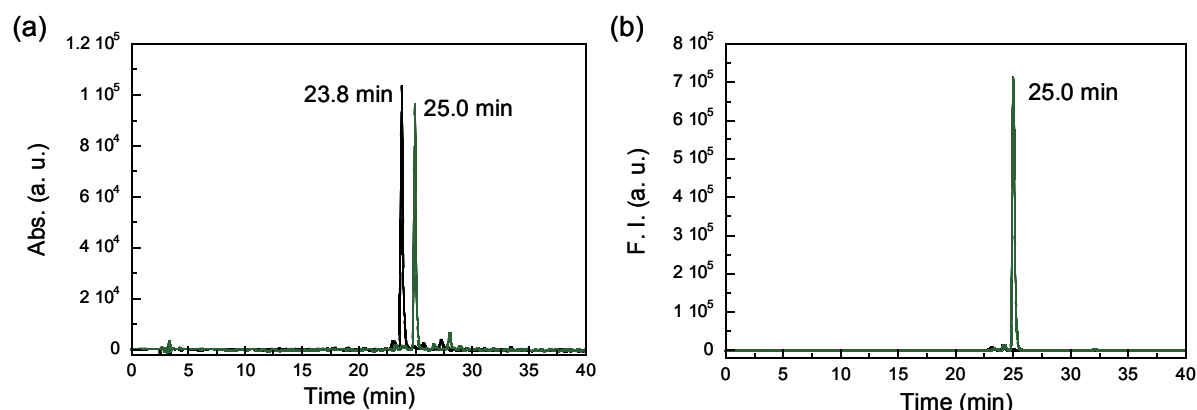


Fig. S2 HPLC confirmation of the enzymatic conversion of complex **1** by NAT. Control solution (black line) and reaction solution (green line) after 8 hours of incubation were injected into a C-18 column. Absorbance at 330 nm (a) or fluorescence at 545 nm (excited at 330 nm) (b) is plotted as a function of retention time. NAT assay was performed at 37 °C in 0.1 M Tris-HCl buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, 0.1 mM acetyl CoA, 2 mM acetylcarnitine, 1 U carnitine acetyltransferase, 0.2 U NAT, and 0.5 mM complex **1**. A linear gradient from A/B = 95/5 to 60/40 over 35 min was used. The retention times of 23.8 min and 25.0 min coincided with those of the standard complexes **1** and **2**, respectively.

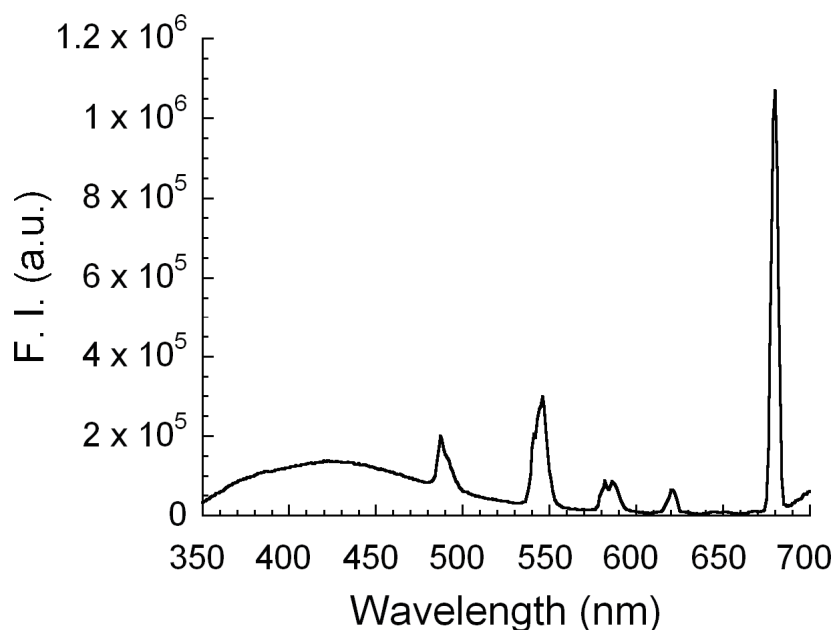


Fig. S3 NAT reaction of complex **1** evaluated with a conventional fluorometer. Emission spectrum of complex **1** at 4 hours after the addition of the probe was plotted. The assay was performed at 37 °C in 0.1 M Tris-HCl buffer (pH 7.4, total 300 μ L), containing 0.5 mM DTT, 0.5 mM EDTA, 0.1

mM acetyl CoA, 2 mM acetylcarnitine, 1 U carnitine acetyltransferase, 0.2 U NAT, and 0.1 mM Tb³⁺ complex. Ex. 340 nm. A broad peak around 400 nm is derived from fluorescence of both the antenna moiety of the complex and unknown fluorescent molecules in the mixture. The sharp peak at 680 nm is due to scattering of excitation light. As shown in Fig. 3, these peaks are completely eliminated by the use of time-resolved measurement.

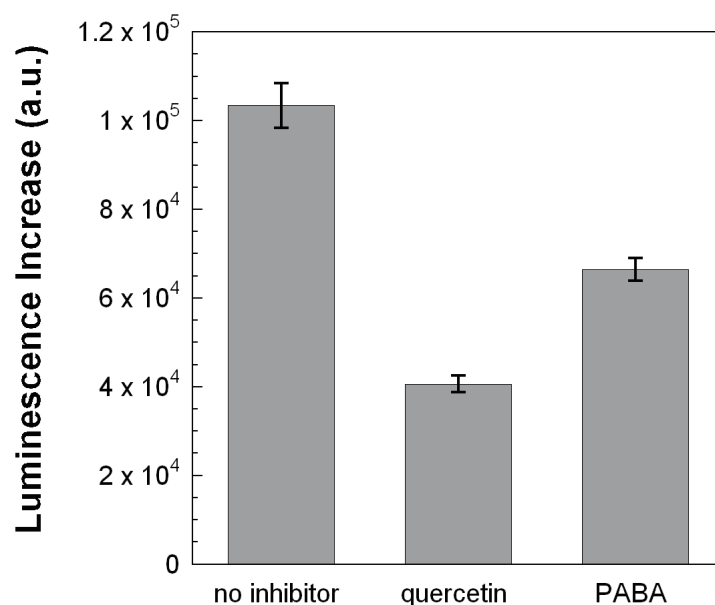


Fig. S4 Inhibition of luminescence increase by quercetin and PABA. The assay was performed on a 96-well microplate and the luminescence increase over 60 min was plotted as mean ± SEM (n = 3). Concentrations of quercetin and PABA were 20 μM and 1 mM, respectively.

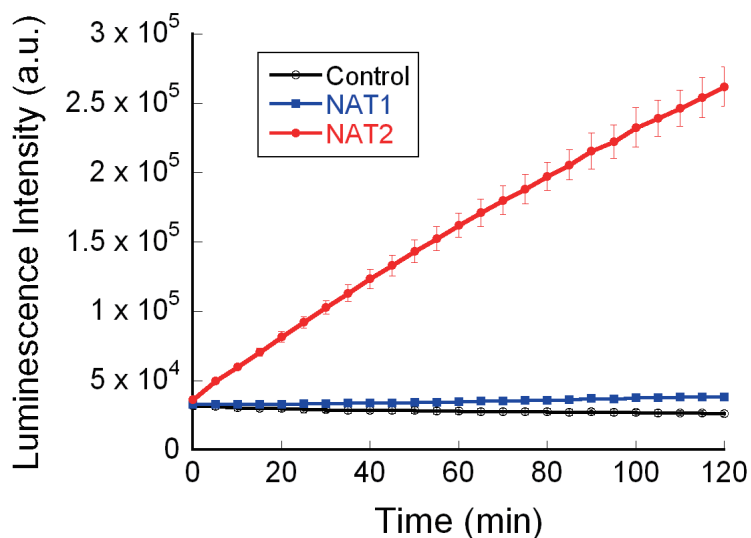


Fig. S5 Reaction of complex **1** with human NATs. Symbols and bars represent mean \pm SEM ($n = 3$). Where no error bars are shown, they are smaller than the symbol. Assay was performed in 0.1 M Tris-HCl buffer (pH 7.4), containing 1 mM DTT, 1 mM EDTA, 0.05 mM acetyl CoA, 5 mM acetylcarnitine, 10 U/mL carnitine acetyltransferase, 5 μ g/mL enzyme, and 1 μ M complex **1**.

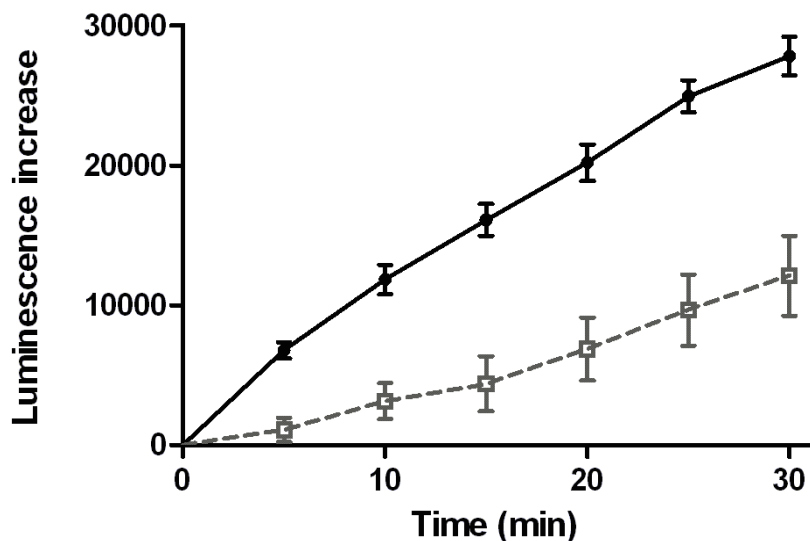


Fig. S6 Time-course of NAT activity measurement using HepG2 cytosol. Shown is the increase of luminescence of the cytosol incubated with complex **1** (black circle), or with complex **1** and quercetin (10 μ M) (gray square). Symbols and bars represent mean \pm SEM ($n = 4$).

3. Supplementary references

- S1. W. H. Melhuish, *J. Phys. Chem.*, 1961, **65**, 229.
- S2. A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. de Sousa, J. A. G. Williams, M. Woods, *J. Chem. Soc., Perkin Trans. 2*, 1999, 493.