Supporting Information

Intracellular Activation of Acetyl-CoA by Artificial Reaction Promoter and Its Fluorescent Detection

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1. General
Starting materials and reagents were purchased from Tokyo Kasei Kogyo (TCI: Tokyo, Japan), Wako (Tokyo, Japan), or Aldrich (Milwaukee, WI). GR grade or dry grade solvents were purchased from Wako, and used without further purification. The $^1$H spectra were recorded using JEOL JNM-ECX500 (500 MHz) FT NMR System in CD$_3$OD or D$_2$O. The $^{13}$C NMR spectra were measured with JEOL JNM-ECX500 (125 MHz) FT NMR System in CD$_3$OD or D$_2$O. Coupling constants are given in hertz. The LR-ESI-MS spectra were recorded on Waters micromass ZQ spectrometer. The HR-ESI-MS spectra were recorded on JEOL The AccuTOF JNM-T100LC spectrometer. The reactions were carried out in oven-dried glasswares under an argon atmosphere with a magnetic stirring. All absorption spectra were recorded using a SHIMADZU UV-1800 UV/Vis spectrophotometer with 1 cm quartz cell. In a similar manner, fluorescence spectra were recorded on a SHIMADZU RF-5300PC spectrofluorophotometer with a 1 cm quartz cell. Phosphate buffered saline (100 mM, pH 7.4, containing 1% DMSO) was used as solvent. HeLa cells were obtained from RIKEN Tsukuba Institute, and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO$_2$. For experimental use, cells were cultured on glass bottom dishes (Iwaki, Tokyo, Japan).

2. Synthesis of probe 3 and thioester 6

![Chemical structures](image)

$^{6,6'}-((4$-Nitrophenyl)methylene)bis(3-(dimethylamino)phenol) (S1)$

3-(Dimethylamino)phenol (825.8 mg, 6.22 mmol, 2.6 equiv), p-toluenesulfonic acid (51 mg, 300 µmol, 0.1 equiv), and AcOH (20 mL) were added to 4-nitrobenzaldehyde (356.3 mg, 2.36 mmol, 1 equiv), and the mixture was stirred for 14 hours at 60 °C. The reaction mixture was cooled to room temperature, and concentrated. Aqueous solution of NaHCO$_3$ was added, and products were extracted with CH$_2$Cl$_2$. The combined organic layer was washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by silica gel column chromatography.
(CH₂Cl₂/MeOH = 30/1 to 20/1) to afford S1 as orange solid (942 mg, 2.31 mmol, 98%). H NMR (500 MHz, CD₂OD): 2.85 (s, 12H), 6.00 (s, 1H), 6.21 (dd, 2H, J = 8.6 Hz, 2.3 Hz), 6.28 (d, 2H, J = 2.3 Hz), 6.55 (d, 2H, J = 8.6 Hz), 7.23 (d, 2H, J = 9.2 Hz), 8.06 (d, 2H, J = 8.6 Hz); C NMR (125 MHz, CD₂OD): 41.2, 43.7, 101.7, 105.9, 120.0, 123.8, 131.0, 131.7, 147.1, 152.3, 155.9, 156.5; LR-ESI-MS: 408 (MH⁺).

**N-((6-(Dimethylamino)-9-(4-nitrophenyl)-3H-xanthen-3-yldiene)-N-methylmethanaminium trifluoroacetate (S2)**

2,3-Dichloro-5,6-dicyano-p-benzoquinone (DDQ) (247.8 mg, 1.09 mmol, 2 equiv) was added to S1 (210.8 mg, 0.517 mmol) in benzene/AcOH (1/1 v/v, 12 mL), and the mixture was stirred for 3 h at room temperature. The resulting mixture was concentrated, and filtered through silica gel column chromatography (CH₂Cl₂/MeOH/TFA = 10/1/0.01) to afford crude product (197.6 mg), which was further purified by reverse phase column chromatography (MeOH) to afford S2 as purple solid (187.3 mg, 0.374 mmol, 72%). H NMR (CD₂OD, 500 MHz): 3.23 (s, 12H), 7.00 (d, 2H, J = 2.3 Hz), 7.11 (dd, 2H, J = 9.2 Hz, 2.3 Hz), 7.30 (d, 2H, J = 9.7 Hz), 7.77 (d, 2H, J = 8.6 Hz), 8.53 (d, 2H, J = 8.6 Hz); C NMR (125 MHz, CD₂OD): 41.1, 97.8, 102.2, 114.3, 114.6, 115.9, 125.0, 130.2, 132.2, 132.3, 140.3, 150.4, 152.8, 159.0; LR-ESI-MS: 388 [M-CF₃COO]⁺; HR-ESI-MS: calcd for C₂₃H₂₂N₃O₄⁺ [M-CF₃COO]⁺ 388.1656, found 388.1674.

**N-((9-(4-Aminophenyl)-6-(dimethylamino)-3H-xanthen-3-yldiene)-N-methylmethanaminium trifluoroacetate (3)**

To a solution of S2 (80.1 mg, 0.160 mmol) in 10 mL of ethanol under argon atmosphere, 10% Pd/C (43.1 mg) was added. The vessel was purged with H₂, and the mixture was stirred for 2 hours at room temperature. The reaction mixture was filtered through Celite and concentrated to afford a crude mixture, which was purified by reverse phase column chromatography (60% MeOH to MeOH) to afford 3 as purple solid (50.5 mg, 0.107 mmol, 67%). H NMR (500 MHz, CD₂OD): 3.34 (s, 12H), 6.85–6.92 (m, 4H), 7.06 (dd, 2H, J = 9.6 Hz, 2.8 Hz), 7.26 (d, 2H, J = 8.2 Hz), 7.63 (d, 2H, J = 9.6 Hz); C NMR (125 MHz, CD₂OD): 40.8, 97.5, 114.4, 115.0, 115.2, 120.9, 125.0, 132.2, 132.3, 133.2, 133.4, 152.7, 158.6, 161.1; LR-ESI-MS 358 [M-CF₃COO]⁺; HR-ESI-MS: calcd for C₂₃H₂₄N₃O⁺ [M-CF₃COO]⁺ 358.1914, found 358.1915.

**N-((9-(4-Acetamidophenyl)-6-(dimethylamino)-3H-xanthen-3-yldiene)-N-methylmethanaminium acetate (4)**

Ac₂O (0.5 mL) and triethylamine (50 µL) were added to 3 (2.7 mg, 5.72 µmol), and the resulting solution was stirred for 5 hours at room temperature. The reaction mixture was concentrated and dried in vacuum. The crude product was purified by reverse phase column chromatography (MeOH) to afford 4 (2.6 mg, 5.66 µmol, 99 %) as purple solid. H NMR (CD₂OD, 500 MHz): 2.14 (s, 3H), 2.27 (s, 3H), 3.24 (s, 12H), 6.85–6.70 (m, 2H), 7.00–7.05 (m, 2H), 7.38–7.42 (m, 4H), 7.79–7.82 (m, 2H); C NMR (CD₂OD, 125 MHz): 20.0, 24.0, 40.9, 97.6, 114.2, 114.6, 115.5, 115.9, 120.9,
131.8, 132.3, 132.9, 158.9, 159.3, 169.8, 172.1; LR-ESI-MS: 400 [M-CH₃COO]⁺; HR-ESI-MS: calcd for C₂₅H₂₆N₃O₂⁺ [M-CH₃COO]⁺ 400.2020, found 400.2028.

**Sodium 3-(acetylthio)propane-1-sulfonate (6)**

To sodium 3-mercapto-1-propanesulfonate (1.38 g, 7.72 mmol), MgBr₂-ether complex (131 mg, 0.50 mmol, 0.07 eq.), 6 mL of Ac₂O, and 6 mL of dioxane were added. The mixture was stirred for 17 hours. After the subsequent addition of MeOH and water to quench the reaction, volatiles were evaporated *in vacuo*. The resulting solid was suspended in MeOH, and was filtered. The filtrate was evaporated to yield sufficiently pure 6 as white solid (1.63 g, 7.41 mmol, 96%).

**3. Measurement of fluorescence quantum yield**

The fluorescence quantum yield ($\Phi_F$) was determined by using rhodamine B, with known $\Phi_F$ value of 0.65 in ethanol as a reference. The quantum yield was calculated according to equation (1), in which $\Phi_{F(S)}$ and $\Phi_{F(R)}$ are the fluorescence quantum yields of the sample and the reference, respectively, the terms $A(S)$ and $A(R)$ are the area under the fluorescence spectra, $(Abs)_{(S)}$ and $(Abs)_{(R)}$ are the optical densities of the sample and reference solutions at the excitation wavelength, and $n(S)$ and $n(R)$ are the refractive indices of the solvents used for the sample and the reference (1.361 for EtOH, 1.333 for water).

$$\Phi_{F(S)}/\Phi_{F(R)} = A(S)/A(R) \times (Abs)_{(R)}/(Abs)_{(S)} \times n(S)^2/n(R)^2$$  \hspace{1cm} (1)

**4. Fluorescence time course measurement**

Time courses of fluorescence during acetylation reactions of 3 were measured using solutions containing 3 (1 µM) and various acyl-transfer promoters (10 mM) in PBS (1% DMSO) (Figure 2b in the text). At 1 min, an acetylating reagent ($N$-methoxy diacetamide 5 or thioester 6 (1 M)) was added. The measurement continued for 5 min or 500 sec. The reaction product 4 was identified with LR-ESI-MS: calcd for C₂₅H₂₆N₃O₂⁺ [M-CH₃COO]⁺ 400.2020, found 400.

**5. Response Selectivity of RH-NH₂ (Figure S1)**

RH-NH₂ (3: 1 µM) was dissolved in PBS, and CaCl₂ (10 mM) or MgSO₄ (10 mM) was added. Also, RH-NH₂ (1 µM) was dissolved in phosphate buffer of pH 6.0, 6.5, or 7.0. Fluorescence intensity was measured at excited 550 nm/detected 575 nm. Results were summarized in Figure S1. It was
found that fluorescence probe 3 selectively responds to N-acetylation, but not to Ca$^{2+}$, Mg$^{2+}$, and N-protonation (i.e. pH). Therefore, 3 is a selective probe that responds to N-acetylation.

6. Assessment of the toxicity of PBu$_3$: Mitochondrial membrane potential change (Figure S2)
To assess possible adverse effects of PBu$_3$ on cells in an analysis time scale, the mitochondrial membrane potential change after the addition of PBu$_3$ (5 mM) was measured by fluorescence of tetramethylrhodamine ethyl ester (TMRE). As shown in Figure S2, there was no significant membrane potential change in the presence and absence of PBu$_3$. This result indicates that there was no fetal effects of PBu$_3$ under the assay conditions.

7. Localization of 3 to the mitochondria of HeLa cells (Figure S3)
For fluorescence imaging, 3 (10 μM) was applied to cells in Hanks’ balanced salt solutions (HBSS) containing (in mM): NaCl, 137; KCl, 5.4; CaCl$_2$, 1.3; MgCl$_2$, 0.5; MgSO$_4$, 0.4; Na$_2$HPO$_4$, 0.3; KH$_2$PO$_4$, 0.4; NaHCO$_3$, 4.2; D-glucose, 5.6; HEPES, 5 (pH adjusted to 7.4 with NaOH) for 30 min at 37 °C. Then, the cells were washed twice with HBSS and observed the fluorescence.
Localization of mitochondria was visualized by expressing TagCFP-Mito to HeLa cells. The pTagCFP-Mito vector (Evrogen, Moscow, Russia) was transfected to HeLa cells with Lipoplectamin LTX (Invitrogen). Fluorescence was observed more than 24 hours after transfection. Fluorescent imaging experiments were performed using a confocal laser scanning microscope system (FluoView FV1000; Olympus, Tokyo, Japan) mounted on an inverted microscope (IX81; Olympus) with a 40× or 60× oil–immersion objective lens. RH-NH$_2$ 3 was excited at 559 nm, and the signal was observed at 580-680 nm. TagCFP-Mito was excited at 440 nm, and the signal was observed at 460-500 nm. Fluorescence images were acquired and analyzed with the FluoView software package (Olympus). Fluorescence intensities were calculated as the mean intensity over a defined region of interest (ROI) containing the cell body of each cell.

8. Effects of PBu$_3$ on reaction rate of intracellular acetylation of 3 (Figure S4)
The time course of the fluorescence enhancement by PBu$_3$-promoted acetylation of 3 in HeLa cells was analyzed at 37 °C. There was only very small fluorescence enhancement in the presence of NMD (5: 10 mM) without adding the reaction promoter, PBu$_3$. To sharp contrast, marked fluorescence enhancement was observed after the addition of PBu$_3$ (5 mM) at 7 min. The result clearly demonstrated that intracellular acetylation of 3 was accelerated by the exogenously-added artificial reaction promoter.

9. Pyruvic acid-induced fluorescence enhancement (Figure S5)
Addition of pyruvic acid (5 mM) at 7 min induced fluorescence enhancement in mitochondria in the
presence of PBu$_3$ and RH-NH$_2$ 3. As a control experiment, we confirmed that the addition of pyruvic acid to probe 3 in the presence of PBu$_3$ did not enhance fluorescence intensity of probe 3 in vitro (Figure S1). This result strongly supports that the cells are functional under the assay conditions, and the observed increase in mitochondrial fluorescence in the current assay conditions is due to acetylation of probe 3 by acetyl-CoA in the mitochondria.

Pyruvic acid is a substrate of tricarboxylic acid cycle (TCA cycle), and pyruvate dehydrogenase synthesizes acetyl-CoA from pyruvate and CoA. The increased acetyl-CoA activates TCA cycle and this results in increase in mitochondrial membrane potential. Therefore, we examined mitochondrial membrane potential. As a result, increase in the mitochondrial membrane potential was observed immediately after the application of pyruvate (Figure S6). This result suggests that the increase in acetyl-CoA was induced immediately after the application of pyruvate, and it is consistent with the time-course of the increase in the fluorescence of RH-NH$_2$ 3 induced by pyruvate.

**Figure S1.** Fluorescence responses of RH-NH$_2$ (3) to various chemical stimulants. (Ex. 550 nm/detect 575nm).
Figure S2. Mitochondrial membrane potential change by PBu₃ (5 mM) of 4 individual cells (Observed by fluorescence of tetramethylrhodamine ethyl ester (TMRE)).

Figure S3. Double-staining of Hela cells with RH-NH₂ 3 and CFP. CFP (Ex. 440 nm/detect 460-500 nm), RH-NH₂ (Ex. 559 nm/detect 570-670 nm).
**Figure S4.** Time course of the fluorescence enhancement by PBu₃-promoted acetylation of 3 in HeLa cells at 37 ºC. NMD (5: 10 mM) was added at 1 min. The reaction promoter PBu₃ (5 mM) was added at 7 min. The enhancement of the fluorescence intensity (F/F₀) was calculated through averaging the results obtained from 5 cells.

**Figure S5.** Time course of the fluorescence enhancement by the PBu₃-promoted acetylation of 3 (10 μM) in HeLa cells at 37 ºC. The reaction promoter PBu₃ (5 mM) was added at 2 min. Pyruvic acid (5 mM) was added at 7 min. The enhancement of the fluorescence intensity (F/F₀) was calculated through averaging the results obtained from 5 cells.
Figure S6. Mitochondrial membrane potential change by the addition of pyruvate (5 mM), which was observed by fluorescence of tetramethylrhodamine ethyl ester (TMRE) in HeLa cells at 37 °C. Pyruvic acid (5 mM) was added at 2 min. Enhancement of the membrane potential was observed immediately after the addition of pyruvic acid. The enhancement of the fluorescence intensity (F/F₀) was calculated through averaging the results obtained from 4 cells.
10. References


11. NMR spectra of compounds

$^1$H-NMR of S2

$^{13}$C-NMR of S2
$^{1}H$-NMR of RH-NH$_2$ 3

$^{13}$C-NMR of RH-NH$_2$ 3
$^1$H-NMR of RH-NHAc 4

$^{13}$C-NMR of RH-NHAc 4