Supporting Information

"Development of Fluorogenic Probe with Transesterification Switch for Detection of Histone Deacetylase Activity"

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1. Experimental Procedures

Materials and instruments

General chemicals were of the best grade available and were supplied by Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), Sigma-Aldrich Chemical Co. (St. Louis, MO), and Peptide Institute, Inc. (Osaka, Japan); and used without further purification.

Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H and at 100.4 MHz for ^{13}C NMR using tetramethylsilane (in $CDCl_3$) as an internal standard. MALDI-LIFT-TOF/TOF MS was performed on a Bruker UltraFlexIII mass spectrometer. High-resolution mass spectra (HRMS) were obtained on a JEOL JMS-700 instrument. ESI-time-of-flight (TOF) MS was performed on a Waters LCT-Premier XE. Silica gel column chromatography was performed using BW-300 (Fuji Silysia Chemical Ltd., Greenville, NC). High-pressure liquid chromatography (HPLC) analysis or purification was performed with an Inertsil ODS-3 column (4.6 or 10.0 mm × 250 mm, GL-Science, Inc. Torrance, CA) using an HPLC system that comprised a pump (PU-2080, JASCO) and a detector (MD-2010 and FP-2020, JASCO). Fluorescence spectra were measured using a Hitachi F4500 spectrometer with a photomultiplier voltage of 700V. The plasmid encoding Sirt1 was kindly provided by Prof. Eric M. Verdin.

Synthesis of compounds (Scheme S1-S4)

Butyl (2,5-dioxopyrrolidin-1-yl) carbonate (**1**)

1-butanol (148 mg, 2.0 mmol) and Et_3N (242 μL, 2.4 mmol) were dissolved in anhydrous CH_3CN (5 mL), and then N,N′-disuccinimidyl carbonate (615 mg, 2.4 mmol) was added. The reaction mixture was stirred for 3 h at room temperature. After the removal of the solvent, the residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 3/7) to give compound **1** as a faint yellow oil (313 mg, 73% yield). ^{1}H NMR

($CDCl_3$, 400 MHz), δ (ppm): 4.33 (t, *J* = 8.0 Hz), 2.84 (s, 4H), 1.74 (quin, *J* = 6.8 Hz, 2H), 1.44 (sext, *J* = 7.6 Hz, 2H), 0.96 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 168.71, 151.56, 71.38, 30.32, 25.43, 18.67, 13.54. HRMS (CI+) *m/z*: calculated for $[M+H]^+$, 216.0866; observed, 216.0873.

4-(chloromethyl)-7-hydroxy-2H-chromen-2-one (**2**)

1,3-dihydroxybenzene (resorcinol) (660 mg, 6.0 mmol) was dissolved in concentrated H_2SO_4 (7 mL), and then ethyl 4-chloroacetoacetate (822 mg, 5.0 mmol) was added to the solution at 0°C. The reaction mixture was stirred at room temperature for 12 h, then poured into crushed ice, and stirred for 1 h. A yellow solid was precipitated, and, after the removal of the solvent by filtration, the precipitate was washed with water and dried under vacuum to afford compound **2** as a faint yellow solid (485 mg, 46% yield). ^{1}H NMR (CD_3CN, 400 MHz), δ (ppm): 8.14 (s, 1H), 7.61 (d, *J* = 8.0 Hz, 1H), 6.84 (dd, J_1 = 8.0 Hz, J_2 = 2.4 Hz, 1H), 6.78 (d, *J* = 2.4 Hz, 1H), 6.32 (s, 1H), 4.74 (s, 2H). ^{13}C-NMR (CD_3CN, 100 MHz) δ (ppm): 161.65, 161.40, 156.58, 156.58, 127.02, 113.64, 112.68, 111.11, 103.73, 42.29. HRMS (EI+) *m/z*: calculated for $[M]^+$, 210.0084; observed, 211.0085.

(7-hydroxy-2-oxo-2H-chromen-4-yl)methanaminium chloride (**3**)

Compound **2** (380 mg, 1.8 mmol) was added under anaerobic conditions to 20 mL of 28% ammonium hydroxide. The resulting yellow solution was stirred at 50°C for 1 h. The progress of the reaction was checked by silica thin-layer chromatography (TLC) with 9/1 acetone/25% ammonium hydroxide as the mobile phase. After the completion of the reaction, the mixture was acidified with 2N HCl and filtered. The filtrate was gently neutralized with 2N NaOH. The precipitate was isolated by filtration and dried under vacuum to afford compound **3** as a faint yellow powder (335 mg, 80% yield). ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.60 (d, *J* = 8.4 Hz, 1H), 6.84 (dd, J_1 = 8.4 Hz, J_2 = 2.8 Hz, 1H), 6.73 (d, *J* = 2.8 Hz, 1H), 6.32 (s, 1H), 3.93 (s, 2H). ^{13}C-NMR (CD_3CN, 100 MHz) δ (ppm): 161.62, 161.21, 156.82, 156.59, 127.05, 113.73, 112.20, 111.15, 103.80, 39.68. HRMS (FAB+) *m/z*: calculated for $[M+H]^+$, 192.0612; observed, 192.0663.

Tert-butyl ((7-hydroxy-2-oxo-2H-chromen-4-yl)methyl)carbamate (**4**)

Compound **3** (191 mg, 0.84 mmol) and Et_3N (120 mg, 1.2 mmol) were dissolved in dimethyl formamide (DMF)/MeOH (9/2, 11 mL), and then $(Boc)_2O$ (262 mg, 1.2 mmol) was added to the solution. The reaction mixture was stirred at room temperature for 18 h. After the completion of the reaction, the solvent was removed *in vacuo* and the residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 7/3) to give compound **4** as a faint yellow solid (216 mg, 88% yield). ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 7.51 (s, 1H), 7.21 (d, *J* = 8.8 Hz, 1H), 6.75 (s, 1H), 6.67(d, *J* = 8.8 Hz, 1H), 6.19 (s, 1H), 5.12 (s, 1H), 4.41 (d, *J* = 6.0 Hz, 2H), 1.51 (s, 9H,). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ (ppm): 161.24, 160.43, 156.22, 155.70, 154.96, 154.17, 125.77, 112.98, 109.96, 107.31, 102.38, 78.46, 28.21. HRMS (CI+) *m/z*: calculated for $[M+H]^+$, 292.1124; observed, 292.1182.

Tert-butyl ((7-((butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)methyl)carbamate (**5**)

Compound **4** (81 mg, 0.28 mmol) was dissolved in anhydrous DMF (3 mL), and then sodium hydride (8.2 mg, 0.34 mmol) was added to the solution. Then, compound **1** (72 mg, 0.34 mmol) dissolved in anhydrous DMF (1 mL) was added, and the mixture was stirred at room temperature for 12 h under an argon atmosphere. *n*-Butanol (1 mL) was added to the reaction mixture on ice and stirred for 30 min to stop any further reaction with the excess sodium hydride. After removing the solvent, the residue was dissolved in 70 mL of AcOEt. The solution was washed with 0.1N HCl aq. (40 mL × 3) and brine (30 mL × 1) and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* and the crude mixture was purified by silica gel flash column chromatography (DCM and then AcOEt/*n*-hexane = 3/7) to give compound **5** as a faint yellow solid (86 mg, 78% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.62 (d, J = 8.8 Hz, 1H), 7.25 (d, J = 2.4 Hz, 1H), 7.17 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H), 6.39(s, 1H), 4.94 (s, 1H), 4.51 (d, J = 6.0 Hz, 2H), 4.29 (t, J = 6.0 Hz, 2H), 1.77-1.71 (m, 2H), 1.49-1.40 (m, 11H), 0.98 (t, J = 7.6 Hz, 3H). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ (ppm): 161.20, 160.52, 156.21, 155.72, 155.02, 154.16, 151.31, 125.69, 112.95, 110.01, 107.33, 102.55, 78.46, 71.10, 29.82, 28.13, 18.16, 13.39. HRMS (FAB+) *m/z*: calculated for $[M+H]^+$, 392.1704; observed, 392.1709.

(7-((butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)methanaminium 2,2,2-trifluoroacetate (**6**)

Compound **5** (86 mg, 0.22 mmol) was dissolved in dichloromethane (DCM)/trifluoroacetic acid (TFA) (1/1, 1 mL). The resulting yellow solution was stirred at room temperature for 2 h. The solvent was removed *in vacuo* to afford compound **6** as a pale yellow powder (88 mg, 99% yield). ^{1}H NMR (CD3CN, 400 MHz) δ (ppm): 7.72 (d, J = 8.8 Hz, 1H), 7.27 (d, J = 2.0 Hz, 1H), 7.22 (dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, 1H), 6.47(s, 1H), 4.37 (s, 1H), 4.25 (t, J = 6.4 Hz, 2H), 1.74–1.67 (m, 2H), 1.47–1.40 (m, 2H), 0.95 (t, J = 7.2 Hz, 3H). ^{13}C-NMR (CD_3CN, 100 MHz) δ (ppm): 160.20, 154.99, 154.69, 153.70, 148.19, 126.33, 118.77, 116.08, 114.71, 110.96, 69.96, 39.81, 31.07, 19.14, 13.78. HRMS (FAB+) *m/z*: calculated for $[M]^+$, 292.1179; observed, 292.1188.

Boc-Lys(me2)-OH (**7**)

Boc-Lys-OH (246 mg, 1.0 mmol) was dissolved in ethanol (10 mL) followed by adding 37% formaldehyde aq. (243 μL, 3 mmol), and this mixture was stirred at 0ºC for 10 min. Then, sodium cyanoborohydride (251 mg, 4 mmol) was added and the resulting mixture was stirred at room temperature for 12 h. The progress of the reaction was checked by silica TLC with 5% H_2O in MeOH as the mobile phase. After the completion of the reaction, the mixture was gently neutralized by 2N HCl, and the solvent was removed *in vacuo*. The residue was dissolved in H_2O (30 mL) and washed with chloroform (20 mL × 10). After removing the solvent, the residue was purified by silica gel flash column chromatography (DCM/MeOH/TEA = 50/50/2) to afford compound **7** as a pale yellow solid (196 mg, 71% yield). ^{1}H NMR (D_2O, 400 MHz) δ (ppm): 3.74 (br, 1H), 2.98 (t, J = 8.0 Hz, 2H), 2.71 (s, 6H), 1.62–1.47 (m, 4H), 1.34–1.22 (m, 11H). MS (ESI+) *m/z*: calculated for $[M+H]^+$, 275.1965; observed, 275.2075.

Boc-Lys(me3)-OH (**8**)

Compound **7** (114 mg, 0.41 mmol) was dissolved in MeCN/MeOH (2/1, 4 mL), and then iodomethane (581 mg, 4.1 mmol) and N, N-diisopropylethylamine (DIEA) (69 mg, 0.54 mmol) were added. The reaction mixture was stirred at room temperature for 1 h. The progress of the reaction was checked by silica TLC with chloroform/MeOH/AcOH (5/4/1) as the mobile phase. After the completion of the reaction, the solvent was removed *in vacuo*. The residue was dissolved in MeOH/0.1N NaOH aq. (1/1, 10 mL) and stirred at room temperature for 1 h. Then, the mixture was gently neutralized with 2N HCl. After the removal of the solvent, the residue was purified by silica gel flash column chromatography (MeOH/TEA = 97/3) to give compound **8** as a white powder (128 mg, 96% yield). ^{1}H NMR (D_2O, 400 MHz) δ (ppm): 3.74 (br, 1H), 3.17 (t, *J* = 8.0 Hz, 2H), 2.96 (s, 9H), 1.70–1.51 (m, 4H), 1.34–1.24 (m, 11H). MS (ESI+) *m/z*: calculated for $[M]^+$, 289.2122; observed, 289.1997.

H_2N-Lys(me3)-OH (**9**)

Compound **8** (128 mg, 0.31 mmol) was dissolved in DCM/TFA (1/1, 6 mL). The resulting yellow solution was stirred at room temperature for 3 h. The solvent was removed *in vacuo* to afford compound **9** as a white powder (125 mg, 97% yield). ^{1}H NMR (D_2O, 400 MHz) δ (ppm): 3.91 (t, *J* = 6.4 Hz, 1H), 3.17 (t, *J* = 8.0 Hz, 2H), 2.92 (s, 9H), 1.87–1.66 (m, 4H), 1.39–1.28 (m, 2H). MS (ESI+) *m/z*: calculated for $[M]^+$, 189.1598; observed, 189.1859.

Fmoc-Lys(me3)-OH

Compound 9 (333 mg, 0.80 mmol) was dissolved in 1,4-dioxan/10% Na_2CO_3 aq. (1/1, 20 mL) followed by the addition of 9-fluorenylmethyl chloroformate (228 mg, 0.88 mmol), and this mixture was stirred at room temperature for 8 h. The progress of the reaction was checked by silica TLC with 5% H_2O in MeOH as the mobile phase. After the completion of the reaction, the mixture was gently acidified with 2N HCl. The solvent was removed and the residue was purified by silica gel flash column chromatography (MeOH/DCM = 7/3) to give Fmoc-Lys(me3)-OH as a white powder (246 mg, 69% yield). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 12.72 (br, 1H), 7.92–7.86 (m, 2H), 7.75–7.64 (m, 3H), 4.32–4.21 (m, 3H), 4.01–3.95 (m, 1H), 3.33–3.29 (m, 2H), 3.07 (s, 9H), 1.78–1.66 (m, 4H), 1.38-1.23 (m, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 173.70, 156.19, 143.85, 140.74, 127.67, 127.10, 125.29, 120.16, 65.60, 64.94, 53.61, 52.07, 46.64, 30.16, 22.57, 21.67. HRMS (FAB+) *m/z*: calculated for $[M]^+$, 411.2225; observed, 411.2294.

Synthesis of DP and K4(Ac)-CCB

For the synthesis of the peptide acids **11** and **14**, the attachment of the first amino acid with 2-chlorotrityl chloride resin was performed by reacting Fmoc-Lys(me3)-OH (89 mg, 0.2 mmol, 2 eq.) and DIEA (139 μL, 0.8 mmol, 8 eq.) with the resin in DMF for 6 h. Both peptide chains were constructed using Fmoc solid-phase chemistry at room temperature. The peptide chains were assembled by activating Fmoc-amino acid (0.4 mmol, 4 eq.) with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 0.4 mmol, 4 eq.), HOBt·H_2O (0.4

mmol, 4 eq.) and N-methylmorpholine (NMM; 0.6 mmol, 6 eq.) in DMF and by reacting the activated amino acid with resins. The Fmoc-amino acids used for the synthesis were as follows: Fmoc-Ala-OH (124 mg, 0.4 mmol), Fmoc-Arg(Pbf)-OH (260 mg, 0.4 mmol), Fmoc-Gln(Trt)-OH (244 mg, 0.4 mmol), Fmoc-Lys(Ac)-OH (164 mg, 0.4 mmol), Fmoc-Lys(Boc)-OH (187 mg, 0.4 mmol), Fmoc-Lys(me3)-OH (89 mg, 0.2 mmol), and Fmoc-Thr(tBu)-OH (159 mg, 0.4 mmol). Completion of the coupling reactions was confirmed using the ninhydrin test. The Fmoc group was removed by using 20% piperidine in DMF. The N-terminal amines of the peptides were acetylated with acetic anhydride (94 μL, 10 eq.) and DIEA (175 μL, 10 eq.) in dichloromethane to afford the peptide-resins **10** and **13** (see Scheme S3 and S4). Each resin was mixed with 0.5% TFA in DCM for 3 h to yield the peptide acids **11** (84 mg, 40 μmol) and **14** (71 mg, 34 μmol), which contained the protecting groups in their side chains. The peptide acid **11** (32 mg, 15 μmol) was coupled with compound **6** (4.9 mg, 12 μmol) using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (WSCD·HCl; 2.9 mg, 15 μmol), HOBt (2.3 mg, 15 μmol), and NMM (4.9 μl, 45 μmol) in DMF for 4 h. The progress of the reaction was checked by HPLC, and, after the completion of the reaction, the crude product was purified by HPLC to afford peptide **12** (23 mg, 65% yield). Peptide acid **14** (25 mg, 12 μmol) was also coupled with compound **6** (5.6 mg, 14 μmol) using WSCD·HCl (3.0 mg, 16 μmol), HOBt (2.4 mg, 16 μmol), and NMM (5.1 μl, 48 μmol) in DMF for 4 h, and purified by HPLC to give peptide **15** (13 mg, 46% yield). Peptides **12** (20 mg, 8.4 μmol) and **15** (10 mg, 4.1 μmol) were treated with a mixture of TFA/TIS (98/2) for 2 h, and were purified by HPLC to afford DP (1.7 mg, 11% yield) and K4(Ac)-CCB (5.3 mg, 71% yield). Each peptide was assessed by HRMS (FAB+). DP: calculated for $[M]^+$, 1416.7958; observed, 1416.7928; K4(Ac)-CCB: calculated for $[M]^+$, 1458.8063; observed, 1458.8090.

Protein expression and purification

E. coli, BL21 (DE3) (Novagen), was transformed with pGEX-4T-1-SIRT1 and grown to an OD_{600} of 0.6–0.8 in Luria-Bertani medium containing 100 μg/mL ampicillin at 37°C. At this point, the temperature was lowered to 25°C and the protein was expressed overnight by adding isopropyl-β-d-thiogalactopyranoside (IPTG) to the medium with a final concentration of 100 μM. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C; resuspended in sonication buffer (5.8 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 10 mM DTT, pH 7.3); and lysed by sonication. The supernatant of the cell lysate was obtained by centrifugation at 15000 rpm for 15 min at 4°C and passed through a column packed with GST-Bind resin. Then, the resin was washed with 5.8 mM sodium phosphate buffer (pH 7.3) containing 137 mM NaCl and 2.7 mM KCl, and eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione in accordance with the manufacturer's protocol. Finally, the buffer exchange was conducted by ultracentrifugation (VIVASPIN 6 PES, molecular weight cut-off 30,000 Da, Sartorius Stedim Biotech, Bohemia, NY) to keep the purified protein in the assay buffer (25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% Tween-20, 20% glycerol). The purity and size of the protein were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of cell lysate

HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2. The cells suspended in the assay buffer (20 mM HEPES (pH 8.0)) were frozen and thawed three times for cell lysis. The cell lysate was prepared from the supernant by centrifugation at 15,000 rpm for 30 min (4°C).

HPLC analyses

HPLC separation was carried out with an increasing ratio of B buffer (0.1% HCOOH in acetonitrile) to A buffer (0.1% HCOOH in H_2O). All the samples were evaluated with a linear gradient of 2–32% B buffer (0.1% HCOOH in acetonitrile) over 30 min.

For the evaluation of transesterification, DP (3 μM) or K4(Ac)-CCB (3 μM) was incubated in 20 mM HEPES buffer (pH 8.0) containing 1.8 mM Tris-HCl, 300 μM NAD^+, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% glycerol at 37ºC. For the examination of the stability of K4(Ac)-CCB in cell lysate, K4(Ac)-CCB (3 μM) was incubated in cell lysate collected from 1.85×10^4 cells at 37ºC. Aliquots were taken at 0, 15, 30, 60, 90, and 120 min, mixed with TFA (final concentration: 0.2%) to stop the reaction, and analyzed by HPLC. The enzyme reaction was conducted by incubating K4(Ac)-CCB (3 μM) with Sirt1 (100 nM) in 20 mM HEPES buffer (pH 8.0) containing 1.8 mM Tris-HCl, 300 μM NAD^+, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% glycerol at 37ºC. Aliquots were treated to stop the reaction and analyzed by HPLC in the same procedure described above. For the inhibition assay, K4(Ac)-CCB (3 μM) was incubated with Sirt1 (100 nM) in the presence of EX527 (150 μM) in 20 mM HEPES buffer (pH 8.0) containing 1.8 mM Tris-HCl, 300 μM NAD^+, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% glycerol at 37ºC. Fluorescence measurements were conducted periodically for 2 h, and the reaction mixture was then mixed with TFA to stop the reaction and analyzed by HPLC.

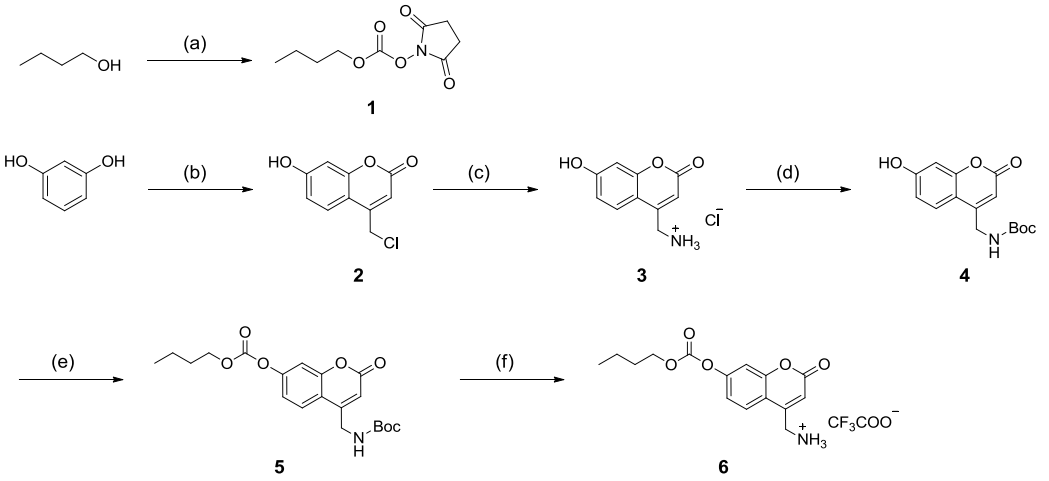
MALDI-LIFT-TOF/TOF MS measurement

DP (0.3 mg) was dissolved in 20 mM HEPES buffer (pH 8.0) containing 150 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$, then incubated at 37ºC for 4 h (final volume: 100 μL). K4(Ac)-CCB (100 μM) was incubated with Sirt1 (500 nM) in 20 mM HEPES buffer (pH 8.0) containing 1.8 mM Tris-HCl, 300 μM NAD^+, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% glycerol at 37ºC for 4 h (final volume: 50 μL) Both of the reaction products were isolated by HPLC and was dissolved in matrix solution, which contains 5 mg/mL α-cyano-4-hydroxycinnamic acid in H_2O/MeCN (3/1) containing 0.05% TFA. MALDI-LIFT-TOF/TOF MS measurement of this sample was made according to the manufacturer's protocol.

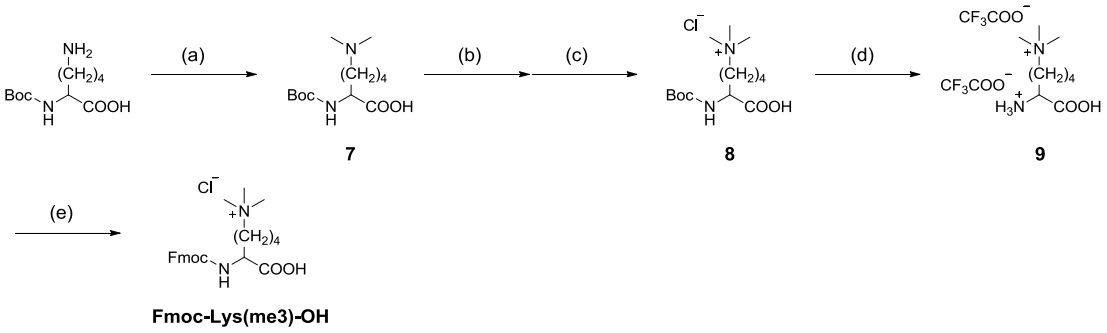
Fluorescence spectroscopy

Fluorescence spectra of K4(Ac)-CCB were recorded with excitation at 371 nm. Spectra were collected every 5 min using a sample containing K4(Ac)-CCB (3 μM) with or without Sirt1 (100 nM) in 20 mM HEPES buffer (pH 8.0) containing 1.8 mM Tris-HCl, 300 μM NAD^+, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% glycerol at 37ºC. For the inhibition assay, EX-527 was added to the reaction mixture at a final concentration of 150 μM.

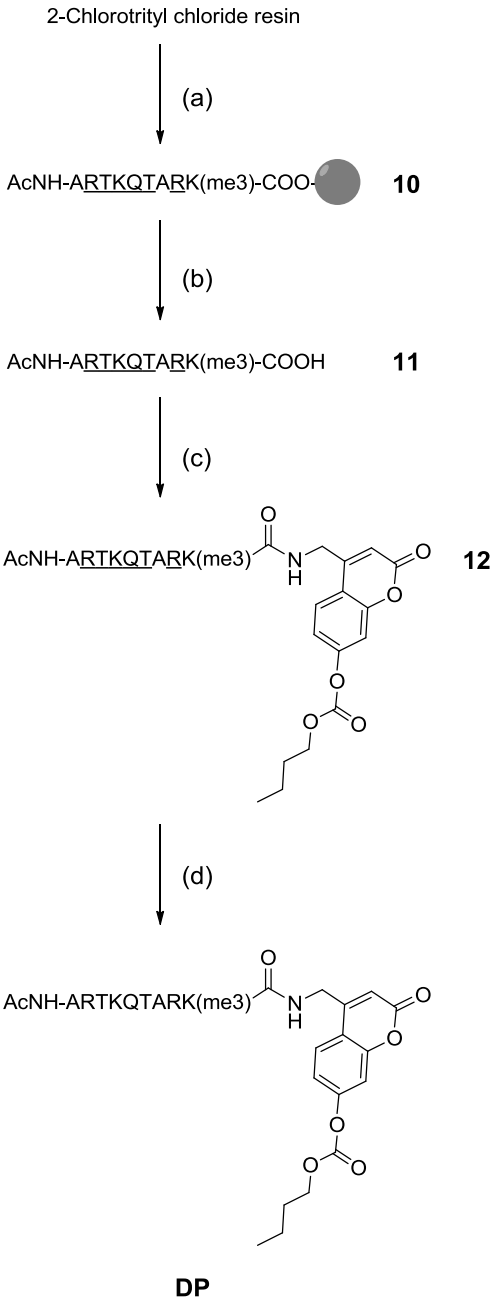
2. Supplementary Schemes



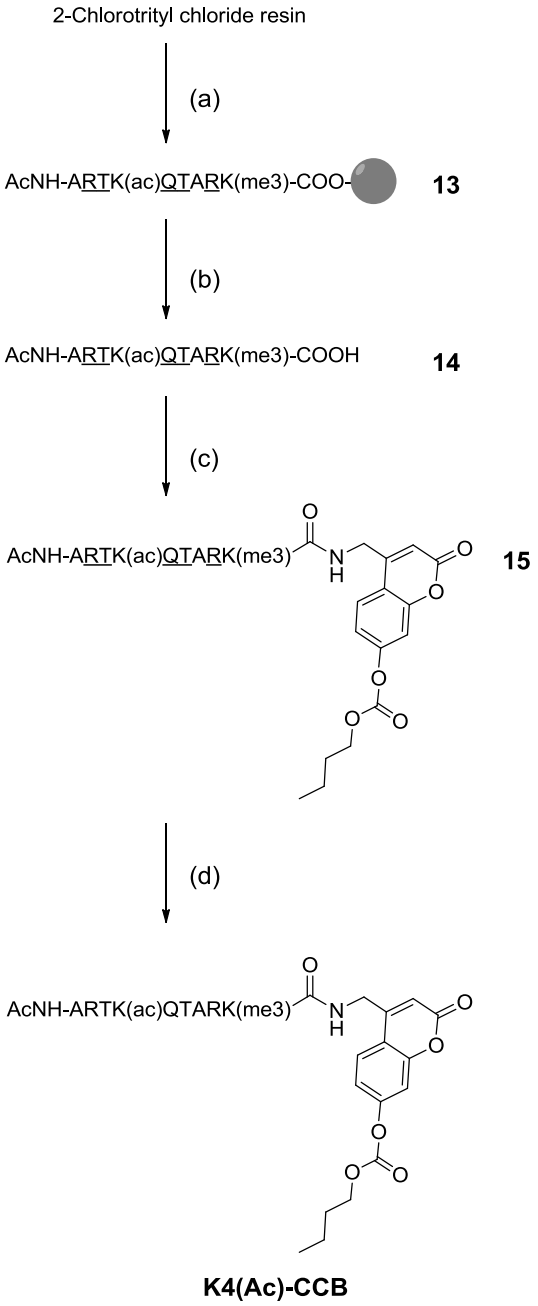
Scheme S1. Synthesis of the coumarin derivative (Compound **6**). (a) N,N'-disuccinimidyl carbonate, TEA, MeCN, RT; (b) Ethyl 4-chloroacetoacetate, H_2SO_4 aq., 0°C to RT; (c) 28% ammonium hydroxide, 50°C; (d) $(Boc)_2O$, TEA, DMF/MeOH, RT; (e) **1**, NaH, DMF, RT; (f) DCM/TFA, RT.



Scheme S2. Synthesis of Fmoc-Lys(me3)-OH. (a) HCHO aq, $NaBH_3CN$, EtOH, 0°C to RT; (b) Iodomethane, DIEA, MeOH/MeCN, RT; (c) MeOH/NaOH aq., RT; (d) DCM/TFA, RT; (e) Fmoc-Cl, 1,4-dioxane/Na_2CO_3 aq., RT.



Scheme S3. Synthesis of DP. Amino acids with protected side chains are underlined. (a) Fmoc solid phase peptide synthesis; (b) 0.5% TFA in DCM, RT; (c) **6**, WSCD·HCl, HOBt, NMM, DMF, RT; (d) 2% TIS in TFA, RT.



Scheme S4. Synthesis of K4(Ac)-CCB. Amino acids with protected side chains are underlined. (a) Fmoc solid phase peptide synthesis; (b) 0.5% TFA in DCM, RT; (c) **6**, WSCD·HCl, HOBt, NMM, DMF, RT; (d) 2% TIS in TFA, RT.

3. Supplementary Figures

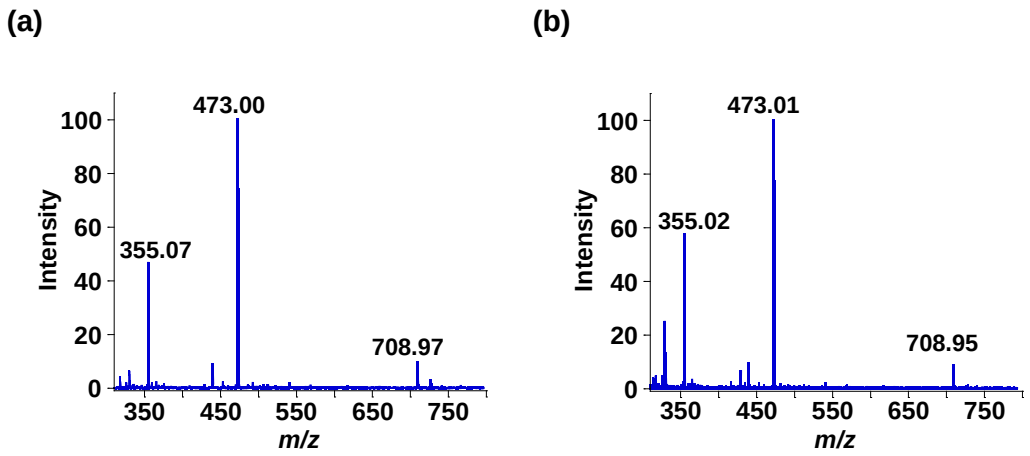


Figure S1. ESI mass spectra of HPLC fractions marked by asterisks 1 (a) and 2 (b), in Figure 2. Calculated mass values for $[M+H]^{2+}$, $[M+2H]^{3+}$, and $[M+3H]^{4+}$ of DP (TP) are 708.90, 472.94, and 354.95, respectively. It is noted that DP and TP have the same mass value.

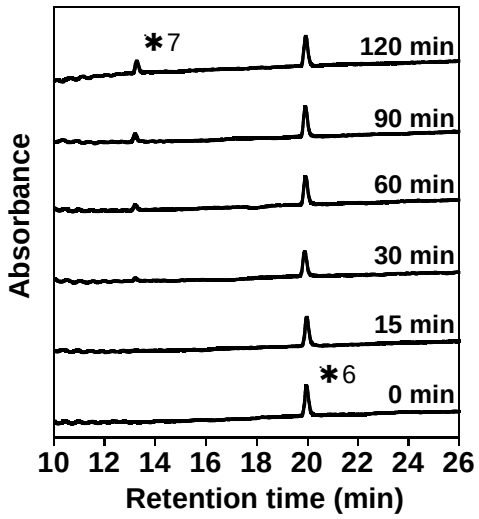


Figure S2. Reversed-phase HPLC analysis of K4(Ac)-CCB in 20 mM HEPES buffer (pH 8.0). Asterisk 6 represents K4(Ac)-CCB. The fraction represented by asterisk 7 was analyzed by ESI-MS (See Figure S3). The absorbance at 305 nm was monitored.

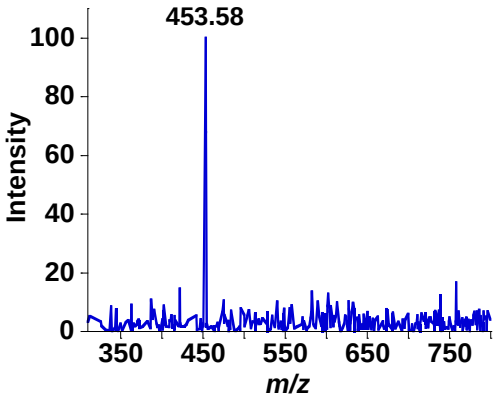


Figure S3. ESI mass spectra of the HPLC fraction marked by asterisk 7 in Figure S2. The calculated mass value for $[M+2H]^{3+}$ of the hydrolysis product of K4(Ac)-CCB is 453.59. ESI mass measurement was conducted using an eluent compound obtained from the peak at the same retention time as asterisk 7 in Figure S2 in the HPLC analysis using 20 μM K4(Ac)-CCB.

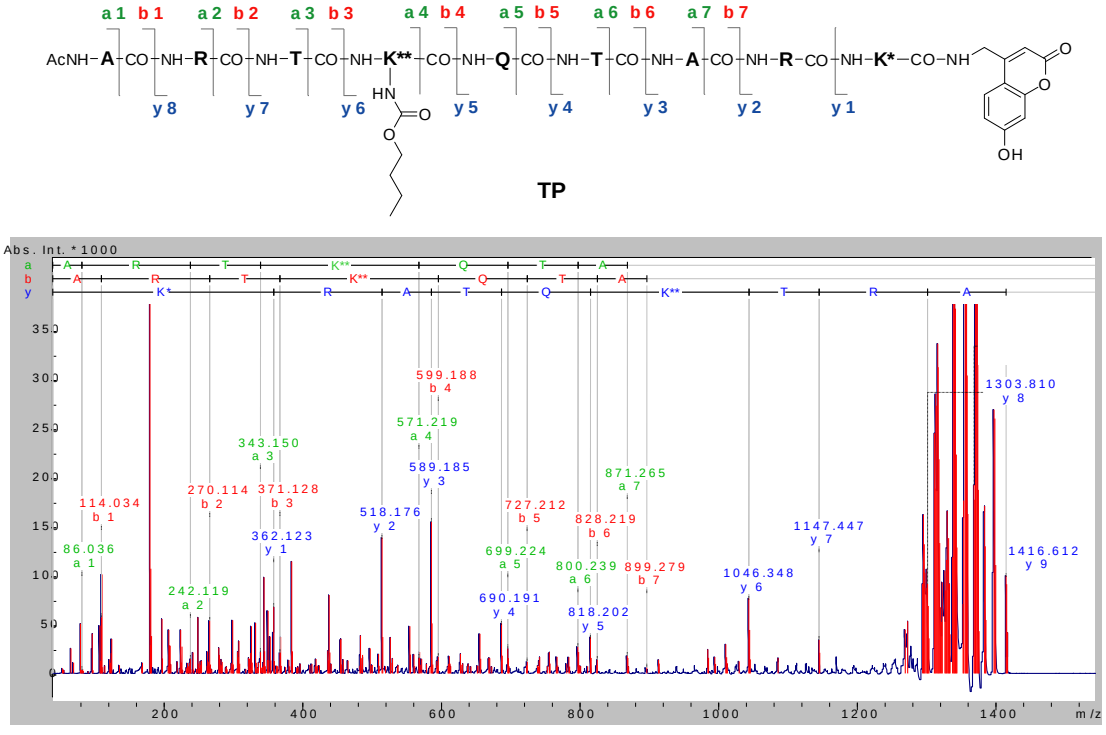


Figure S4. MALDI-LIFT-TOF/TOF MS analysis of compound obtained by the transesterification reaction of DP. K* represents trimethylated lysine at the ninth position with a coumarin-derivative at the C-terminal. K** represents acylated lysine at the fourth position. y 9 indicates the parent mass value.

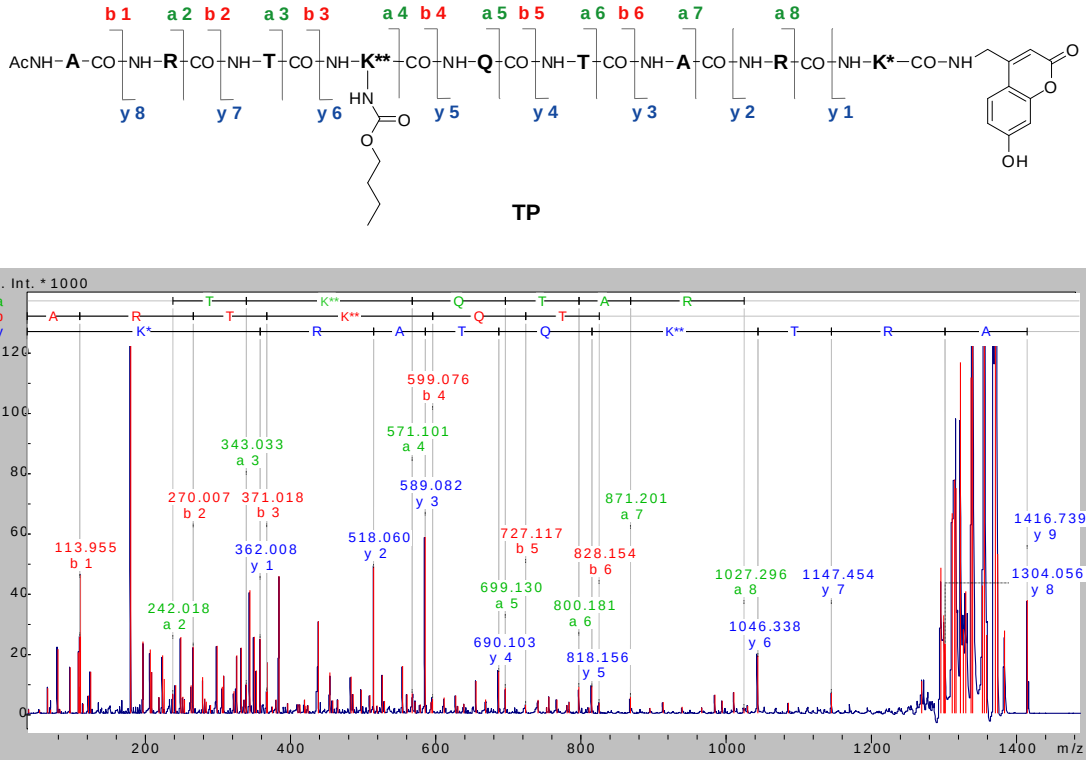


Figure S5. MALDI-LIFT-TOF/TOF MS analysis of compound obtained by the enzymatic reaction of K4(Ac)-CCB with Sirt1. K* represents trimethylated lysine at the ninth position with a coumarin-derivative at the C-terminal. K** represents acylated lysine at the fourth position. y 9 indicates the parent mass value.

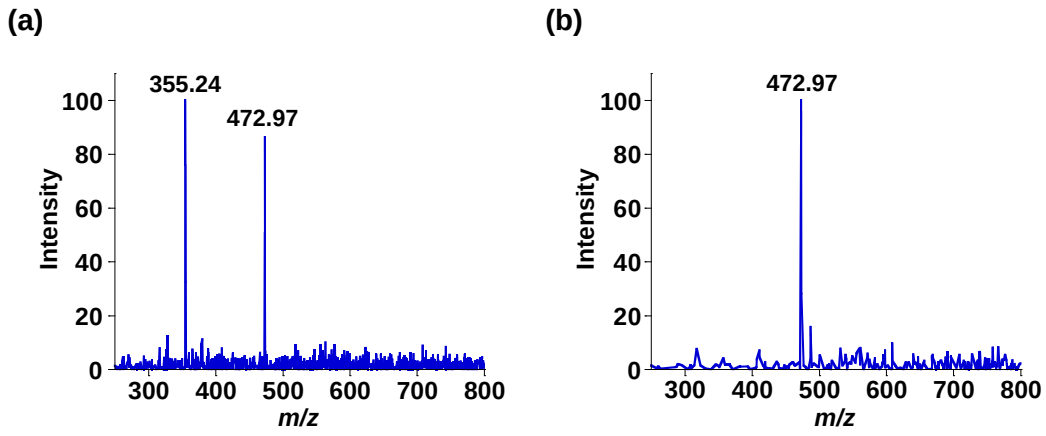


Figure S6. ESI mass spectra of the HPLC fractions marked by asterisks 4 (a) and asterisk 5 (b), in Figure 3. Calculated mass values for $[M+2H]^{3+}$ and $[M+3H]^{4+}$ of DP (TP) are 472.94 and 354.95, respectively.

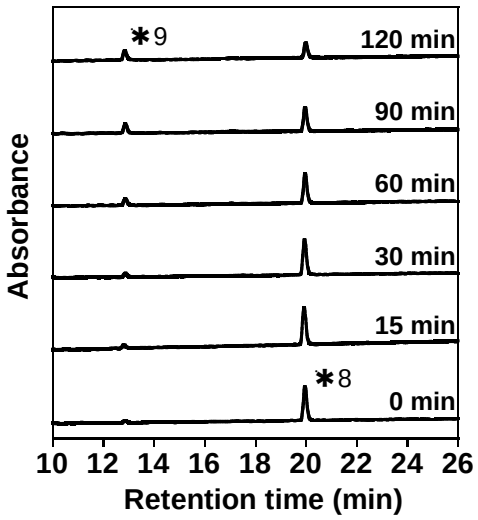


Figure S7. Reversed-phase HPLC analysis of K4(Ac)-CCB in cell lysate (pH 8.0). Asterisk 8 and 9 represent K4(Ac)-CCB and the hydrolysis product of K4(Ac)-CCB, respectively. The hydrolysis rate is estimated to be 0.27% / min. The absorbance at 305 nm was monitored.

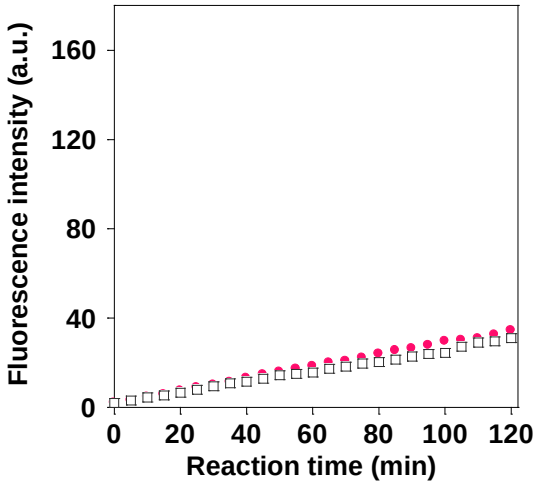


Figure S8. Time course of fluorescence intensity of K4(Ac)-CCB in the presence (circles) of heat-denatured Sirt1 or absence (squares) of Sirt1. Fluorescence measurement was made at 37ºC with the excitation and emission wavelengths of 371 and 466 nm, respectively.

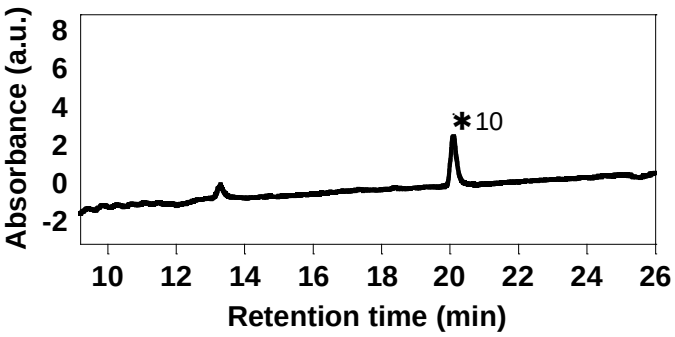


Figure S9. Reversed-phase HPLC analysis of the enzyme reaction mixture in the presence of EX-527. Asterisk 10 represents K4(Ac)-CCB.