Supporting Information

"Fluorogenic Probes for Detecting Deacylase and Demethylase Activity Towards Post-Translationally-Modified Lysine Residues"

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1. Materials and Methods

Materials and instruments

General chemicals were supplied by Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemicals (Osaka, Japan), Sigma-Aldrich Chemical Co. (St. Louis, MO) and the Peptide Institute, Inc. (Osaka, Japan) and used without further purification. Compounds, 2-(7-((*tert*-butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)acetic acid and (7-((*tert*-butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)methanaminium 2,2,2-trifluoroacetate were synthesized as reported previously.[S1] The plasmid encoding human JMJD2E was kindly provided by Prof. Christopher J. Schofield (University of Oxford).

^{1}H and ^{13}C NMR spectra were recorded on a BRUKER AscendTM 500 instrument at 500 MHz for and 125 MHz respectively, using tetramethylsilane as an internal standard. ESI-time-of-flight (TOF) MS was performed on a Waters LCT-Premier XE. MALDI-LIFT-TOF/TOF MS using a Bruker UltraFlexIII mass spectrometer. High-pressure liquid chromatography (HPLC) purification/analyses experiments were performed using an HPLC system comprised of a pump (PU-2080, JASCO), detectors (MD-2010 and FP-2020, JASCO) and Inertsil ODS-3 column (4.6 or 10.0 mm × 250 mm,

GL-Science, Inc. Torrance, CA). Time-course HPLC analysis was conducted using an HPLC system comprised of an auto sampler (5210, HITACHI), a pump (5110, HITACHI) and UV detectors (5430 and 5440, HITACHI). Fluorescence measurements were conducted using a Hitachi F7000 spectrometer with a photomultiplier voltage of 700V or a PerkinElmer ARVO MX 1420 multilabel counter.

Syntheses of compounds

Fmoc-Lys(Mal-O^{t}Bu)-OH (1)

Dicyclohexylcarbodiimide (DCC) (2.06 g, 10.0 mmol) in anhydrous THF (20 mL) was added to a stirred solution of mono-*tert*-butyl malonate (1.60 g, 10.0 mmol) and *N*-hydroxysuccinimide (NHS) (1.15 g, 10.0 mmol) in anhydrous THF (10 mL) at 0 ^{o}C and the resultant mixture stirred for 16 h . The reaction mixture was filtered and the filtrate evaporated *in vacuo* to afford a crude residue that was added to a stirred solution of Fmoc-Lys-OH (921 mg, 2.5 mmol) and *N,N*-diisopropylethylamine (DIEA) (446 uL, 2.5 mmol) in anhydrous CH_2Cl_2 (10.0 mL) at rt, with the resultant mixture then stirred for 17 h. The solvent was then removed *in vacuo* to afford a crude residue that was purified by silica chromatography using CH_2Cl_2/methanol (98:2) as an eluent to afford the title compound **1** (562 mg, 44% yield). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 7.99 (t, J = 5.5 Hz, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.72 (d, J = 7.5 Hz, 2H), 7.43-7.40 (m, 3H), 7.33 (t, J = 7.5 Hz, 2H), 4.28-4.21 (m, 3H), 3.89-3.84 (m, 1H, e), 3.07-3.01 (m, 4H), 1.74-1.55 (m, 2H), 1.44-1.35 (m, 13H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 174.0, 167.3, 165.2, 156.2, 143.8, 140.7, 127.7, 127.1, 125.3, 120.1, 80.4, 65.6, 53.7, 46.7, 43.7, 38.4, 30.4, 28.5, 27.7, 23.0. HRMS (Fab$^+$) m/z: calcd for $[M+H]^+$ 511.2439, Found for 511.2443.

Fmoc-Lys(Suc-O^{t}Bu)-OH (2)

DCC (618 mg, 3.0 mmol) in anhydrous THF (10.0 mL) was added to a stirred solution of mono-*tert*-butyl succinate (522 mg, 3 mmol) and NHS (342 mg, 3.0 mmol) in anhydrous THF (10.0 mL) at 0 ^{o}C and the resultant mixture stirred for 5 h. The reaction mixture was filtered and the filtrate evaporated *in vacuo* to afford a crude residue that was added to a stirred solution of Fmoc-Lys-OH (552 mg, 1.5 mmol) and DIEA (267 μL, 1.5 mmol) in anhydrous DCM (10.0 mL) at rt, with the resultant mixture then stirred for 17 h. The solvent was then removed *in vacuo* to afford a crude residue that was purified by silica gel chromatography using CH_2Cl_2/methanol (98:2) to afford the title compound **2** (32 mg, 28% yield). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 7.89 (d, J = 7.5 Hz, 2H), 7.81 (t, J = 5.5 Hz, 1H), 7.72 (d, J =7.5 Hz, 2H), 7.54 (br, 1H), 7.42 (t, J =7.5Hz, 3H), 7.33 (t, J=7.5 Hz, 2H,), 4.28-4.21 (m, 3H), 3.90-3.85 (m, 1H), 3.03-3.00 (m, 2H), 2.38 (t, J = 7.0 Hz, 2H), 2.27 (t, J = 7.0 Hz, 2H), 1.72-1.55 (m, 2H), 1.39-1.28 (m, 13H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 174.4, 172.1, 171.0, 156.6, 144.3, 144.3, 144.2, 128.1, 127.5, 125.8, 120.6, 80.0, 66.1, 54.2, 47.1, 38.7, 30.9, 30.8, 30.6, 29.2, 23.5. HRMS (Fab$^+$) m/z: calcd for $[M+H]^+$ 525.2595, Found for 525.2607.

Fmoc-solid phase synthesis of peptides or probes.

Peptides **P-N(H)Mal**, **P-N(H)Suc**, **P-NHMe** and **P-NMe$_2$** were synthesized using solid-phase peptide coupling reactions using Rink-amide MBHA resin LL [**(P-N(H)Mal/P-N(H)Suc**: 0.1 mmol; **P-N(H)Me/P-NMe$_2$)**: 0.025 mmol]. Solid phase peptides were assembled by reacting the unprotected amino group of a resin bound peptide with a solution of an activated Fmoc-amino acid. Each Fmoc-amino acid [**P-N(H)Mal/ P-N(H)Suc)**: 0.4 mmol, 4eq.; **P-N(H)Me/P-NMe$_2$**: 0.1 mmol, 4 eq.] was activated through reaction with PyBOP [**P-N(H)Mal/P-N(H)Suc**: 0.4 mmol, 4eq.; **P-N(H)Me/P-NMe$_2$**: 0.1 mmol, 4 eq.], HOBt·H_2O [**P-N(H)Mal/P-N(H)Suc**: 0.4 mmol, 4eq.; **P-N(H)Me/P-NMe$_2$**: 0.1 mmol, 4 eq.] and NMM [**P-N(H)Mal/P-N(H)Suc**: 0.6 mmol, 6 eq.; **P-N(H)Me/P-NMe$_2$**: 0.15 mmol, 6 eq.)] in DMF. The Fmoc-amino acids used in these peptide syntheses were: Fmoc-Glu-OAll, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Lys(Ac)-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Lys(Mal/Suc, tBu)-OH, Fmoc-Lys(Me, ε-N-Boc)-OH, Fmoc-Lys(NMe_2)-OH·HCl, and Fmoc-Arg(Pbf)-OH. Completion of peptide coupling reactions were confirmed using a ninhydrin test. Fmoc groups were removed through treatment of Fmoc peptides with 20% piperidine in DMF.

P-N(H)Mal and P-N(H)(Suc)

Treatment of the *N*-terminal amino groups of peptide resins **3**/**4** with 2-(7-((*tert*-butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)acetic acid (Scheme S1) (64.0 mg, 2 eq.), PyBOP (104 mg, 2 eq.) and NMM (33.0 μL, 2eq.) in DMF for 16 h gave peptide resins **5**/**6**. Deprotection of peptide resins **5**/**6** was achieved via their treatment with a mixture of TFA/TIS (95:5, v/v) for 3 h, which gave crude peptides that were purified by HPLC to afford **P-N(H)Mal** and **P-N(H)Suc** whose purities were assessed by HPLC and ESI-TOF MS. **P-N(H)Mal** : calculated for $[M-H]^-$, 990.42; observed, 990.20; **P-N(H)Suc**: calculated for $[M-H]^-$, 1004.43; observed, 1004.23.

P-N(H)Me and P-NMe$_2$

The *N*-terminal amino residues of peptide resins **7** and **8** were acetylated with acetic anhydride (24 μL, 0.25 mmol) and DIEA (44 μL, 0.25 mmol) in dichloromethane to afford peptide resins **9** and **10** (Scheme S2). Selective removal of the allyl group at the C-terminus was achieved by mixing each resin with tetrakis(triphenylphosphine)palladium(0) [($Pd(PPh_3)_4$; 86.6 mg, 0.075 mmol in CH_3Cl/AcOH/NMM (37:2:1, 5 mL) for 4 h under anaerobic conditions to afford peptide resins **11** and **12** (Scheme S1). (7-((*tert*-Butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)methanaminium 2,2,2-trifluoroacetate[S1] (50.7 mg, 0.125 mmol) was coupled with peptide-resins **11** and **12** using PyBOP (65.0 mg, 0.125 mmol) and NMM (20 μL, 0.2 mmol) in DMF for 6 h to afford peptide resins **13** and **14**. The final deprotection steps were carried out using peptides **11**/**12** with a TFA/TIS mixture (95:5, v/v) for 2 h. The crude peptides were precipitated using cold diethylether, lyophilized and purified by HPLC to afford **P-N(H)Me** (4.1 mg, 10% yield) and **P-NMe$_2$** (9.3 mg, 22% yield). The fidelity of the purified peptides and probes were confirmed by HPLC and ESI-TOF MS. **P-N(H)Me**: calculated for $[M+2H]^{2+}$, 736.38; observed, 736.37; **P-NMe$_2$**: calculated for $[M+2H]^{2+}$, 743.39; observed, 743.39.

Preparation of plasmid and recombinant proteins

E. coli, BL21 (DE3) (Novagen), was transformed with pNIC28-Bsa4-JMJD2E and grown to an OD_{600} of 0.6–0.8 in Luria-Bertani medium containing 25 μg/mL Kanamycin at 37 °C. At this point, the temperature was lowered to 25 °C and the protein expressed for 12 h by adding isopropyl-β-d-thiogalactopyranoside (IPTG) to the medium at a final concentration of 100 μM. The cells were harvested by centrifugation at 5000 rpm for 5 min at 4 °C, resuspended in the sonication buffer (50 mM Na_2HPO_4, 300 mM NaCl, 1 mM DTT, pH 8.0) 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 cOmplete His-Tag Purification Resin (Roche). The resin was then washed with 50 mM Na_2HPO_4 buffer (pH 8.0) containing 300 mM NaCl, 1 mM DTT, and 5 mM imidazole, and then eluted with 50 mM Na_2HPO_4 buffer (pH 8.0) containing 300 mM NaCl, 1 mM DTT, and 250 mM imidazole according to the manufacturer's protocol. The protein was then applied to Superdex^{TM} 75 10/300 GL (GE Healthcare) equilibrated with 10 mM HEPES·NaOH (pH 8.0) containing 500 mM NaCl, 1 mM DTT, 5% glycerol and eluted using a NGC^{TM} Chromatography System (Bio-Rad). The purity of the protein was then checked by SDS-PAGE analysis.

HPLC analyses of enzyme and transesterification reactions

Enzymatic deacylation reactions were analysed by incubating **P-N(H)Mal** (5 μM) or **P-N(H)Suc** (5 μM) with Sirt5 (100 nM) in analysis buffer 1 (20 mM HEPES, 300 μM NAD^+, 0.15% glycerol and 2.0% DMSO, pH 8.0) at 37 °C for 5, 10, 15, 20, 30 and 45 min. Transesterification reactions were evaluated by incubating **P-NHMe** (5 μM) in analysis buffer 2 (48 mM HEPES, 200 mM NaCl, 100 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM ascorbate, 1 μM 2-oxoglutarate (2-OG), 40 μM DTT, and 0.2% glycerol, pH 8.0) at 37 °C for 0, 15, 30, 60, 120, and 180 min. Demethylation reactions were analysed by incubating **P-NMe$_2$** (5 μM) with JMJD2E (5 μM) in analysis buffer 2 at 37 °C for 0, 15, 30, 60, 120, and 180 min. A relatively high concentration of JMJD2E was required for detection of the enzyme reactions, as reported previously.[S2] All the reactions were stopped by adding the mixture to TFA (final concentration: 0.2 or 0.5%) and then analysed by HPLC.

HPLC analyses were performed with an increasing ratio of buffer B (0.1% HCOOH in acetonitrile) to buffer A (0.1% HCOOH in H_2O). Enzyme reactions of **P-N(H)Mal** and **P-N(H)Suc** were analysed using a linear gradient of 10–55% buffer B (0.1% HCOOH in acetonitrile) over 30 min. Enzyme and transesterification reactions of **P-NHMe** and **P-NMe$_2$** were analysed using 2–42% buffer B (0.1% HCOOH in acetonitrile) over 40 min. The absorption, excitation and emission wavelength for HPLC fluorescence monitoring were 305, 338 and 458 nm, respectively.

MALDI-LIFT-TOF/TOF MS measurement

P-N(H)Mal (100 μM) or **P-N(H)Suc** (100 μM) was incubated with Sirt5 (500 nM) in analysis buffer 1 at 37 ºC for 6 h. **P-NHMe** (20 μM) was incubated in analysis buffer 2 at 37 ºC for 6 h. **P-NMe$_2$** (20 μM) was reacted with JMJD2E (10 μM) in analysis buffer 2 at 37 ºC for 6 h. Each reaction was purified by HPLC and peaks mixed with a matrix solution, comprised of 5 mg/mL α-cyano-4-hydroxycinnamic acid in H_2O/MeCN (1/1) and 0.05% or 0.1% TFA. MALDI-LIFT-TOF/TOF MS measurements were conducted according to the manufacturer's protocol.

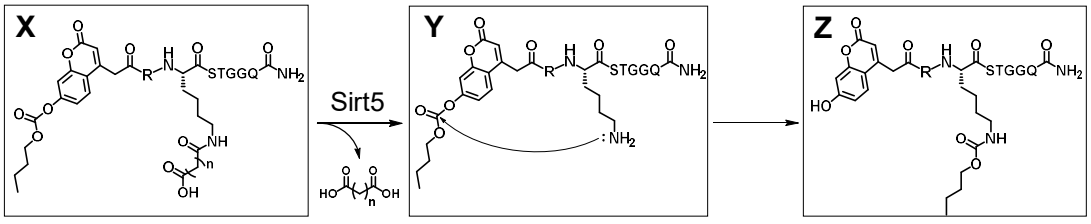
Fluorescence spectroscopy

P-N(H)Mal (5 μM) or **P-N(H)Suc** (5 μM) were incubated with or without Sirt5 (100 nM) in analysis buffer 1 at 37 °C. **P-NMe$_2$** (5 μM) was incubated with or without JMJD2E (5 μM) in analysis buffer 2 at 37 °C. Control reactions were carried out by substituting heat-inactivated enzymes in the assay. The fluorescence intensity of the probes was measured with an excitation wavelength of 371 nm and an emission wavelength of 466 nm.

Kinetic analyses of Sirt5-catalysed reactions

P-N(H)Mal or **P-N(H)Suc** (1.25, 2.5, 5, 7.5, 10, 15, 20 μM) was incubated with Sirt1 (100 nM) in analysis buffer 1 at 37 °C. Fluorescence intensities of the probes was recorded every 0.1 sec after the probes had been mixed with the enzyme. Excitation and emission wavelengths were 371 nm and 466 nm, respectively. The kinetic parameters for enzymatic reactions were obtained using a modified Michaelis-Menten equation.

Since fluorescence output is only obtained after the transesterification reaction has been triggered by enzymatic reactions, any delay in appearance of the fluorescence increase is considered using the following kinetic analysis.



In the above scheme, X, Y, Z and E represent the probes, deacylated intermediates, transferred products and Sirt5, respectively. The kinetic parameters, k_X, k_X', k_Y, and k_Z, represent rate constants for association of E and X, dissociation of EX, deacylation of X and transesterification of Y, respectively. The analysis carried out is based on the assumption that Michaelis-Menten conditions are operating in the *N*-deprotection reactions, where an excess of substrate is present in the early stage of enzyme reactions and the concentration of EX occurs in the steady state.

An equation that combines the transesterification kinetics and the Michaelis-Menten equation was derived as follows:

$$\frac{d[EX]}{dt} = k_X[E][X] - k_X'[EX] - k_Y[EX] = 0 \quad \text{(Eq-1)}$$

$$\longleftrightarrow [EX] = \frac{[E]_0[X]}{K_m + [X]} \quad \text{(Eq-2)} \qquad \left([E]_0 = [E] + [EX],\ K_m = \frac{k_X' + k_Y}{k_X} \right)$$

$$\longleftrightarrow \frac{d[Y]}{dt} + \frac{d[Z]}{dt} = \frac{k_Y[E]_0[X]}{K_m + [X]} \quad \text{(Eq-3)} \qquad \left(\frac{d[Y]}{dt} = k_Y[EX] - k_Z[Y] \right)$$

$$\longleftrightarrow \frac{1}{k_Z}\frac{d^2[Z]}{dt^2} + \frac{d[Z]}{dt} = \frac{k_Y[E]_0[X]}{K_m + [X]} \quad \text{(Eq-4)} \quad \left(\frac{d[Z]}{dt} = k_Z[Y] \longleftrightarrow \frac{d^2[Z]}{dt^2} = k_Z\frac{d[Y]}{dt} \right)$$

The differential equation (Eq-4) is solved to give the concentration of Z, which is represented as a function of *t*.

$$[Z] = Ae^{-k_Z t} + Bt - A \qquad A : \text{constant} \qquad \text{(Eq-5)}$$

$$B = \frac{k_Y[E]_0[X]}{K_m + [X]} \quad \text{(Eq-6)}$$

The concentration of Z can be obtained from fluorescence intensity values as follows:

$$[Z] = \frac{\Delta F_t}{\Delta F_{max}}[X]_0 \quad \text{(Eq-7)}$$

where ΔF_t, ΔF_{max} and $[X]_0$ represent observed change in fluorescence intensity of Z, maximum change in fluorescence intensity of Z, and total concentration of X.

Substitution of Eq-5 for Eg-7 gives the following equation:

$$F_t = F_0 + (F_{max} - F_0)(Ae^{-k_Z t} + Bt - A) / [X]_0 \quad \text{(Eq-8)}$$

where F_t, F_0 and F_{max} represent observed, initial and maximum fluorescence intensity values for Z, and k_z is 3.1×10^{-3} sec^{-1}, as previously reported.[S1]

The fluorescence intensity recorded after mixing various concentrations of the probes with Sirt5 was plotted against time and fitted to Eq-8 to give a value for *B*, which is represented by Eq-6. The values of *B* were then plotted against the concentration of the probe. By fitting the plots to Eq-6, k_y and K_m were obtained as the turnover number (k_{cat}) and the Michaelis constant for the enzyme reactions of the probes, respectively.

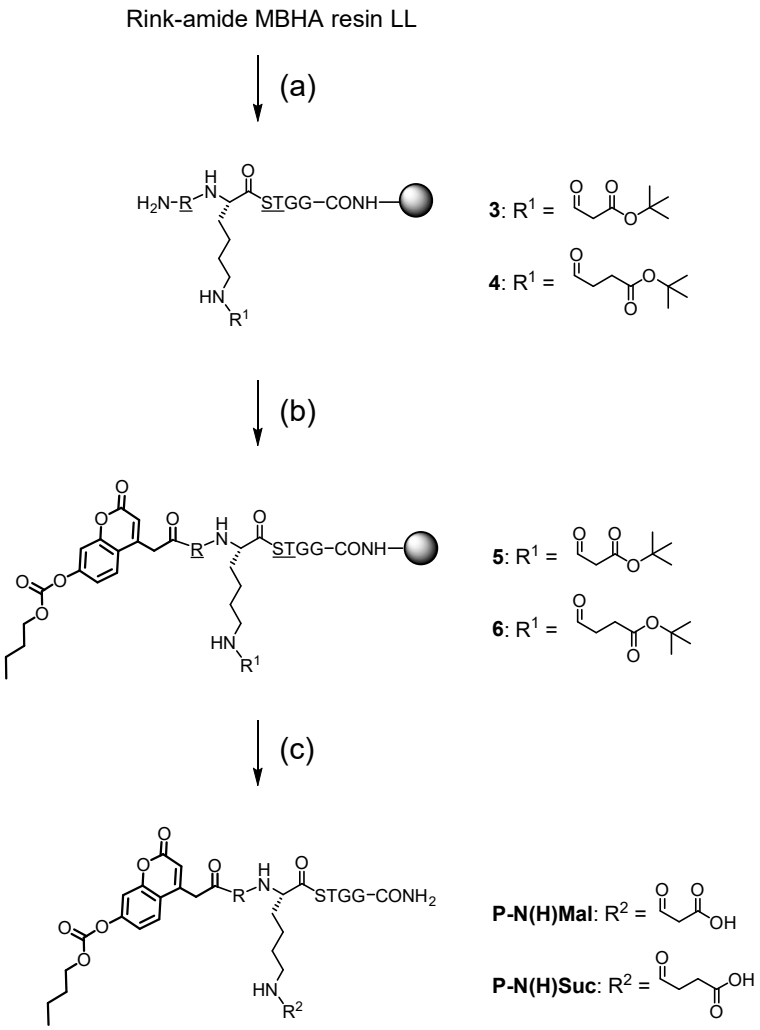
The carbonate ester of the probes underwent partial hydrolysis over extended periods of time, however, this background hydrolysis reaction did not affect the kinetic analyses, because fluorescence data was acquired in the first 2 min after mixing the probe with the enzyme, meaning there was insufficient time for significant probe hydrolysis to occur. The possibility that intermolecular cleavage

reactions were responsible for the assay data was not considered, because our previous report had shown that intermolecular reactions do not occur using these experimental conditions.[S1]

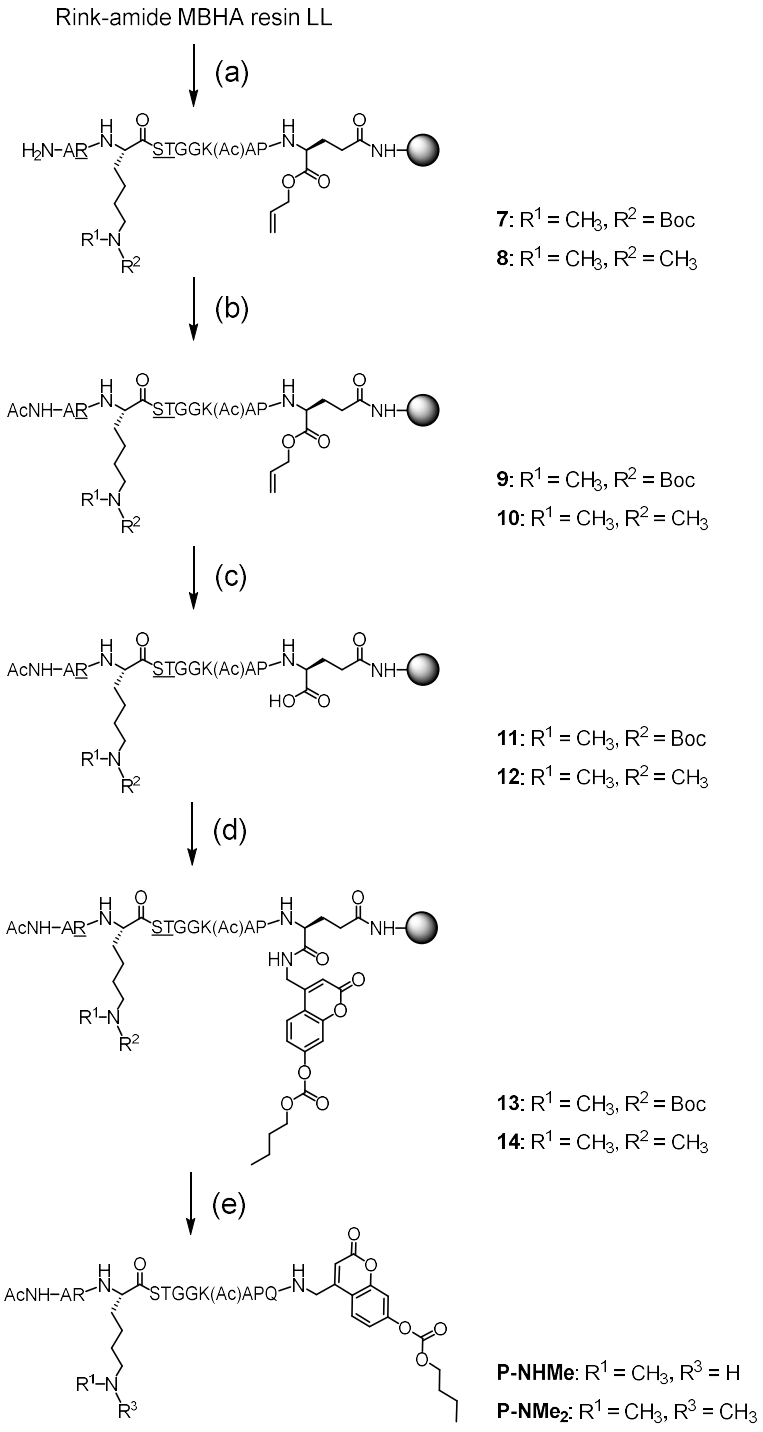
Plate reader analyses

The different enzyme selectivity profiles towards **P-N(H)Mal** and **P-N(H)Suc** were investigated using an ARVO MX 1420 multilabel counter. **P-N(H)Mal** and **P-N(H)Suc** were incubated with HDACs (HDAC1, HDAC2, HDAC3, HDAC6 and HDAC11) and Sirtuins (Sirt1, Sirt3, Sirt5 and Sirt6) at 37 °C for 30 min in 20 mM HEPES buffer (pH 8.0) and analysis buffer 1, respectively. The concentration of the probes and the enzymes were 5 μM and 100 nM, respectively. Carboxyesterases 1 and 2 (CES1 and CES2) (Sigma-Aldrich, #E0287 and #E0412) (100 nM) were reacted with each of **P-N(H)Mal** (5 μM), **P-N(H)Suc** (5 μM) and **P-NMe$_2$** (5 μM) at 37 °C for 30 min. Fluorescence detection of enzymatic activity in cell lysate was achieved using HeLa cells, which were washed with PBS three times, suspended in analysis buffer 1, and lysed using a freeze-thaw cycle (5 repeats) followed by centrifugation and extraction of the combined supernatants to afford a total concentration of 0.83 mg/mL of cellular proteins. The lysate was diluted by analysis buffer 1 to a concentration of 0.32 mg/mL proteins and then used for fluorescence detection. Sirt5 (100 nM, 3.2 μg/mL) was incubated with either **P-N(H)Mal** (5 μM) or **P-N(H)Suc** (5 μM) in the cell lysate at 37 °C for 20 min. JMJD2E (10 μM, 0.65 mg/mL) was mixed with the cell lysate and incubated with **P-NMe$_2$** (5 μM) in the absence or presence of *N*-EthylMaleimide (NEM) (1 mM) at 37 °C for 60 min. All fluorescence measurements were acquired using excitation and emission wavelengths of 350 ± 40 nm and 460 ± 40 nm, respectively.

2. Supplementary Schemes



Scheme S1. Synthesis of **P-N(H)Mal** and **P-N(H)Suc**. The underlined amino acids indicate that the side chains are protected. (a) Fmoc solid-phase peptide synthesis; (b) 2-(7-((*tert*-butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)acetic acid, PyBOP, NMM, DMF; (c) 5% TIS in TFA.



Scheme S2. Synthesis of **P-NHMe** and **P-NMe$_2$**. The underlined amino acids indicate that the side chains are protected. (a) Fmoc solid-phase peptide synthesis; (b) $Pd(PPh_3)_4$ in CH_3Cl/AcOH/NMM; (c) (7-((*tert*-butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl)methanaminium 2,2,2-trifluoroacetate, PyBOP, NMM, DMF; (d) 5% TIS in TFA.

3. Supplementary Tables

Table S1. *B* values obtained using various concentrations of probes.

Probe concentration (μM)	$B \times 10^2$ ($M s^{-1}$)	
	P-N(H)Mal	**P-N(H)Suc**
1.25	0.53 ± 0.12	0.91 ± 0.18
2.5	0.89 ± 0.14	1.35 ± 0.05
5	1.05 ± 0.15	1.81 ± 0.16
7.5	1.21 ± 0.08	1.87 ± 0.04
10	1.41 ± 0.06	2.00 ± 0.23
15	1.46 ± 0.05	2.16 ± 0.17
20	1.46 ± 0.13	2.12 ± 0.21

Table S2. Kinetic parameters for enzyme reactions of Sirt5 and probes.

	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} s^{-1}$)
P-N(H)Mal	1.7×10^{-1}	2.6×10^{-6}	6.5×10^{4}
P-N(H)Suc	2.4×10^{-1}	1.9×10^{-6}	1.3×10^{5}

4. Supplementary Figures

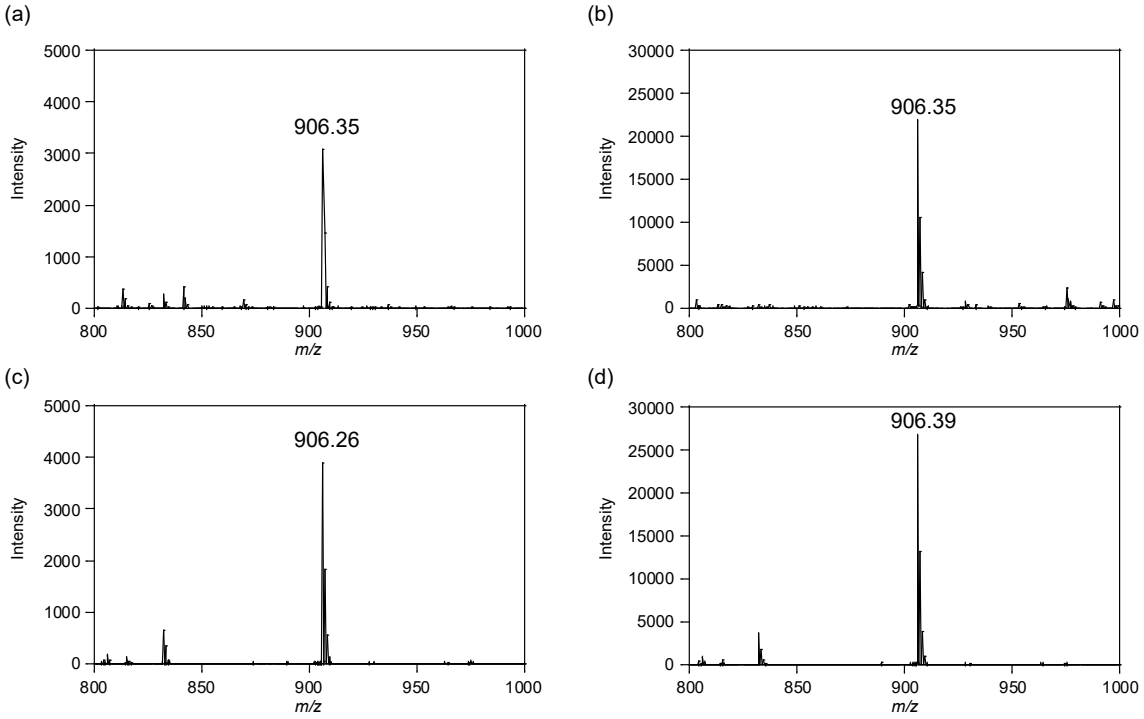


Figure S1. ESI-MS analyses of HPLC fractions of Sirt5 catalyzed reactions of **P-N(H)Mal** and **P-N(H)Suc**. (a, b) Mass spectra of fluorescent transferred products derived from (a) **P-N(H)Mal** and (b) **P-N(H)Suc**. (c, d) Mass spectra of deacylated intermediates (**P-NH$_2$**) derived from (c) **P-N(H)Mal** and (d) **P-N(H)Suc**. After **P-N(H)Mal** (5 μM) or **P-N(H)Suc** (5 μM) was reacted with Sirt5 (100 nM) at 37 °C for (a, b) 5 min; (c, d) 5 min. HPLC fractions at retention times of ~12 min and ~9.5 min were isolated as deacylated intermediates (**P-NH$_2$**) and transferred products (**P-NHCO$_2$Bu**), respectively. Identical *m/z* values for both the deacylated intermediates (**P-NH$_2$**) and the transferred products (**P-NHCO$_2$Bu**) of 906.43 were found.

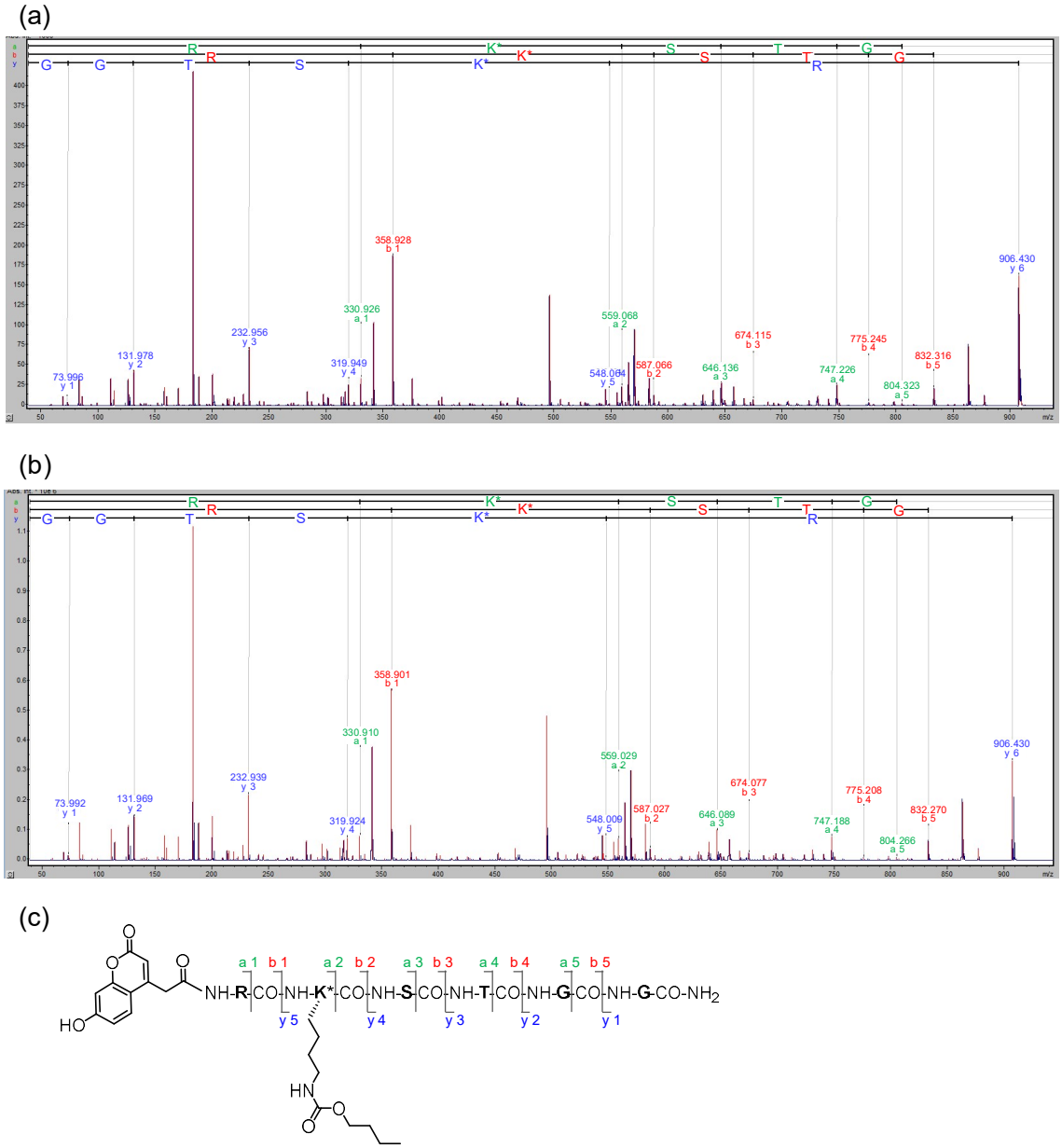


Figure S2. MALDI-TOF/TOF MS analyses of transferred products (**P-NH-CO$_2$Bu**) of Sirt5-reacted probes. (a, b) MS/MS spectra of transferred products derived from (a) **P-N(H)Mal** and (b) **P-N(H)Suc**. (c) Fragmentation patterns of transferred products. K* represents *N*-acylated lysine. The *m/z* value of y 6 is for the parent compound.

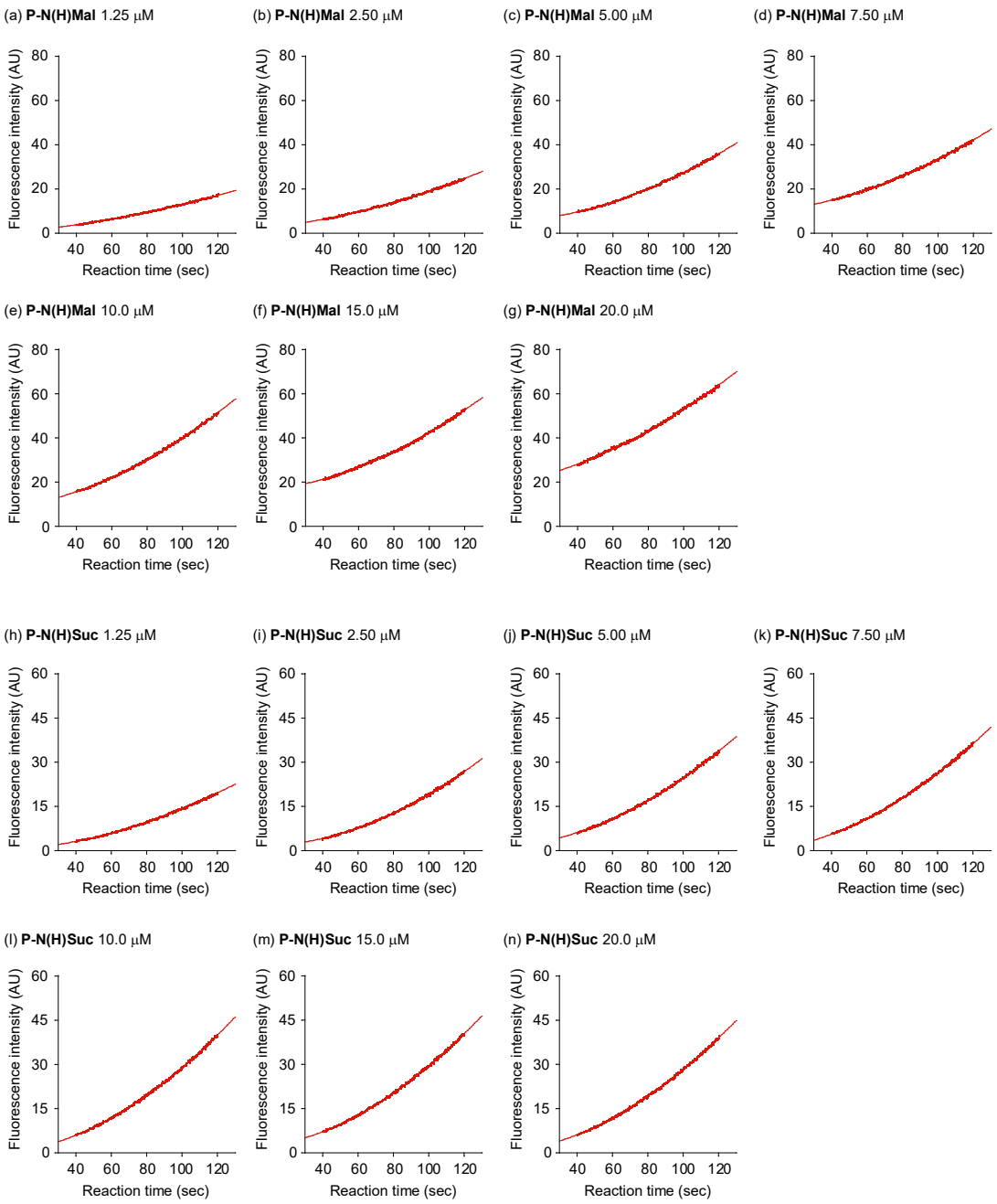


Figure S3. Fluorescence measurements of **P-N(H)Mal** and **P-N(H)Suc** in early stages of enzyme reactions. Fluorescence intensities for **P-N(H)Mal** (a-g) and **P-N(H)Suc** (h-n) were recorded during the period from 40 to 120 sec after addition of Sirt5 and fitted using Eq-8 described in the Sirt5 kinetic analysis section (vide supra). Probe concentrations used were 1.25 μM (a, h), 2.50 μM (b, i), 5.00 μM (c, j), 7.50 μM (d, k), 10.0 μM (e, l), 15.0 μM (f, m) and 20.0 μM (g, n). As a result of the curve fitting, *B* values described in the in the Sirt5 kinetic analysis section were obtained and are shown in Table S1.

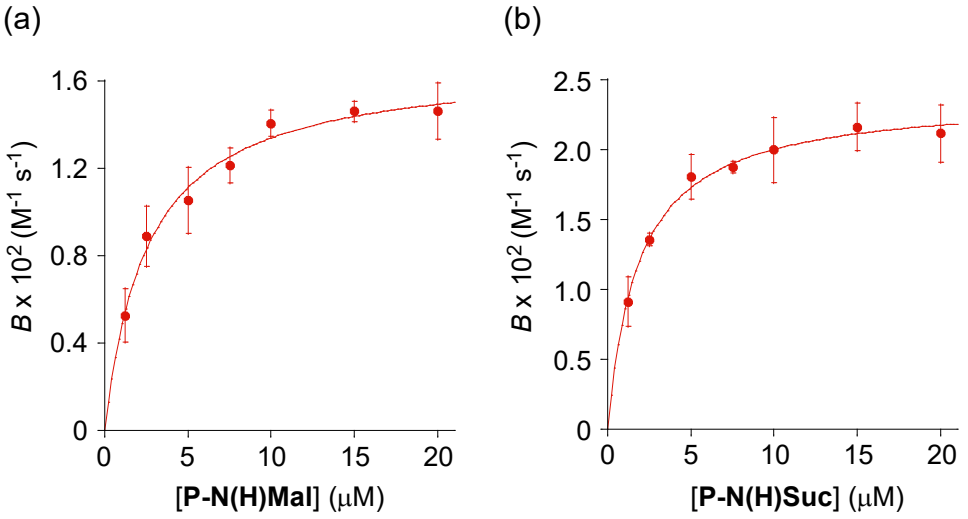


Figure S4. Determination of turnover number (k_{cat}) and Michaelis constant (K_m). The B values of **P-**(a) **N(H)Mal** and (b) **P-N(H)Suc** were obtained by three independent repeats of the experiments described in Figure S3 and Table S1, and their values plotted against probe concentration. The plots were fitted to Eq-6, which is described in the Sirt5 kinetic analysis section to give k_{cat}, K_m and k_{cat}/K_m, the results of which are summarized in Table S2.

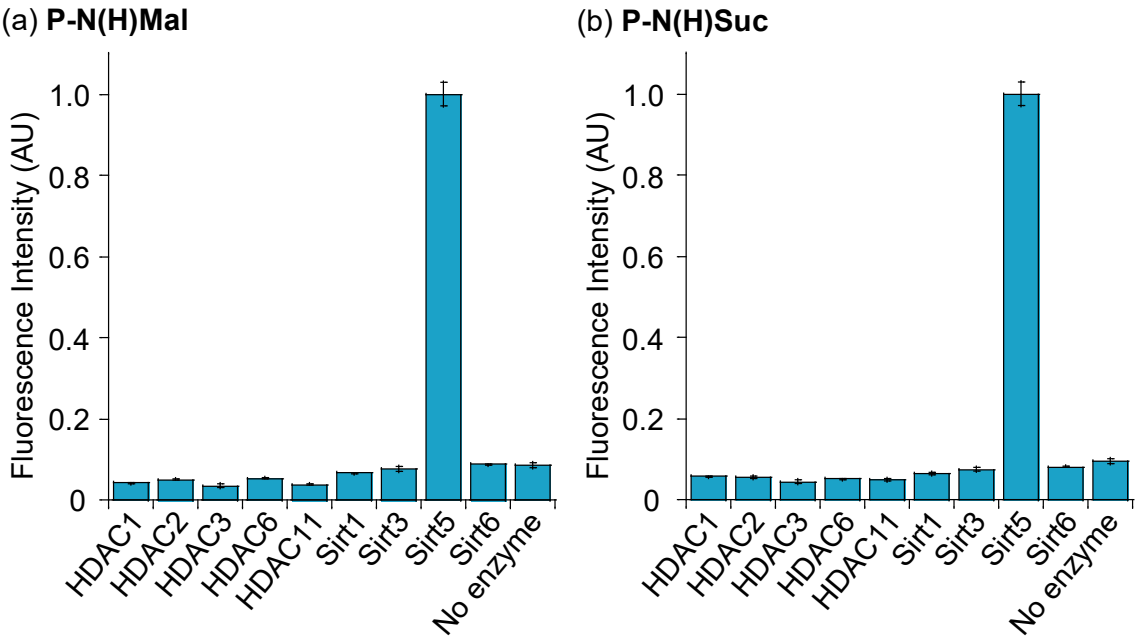


Figure S5. Enzyme selectivities of **P-N(H)Mal** and **P-N(H)Suc**. Fluorescence intensities at 460 nm were detected after each enzyme (100 nM) was incubated with (a) **P-N(H)Mal** or (b) **P-N(H)Suc** (5 μM) at 37 °C for 30 min.

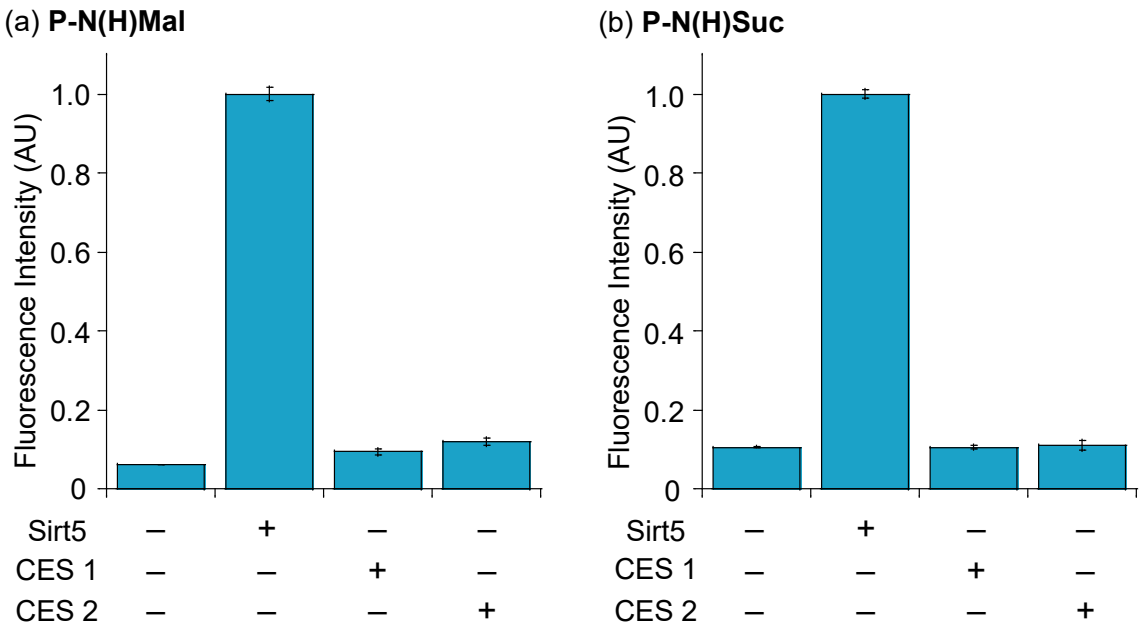


Figure S6. Reactivites of **P-N(H)Mal** and **P-N(H)Suc** with CES 1 or 2. Fluorescence intensities at 460 nm were detected after Sirt5 or each esterase (100 nM) was incubated with (a) **P-N(H)Mal** or (b) **P-N(H)Suc** (5 μM) at 37 °C for 30 min.

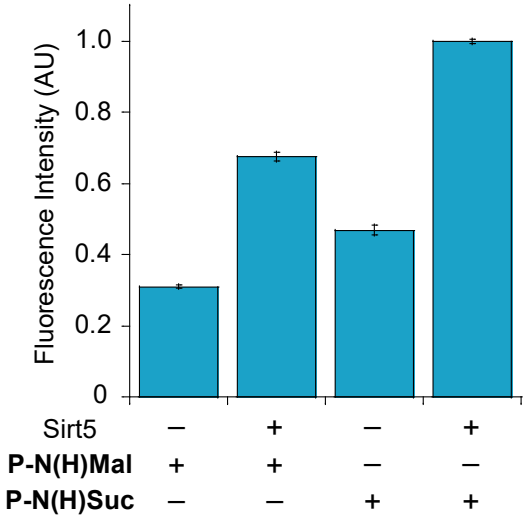


Figure S7. Fluorescence detection of Sirt5 activity in cell lysate using **P-N(H)Mal** and **P-N(H)Suc**. Fluorescence intensities at 460 nm was detected after Sirt5 (100 nM) was incubated with **P-N(H)Mal** or **P-N(H)Suc** (5 μM) at 37 °C for 20 min in cell lysate containing 0.32 mg/mL cellular proteins.

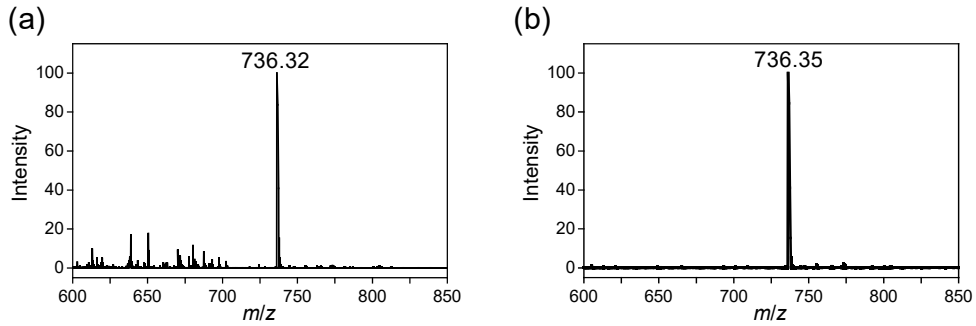


Figure S8. ESI mass spectra of HPLC fractions collected at retention times of (a) ~23 min and (b) ~26 min shown in Figure 3. The value calculated for *m/z* for the $[M+2H]^{2+}$ ion of **P-NHMe** or its transferred product (**-N(Me)CO$_2$Bu** was 736.38.

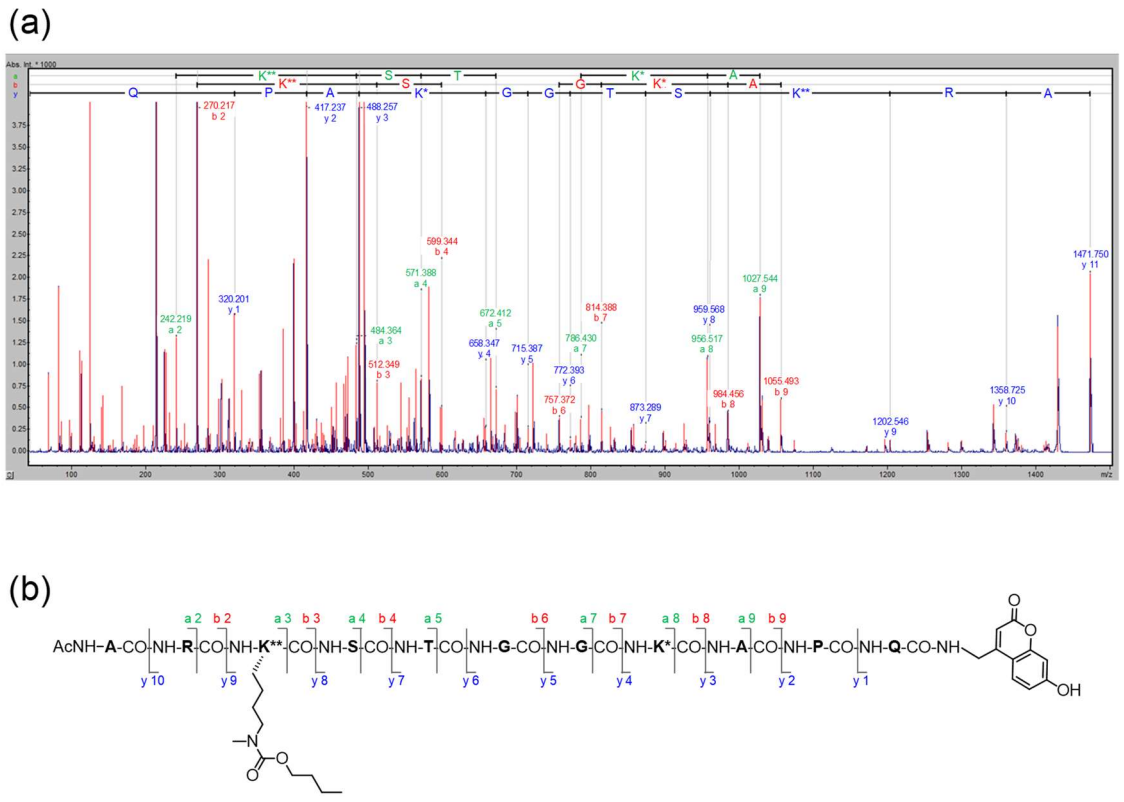


Figure S9. MALDI-TOF/TOF MS analysis of compound obtained from the *O*- to *N*- transesterification reaction of **P-NHMe**. (a) MS/MS spectrum of transfer product **P-N(Me)CO$_2$Bu** produced from **P-NHMe**. (b) Fragmentation patterns of the transferred product **P-N(Me)CO$_2$Bu**. K* represents acetylated lysine at the eighth position. K** represents carbomylated lysine at the third position. The *m/z* value of y 11 indicates the parent compound.

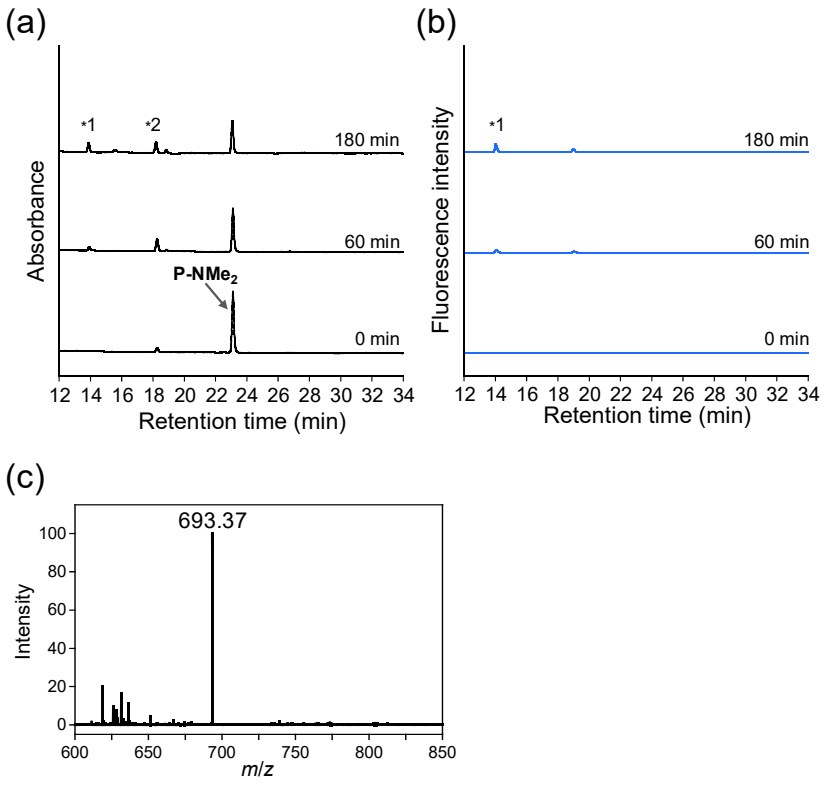


Figure S10. HPLC and ESI-MS analyses of **P-NMe$_2$** in the absence of JMJD2E. (a) Absorption and (b) fluorescence chromatograms of **P-NMe$_2$** incubated in HEPES buffer (analysis buffer 2) at 37 °C. The small peaks marked with asterisks 1 and 2 originated from a peptide hydrolysis product and DTT in the buffer, respectively. (c) ESI-MS spectrum of the HPLC fraction marked with asterisk 1. The $[M+2H]^{2+}$ value calculated for a hydrolysed **P-NMe$_2$** peptide containing a free 7-hydroxycoumarin fragment is 693.36.

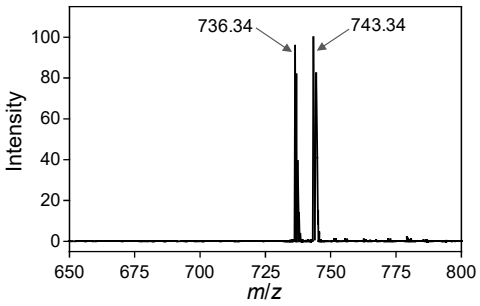


Figure S11. ESI mass spectra of the HPLC peak collected at ~23 min that was obtained from a 15 min demethylase reaction (see Fig 3 of main paper). Calculated *m/z* values for $[M+2H]^{2+}$ values of **P-NMe$_2$** and **P-NHMe** are 743.39 and 736.38, respectively.

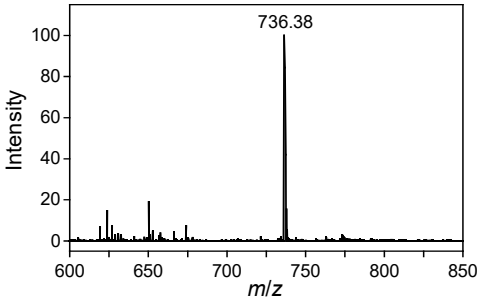
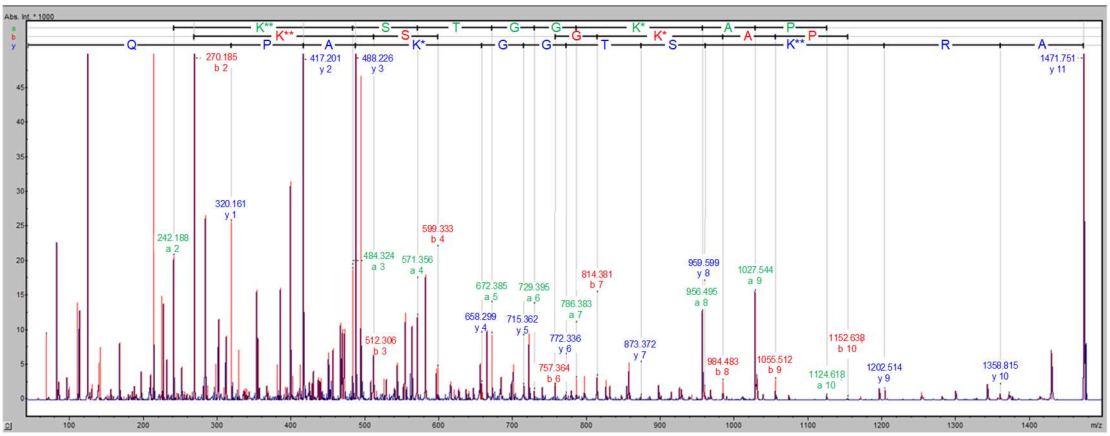


Figure S12. ESI mass spectrum of HPLC fraction (retention time = 26.0 min) in Figure 3. Calculated *m/z* value for $[M+2H]^{2+}$ of **P-NHMe** and its transferred product is 736.38. It should be noted that **P-NHMe** and its transferred product **P-N(Me)CO$_2$Bu** have the same mass values.

(a)



(b)

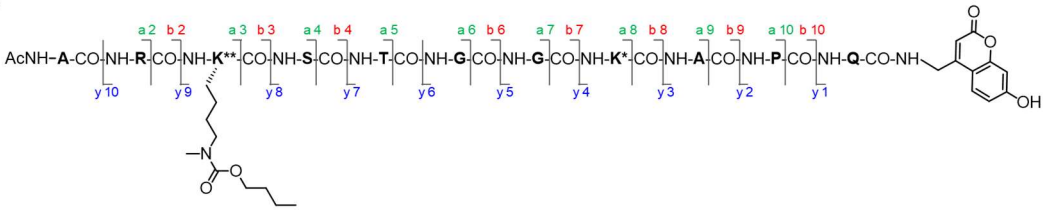


Figure S13. MALDI- TOF/TOF MS analysis of the HPLC purified peptide product **P-N(Me)CO$_2$Bu** produced from the *N*-deprotection reaction of **P-Me$_2$** with JMJD2E. K* corresponds to an acetylated lysine at the eighth residue from the *N*-amino terminus. K** corresponds to a carbomylated lysine at the third residue from the *N*-amino terminus. The *m/z* value for y 11 of 1471.751 corresponds to the molecular ion for **P-N(Me)CO$_2$Bu**.

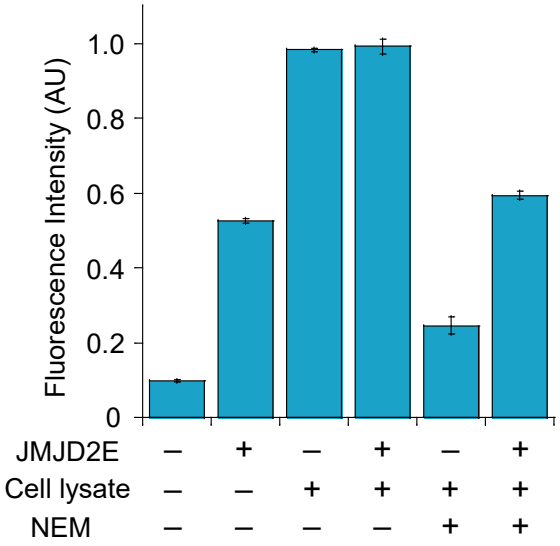


Figure S14. Fluorescence detection of JMJD2E activity in cell lysate using **P-NMe$_2$**. Fluorescence intensities at 460 nm was detected after JMJE2E (10 μM) was mixed with cell lysate containing 0.32 mg/mL cellular proteins and incubated with **P-NMe$_2$** (5 μM) in the absence or presence of *N*-EthylMaleimide (NEM) (1 mM) at 37 °C for 60 min.

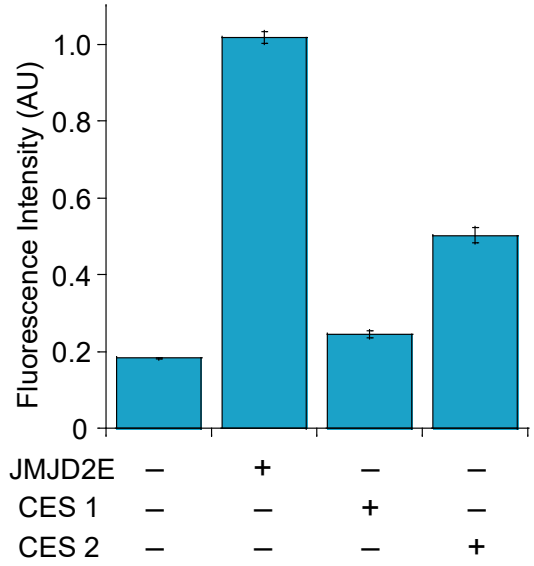


Figure S15. Reactivity of **P-NMe$_2$** with human carboxyesterase 1 or 2. Fluorescence intensities at 460 nm were detected after **P-NMe$_2$** (5 μM) was incubated with CES1 (100 nM), CES2 (100 nM) or JMJD2E (10 μM) at 37 °C for 60 min.

5. Supplementary References

[S1] R. Baba, Y. Hori and K. Kikuchi, *Chem. Eur. J.*, **2015**, *21*, 4695–4702.

[S2] N. R. Rose, S. S. Ng, J. Mecinovic, B. M., S. H. Bello, Z. Sun, M. A. McDonough, U. Oppermann, C. J. Schofield, *J. Med. Chem.*, **2008**, *51*, 7053–7056.