Supplementary Information for:

Potent sirtuin inhibition bestowed by L-2-amino-7-carboxamidoheptanoic acid (L-ACAH), a N^{ε}-acetyl-lysine analog

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Contents:

Experimental

General. The following are the materials obtained for the compound synthesis. All the materials obtained from commercial sources were used as received without further purification. Sigma-Aldrich: anhydrous MeOH, silica gel (70–230 mesh, 60 Å), N-methylmorpholine (NMM), piperidine, phenol, thioanisole, ethanedithiol; EMD biosciences: dichloromethane (DCM), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), acetonitrile, acetic anhydride; Novabiochem: all the N^{α}-Fmoc-protected amino acids except L-α-(9-fluorenylmethoxycarbonyl-amino)-suberic acid 8-methyl ester (**Scheme 1**) that was synthesized in the current study, Rink Amide AM resin for the solid phase peptide synthesis (SPPS), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU, the coupling reagent used for SPPS), N-hydroxybenzotriazole (HOBt); Fisher: anhydrous diethyl ether and anhydrous Na_2SO_4; Bachem: L-α-amino suberic acid; BDH: 28-30% (w/w) NH_4OH; Alfa Aesar: thionyl chloride, aniline, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochoride (EDC•HCl).

^{1}H and ^{13}C NMR spectra were obtained on a Varian Mercury 300 spectrometer or a Varian GEMINI 300 spectrometer. Mass spectra for the peptides synthesized in the current study were recorded on a Bruker Esquire-LC ion trap mass spectrometer (with electrospray ionization (ESI)). High resolution mass spectrometry (HRMS) was performed at the high resolution mass spectrometry facility of the University of California, Riverside. The samples were analyzed by the Agilent 6210 LCMS instrument that was operated in the flow injection mode and the "Multimode" ionization technique was used.

The following are the materials obtained for the biochemical assays used in the current study, including the SIRT1/2/3 inhibition assays, the mass spectrometry-based sirtuin assays for the detection of catalytic species from peptide **1**, HDAC8 time course and inhibition assays: GST-SIRT1 was expressed and purified from *Escherichia coli* as described previously using pGEX2TK-P•SIRT1 (human full length) (a kind gift from Prof. Tony Kouzarides).[1] Sir2Tm protein was a kind gift from Prof. Cynthia Wolberger. SIRT2 (human full length), SIRT3 (human amino acid 102-399), and HDAC8 (human full length) were purchased from Enzo Life Sciences (Product Nos. BML-SE251-0500, BML-SE270-0500, and BML-SE145-0100, respectively). The following peptides were used in the current study as the sirtuin substrates. Specifically, H_2N-HK-AcK-LM-COOH corresponding to amino acids 380-384 of the K^{382}-acetylated human p53 protein was used as the SIRT1 substrate; H_2N-MPSD-AcK-TIGG-COOH corresponding to amino acids 36-44 of the K^{40}-acetylated human α-tubulin was used as the SIRT2 substrate; H_2N-KRLPKTRSG-AcK-VMRRLLRKII-COOH corresponding to amino acids 633-652 of the
K^{642}-acetylated human acetyl-coenzyme A synthetase 2 (AceCS2) was used as the SIRT3 substrate. Of note, these peptides were also used in our previous studies as the sirtuin substrates.[1,2] NaCl, KCl, formic acid, and $H_2{}^{18}O$ were purchased from Fisher. Trizma, β-NAD^+, DMSO (molecular-biology grade), DL-dithiothreitol (DTT), pyridine, α-cyano-4-hydroxycinnamic acid (CHCA), and a 1.0 M solution of $MgCl_2$ (molecular-biology grade) were purchased from Sigma. The methanol for the methanolysis experiment was purchased from EMD Biosciences. All the materials obtained from commercial sources were used as received without further purification.

Synthesis of L-α-(9-fluorenylmethoxycarbonyl-amino)-suberic acid 8-methyl ester (Scheme 1).[3] **(a)** To a stirred solution of L-α-amino-suberic acid (450 mg, 2.4 mmole) in anhydrous methanol (6 mL) was added dropwise at -20 °C thionyl chloride (173 µL, 2.4 mmole). After the addition was complete, the reaction mixture was stirred at room temperature until the consumption of the starting material, as judged by thin-layer chromatography (TLC). The reaction mixture was then concentrated under reduced pressure, affording a white solid. **(b)** This solid material was then suspended in double deionized water (ddH_2O) (24 mL) and the pH was adjusted to ~10 with a 10% (w/v) aqueous solution of Na_2CO_3. To this stirred suspension was added dropwise a solution of N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) (1.619 g, 4.8 mmole) in 1,4-dioxane (12 mL) at room temperature. After the addition was complete, the reaction mixture was stirred at room temperature for 2.5 h before the excess Fmoc-OSu was extracted away with diethyl ether (4 x 200 mL). The aqueous layer was acidified with a 6 M aqueous HCl solution at 0 °C, and the product was extracted with ethyl acetate (3 x 100 mL). The combined organics were concentrated under reduced pressure to afford an oily residue from which the desired product was isolated *via* silica gel column chromatography as a white solid (259 mg, 25% after two steps): ^{1}HNMR (300 MHz, $CDCl_3$) δ (ppm) 9.01 (br, 1H, COOH), 7.74 (d, J = 7.2 Hz, 2H, H_{arom}), 7.59 (d, J = 6.3 Hz, 2H, H_{arom}), 7.38 (t, J = 7.2 Hz, 2H, H_{arom}), 7.29 (t, J = 7.2 Hz, 2H, H_{arom}), 5.39 (d, J = 8.1 Hz, 1H, NHC(=O)O), 4.54-4.32 (m, 3H, CH_2O and H_α), 4.21 (t, J = 6.6 Hz, 1H, Fluorenyl H_9), 3.65 (s, 3H, OCH_3), 2.29 (t, J = 7.2 Hz, 2H, $CH_2C(=O)O$), 1.98-1.18 (m, 8H, $CH_2CH_2CH_2CH_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 177.1 (COOH), 174.5 ($COOCH_3$), 156.3 (NHC(=O)O), 143.9 (C_{arom}), 141.5 (C_{arom}), 127.9 (C_{arom}), 127.3 (C_{arom}), 125.3 (C_{arom}), 120.2 (C_{arom}), 67.3 (CH_2O), 53.8 (C_α), 51.8 (OCH_3), 47.3 (Fluorenyl C_9), 34.1 ($CH_2C(=O)O$), 32.3 (CH_2), 28.7 (CH_2), 25.1 (CH_2), 24.7 (CH_2). HRMS (Multimode)

calcd. for $C_{24}H_{28}NO_6$ ($[M + H]^+$) 426.1917; found: 426.1919. Of note, the above NMR data agrees with that reported by Glenn et al.[3] for DL-α-(9-fluorenylmethoxycarbonyl-amino)-suberic acid 8-methyl ester.

Synthesis of 1 and 2 (Scheme 1), and 4 (Scheme 3). The solid phase synthesis part of the synthesis of these three compounds was all performed with the Fmoc chemistry-based solid phase peptide synthesis (SPPS) on a PS3 peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) from Rink Amide AM resin. For each amino acid coupling reaction, 4 (or 2) equivalents of a Fmoc-protected amino acid, 3.8 (or 1.9) equivalents of the coupling reagent HBTU and the additive HOBt were used in the presence of 0.4 M NMM/DMF, and the coupling reaction was allowed to proceed at room temperature for 1 (or 2) h. A 20% (v/v) piperidine/DMF solution was used for Fmoc removal. Each of the resin-bound intermediates was treated with acetic anhydride (to acetylate the N-terminal α-amino group) before it was cleaved from the resin at room temperature for 4 h by a trifluoroacetic acid (TFA)-containing cleavage cocktail (83.6% (v/v) TFA, 5.9% (v/v) phenol, 4.2% (v/v) ddH_2O, 4.2% (v/v) thioanisole, 2.1% (v/v) ethanedithiol) and precipitated in cold diethyl ether. Each of the methylester intermediates thus obtained was then lyophilized down to a white solid and dissolved in a 28-30% (w/w) NH_4OH aqueous solution (20 mL), and the solution was thoroughly mixed at room temperature for 8 hours. This solution was then re-lyophilized and the desired product (i.e. **1** or **4**) was isolated by the reversed-phase HPLC on a preparative C18 column (100 Å, 2.14 x 25 cm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 10 mL/min and monitored at 214 nm. The pooled HPLC fractions were stripped of acetonitrile and lyophilized to give **1** or **4** as a puffy white solid. The purified **1** and **4** were > 95% pure as verified by the reversed-phase HPLC on an analytical C18 column (100 Å, 0.46 x

25 cm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min and monitored at 214 nm. In the same manner, the purified methylester intermediate **2** (> 95%) was also obtained and subjected to the SIRT1 inhibition assay. The exact masses of the purified peptides **1** and **2** were confirmed by the ESI-MS analysis. The exact mass of the purified peptidomimetic **4** was confirmed by the HRMS analysis.

Peptide **1**: MS (ESI) calcd. for $C_{33}H_{59}N_{10}O_7S$ ($[M + H]^+$) 739.4; found: 739.5.

Peptide **2**: MS (ESI) calcd. for $C_{34}H_{60}N_9O_8S$ ($[M + H]^+$) 754.4; found: 754.4.

Peptidomimetic **4**: MS (Multimode) calcd. for $C_{26}H_{48}N_7O_7$ ($[M + H]^+$) 570.3615; found: 570.3588.

Synthesis of CH_3CONH-RH-AcK-(AcK)-$CONH_2$ and CH_3CONH-RH-AcK-(L-ACAH)-$CONH_2$. While the latter peptide was synthesized in the current study, the former peptide used in the current study was that synthesized and used in our previous study.[4] However, both peptides were synthesized in the same manner as that described above for the solid phase synthesis of the methylester intermediates on the Rink Amide AM resin. The HPLC purified latter peptide (> 95%) was also confirmed by the exact mass measurement with ESI-MS analysis: MS (ESI) calcd. for $C_{30}H_{53}N_{12}O_7$ ($[M + H]^+$) 693.4; found: 693.4.

Synthesis of 3. (a) To a stirred solution of L-α-amino-suberic acid (450 mg, 2.4 mmole) in anhydrous methanol (6 mL) was added dropwise at -20 °C thionyl chloride (173 μL, 2.4 mmole). After the addition was complete, the reaction mixture was stirred at room temperature until the consumption of the starting material, as judged by TLC. The reaction mixture was then concentrated under reduced pressure, affording a white solid. **(b)** A portion of the above-obtained solid material (1.0 mmole) was dissolved in a 28-30% (w/w) NH_4OH aqueous solution

(20 mL), and the solution was thoroughly mixed at room temperature for 8 hours before lyophilization. **(c)** The residue from lyophilization was suspended in ddH_2O (10 mL) and its pH was adjusted to ~10 with a 10% (w/v) aqueous solution of Na_2CO_3. A solution of N-(benzyloxycarbonyl-oxy)succinimide (Z-OSu) (498 mg, 2.0 mmole) in 1,4-dioxane (5 mL) was then added dropwise at room temperature. The reaction mixture was stirred at room temperature for 5 hours before ddH_2O (50 mL) was added and the residual Z-OSu was removed by diethyl ether extraction (3 x 200 mL). The aqueous layer was acidified at 0 °C with a 6 M aqueous HCl solution to pH ~1, and the product was extracted with ethyl acetate (3 x 100 mL). The combined organics were concentrated under reduced pressure to afford an oily residue. **(d)** This residue was then dissolved in DMF (9 mL), and to the resulting solution was added while stirring at room temperature aniline (182 uL, 2.0 mmole), EDC•HCl (288 mg, 1.5 mmole), and HOBt (203 mg, 1.5 mmole). The reaction mixture was stirred at room temperature for 24 hours before DCM (450 mL) was added. The organic layer was then washed with 0.1 M HCl (2 x 225 mL), 0.5 M NaOH (2 x 225 mL), and ddH_2O (2 x 650 mL), dried over anhydrous Na_2SO_4, and concentrated to give a residue from which the desired product was isolated by silica gel chromatography as an off-white solid (45 mg, 12% after four steps): ^{1}HNMR (300 MHz, DMSO-d_6) δ (ppm) 9.97 (s, 1H, C(=O)NH), 7.64-7.55 (m, 3H, C(=O)NH_2 and H_{arom}), 7.51 (d, J = 7.5 Hz, 1H, H_{arom}), 7.45-7.26 (m, 6H, H_{arom}), 7.23-7.15 (m, 1H, H_{arom}), 7.04 (t, J = 7.2 Hz, 1H, H_{arom}), 6.64 (br, 1H, NHC(=O)O), 5.03 (s, 2H, CH_2O), 4.18-4.03 (m, 1H, H_α), 2.01 (t, J = 7.2 Hz, 2H, $CH_2C(=O)$), 1.73-1.54 (m, 2H, CH_2), 1.54-1.40 (m, 2H, CH_2), 1.40-1.18 (m, 4H, CH_2CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 174.2 (C(=O)NH_2), 171.1 (C(=O)NH), 156.0 (NHC(=O)O), 138.9 (C_{arom}), 137.0 (C_{arom}), 128.7 (C_{arom}), 128.3 (C_{arom}), 127.7 (C_{arom}), 127.6 (C_{arom}), 123.2 (C_{arom}),

119.2 (C_{arom}), 65.4 (CH_2O), 55.4 (C_α), 35.0 ($CH_2C(=O)$), 31.7 (CH_2), 28.4 (CH_2), 25.3 (CH_2), 24.9 (CH_2). HRMS (Multimode) calcd. for $C_{22}H_{28}N_3O_4$ ($[M + H]^+$) 398.2080; found: 398.2082.

Sirtuin inhibition assay (IC_{50} determination). The HPLC-based sirtuin inhibition assay was performed as described previously.[1,5-7] Briefly, a sirtuin inhibition assay solution (100 μL) contained the following components: 25 mM (or 50 mM for the SIRT2 assay) Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA (Sigma Cat. No. A3803 with reduced fatty acid content, for the SIRT2 assay only), β-NAD^+ (0.5 mM for the SIRT1 and the SIRT2 assay, 3.5 mM for the SIRT3 assay), a peptide substrate (0.3 mM of the SIRT1 substrate: H_2N-HK-AcK-LM-COOH; 0.5 mM of the SIRT2 substrate: H_2N-MPSD-AcK-TIGG-COOH; or 0.03 mM of the SIRT3 substrate: H_2N-KRLPKTRSG-AcK-VMRRLLRKII-COOH), a sirtuin enzyme (GST-SIRT1, 350 nM; SIRT2, 150 nM; or SIRT3, 215 nM), and a potential inhibitor (**1**, **2**, **3**, or **4**) with varied concentrations including 0. Of note, while compounds **1-4** were all evaluated with SIRT1, only compound **4** was also evaluated for its inhibitory potency against SIRT2 and SIRT3. The same [S]/Km ratios for both substrates (~3.2 for the peptide substrates and ~5.6 for NAD^+) were used for the inhibition assays with SIRT1, SIRT2, and SIRT3 when assessing the inhibitory selectivity of **4** among these three human sirtuins. An enzymatic reaction was initiated by the addition of a sirtuin enzyme at 37 °C and was allowed to be incubated at 37 °C for 5 min (for the SIRT1 assay), or 60 min (for the SIRT2 assay), or 10 (or 20) min (for the SIRT3 assay) until quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (100 Å, 5 μm, 0.46 x 25 cm) or a reversed-phase Pursuit XRs Ultra 2.8 C18 column (100 Å, 2.8 μm, 100 x 2.0 mm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min or 0.5 mL/min, respectively, and UV monitoring at 214 nm. The

enzymatic deacetylation product was quantified with HPLC peak integration. Turnover of the limiting substrate was maintained at ≤ 13%. Stock solutions of **1**, **2**, and **4** were prepared in ddH_2O, and that for **3** was prepared in DMSO. The final DMSO concentration in an assay mixture was maintained at ≤ 5% (v/v). No deleterious effect on sirtuin's deacetylase activity was observed at this DMSO concentration. IC_{50} values were estimated from the Dixon plots ($1/v_0$ *vs.* [inhibitor])[8] as an indication of the inhibition potency.

SIRT1 inhibition kinetics of 1 (inhibition pattern determination). A SIRT1 inhibition assay solution (100 μL) contained the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM β-NAD^+, 350 nM GST-SIRT1, the SIRT1 peptide substrate H_2N-HK-AcK-LM-COOH varied from 0 to 1,000 μM, and peptide **1** (0, 5, or 10 μM). An enzymatic reaction was initiated by the addition of GST-SIRT1 at 37 °C and was allowed to be incubated at 37 °C for 5 min before quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (100 Å, 5 μm, 0.46 x 25 cm) eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min and UV monitoring at 214 nm. The enzymatic deacetylation product was quantified with HPLC peak integration. Turnover of the limiting substrate was maintained at ≤ 16%. Data were analyzed by the Enzyme Kinetics module of SigmaPlot® 11 (Systat Software, Inc.; San Jose, CA, USA), and were best fit to the linear competitive mode of SIRT1 inhibition by peptide **1** *versus* the acetylated substrate H_2N-HK-AcK-LM-COOH, as revealed by the pattern of intersecting double-reciprocal plots at the $1/v_0$ axis (**Fig. 3a**).[9]

SIRT1 inhibition kinetics of 1 (time-dependence of inhibition). A SIRT1 inhibition assay solution contained the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl,

2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM β-NAD^+, 0.3 mM H_2N-HK-AcK-LM-COOH (the SIRT1 substrate), peptide **1** (0, 5, or 10 μM), and 350 nM GST-SIRT1. An enzymatic reaction was initiated by the addition of GST-SIRT1 at 37 °C and was allowed to be incubated at 37 °C until quenched at different time points (0, 2, 4, 8, 16, 30 min) with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (100 Å, 5 μm, 0.46 x 25 cm) eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min and UV monitoring at 214 nm. The enzymatic deacetylation product was quantified at each of the time points with HPLC peak integration. Turnover of the limiting substrate was maintained at < 20%. The data from the experiment in the absence of peptide **1** were analyzed with a linear regression approach (**Fig. 3b**), whereas the data from the experiment in the presence of peptide **1** were fit to the following equation (Eq. 1)[10] using SigmaPlot® 11 (**Fig. 3b**), wherein v_0 is the initial velocity, v_s is the steady state velocity, t is the time following the initiation of the enzymatic reaction, k is the apparent first-order rate constant to reach the steady state, [P] is the concentration of H_2N-HK-K-LM-COOH (the deacetylated SIRT1 substrate) produced at the different time points following the start of an enzymatic reaction.

$$[P] = v_s\, t + (v_0 - v_s)\,(1 - e^{-k\,t}) / k \qquad \text{(Eq. 1)}$$

SIRT1 inhibition kinetics of 1 (activity recovery of SIRT1 inhibited by 1). A SIRT1 deacetylation assay solution contained the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM β-NAD^+, 0.3 mM H_2N-HK-AcK-LM-COOH (the SIRT1 substrate), and 350 nM GST-SIRT1 (either free or the complex with peptide **1**). While the free GST-SIRT1 was used in the control experiment, the pre-formed GST-SIRT1•Peptide **1** complex was used to determine if there was a SIRT1 deacetylase activity

recovery from the inhibition by peptide **1**. Specifically, GST-SIRT1 (8 μM) was pre-incubated with peptide **1** (150 μM) for 30 min at 37 ºC before 6.3 μL of such pre-incubation sample was rapidly diluted by 95-fold into the SIRT1 deacetylation assay solution. For the control experiment, GST-SIRT1 (8 μM) was incubated in the absence of peptide **1** for 30 min at 37 ºC before 6.3 μL of this sample was also rapidly diluted by 95-fold into the SIRT1 deacetylation assay solution. Following the initiation of an enzymatic reaction with either the free GST-SIRT1 or the pre-formed GST-SIRT1•Peptide **1** complex, the enzymatic reaction was allowed to be incubated at 37 °C before quenched at different time points (0, 2, 4, 8, 12, 16, 24, 30 min) with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (100 Å, 5 μm, 0.46 x 25 cm) eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min amd UV monitoring at 214 nm. The enzymatic deacetylation product was quantified at each of the time points with HPLC peak integration. The data from the control experiment were analyzed with a linear regression approach (**Fig. 3c**), whereas the data from the enzymatic reactions initiated with the pre-formed GST-SIRT1•Peptide **1** complex were fit to the following equation (Eq. 2) using SigmaPlot® 11 (**Fig. 3c**), wherein v_s is the steady state velocity, t is the time following the initiation of the enzymatic reaction, k is the first-order off rate constant for the GST-SIRT1•Peptide **1** complex. [P] is the concentration of H_2N-HK-K-LM-COOH (the deacetylated SIRT1 substrate) produced at the different time points following the start of an enzymatic reaction.

$$[P] = v_s (t - (1 - e^{-k\,t}) / k) \qquad \text{(Eq. 2)}$$

Mass spectrometry detection of the catalytic species with peptide 1 and SIRT1 or Sir2Tm. (a) An assay solution (20 μL) contained the following components: 20 mM pyridinium

formate (pH 7.0), 1 mM DTT, 0.5 mM β-NAD^+, 0.4 mM peptide **1**, and an enzyme (GST-SIRT1 or Sir2Tm with the final concentration of 2 μM or 25 μM respectively). An enzymatic reaction was initiated by the addition of the enzyme at 25°C and was allowed to be incubated at 25°C for 30 min before the reaction mixture was snap frozen in liquid nitrogen and stored at -70 °C until the mass spectral analysis by matrix-assisted laser desorption ionization (MALDI)-MS (negative reflector mode with α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix). **(b)** For the methanolysis experiment, methanol was included in the above assay mixture with GST-SIRT1 to achieve a final concentration of 20% (v/v) in the 20 μL of the assay mixture. The enzymatic reaction was initiated at 25 °C with the addition of GST-SIRT1 (final concentration of 2 μM), and was allowed to be incubated at 25 °C for 30 min before the addition of TFA to a final concentration of 1% (v/v) and the immediate snap freezing in liquid nitrogen. The sample was stored at -70 °C until the mass spectral analysis by MALDI-MS (negative reflector mode with CHCA as the matrix) and ESI-MS (positive mode). **(c)** For the ^{18}O labeling experiment, $H_2{}^{18}O$ was included in the assay mixture in **(a)** with GST-SIRT1 to achieve a final concentration of 63% (v/v) in the 20 μL of the assay mixture. Of note, the stock solutions of DTT and β-NAD^+ were prepared in $H_2{}^{18}O$. The enzymatic reaction was initiated at 25 °C with the addition of GST-SIRT1 (final concentration of 2 μM), and was allowed to be incubated at 25°C for 30 min before the reaction mixture was snap frozen in liquid nitrogen and stored at -70 °C until the mass spectral analysis by MALDI-MS (negative reflector mode with CHCA as the matrix). **(d)** The MALDI-MS experiments were carried out on a Bruker Ultraflex III tandem time-of-flight (ToF/ToF) mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with a Nd:YAG laser emitting at a wavelength of 355 nm. All spectra were recorded in a negative reflector mode. The solution of the matrix CHCA was prepared in acetonitrile/water (1/1 (v/v)) at 5 mg/mL. The

sample preparation followed the sandwich method. Briefly, 0.5 μL of the matrix solution was initially deposited on the wells of a 384-well ground-steel plate, allowing the spots to dry in the air. A sirtuin assay sample was thawed and 0.5 μL of it was deposited on the top of a dried matrix spot, and the plate was then placed in a vacuum for 1 hour at room temperature to dry the spot. Another 0.5 μL of the matrix solution was then deposited on the top of the dried sample spot. Data analysis was conducted with the flexAnalysis software. **(e)** The ESI-MS experiments were performed with a Waters Synapt HDMS quadrupole/time-of-flight (Q/ToF) mass spectrometer (Waters, Milford, MA). The following ESI parameters were selected: ESI capillary voltage, 3.5 kV; sample cone voltage, 35 V; extraction cone voltage, 3.2 V; desolvation gas flow, 500 L/h (N_2); trap collision energy (CE), 6 eV; transfer CE, 4 eV; trap gas flow, 1.5 mL/min (Ar); IM gas flow, 22.7 mL/min (N_2); sample flow rate, 5 μL/min; source temperature, 120 °C; desolvation temperature, 150 °C. A thawed sirtuin assay mixture was analyzed in a positive mode. Data analysis was conducted with the MassLynx 4.1 programs of Waters.

HDAC8 time course assay. This assay was also performed as described previously.[4,7] Briefly, a HDAC8 assay solution had the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA (Sigma Cat. No. A3803 with reduced fatty acid content), 0.3 mM of a test compound (CH_3CONH-RH-AcK-(AcK)-$CONH_2$ or CH_3CONH-RH-AcK-(L-ACAH)-$CONH_2$), and 375 nM HDAC8. An enzymatic reaction was initiated by the addition of HDAC8 at room temperature and was allowed to be incubated at room temperature until quenched with the following stop solution at different time points (0, 1, 2, 4h): 1.0 M HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase HPLC C18 column (100 Å, 0.46 x 25 cm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min,

and UV monitoring at 214 nm. The enzymatic deacetylation product was quantified at each of the time points with HPLC peak integration.

HDAC8 inhibition assay. The HDAC8 inhibition assay solution had the following components: 25 mM Tris•HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA (Sigma Cat. No. A3803 with reduced fatty acid content), 0.3 mM of the HDAC8 peptide substrate CH_3CONH-RH-AcK-(AcK)-$CONH_2$, 375 nM HDAC8, and the test peptide CH_3CONH-RH-AcK-(L-ACAH)-$CONH_2$ with varied concentrations including 0. An enzymatic reaction was initiated by the addition of HDAC8 at room temperature and was allowed to be incubated at room temperature for 1 h before quenched with the following stop solution: 1.0 M HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase HPLC C18 column (100 Å, 0.46 x 25 cm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min, and UV monitoring at 214 nm. The enzymatic deacetylation product was quantified with HPLC peak integration.

Western blot analysis. This assay was also performed as described previously.[7] HCT116 human colon cancer cells were cultured in McCoy5A culture medium containing 10% FBS with penicillin and streptomycin. HCT116 cells (5×10^5) were treated for 8 h with compound **4** at different concentrations (0, 5, 50, and 500 μM) and then collected and extracted with RIPA buffer (50 mM Tris•HCl (pH 8.0), 0.5% triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM NaF). Equivalent amounts of proteins from each lysate were resolved in 10% SDS-polyacrylamide gel and then transferred onto PVDF membranes (Bio-Rad Laboratories). After having been blocked with Tris-buffered saline (TBS) containing 5% milk, the transblotted membrane was incubated overnight at 4 °C with acetylated p53 antibody (Cell Signaling) (1:1000 dilution). After washed twice with water, the membranes were incubated with

the rabbit antimouse IgG-horseradish peroxidase conjugate (diluted 1:2500) for 2 h at room temperature, and again washed twice with water. The immunoblots were visualized by enhanced chemiluminescence. Stripping the membrane and then blot it with p53 antibody (Cell Signaling) (1:1000 dilution).

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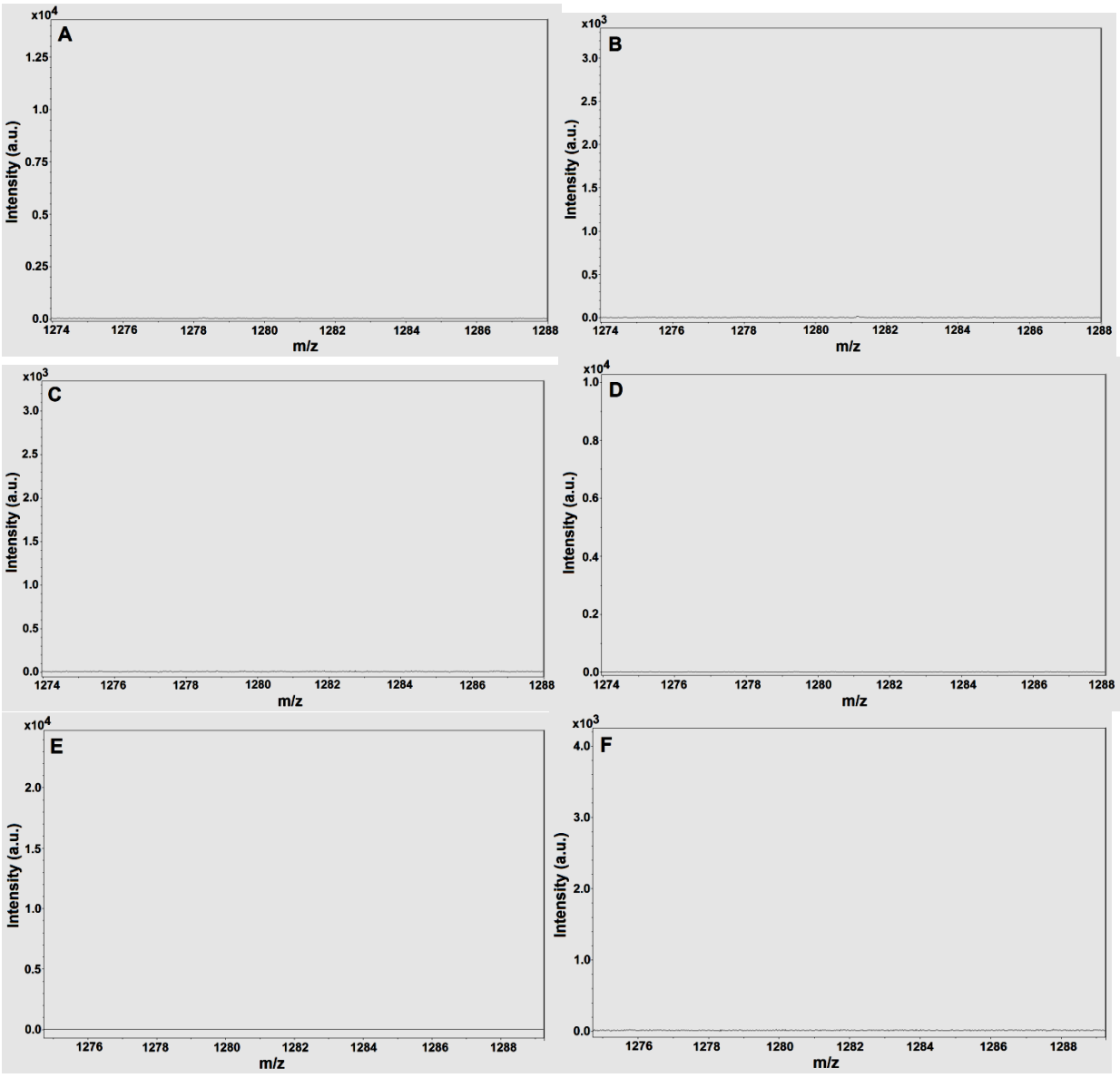

A
x10^4
Intensity (a.u.)
m/z
B
x10^3
Intensity (a.u.)
m/z
C
x10^3
Intensity (a.u.)
m/z
D
x10^4
Intensity (a.u.)
m/z
E
x10^4
Intensity (a.u.)
m/z
F
x10^3
Intensity (a.u.)
m/z

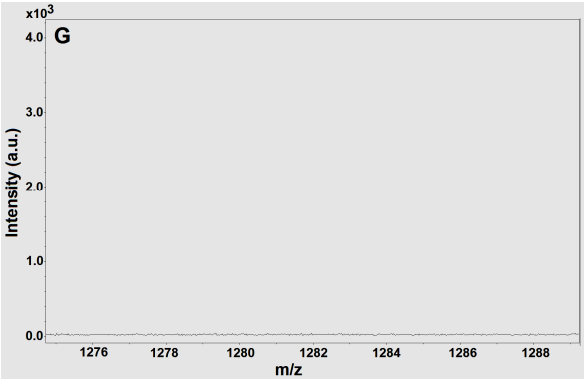


Figure S1. The lack of a detectable peak cluster starting at m/z 1278.3 from the mass spectral analysis by MALDI-MS (negative reflector mode with CHCA as the matrix) of the incubation mixture in which one of the following components was omitted from that generating the data in **Fig. 4A** (with SIRT1) or **Fig. 4D** (with Sir2Tm). **(A)** SIRT1 was omitted; **(B)** peptide **1** was omitted in the SIRT1 incubation; **(C)** NAD^+ was omitted in the SIRT1 incubation; **(E)** SirTm was omitted; **(F)** peptide **1** was omitted in the Sir2Tm incubation; **(G)** NAD^+ was omitted in the Sir2Tm incubation. Experimental conditions were same as those used to generate the data in **Fig. 4A** or **Fig. 4D**. The peak cluster starting at m/z 1278.3 was also undetectable from the matrix CHCA **(D)** under the same mass spectral analysis condition.

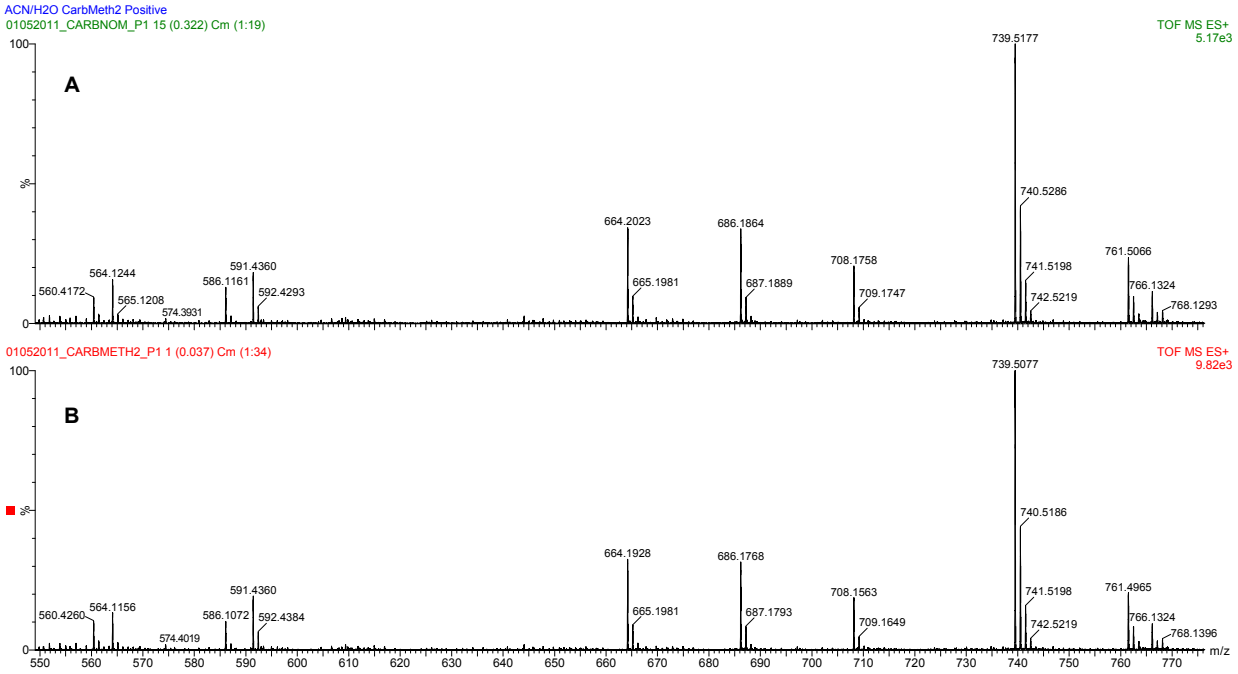
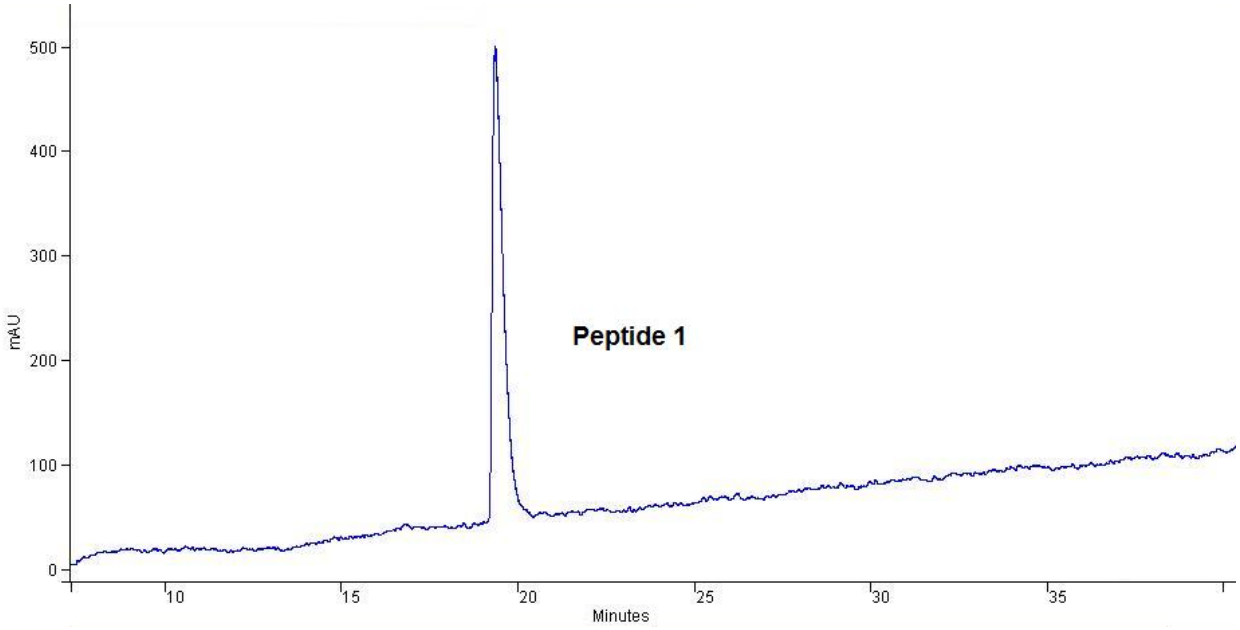
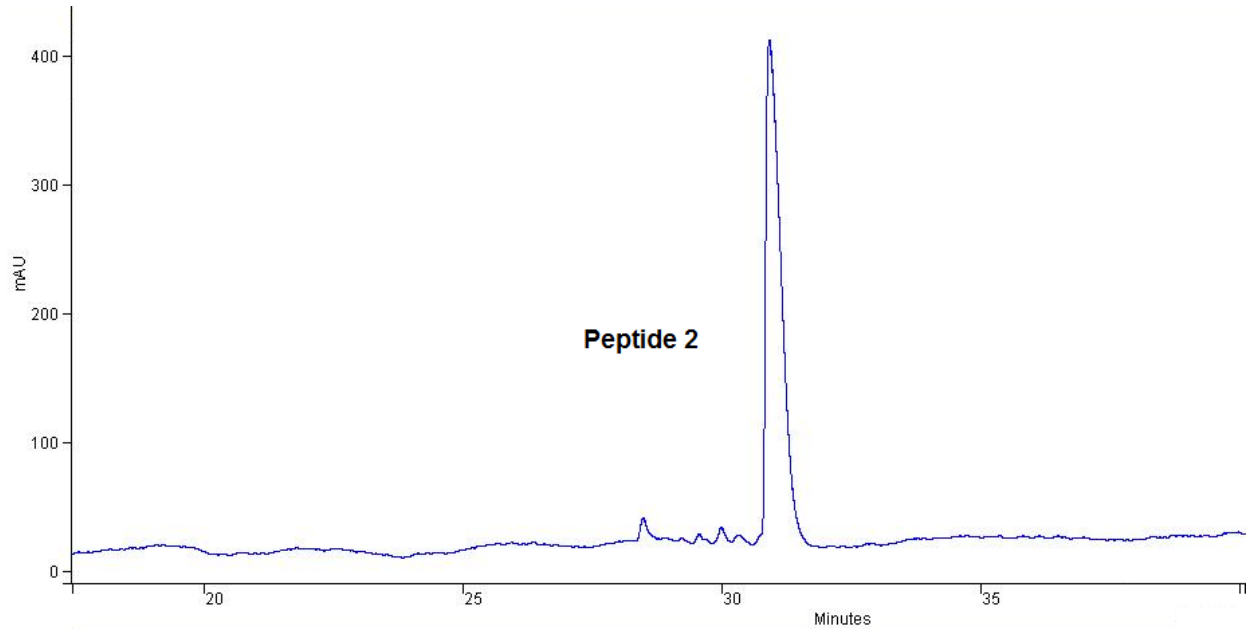
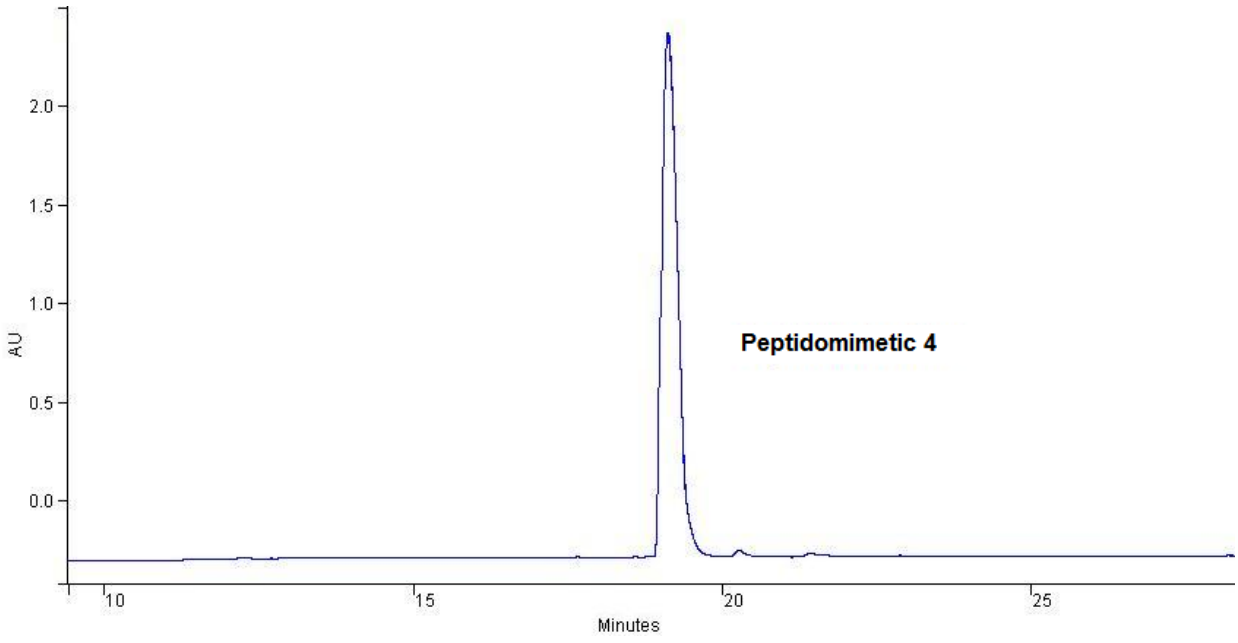
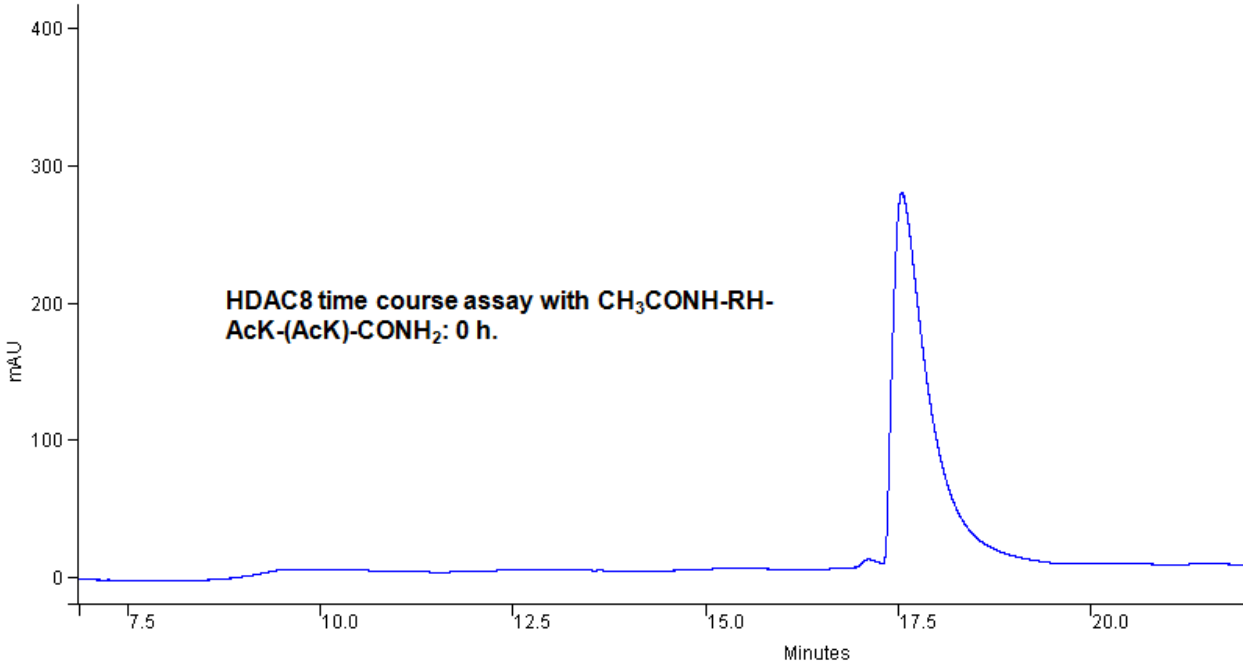


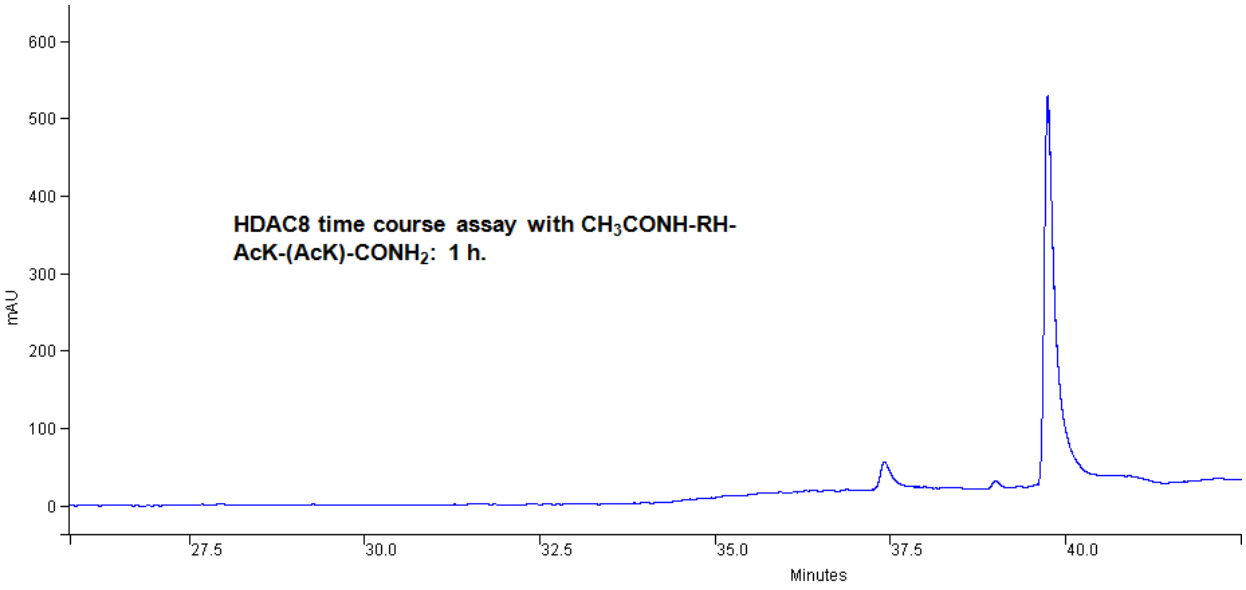
Figure S2. Comparative mass spectral analysis by ESI-MS (positive mode) of the incubation mixture of peptide **1** with SIRT1 in the absence (**A**) or presence (**B**) of 20% (v/v) of methanol. Spectrum **A** is essentially same as the spectrum **B**. On both spectra, the peaks at m/z 739.5 and 761.5 represent respectively the $[M+H]^+$ and $[M+Na]^+$ ions derived from peptide **1**. The peaks at m/z 664.2, 686.2, and 708.2 represent respectively the M^+, $[M+Na-H]^+$, and $[M+2Na-2H]^+$ ions from NAD^+.

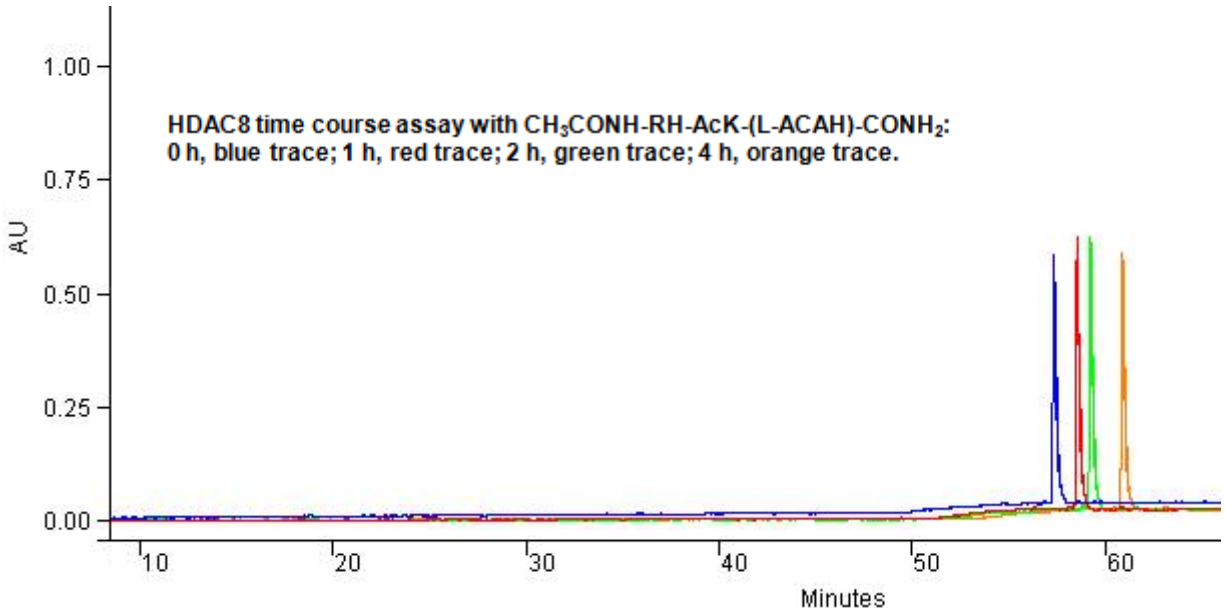

500
400
300
200
100
0
mAU
Peptide 1
10
15
20
25
30
35
Minutes


400
300
200
100
0
mAU
Peptide 2
20
25
30
35
Minutes


2.0
1.5
1.0
0.5
0.0
AU
Peptidomimetic 4
10
15
20
25
Minutes


HDAC8 time course assay with CH3CONH-RH-AcK-(AcK)-CONH2: 0 h.
mAU
400
300
200
100
0
7.5
10.0
12.5
15.0
17.5
20.0
Minutes


HDAC8 time course assay with CH3CONH-RH-AcK-(AcK)-CONH2: 1 h.
mAU
600
500
400
300
200
100
0
27.5
30.0
32.5
35.0
37.5
40.0
Minutes


HDAC8 time course assay with CH3CONH-RH-AcK-(L-ACAH)-CONH2:
0 h, blue trace; 1 h, red trace; 2 h, green trace; 4 h, orange trace.
AU
1.00
0.75
0.50
0.25
0.00
10
20
30
40
50
60
Minutes