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A mechanism-based potent sirtuin inhibitor containing N^ε-thiocarbamoyl-lysine (TuAcK)

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Abstract

In the current study, we have identified N^ε-thiocarbamoyl-lysine (TuAcK) as a general sirtuin inhibitory warhead which was shown to be able to confer potent sirtuin inhibition. This inhibition was also shown to be mechanism-based in that the TuAcK residue was able to be processed by a sirtuin enzyme with the formation of a stalled S-alkylamidate intermediate.

Keywords

sirtuin; deacetylase; mechanism-based; inhibitor; N^ε-thiocarbamoyl-lysine

Sirtuin enzymes are a family of cellular proteins able to catalyze the acetyl group removal from the specific N^ε-acetyl-lysine (AcK, Fig. 1) side chains on both histone and non-histone proteins.^{1–7} These enzymes have been found in all the living organisms from bacteria to humans, with the yeast silent information regulator (Sir2) as the founding family member whose protein lysine deacetylase activity was discovered about one decade ago.^{3,8–12} The available research indicated that the lower prokaryotic species have less sirtuin isoforms than the higher eukaryotic species such as humans that have 7 identified sirtuin isoforms (SIRT1–7). As illustrated in Figure 1, this enzymatic acetyl group removal (or deacetylation) reaction employs β-nicotinamide adenine dinucleotide (β-NAD⁺ or NAD⁺) as its cosubstrate. Because of this use of NAD⁺, in addition to regulating cellular processes such as gene transcription *via* the functional modulation of histone proteins and various transcription factors and coregulators, the sirtuin-catalyzed lysine deacetylation reaction has also been shown to be important in regulating metabolism *via* the functional modulation of multiple metabolic enzymes in response to the cellular [NAD⁺]/[NADH] ratio fluctuations.^{11,13–20} The studies performed in the past a few years have also started revealing the therapeutic benefits of the chemical modulation of this enzymatic deacetylation reaction for such disease states as diabetes, cancer, and neurodegenerative

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diseases.^{11,13,21–34} In order to achieve a fuller exploration of the sirtuin pharmacology as well as biology, there is still a need for a continued search for novel chemical modulators for the sirtuin-catalyzed protein lysine deacetylation reaction that was discovered not long ago, yet has already revealed its (path)physiological prominence.

Because the AcK residue could be regarded as the “minimal” substrate for the sirtuin-catalyzed lysine deacetylation reaction, our laboratory has been interested in identifying AcK analogs as the inhibitory warheads for this enzymatic deacetylation reaction.^{35–39} Previously, we found that very potent sirtuin inhibition could be obtained by a series of peptidic and peptidomimetic compounds containing N^ε-thioacetyl-lysine (ThAcK, Fig. 1).³⁹ The mechanistic studies by us and others demonstrated that ThAcK bestowed this potent sirtuin inhibition *via* a mechanism-based fashion in that the ThAcK-containing inhibitor could be recognized by a sirtuin enzyme as a substrate and processed to furnish a longer-lived (or stalled) α -1'-S-alkylamidate intermediate (Fig. 1a, X=S) that presumably behaves as a bisubstrate sirtuin inhibitory species.^{35,39–42} While the use of the thioamide-containing ThAcK has represented a simple yet very powerful technology to quickly identify very potent sirtuin inhibitors, the therapeutic use of a thioamide compound could be compromised by the potential cellular toxicity, especially the hepatotoxicity, after the metabolic S-oxidation and activation of such compound.^{43–47} It is possible to ameliorate this potential toxic side effect to certain extent *via* managing the drug administration route (e.g. *i.v. versus p.o.*),⁴⁸ developing alternative potent sirtuin inhibitory AcK analogs would be another avenue to take.

In the current study, we have identified the novel AcK analog N^ε-thiocarbamoyl-lysine (TuAcK, Fig. 1b) as a potent sirtuin inhibitory warhead.

Our pursuit of TuAcK as a potential novel sirtuin inhibitory warhead was based on the following considerations. First, since N^ε-carbamoyl-lysine (or homocitrulline, Fig. 1b) was shown previously to be processed by the yeast sirtuin Hst2 to form the corresponding analog of α -1'-O-alkylamidate intermediate shown in Figure 1a following the Hst2-catalyzed nicotinamide cleavage from NAD⁺,^{49,50} we reasoned that TuAcK could also be processed similarly. However, unlike the intermediate formed from N^ε-carbamoyl-lysine which was suggested to be intercepted by water to regenerate N^ε-carbamoyl-lysine,^{49,50} the corresponding intermediate formed from TuAcK could be longer-lived just like that formed from ThAcK, thus exerting a potent sirtuin inhibition also presumably as a bisubstrate analog inhibitor. Secondly, some thiourea compounds have been known to be strong scavengers of the hydroxyl radical or the superoxide radical anion.^{51–53} This potential anti-oxidant effect of a thiourea compound constitutes another appeal for developing the thiourea-containing TuAcK as a basic sirtuin inhibitory motif since a compromised cellular anti-oxidation defense system could occur following the inhibition of the sirtuin-catalyzed deacetylation of the FoxO family of transcription factors. Specifically, the SIRT1- and SIRT2-catalyzed deacetylation of FoxO3/4 and FoxO3a, respectively, was shown to lead to the activation of these transcription factors and the consequent enhanced expression of their target genes whose products are components of the cellular anti-oxidation defense system, e.g., manganese superoxide dismutase.^{54–56} This sirtuin inhibition-induced potential attenuation of the cellular anti-oxidation defense system could be of a particular concern when developing therapeutics for the neurodegenerative diseases, for example SIRT2 inhibitors for the Parkinson's disease.⁵⁷ Within this context, a diminished cellular ATP level has been recently observed in PC12 cells following the SIRT2 silencing by RNAi, and the pharmacological SIRT2 inhibition has also been shown in the same study to exacerbate the ATP level diminishing from the H₂O₂ treatment.⁵⁸

To assess the sirtuin inhibitory power of TuAcK, we prepared compound **1** as shown in Scheme 1.⁵⁹ Following the assembly of a tripeptide on the Rink Amide resin *via* the 9-fluorenylmethoxycarbonyl (Fmoc) chemistry-based solid phase peptide synthesis (SPPS),⁶⁰ the corresponding unprotected tripeptide amide was released from the resin with an 85% (v/v) solution of trifluoroacetic acid (TFA) in dichloromethane (DCM). This intermediate was then treated with thiophosgene followed by a 7 M solution of ammonia in MeOH, as literature procedures.^{61,62} The resulting product was isolated with the preparative high pressure liquid chromatography (HPLC).

It should be pointed out that the structural context of TuAcK in compound **1** was the peptidomimetic scaffold that we used previously, i.e. the tripartite structure with a central AcK analog and two immediately flanking N^δ-acetyl-ornithine (AcOrn).^{37,39} The use of this peptidomimetic scaffold in compound **1** also allowed us to examine whether this compound would also be able to inhibit a human sirtuin enzyme inside cells since this peptidomimetic scaffold was shown by us previously to be proteolytically stable and to be able to confer cell membrane permeability on the compound harboring ThAcK at the central position.³⁷

When compound **1** was subjected to a HPLC-based SIRT1 inhibition assay,⁶³ we obtained an IC₅₀ of 89.5 ± 16.1 μM, a value which is ~15-fold greater than that of its ThAcK counterpart (Table 1). However, this IC₅₀ value was ~3-fold lower than that for another peptidomimetic sirtuin inhibitor that we reported previously, i.e. CH₃CONH-AcK-(L-ACAH)-AcK-CONH₂³⁸ in which L-ACAH stands for L-2-amino-7-carboxamidoheptanoic acid (Fig. 1b) (Table 1). Of note, this AcK-based peptidomimetic scaffold is structurally very similar to the AcOrn-based scaffold in compound **1**, since the AcOrn side chain is only one methylene (CH₂) less than that of AcK. Considering that the AcOrn-based peptidomimetic scaffold was found by us previously to be ~3-fold weaker than the AcK-based one in bestowing SIRT1 inhibition,³⁷ in our opinion, TuAcK is a more powerful SIRT1 inhibitory warhead than L-ACAH.

Encouraged by this *in vitro* SIRT1 inhibition result, compound **1** was further assessed for its capability of inhibiting the SIRT1 inside the HCT116 human colon cancer cells that express the wild-type p53 protein.⁶⁵ The SIRT1 inhibitory potency in this cellular assay was assessed by the extent of the acetylation level increase for this SIRT1 endogenous substrate protein at the K382 position⁶⁶ following the treatment with compound **1**. As shown in Figure 2, compound **1** was observed to be able to enhance the p53 K³⁸² acetylation level in a dose-dependent manner. Specifically, while a slight acetylation level increase was observed at 5 μM, compound **1** was found to be able to more dramatically enhance the p53 acetylation level at higher concentrations (50 and 250 μM).

As mentioned above, it is possible that TuAcK could also be processed as ThAcK by a sirtuin enzyme to form a stalled alkylamidate intermediate following the nicotinamide cleavage from NAD⁺. To explore this possibility, we used mass spectrometry in an attempt to detect any possibly longer-lived catalytic species from an incubation of compound **1** with NAD⁺ in the presence of SIRT1.⁶⁷ When the incubation mixture was subjected to a matrix-assisted laser desorption ionization (MALDI) mass spectral analysis in negative reflector mode, a species with m/z 1097.8 was observed, as shown in Figure 3a, from this incubation mixture under a non-quenching condition which was also used by Smith and Denu to detect the stalled α-1'-S-alkylamidate intermediate from an incubation of a ThAcK-containing peptide and NAD⁺ in the presence of the yeast sirtuin Hst2.⁴⁰ We further found that the cluster of the peaks starting at m/z 1097.8 were not detectable under the same experimental conditions by omitting SIRT1 or compound **1** from the incubation mixture (Figs. 3b and 3c), and were not detectable from the MALDI matrix 3-Hydroxypicolinic acid (3-HPA) either (Fig. 3d). Therefore, the species with m/z 1097.8 appeared to be exclusively formed from

the SIRT1-catalyzed reaction of NAD^+ with compound **1**. The species with m/z 1097.8 could well represent the $[\text{M-H}]^-$ anion derived from the corresponding alkylamidate intermediate (chemical formula (neutral form): $\text{C}_{38}\text{H}_{63}\text{N}_{13}\text{O}_{19}\text{P}_2\text{S}$; exact mass (neutral form): 1099.4 Da) which is an analog of the α -1'-O-alkylamidate intermediate in Figure 1a, just as the α -1'-S-alkylamidate intermediate was detected with the MALDI-MS analysis in the study of Smith and Denu.⁴⁰

In order to assess whether TuAcK could be a general sirtuin inhibitory warhead, we subsequently evaluated the inhibitory potency of compound **1** against human SIRT2 and SIRT3. As shown in Table 1, compound **1** was found to exhibit a more or less comparable inhibitory potency (within ~3-fold) against SIRT1, SIRT2, and SIRT3, thus suggesting that TuAcK could confer potent sirtuin inhibition as a general inhibitory warhead. Table 1 also shows the comparable inhibitory potency against SIRT1, SIRT2, and SIRT3 that we previously found for the peptidomimetic sirtuin inhibitors containing ThAcK or L-ACAH.^{37,38} However, a closer inspection of the inhibitory data for compound **1** and the ThAcK-containing inhibitor in Table 1 revealed their different rank order of potency. In specific, while both compounds were shown to exhibit a slightly stronger inhibition against SIRT3 than SIRT1, compound **1** exhibited the strongest inhibition against SIRT3, whereas the ThAcK inhibitor exhibited the strongest inhibition against SIRT2. Since these two inhibitors have the same peptidomimetic scaffold, their different rank order of sirtuin inhibitory potency suggested that different sirtuin active sites respond differently to the AcK side chain structural variations.

Taken together, in the current study, we have identified TuAcK as another general sirtuin inhibitory warhead which was shown to be able to confer potent sirtuin inhibition. Experiments with SIRT1 suggested that a TuAcK sirtuin inhibitor also behaved as a mechanism-based inhibitor in that the TuAcK residue was also able to be processed by a sirtuin enzyme with the formation of a stalled S-alkylamidate intermediate, like that for ThAcK. Given the potential for a thiourea compound to be a strong anti-oxidant, further development of peptidomimetic or non-peptidic human sirtuin inhibitors containing TuAcK or its homologs should be warranted in the continued search for novel sirtuin inhibitors. For the TuAcK homologs, those bearing alkyl groups (methyl, ethyl, etc.) on the terminal NH_2 group of TuAcK side chain could be considered, given the capability of the sirtuin active site to accommodate bulkier acyl groups than the acetyl group of AcK side chain.³⁹ The study on TuAcK homologs is under way in our laboratory and the corresponding findings will be reported in due course.

Acknowledgments

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59. Synthesis of compound **1** (Scheme 1). (a) The solid phase synthesis part of the synthesis of this compound was performed with the Fmoc chemistry-based solid phase peptide synthesis (SPPS)⁶⁰ at a 0.05 mmol scale on a PS3 peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) from Rink Amide AM resin. For the amino acid coupling reactions, 4 equivalents of N^α-Fmoc-N^ε-Boc-L-lysine or 2 equivalents of N^α-Fmoc-N^δ-acetyl-ornithine (synthesized as described previously³⁷) were used together with 3.8 or 1.9 equivalents, respectively, of the coupling reagent HBTU and the additive HOBt in the presence of 0.4 M NMM/DMF, and the corresponding coupling reaction was allowed to proceed at room temperature for 1h or 2h, respectively. A 20% (v/v) piperidine/DMF solution was used for Fmoc removal. The resin-bound intermediate 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 solution of TFA in DCM (85% v/v). This solution was then evaporated at room temperature on a Rotavapor to dryness. The resulting residue was further dried overnight under high vacuum (oil pump) and used for the next reaction without further purification. (b) To a cooled (ice-water bath) and stirred solution of the above-obtained residue (~ 0.05 mmol) in ddH₂O (373 μL) was added dropwise triethylamine to neutralize the solution, which was followed by the further addition of 28 μL (0.2 mmol) of triethylamine dropwise at 0 °C. A solution of thiophosgene (CSCl₂) (5.7 μL, 0.0744 mmol) in acetone (373 μL) was then added dropwise at 0 °C. After the addition was complete, the reaction mixture was stirred at room temperature for 3 h, and was concentrated to dryness with a Rotavapor. The resulting residue was used for the next reaction immediately. (c) The residue from the previous step was cooled down with an ice-water bath, and to which was added dropwise while stirring 1.5 mL of a 7 N solution of ammonia in methanol. The whole reaction mixture was then stirred at 0 °C for 3 h, and then concentrated to dryness with a Rotavapor. Compound **1** was isolated from the resulting residue by the reversed-phase HPLC on a preparative C18 column (100 Å, 2.14 × 25 cm). The column was eluted with a gradient of ddH₂O 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 compound **1** as a puffy white solid. The purified compound **1** was >95% pure as verified by the reversed-phase HPLC on an analytical C18 column (100 Å, 0.46 × 25 cm). The column was eluted with a gradient of ddH₂O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min and monitored at 214 nm. The exact mass of the purified compound **1** was confirmed by the electrospray ionization

(ESI)-MS analysis with a Bruker Esquire-LC ion trap mass spectrometer at the University of Akron and the high resolution mass spectrometry (HRMS) analysis with an Agilent 6210 LCMS instrument at the high resolution mass spectrometry facility of the University of California, Riverside: MS (ESI) calcd. for $C_{23}H_{43}N_8O_6S$ ($[M + H]^+$) 559.3; found: 559.6. HRMS (Multimode) calcd. for $C_{23}H_{43}N_8O_6S$ ($[M + H]^+$) 559.3026; found: 559.3010. Calcd. for $C_{23}H_{42}N_8O_6SNa$ ($[M + Na]^+$) 581.2846; found: 581.2846. Calcd. for $C_{23}H_{42}N_8O_6SK$ ($[M + K]^+$) 597.2585; found: 597.2561.

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63. **Sirtuin inhibition assay.** The HPLC-based sirtuin inhibition assay was performed as described previously.^{35–38,64} Briefly, a sirtuin inhibition assay solution (50 μ L) 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 MgCl₂, 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: H₂NHK-AcK-LM-COOH; 0.5 mM of the SIRT2 substrate: H₂N-MPSD-AcK-TIGG-COOH; or 0.03 mM of the SIRT3 substrate: H₂N-KRLPKTRSG-AcK-VMRLLLRKII-COOH), a sirtuin enzyme (GST-SIRT1, 350 nM; SIRT2, 150 nM; or SIRT3, 500 nM), and compound **1** with varied concentrations including 0. Of note, the above peptides were also used in our previous studies as the sirtuin substrates.^{64,68} While 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),⁶⁴ SIRT2 (human full length) and SIRT3 (human amino acid 102–399) were purchased from Enzo Life Sciences (Product Nos. BML-SE251-0500 and BML-SE270-0500, respectively). It should be pointed out that 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 for assessing the inhibitory selectivity of compound **1** 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 and the SIRT3 assay) or 60 min (for the SIRT2 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 μ m, 0.46 \times 25 cm), eluting with a gradient of ddH₂O 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 \leq 15%. The stock solution of compound **1** was prepared in ddH₂O. IC₅₀ values were estimated from the Dixon plots (1/v₀ vs. [inhibitor])⁶⁹ as an indication of the inhibition potency.
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65. **Western blot analysis.** This assay was also performed as described previously.^{37,38} HCT116 human colon cancer cells were cultured in McCoy5A culture medium containing 10% FBS with penicillin and streptomycin. HCT116 cells (2×10^5) were treated for 10 h with compound **1** at different concentrations (0, 5, 50, and 250 μ M) 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, 1 mM PMSF, 1x Roche complete protease inhibitor cocktail). 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 for 1 h, 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 anti-mouse IgG-horseradish peroxidase conjugate (diluted 1:5000) 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) overnight at 4 °C.
66. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. *Cell.* 2001; 107:149. [PubMed: 11672523]
67. **Mass spectrometry detection of the catalytic species with compound 1 and SIRT1.** This assay was also performed as described previously.^{37,38} (a) An assay solution (20 μ L) contained the following components: 20 mM pyridinium formate (pH 7.0), 1 mM DTT, 0.5 mM β -NAD⁺, 0.6

mM compound 1, and 4 μ M GST-SIRT1. An enzymatic reaction was initiated by the addition of GST-SIRT1 at 25 °C and was allowed to be incubated at 25 °C for 1 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 3-HPA as the matrix). (b) 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 3-HPA was prepared in acetonitrile/water (1/1 (v/v)) at 5 mg/mL. The sample preparation followed the sandwich method. Briefly, 0.5 μ L 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 SIRT1 assay sample was thawed and 0.5 μ L of it was deposited on the top of a dried matrix spot. Another 0.5 μ L of the matrix solution was then deposited on the top of the air-dried sample spot. Data analysis was conducted with the flexAnalysis software.

68. Robinson, S.J.; Hirsch, B.M.; Jamonnak, N.; Zheng, W. Undergraduate Student Research Poster Session, 41st ACS Central Regional Meeting; May 2009; Cleveland, Ohio, USA.
69. Dixon M. *Biochem J.* 1953; 55:170. [PubMed: 13093635]



Figure 1.

(a) The proposed chemical mechanism for the sirtuin-catalyzed lysine N^ε de(thio)acetylation reaction. When a ThAcK-containing suicide substrate (or mechanism-based inhibitor) (X = S) is used, the corresponding α-1'-S-alkylamidate intermediate is stalled along the reaction coordinate. ADP, adenosine diphosphate; B: refers to a general base. **(b)** Structural comparison of N^ε-acetyl-lysine (AcK) and its analogs.

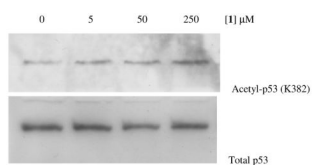


Figure 2. Western blot analysis of the p53 protein acetylation level change in HCT116 human colon cancer cells following the treatment with compound **1**.

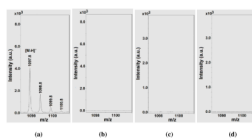
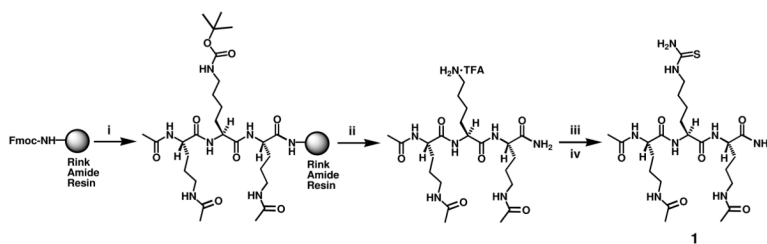


Figure 3. The MALDI-MS analysis of the following samples: **(a)** The assay mixture from compound **1** with SIRT1 and NAD^+ ; **(b)** The assay mixture from compound **1** with NAD^+ in the absence of SIRT1; **(c)** The assay mixture from NAD^+ with SIRT1 in the absence of compound **1**; **(d)** the MALDI matrix 3-hydroxypicolinic acid (3-HPA) only. All the MALDI-MS analyses were performed in a negative reflector mode. See ref. 67 for further experimental conditions.

**Scheme 1.**

Synthesis of the TuAcK-containing compound **1**. Reagents: (i) Fmoc-based SPPS; (ii) 85% (v/v) TFA in DCM; (iii) Thiophosgene (CSCl₂), Et₃N; (iv) 7 N NH₃ in MeOH.

Table 1Sirtuin Inhibition^a

Compound	1	ThAcK ^b	L-ACAH ^c	
SIRT1	89.5 ± 16.1	5.9 ± 1.6 ^d	290.0 ± 88.0 ^e	
IC ₅₀ (μM)	SIRT2	159.1 ± 32.8	2.9 ± 1.3 ^d	190.2 ± 1.7 ^e
	SIRT3	57.2 ± 17.6	4.3 ± 0.1 ^d	213.5 ± 31.2 ^e

^aSee ref. 63 for the SIRT1/2/3 inhibition assay details.

^bThAcK, here referring to CH₃CONH-AcOm-ThAcK-AcOm-CONH₂.³⁷

^cL-ACAH, here referring to CH₃CONH-AcK-(L-ACAH)-AcK-CONH₂.³⁸

^dTaken from ref. 37.

^eTaken from ref. 38.