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N^{ε}-Thioacetyl-lysine: A multi-facet functional probe for enzymatic protein lysine N^{ε}-deacetylation

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Abstract—Peptides containing N^{ε}-thioacetyl-lysine and N^{ε}-acetyl-lysine were evaluated for their de(thio)acetylation catalyzed by human HDAC8 and SIRT1, two distinct protein deacetylases. N^{ε}-Thioacetyl-lysine was found to be a mimic of N^{ε}-acetyl-lysine for HDAC8-catalyzed reaction, but to confer inhibition against SIRT1. These results point to further diverse applications based on N^{ε}-thioacetyl-lysine.

Protein posttranslational lysine N^{ε}-acetylation has been identified in an increasing list of intracellular proteins, such as the core histone proteins, various transcription factors, and α-tubulin that are, respectively, involved in gene transcriptional and cytoskeletal control.[1–3] This type of protein posttranslational modification has emerged as an intracellular signaling mechanism rivaling protein posttranslational phosphorylation on serine, threonine, and tyrosine side chain OH groups. Multiple protein acetyltransferases and protein deacetylases have been identified and shown to catalyze the lysine N^{ε}-acetylation and deacetylation reactions, respectively, on proteins (Fig. 1).[4–6]

Based on homology with yeast transcriptional repressors, phylogenetic analysis, and different cofactor requirements, protein deacetylase enzymes have been categorized into class I, II, III, and IV (HDAC11 and its related enzymes) subfamilies.[5,7] Class I, II, and IV enzymes all require a catalytic zinc (Zn^{2+}), whereas class III enzymes (also named sirtuins) require coenzyme NAD^{+} for activity. The past several years have witnessed significant advances in the fundamental understanding and pharmacological modulation of the protein deacetylation reactions, in part fueled by the observation that several inhibitors of class I and II enzymes have anti-proliferation effects on cancer cell lines.[8,9] Indeed, several small molecule inhibitors for class I and II enzymes are being evaluated in clinical trials for their anti-cancer potentials.[8,9]

Furthermore, various types of enzymatic assays and further small molecule modulators have been developed for many protein deacetylases.[8–16] X-ray crystal structures have also been solved for several protein deacetylases.[6,17–20] Despite these past accomplishments, we are still far from having a complete fundamental understanding of protein deacetylase-catalyzed deacetylation reactions. More research is further warranted given the critical roles played by these enzymes in gene transcriptional and cytoskeletal control, with the aim of further realizing the pharmacological potential through modulating protein deacetylase-catalyzed deacetylation reactions. In this report, we disclose the synthesis and characterization of N^{ε}-thioacetyl-lysine as a multi-facet functional probe for enzymatic protein lysine N^{ε}-deacetylation (Fig. 2). We hypothesized that the thioacetyl group would be a close structural mimic for acetyl group, but would also be able to reveal its own functional uniqueness that can be exploited to develop novel means for studying and modulating protein deacetylase-catalyzed deacetylation reactions.

We set out to test our hypothesis by assessing the possible enzymatic dethioacetylation reaction by protein deacetylases. For our analysis, we chose human HDAC8 (histone deacetylase 8 named after its first discovered protein substrate histone) and human SIRT1

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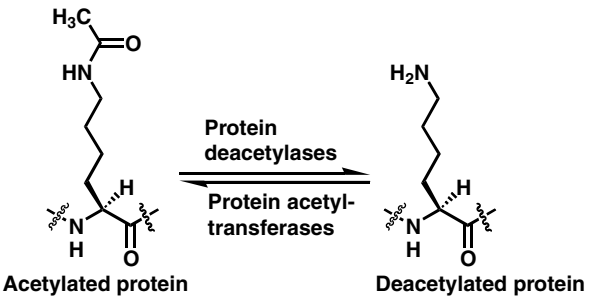


Figure 1. The lysine N^{ε}-acetylation and deacetylation reactions catalyzed, respectively, by protein acetyltransferases and protein deacetylases.

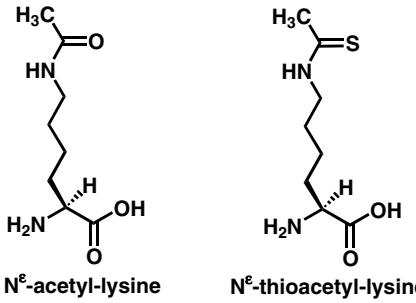


Figure 2. Structural comparison of N^{ε}-acetyl-lysine and N^{ε}-thioacetyl-lysine.

(Sirtuin type 1) as representative members, respectively, from the zinc-dependent and the NAD^+-dependent subfamilies of protein deacetylase enzymes.[5,7] Previous studies have demonstrated that C-terminal lysine N^{ε}-acetylated human tumor suppressor p53 protein could be deacetylated by human HDAC1 and sirtuins including human SIRT1 in vitro and in vivo.[21–25] Furthermore, a peptide derived from the C-terminal region of the human p53 protein (amino acid residue 372–389) N^{ε}-acetylated at Lys382 has been shown to serve as an in vitro substrate for sirtuins including human SIRT1.[24,25] We therefore used this peptide template to incorporate N^{ε}-thioacetyl-lysine into position 382 (peptide **3** in Fig. 3). For this purpose, N^{α}-Fmoc-N^{ε}-thioacetyl-lysine was synthesized as shown in Scheme 1 for its subsequent incorporation into a peptide using Fmoc-chemistry-based solid-phase peptide synthesis (SPPS).[26–28] As indicated in Figure 3, two additional peptides, that is, peptides with N^{ε}-acetylated or non-acetylated Lys residue at position 382, were also synthesized by Fmoc-chemistry based SPPS. Peptide **1** was used as the synthetic authentic de(thio)acetylation peptide product, whereas peptide **2** was evaluated side-by-side with peptide **3**.

Peptides **2** and **3** were first evaluated as potential substrates for human HDAC8. In a typical HDAC8 assay,[29–31] the de(thio)acetylated peptide product (peptide **1**) was formed from both peptides **2** and **3** following a 1-h enzymatic reaction at 37 °C, as shown by the representative HPLC assay chromatograms (Fig. 4a). The absence of signal for peptide **1** above the HPLC reliable detection limit (~1 μM) from non-enzymatic reaction and from a HDAC8 assay with a p53 C-terminal peptide (amino acid residue 372–389) N^{ε}-thioacetylated at Lys381 within the same reaction time frame (data not shown) further corroborated the genuine enzymatic de(thio)acetylation as represented in Fig. 4a. The intriguing finding from our assay is that peptides **2** and **3** were comparably de(thio)acetylated by human HDAC8 to form peptide **1** with estimated k_{obs} being 0.38 min^{-1} and 0.41 min^{-1}, respectively. This result suggests that the thioacetyl group can serve as a functional mimic for the acetyl group for the enzymatic deacetylation reactions catalyzed by HDAC8, and likely by all the zinc-dependent protein deacetylase enzymes, because these enzymes share a highly conserved catalytic domain based on sequence homology.[5,7] We are currently expanding this finding to develop a novel spectrophotometric HDAC assay via quantifying thioacetic acid product released from the enzymatic reaction.

On the other hand, when peptides **2** and **3** were subjected to human SIRT1 assay,[32,33] the deacetylated peptide product (peptide **1**) was obviously formed from peptide **2**, but not from peptide **3** (i.e., no peptide **1** signal above the reliable detection limit of the HPLC assay (~1 μM) was observed) following a 10-min enzymatic reaction at 37 °C, as indicated by the representative HPLC assay chromatograms (Fig. 4b). The k_{obs} for the SIRT1-catalyzed deacetylation of peptide **2** was estimated to be 9.29 min^{-1}. Through more extensive time course analysis of SIRT1-catalyzed dethioacetylation of peptide **3**, the k_{obs} was estimated to be 0.023 min^{-1}. Peptide **3** is thus ~400-fold less efficiently processed by SIRT1 as compared to peptide **2**. Based on the currently available structural and biochemical evidence, a catalytic mechanism has been proposed for sirtuins,[6,19,36–39] in that it is generally believed that the first step along the reaction coordinate is the cleavage of nicotinamide from coenzyme NAD^+ with the formation of a high-energy *O*-alkyl amidate intermediate. In this regard, two factors may have contributed to our observed dramatic difference in SIRT1-catalyzed deacetylation of peptide **2** and dethioacetylation of peptide **3**: (i) the perturbed nicotinamide cleavage upon replacing acetyl with thioacetyl group; (ii) the perturbed catalytic formation of peptide **1** following nicotinamide cleavage upon replacing acetyl with thioacetyl group. To preliminarily address these two possibilities, we used an HPLC assay to monitor the time-dependent enzymatic production of nicotinamide from peptides **2** and **3**. Under the same assay conditions as those for assessing SIRT1-catalyzed de(thio)acetylation reactions, the k_{obs} for the SIRT1-catalyzed nicotinamide production from peptide **2** was estimated to be 5.60 min^{-1}, whereas the k_{obs} for SIRT1-catalyzed nicotinamide production from peptide **3** was estimated to be 0.46 min^{-1}. This 7.4-fold difference in k_{obs}s for nicotinamide formation from peptides **2** and **3** thus suggested that the above second factor may be contributing more for the observed difference in SIRT1-catalyzed deacetylation and dethioacetylation. While further studies are needed to unambiguously account for the observed difference in SIRT1-catalyzed deacetylation and dethioacetylation, our current studies suggested that, due to the close structural similarity between acetyl and thioacetyl moieties, peptide **3** is

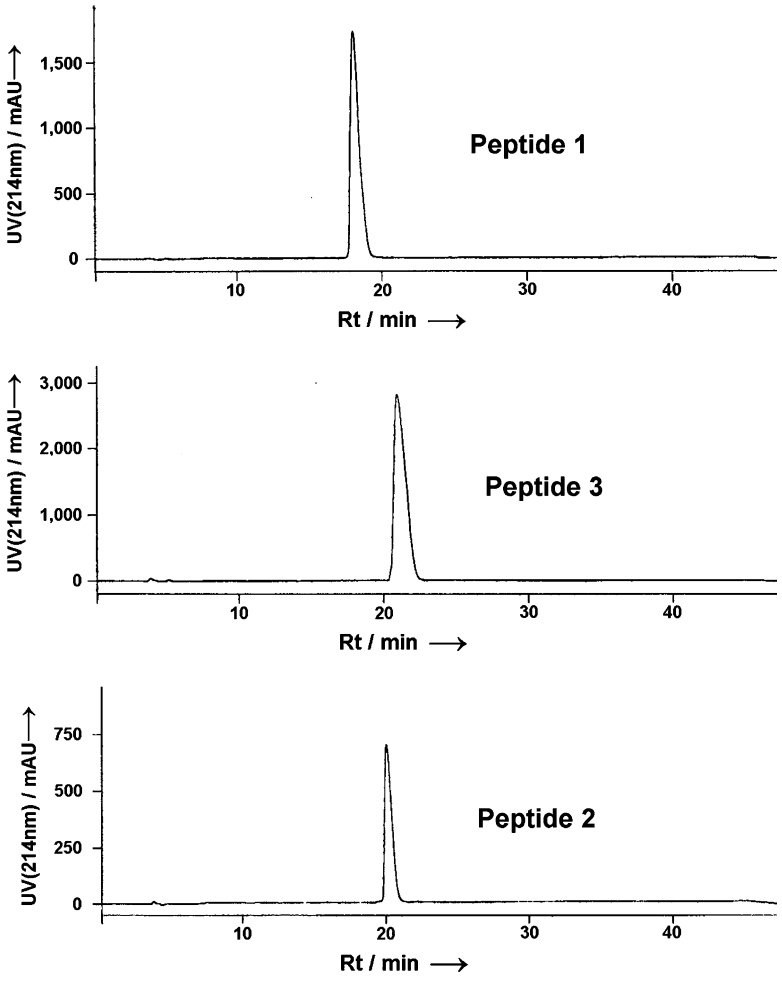
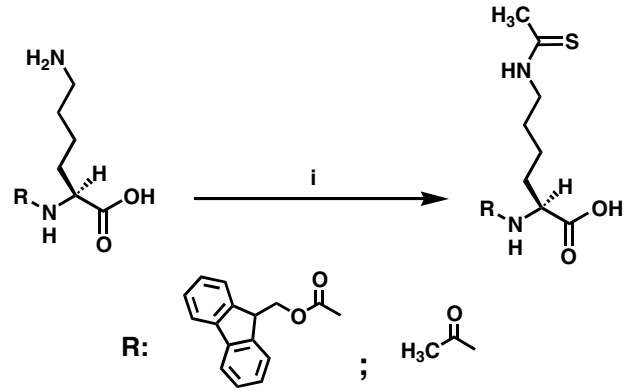


Figure 3. Analytical RP-HPLC profiles for the purified peptides used in this study. Peptide sequences are H_2N-KKGQSTSRHK**X**LMFKTEG-COOH with **X** = Lys (peptide **1**), N^{ε}-acetyl-lysine (peptide **2**), and N^{ε}-thioacetyl-lysine (peptide **3**).



Scheme 1. Reagents: (i) ethyl dithioacetate, ethanol: 5% (w/v) aq Na_2CO_3 (1:1 (v/v)).

able to be processed by SIRT1, but to form a catalytically less competent longer-lived intermediate following the nicotinamide cleavage step along the reaction coordinate, as compared to the normal processing of peptide **2** by SIRT1. Therefore, the N^{ε}-thioacetyl-lysine-containing peptide (e.g., peptide **3**) could be an invaluable biochemical/biophysical probe for dissecting the intermediate events for sirtuin-catalyzed deacetylation reaction.

Peptide **3** was further evaluated as a potential inhibitor for SIRT1-catalyzed deacetylation reaction. As shown in Table 1, peptide **3** was a ~260-fold stronger SIRT1 inhibitor than nicotinamide, a known inhibitor of SIRT1.[40,41] Prompted by this result, we also tested the inhibition potency of two extremely truncated peptide **3** analogs, that is, N^{α}-Fmoc-N^{ε}-thioacetyl-lysine and N^{α}-acetyl-N^{ε}-thioacetyl-lysine shown in Scheme 1.[42] As shown in Table 1, both of these simple compounds behaved as much weaker inhibitors of SIRT1 as compared to peptide **3**. This finding suggested that the amino acid residues surrounding the N^{ε}-thioacetyl-lysine residue contribute to a strong binding interaction with SIRT1, thus enhancing the inhibition potency dramatically. Given the observed potent inhibition of SIRT1 by peptide **3**, its structure–activity relationship (SAR) studies will be of great interest to investigate the structural determinants for the potent inhibition of peptide **3** against SIRT1, aiming to develop potent and novel types of SIRT1 inhibitors. These inhibitors will be not only invaluable chemical tools to study SIRT1 biology, but also potential therapeutic agents for human disorders including cancer.[43]

Taken together, we have identified N^{ε}-thioacetyl-lysine as a multi-facet functional probe for enzymatic protein

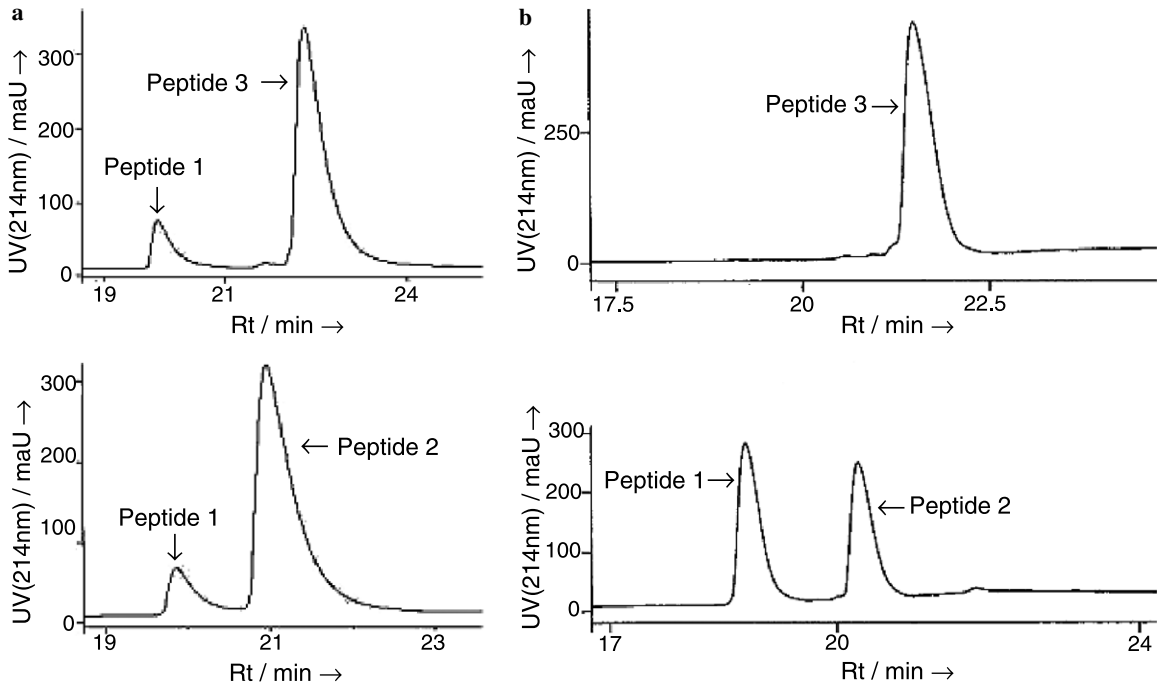


Figure 4. Enzymatic de(thio)acetylation of peptides **2** and **3**. (a) HPLC chromatograms from a HDAC8 assay; (b) HPLC chromatograms from a SIRT1 assay.

Table 1. Inhibition of SIRT1[a]

Compound	IC_{50} (μM)
Nicotinamide	520
Peptide **3**	2
N^{α}-Fmoc-N^{ε}-thioacetyl-lysine	2000 (IC_{25})
N^{α}-Acetyl-N^{ε}-thioacetyl-lysine	No inhibition at 2 mM

[a] Substrate concentrations used, 0.5 mM β-NAD^+, 0.3 mM peptide **2**.

lysine N^{ε}-deacetylation. The use of this novel unnatural amino acid will provide us with opportunities for developing novel HDAC assays and novel types of sirtuin inhibitors, as well as for an enhanced mechanistic understanding of the class III protein deacetylase enzymes. Given the critical roles played by protein deacetylase enzymes in gene transcriptional and cytoskeletal control, as well as in regulating HIV transcription as recently identified,[44] the study reported here will have far-reaching impact. Furthermore, thioacetyl replacement for acetyl group may also be an appealing strategy for studying other deacetylases that accept non-protein substrates.[45]

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26. Synthesis of N^{α}-Fmoc-N^{ε}-thioacetyl-lysine. To a stirred suspension of N^{α}-Fmoc-lysine (368 mg, 1.0 mmol) in EtOH (2.12 mL) was added dropwise at 0 °C a 5% (w/v) aqueous solution of Na_2CO_3 (2.12 mL). Ethyl dithioacetate (126 μL, 1.1 mmol) was then added dropwise at 0 °C. After the addition was complete, the reaction mixture was stirred at room temperature for 5 h before the addition of a 50% (v/v) solution of EtOH in double deionized water (3 mL). The ethanol was removed under reduced pressure and the residual aqueous solution was acidified with 6 N HCl to pH ~1–2 and extracted with dichloromethane. The combined organics were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure, affording an oily residue from which the product was isolated via silica gel column chromatography as an off white solid (302 mg, 71 %): ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.33 (br, 1H, C(=S)NH), 7.97–7.30 (m, 8H, H_{arom}), 5.73 (br d, 1H, J = 6.6 Hz, OC(=O)NH), 4.50–4.05 (m, 4H, fluorenyl H_9, CH_2O, and H_α), 3.59 (br, 2H, CH_2NH), 2.49 (s, 3H, CH_3), 1.89–1.28 (m, 6H, $CH_2CH_2CH_2$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.9 (C(=S)NH), 176.2 (COOH), 156.7 (NHC(=O)O), 143.5 (C_{arom}), 141.3 (C_{arom}), 128.0 (C_{arom}), 127.3 (C_{arom}), 125.1 (C_{arom}), 120.2 (C_{arom}), 67.4 (CH_2O), 53.5 (C_{alpha}), 47.1 (fluorenyl C_9), 46.1 (CH_2NH), 33.9 (CH_2), 32.0 (CH_2), 27.1 (CH_2), 22.8 (CH_3); HRMS (ESI) calcd for $C_{23}H_{26}N_2NaO_4S$ ([M+Na]$^+$) 449.15055; found: 449.14955.
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30. Product data, BIOMOL Research Laboratories, Inc.
31. HDAC8 assay. A typical 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. # A3803 with reduced fatty acid content), 0.3 mM peptide **2** or **3**, and 1.5 μM human HDAC8 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA). An enzymatic reaction was initiated by the addition of enzyme at 37 °C and was allowed to be incubated at 37 °C until quenched at different time points with the following stop solution: 1.0 M HCl and 0.16 M acetic acid. The quenched assay solutions were analyzed by reversed-phase HPLC with a C18 analytical column, eluting with the following gradient of double deionized water containing 0.05% (v/v) trifluoroacetic acid (mobile phase A) and acetonitrile containing 0.05% (v/v) trifluoroacetic acid (mobile phase B): linear increase from 0% B to 50% B from 0–40 min (1 mL/min), and UV monitoring at 214 nm. It was observed that peptides **1**, **2**, and **3** were well separated with this HPLC gradient. The enzymatically formed peptide **1** was confirmed by its comigration with the chemically synthesized authentic peptide **1** and by MALDI-TOF mass spectrometric analysis, and was quantified by HPLC peak integration and comparison with that of synthetic authentic peptide **1**. Under the same assay conditions, no peptide **1** formation above the HPLC reliable detection limit (~1 μM) was observed for non-enzymatic reaction.
32. The SIRT1 assay data presented in this report are from the assays employing GST-SIRT1. However, we have demonstrated the comparable activities of SIRT1 and GST-SIRT1 toward both peptides **2** and **3**. GST-SIRT1 was expressed and purified from *Escherichia coli* as described previously.[21,34,35] Human SIRT1 was purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA.
33. SIRT1 assay. A typical time course assay solution had 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 peptide **2** or **3**, and 1.4 μM human GST-SIRT1 (or GST-free SIRT1). An enzymatic reaction was initiated by the addition of enzyme at 37 °C and was allowed to be incubated at 37 °C until quenched at different time points with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were analyzed by reversed-phase HPLC with a C18 analytical column, as with the HDAC8 assays described above. Under the same assay conditions, no peptide **1** formation above the HPLC reliable detection limit (~1 μM) was also observed for non-enzymatic reaction. For assessing SIRT1-catalyzed nicotinamide production, the C18 analytical column was eluted with the following gradient of double deionized water containing 0.05% (v/v) trifluoroacetic acid (mobile phase A) and acetonitrile containing 0.05% (v/v) trifluoroacetic acid (mobile phase B): linear increase from 0% B to 15% B from 0 to 40 min (1 mL/min), and monitored with UV at 261 nm. The nicotinamide formed was identified and quantified by using the commercially available authentic material. SIRT1 inhibition assay solutions had 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^+, and 0.3 mM peptide **2**, an inhibitor with varied concentrations including 0, 1.4 μM human GST-SIRT1. An enzymatic reaction was initiated by the

addition of enzyme at 37 °C and was allowed to be incubated at 37 °C for 10 min until quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The peptide **1** formation was identified and quantified with analytical reversed-phase HPLC. Stock solutions of peptide **3** and nicotinamide were prepared in double deionized water. Stock solutions of N^{α}-Fmoc-N^{ε}-thioacetyl-lysine and N^{α}-acetyl-N^{ε}-thioacetyl-lysine were prepared in DMSO, so that a final DMSO concentration of 4% (v/v) in an assay solution was obtained. No effect on deacetylase activity was observed at this DMSO concentration. IC_{50} values were estimated as an indication of the inhibition potency.

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