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Novel thiourea-based sirtuin inhibitory warheads

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ABSTRACT

N^{ε}-Thiocarbamoyl-lysine was recently demonstrated by our laboratory to be a potent catalytic mechanism-based SIRT1/2/3 inhibitory warhead, in the current study, among the prepared analogs of N^{ε}-thiocarbamoyl-lysine with its terminal NH_2 mono-substituted with alkyl and aryl groups, we found that N^{ε}-methyl-thiocarbamoyl-lysine and N^{ε}-carboxyethyl-thiocarbamoyl-lysine, respectively, also behaved as strong inhibitory warheads against SIRT1/2/3 and SIRT5, typical deacetylases and deacylase in the human sirtuin family, respectively. Moreover, N^{ε}-methyl-thiocarbamoyl-lysine was found in the study to be a ~2.5–18.4-fold stronger SIRT1/2/3 inhibitory warhead than its lead warhead N^{ε}-thiocarbamoyl-lysine.

Sirtuins are a family of protein N^{ε}-acyl-lysine deacylase enzymes evolutionarily conserved in all the three domains of life.[1–4] These enzymes catalyze a type of deacylation reaction in which the N^{ε}-acyl-lysine substrate is condensed with β-NAD^+ with the formation of the deacylated product, along with another two products, that is, nicotinamide and 2′-*O*-AADPR (Fig. 1). The N^{ε}-acyl groups to be removed range from the simple acetyl group to the bulkier succinyl, glutaryl, and myristoyl groups. This enzymatic reaction represents one way of reversing the installation of the N^{ε}-acyl groups on specific lysine side chains on proteins, which can be accomplished enzymatically or non-enzymatically.[5,6] Another family of protein N^{ε}-acyl-lysine deacylases are metalloenzymes that harbor a catalytically essential Zn^{2+} for the hydrolysis of the N^{ε}-acyl-lysine side chain amide functionality.[7,8]

Since the discovery in 2000 of the protein N^{ε}-acetyl-lysine deacetylase activity for the sirtuin family founding member, that is, the yeast silent information regulator 2 (sir2) protein,[9] there have been ever increasing research endeavors on the sirtuin-catalyzed deacylation reaction, with three primary focuses being (1) the elucidation of its sophisticated catalytic mechanisms,[1,10] (2) the interrogation of its (patho)physiological functions,[11–15] and (3) the exploration of the therapeutic potentials via targeting this enzymatic reaction.[16,17] The ultimate goal of these endeavors would be to develop novel therapeutic agents via targeting (inhibiting or activating) the sirtuin-catalyzed deacylation reaction. Therefore, there has been an active research on developing chemical modulators (inhibitors and activators) for this enzymatic reaction.[1,16,18–21] It is worth noting that such chemical entities would also be valuable chemical biological or pharmacological research tools.

Among the currently existing sirtuin chemical modulators, the catalytic mechanism-based modulators, mostly inhibitors, represent a class of intriguing double-edged compounds in that they have also been instrumental in helping dissect the chemical mechanism of the sirtuin deacylation reaction (Fig. 1).[1,18,22] The current catalytic mechanism-based sirtuin inhibitors are primarily based on the N^{ε}-thioacyl-lysine inhibitory warheads,[1,18,22,23] with N^{ε}-thioacetyl-lysine being the first such warhead that our laboratory and that of Denu developed during 2006–2007.[24,25] In order to address the concern over a thioamide compound's potential cellular toxicity (especially the hepatotoxicity) following its metabolic

Abbreviations: β-NAD^+, β-nicotinamide adenine dinucleotide; 2′-*O*-AADPR, 2′-*O*-acyl-ADP-ribose; IC_{50}, the inhibitor concentration at which an enzymatic reaction velocity is reduced by 50%; SPPS, solid phase peptide synthesis; RP-HPLC, reversed-phase high pressure liquid chromatography; HRMS, high-resolution mass spectrometry.

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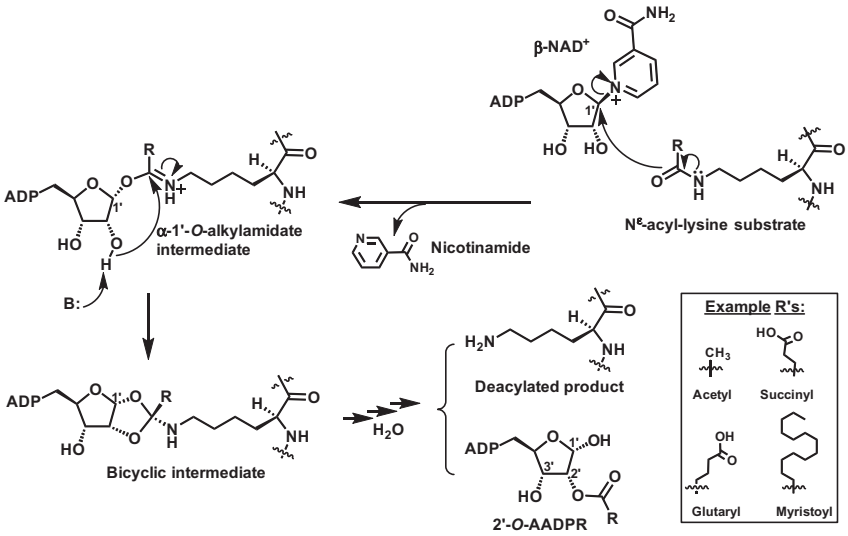

Figure 1. The current version of the chemical mechanism for the sirtuin-catalyzed deacylation of a N^{ε}-acyl-lysine substrate. β-NAD$^+$, β-nicotinamide adenine dinucleotide; 2′-*O*-AADPR, 2′-*O*-acyl-ADP-ribose; ADP, adenosine diphosphate; B: refers to a general base.

S-oxidation and activation thereafter,[26–30] our laboratory previously developed N^{ε}-thiocarbamoyl-lysine as a potent and general SIRT1/2/3 inhibitory warhead, with its catalytic mechanism-based nature directly demonstrated with compound **1** and SIRT1, a typical deacetylase in the human sirtuin family (Fig. 2).[31] It was found in our study that SIRT1 was able to process compound **1** as a substrate yet with the formation of the depicted α-1′-*S*-alkylamidate intermediate which was apparently longer-lived so that it was detectable with mass spectrometry. In order to improve its inhibitory potency and to extend this design concept to other human sirtuin family members, in the current study, we prepared seven analogs of N^{ε}-thiocarbamoyl-lysine with its terminal NH_2 mono-substituted with alkyl and aryl groups (Figs. 3 and 4). Among these analogs, N^{ε}-methyl-thiocarbamoyl-lysine (the central residue in compound **3**, Fig. 3) was found to be also a strong SIRT1/2/3 inhibitory warhead. Moreover, N^{ε}-carboxyethyl-thiocarbamoyl-lysine (the central residue in compound **8**, Fig. 4) was found to be a potent inhibitory warhead against SIRT5, a typical deacylase in the human sirtuin family. The addition of these novel thiourea-based sirtuin inhibitory warheads into the arsenal would facilitate the development of advanced members (being both potent and safe) in the family of the catalytic mechanism-based inhibitors which have become a major type of the inhibitors for the sirtuin-catalyzed deacylation reaction.

Our initial effort in the current study was to improve the inhibitory power of the SIRT1/2/3 warhead N^{ε}-thiocarbamoyl-lysine via introducing alkyl and aryl mono-substituents at its terminal NH_2. The rationale behind this was the previous demonstration that the active sites of those sirtuins with a robust N^{ε}-acetyl-lysine deacetylase activity were also able to accommodate the N^{ε}-acyl groups bulkier than acetyl, such as propionyl, butyryl, and even long-chain fatty acyl groups like myristoyl.[1,18,32] However, unlike the lysine N^{ε}-deacylation of the short-chain fatty acyl groups, that of the long-chain fatty acyl groups seems to be a shared feature of many sirtuins including both the deacetylase members (e.g., SIRT1-3) and the deacylase members (e.g., SIRT5 and SIRT6) in the human sirtuin family. Therefore, the mono-substituents at the terminal NH_2 of N^{ε}-thiocarbamoyl-lysine would not include long-chain fatty acyl groups, instead, methyl and ethyl groups (simple alkyl chain homologation), isopropyl group (alkyl chain branching), and phenyl group (an aryl ring) were employed.

To assess the inhibitory power of the newly designed analogs of N^{ε}-thiocarbamoyl-lysine, they were incorporated as the central residue into a tripeptidic scaffold with N^{ε}-acetyl-lysine at both of its −1 and +1 positions (Fig. 3). This scaffold is one of the two proteolytically stable and cell permeable scaffolds that our laboratory discovered and has been using over the past few years when evaluating the performance of a sirtuin inhibitory warhead, which also

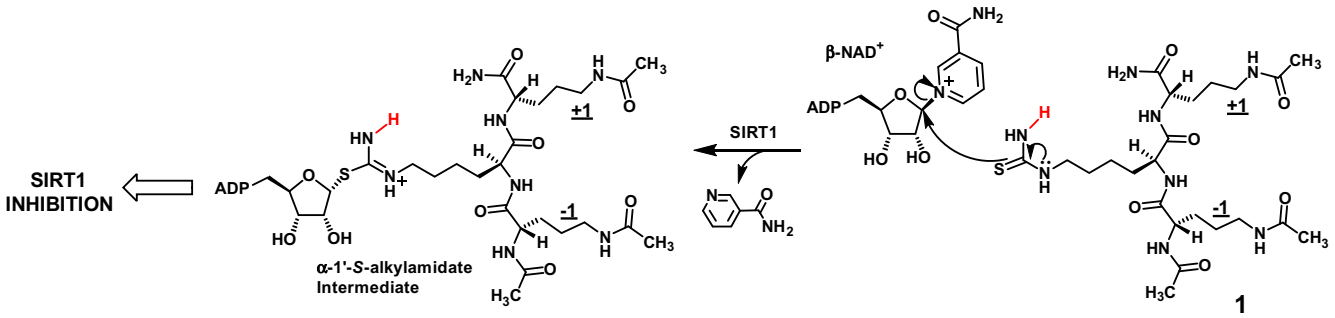

Figure 2. The catalytic mechanism-based SIRT1 inhibition by the tripeptidic compound **1** that harbors the lead inhibitory warhead N^{ε}-thiocarbamoyl-lysine at its central position. SIRT1 was found previously in our laboratory to be able to process compound **1** as a substrate yet with the formation of the longer-lived α-1′-*S*-alkylamidate intermediate.[31] *Note:* the N^{ε}-thiocarbamoyl-lysine-containing compound **1** has N^{δ}-acetyl-ornithine at both −1 and +1 positions.

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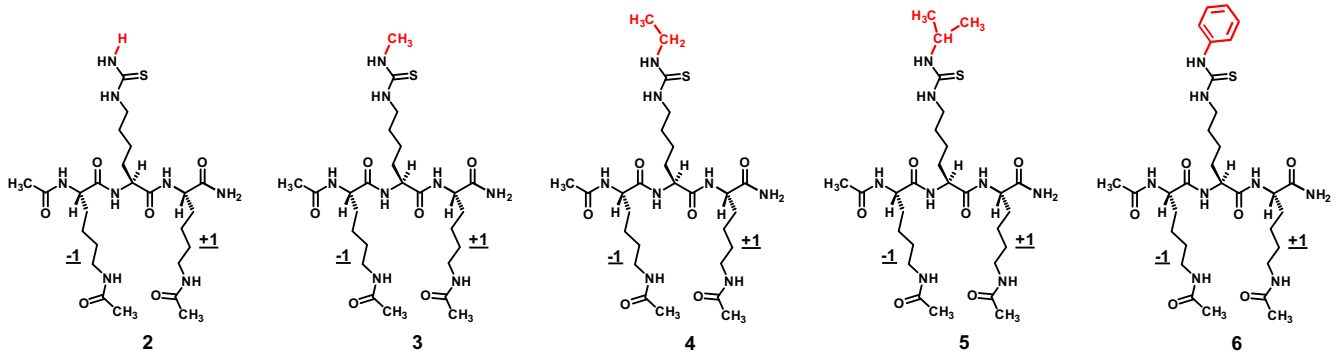


Figure 3. The chemical structures of compound **2** and the initial four analogs of N^{ε}-thiocarbamoyl-lysine embedded in the same tripeptidic scaffold as the central residue. The side chain terminal NH-substituents were colored in red. These analogs were designed to assess their SIRT1/2/3 inhibitory power. *Note:* the N^{ε}-thiocarbamoyl-lysine-containing compound **2** has N^{ε}-acetyl-lysine at both −1 and +1 positions.

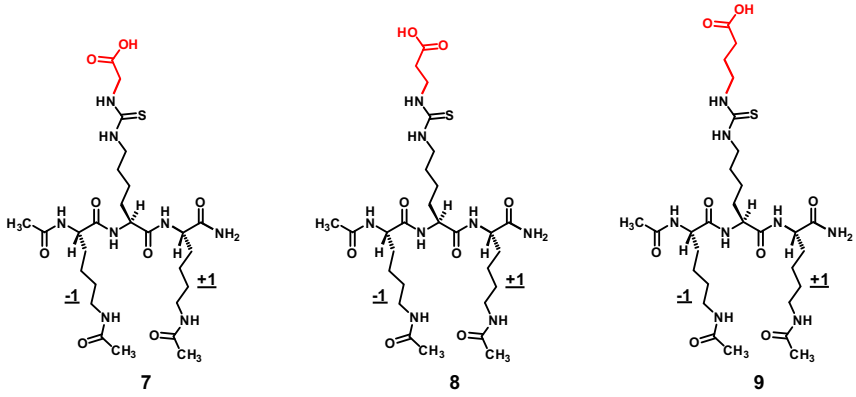
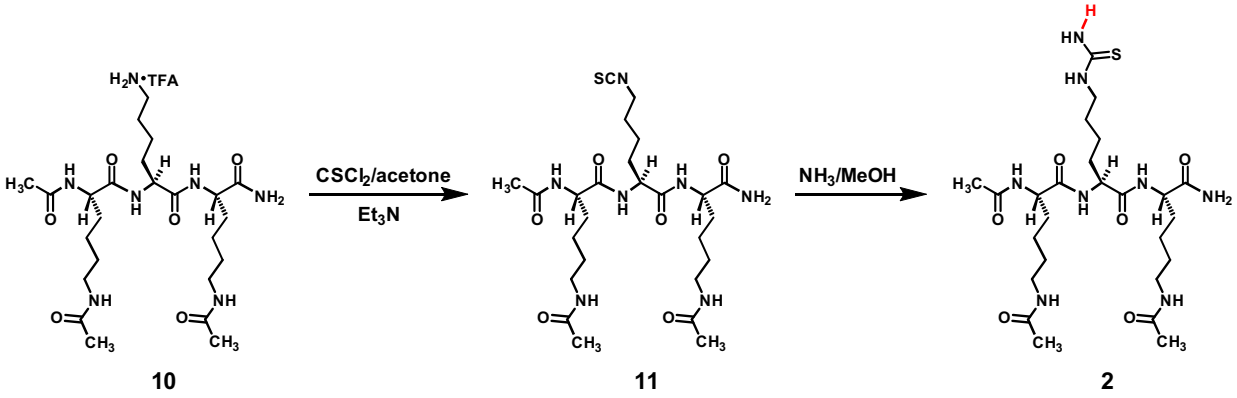


Figure 4. The chemical structures of the three further analogs of N^{ε}-thiocarbamoyl-lysine with their side chain terminal NH-substituents (colored in red) all ending with carboxyl. These analogs were embedded as the central residue in the same tripeptidic scaffold as that in Figure 3, and were designed to primarily assess their SIRT5 inhibitory power.

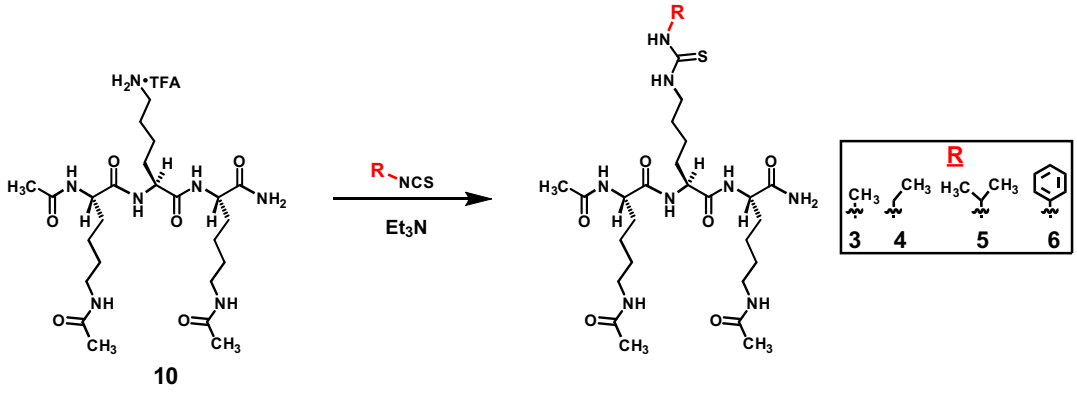


Scheme 1. The synthetic scheme for compound **2**. The tripeptidic compound **10** was obtained from China Peptides Co., Ltd via a custom synthesis order; its purity was ⩾98% and its identity was confirmed by ESI-MS analysis.

includes a similar tripeptidic scaffold but with N^{δ}-acetyl-ornithine at both −1 and +1 positions (the one present in compound **1**).[18] Because it was found previously in our laboratory that the di-acetyl-lysine scaffold was able to confer a stronger SIRT1 inhibition than the di-acetyl-ornithine scaffold,[33] we opt to use the former scaffold in the current study. Accordingly, for a direct sirtuin inhibitory potency comparison with analogs **3**–**6**, we also prepared the analog of compound **1** with N^{ε}-acetyl-lysine at both −1 and +1 positions (i.e., compound **2**, Fig. 3).

The tripeptidic compounds **2**–**6** (Fig. 3) were prepared according to Schemes 1 and 2. They were isolated from the respective reaction mixtures by semi-preparative reversed-phase high pressure liquid chromatography (RP-HPLC), and were shown to be >95% pure based on analytic RP-HPLC analysis. Their identities were confirmed by high-resolution mass spectrometry (HRMS) analysis (Table 1).

When compound **2** was assessed for its SIRT1/2/3 inhibitory potencies, as expected, it exhibited a ∼1.8-fold stronger SIRT1



Scheme 2. The synthetic scheme for compounds **3**–**6**.

Table 1
HRMS analysis of compounds **2**–**9**[a]

Compound	Ionic formula	Calculated *m*/*z*	Observed *m*/*z*
2	$[C_{25}H_{47}N_8O_6S]^+$	587.3339	587.3326
3	$[C_{26}H_{48}N_8O_6SNa]^+$	623.3310	623.3285
4	$[C_{27}H_{50}N_8O_6SNa]^+$	637.3466	637.3476
5	$[C_{28}H_{52}N_8O_6SNa]^+$	651.3623	651.3591
6	$[C_{31}H_{50}N_8O_6SNa]^+$	685.3466	685.3460
7	$[C_{27}H_{48}N_8O_8SNa]^+$	667.3208	667.3187
8	$[C_{28}H_{50}N_8O_8SNa]^+$	681.3365	681.3377
9	$[C_{29}H_{52}N_8O_8SNa]^+$	695.3521	695.3530

[a] All the compounds were measured with the positive mode of electro-spray ionization (ESI).

Table 2
SIRT1/2/3 inhibition by compounds **2**–**6**[a]

Compound	IC_{50} (μM)		
	SIRT1	SIRT2	SIRT3
1[b]	89.5 ± 16.1	159.1 ± 32.8	57.2 ± 17.6
2	49.9 ± 3.68	30.4 ± 3.6	5.4 ± 0.5
3	5.6 ± 3.4	1.65 ± 0.64	2.2 ± 0.07
4	83.4 ± 47.4	N.D.[c]	N.D.
5	117.9 ± 75.6	N.D.	N.D.
6	62.9 ± 5.7	N.D.	N.D.

[a] While the sirtuin inhibition assay details are fully described in 'Supplementary material', the following is the information on the substrate concentration, assay time, and sirtuin concentration, the three key assay parameters employed. [β-NAD^+] used: 0.5 mM for the SIRT1 and SIRT2 assays, 3.5 mM for the SIRT3 assay. [Peptide substrate] used: 0.3 mM, 0.39 mM, and 0.105 mM of H_2N-HK-(N^{ε}-acetyl-lysine)-LM-COOH for the SIRT1, SIRT2, and SIRT3 assay, respectively. [Sirtuin] used: His_6-SIRT1 or GST-SIRT1, 320 nM; His_6-SIRT2, 297 nM; His_6-SIRT3, 369 nM. Enzymatic reaction times used: 10 min for the SIRT1 and SIRT2 assays, 8 min for the SIRT3 assay.

[b] The SIRT1/2/3 inhibitory data for compound **1** were taken from Ref. 31, which were acquired under the same assay condition as that employed in the current study.

[c] N.D., not determined in the current study.

inhibition than compound **1** (Table 2). Moreover, a ~5.2-fold and a ~10.6-fold stronger inhibition, respectively, against SIRT2 and SIRT3 were also observed for **2** than **1**. When compounds **3**–**6** were evaluated for their inhibitory potencies against SIRT1, the three N^{ε}-thiocarbamoyl-lysine analogs, respectively, presented by compounds **4**–**6** (i.e., N^{ε}-ethyl-, N^{ε}-isopropyl-, and N^{ε}-phenyl-thiocarbamoyl-lysine) seemed to be about 1.3–2.4-fold weaker SIRT1 inhibitory warheads than N^{ε}-thiocarbamoyl-lysine. However, N^{ε}-methyl-thiocarbamoyl-lysine presented by compound **3** was found to be an about 8.9-fold stronger SIRT1 inhibitory warhead than N^{ε}-thiocarbamoyl-lysine (Table 2). Therefore, compound **3** was further assessed for its inhibitory potencies against SIRT2 and SIRT3. As can be seen in Table 2, N^{ε}-methyl-thiocarbamoyl-lysine seemed to be an ~18.4-fold and an ~2.5-fold stronger SIRT2 and SIRT3 inhibitory warhead, respectively, than N^{ε}-thiocarbamoyl-lysine. Of note, the above sirtuin inhibitory power differences were inferred from a comparison of the sirtuin inhibitory potencies of analogs **3**–**6** with that of compound **2**.

Since some thiourea compounds were previously reported to be effective scavengers of the superoxide radical anion ($O_2^{\cdot-}$),[34] we were also interested in determining the anti-oxidant potential of N^{ε}-methyl-thiocarbamoyl-lysine whose side chain harbors a thiourea functionality. In the experiment, compound **3** was assessed side-by-side with a N^{ε}-thioacetyl-lysine-containing compound with the same tripeptidic scaffold (shown in Fig. 5). As depicted in Figure 5, while this latter compound did not exhibit a significant anti-oxidant activity against the superoxide radical anion under our experimental condition, with only a ~6% of inhibition at 200 μM of the compound; **3** exhibited a much more significant anti-oxidant activity under the same experimental condition, with a 50% of inhibition at 200 μM of the compound.

Compound **3** was also subjected to a pronase digestion assay to assess its proteolytical stability. As shown in Figure 6, as compared to the linear peptide control, compound **3** was found to be much more proteolytically stable. Specifically, while the control peptide was degraded almost completely within 3 min of incubation with pronase, there was still ~88% remaining for compound **3** even after 60 min of incubation with pronase under the same experimental condition. Of note, pronase was employed in this experiment because it has a very broad substrate specificity due to the presence in it of a variety of different types of the proteases/peptidases.[35]

Compound **3** was further examined for its ability to inhibit intracellular SIRT1. For this purpose, a convenient Western blot analysis[33] that our laboratory established and has been using in the past few years was employed. As shown in Figure 7, treating human colon cancer HCT116 cells with compound **3** at increasing concentrations (0–250 μM) was able to bring about a dose-dependent increase of the p53 protein acetylation at K^{382}, a native deacetylation site of SIRT1. In Figure 7, the immunoreactivity of the total p53 protein blotted with a total-p53 antibody was also included to exclude the possibility that the observed p53 K^{382} acetylation level increase was due to an increase in the total p53 protein level.

Due to the catalytic mechanism (especially the chemical mechanism) conservation for the sirtuin-catalyzed deacylation (including deacetylation)[1,10,18] and the demonstrated (with SIRT1) catalytic mechanism-based nature for the lead warhead N^{ε}-thiocarbamoyl-lysine,[31] we were interested in extending the design

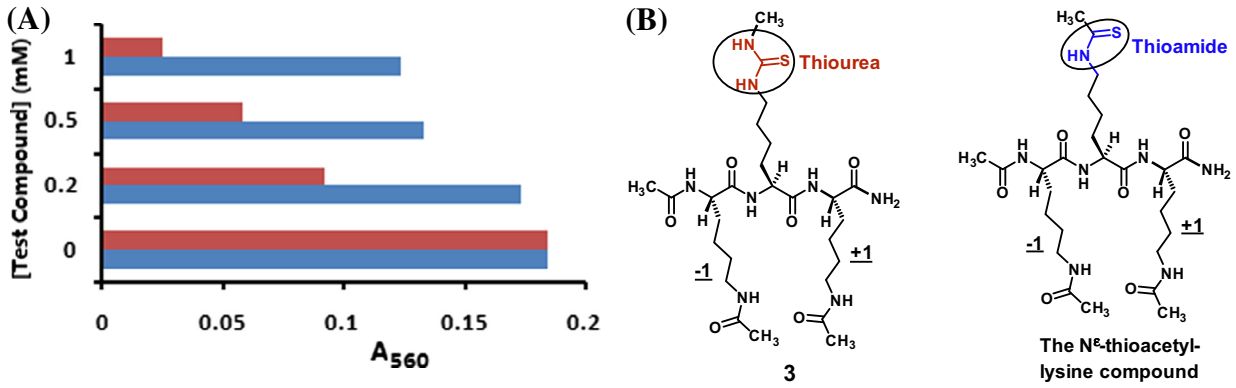


Figure 5. The superoxide radical anion scavenging activities of compound **3** and the depicted N^{ε}-thioacetyl-lysine compound. (A) The bar graph indicating the stronger antioxidant activity of compound **3** than the N^{ε}-thioacetyl-lysine compound. ■, compound **3**; ■, N^{ε}-thioacetyl-lysine compound. See 'Supplementary material' for experimental details. (B) A structural comparison of compound **3** and the N^{ε}-thioacetyl-lysine compound.

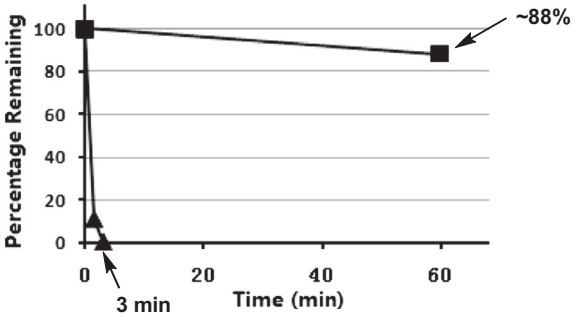


Figure 6. The proteolytic stability of **3**, as assessed by the pronase digestion assay. ■, the degradation profile of **3**; ▲, the degradation profile of the control: H_2N-HK-(N^{ε}-acetyl-lysine)-LM-COOH.

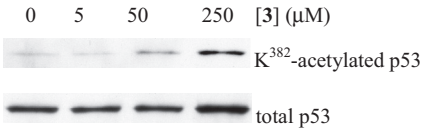
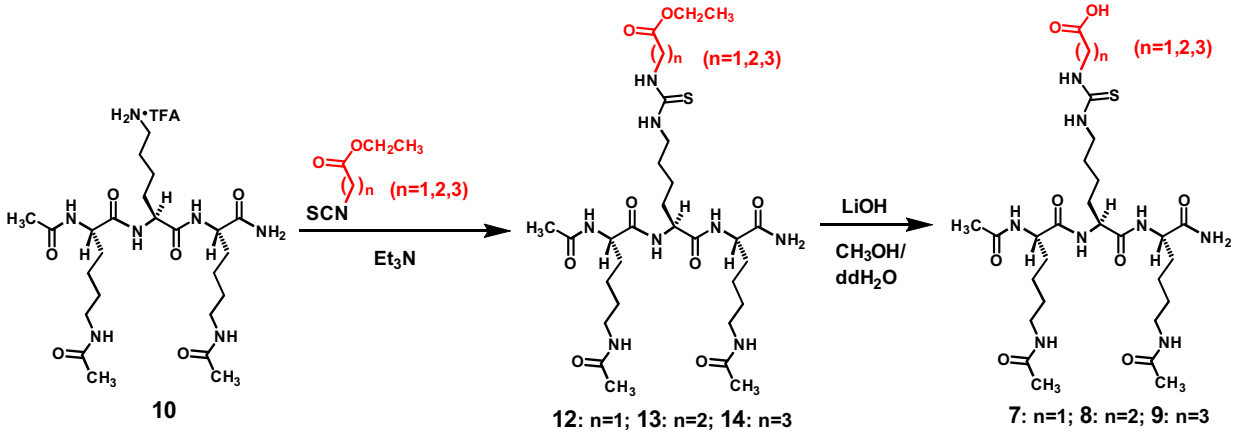


Figure 7. The cell permeability of **3**, as assessed by the Western blot analysis of the p53 protein Lys^{382} acetylation level change in HCT116 human colon cancer cells treated with **3**.

of the thiourea-based sirtuin inhibitory warhead to other members of the human sirtuin family, especially those possessing a strong deacylase activity but a weak deacetylase activity. For this purpose, we picked the typical deacylase SIRT5. Three N^{ε}-carboxyalkyl-thiocarbamoyl-lysines, respectively, embedded in compounds **7**, **8**, and **9** as the central residue (Fig. 4) were designed based on the previous observations that SIRT5 possessed stronger demalonylase, desuccinylase, and deglutarylase activities than its deacetylase activity.[1,3] Of note, compounds **7**–**9** have the same di-acetyl-lysine tripeptidic scaffold as that in compounds **2**–**6**.

The designed tripeptidic compounds **7**–**9** were synthesized according to Scheme 3, isolated from reaction mixtures by semi-preparative RP-HPLC, and were also shown to be >95% pure based on analytic RP-HPLC analysis. The identities of the compounds **7**-**9** isolated were also confirmed by HRMS analysis (Table 1).

When compounds **7**–**9** were evaluated with the human SIRT5, compound **8** was found to be a significantly stronger SIRT5 inhibitor than compounds **7** and **9** (Table 3). Therefore, the central residue of compound **8**, that is, N^{ε}-carboxyethyl-thiocarbamoyl-lysine, would represent a potent inhibitory warhead against SIRT5. It should be pointed out that the intermediate **13** during the synthesis of compound **8** (Scheme 3), that is, the ethyl ester of **8**, was found to be a weak SIRT5 inhibitor with an IC_{50} value greater than 100 μM. This finding reinforced the notion that the free terminal carboxyl in a N^{ε}-succinyl-lysine substrate is necessary for its tight



Scheme 3. The synthetic scheme for compounds **7**–**9**.

Table 3
Sirtuin inhibitory potencies of compounds **7**–**9**[a]

Compound	IC_{50} (μM)		
	SIRT5	SIRT6	SIRT1
7	>100	–	–
8	5.0 ± 1.9	~2400	>100
9	>100	–	–

[a] While the sirtuin inhibition assay details are fully described in 'Supplementary material', the following is the information on the substrate concentration, assay time, and sirtuin concentration, the three key assay parameters employed. [β-NAD^+] used: 0.5 mM for the SIRT1 assay, 0.8 mM for the SIRT5 assay, 0.2 mM for the SIRT6 assay. [Peptide substrate] used: 0.3 mM of H_2N-HK-(N^{ε}-acetyl-lysine)-LM-COOH for the SIRT1 assay, 0.88 mM of CH_3CONH-AR-(N^{ε}-succinyl-lysine)-ST-$CONH_2$ for the SIRT5 assay, 0.02 mM of H_2N-EALPK-(N^{ε}-myristoyl-lysine)-TGGPQ-$CONH_2$ for the SIRT6 assay. [Sirtuin] used: His_6-SIRT1 or GST-SIRT1, 320 nM; GST-SIRT5, 370 nM; His_6-SIRT6, 313 nM. Enzymatic reaction times used: 10 min for the SIRT1 assay, 5 min for the SIRT5 assay, 12 min for the SIRT6 assay.

binding at SIRT5 active site via promoting a salt bridge formation with a catalytically essential arginine side chain.[36]

Since SIRT6 was also recently found to possess a stronger deacylase (demyristoylase) activity than its ability to catalyze deacetylation,[1,3] we were interested in examining whether N^{ε}-carboxyethyl-thiocarbamoyl-lysine could also be an inhibitory warhead against SIRT6 which is another known deacylase member in the human sirtuin family. However, in contrast to its strong inhibition against SIRT5, compound **8** was found to be a weak SIRT6 inhibitor with an IC_{50} value around 2.4 mM (Table 3). Moreover, compound **8** was also found to be a weak inhibitor against SIRT1 with an IC_{50} value greater than 100 μM (Table 3). These findings are consistent with the past observation that the succinyl removal from N^{ε}-succinyl-lysine that N^{ε}-carboxyethyl-thiocarbamoyl-lysine mimics is unique to SIRT5 as compared to SIRT6 and the canonical deacetylase members (SIRT1/2/3) in the human sirtuin family.[32]

To summarize, in the current study, built upon the lead sirtuin inhibitory warhead N^{ε}-thiocarbamoyl-lysine, we identified N^{ε}-methyl-thiocarbamoyl-lysine as a novel thiourea-based SIRT1/2/3 inhibitory warhead that is ~2.5–18.4-fold stronger than N^{ε}-thiocarbamoyl-lysine and possesses a significant anti-oxidant activity against the superoxide radical anion. We have also identified N^{ε}-carboxyethyl-thiocarbamoyl-lysine as a strong and selective (vs SIRT6 and SIRT1) thiourea-based SIRT5 inhibitory warhead.

The availability of these novel warheads would broaden the horizon in developing more effective inhibitors against the therapeutically important sirtuin-catalyzed deacylation reaction as potential therapeutic agents. In addition to being still potent, the class of the thiourea-based warheads would potentially be able to circumvent the cytotoxicity problem associated with the use of the currently most potent warheads N^{ε}-thioacyl-lysines, rendering the class of the sirtuin inhibitors harboring the thiourea-based warheads potentially safer future sirtuin-inhibiting therapeutics. Moreover, the observed strong anti-oxidant activity of the N^{ε}-methyl-thiocarbamoyl-lysine-containing compound **3** in the current study suggested that the thiourea-based sirtuin inhibitors would be protective toward normal cells; however, in the meantime, this anti-oxidant activity could possess an anti-cancer potential according to the literature precedents.[37,38]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.058.

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