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# Peptides and Pseudopeptides as SIRT6 Deacetylation Inhibitors

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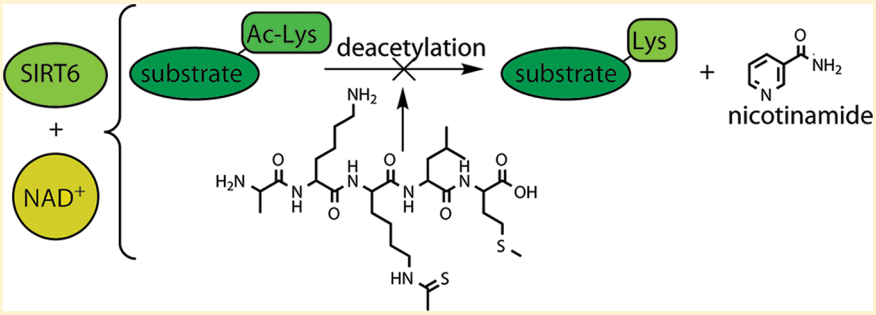
Supporting Information




**ABSTRACT:** SIRT6 belongs to the family of histone deacetylases (class III), but it also has mono-ADP-ribosyltransferase activity. SIRT6 is a nuclear sirtuin that has been associated with aging, cellular protection, and sugar metabolism. Despite these important roles for SIRT6, thus far, there are only a few weak SIRT6 inhibitors available, and no structure−activity relationship (SAR) studies have been published. This is the first study concerning peptides and pseudopeptides as SIRT6 deacetylation inhibitors and the first SAR data concerning SIRT6. We also investigated the molecular interactions using a homology model. We report three compounds exhibiting 62−91% SIRT6 inhibition at 200 $\mu$M concentration. These compounds can serve as starting points for systematic SAR studies and SIRT6 inhibitor design.

**KEYWORDS:** *inhibitor, peptide, pseudopeptide, sirtuin, SIRT6, substrate-based inhibitor*

Sirtuins or class III histone deacetylaces (SIRT1-SIRT7) catalyze the deacetylation of $N^{\varepsilon}$-acetyllysine residues using the cofactor nicotinamide adenine dinucleotide ($NAD^+$). The involvement of sirtuins in fundamental metabolic processes, especially their interesting association with longevity,[1] has triggered the search for sirtuin regulators.[2] Sirtuin isoforms have different subcellular localizations and substrate specificities, and SIRT6 is a nuclear sirtuin with deacetylase and mono-ADP-ribosyltransferase activities, although both activities have been reported to be weak.[3,4] These reactions of SIRT6 are independent of each other, but both are inhibited by the physiological sirtuin inhibitor nicotinamide.[5,6] SIRT6 knockout mice display a phenotype of premature aging and suffer severe metabolic defects,[7] whereas male mice with SIRT6 overexpression enjoy a 15% longer lifespan than their wildtype counterparts.[8] SIRT6 promotes DNA repair,[6,9,10] prevents the side effects of obesity,[11] and protects cardiomyocytes from hypertrophy.[12] All of these benefits are at least partially mediated through SIRT6 deacetylation reactions. SIRT6 modulates the telomeric chromatin structure by deacetylating $N^{\varepsilon}$-acetyllysines 9 and 56 of histone 3 (H3K9Ac and H3K56Ac, respectively).[13−15] It has no activity on at least a dozen other histone tail residues, meaning that SIRT6 might have a high degree of intrinsic substrate selectivity.[13] SIRT6 also possesses deacetylase activity on DNA endonuclease RBBP8 (retinoblastoma-binding protein 8), which promotes DNA resection after DNA damage.[10] Because of its important roles in aging and cell protection, SIRT6 is recognized as an interesting biochemical target. However, only a limited amount of research has been focused on the physiological role of SIRT6. Although activation seems to be the more beneficial mode of SIRT6 regulation, it has been proposed that SIRT6 inhibition could be useful in the treatment of type II diabetes[16] or immune-mediated disorders.[17] Understanding the structure−activity relationship (SAR) of SIRT6 inhibition is extremely important. So far, published regulators of SIRT6 are scarce, and there are no data on the SAR concerning activation or inhibition of SIRT6. In fact, the only published compounds that have some inhibitory activity against SIRT6 are fenugreek seed extract[5] and five small molecules (Chart 1) reported as semispecific inhibitors exhibiting around 25−50% inhibition at 100 $\mu$M.[18]

The aim of this study was to provide the starting point for the development of SIRT6 inhibitors and to conduct the first structure−activity analysis of SIRT6 inhibition. Without any prior knowledge of SIRT6 inhibitors, we chose to study a varied set of peptides and pseudopeptides (Figure 1) that were known to inhibit other sirtuin subtypes, namely, SIRT1 and SIRT2.[19−21] These substrate-based compounds possess an

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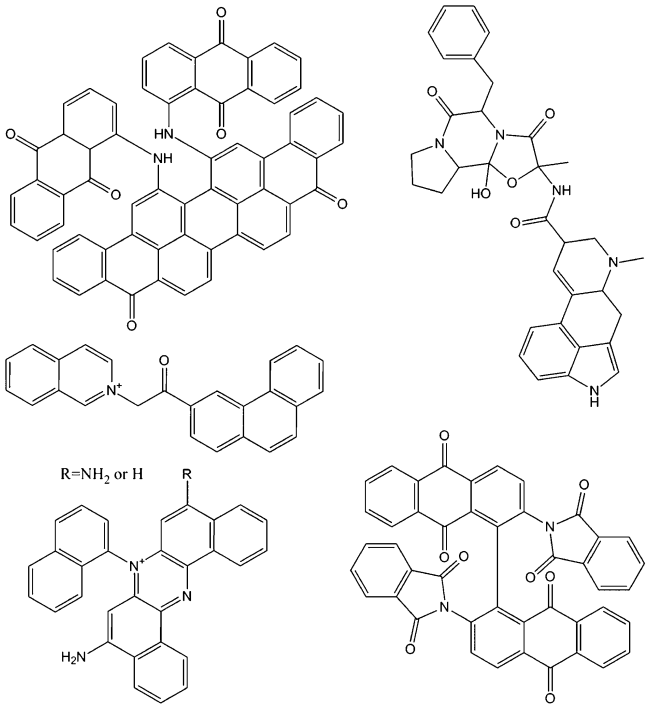
**Chart 1. Previously Published SIRT6 Inhibitors[18]**




$N^{\epsilon}$-modified lysine residue and utilize the conserved $N^{\epsilon}$-acetyllysine binding site.

The compounds were tested in a commercial in vitro SIRT6 deacetylation activity assay (Figure 2; see the Supporting Information for details). At 200 $\mu$M concentration, all compounds were able to inhibit SIRT6 deacetylation activity. The inhibition activity of compounds **2**, **3**, and **14** exceeded 60%, and their $IC_{50}$ values were determined (Table 1). In addition, a SIRT6 homology model, suitable to study the enzyme−inhibitor interactions, was constructed using the substrate-bound SIRT3 crystal complex[22] as the template.

Compounds **1**−**7** form the first compound group. These peptides possess an $N^{\epsilon}$-thioacetyllysine residue that can react with $NAD^+$ to form a thioimidate intermediate.[23] Three of the peptides are pentapeptides (**1**−**3**), which are based on sequences of SIRT1 and SIRT2 substrate p53 (**2** and **3**) or $\alpha$-tubulin (**1**). Compound **1** has a methionine in its carboxy terminus instead of isoleucine in $\alpha$-tubulin, while compounds **2** and **3** differ on their amino terminal properties. Compounds **1**−**3** are all potent SIRT1 and SIRT2 inhibitors,[21] but their ability to inhibit SIRT6 is weaker with inhibition percentages of 91% (**3**), 62% (**2**), and 35% (**1**). The small change in the peptide sequence from changing the histidine (**2**) to alanine (**3**) increased the inhibition percentage from 62 to 91%, although it did not have any effect on SIRT1 or SIRT2 inhibition. Nonetheless, compound **3** seems to be a more potent SIRT1 and SIRT2 inhibitor; the $IC_{50}$ values for SIRT1,[21] SIRT2,[21] and SIRT6 are 0.38, 8.5, and 47 $\mu$M, respectively.

We also studied a set of shorter peptides (**4**−**7**). Compound **4** has a *tert*-butoxycarbonyl (boc)-protected amino terminus, whereas compounds **5** and **6** have acetylated amino termini. Compound **7** is an unprotected tripeptide with D-phenylalanine in the amino terminus. Among the shorter peptides, compound **5** exhibited the highest SIRT6 inhibition, 54%. This may indicate that longer peptides with the correct set of side chains are beneficial for the binding to SIRT6, as two of the pentapeptides were more potent. In addition, replacement of the acetyl group (**5**) by a boc group (**4**) did not improve inhibition activity, and also, the *N*-Ac-proline (**6**) displayed decreased inhibition activity as compared to the *N*-Ac-alanine (**5**). Both boc and proline groups are sterically demanding, bulky, and hydrophobic groups, which may be the reason for the weaker binding of compounds **4** and **6**. Tripeptide **7** was also less active than compound **5**, which may be due to the shorter sequence or the unnatural D-phenylalanine. Interestingly, compound **7** seems to exhibit selectivity toward SIRT1.

The second compound group consisted of peptides with different $N^{\epsilon}$-modifications on the lysine residue (**8**−**10**). Compound **8** with a bulky and lipophilic 3,3-dimethylacrylic moiety caused only low 26% inhibition. An increase in flexibility by removal of the double bond (**9**) did not increase the SIRT6 inhibitory activity, which differs from the situation with SIRT2, where the increase in flexibility clearly improved binding. The most active compound in this group was compound **10** with an isothiovaleryl moiety, indicating that also SIRT6 inhibition is improved by thioamide groups as has been shown for other sirtuins.[24] However, compound **10** was slightly less active than compound **5** with the smaller thioacetyl group. These results indicate that SIRT6 may be more sterically limited in the hydrophobic $N^{\epsilon}$-acetyllysine binding pocket than SIRT1 or SIRT2. This is also supported by structural data; SIRT6 has a tryptophan residue in the binding site where as the corresponding residue in SIRT1 and SIRT2 is phenylalanine, which can cause their binding sites to have more space and may provide a way to gain selectivity between different sirtuin subtypes.

The third compound group consisted of pseudopeptides (**11**−**16**). Pseudopeptides **11** and **12** share an alanine residue in their carboxy termini, but they do not have natural amino acid residues in their amino termini. Interestingly, despite the diverse structures in their amino termini, these pseudopeptides displayed almost equipotent inhibitory activity to compound **5**. This implies that SIRT6 allows diverse structures in the amino terminuses of its inhibitors. The bulky *N*-boc-piperidin-3-carbonyl group of pseudopeptide **11** is sterically demanding. However, this pseudopeptide is more active than peptides **4** and **6**, which also have large bulky groups, suggesting that SIRT6 might be sensitive to the shape of the group. In addition, pseudopeptide **12** with 3-phenylpropanoyl group exhibited better inhibitory activity than peptide **7** with D-phenylalanine group, further supporting this view. Apparently, the chiral center in compound **7** distorts the phenyl group into an unfavorable position, whereas the freely rotatable phenyl ring of compound **12** can find more a optimal binding conformation.

Pseudopeptides **13**, **15**, and **16** share benzyloxycarbonyl (cbz)-protected $N^{\epsilon}$-thioacetyllysine that has been coupled with different groups at the carboxy terminus. The placement of the C-terminal ring system seems to affect the binding because compound **13** with an extended carboxy terminal was a more potent inhibitor than compounds **15** and **16**. The change from $N^{\epsilon}$-thioacetylation of compound **15** (36%) to $N^{\epsilon}$-selenoacetylation of compound **14** (62%) increased the inhibitory activity significantly. This change was also observed with SIRT1 and SIRT2.[20] Although closely related, sulfur and selenium have slightly different electrostatic properties and sizes, which may be responsible for the differences in the inhibition activity of compounds **14** and **15**. Interestingly, the extent of SIRT6 inhibition did not significantly differ between compounds **15**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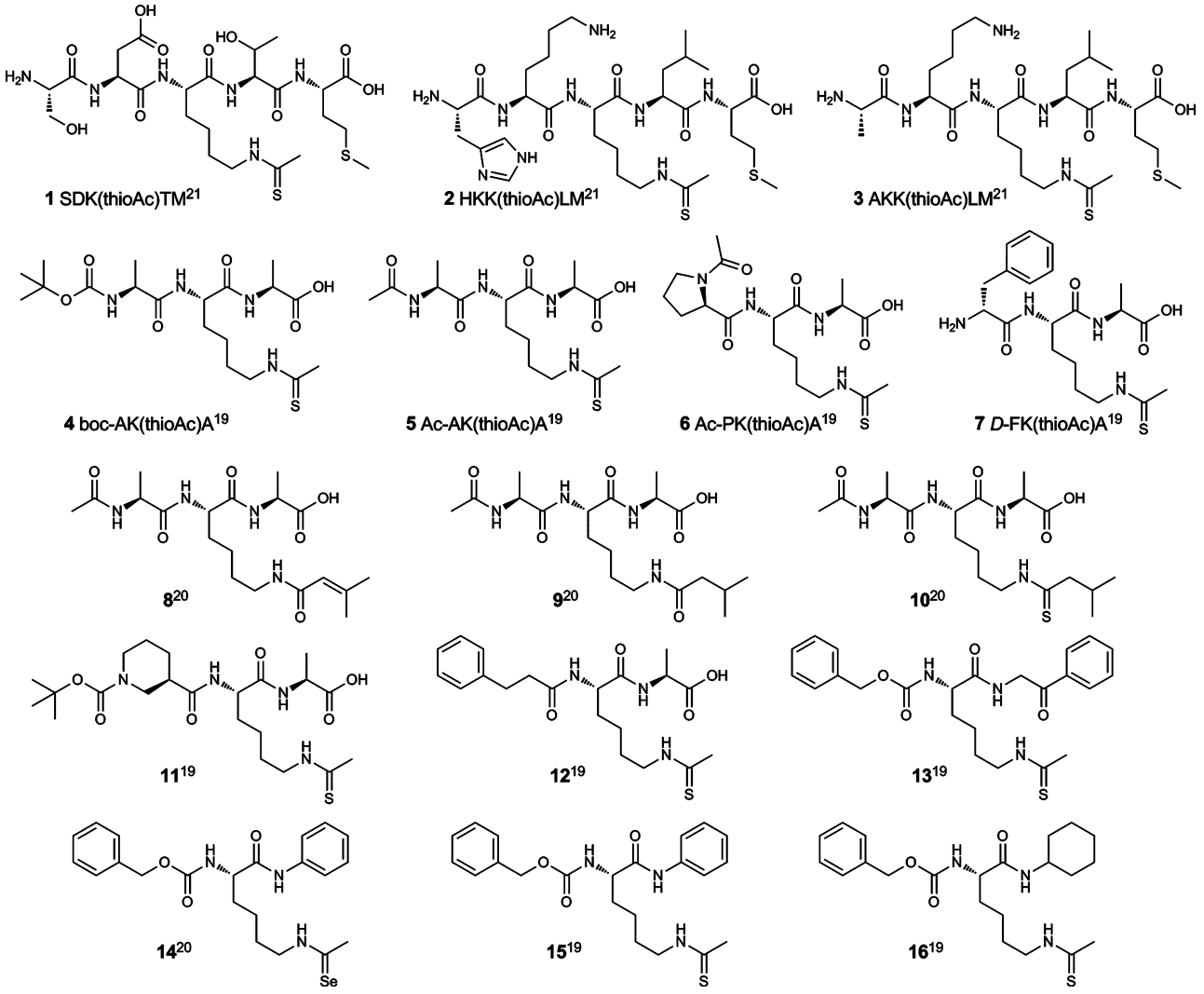


**Figure 1.** Structures of the compounds that were studied for their ability to inhibit SIRT6.

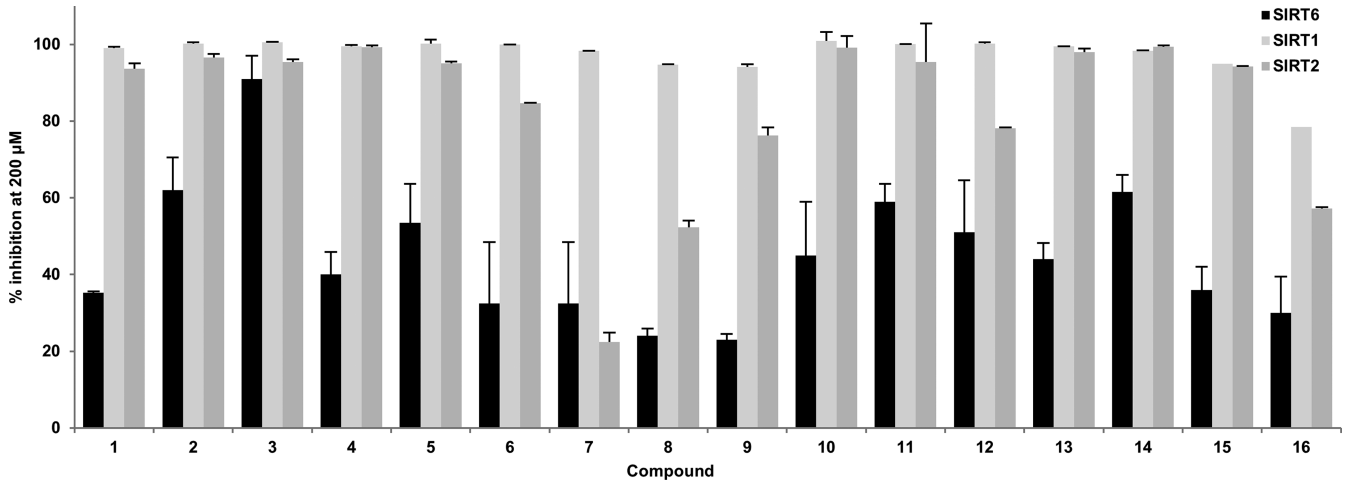


**Figure 2.** Inhibition activity of the tested compounds against SIRT6, SIRT1, and SIRT2, including the standard deviation. All measurements were performed using a Fluor de Lys assay and repeated at least three times. The data for SIRT1 and SIRT2 were first reported in our previous studies.[19−21]

and **16**, when the phenyl group had been replaced by a cyclohexyl group. This change reduced the inhibitory activity against SIRT2 and, to a lesser extent, against SIRT1.[19] The phenyl ring and cyclohexyl have distinctive electrostatical properties, but it seems that neither of these structures can undertake favorable interactions with SIRT6.


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**Table 1. $IC_{50}$ Values of Compounds with Inhibitory Activity Exceeding 60% at 200 $\mu$M**

| compd | $IC_{50}$ ($\mu$M)[a] |
|---|---|
| **2** | 78 (57−106) |
| **3** | 47 (34−60) |
| **14** | 285 (202−368) |

[a]The measurements were performed with a Fluor de Lys assay. The assay was performed twice, each time in triplicate, and $IC_{50}$ represents the average of these six measurements. The 95% confidence intervals are in parentheses.

Similar to the other sirtuins, SIRT6 contains a conserved catalytic core: a Rossmann fold domain, which binds $NAD^+$ and a smaller zinc-binding domain (Figure 3). These domains are

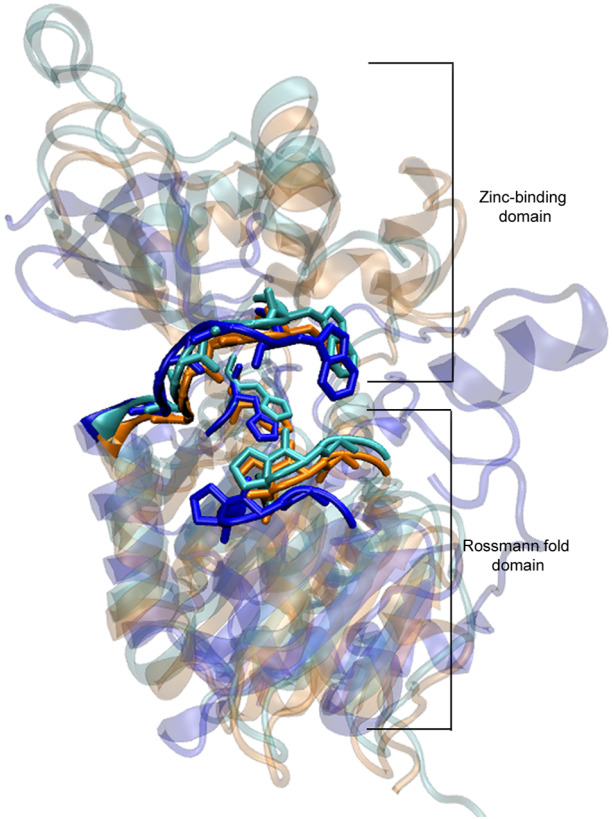


**Figure 3.** SIRT6 crystal structure[3] (3PKJ, blue), SIRT3 crystal structure[22] (3GLR, orange), and the constructed homology model of SIRT6 (cyan) superimposed to demonstrate the differences between structures. The highlighted area consists of Trp187−Asp193, His131, and Ile217−Pro219 (SIRT6 3PKJ numbering) and shows the substrate binding site between the two domains of the enzyme. The picture was generated using the Visual Molecular Dynamics[25] (VMD) software version 1.9.2.

connected with several loops, and the substrate binding region is in a cleft between the two domains. The backbone of the substrate peptide forms $\beta$-sheetlike interactions with both domains (Figure 4), which is called the $\beta$-staple binding.

The crystal structure of SIRT6[3] (Figure 3, blue) has a larger cleft between the two domains than the crystal structure of SIRT3 with a bound peptide[22] (Figure 3, orange). This open conformation is also seen in other apo sirtuin structures, which are not suitable for molecular docking studies,[19] because it prevents the formation of the hydrogen-bonding network.

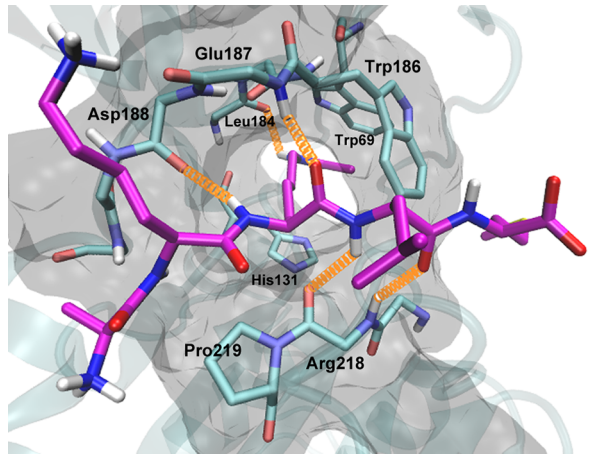


**Figure 4.** Compound **3** showing the $\beta$-staple binding conformation with the homology model of SIRT6. For clarity, only hydrogen bonds depicting the $\beta$-staple binding conformation are shown. The picture was generated using the VMD[25] software version 1.9.2.

Binding of the substrate causes the two domains to move closer to one another,[26] thus enabling the hydrogen-bonding network. The crystal structure of SIRT6 also has a splayed zinc-binding domain, and it is possible that the conformation is not optimal for the deacetylation reaction[3] (see the Supporting Information). Therefore, we proceeded to build a homology model using the ORCHESTRAR tool in Sybyl-X[27] version 1.2 (see the Supporting Information for details).

The interactions between the substrate-mimicking compounds and the SIRT6 were studied with all tested compounds being docked in the homology model of SIRT6 using Glide SP version 5.7.[28] The compounds, except compounds **1** and **14**, were successfully docked and displayed the $\beta$-staple binding conformation seen in Figure 4, forming at least hydrogen bonds with Leu184, Glu187, and carbonyl oxygen of Arg218 (see the Supporting Information for individual pictures). For compound **1**, no docking poses were returned in the output file of the docking, and compound **14** displayed distorted binding conformation, most probably due to the missing selene parameters.

Pentapeptides **2** and **3** exhibited multiple plausible $\beta$-staple binding conformation-containing poses, with varying side chain interactions. Compounds **3** and **2** possess a nonpolar methionine at position +2 (calculating from the $N^{\varepsilon}$-acetyllysine, the second residue toward the carboxy terminus). This methionine was not able to undergo favorable hydrophobic interactions because of hydrophilic Arg218 in SIRT6 and was thus flanking outside the binding site.

The hydrophobic boc group of compound **4** was positioned near hydrophilic residue Ser190, impairing the formation of hydrogen bond to Asp188, which may explain the weaker inhibitory activity as compared to compound **5**.

In addition to the hydrogen bonds with Leu184, Glu187, and the carbonyl oxygen of Arg218, compound **5** possessed an additional hydrogen bond between the carboxy terminus and the Arg218. Increasing conformational rigidity with the proline residue in compound **6** did not allow optimal interactions during the binding; therefore, compound **6** could not form a hydrogen bond with Arg218. Although compound **7** did possess an additional hydrogen bond between its amino terminal and Glu187 due to D-phenylalanine, the phenyl group could not undertake favorable interactions.


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Compounds **8**–**10** displayed a similar hydrogen-bonding pattern as compound **5**. The bulky and hydrophobic $N^{\epsilon}$-modifications of these compounds are close (<4 Å) to the Trp69 at the back of the $N^{\epsilon}$-acetyllysine binding pocket and may thus not properly fit in this hydrophobic pocket.

The bulky piperidine derivative of compound **11** protruded away from the enzyme and turns the boc group near the backbone of the zinc-binding domain. Instead, the amino terminal phenyl groups of compounds **12** and **13** were positioned in the cleft between the two domains of SIRT6. The carboxy terminal phenyl group of compound **13** could reach to form $\pi$–$\pi$ interactions with Trp186, but this was not observed in the docking results. Instead, the phenyl ring is protruding away from the enzyme.

The carboxybenzyl moiety of compounds **15** and **16** did not allow the formation of hydrogen bond to Arg218 (Figure 4). The phenyl ring of **15** and the cyclohexyl ring of **16** shared a similar position in space, slightly protruding away from the enzyme. The backbone of compound **16** was too short to form $\pi$–$\pi$ interactions between the phenyl group and the Trp186.

In conclusion, we have shown that peptides and pseudopeptides can inhibit the deacetylation reaction of SIRT6; the most potent compound had a 47 $\mu$M $IC_{50}$ value. In general, the potency of the studied compounds was lower toward SIRT6 than toward SIRT1 or SIRT2. It is possible that the weak deacetylation activity of SIRT6 combined to the high substrate selectivity sets challenges to the substrate-based approach. It also seems that SAR of SIRT6 does not slavishly follow the SAR of SIRT1 or SIRT2. While the SIRT6 inhibitory potency of the best compounds reported is still rather weak, this SAR study offers a starting point for optimization work to obtain compounds with improved potency, which are needed for studies on the physiological role of SIRT6.

## ■ ASSOCIATED CONTENT

### Supporting Information

In vitro assay and molecular modeling methods, the pictures, and the PDB files of the docking poses. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

Ac, acetyl; boc, *tert*-butoxycarbonyl; cbz, carbonyloxybenzyl; $NAD^+$, nicotinamide adenine dinucleotide; SAR, structure–activity relationship; SIRT1–7, human silent informatory regulator 1–7

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## ■ NOTE ADDED AFTER ASAP PUBLICATION

This paper published ASAP on October 26, 2012 with an error in Figure 1. The corrected version was reposted on November 12, 2012.