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Bivalent SIRT1 inhibitors

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Abstract

In the current study, bivalent compounds **1**-**17** constructed by covalently linking the ε-amino group of N^{ε}-acetyl-lysine in a tripeptidic scaffold to a functionality *via* a linker were prepared and examined for their inhibitory potencies against SIRT1, a prototypical member of the β-nicotinamide adenine dinucleotide (β-NAD^+)-dependent sirtuin family of protein N^{ε}-acyl-lysine deacylases. A few of them were found to be stronger SIRT1 inhibitors than the N^{ε}-acetyl-lysine-containing monovalent counterparts **18** and **19**. As exemplified with compounds **6** and **18**, a bivalent SIRT1 inhibitor could exhibit a greater degree of inhibitory selectivity among SIRT1/2/3 than the corresponding monovalent counterpart. This study has laid a foundation for the future development of superior bivalent inhibitors against the (patho)physiologically and therapeutically important sirtuin family of deacylase enzymes.

Key words: bivalent, SIRT1, sirtuin, inhibitor.

Abbreviations:

1. β-NAD^+, β-nicotinamide adenine dinucleotide

2. NAM, nicotinamide

3. 2'-*O*-AADPR, 2'-*O*-acyl-ADP-ribose

4. RP-HPLC, reversed-phase high performance liquid chromatography

5. HRMS, high-resolution mass spectrometry

6. EDC-MeI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide

7. DIC, N,N'-diisopropylcarbodiimide

8. HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate

9. HOBt, N-hydroxybenzotriazole

10. NMM, N-methylmorpholine

11. EDC-HCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

12. TFA, trifluoroacetic acid

13. IC_{50}, the inhibitor concentration at which an enzymatic reaction velocity is reduced by 50%

14. DIPEA, N,N-diisopropylethylamine

The sirtuins refer to a family of intracellular enzymes able to catalyze the protein N^{ε}-acyl-lysine deacylation in a β-nicotinamide adenine dinucleotide (β-NAD^{+})-dependent manner (**Figure 1**).[1,2] Sirtuins are evolutionarily conserved ancient enzymes and are found in both prokaryotes and eukaryotes.[3] In eukaryotes, while the original native sirtuin substrates identified were histone proteins, a large number of non-histone proteins have also been identified later on as native sirtuin substrates.[4] These non-histone sirtuin substrates are not only found in nucleus where histone proteins also reside, but also found in cytoplasm and mitochondrion. Moreover, all the currently know eukaryotic sirtuins also reside in these three intracellular compartments.[5] These observations are consistent with the increasingly demonstrated importance of the sirtuin-catalyzed deacylation reaction in regulating not only nuclear events (e.g. transcription, DNA damage repair) but also cellular events occurring in cytoplasm and mitochondrion (primarily metabolism).[6-8] The sirtuin-catalyzed deacylation reaction has also been regarded as a therapeutic target for human diseases including cancer, metabolic diseases, and neurodegeneration.[9-11] Therefore, the chemical modulators (inhibitors and activators) for the sirtuin-catalyzed deacylation reaction have been actively pursued during past few years,[1,9,12-14] with a hope of developing novel therapeutic agents for the above-mentioned diseases. A potent, selective, metabolically stable, and cell permeable chemical modulator for the sirtuin-catalyzed deacylation reaction can also be employed to further explore the therapeutic potentials of this enzymatic reaction. To facilitate the exploration and exploitation of this enzymatic reaction, developing its effective chemical modulators with the afore-mentioned calibers seems to represent a bottleneck step.

In terms of the development of sirtuin inhibitors, chemical library screening and

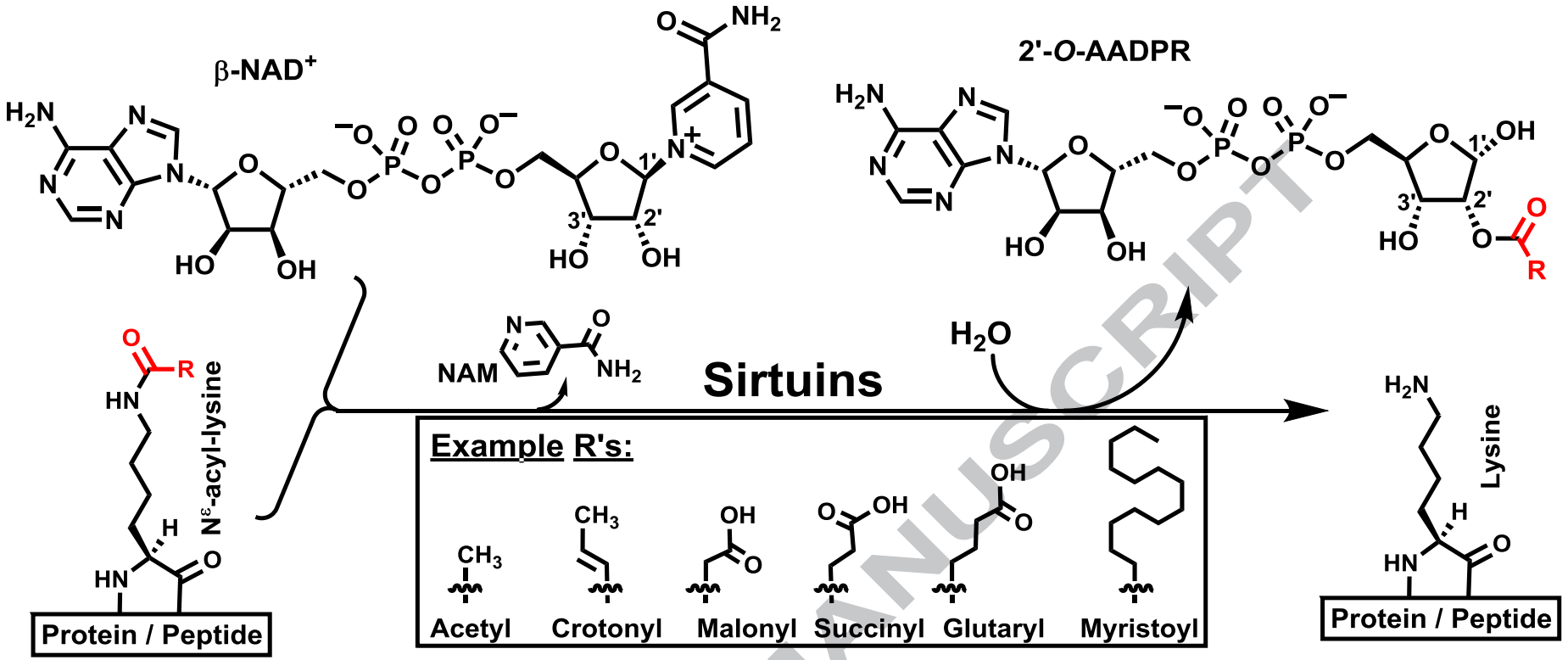


Figure 1. The sirtuin-catalyzed β-NAD$^+$-dependent N$^\varepsilon$-acyl-lysine deacylation reaction. β-NAD$^+$, β-nicotinamide adenine dinucleotide; NAM, nicotinamide; 2'-*O*-AADPR, 2'-*O*-acyl-ADP-ribose.

catalytic mechanism-based design have been two primary approaches employed, with the identification of a few fairly potent and/or selective inhibitors.[15-23] As an effort in developing a novel avenue for sirtuin inhibitor design, in the current study, we performed a preliminary study on

bivalent compounds as for their sirtuin inhibitory efficacy. We found that this design was able to furnish strong bivalent inhibitors against the deacetylation reaction catalyzed by the prototypical sirtuin SIRT1, which were also stronger than their monovalent counterparts. We further found that a bivalent SIRT1 inhibitor could also exhibit a greater inhibitory selectivity among SIRT1/2/3 than the corresponding monovalent counterpart.

Our design of the bivalent sirtuin inhibitors was inspired by the observed presence of an open space in sirtuin 3-dimensional structures that extends from the side chain of the bound substrate's N^{ε}-acetyl-lysine residue (depicted in **Figure 2A**).[24,25] We reasoned that, if a functionality (R) is covalently anchored onto a N^{ε}-acetyl-lysine substrate *via* a linker, as depicted in **Figure 2B**, the resulting bivalent compound could be a stronger sirtuin inhibitor than a N^{ε}-acetyl-lysine-containing inhibitor (a monovalent counterpart) if the linker and R could also be accommodated at a sirtuin active site. This potency enhancement would be due to a decreased entropic penalty associated with the binding of a bivalent compound to a sirtuin active site, as compared with the binding of its monovalent counterpart. Also from the depiction in **Figure 2A**, the binding of the linker and R of a bivalent compound could also prevent β-NAD^+ from binding to the same sirtuin active site, due to a steric clash with the nicotinamide moiety of β-NAD^+. Therefore, such a bivalent compound would not be deacylated by a sirtuin. It should be noted that, except for the different conformations of the cyclopentane ring of carba-NAD^+ (depicted in **Figure 2A**) and the N-ribose of β-NAD^+ bound in a Sir2Tm/β-NAD^+/N^{ε}-acetyl-lysine substrate Michaelis complex, the binding modes of carba-NAD^+ and β-NAD^+ are similar, especially concerning the nicotinamide and adenine moieties of these two molecules.[26]

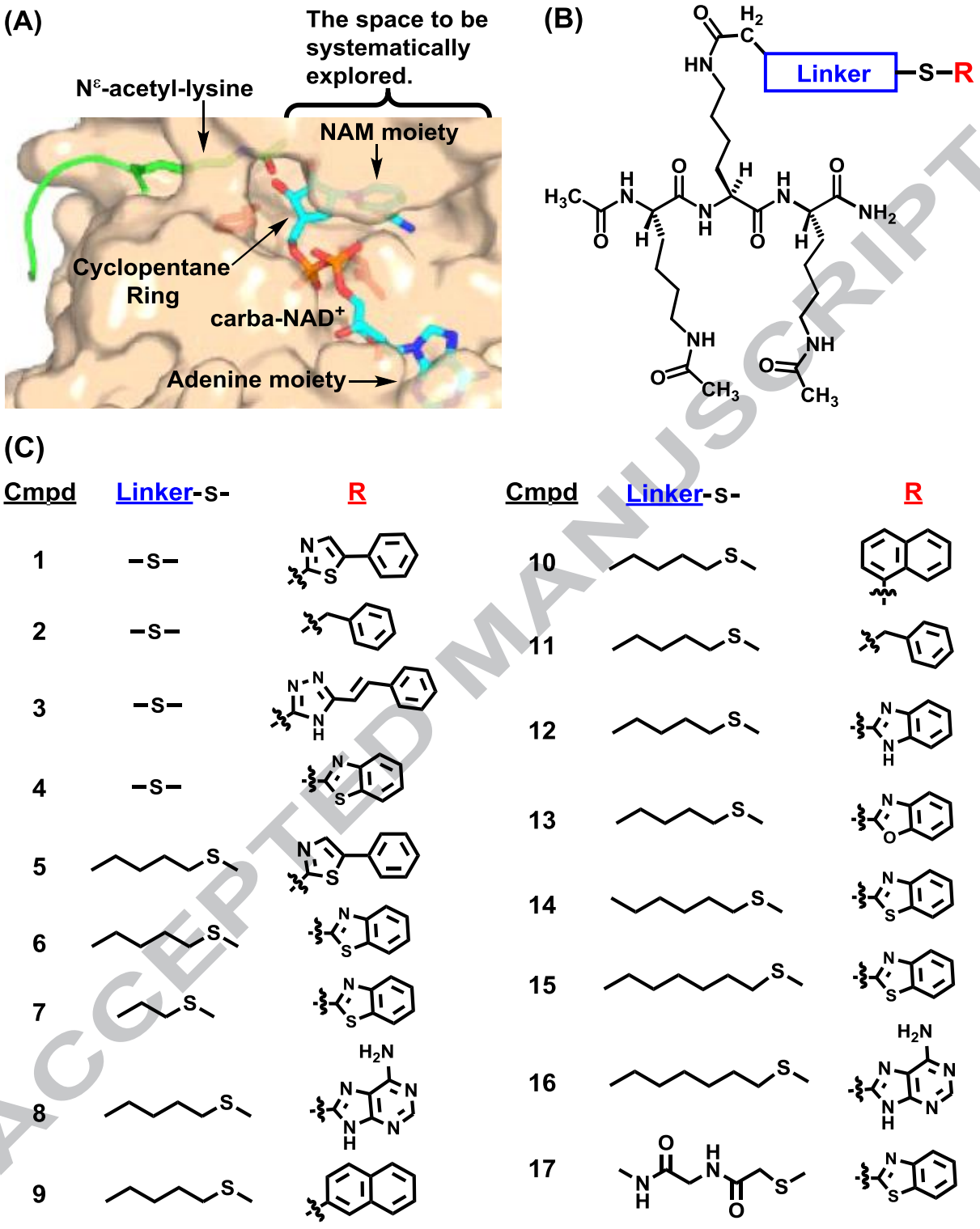
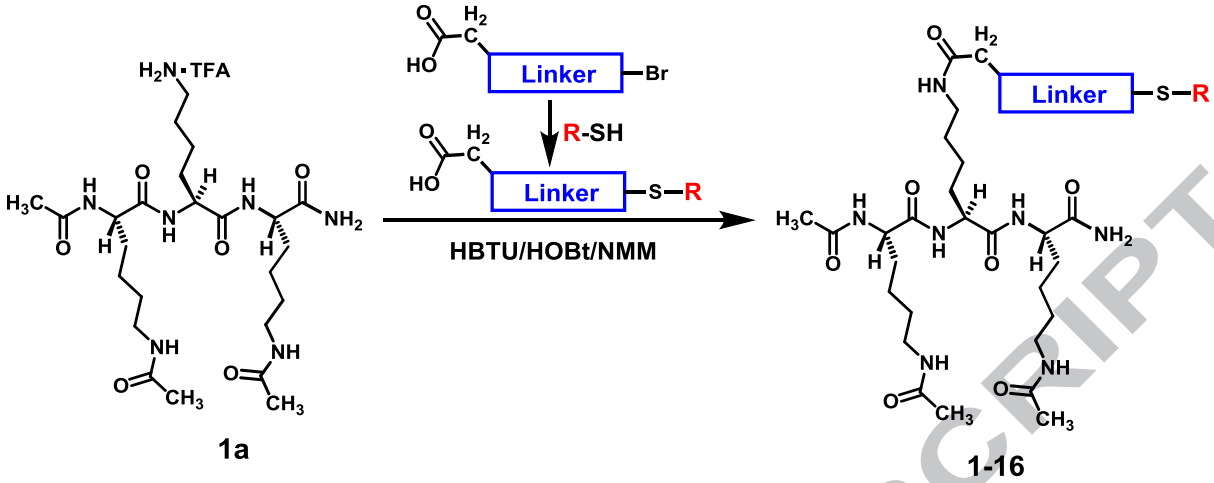


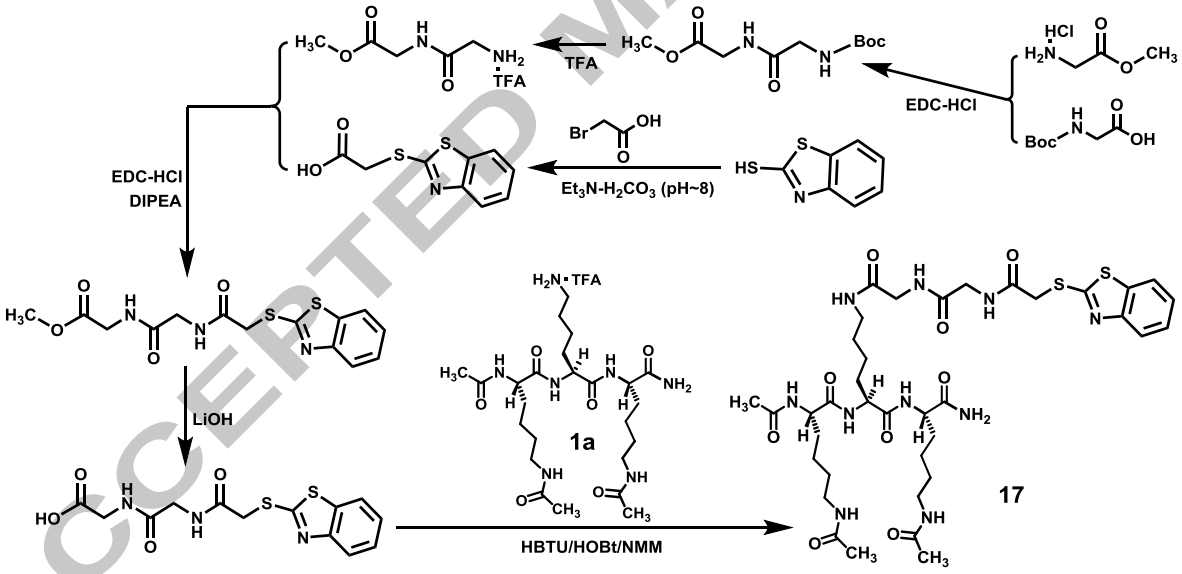
Figure 2. The design of bivalent compounds **1-17**. **(A)** A 3-dimensional rendering of the X-ray crystallographically determined structure of the ternary complex of the yeast sirtuin Hst2 with carba-NAD^+ and a K^{16} N^{ε}-acetylated peptide substrate derived from the histone H4 protein (Protein Data Bank code: 1SZC) (adapted from ref. 25 and used with permission), illustrating the presence of an open space extending from the side chain of the bound substrate's N^{ε}-acetyl-lysine residue. **(B, C)** The chemical structures of compounds **1-17** designed in the current study. NAM, nicotinamide; Cmpd, Compound.

As shown in **Figures 2B** and **2C**, we used the depicted tripeptidic scaffold to construct bivalent compounds **1**-**17** by covalently linking the side chain ε-amino group of its central residue to a distal functionality *via* a linker. Compounds **1**-**17** were designed to primarily assess their inhibitory power against SIRT1, a prototypical sirtuin family member. For this set of seventeen compounds, we used simple aliphatic chains with different lengths (numbers of methylene unit) as their linkers; as for the R groups, we used aromatic instead of aliphatic groups due to the considerations on structural bulkiness and ease of manifestation of different electrosteric properties. With aromatic groups, unique π-electron-mediated interactions at sirtuin active site can also be introduced. Given the demonstrated capability of SIRT1 to also robustly catalyze the lysine N^{ε}-defatty acylation, e.g. the catalytic removal of the slim myristoyl group from N^{ε}-myristoyl-lysine,[27,28] the use of the bulkier aromatic R groups would also help to prevent the SIRT1-catalyzed deacylation of compounds **1**-**17**. It should be noted that the tripeptidic scaffold in compounds **1**-**17** is the one that has also been used in our laboratory over the past few years to evaluate the performance of a sirtuin inhibitory warhead embedded in the scaffold.[1,14]

Compounds **1**-**17** were synthesized according to **Schemes 1** and **2**. The overall synthetic strategy is to prepare the conjugate of R and the linker with a terminal carboxyl for the subsequent condensation with the tripeptidic starting material **1a** under peptide coupling reaction condition. For compounds **1**-**16**, the linker-R conjugate was prepared by an alkylation reaction between commercially available R-SH and a bromoalkanoic acid. For compound **17**, the linker-R conjugate was prepared by a more extensive series of synthetic manipulation, as depicted in **Scheme 2**. The crude **1**-**17** from respective reaction mixtures were purified by



Scheme 1. The synthesis of compounds **1**-**16**. HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HOBt, N-hydroxybenzotriazole; NMM, N-methylmorpholine. The tripeptidic compound **1a** was purchased from China Peptides Co., Ltd. through a custom synthesis order; its purity was ⩾98% based on RP-HPLC analysis and its exact mass was confirmed by ESI-MS analysis.



Scheme 2. The synthesis of compound **17**. EDC-HCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; TFA, trifluoroacetic acid; DIPEA, N,N-diisopropylethylamine; HBTU/HOBt/NMM, as defined in **Scheme 1**. The tripeptidic compound **1a** came from the same source with the same quality as that used for the synthesis described in **Scheme 1**.

semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC). The purified **1**-**17** were each shown to be >95% pure based on analytic RP-HPLC analysis, and

their exact masses were confirmed by high-resolution mass spectrometry (HRMS) analysis (**Table 1**).

Table 1. HRMS analysis of compounds **1-19**[a]

Compound	Ionic Formula	Calculated m/z	Observed m/z
1	$[C_{35}H_{53}N_8O_7S_2]^+$	761.3473	761.3469
2	$[C_{33}H_{54}N_7O_7S]^+$	692.3800	692.3806
3	$[C_{36}H_{55}N_{10}O_7S]^+$	771.3970	771.3972
4	$[C_{33}H_{50}N_8O_7S_2Na]^+$	757.3136	757.3111
5	$[C_{39}H_{61}N_8O_7S_2]^+$	817.4099	817.4092
6	$[C_{37}H_{58}N_8O_7S_2Na]^+$	813.3762	813.3788
7	$[C_{35}H_{55}N_8O_7S_2]^+$	763.3630	763.3635
8	$[C_{35}H_{59}N_{12}O_7S]^+$	791.4345	791.4340
9	$[C_{40}H_{62}N_7O_7S]^+$	784.4426	784.4420
10	$[C_{40}H_{62}N_7O_7S]^+$	784.4426	784.4411
11	$[C_{37}H_{62}N_7O_7S]^+$	748.4426	748.4412
12	$[C_{37}H_{60}N_9O_7S]^+$	774.4331	774.4323
13	$[C_{37}H_{59}N_8O_8S]^+$	775.4171	775.4168
14	$[C_{38}H_{61}N_8O_7S_2]^+$	805.4099	805.4084
15	$[C_{39}H_{63}N_8O_7S_2]^+$	819.4256	819.4226
16	$[C_{37}H_{63}N_{12}O_7S]^+$	819.4658	819.4652
17	$[C_{37}H_{57}N_{10}O_9S_2]^+$	849.3746	849.3732
18	$[C_{26}H_{46}IN_7O_7Na]^+$	718.2396	718.2390
19	$[C_{28}H_{50}IN_8O_8]^+$	753.2791	753.2788

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

When compounds **1**-**4** were initially prepared and screened for their SIRT1 inhibitory potency, we found that the R groups in compounds **1** and **4** seemed to confer stronger SIRT1 inhibition than those in compounds **2** and **3** (**Table 2**). Therefore, the two R groups in **1** and **4** were selected to construct compounds **5** and **6** whose linker chain was more extended than that in compounds **1** and **4**. We would like to see if stronger SIRT1 inhibition could be realized by this linker chain extension. Indeed, while **5** was found to be slightly stronger than **1**, **6** exhibited a much stronger SIRT1 inhibition than **4**. More importantly, **6** was also found to be modestly stronger than **5** (**Table 2**). Encouraged by this finding, we then prepared **7** whose R group was the same as that in **6**, yet linker chain was two methylene units less than that in **6**. We found that **7** was a weaker SIRT1 inhibitor than **6** (**Table 2**), suggesting that linker chain shortening from that in **6** had a deleterious effect on SIRT1 inhibition. We subsequently prepared **8**-**13** that had the same linker chain as that in **6**, yet different R groups from that in **6**. We found that, while **9** and **10** were slightly weaker SIRT1 inhibitors than **6**, all the remaining compounds (i.e. **8** and **11**-**13**), especially **8**, were much weaker SIRT1 inhibitors than **6** (**Table 2**), suggesting that the binding pocket for R group is mostly hydrophobic in nature yet is able to confer a specific interaction environment for R group. We further explored the effect of linker chain elongation from that in **6** on SIRT1 inhibitory potency. For this, we prepared and assessed **14** and **15** that had the same R group as that in **6**, yet had linker chains one and two methylene units, respectively, more than that in **6**. We found that, while **14** was a weaker SIRT1 inhibitor than **6**, **15** exhibited a ~2.3-fold enhancement in SIRT1 inhibitory potency as compared with **6** (**Table 2**). Prompted by this finding, we further prepared and assessed **16** (same linker yet different R as compared with **15**)

and **17** (same R yet different linker as compared with **15**). Both compounds were found to be much weaker SIRT1 inhibitors than **15** (**Table 2**), suggesting that the binding regions for both R and the linker of a bivalent compound are hydrophobic in nature. Of note, the hydrophilic R in **16** is the one also found in **8**, and the linker in **17** can be regarded as the hydrophilic counterpart of the hydrophobic linker in **15** with roughly same length. It is worth noting that compounds **1-17** were all found not to be deacylated under our assay condition.

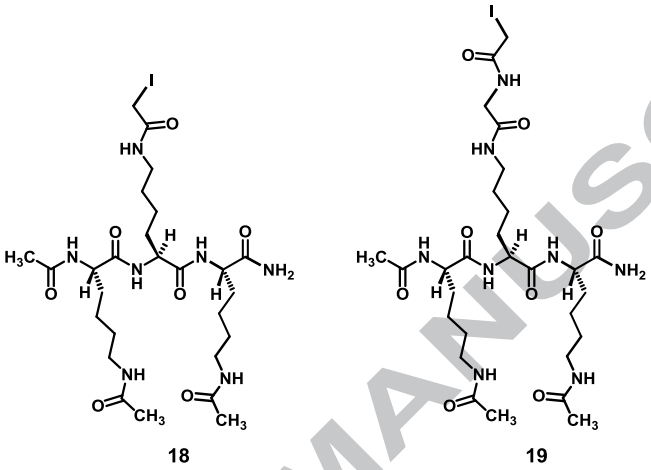
Table 2. The SIRT1 inhibition of compounds **1-19**[a]

Compound	IC_{50}(μM)	Compound	IC_{50}(μM)
1	18	**11**	57.7
2	171	**12**	187.3
3	149.5	**13**	133.1
4	77.5	**14**	30.0
5	15.4	**15**	5.3
6	12.4	**16**	45.8
7	27.0	**17**	116
8	>900	**18**	39.3
9	14.6	**19**	>200
10	14.6		

[a]See "Supplementary Material" for SIRT1 inhibition assay details.

Since a bivalent ligand is known to exhibit stronger target binding affinity than its monovalent counterpart, due to the decreased entropic penalty for the binding of a bivalent ligand than its monovalent counterpart to the same target,[29] we also prepared and assessed the SIRT1 inhibitory potency of the two compounds shown in **Figure 3**. Our use of the iodoacetyl compounds **18** and **19** as the monovalent controls to be evaluated along with compounds **1-17**

were not only based on a structural intuition that **18** and **19** are R-less, but also based on our fortuitous finding that these two compounds did not behave as irreversible SIRT1 inhibitors nor behave as SIRT1 substrates under our assay condition; they behaved as simple reversible SIRT1 inhibitors.

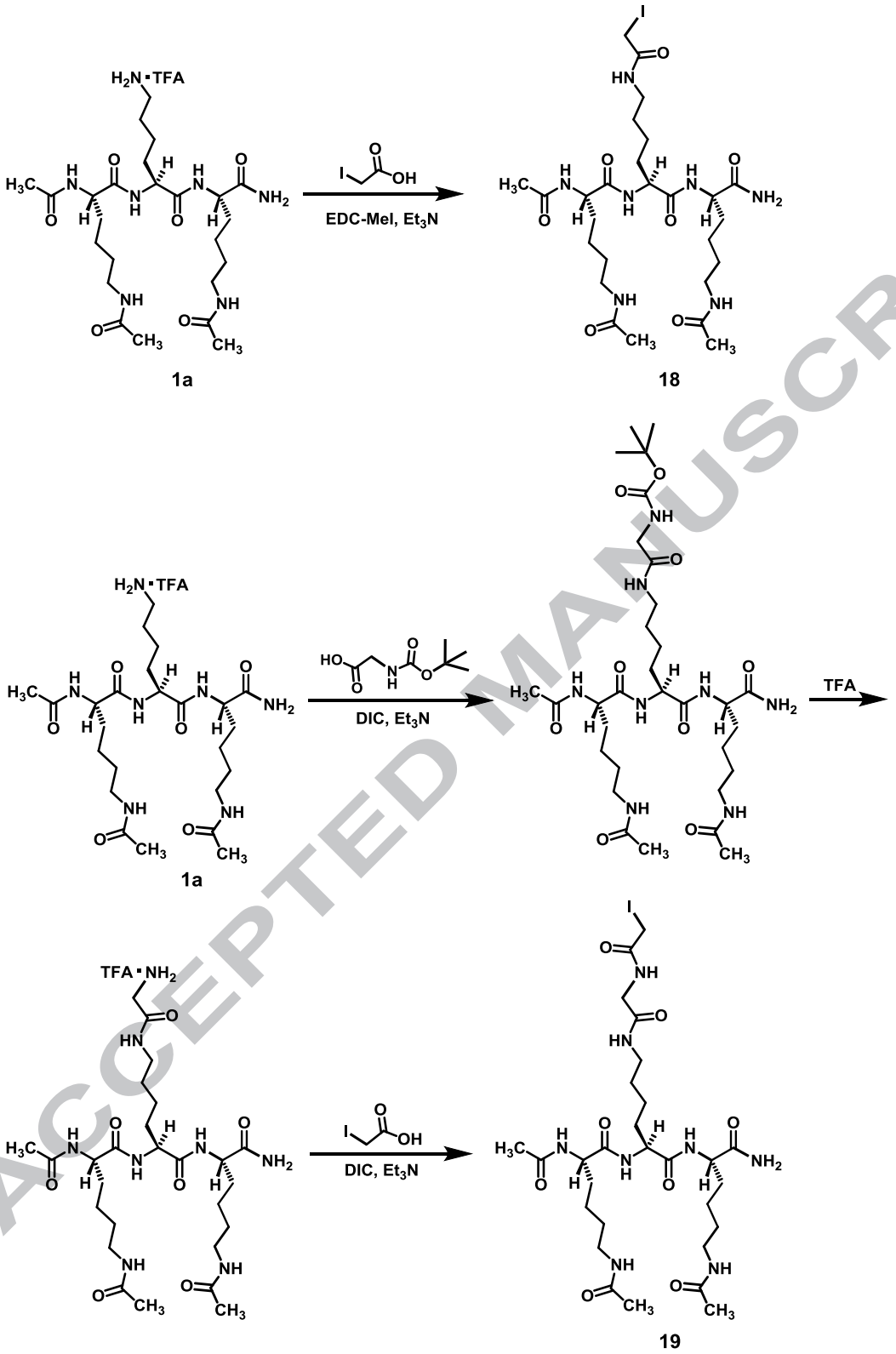


18 **19**

Figure 3. The chemical structures of the two monovalent compounds used in the current study.

As shown in **Table 2**, **18** and **19** were found to be weaker SIRT1 inhibitors than most compounds among **1**-**17**, especially compounds **5**, **6**, **9**, **10**, and **15**. Therefore, the screen of compounds **1**-**17** for their SIRT1 inhibitory potency generated a few compounds that were found to be stronger than their monovalent counterparts **18** and **19**.

Compounds **18** and **19** were prepared according to **Scheme 3**. The iodoacetylation of a precursor amine was performed under the amide bond formation condition with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC-MeI) or N,N'-diisopropylcarbodiimide (DIC) as the coupling reagent. The crude **18** and **19** from respective reaction mixtures were also purified by semi-preparative RP-HPLC. The purified **18** and **19** were also each shown to be >95% pure based on analytic RP-HPLC analysis, and their exact masses were also confirmed by HRMS analysis (**Table 1**).



Scheme 3. The synthesis of compounds **18** and **19**. EDC-MeI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide; DIC, N,N'-diisopropylcarbodiimide; TFA, as defined in **Scheme 2**. The tripeptidic compound **1a** also came from the same source with the same quality as that used for the synthesis described in **Scheme 1**.

In order to shed light on the mechanism of SIRT1 inhibition by the bivalent compounds, we next determined the SIRT1 inhibition kinetics for **6**. It should be noted that, even though **15** is the strongest bivalent SIRT1 inhibitor among **1**-**17**, due to its lower aqueous solubility than the second strongest compound (i.e. **6**), we therefore picked the more manageable **6** for the SIRT1 inhibition kinetics determination. As indicated in **Figure 4**, **6** was found to be competitive *versus* the N^{ε}-acetyl-lysine substrate and non-competitive *versus* β-NAD^+. This finding would be consistent with an ordered sequential kinetic mechanism for the SIRT1-catalyzed deacetylation reaction, in which the N^{ε}-acetyl-lysine substrate binding precedes the binding of β-NAD^+.[30] This finding also suggested that the bivalent SIRT1 inhibitors seemed indeed to be able to occupy the open space observed in sirtuin structural analysis and thus prevent the binding of the N^{ε}-acetyl-lysine substrate (to free sirtuin) and β-NAD^+ (to its binding site created on sirtuin following the binding of the N^{ε}-acetyl-lysine substrate to free sirtuin).

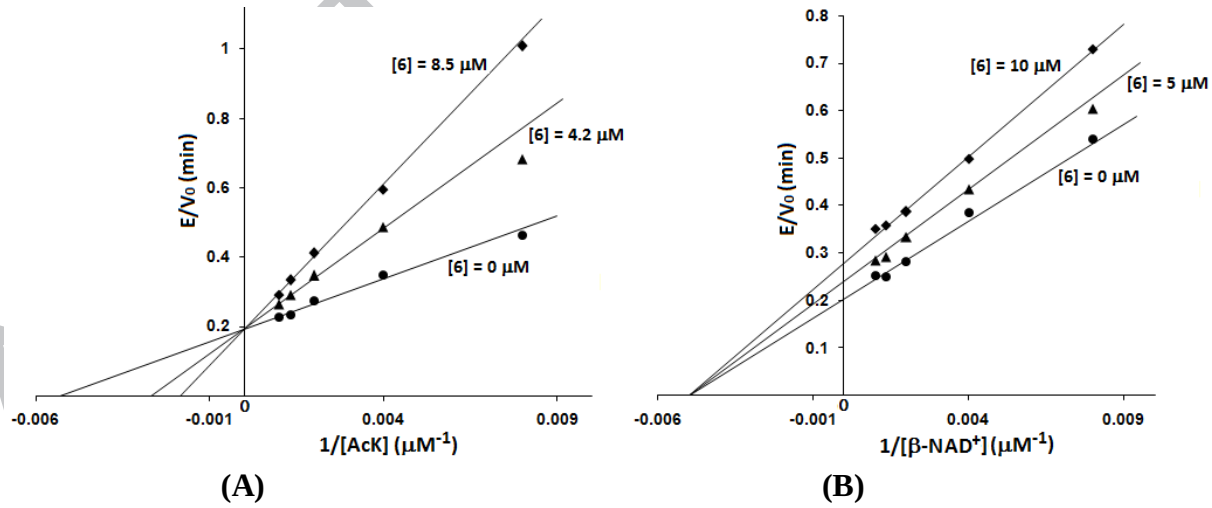


Figure 4. SIRT1 inhibition kinetics of compound **6**. **(A)** Competitive inhibition of **6** *versus* the SIRT1 substrate H_2N-HK-(N^{ε}-acetyl-lysine)-LM-COOH (AcK). K_{is} and the apparent K_m for AcK were determined to be 6.4 ± 3.9 µM and 200.4 ± 15.2 µM, respectively. **(B)** Non-competitive inhibition of **6** *versus* β-NAD^+. K_{ii} and the apparent K_m for β-NAD^+ were determined to be 31.1 ± 5.4 µM and 168.9 ± 46.7 µM, respectively. See "Supplementary Material" for assay details.

Since a bivalent ligand is also known to be able to exhibit an enhanced binding selectivity than its monovalent counterpart toward a desired target *versus* other homologous targets, due to the possible existence of different distances between the two binding pockets engaged by a bivalent ligand among different targets,[29] we also performed a comparative assessment of the bivalent compound **6** and its monovalent counterpart **18** for their inhibitory potencies against SIRT2 and SIRT3, two sirtuins closely related to SIRT1 in terms of the substrate N^{ε}-acyl head specificity.[1,27,28,31] As shown in **Table 3**, while **18** was found to be essentially a pan-SIRT1/2/3 inhibitor, **6** exhibited an appreciable degree of inhibitory selectivity toward SIRT1 *versus* SIRT2 and SIRT3.

Table 3. The comparative assessment of SIRT1/2/3 inhibition for compounds **6** and **18**[a]

Compound	IC_{50}(μM)				
	SIRT1	SIRT2	SIRT3	SIRT1/ SIRT2[b]	SIRT1/ SIRT3[c]
6	12.4 ± 2.0	>200	>1,000	>16	>81
18	39.3 ± 18.0	22.2 ± 7.4	54.2 ± 16.3	0.56	1.38

[a]See "Supplementary Material" for SIRT1/2/3 inhibition assay details. [b]The inhibitory selectivity of SIRT1 *versus* SIRT2. [c]The inhibitory selectivity of SIRT1 *versus* SIRT3.

To summarize, in the current study, we preliminarily explored the feasibility of developing bivalent-type sirtuin inhibitors with SIRT1 as the model sirtuin. As compared with the monovalent counterpart, several bivalent compounds among **1**-**17** were found to be stronger SIRT1 inhibitors. Moreover, as demonstrated with compounds **6** and **18**, as well as human SIRT1/2/3, a bivalent compound could also exhibit a higher degree of inhibitory selectivity among different sirtuins than its monovalent counterpart. Our findings suggested that, when trying to develop potent and selective sirtuin inhibitors, bivalent compounds could

be also considered as another class of inhibitors to pursue.

Acknowledgements

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Graphical Abstract

Bivalent SIRT1 inhibitors

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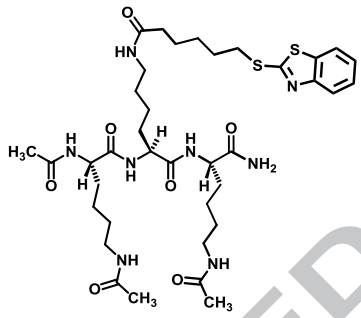
A study on seventeen bivalent compounds (**1**-**17**) and their monovalent counterparts (**18** and **19**) with SIRT1/2/3 supported the feasibility of developing bivalent-type sirtuin inhibitors able to furnish a stronger and more selective sirtuin inhibition than their monovalent counterparts.

Compound	$IC_{50}(\mu M)$		
	SIRT1	SIRT2	
6	12.4 ± 2.0	>200	
18	39.3 ± 18.0	22.2 ± 7.4	54



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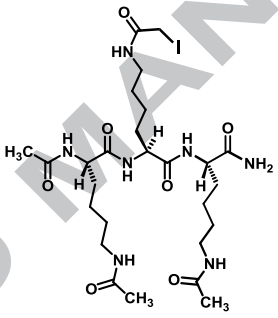

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