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Mechanism-based Modulator Discovery for Sirtuin-catalyzed Deacetylation Reaction

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Abstract: Silent information regulator 2 (Sir2) enzymes or sirtuins are a family of evolutionarily conserved intracellular protein deacetylases that can catalyze the acetyl group removal from the specific N^{ε}-acetyl-lysine (AcK) side chains on a variety of proteins from all kingdoms of life. Yeast Sir2 was the first sirtuin identified, and so far seven sirtuins (i.e. SIRT1-7) have been found in mammals including humans. The sirtuin-catalyzed deacetylation reaction has captured tremendous interest during the past a few years because of (i) its increasingly demonstrated importance in many crucial biological processes such as gene transcription, metabolism, and aging, and thus its therapeutic potential for metabolic and age-related diseases and cancer, and (ii) its unique deacetylation chemistry. Specifically, the sirtuin-catalyzed AcK side chain deacetylation is not merely an amide hydrolysis reaction, instead is coupled to the nicotinamide cleavage from β-nicotinamide adenine dinucleotide (β-NAD^+ or NAD^+) with the generation of three enzymatic products, i.e. the deacetylated protein species, nicotinamide, and 2'-O-acetyl-ADP-ribose (2'-O-AADPR). Here the author would like to review the past endeavors on developing mechanism-based sirtuin modulators (inhibitors and activators). The first part of this article will provide an updated mechanistic picture of the sirtuin-catalyzed deacetylation reaction. The second part will be focused on how the mechanistic knowledge has been exploited for the design of effective sirtuin modulators.

Keywords: Activator, Inhibitor, Mechanism, Mechanism-based, Protein deacetylase, Sirtuin.

1. INTRODUCTION

N^{ε}-acetylation and deacetylation on specific lysine side chains have been recognized as an important post-translational modification to exert structural and functional regulation of proteins [1-23]. Both histone and non-histone proteins can be regulated by this acetylation/deacetylation switch. While this regulatory switch can specifically alter the structure and function of the modified protein itself, it can also exert an extended functional impact *via* the specific recruitment of bromodomain-containing proteins following lysine N^{ε}-acetylation [24,25]. Therefore, as shown in (Fig. **1**), the creation, destruction, and specific recognition of N^{ε}-acetyl-lysine (AcK) define the three central molecular events of the signaling mechanism *via* the acetylation/ deacetylation of specific lysine side chains.

Since mid-1990s, many protein lysine acetyltransferases and protein acetyl-lysine deacetylases have been identified, which respectively catalyze the lysine N^{ε}-acetylation and deacetylation [2,3,5,6,8-12,15,16,19,20,22,23]. For example, multiple transcriptional co-activators such as p300, cAMP-response element binding protein (CREB) binding protein (CBP), and p300/CBP-associated factor (PCAF) were shown to possess an intrinsic protein lysine acetyltransferase activity. Furthermore, the yeast transcriptional repressors reduced potassium dependency 3 (Rpd3), histone deacetylase 1 (Hda1), and silent information regulator 2 (Sir2) were shown to possess an intrinsic protein acetyl-lysine deacetylase activity. The bromodomain represents a family of AcK binding protein modules that contain ~110 amino acid residues and are present in many chromatin-associated proteins including transcriptional co-activators, bromodomain and extra-terminal domain (BET) family of nuclear proteins, and ATP-dependent chromatin-remodeling complexes [24,25].

For the enzymes catalyzing the lysine N^{ε}-acetylation or deacetylation, all the currently known protein lysine acetyltransferases use acetyl-coenzyme A as the co-substrate to have its acetyl group transferred onto the lysine side chain ε-amino group (Fig. **2**). However, the currently known protein acetyl-lysine deacetylases can use either Zn^{2+} or β-nicotinamide adenine dinucleotide (β-NAD^+ or NAD^+) as the catalytic cofactor to achieve the acetyl group removal from the AcK side chain. Specifically, those deacetylases in the classical family of Zn^{2+}-containing metalloenzymes (with yeast Rpd3 and Hda1 as the founding members) utilize the catalytic Zn^{2+} to facilitate the hydrolysis of the AcK side chain acetamide to afford the deacetylated protein species and acetic acid (Fig. **2**). However, those in the sirtuin family of enzymes (with the yeast Sir2 as the founding member) utilize NAD^+ as the co-substrate to achieve the AcK side chain deacetylation. The sirtuin-catalyzed acetyl group removal from the AcK side chain on a protein is coupled to the nicotinamide cleavage from NAD^+ with the generation of three enzymatic products, i.e. the deacetylated protein species, nicotinamide, and 2'-O-acetyl-ADP-ribose (2'-O-AADPR) whose 2'-acetyl group is derived from the side chain acetyl of the AcK residue on a sirtuin substrate protein (Fig. **2**).

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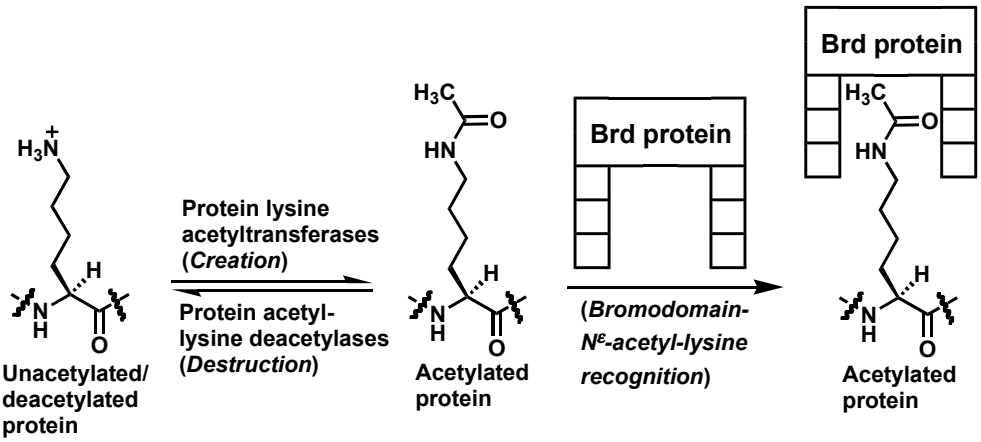

Fig. (1). Protein lysine acetyltransferase-catalyzed creation, acetyl-lysine deacetylase-catalyzed destruction, and bromodomain-mediated specific recognition of N^{ε}-acetyl-lysine on proteins. Brd protein: bomodomain-containing protein.

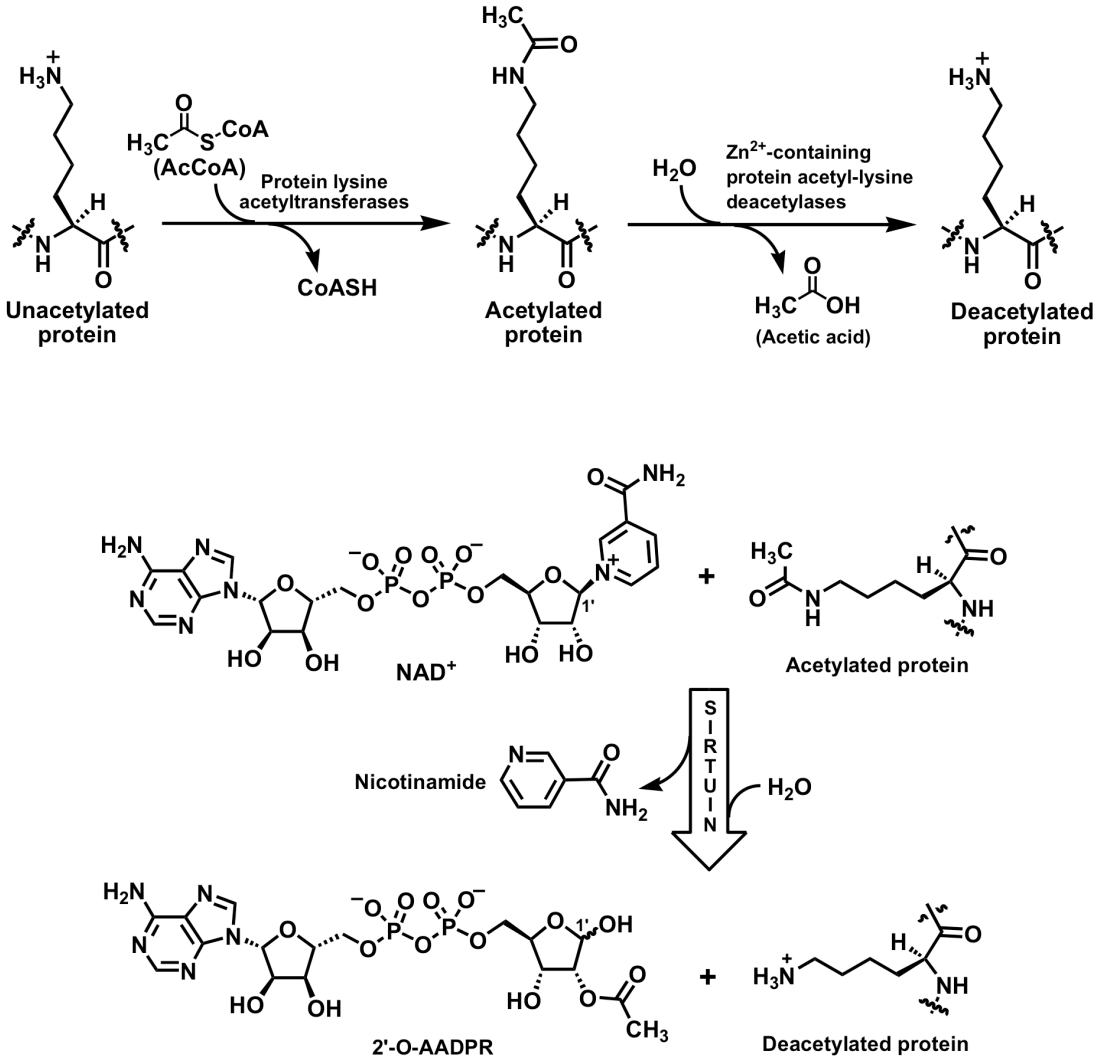

Fig. (2). The protein lysine N^{ε}-acetylation and deacetylation reactions catalyzed respectively by protein lysine acetyltransferases and the two families of protein acetyl-lysine deacetylases. AcCoA, acetyl-coenzyme A; CoASH, coenzyme A; NAD^{+}, β-nicotinamide adenine dinucleotide; 2'-O-AADPR, 2'-O-acetyl-ADP-ribose. The stereochemistry at the C1' position of 2'-O-AADPR is not specified since it was shown to be present in solution as the α- and β-anomers (~1:1 molar ratio) due to the fast epimerization at the C1' position [26], and this designation was used throughout this manuscript.

As the topic enzymes for this review, the sirtuin family of enzymes have been identified in organisms from all kingdoms of life [6,8,9,11,27-42]. Examples include the mammalian sirtuins (SIRT1-7), the bacterial sirtuins (CobB isoforms, Sir2Tm, as well as Rv1151c and another Sir2-like protein found in mycobacteria that may function in the nonhomologous end-joining pathway in this bacteria), the archaeal sirtuins (Sir2Af1 and Sir2Af2), the parasitic sirtuins (PfSir2A, PfSir2B, EhSir2a, and the Sir2 orthologs from Cryptosporidium, Trypanosoma, and Leishmania parasites), and the yeast sirtuins (Sir2, Hst1-4, and the Sir2 orthologs from other fungal species). For the eukaryotic sirtuins, they have also been found to be present in different sub-cellular compartments, including the nuclear sirtuins (e.g. mammalian SIRT1, SIRT6, and SIRT7), the mitochondrial sirtuins (e.g. mammalian SIRT3, SIRT4, and SIRT5), and the cytoplasmic sirtuins (e.g. mammalian SIRT1, SIRT2 and yeast Hst2). Moreover, the seven human sirtuins have also been found to be ubiquitously expressed in different organs/tissues [43,44].

Endogenous lysine N^{ε}-acetylated protein substrates have been identified for most prokaryotic and eukaryotic sirtuin enzymes [23,27,29,30,33-35,38,39,44-106]. For example, the acetylated bacterial proteins acetyl-coenzyme A synthetase and CheY are known to be deacetylated by CobB. The mycobacterial acetyl-coenzyme A synthetase was also shown recently to be a substrate for Rv1151c. Besides the acetylated histone proteins, a variety of non-histone proteins, when acetylated, have also been known to be substrates for the eukaryotic sirtuins. For example, the parasitic sirtuin EhSir2a was found to deacetylate the acetylated microtubule protein α-tubulin, and the *Plasmodium falciparum* sirtuin PfSir2A was shown to be able to deacetylate the acetylated PfAlba3 which is an Alba-family DNA-binding protein found in this parasite. In the case of the seven mammalian enzymes, acetylated physiological substrates have been identified for SIRT1-3 and SIRT5-6. Being the most studied mammalian sirtuin, SIRT1 has been shown to accept more than two dozens of acetylated proteins as its physiological substrates, for example, the core histone proteins H3 and H4, the linker histone protein H1, various transcription factors (e.g. p53, FoxO3, FoxO4, BCL6, NF-κB, and MyoD), proteins involved in DNA repair (e.g. Ku70, APE1, and XPA), the HIV-1 Tat protein, and other non-histone proteins such as the cytoplasmic metabolic enzymes acetyl-coenzyme A synthetase 1 (AceCS1), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1), and phosphoglycerate mutase-1, as well as the F-actin-binding protein cortactin. Interestingly, SIRT1 was also shown to be able to catalyze the deacetylation of the lysine acetylated protein lysine acetyltransferases including p300, hMOF, and Tip60, so as to modulate their acetyltransferase activity [49,66-68]. This observation implied that, in addtion to catalyzing the direct acetyl group removal from the lysine acetylated protein product of a lysine acetyltransferase-catalyzed reaction, a sirtuin can also directly modulate the enzymatic activity of the acetyltransferase itself to counteract the enzymatic lysine acetylation reaction. SIRT1 was also recently shown to be able to catalyze the deacetylation of the AcK side chains on the acetylated DNMT1 which is one of the DNA methyltransferase enzymes important for the epigenetic transcriptional control [69]. This interesting finding suggested the presence of a functional cross-talk between the two major types of the epigenetic mechanisms, i.e. DNA methylation/demethylation and histone acetylation/ deacetylation.

As compared to SIRT1, a shorter list of acetylated proteins have been identified as physiological substrates for the other mammalian sirtuins. However, for SIRT3, since the discovery of its first endogenous protein substrate (i.e. the acetylated AceCS2 catalyzing the formation of acetyl-coenzyme A from acetate) in 2006, we have seen a steady increase in the number of its endogenous protein substrates discovered over the past a few years. Since the mature SIRT3 protein resides in the mitochondrial matrix, its currently known protein substrates are also all mitochondrial matrix proteins, and include the acetylated version of AceCS2 (as mentioned above), HMGCS2, the respiratory chain complexes I and II, aldehyde dehydrogenase 2, glutamate dehydrogenase, ornithine transcarbamoylase (OTC), long-chain acyl-coenzyme A dehydrogenase (LCAD), LKB1 (the primary upstream kinase for the energy metabolic regulator AMPK), isocitrate dehydrogenase 2 (IDH2), manganese superoxide dismutase (MnSOD), the mitochondrial ribosomal protein MRPL10, and cyclophilin D. Given the known functions of these substrate proteins, it could be concluded that the reversible lysine acetylation of the SIRT3 substrate proteins plays an important role in regulating mitochondrial oxidative metabolism, the mitochondrial reactive oxygen species (ROS) abundance, the mitochondrial protein synthesis, and the mitochondrial structural/chemical homeostasis.

As for the remaining mammalian sirtuins, not that many endogenous protein substrates have been identified. Specifically, the acetylated α-tubulin, histone H4 protein, the gluconeogenesis enzyme phosphoenolpyruvate carboxykinase (PEPCK1), cortactin, the cytoplasmic loop of the prolactin receptor, three transcription factors (i.e. p53, FoxO3a, and NF-κB), as well as CDH1 and CDC20 which are the two coactivators of anaphase-promoting complex/cyclosome (APC/C) have been identified as the substrates for SIRT2. It should be pointed out that while SIRT2 resides primarily in the cytoplasm, it was also observed to be localized on chromatin during the G2/M transition in the mammalian cell cycle where it can catalyze the deacetylation of histone H4, which is critical for the formation and maintenance of heterochromatin during the G2/M transition. For the mitochondrial sirtuin SIRT5, the acetylated urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) is the only currently known physiological substrate, even though the acetylated cytochrome c was also shown to be an *in vitro* SIRT5 substrate. Interestingly, two very recent studies suggested that SIRT5 was also able to accept the lysine N^{ε}-malonylated and the lysine N^{ε}-succinylated proteins as its endogenous substrates and to catalyze the removal of the corresponding side chain N^{ε}-acyl groups [107,108]. Despite the speculation that SIRT4 may also possess a protein acetyl-lysine deacetylase activity, the mono-ADP-ribosyltransferase has been the only activity identified so far for this mitochondrial sirtuin, and glutamate dehydrogenase has been

the only physiological substrate identified for this activity of SIRT4 [109]. A couple of physiological substrates have also been identified for SIRT6's acetyl-lysine deacetylase activity, including the acetylated histone H3 protein and C-terminal binding protein (CtBP) interacting protein (CtIP). SIRT6 was also shown to be a mono-ADP-ribosyltransferase being able to accept itself and other proteins (e.g. poly(ADP-ribose)polymerase-1 (PARP1)) as the substrate [110,111]. The p53 protein was thought to also serve as an endogenous substrate for SIRT7 based on the *in vitro* experiments with acetylated p53 peptides.

Not only the endogenous acetylated protein substrates have been identified for sirtuins, sites of deacetylation have also been pinpointed on many of these protein substrates. (Fig. **3**) presents an alignment of the AcK-containing target sequences derived from the currently known physiological protein substrates whose deacetylation sites have been identified for human SIRT1 [50,51,53-57,59,60], human SIRT2 [90,91,99], human SIRT3 [70,86], and human SIRT6 [102-104]. The four positions immediately flanking AcK are indicated as the -2, -1, +1, and +2 positions.

The sirtuin-catalyzed deacetylation reaction has been shown to play a regulatory role in a variety of important biological processes such as gene transcription, apoptosis, DNA repair, metabolism, aging, neurodegeneration, and HIV-1 replication [10,11,112-129], the past a few years have thus seen the identification of different types of the chemical modulators (inhibitors and activators) for this enzymatic deacetylation reaction, which could lead to the development of novel therapeutics for metabolic and age-related diseases and cancer [49,130-148]. These chemical modulators could also be exploited to help further explore the sirtuin biology and pharmacology. The unique way of the AcK side chain deacetylation catalyzed by a sirtuin enzyme also attracted a lot of mechanistic investigation into the nature of the sirtuin deacetylation chemistry [6,8,9,12,49,149-155]. In this review, an updated mechanistic picture of the sirtuin-catalyzed deacetylation reaction will be initially presented. In the second part of this review, the author will present and discuss the development of one unique class of sirtuin-selective modulators, i.e. the mechanism-based sirtuin inhibitors and activators, *via* the judicious exploitation of the unique features of the sirtuin deacetylation chemistry.

2. CATALYTIC MECHANISMS OF THE SIRTUIN-CATALYZED DEACETYLATION REACTION

2.1. Kinetic Mechanism

Since the sirtuin-catalyzed deacetylation reaction utilizes NAD^+ to achieve the AcK side chain acetyl group removal from the AcK-containing protein substrate (Fig. **2**), the first kinetic mechanistic question is whether this reaction proceeds *via* the ping-pang or the sequential kinetic mechanism. By using the yeast sirtuin Hst2 and the human sirtuin SIRT2 as the model sirtuin enzymes, Denu and co-workers demonstrated that the sirtuin deacetylation reaction obeyed an ordered ternary complex sequential kinetic mechanism in that the AcK substrate binds to sirtuin active site prior to NAD^+ with the formation of the ternary complex of these two substrates with the sirtuin enzyme, and the first chemical step occurs only after the formation of this ternary complex [156]. This study further revealed that this first chemical step was the nicotinamide cleavage from NAD^+ since nicotinamide was determined to be the first product formed and released, followed by the random release of the other two products (i.e. the deacetylated species and 2'-O-AADPR). Another study by Smith and Denu determined with the yeast sirtuin Hst2 that the product release is the rate-limiting step for the overall sirtuin catalysis [157]. However, this phenomenon seems not to be conserved among different sirtuins since the overall catalytic velocity of the deacetylation reaction catalyzed by other sirtuins such as the *Plasmodium falciparum* sirtuin PfSir2A was demonstrated to be limited by the further chemical transformation of the α-1'-O-alkylamidate intermediate formed after the nicotinamide cleavage from NAD^+ (*vide infra*) [158].

Substrate	Target sequence (-2 -1 +1 +2)	Enzyme
Human BCL6:	…WK-K-Y-**AcK**^{379}-F-I-VL…	SIRT1
Human Ku70:	…NP-E-G-**Ack**^{539}-V-T-**AcK**^{542}-R-K-HD…	SIRT1*
Human NF-κB:	…YE-T-F-**AcK**^{310}-S-I-MK…	SIRT1
Human p53:	…SR-H-K-**AcK**^{382}-L-M-FK…	SIRT1
Human MyoD:	…W-A-C-**AcK**^{99}-A-C-**AcK**^{102}-R-K-TT…	SIRT1*
Human APE1:	…KR-G-K-**AcK**^{7}-G-A-VA…	SIRT1
Human XPA:	…MA-N-V-**AcK**^{63}-A-A-P-**AcK**^{67}-I-I-DT…	SIRT1*
Human α-tubulin:	…MP-S-D-**AcK**^{40}-T-I-GG…	SIRT2
Human histone H4:	…KG-G-A-**AcK**^{16}-R-H-RKV…	SIRT2
Human NF-κB:	…YE-T-F-**AcK**^{310}-S-I-MK…	SIRT2
Human AceCS2:	…TR-S-G-**AcK**^{642}-V-M-RR…	SIRT3
Human MnSOD:	…VT-E-E-**AcK**^{68}-Y-Q-EA…	SIRT3
Human IDH2:	…GA-M-T-**AcK**^{413}-D-L-AG…	SIRT3
Human histone H3:	…QT-A-R-**AcK**^{9}-S-T-GG…	SIRT6
Human histone H3:	…RR-Y-Q-**AcK**^{56}-S-T-EL…	SIRT6

Fig. (3). An alignment of AcK-containing target sequences derived from the currently known physiological substrates for SIRT1, SIRT2, SIRT3, and SIRT6. (*The site preference has not been established).

2.2. Chemical Mechanism

Even though the sirtuin-catalyzed deacetylation reaction is known to utilize NAD^+ to achieve the AcK side chain deacetylation from the AcK-containing protein substrate, water was also demonstrated to be involved in this enzymatic reaction, as supported by the isotopic labeling experiments with the use of $H_2{}^{18}O$ [26,157]. However, this water participation is conceivably in a different manner than that in the AcK deacetylation reaction catalyzed by the classical HDACs which are Zn^{2+}-containing metalloenzymes and use Zn^{2+} to activate the water molecule for the subsequent nucleophilic attack onto the electrophilic AcK side chain amide carbonyl [159]. Instead, it could serve a similar role as that in the reaction catalyzed by the serine (or cysteine) proteases in that water was employed to hydrolyze a covalent enzyme intermediate along the reaction coordinate [160]. If this were the case, then the sirtuin-catalyzed deacetylation reaction could also proceed *via* the ping-pong kinetic mechanism, as that for the serine (or cysteine) protease-catalyzed reaction. However, the above-described kinetic study by Denu's group demonstrating that the sirtuin deacetylation reaction obeys the ordered ternary complex sequential kinetic mechanism indicated that the water use in the sirtuin-catalyzed AcK side chain deacetylation reaction is definitely in a different manner from that in the serine (or cysteine) protease-catalyzed peptide bond hydrolysis reaction.

Consistent with the ternary complex sequential kinetic mechanism, there must be a direct chemical reaction between the two substrates (AcK-containing substrate and NAD^+). However, how do these two molecules interact with each other once they have both bound to the sirtuin active site with the formation of the corresponding Michaelis complex? As shown in (Fig. **4**), these two substrates react with each other *via* a nucleophilic substitution reaction in that the AcK side chain amide oxygen nucleophilically attacks the electrophilic anomeric C1' of NAD^+, leading to the formation of the depicted α-1'-O-alkylamidate intermediate. The formation of this high energy intermediate *via* the depicted reaction route has received various informative biochemical and structural supports, however, the more direct support came from the studies with the use of the following two peptides: H_2N-KSTGG([^{18}O]AcK)APRKQCONH$_2$ and H_2N-KKGQSTSRHK(ThAcK)LMFKTEG-COOH (Fig. **5**) [157, 161]. Of note, the former peptide sequence was derived from the histone H3 protein and [^{18}O]AcK refers to the AcK analog with the side chain amide oxygen being replaced with ^{18}O; the latter peptide sequence was derived from the human p53 tumor suppressor protein and ThAcK refers to the AcK analog with the side chain amide oxygen being replaced with S. When the [^{18}O]AcK-containing histone H3 peptide was used in a sirtuin deacetylation assay with yeast Hst2, the ^{18}O label on this acetylated substrate was found to be transferred onto the C1' position of NAD^+, thus yielding the 2'-O-AADPR analog with the 1'-OH replaced with ^{18}OH [157]. This experimental observation strongly argued for the presence of an obligatory covalent bond formation between the side chain amide oxygen of AcK and the C1' of NAD^+ for the sirtuin-catalyzed deacetylation reaction. When the crystal of the bacterial sirtuin Sir2Tm with the bound p53 peptide containing ThAcK was soaked with a cryoprotective solution containing NAD^+, the catalytically active Sir2Tm in the crystal state was found to be capable of catalyzing the covalent bond formation between the side chain thioacetyl sulfur of ThAcK and the C1' of NAD^+, with the accompanying nicotinamide cleavage from NAD^+. However, the formed α-1'-S-alkylamidate intermediate was long-lived enough to be trapped within the crystal without further catalytic conversions (Fig. **5**) [161]. The successful solution of this 2.5 Å Sir2Tm crystal structure provided the conclusive evidence for the existence of the α-1'-S-alkylamidate intermediate that is stalled following the Sir2Tm-catalyzed nicotinamide cleavage, thus providing the first structural evidence for the formation of the α-1'-O-alkylamidate intermediate along the sirtuin catalytic coordinate *via* the nucleophilic substitution reaction route depicted in (Fig. **4**).

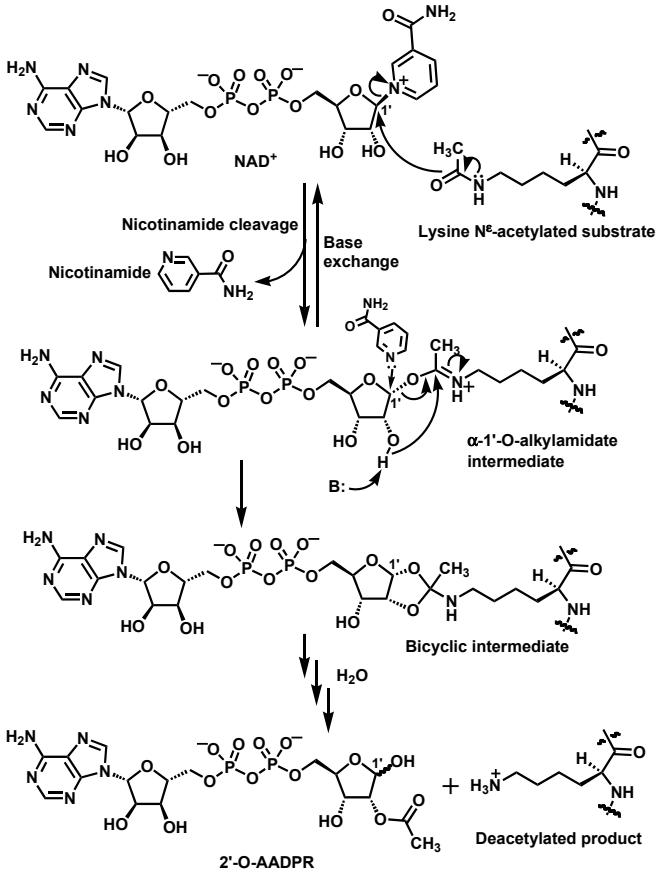

Fig. (4). The proposed chemical mechanism for sirtuin-catalyzed deacetylation reaction. B: refers to a general base.

Since it is known that a nucleophilic substitution reaction could follow a S_N1 or a S_N2 mechanism, in the case of the sirtuin-catalyzed nicotinamide cleavage and the formation of the α-1'-O-alkylamidate intermediate, the S_N1-like mechanism would involve a fully dissociated and enzyme stabilized oxacarbenium ion intermediate, whereas the S_N2-like mechanism would be a concerted mechanism with nucleophilic participation. The results from recent

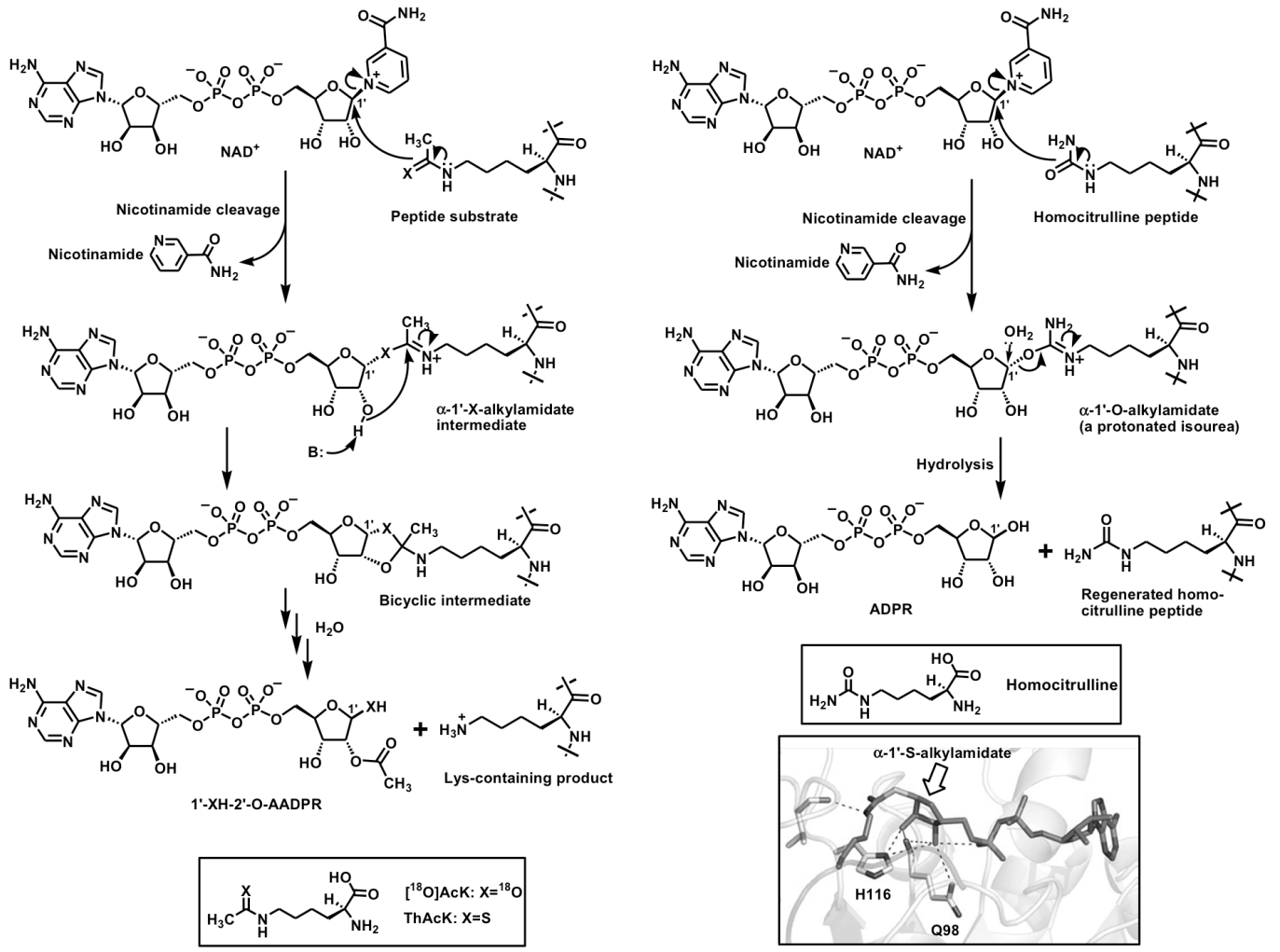

Fig. (5). The proposed sirtuin processing of the peptides containing [^{18}O]AcK, ThAcK, or homocitrulline. The [^{18}O]AcK-containing peptide used in ref. 157 was H_2N-KSTGG([^{18}O]AcK)APRKQCONH$_2$; The ThAcK-containing peptide used in ref. 161 was H_2N-KKGQSTSRHK-(ThAcK)LMFKTEG-COOH; The homocitrulline-containing peptide used in ref. 170 was H_2N-KSTGG(homocitrulline)APRKQCONH$_2$, and that used in ref. 169 was carbamoylpoly-L-lysine. When the ThAcK-containing peptide was used, the corresponding α-1'-S-alkylamidate intermediate was stalled along the reaction coordinate, so that it could be trapped within a sirtuin active site, as illustrated by that trapped within the Sir2Tm active site whose partial structure is shown here as the stick model. When a homocitrulline-containing peptide was used, the resulting α-1'-O-alkylamidate intermediate analog was unable to be converted to the corresponding bicyclic intermediate, instead it was hijacked by water to afford ADP-ribose (ADPR) and the regenerated homocitrulline peptide. B: refers to a general base.

experimental and computational studies tend to favor a concerted S_N2-like mechanism involving a highly dissociative transition state with a strong oxacarbenium ion character and a modest nucleophilic participation of the acetyl-lysine side chain amide oxygen [162-164].

As alluded from the above description, the water molecule would likely attack a catalytic intermediate after the nicotinamide cleavage step along the sirtuin-catalyzed deacetylation reaction coordinate. However, it seems to be unlikely that this attack would occur on the α-1'-O-alkylamidate intermediate since it would otherwise lead to the generation of ADP-ribose (ADPR) and the regeneration of the AcK-containing substrate, and thus preventing this intermediate from committing to the deacetylation chemistry (*vide infra*). Yet, the regeneration of the starting substrate(s) has been experimentally observed when a sirtuin deacetylation assay was incubated with exogenous nicotinamide at increasing concentrations [165,166] (Fig. **4**), or if the α-1'-O-alkylamidate intermediate or its close analog has a longer half-life within the sirtuin active site thus granting the water molecule a greater opportunity for its attack on this high energy intermediate (Fig. **5**). While the former phenomenon offers a satisfying explanation for the fact that nicotinamide behaves as a non-competitive inhibitor against sirtuin-catalyzed deacetylation reaction [167,168], the latter phenomenon was observed when the *Plasmodium falciparum* sirtuin PfSir2A was assayed [158] and when the sirtuin peptide substrate containing homocitrulline instead of AcK was examined under the sirtuin deacetylation assay condition [169,170], and could be explained by the greater half-lives of the α-1'-O-alkylamidate intermediate and its close analog

formed from the homocitrulline peptide. As depicted in (Fig. **4**) for the reaction of nicotinamide with the α-1'-O-alkylamidate intermediate, this nucleophilic substitution reaction could well account for how the two sirtuin substrates are regenerated. When nicotinamide is replaced with water, the corresponding nucleophilic substitution reaction depicted in (Fig. **5**) would account for how the peptide substrate is regenerated and how ADPR is produced from this reaction.

The next question is how the α-1'-O-alkylamidate intermediate can be transformed ultimately to the deacetylated product and 2'-O-AADPR. As proposed in (Fig. **4**), this intermediate is converted to the depicted bicyclic intermediate following the intra-molecular nucleophilic attack of the activated 2'-OH onto the iminium carbon of the α-1'-O-alkylamidate intermediate. Since an intra-molecular reaction is kinetically more favorable than the corresponding inter-molecular reaction, the proposed transformation of the α-1'-O-alkylamidate intermediate to the bicyclic intermediate in (Fig. **4**) also explains that the α-1'-O-alkylamidate intermediate was capable of committing to the deacetylation chemistry instead of being hydrolyzed once it is formed within a sirtuin's active site.

The intra-molecular nucleophilic attack of the 2'-OH onto the iminium carbon of the α-1'-O-alkylamidate intermediate with the formation of the 5-membered dioxo ring in the bicyclic intermediate is also expected to be favored considering the following: (i) the thermodynamically favored 5-membered ring transition state structure for the reaction and (ii) the geometrically favored relative positioning of the nucleophile and the electrophile, i.e. the 2'-OH and the anomeric position of α-1'-O-alkylamidate both assume the α-configuration. Moreover, the de-protonation of the 2'-OH by a general base is expected to further facilitate this intra-molecular reaction due to the enhanced nucleophilicity of 2'-OH.

The proposed transformation of the α-1'-O-alkylamidate intermediate into the bicyclic intermediate has also received experimental support, for example, the origin of the acetyl carbonyl oxygen of 2'-O-AADPR has been shown to be water as evidenced by the transfer of the ^{18}O label in $H_2{}^{18}O$ onto this position [26,157]. Moreover, as compared to the wild-type yeast Hst2, the Hst2[H135A] mutant was found to have a significantly diminished (~100-fold) deacetylase activity since it lost the catalytically essential H135 side chain that was proposed to serve as a general base to activate the 2'-OH group of α-1'-O-alkylamidate *via* proton abstraction, presumably for its intra-molecular nucleophilic addition reaction with α-1'-O-alkylamidate [166]. Another piece of the support for this enzymatic transformation route came from the above-described ability of water to intercept at the C1' position of the α-1'-O-alkylamidate intermediate that was formed from NAD^+ and a homocitrulline peptide because this intermediate has a weakly electrophilic "iminium" carbon [169,170].

However, a piece of the relatively more direct experimental evidence for this proposed enzymatic transformation came from a recent study from my laboratory [171]. Specifically, we discovered that, when using the p53 peptide CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$ in a sirtuin deacetylation assay, the corresponding bicyclic intermediate (Intermediate II in Fig. **6**) had a longer half-life such that it could be detected with mass spectrometry. Of note, L-ACAH refers to L-2-amino-7-carboxamidoheptanoic acid which is a close structural analog of AcK.

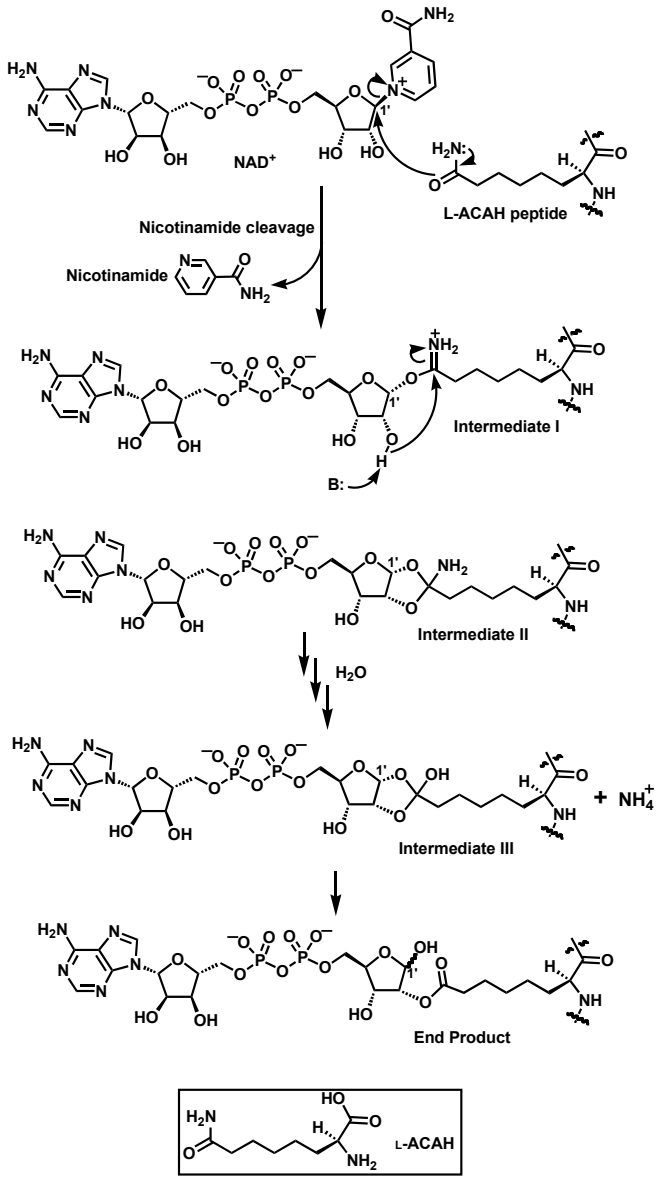

Fig. (6). The proposed sirtuin processing of the L-ACAH peptide CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$. In the study in ref. 171, Intermediate II and (Intermediate III and/or End Product) were shown to be the stalled species along the sirtuin-catalyzed reaction coordinate.

As depicted in (Fig. **4**), the water molecule was proposed to be involved in the collapse of the bicyclic intermediate. The afore-mentioned ^{18}O label transfer from $H_2{}^{18}O$ onto the acetyl carbonyl oxygen of 2'-O-AADPR observed during the sirtuin deacetylation assay in $H_2{}^{18}O$ [26,157] provided a piece of convincing evidence for this proposal. This observation with the use of $H_2{}^{18}O$ also suggested that this

water attack occurred regioselectively at the tetrahedral carbon of the dioxo ring in the bicyclic intermediate, rather than at the C1' position which is another plausible position susceptible to a nucleophilic attack. Another experimental support for this regioselective water attack came from the afore-mentioned study with the [^{18}O]-acetylated H3 peptide with the yeast Hst2 enzyme [157]. Since the ^{18}O label of the [^{18}O]-acetylated H3 peptide was found to be transferred onto the C1' position of 2'-O-AADPR, it could be concluded that the C1'-^{18}O bond stays throughout the reaction coordinate once it is formed in the α-1'-O-alkylamidate intermediate from NAD^+ and the [^{18}O]-acetylated H3 peptide.

Even though several pathways have been proposed to explain how the bicyclic intermediate could be resolved in the presence of water to afford the deacetylated product and 2'-O-AADPR [169,172], further studies are still needed to pinpoint the responsible pathway(s). Toward this end, the afore-described discovery with CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$ that the corresponding bicyclic intermediate (Intermediate II in Fig. **6**) had a longer half-life [171] could also be of a great value. In specific, given the close structural similarity between the side chains of AcK and L-ACAH, a L-ACAH-containing peptide (e.g. CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$) could be an invaluable biochemical/ biophysical probe for further dissecting the intermediate events for the sirtuin-catalyzed deacetylation reaction, *via* trapping the longer-lived Intermediate II (Fig. **6**) within the active site of a sirtuin crystal, and solving the corresponding co-crystal structure by X-ray crystallography. This type of study will not only be able to provide direct evidence for the existence of the proposed bicyclic intermediate (depicted in Fig. **4**) but also be able to shed light on how the bicyclic intermediate collapses in the presence of water toward the deacetylated product and 2'-O-AADPR, which is currently still unknown. The afore-mentioned previous success in using a ThAcK-containing peptide to structurally elucidate the sirtuin deacetylation reaction intermediate steps involving α-1'-O-alkylamidate *via* solving the co-crystal structure of Sir2Tm (a bacterial sirtuin) with a molecule of the stalled α-1'-S-alkylamidate intermediate trapped within its active site [161] should encourage similar endeavors using a L-ACAH-containing peptide.

It should be pointed out that, in the study with CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$ [171], it was also found that a close analog (Intermediate III in Fig. **6**) of a possible intermediate in the path of the collapse of the bicyclic intermediate toward the deacetylated product and 2'-O-AADPR was also likely relatively longer-lived such that it could also be detected by mass spectrometry. Therefore, the structural study with a L-ACAH-containing peptide may also be able to provide direct evidence for the existence of an intermediate in the path of the collapse of the bicyclic intermediate toward the deacetylated product and 2'-O-AADPR, thus further detailing how this enzymatic transformation takes place.

3. SUBSTRATE SPECIFICITY FOR THE SIRTUIN-CATALYZED DEACETYLATION REACTION

As illustrated in (Fig. **7**), it was suggested from previous structural and biochemical studies [173-177] that (i) AcK and the two immediately flanking amino acid residues on each side of AcK were the peptide residues that made predominant binding interactions with a sirtuin enzyme, (ii) AcK and the two immediately flanking amino acid residues on each side of AcK formed a β-strand in a sirtuin-bound peptide substrate, and that this β-strand formed an anti-parallel β-sheet with two β-strands from a sirtuin enzyme. In addition to the observed peptide main-chain interactions within the β-sheet and the sirtuin interactions with the side chain of AcK within its binding pocket, the amino acid side chains immediately flanking AcK were also demonstrated to participate in selective substrate recognition at the sirtuin active site.

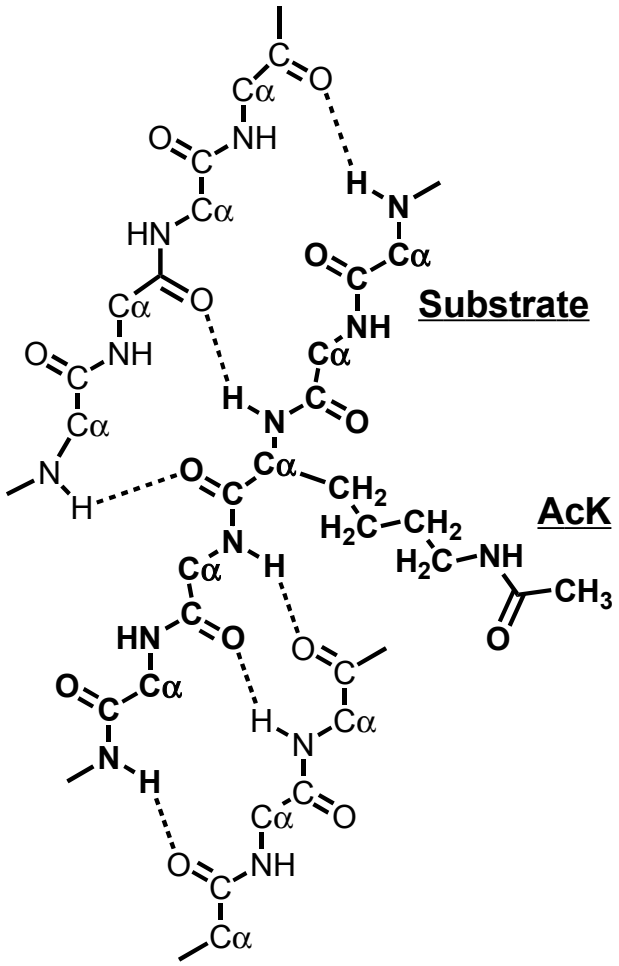

Fig. (7). (Modified from ref. 173) A schematic illustration of the key enzyme interactions of an AcK-containing peptide substrate bound to a sirtuin active site. The peptide substrate and its AcK residue are shown in bold style. The side chains are not shown for the substrate residues flanking AcK and the sirtuin active site residues (shown in plain style) that are involved in the β-sheet formation with the bound peptide substrate. Hydrogen bonds are indicated by dashed lines.

By employing a series of histone H3 and H4 peptides with different amino acid sequences yet all containing a single AcK residue, Denu and co-workers found that a sirtuin (yeast Sir2, yeast Hst2, or human SIRT2) displayed

varying substrate activities (k_{cat}/K_m) among these mono-acetylated peptides [156]. Moreover, for a given peptide, varying substrate activities were also shown for the three sirtuins used in the study.

A recent structural and biochemical study using the bacterial enzyme Sir2Tm as a model system demonstrated the significant role of the side chain interactions at the -1 and +2 positions relative to AcK in the target sequence [175]. The results from this study elegantly explained some of the observed enzyme-substrate specific recognition for not only the model enzyme Sir2Tm, but also for the yeast sirtuin Hst2. Specifically, because of the presence of a negatively charged aspartate residue at the -1 position of the acetylated α-tubulin peptide, the previously observed very inefficient processing of this peptide by Hst2 [178] could be due to the expected charge repulsion with an aspartate residue of Hst2. The context-dependent substrate specificity was recently demonstrated for SIRT1 based on the screening of a 5-amino acid AcK-containing peptide library [176]. Specifically, it was shown in this study that the SIRT1 preference for the side chain at a given position relative to AcK depends on the identities of the side chains at the neighboring positions.

The important role that amino acid side chains immediately flanking AcK play in sirtuin-substrate recognition can also be appreciated from the different sets of AcK-harboring segments shown in (Fig. **3**) that are recognized by different human sirtuins. It can be further appreciated from the fact that the different human sirtuins have their own preferred commercially available *in vitro* substrates, i.e. peptide-AMC conjugates with different peptide sequences (AMC stands for 7-amino-4-methylcoumarin). For example, among the human sirtuin *in vitro* substrates available from Enzo Life Sciences (http://www.enzolifesciences.com), SIRT1 prefers (within 2-fold of difference) H_2N-KGGA(AcK)-AMC, H_2N-RHK(AcK)-AMC, and H_2N-RH(AcK)(AcK)-AMC; SIRT2 prefers (within 2-fold of difference) H_2N-KGGA(AcK)-AMC, H_2N-RHK(AcK)-AMC, H_2N-QPK(AcK)-AMC, H_2N-RH(AcK)(AcK)-AMC, and H_2N-(AcK)SK(AcK)-AMC; SIRT3 prefers (within 2-fold of difference) H_2N-(AcK)QTAR(AcK)-AMC and H_2N-QPK(AcK)-AMC.

In addition to the above-described efforts to probe whether and how the amino acid side chains immediately flanking the AcK residue affect the substrate recognition for a sirtuin enzyme, researchers have also been interested in examining the substrate specificity at the AcK binding tunnel. Even though sirtuins were initially identified as a class of AcK deacetylase enzymes, interestingly, several sirtuins were later on found to be also capable of catalyzing the removal of acyl groups bulkier than the acetyl group on the side chain of AcK. Specifically, multiple sirtuins (e.g. SIRT1, Sir2Tm, and Hst2) have been shown to be able to catalyze the removal of the side chain thioacetyl group of ThAcK [161,179,180]. A ThAcK-containing sirtuin substrate was also shown to be capable of supporting a robust enzymatic nicotinamide cleavage from NAD^+ with comparable reaction velocity to that supported by the AcK-containing substrate [179,180]. Moreover, a depropionylase activity catalyzing the N^ε-depropionylation of propionyl-lysine, a debutyrylase activity catalyzing the N^ε-debutyrylation of butyryl-lysine, and/or a dephenylacetylase activity catalyzing the N^ε-dephenylacetylation of phenylacetyl-lysine were also reported for several sirtuins, including the human SIRT1, SIRT2, SIRT3, the yeast Hst2, and the bacterial sirtuins Sir2Tm and CobB [170,181-184]. It should be pointed out that, while sirtuin's debutyrylase and dephenylacetylase activities were only observed in the *in vitro* activity assays, sirtuin's depropionylase activity was observed in both *in vitro* and *in vivo* (cellular) assays. In specific, the SIRT1 depropionylase activity was also demonstrated in a recent cellular study with 293T cells [182]. In this study, SIRT1 was shown to significantly depropionylate the lysine N^ε-propionylated p53 and p300 proteins inside the cell, based on a Western blotting analysis.

Very recently, the mitochondrial sirtuin SIRT5 was shown to be also able to catalyze the removal of the malonyl and succinyl groups respectively from N^ε-malonyl-lysine and N^ε-succinyl-lysine residues on peptides and proteins in both *in vitro* and *in vivo* assays [107,108]. In addition, the *Plasmodium falciparum* sirtuin PfSir2A was very recently shown to be able to more efficiently catalyze the removal of the medium and long chain fatty acyl groups (from N^ε-octanoyl-lysine and N^ε-myristoyl-lysine) than the removal of the short chain fatty acyl groups (from N^ε-butyryl-lysine and N^ε-acetyl-lysine) [185].

In another recent study, a small molecule containing ethyl N^ε-malonyl-lysine was also shown to be capable of supporting the SIRT1-catalyzed nicotinamide cleavage from NAD^+, and the resulting stable lysine-ADP-ribose conjugate was detected by mass spectrometry [186]. The authors of this study also proposed that the SIRT1-catalyzed formation of this stable covalent conjugate accounted for the mechanism-based SIRT1 inhibition by this small molecule. My laboratory recently found that a sirtuin (e.g. SIRT1) was also able to catalyze the formation of a stalled S-alkylamidate intermediate, like that for ThAcK, from a TuAcK-containing peptidomemetic compound, indicating the capability of TuAcK to support the nicotinamide cleavage from NAD^+ [187]. Of note, TuAcK refers to N^ε-thiocarbamoyl-lysine. It should be pointed out that the two examples of the AcK analogs cited in this paragraph presumably were not able to support a deacylation reaction, yet their capability to support the nicotinamide cleavage from NAD^+ argued for the capability of the AcK binding tunnel to accommodate these two AcK analogs.

The above findings therefore indicated that, as a general feature, the AcK binding tunnel of the sirtuin active site is also able to accommodate a N^ε-acyl group that is bulkier than the N^ε-acetyl group of AcK. (Fig. **8**) depicts those accommodatable AcK analogs with bulkier side chain acyl groups than the acetyl group in AcK side chain.

Importantly, the above-described studies also suggested that the AcK binding tunnel in different sirtuins have different preferences for the N^ε-acyl groups to be accommodated. For example, while the yeast sirtuin Hst2 was found to exhibit comparable depropionylase and deacetylase activities, human SIRT1, SIRT2, and SIRT3 all

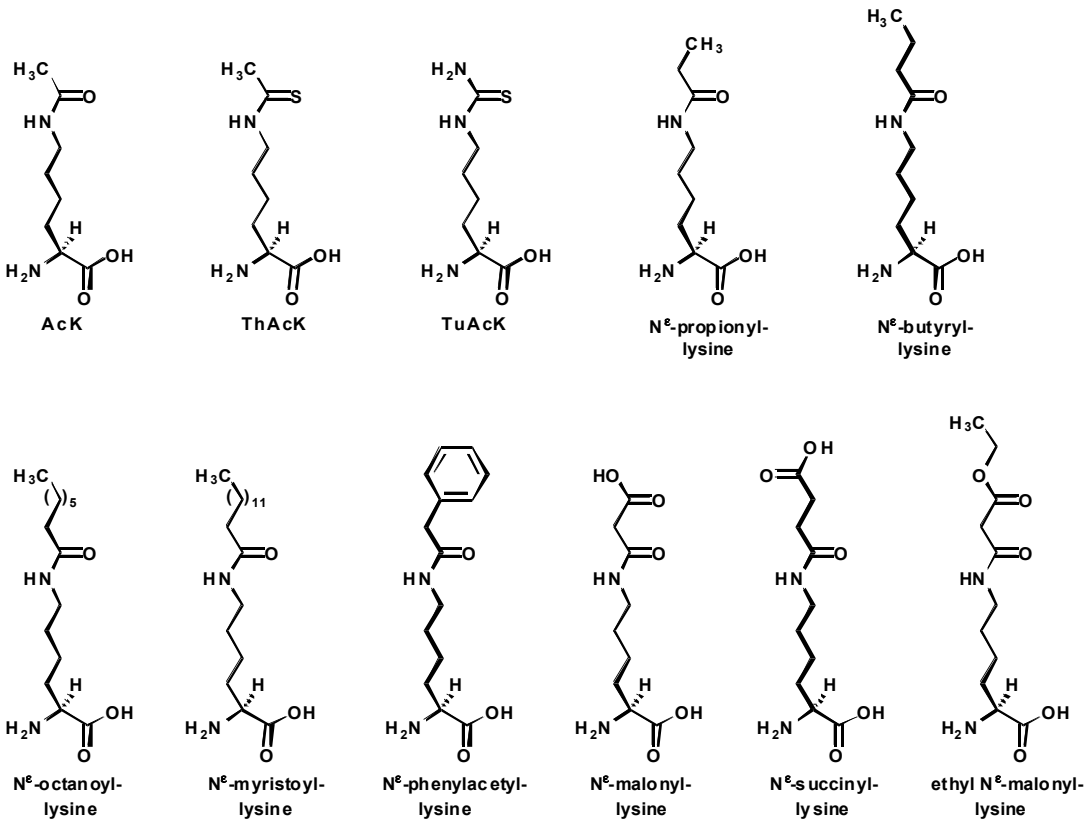

Fig. (8). Structural comparison of AcK and its analogs with bulkier side chain N$^{\varepsilon}$-acyl groups than the N$^{\varepsilon}$-acetyl group in AcK side chain. These analogs were also found to be accommodatable by the sirtuin active site AcK binding tunnel. As a typical member of this panel of the AcK analogs, ThAcK is also shown here for a structural comparison with AcK and its other analogs.

displayed a lower depropionylase activity than the corresponding deacetylase activity by ~2-3-fold. However, while Hst2 was found to only possess a negligible debutyrylase activity (~2% of its corresponding deacetylase activity), the above three human sirtuins were found to still possess an appreciable debutyrylase activity which was ~20% of their corresponding deacetylase activity [170, 183]. Furthermore, the afore-mentioned SIRT5-catalyzed demalonylation and desuccinylation reactions seemed to be specific to SIRT5 because none of the remaining human sirtuins was found to be able to catalyze these two deacyl reactions [107,108]. The authors of the studies also found that SIRT5's demalonylase and desuccinylase activities were much greater than its deacetylase activity. In this regard, the mitochondrial sirtuins SIRT3 and SIRT5 could be used in nature as the enzymes dedicated primarily toward different pools of the lysine N$^{\varepsilon}$-acylated proteins, with the former so-called mitochondrial global deacetylase [77] catalyzing the N$^{\varepsilon}$-deacetylation of AcK formed from the enzymatic acetylation reaction with acetyl-coenzyme A, and the latter catalyzing the removal of the N$^{\varepsilon}$-acyl groups from malonyl- and succinyl-lysine residues formed from the enzymatic acylation reactions with malonyl- and succinyl-coenzyme A, respectively. As mentioned above, the deoctanoylase and the demyristoylase activities of PfSir2A seem to be greater than its debutyrylase and deacetylase activities [185].

To further address the substrate specificity of the sirtuin-catalyzed deacetylation reaction, my laboratory prepared a series of pentapeptides with the p53 peptide sequence of H_2N-HK-X-LM-COOH in which X refers to AcK or one of its close structural analogs shown in (Fig. **9**). When these peptides were examined for their ability to support the nicotinamide cleavage from NAD^+ and the deacetylation with the human SIRT1, robust SIRT1-catalyzed nicotinamide cleavage and deacetylation were observed with the AcK-containing peptide, however none of the AcK analog peptides was found to be able to support the nicotinamide cleavage or the deacetylation under the experimental condition of the study [188]. It should be pointed out that no nicotinamide cleavage or deacetylation was also observed when acetyl-poly-ornithine was assayed with Hst2 [169]. It can thus be concluded from these observations that the sirtuin (e.g. SIRT1 or Hst2)-catalyzed nicotinamide cleavage and deacetylation have a very stringent requirement for the distance between the α-carbon and the side chain acetamido group, with that found in AcK being optimal.

Interestingly, it was also found in our study with peptides H_2N-HK-X-LM-COOH that the SIRT1 inhibitory potency increased when the distance between the side chain carbonyl oxygen atom and the α-carbon became closer to that in AcK [188]. Specifically, among all the analogs, that distance in S-Car-K is closest to that in AcK, followed by S-AcK. Interestingly, H_2N-HK-(S-AcK)-LM-COOH was found to be

H_2N-HK-(**AcK**)-LM-COOH
H_2N-HK-(**AcOrn**)-LM-COOH
H_2N-HK-(**CysAcm**)-LM-COOH
H_2N-HK-(**O-AcK**)-LM-COOH
H_2N-HK-(**S-AcK**)-LM-COOH
H_2N-HK-(**S-Car-K**)-LM-COOH

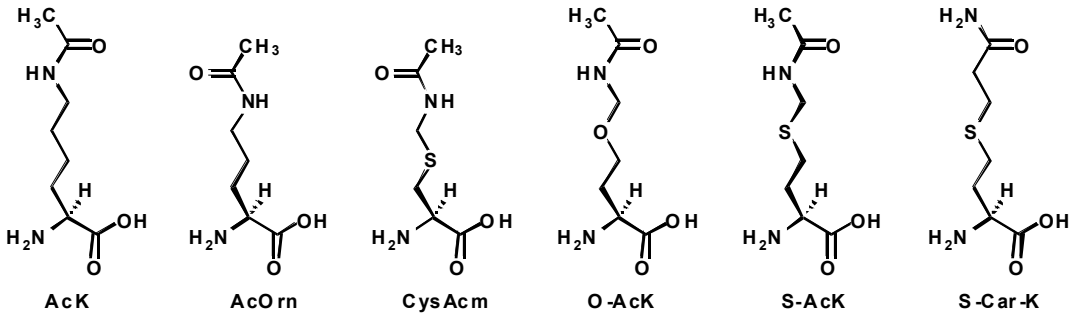

Fig. (9). The series of the pentapeptides H_2N-HK-X-LM-COOH in which X refers to AcK or one of its close structural analogs with subtly different distances from the α-carbon to the side chain amide oxygen from that in AcK. The name codes given to these AcK analogs are those also used in our original report.

a weaker SIRT1 inhibitor than H_2N-HK-(S-Car-K)-LM-COOH. This finding implied that the side chain of S-Car-K could be better accommodated by the AcK binding tunnel than that of S-AcK.

4. MECHANISM-BASED INHIBITORS OF THE SIRTUIN-CATALYZED DEACETYLATION REACTION

As mentioned above, the sirtuin-catalyzed deacetylation reaction has been shown to be involved in a variety of important biological processes such as gene transcription, apoptosis, DNA repair, metabolism, aging, neurodegeneration, and HIV-1 replication [10,11,112-129], as such the chemical modulation (inhibition and activation) of this enzymatic reaction could have therapeutic benefits in managing metabolic and age-related diseases and cancer. Furthermore, the corresponding chemical modulators could also be invaluable tools for helping explore the sirtuin biology and pharmacology.

The past a few years have witnessed a tremendous interest in developing various types of the chemical modulators (inhibitors and activators) for the deacetylase activity of sirtuins [49,130-148]. One technique that has been employed during this enterprise was the lead discovery *via* high throughput screening of chemical compound libraries against different sirtuin isoforms. In terms of developing sirtuin inhibitors, potent and/or selective compounds with different structural classes have been identified *via* this approach and its integration with the downstream lead optimization *via* traditional medicinal chemistry effort. However, another approach known as the mechanism-based lead discovery has also exhibited its great power during the past a few years per its capability of quickly furnishing potent lead compounds against the deacetylase activity of sirtuins. While the lead discovery *via* the above two approaches both do not rely on the structural information of sirtuin enzymes which is still not that rich particularly for the human sirtuins in the ligand-bound form, the latter approach has additional advantages of being able to cost-effectively and quickly furnish lead compounds and being applicable to the entire sirtuin family of deacetylase enzymes since the sirtuin family members are believed to have a well-conserved deacetylation catalytic mechanism.

One type of the mechanism-based enzyme inhibitor refers to an analog of an enzymatic reaction substrate that can be processed by the enzyme with the formation of a true enzyme inhibiting species whose mode of inhibition could be covalent or non-covalent [189]. As such, this type of the mechanism-based inhibitor is alternatively known as a suicide substrate. By inspecting the proposed chemical mechanism for the sirtuin-catalyzed deacetylation reaction (Fig. **4**), the close structural analogs of NAD^+ or AcK (as a "minimal substrate") could be both potentially processed by a sirtuin with the formation of a true inhibiting species. One kind of such species would be a stalled catalytic intermediate resembling the α-1'-O-alkylamidate or the bicyclic intermediate, which could behave as a bi-substrate analog inhibiter. However, the close analogs of NAD^+ could be less favored compounds to develop than the close analogs of AcK since (i) NAD^+ is also involved in a plethora of biochemical reactions in a biological system in addition to the sirtuin-catalyzed reaction, such as a variety of oxidation-reduction reactions, and (ii) these compounds could be not that cell permeable due to the richly charged nature of NAD^+ and its anticipated close structural analogs under the physiological pH. Therefore, the close structural analogs of AcK became a focus during the past a few years as mechanism-based sirtuin inhibitory warheads. The first such warhead was ThAcK that was first reported in my laboratory at University of Akron [179].

4.1. The ThAcK-containing Sirtuin Inhibitors

As described above, a ThAcK-containing peptide could be accepted by a sirtuin (e.g. SIRT1, Sir2Tm, or Hst2) as a substrate to support the nicotinamide cleavage with the formation of a stalled species, i.e. α-1'-S-alkylamidate [161,179,180]. Since this stalled intermediate can be

regarded as a covalent conjugate between the two substrates (i.e. NAD^+ and the ThAcK-containing peptide), it likely behaves as a tight-binding bi-substrate analog sirtuin inhibitor. Therefore, the ThAcK-containing peptide substrate could be a *bona fide* mechanism-based sirtuin inhibitor or suicide substrate.

Since the initial disclosure of our discoveries that the ThAcK-containing human p53 peptide H_2N-KKGQSTSRHK-(ThAcK)-LMFKTEG-COOH exhibited a very potent SIRT1 inhibition and that this peptide could be accepted by SIRT1 as a substrate, leading to the nicotinamide formation at an appreciable rate but a very sluggish formation of the dethioacetylated peptide substrate [179], quite a few ThAcK-containing peptides have also been shown to be potent inhibitors against different sirtuins including Hst2, SIRT1, SIRT2, and SIRT3 [180,190,191]. Interestingly, some of these reported ThAcK peptides also displayed various degrees of inhibitory selectivity among different sirtuins (e.g. among SIRT1, SIRT2, and SIRT3). Since a ThAcK peptide can behave as a mechanism-based sirtuin inhibitor in that it would be first recognized as a sirtuin substrate, the observed selective inhibition among different sirtuins by a ThAcK peptide therefore further strengthened the notion that different sirtuins could have quite different modes of substrate side chain recognition at their active sites even though they all catalyze the NAD^+-dependant deacetylation reaction. Through in-depth kinetic studies with the yeast Hst2, the ThAcK-containing histone H3 peptide H_2N-KSTGG-(ThAcK)-APRKQ-COOH was found by Smith and Denu to be a competitive inhibitor *versus* the AcK peptide substrate with an inhibition constant (K_{is}) of 17 nM [180].

Since a linear peptide is susceptible to peptide bond cleavage catalyzed by peptidases/proteases and is not cell permeable in general [192], developing proteolytically stable and cell permeable ThAcK-containing peptidomimetic or non-peptide sirtuin inhibitors has become a recent interest. One such compound (compound **1** in Fig. **10**) reported by Miyata and co-workers represents the first reported non-peptidic ThAcK-containing sirtuin inhibitor [193]. Under their assay condition, the authors reported that (i) this compound behaved as a potent SIRT1 inhibitor (IC_{50} ~2.7 μM) with 8.5-fold and >37-fold inhibitory selectivity *versus* SIRT2 and SIRT3, respectively, and (ii) this compound was able to inhibit SIRT1 inside the human colon cancer cell line HCT116 in a concentration-dependent manner, as evidenced by the concentration-dependent elevation of the Lys^{382} acetylation level of p53 in the HCT116 cells following the compound treatment.

My laboratory has identified two ThAcK-based proteolytically stable and cell permeable peptidomimetic inhibitors (compounds **2** and **3** in Fig. **10**) against the human sirtuins [194]. We found that these two compounds were both stronger SIRT1 inhibitors than the compound reported by Miyata and co-workers by 67-fold and 24-fold for **2** and **3**, respectively. A concentration-dependent increase in the p53 protein Lys^{382} acetylation level inside the human colon cancer cell line HCT116 was also observed following the treatment with these two compounds, suggesting that they were also able to inhibit the SIRT1 deacetylase activity inside the HCT116 cells.

Very recently, Huhtiniemi et al. reported that their ThAcK-containing non-peptidic compound (**4** in Fig. **10**) and peptidomimetic compounds (**5** and **6** in Fig. **10**) were also stronger SIRT1 inhibitors than compound **1** in (Fig. **10**) by 6.2-fold, 7.6-fold, and 10.2-fold for **4**, **5**, and **6**, respectively [195]. Furthermore, they showed that these three compounds were also able to inhibit the SIRT1 deacetylase activity inside different cells including the normal human astrocyte (NHA) cells, the ARPE retinal pigment epithelial cells, and the SH-SY5Y neuroblastoma cells. However, the lack of the Western blotting signal for the total p53 protein level with which the signal for the acetylated p53 protein level can be compared makes it difficult to judge exactly how efficient these three compounds inhibit the intracellular SIRT1. And for the peptidomimetic compounds **5** and **6**, their proteolytical stability is yet to be assessed.

It thus seems that our peptidomimetic scaffolds are able to confer the strongest sirtuin inhibition among all the currently reported non-peptidic or peptidomimetic scaffolds.

4.2. L-ACAH-containing Sirtuin Inhibitors

As described in the last section, the discovery of the very potent sirtuin inhibition by a compound containing the sirtuin inhibitory warhead ThAcK truly ushers in a very simple yet very effective sirtuin inhibitor lead discovery approach. However, since ThAcK contains a thioamide functionality on its side chain, the potential liability of a thioamide compound to the metabolic activation and the ensuing cytotoxicity [196-200], especially the hepatotoxicity, could be a drawback for the ThAcK-based therapeutics, especially those intended to be administered orally due to the first-pass effect [201]. This toxicity was thought to be derived from the metabolic S-oxidation of the thioamide functionality with the formation of the reactive S,S-dioxide electrophile that can react with nucleophilic species on biological macromolecules [196-200]. Therefore, it has also been our interest to identify other close structural analog(s) of AcK that could also confer potent sirtuin inhibition.

One such analog that we discovered recently is L-ACAH (Fig. **6**) that resulted from the isosteric swapping of the NH and the CH_3 groups of the acetamide portion in AcK. This structural change was made on the following considerations: (i) carboxamide is a common functional group in drug molecules [202]; (ii) as described above in Section 3, the pair-wise SIRT1 inhibitory study with peptides H_2N-HK-(S-AcK)-LM-COOH and H_2N-HK-(S-Car-K)-LM-COOH (Fig. **9**) implied that the side chain of S-Car-K could be better accommodated by the AcK binding tunnel than that of S-AcK, which is consistent with the fact that the extended distance between the α-carbon and the side chain amide oxygen of S-Car-K is closer to that in AcK than S-AcK. Therefore, the side chain of L-ACAH could be accommodated even better by the AcK binding tunnel since that distance of L-ACAH seems to be even closer to that of AcK

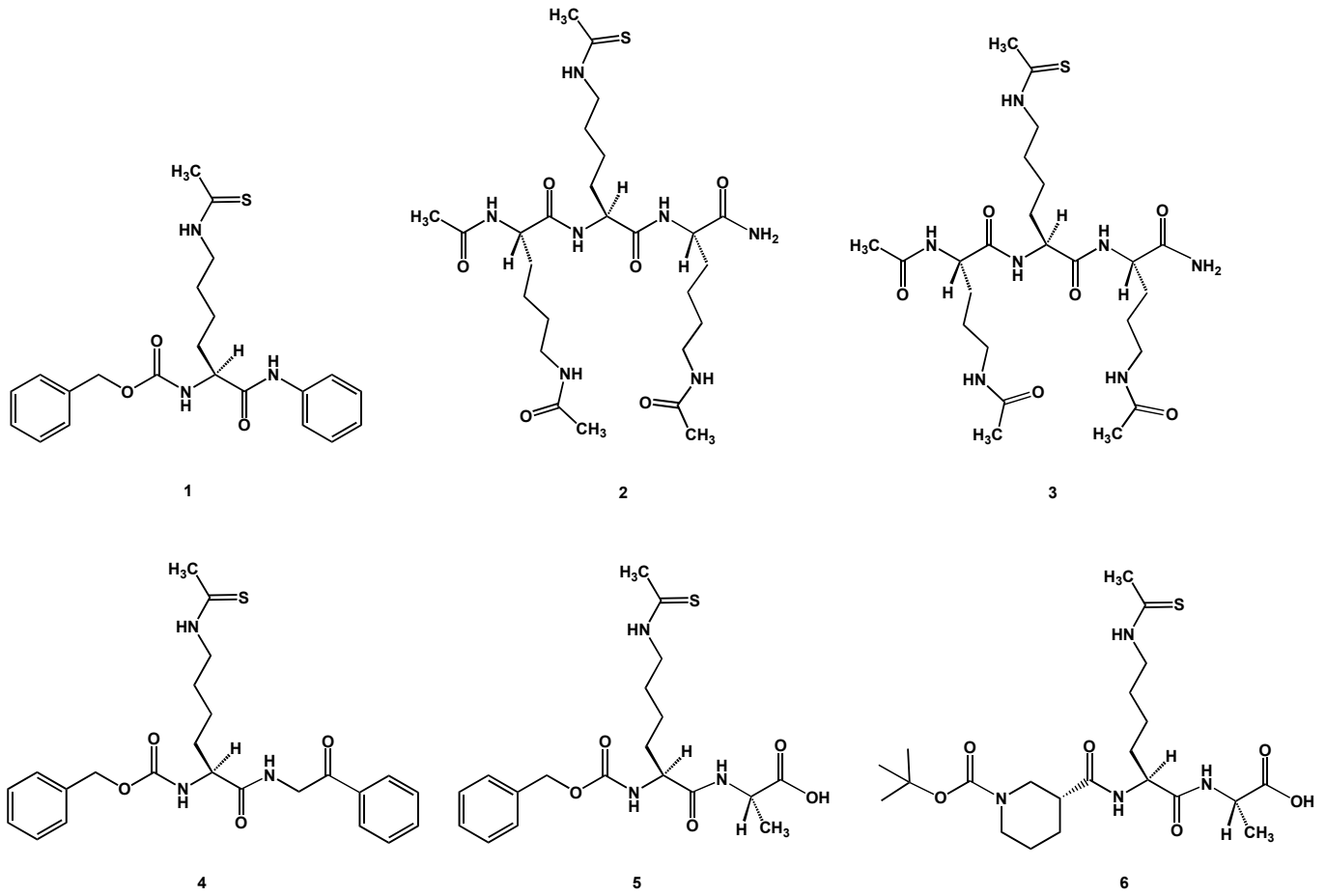

Fig. (10). The chemical structures of the non-peptidic or peptidomimetic ThAcK-containing sirtuin inhibitors.

than S-Car-K. And as such, a strong sirtuin inhibition could be reasonably expected for a compound containing L-ACAH.

When L-ACAH was replaced for the AcK residue in the following SIRT1 peptide substrate: CH_3CONH-HK-AcK-LM-$CONH_2$, we found that the resulting pentapeptide CH_3CONH-HK-(L-ACAH)-LM-$CONH_2$ potently inhibited SIRT1 (IC_{50} ~ 12 μM) under our HPLC-based assay condition [171]. The *in vitro* studies with this peptidic inhibitor revealed that (i) the inhibitor was competitive *versus* the AcK-containing SIRT1 peptide substrate H_2N-HK-AcK-LM-COOH with an inhibition constant (K_{is}) of 2.6 μM, (ii) the inhibitor was of the slow, tight-binding type as suggested by the time-dependent inhibition and a lag in the formation of the deacetylated product during the activity recovery of the inhibited SIRT1 toward a full regain of its deacetylase activity, and (iii) as above described in Section 2.2 and that depicted in (Fig. **6**), the inhibitor was able to be converted to at least one longer-lived catalytic intermediate (Intermediate II and likely Intermediate III as well) and perhaps all the way to the end product (similar to 2'-O-AADPR) as well.

In order to explore the feasibility of developing the L-ACAH-containing non-peptidic or peptidomimetic sirtuin inhibitors, as has been done by us and others for the development of non-peptidic or peptidomimetic ThAcK-containing sirtuin inhibitors, the two compounds shown in (Fig. **11**) were designed, synthesized, and evaluated for their inhibitory potency against sirtuins [171]. Of note, in the first compound, L-ACAH was incorporated into the non-peptidic scaffold that Miyata and co-workers used previously to develop their non-peptidic ThAcK-containing human sirtuin inhibitor [193]; in the second compound, L-ACAH was incorporated into the peptidomimetic scaffold that we used previously to develop our peptidomimetic ThAcK-containing human sirtuin inhibitor [194]. We found that while the first compound was a fairly weak inhibitor against SIRT1-catalyzed deacetylation of H_2N-HK-AcK-LM-COOH with an IC_{50} > 1 mM, the second compound behaved as a reasonably potent SIRT1 inhibitor with an IC_{50} ~290 μM. This difference in the SIRT1 inhibitory potency of these two compounds further reinforced the notion afore-mentioned in Section 4.1 that our peptidomimetic scaffolds are able to confer the strongest sirtuin inhibition among all the currently reported non-peptidic or peptidomimetic scaffolds. In this study, we also found the second compound to be an *in vitro* inhibitor against SIRT2 and SIRT3 with comparable potency to that against SIRT1. The subsequent *in vivo* characterization of the above second compound revealed its capability of inhibiting the SIRT1 deacetylase activity inside the human colon cancer cell line HCT116.

Taken together, the above findings suggested that the sirtuin deacetylase inhibition by a L-ACAH-containing compound is mechanism-based and a general inhibitory strategy against sirtuin-catalyzed deacetylation reaction. They also pointed to the potential of developing further potent and cell permeable L-ACAH-based peptidomimetic or non-peptidic inhibitors for sirtuins.

4.3. Other mechanism-based Sirtuin Inhibitors

As depicted in (Fig. **4**), one unique mechanistic feature along the sirtuin deacetylation reaction coordinate is the transglycosidation (or the base exchange) reaction with the free nicotinamide to regenerate NAD^+ and the AcK-containing substrate. As such, nicotinamide could be also a mechanism-based sirtuin inhibitor. Indeed, nicotinamide was found to be a universal inhibitor against the sirtuin-catalyzed deacetylation reaction, and the kinetics of this inhibition was also determined to be non-competitive *versus* the AcK substrate [167,168]. Since nicotinamide is present as an endogenous small molecule, sirtuin deacetylase activity is thus subjected to an *in vivo* modulation by nicotinamide. However, it should be pointed out that differential inhibitory potency has been observed for nicotinamide against the deacetylation reaction catalyzed by different sirtuins. Specifically, in the study of Sauve and Schramm with murine SIRT1, yeast Sir2p, and archeal Sir2-Af2 [165], nicotinamide was found to most potently inhibit the SIRT1-catalyzed deacetylation reaction, whereas Sir2-Af2 was found to be least inhibitable by nicotinamide among the three sirtuins. This differential inhibitory efficiency of nicotinamide is consistent with the decreasing capability of the saturating nicotinamide to cause the return of the α-1'-O-alkylamidate intermediate to NAD^+ and the acetylated substrate in the order of SIRT1, Sir2p, and Sir2-Af2. In the study of Denu and co-workers with yeast Hst2 and human SIRT2 [166], nicotinamide was found to inhibit Hst2- and SIRT2-catalyzed deacetylation reaction with apparent K_i values of 298 and 33.9 μM, respectively, which is consistent with the k_{cat}/K_m ratios for the nicotinamide base exchange reaction catalyzed by these two sirtuins: 7.4 x 10^3 M^{-1} s^{-1} for Hst2 and 6.3 x 10^4 M^{-1} s^{-1} for SIRT2.

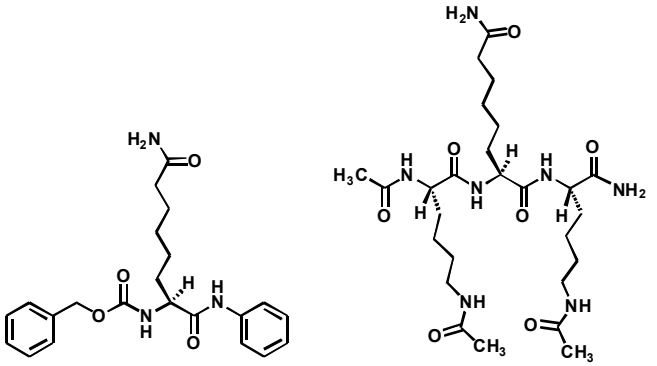

Fig. (11). The chemical structures of the designed non-peptidic and peptidomimetic L-ACAH-containing compounds.

As described above in Section 3, the SIRT1 active site was recently shown by Miyata and co-workers to be also able to accommodate ethyl N^{ε}-malonyl-lysine (Fig. **8**) to support the nicotinamide cleavage from NAD^+, which led to the formation of a stable lysine-ADP-ribose conjugate from a small molecule containing ethyl N^{ε}-malonyl-lysine [186]. (Fig. **12**) depicts the proposed route of the enzymatic formation of this conjugate whose presence in the SIRT1 assay mixture was confirmed by mass spectral detection. Since this stable conjugate formed within the SIRT1 active site can be regarded as a compound simultaneously occupying the binding sites for NAD^+ and AcK substrate, it also likely behaves as a tight-binding bi-substrate analog SIRT1 inhibitor. Therefore, the ethyl N^{ε}-malonyl-lysine-containing small molecule could also be a *bona fide* mechanism-based SIRT1 inhibitor or suicide substrate, with the stable lysine-ADP-ribose conjugate as the true SIRT1 inhibiting species. This small molecule was also found to be a competitive SIRT1 inhibitor *versus* the AcK substrate with a K_{is} of 9.5 μM, suggesting that ethyl N^{ε}-malonyl-lysine was able to compete directly with AcK for binding to its binding tunnel. Under authors' assay condition, this small molecule was further shown to be a selective SIRT1 inhibitor with 17-fold and >77-fold inhibitory selectivity *versus* SIRT2 and SIRT3, respectively. It was also shown to be capable of inhibiting SIRT1 inside the human colon cancer cell line HCT116 in a concentration-dependent manner, as evidenced by the concentration-dependent elevation of the Lys^{382} acetylation level of p53 in the HCT116 cells following the compound treatment.

As above mentioned in Section 3, my laboratory previously at University of Akron very recently also developed TuAcK (Fig. **8**) as another close AcK analog that could also confer potent mechanism-based sirtuin inhibition [187]. This development represents another significant step in our ongoing effort to develop potent sirtuin inhibitory AcK analogs alternative to ThAcK.

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. **5**) was shown previously to be processed by the yeast sirtuin Hst2 to form the corresponding analog of α-1'-O-alkylamidate intermediate shown in (Fig. **5**) following the Hst2-catalyzed nicotinamide cleavage from NAD^+ [169,170], we reasoned that TuAcK could also be processed similarly. However, unlike the intermediate formed from homocitrulline which was suggested to be intercepted by water to regenerate homocitrulline (as depicted in Fig. **5**) [169,170], the corresponding intermediate formed from TuAcK could be longer-lived just like that formed from ThAcK; this intermediate may also be able to exert a potent sirtuin inhibition as a bi-substrate analog inhibitor. Secondly, some thiourea compounds have been known to be strong scavengers of the hydroxyl radical or the superoxide radical anion [203-205]. This potential anti-oxidant effect of a thiourea compound constitutes another strong argument 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 [52,98,206]. The sirtuin inhibition-induced potential attenuation of the cellular anti-oxidation defense system could be of a particular concern when developing therapeutics for the diseases of aging (e.g. SIRT2

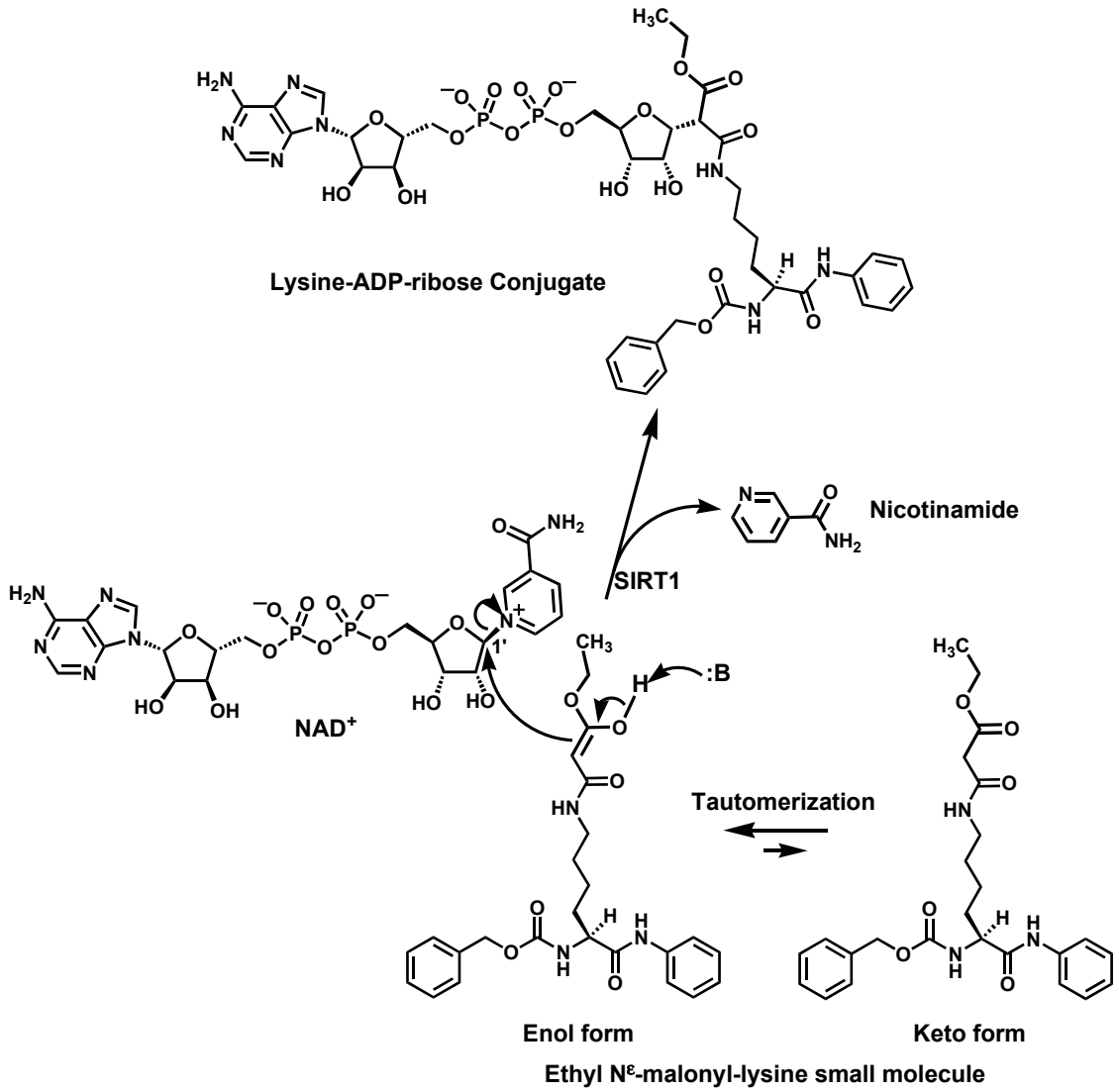

Fig. (12). The proposed route of the SIRT1-catalyzed formation of the stable lysine-ADP-ribose conjugate from NAD^+ and the depicted small molecule containing ethyl N^{ε}-malonyl-lysine. B: refers to a general base.

inhibitors for the Parkinson's disease [207]). In this regard, it has been recently reported that SIRT2 silencing by RNAi and pharmacological inhibition of the SIRT2 deacetylase activity resulted in a diminished cellular ATP level in PC12 cells [208].

To assess the sirtuin inhibitory power of TuAcK, the peptidomimetic compound (i.e. TuAcK Trimer in Fig. **13**) was prepared [187]. The structural context of TuAcK in this compound is the same as that of ThAcK in compound **3** in (Fig. **10**), i.e. the AcOrn-based peptidomimetic scaffold that was shown by us previously to be proteolytically stable and to be able to confer cell membrane permeability on compound **3** [194].

When TuAcK Trimer was evaluated by a HPLC-based SIRT1 inhibition assay, we found that it was ~15-fold weaker than its ThAcK counterpart (i.e. **3** in Fig. **10**) as a SIRT1 inhibitor. However, it was a ~3-fold stronger SIRT1 inhibitor than the second compound in (Fig. **11**) (i.e. the L-ACAH compound with the AcK-based peptidomimetic scaffold) [187]. Since we previously also found that the AcOrn-based peptidomimetic scaffold was ~3-fold weaker than the AcK-based one in bestowing SIRT1 inhibition [194], TuAcK thus seems to be a more powerful SIRT1 inhibitory warhead than L-ACAH.

In the study with TuAcK Trimer [187], we also found that it exhibited a more or less comparable inhibitory potency (within ~3-fold) against SIRT1, SIRT2, and SIRT3, suggesting that TuAcK could confer potent sirtuin inhibition as a general inhibitory warhead. By using mass spectrometry in the same study, we also found that the TuAck residue in TuAcK Trimer was also able to be processed by SIRT1 with the formation of a stalled S-alkylamidate intermediate, like that for ThAcK. (Fig. **13**) depicts how the SIRT1-catalyzed condensation of TuAcK Trimer with NAD^+ could form the α-1'-S-alkylamidate intermediate, the likely true SIRT1 inhibiting species.

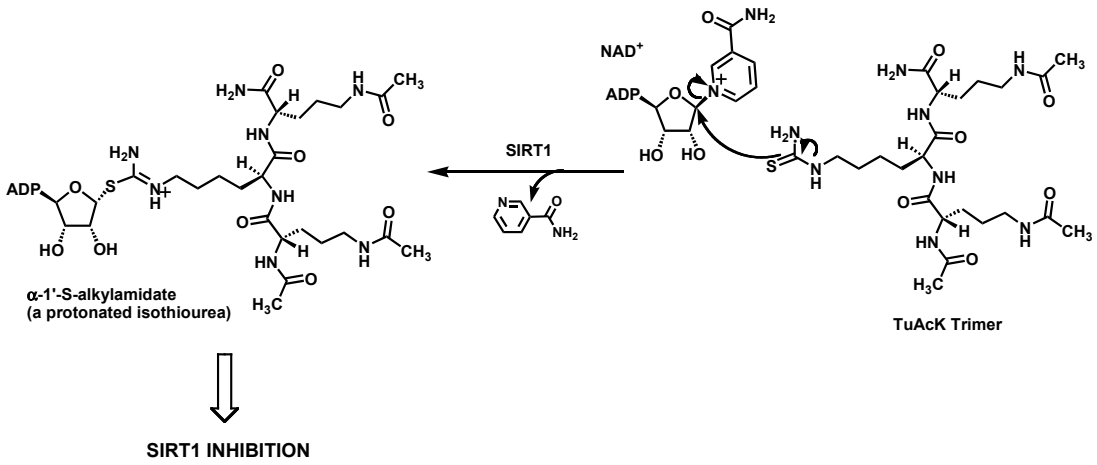

Fig. (13). Proposed SIRT1 processing of TuAcK trimer with the formation of the depicted α-1'-S-alkylamidate intermediate, leading to SIRT1 inhibition.

Taken together, the above findings suggested that TuAcK represented another general and strong sirtuin inhibitory warhead, and that a TuAck sirtuin inhibitor also behaved as a mechanism-based inhibitor. Considering the potential of a thiourea compound as 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 sirutin inhibitors. For the TuAcK homologs, those bearing alkyl groups (e.g. methyl, ethyl) on the terminal NH_2 group of TuAcK side chain may be considered, given the capability of the sirtuin active site to accommodate bulkier N^{ε}-acyl groups than the N^{ε}-acetyl group in AcK side chain, as afore-described in Section 3. The study on TuAcK homologs is under way in my laboratory currently at Jiangsu University and the corresponding findings will be reported in due course.

N^{ε}-selenoacetyl-lysine, N^{ε}-isothiovaleryl-lysine, and N^{ε}-thiosuccinyl-lysine were also found in *in vitro* assays to be able to confer strong sirtuin inhibition [209,210]. Specifically, the compounds shown in (Fig. **14**) that contained either N^{ε}-selenoacetyl-lysine or N^{ε}-isothiovaleryl-lysine were recently found to be SIRT1 and SIRT2 inhibitors with comparable potency to their ThAcK counterparts; and the N^{ε}-thiosuccinyl-lysine-containing peptide shown in (Fig. **14**) was very recently found to be a potent and most selective (*versus* SIRT1-3) SIRT5 inhibitor identified to date. Presumably these sirtuin inhibitors work *via* a similar mechanism-based manner to that by the ThAcK-containing sirtuin inhibitors.

5. MECHANISM-BASED ACTIVATORS OF THE SIRTUIN-CATALYZED DEACETYLATION REACTION

In addition to the interest in developing sirtuin inhibitors, developing the activators for the deacetylase activity of sirtuins has also attracted certain degree of attention primarily due to the potential of SIRT1 activation in treating type 2 diabetes [211]. Among the claimed SIRT1 activators are the natural product resveratrol and some synthetic compounds developed by Sirtris (a GSK company) *via* the chemical library screening [141,147]. However, how these compounds achieve SIRT1 activation has been debated [147], and resveratrol has been recently found to be an indirect activator of SIRT1, *via* its direct inhibition of cAMP-dependent phosphodiesterases, especially PDE4 [212,213]. It should be pointed out that the sirtuin deacetylation mechanistic features have been so far primarily exploited for developing the inhibitors rather than the activators. But still isonicotinamide (Fig. **15**) is a *bona fide* mechanism-based activator for the sirtuin-catalyzed deacetylation reaction.

Isonicotinamide was designed as a competitive inhibitor for the nicotinamide base-exchange reaction, thereby relieving the nicotinamide inhibition of the sirtuin deacetylation reaction. Indeed, isonicotinamide was found by Sauve et al. to be a selective and competitive inhibitor against the nicotinamide base-exchange reaction catalyzed by the yeast Sir2 [214]. This mode of inhibition could be appreciated from the fact that isonicotinamide is a close structural analog of nicotinamide resulting from the CH and N isosteric swapping, so that it could be accommodated by the nicotinamide binding pocket within a sirtuin active site. When bound to the nicotinamide binding pocket, the primary carboxamide group of isonicotinamide is expected to behave similarly to that of nicotinamide which was observed previously to act as an anchor for the whole nicotinamide molecule *via* its two critical hydrogen bond interactions within the nicotinamide binding pocket [215]. Moreover, the pyridine ring of the bound isonicotinamide is also expected to be able to rotate about the primary carboxamide anchor, as that observed previously for the bound nicotinamide [215]. It should be pointed out that, whereas two consequent low-energy rotamers were observed for the asymmetric nicotinamide molecule [215], the pyridine ring rotation should still give rise to a single rotamer for the symmetric isonicotinamide molecule. However, the bound isonicotinamide and nicotinamide very likely still have a same binding pose for their pyridine rings. Then the pyridine N of the bound isonicotinamide should be positioned too far away from the electrophilic C1' position of the α-1'-O-alkylamidate

intermediate to react with this intermediate (Fig. **15**). Therefore, isonicotinamide should also lack any inhibitory action toward the sirtuin deacetylation chemistry, which has been demonstrated with the yeast Sir2 [214]. Isonicotinamide has also been shown to be able to increase both the apparent K_m value of nicotinamide for the yeast Sir2-catalyzed base-exchange reaction and the apparent K_i value for nicotinamide's inhibition against the yeast Sir2 deacetylation reaction [214].

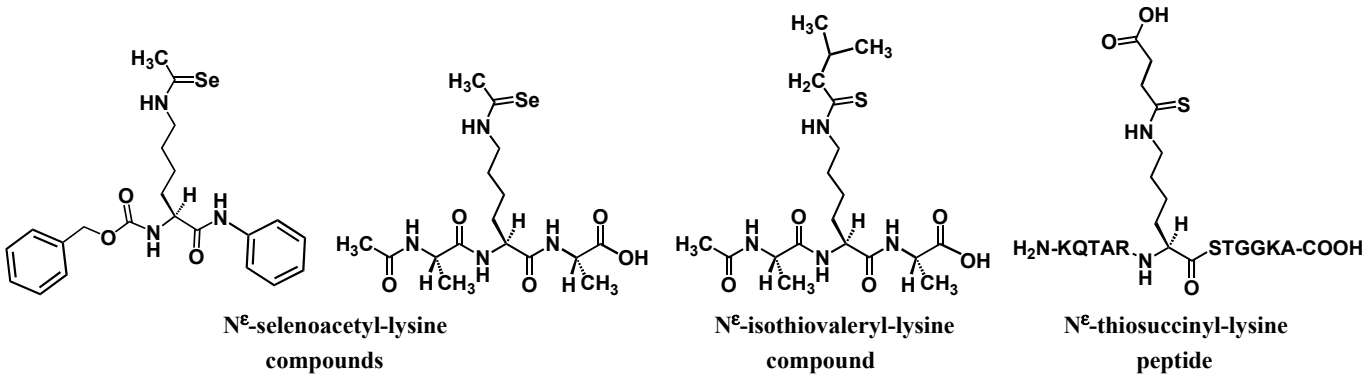

Fig. (14). The putative mechanism-based sirtuin inhibitors containing N^{ε}-selenoacetyl-lysine, N^{ε}-isothiovaleryl-lysine, or N^{ε}-thiosuccinyl-lysine.

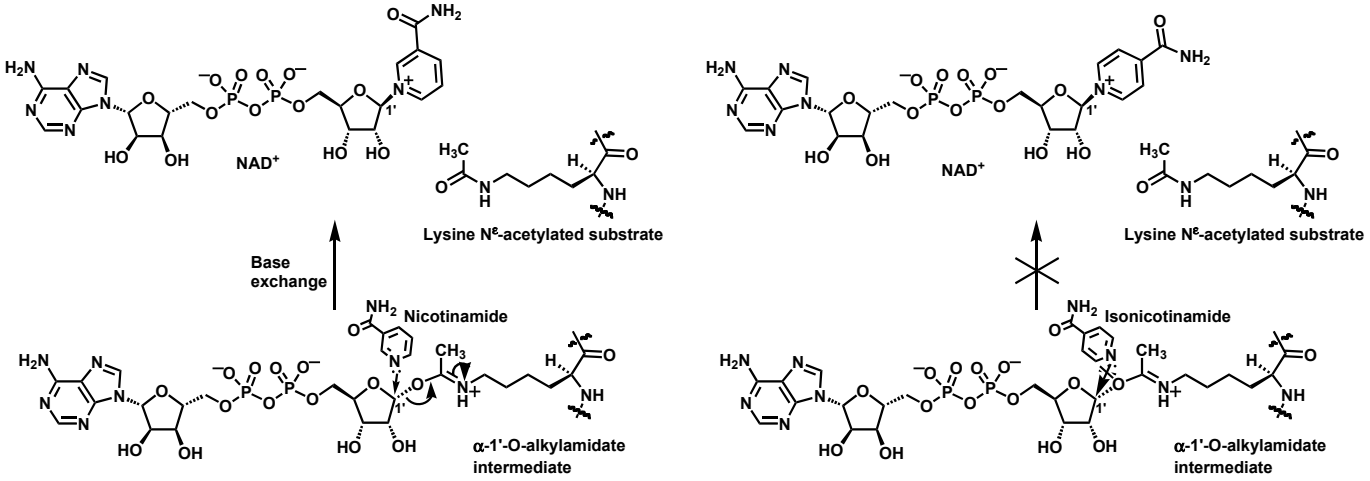

Fig. (15). A comparative illustration of the nicotinamide-enabled and the isonicotinamide-disabled sirtuin base-exchange reaction.

As afore-mentioned in Section 4.3, different sirtuins have different degrees of sensitivity toward the nicotinamide inhibition against their deacetylation reaction, it would thus be conceivable that different sirtuins will also correspondingly be differentially activated by isonicotinamide. In specific, if one sirtuin-catalyzed deacetylation reaction is more sensitive than another toward the nicotinamide inhibition, the former will also be more sensitive toward the isonicotinamide activation.

6. CONCLUSIONS AND PERSPECTIVES

Since the discovery of the AcK N^{ε}-deacetylase activity of the yeast Sir2 protein about 10 years ago [216], more and more Sir2 homologs collectively forming the sirtuin family of the deacetylase enzymes have been identified from yeast and other organisms including humans. Concomitantly, more and more physiological targets for these enzymes have been discovered. Significantly, these targets are involved in an ever increasing array of crucial physiological processes such as gene transcription, apoptosis, and metabolism, just name a few. As such, tremendous interest has been demonstrated for exploring the therapeutic benefits by inhibiting or activating the sirtuin-catalyzed deacetylation reaction. While this exploration is still ongoing, past genetic and pharmacological studies have already suggested that human SIRT1 activation may alleviate type 2 diabetes, SIRT1/2 inhibition may be an avenue for developing novel anti-cancer agents, and SIRT2 inhibition constitutes a novel therapeutic approach to Parkinson's disease. Within this context, a rigorous pursuit of the inhibitors and the activators for the sirtuin deacetylation reaction is not surprising since these molecular entities could not only lead to novel therapeutics but also help to further decipher the biology and pharmacology of this deacetylation reaction.

In terms of developing inhibitors and activators for sirtuin deacetylation reaction, theoretically multiple lead discovery approaches could be taken, such as chemical

library screening, structure-based design, virtual library screening, etc. Considering that the structural information of sirtuin enzymes is still not that rich at present time particularly for the human sirtuins in the ligand-bound form, the structure-based approaches have been overall of limited use despite two very recent reports toward the structure-based sirtuin inhibitor lead discovery and derivatization [195,217]. Instead the library screening approach has been successful in furnishing sirtuin inhibitors with different structural classes. Several claimed activators have also been discovered by such approach. However, the mechanism-based lead discovery approach has also exhibited its great power during the past a few years since it could quickly and cost-effectively furnish lead inhibitors and activators for all the deacetylase enzymes within the entire sirtuin family due to the well-conserved catalytic mechanism for all the sirtuin deacetylase enzymes.

As for the lead inhibitor discovery, the mechanism-based approach has been primarily about the discovery of AcK close structural analogs as effective mechanism-based sirtuin inhibitory warheads. During the past a few years, a variety of such warheads have been discovered including ThAcK, L-ACAH, TuAcK, and ethyl N^{ε}-malonyl-lysine. The ThAcK close structural derivatives N^{ε}-selenoacetyl-lysine, N^{ε}-isothiovaleryl-lysine, and N^{ε}-thiosuccinyl-lysine presumably are also able to confer a mechanism-based sirtuin inhibition in the same manner as that of ThAcK. Amazingly, the use of ThAcK and L-ACAH could lead to the stalling of different catalytic intermediates, i.e. the α-1'-S-alkylamidate intermediate and a bicyclic intermediate closely resembling, respectively, the α-1'-O-alkylamidate intermediate and the bicyclic intermediate along the reaction coordinate of the sirtuin deacetylation reaction (Fig. **4**). As such, one more significant advantage of the mechanism-based lead discovery approach over that based on the chemical library screening is the possibility to use the resulting compounds as effective sirtuin deacetylation mechanistic probes.

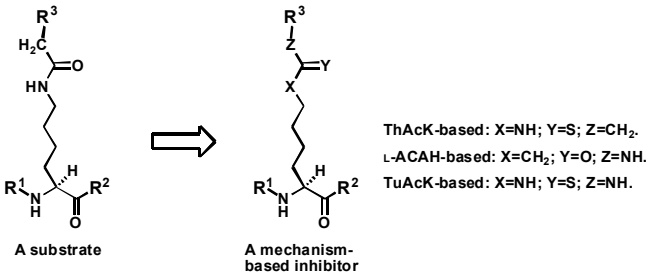

Fig. (16). The schematic illustration of the transformation of a sirtuin substrate into a mechanism-based sirtuin inhibitor. The substrate specificity for a given sirtuin is specified by the identities of the substituents R^1, R^2, and R^3.

With the ever enhanced understanding of the catalytic mechanism of the sirtuin deacetylation reaction and the substrate specificity for this enzymatic reaction, the judicious integration of the mechanism-based lead discovery approach and this substrate specificity knowledge could be a very powerful lead optimization approach. (Fig. **16**) conceptually illustrates such approach when applied to the development of potent and selective sirtuin inhibitors. Replacing AcK or its analog in a sirtuin substrate with a mechanism-based sirtuin inhibitory warhead (ThAcK-, L-ACAH-, or TuAcK-based) would lead to a potent sirtuin inhibitor. It should also be pointed out that, besides the substrate specificity elements surrounding the AcK residue defined by R^1 and R^2, the presence of an additional substrate specificity element at the AcK binding tunnel defined by R^3 would lead to a tremendous amplification of the recognition discrimination for a given substrate among different sirtuins and to a mechanism-based inhibitor also selective among different sirtuins. The very recent successful identification of the N^{ε}-thiosuccinyl-lysine peptide shown in (Fig. **14**) as a potent and very selective SIRT5 inhibitor [210] has already attested to the power of this approach in that the previous discovery that N^{ε}-succinyl-lysine (R^3 = CH_2COOH in Fig. **16**) is preferentially (*versus* N^{ε}-acetyl-lysine) and uniquely (among all human sirtuins) recognized by SIRT5 [107,108] has been exploited to give rise to this ThAcK-based inhibitor (R^3 = CH_2COOH in Fig. **16**) with exceptional SIRT5 inhibitory profile (potency and selectivity).

Since our mechanistic understanding of the sirtuin deacetylation chemistry is still not complete, it is expected that novel mechanism-based sirtuin inhibitors and activators will be developed from the exploitation of the further mechanistic details of this fascinating NAD^+-dependent enzymatic AcK N^{ε}-deacetylation reaction.

CONFLICT OF INTEREST

The author confirms that this article content has no conflicts of interest.

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