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Phosphorus-Based SAHA Analogues as Histone Deacetylase Inhibitors†

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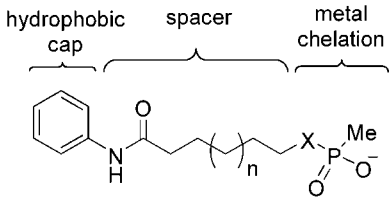
ABSTRACT



1: n=1, X = NH, **2:** n=1, X = O, **3:** n=0, X = CH_2

Three analogues of suberoyl anilide hydroxamic acid (SAHA) with phosphorus metal-chelating functionalities were synthesized as inhibitors of histone deacetylases (HDACs). The compounds showed weak activity for HeLa nuclear extracts (IC_{50} = 0.57–6.1 mM), HDAC8 (IC_{50} = 0.28–0.41 mM), and histone-deacetylase-like protein (HDLP, IC_{50} = 0.33–1.9 mM), suggesting that the transition state of HDAC is not analogous to zinc proteases. Antiproliferative activity against A2780 cancer cells (IC_{50} = 0.11–0.12 mM), comparable to SAHA (0.15 mM), was observed.

Histone deacetylases (HDACs), a family of enzymes that regulate chromatin remodeling and gene transcription via the dynamic process of acetylation and deacetylation of core histones, have been recognized as attractive therapeutic targets for anticancer,[1] antifungal, antiviral, and antiinflammatory treatment.[2]

Activity in the field of HDACs accelerated when crystal structures of *A. aeolicus* histone deacetylase homologue histone-deacetylase-like protein (HDLP) and HDLP bound to the inhibitors, trichostatin A and suberoyl anilide hydroxamic acid (SAHA), were reported. [3] The structures revealed that the enzyme contains a Zn ion at the bottom of the active site and that the Zn was chelated by the hydroxamic acid. The transition state (TS) of the deacetylation was proposed to be similar to that known for Zn metalloproteases, where the reaction proceeds through formation of a tetrahedral oxyanion intermediate (Figure 1).[3b] Although the sequence identity of HDLP with human HDAC1 is only 35%, the catalytic domain of HDLP is very closely related to known

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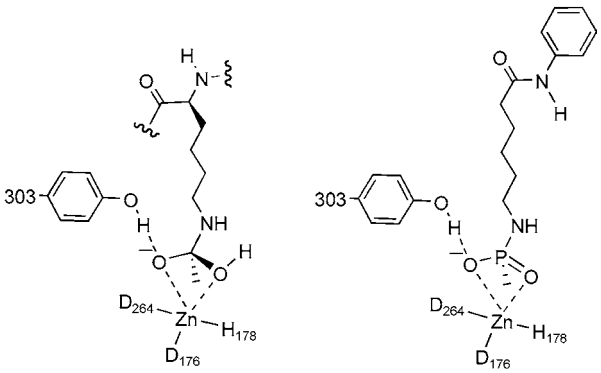

Figure 1. Left: Transition state proposed for human HDAC1.[3b] Right: Model for binding of phosphonamidate **1**.

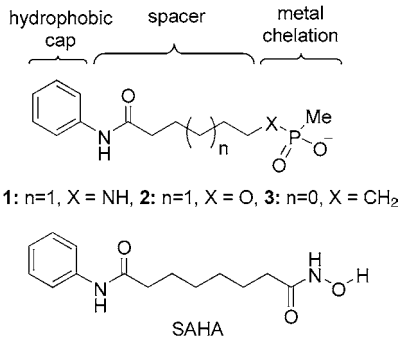

Figure 2. Structures of synthetic inhibitors compared with the known inhibitor, SAHA,[3a] with the structural motifs required for HDAC inhibition.[2a]

human HDAC isoforms and, thus, the mechanism of deacetylation may be conserved as well.[1c,3b]

Both hydroxamic acid and phosphoramidate motifs are found in known inhibitors of zinc proteases such as thermolysin and carboxypeptidase A. For example, thermolysin is inhibited by both Leu−NHOH ($K_i = 190\ \mu$M) and $^{2-}O_3P$−Leu−NH_2 ($K_i = 1.3\ \mu$M).[4] Leu−NHOH binds "backwards" in the active site, with the side chain in the P1′ site; thus, the binding contribution due to the leucine side chain is approximately the same for both inhibitors.[5] The anionic form of the hydroxamic acid ($RNOH^-$) binds to thermolysin,[5] and in both cases, the bidentate anion is coordinated to the zinc. Therefore, the phosphoramidate is a better TS analogue of the Zn protease reaction than the hydroxamic acid.

Phosphorus-containing compounds are among the most potent known inhibitors of metalloproteases.[6] Bartlett and Marlowe demonstrated that phosphonamidates are transition-state analogue inhibitors of thermolysin.[6c] The phosphorus center is tetrahedral with heteroatoms appropriately positioned to mimic the tetrahedral TS of amide bond hydrolysis (Figure 1). The phosphorus−heteroatom bonds are longer than ground-state carbon−heteroatom bonds and thus able to mimic the longer bonds of the TS.[6c]

The mechanism of HDAC deacetylation, simply an amide bond hydrolysis, was proposed to have certain analogies to zinc protease mechanisms, including a tetrahedral TS bound to Zn.[3b] This suggested that phosphorus-based TS analogues could act as potent HDAC inhibitors. To investigate the analogies between HDAC and Zn protease TSs, phosphonamidate, phosphonate, and phosphinate SAHA analogues **1**−**3** (Figure 2) were synthesized and tested as HDAC inhibitors.

The structural motifs that are significant for inhibitory activity from structure−activity relationship studies[2a] and observed in the X-ray structure[3b] are (1) a hydrophobic capping group binding to the outer rim of the tunnel, (2) a spacer interacting with the hydrophobic walls of the tunnel, and (3) a functional group serving to chelate the metal cation in the active site (Figure 2).

The syntheses of phosphonamidate **1**, phosphonate **2**, and phosphinate **3** are outlined in Scheme 1. Each synthesis began with aniline reacting with a six-carbon acid via the acyl chloride to form anilides **4**−**6**. *N*-Carbobenzyloxy-6-amino-hexanoic acid was the starting material for phosphonamidate **1**; the monomethyl ester of pimelic acid was used for phosphonate **2**, and 6-bromo-hexanoic acid was used for phosphinate **3**.

The anilide **4** was deprotected by catalytic hydrogenation to afford the free amine **7**, which was coupled with methyl phosphonic acid methyl ester **9** using EDC to give the phosphonamidate methyl ester **10**. Phosphonamidates are known to be labile under acidic conditions.[6e] It is known that Li^+ as a counterion stabilizes phosphonamidates.[6b,f] To avoid hydrolysis of phosphonamidates **10** and **1**, saponification followed by neutralization was monitored by ^{31}P NMR. The best results were achieved using 15 equiv of 1.5 M LiOH in MeOH or CH_3CN for ester hydrolysis and careful neutralization to pH 7.1. The lithium salt **1** was stable as a solid at room temperature under anhydrous conditions. It was used in the bioassays without further purification.

The phosphonate precursor, alcohol **8**, was obtained by reduction of the methyl ester **5** with $LiAlH_4$ at −10 °C. Alcohol **8** was then reacted with methylphosphonic acid **9** using PyBOP as the activating reagent and DIEA as base.[7]

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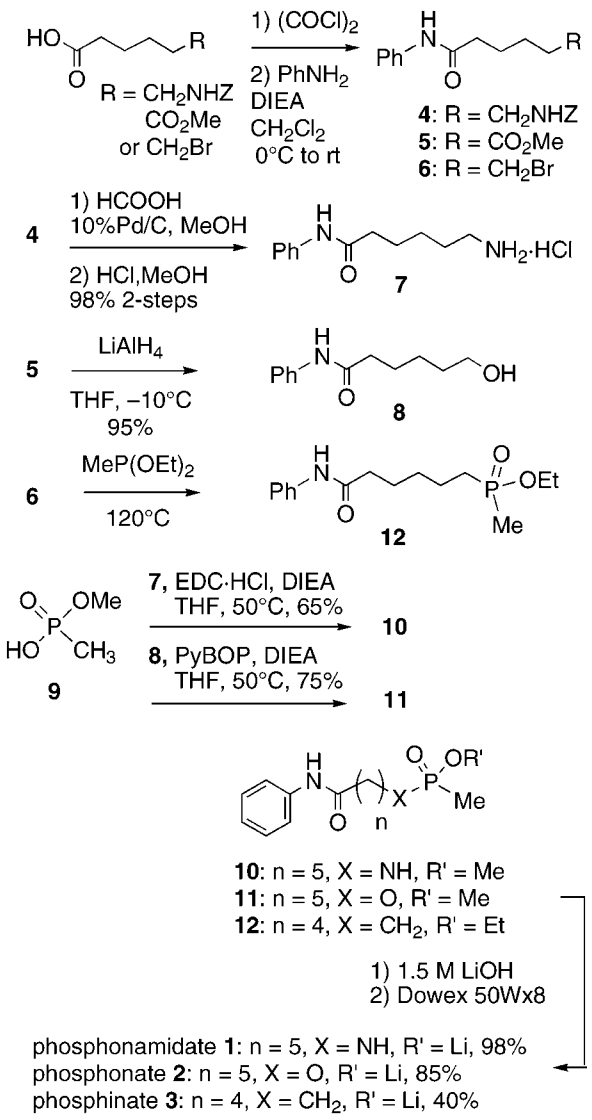
Scheme 1. Synthesis of Phosphorus-Based SAHA Analogues **1**−**3**



We found that reaction of phosphonate ester **11** with tributylamine[8] or TMSBr[7] led to complete hydrolysis with formation of alcohol **8**. Treatment of ester **11** with 10 equiv of 1.5 N LiOH in MeOH for 2 h afforded the phosphonate **2** with only partial hydrolysis (15% of **8**).

The phosphinate ester **12** was prepared by the Arbuzov reaction of bromide **6** with methyl diethyl phosphite, followed by saponification with 1.5 N LiOH. Compounds **1**−**3** were shown to be stable to hydrolysis in 25 mM Tris buffer, pH 8 at 37 °C by HPLC.

The phosphonamidate, phosphonate, and phosphinate **1**−**3**, respectively, were evaluated in HDAC enzyme inhibition assays using hyperacetylated core histones purified from HeLa cells,[9] modified by preincubation of inhibitors with enzyme for 10 h at room temperature. Preincubation was used to test for possible slow-binding behavior of the inhibitors. Slow-binding inhibition has been observed previously for all three types of phosphorus inhibitors with thermolysin.[6d,h] For the phosphonamidate **1**, a slight difference in inhibition was observed with preincubation; for **2** and **3**, the difference was negligible (Table 1). The phosphonamidate **1** was approximately 10-fold better in the acetylated histone assay than **2** or **3** (Table 1). Compounds **1**−**3** were stable under the assay conditions with nuclear extracts and unlabeled histones, with preincubation for 10 h at room temperature, as shown by HPLC.

To assay the compounds against a single enzymatic species, recombinant HDAC8 was used in a functional fluorescence assay (Supporting Information). Compounds **1**−**3** had IC_{50} values against HDAC8 in the 0.3 to 0.4 mM range (Table 1). These IC_{50} values are significantly lower for HDAC8 than for the mixture of nuclear HDACs used in the first assay. This may imply a measure of specificity for human HDAC8. SAHA is still a significantly more potent inhibitor of HDAC8 (greater than 1000-fold). The stability of HDAC8 was such that we could not test for slow-binding behavior.

Because the crystal structure showed SAHA bound to bacterial HDLP, compounds **1**−**3** were also assayed against HDLP. Phosphonamidate **1** showed moderate activity against HDLP that improved slightly with preincubation (Table 1). Phosphonate **2** and phosphinate **3** were weak inhibitors that did not show slow-binding behavior with HDLP, consistent with the behavior with nuclear extracts (Table 1).

Compounds **1**−**3** were also tested in an antiproliferative assay against the A2780 human ovarian cancer cell line. The IC_{50} values in this assay were all in the 110−120 μM range and were comparable to SAHA (Table 1).

We have synthesized phosphorus-based compounds that are structurally analogous to the hydroxamic acid, SAHA, except for the chelating moiety, and thus their potency can be directly compared. Phosphinate **3** is one atom shorter than **1**, **2**, and SAHA, and it might be argued that its weak inhibition is the result of that structural difference. However, the difference between the phosphorus-based inhibitors and the hydroxamic acid SAHA is much more significant. SAHA is consistently 3 orders of magnitude more potent in the enzyme inhibition assays. The greater negative charge on the phosphorus-based inhibitors compared with the charge on the hydroxamic acids may hinder binding to HDACs as opposed to metalloproteases.

Our results demonstrate that there is a distinct difference in reactivity profile between zinc proteases and HDACs. Given the successful history of phosphorus-based TS analogues as inhibitors of zinc proteases, we hypothesize that the TS state of HDAC enzymatic activity is different from that of zinc proteases. Moreover, the reactivity profile suggests that the 5- to 55-fold improvement in inhibition in the cell-based assay vs the nuclear extracts assay may be due to a more pronounced effect on additional isoforms, one or more of which influences proliferation. For compounds

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Table 1. HDAC Enzymatic Inhibition and Antiproliferative Activity of **1**−**3** and SAHA

assay:	HeLa nuclear extracts, (^{3}H)−hyperacetylated histones[a] IC_{50} (mM)		recombinant human HDAC8[b] IC_{50} (mM)	*A. aeolicus* recombinant HDLP[b] IC_{50} (mM) and/or (% inhibition at 1 mM)		human ovarian A2780 cell growth inhibition IC_{50} (mM)
preincubation:	0 h	10 h	0 h	0 h	15 h	0 h
1	2.0 ± 0.3	0.57 ± 0.02	0.28 ± 0.05	0.52 ± 0.05 (89.2%)	0.33 ± 0.04 (80.7%)	0.118 ± 0.002
2	6.1 ± 1.2	6.1 ± 0.8	0.39 ± 0.04	1.8 ± 0.3 (46.7%)	NA[c] (42.6%)	0.11 ± 0.01
3	8.2 ± 0.1	6.1 ± 0.2	0.39 ± 0.04	1.3 ± 0.1 (76.2%)	NA[c] (69.2%)	0.12 ± 0.01
SAHA	0.11 ± 0.02 × 10^{-3}	NA[c]	0.27 ± 0.09 × 10^{-3}	0.12 ± 0.05 × 10^{-3} (100%)	NA[c] (100%)	0.151 ± 0.004

[a] Concentration of ^{3}H−hyperacetylated histones was 0.50 μg/μL. [b] Concentration of Fluor de Lys substrate was 250 μM. [c] NA = not assayed.

1−**3**, the cell-based assay results are most consistent with HDAC8 inhibition.

In conclusion, three tetrahedral-phosphorus-containing analogues of SAHA were synthesized by rapid three- or four-step procedures and assayed as inhibitors of HDAC. All three compounds are weak inhibitors of the HDACs tested, suggesting that tetrahedral phosphorus analogues of SAHA do not mimic the TS for HDAC. We hypothesize that the TS for HDACs is not analogous to zinc-proteases as proposed,[3b] and mechanistic studies of HDACs are required to elucidate the key differences. In the future, these differences may be exploited to obtain specificity in the design and development of therapeutic inhibitors.

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Supporting Information Available: Experimental procedures for the synthesis, characterization, and bioassays of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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