

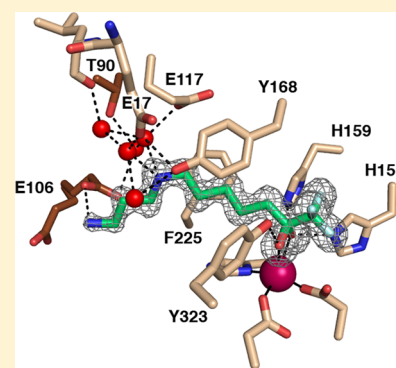
Design, Synthesis, and Evaluation of Polyamine Deacetylase Inhibitors, and High-Resolution Crystal Structures of Their Complexes with Acetypolyamine Amidohydrolase

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S Supporting Information

ABSTRACT: Polyamines are essential aliphatic polycations that bind to nucleic acids and accordingly are involved in a variety of cellular processes. Polyamine function can be regulated by acetylation and deacetylation, just as histone function can be regulated by lysine acetylation and deacetylation. Acetypolyamine amidohydrolase (APAH) from *Mycoplana ramosa* is a zinc-dependent polyamine deacetylase that shares approximately 20% amino acid sequence identity with human histone deacetylases. We now report the X-ray crystal structures of APAH–inhibitor complexes in a new and superior crystal form that diffracts to very high resolution (1.1–1.4 Å). Inhibitors include previously synthesized analogues of *N*⁸-acetylspermidine bearing trifluoromethylketone, thiol, and hydroxamate zinc-binding groups [Decroos, C., Bowman, C. M., and Christianson, D. W. (2013) *Bioorg. Med. Chem.* 21, 4530], and newly synthesized hydroxamate analogues of shorter, monoacetylated diamines, the most potent of which is the hydroxamate analogue of *N*-acetylcadaverine (IC₅₀ = 68 nM). The high-resolution crystal structures of APAH–inhibitor complexes provide key inferences about the inhibition and catalytic mechanism of zinc-dependent deacetylases. For example, the trifluoromethylketone analogue of *N*⁸-acetylspermidine binds as a tetrahedral gem-diol that mimics the tetrahedral intermediate and its flanking transition states in catalysis. Surprisingly, this compound is also a potent inhibitor of human histone deacetylase 8 with an IC₅₀ of 260 nM. Crystal structures of APAH–inhibitor complexes are determined at the highest resolution of any currently existing zinc deacetylase structure and thus represent the most accurate reference points for understanding structure–mechanism and structure–inhibition relationships in this critically important enzyme family.



In all forms of life, the reversible covalent modification of proteins and other biologically important molecules is a critical chemical strategy for the regulation of molecular and cellular function. For example, proteins can undergo methylation, phosphorylation, acetylation, glycosylation, ADP ribosylation, farnesylation, myristoylation, or ubiquitination in the course of their biological functions.¹ In particular, acetylation targets primary amino groups, as found in the side chain of lysine, the N-terminus of a protein, or polyamines such as spermidine and spermine. Lysine acetylation was first identified in histone proteins,² where it plays a crucial role in transcriptional regulation.³ Histone acetylation can alter nucleosome structure, making it less condensed to facilitate transcription. Significantly, non-histone proteins also serve as targets for lysine acetylation.⁴ In total, the mammalian acetylome comprises more than 3600 lysine acetylation sites that regulate myriad cellular functions.^{5,6}

Acetyltransferases utilize acetyl-CoA to generate acetyllysine, whereas histone deacetylases (HDACs) catalyze the hydrolysis of acetyllysine to regenerate free lysine and acetate.⁷ The HDAC family contains 18 isoforms divided into four different classes. The class I (HDAC1, -2, -3, and -8), class IIa (HDAC4, -5, -7, and -9), class IIb (HDAC6 and -10), and class IV (HDAC11) enzymes are metal-dependent deacetylases that

share a common α/β fold.⁸ In contrast, the class III enzymes (sirtuins 1–7) are NAD-dependent deacetylases that are evolutionarily and mechanistically distinct.⁹ Notably, metal-dependent HDACs adopt the α/β fold originally observed for the binuclear manganese metallohydrolase arginase.¹⁰ The mechanism of HDAC catalysis has been established on the basis of biochemical and structural studies of the histone deacetylase-like protein from *Aquifex aeolicus* and human HDAC8.^{11–17}

In addition to proteins, small molecules are also subject to reversible acetylation in the course of their biological function. For example, polyamines such as putrescine, spermidine, and spermine are essential cationic metabolites involved in numerous cellular processes in all forms of life and are subject to acetylation (Figure 1).¹⁸ Enzymes of polyamine biosynthesis are tightly regulated,^{18,19} and dysregulation of polyamine metabolism is often associated with certain disease pathologies such as cancer.^{20,21} As with acetyllysine residues, the decreased overall charge of acetylpolyamines attenuates their affinity for negatively charged nucleic acids. In eukaryotes, two enzymes

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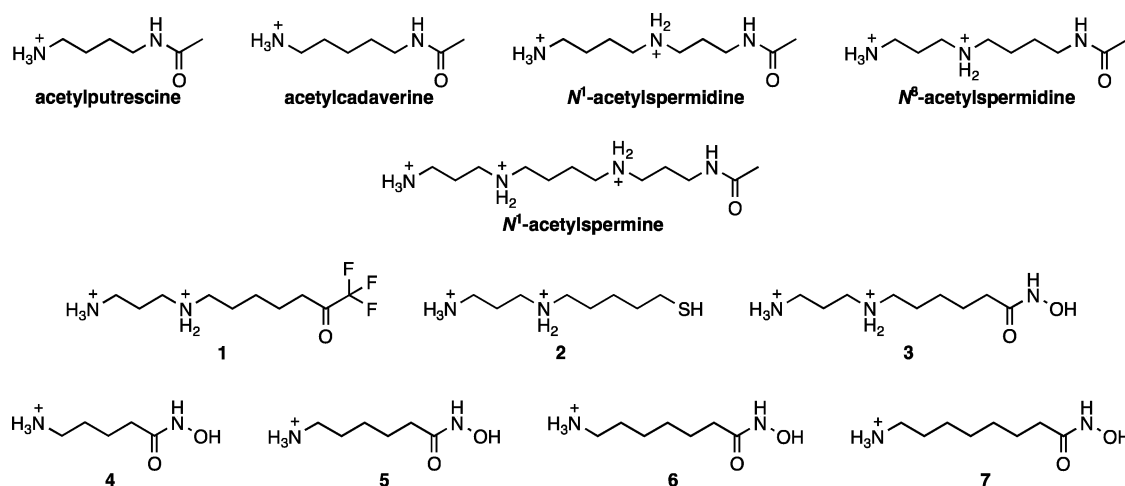


Figure 1. Substrates and inhibitors of APAH from *Mycoplana ramosa*. Substrates include acetylputrescine, acetylcadaverine, N^1 -acetylspermidine, N^8 -acetylspermidine, and N^1 -acetylspermine. Previously described³³ inhibitors of APAH are analogues of the substrate N^8 -acetylspermidine bearing different metal-binding groups: trifluoromethylketone (1), thiol (2), or hydroxamic acid (3). New inhibitors reported in this study (4–7) are shorter hydroxamic acids with varying chain lengths.

are responsible for polyamine acetylation: the cytosolic spermine/spermidine N^1 -acetyltransferase²² and the nuclear N^8 -acetyltransferase.²³ Differences in selectivity and subcellular localization of these enzymes reflect different roles for the corresponding acetylated polyamines. For instance, N^1 -acetylspermidine, produced in the cytosol, is converted back to the shorter diamine putrescine through an oxidative deamination reaction catalyzed by polyamine oxidase.²⁴ In contrast, N^8 -acetylation of spermidine in the nucleus allows the export of N^8 -acetylspermidine to the cytosol.²⁵ Deacetylation of N^8 -acetylspermidine, but not N^1 -acetylspermidine, occurs in the cytosol to recycle spermidine, which either reenters the nucleus or serves as a substrate for spermine synthase.^{25,26} The mammalian polyamine deacetylase responsible for the specific deacetylation of N^8 -acetylspermidine remains unidentified, even though its activity has been detected in subcellular fractions and in cells.^{25–28}

Acetypolyamine amidohydrolase (APAH) is a polyamine deacetylase identified in the Gram-negative bacterium *Mycoplana ramosa*.²⁹ APAH exhibits broad substrate specificity in that it catalyzes the deacetylation of all short- and long-chain acetypolyamines shown in Figure 1, although it exhibits a modest preference for short-chain polyamines.^{29,30} Like the metal-dependent HDACs, APAH is a zinc metallohydrolase that adopts the α/β arginase fold.³¹ However, APAHs are dimeric enzymes, as revealed by the crystal structures of APAH from *M. ramosa* and *Burkholderia pseudomallei*,^{31,32} whereas HDACs such as HDAC8 are monomers.^{12,13} Assembly of the APAH dimer results in a narrow “L”-shaped active site at the dimer interface that confers specificity for long, flexible, and positively charged acetypolyamine substrates.

We recently reported the synthesis and evaluation of N^8 -acetylspermidine analogues as APAH inhibitors.³³ These analogues differ by the nature of the functional group targeting Zn^{2+} coordination in the active site. The most potent inhibitors contain either a trifluoromethylketone or a hydroxamate Zn^{2+} -binding group (Figure 1) and exhibit nanomolar inhibitory potency. Here, we report the synthesis and evaluation of new hydroxamate analogues of short-chain acetyldiamines, compounds 4–7 (Figure 1). These compounds exhibit increased inhibitory potency against APAH and also inhibit HDAC8. We

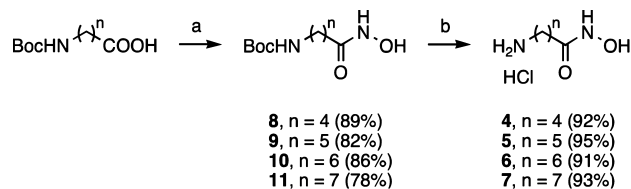
additionally report high-resolution X-ray crystal structures of APAH complexed with inhibitors 1–6 (Figure 1) in a new crystal form. These results provide new insight into catalysis and inhibition in the greater family of metal-dependent deacetylases.

METHODS

Chemistry. General Remarks. All reagents were at least 95% pure, purchased from Fisher Scientific, Alfa Aesar, Sigma-Aldrich, or Bachem, and used without any further purification. All solvents were of high-performance liquid chromatography grade and purchased from Fisher Scientific or Sigma-Aldrich. For reactions requiring anhydrous conditions, solvents were purchased as anhydrous grade from Fisher Scientific except for CH_2Cl_2 , which was freshly distilled under N_2 from P_2O_5 . Reactions were monitored by thin layer chromatography with Sigma-Aldrich aluminum plates (silica gel, 60 Å, 200 μm) and visualized after the plates had been stained with a ninhydrin solution. Flash column chromatography was performed using Fisher Scientific silica gel 60 (230–400 mesh). High-resolution mass spectrometry (HRMS) was performed on a Waters LC-TOF mass spectrometer (model LCT-XE Premier) using electrospray ionization (ESI) in positive mode. 1H and ^{13}C spectra were recorded on a Bruker DRX 500 spectrometer operating at 500 and 125.6 MHz for 1H and ^{13}C NMR, respectively. 1H and ^{13}C NMR chemical shifts (δ) are reported in parts per million relative to the residual solvent peak. NMR coupling constants (J) are reported in hertz, and multiplicities are denoted as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; dt, doublet of triplets.

The synthesis of compounds 1–3 as dihydrochloride salts has already been described.³³ The synthesis of compounds 4–7 is summarized in Scheme 1. Briefly, *N*-Boc-protected carboxylic acid starting materials were activated with carbonyldiimidazole (CDI) before reaction with unprotected hydroxylamine to form hydroxamic acids 8–11.^{33,34} Deprotection of compounds 8–11 was achieved under acidic conditions [anhydrous HCl (1 M) in ethyl acetate (AcOEt)] to yield inhibitors 4–7 as hydrochloride salts. Experimental details and characterization are given below for each new compound.

Scheme 1. Synthesis of New APAH Inhibitors 4–7^a



^aReagents and conditions: (a) carbonyldiimidazole (1.5 equiv) in tetrahydrofuran (room temperature, 1 h), then $\text{NH}_2\text{OH} \cdot \text{HCl}$ (2 equiv, room temperature, overnight); (b) anhydrous HCl (1 M) in ethyl acetate (room temperature, overnight).

5-(tert-Butoxycarbonylamino)-N-hydroxypentanamide (8). To a solution of *N*-(tert-butoxycarbonyl)-5-aminovaleric acid (1.00 g, 4.60 mmol) under argon in dry tetrahydrofuran (30 mL) was added CDI (1.12 g, 6.91 mmol). After 1 h at room temperature, hydroxylamine hydrochloride (640 mg, 9.21 mmol) was added to the solution. After being stirred overnight, the reaction mixture was diluted with a 5% KHSO_4 aqueous solution (100 mL) and extracted with AcOEt . Combined organic extracts were washed with brine and dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The solid was further purified by flash chromatography on silica gel with hexanes/ AcOEt gradients followed by AcOEt/MeOH gradients to afford hydroxamic acid **8** as a white solid (953 mg, 89%): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.32 (s, 1H), 8.68 (s, 1H), 6.77 (t, $J = 5.0$ Hz, 1H), 2.88 [apparent q (dt), $J = 6.5$ Hz], 1.92 (t, $J = 7.3$ Hz, 2H), 1.49–1.40 (m, 2H), 1.36 (s, 9H), 1.35–1.29 (m, 2H); ^{13}C NMR (125.6 MHz, $\text{DMSO}-d_6$) δ 169.0, 155.6, 77.4, 39.6, 32.0, 29.2, 28.3 (3C), 22.6; HRMS (ESI) calcd for $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_4\text{Na}$ [$\text{M} + \text{Na}$]⁺ 255.1321, found 255.1327.

6-(tert-Butoxycarbonylamino)-N-hydroxyhexanamide (9). Reaction of *N*-(tert-butoxycarbonyl)-6-aminocaproic acid (1.00 g, 4.32 mmol) with CDI (1.05 g, 6.48 mmol) and hydroxylamine hydrochloride (601 mg, 8.65 mmol) was performed under the same conditions that were used for **8** and afforded after purification hydroxamic acid **9** as a white solid (871 mg, 82%): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.31 (s, 1H), 8.64 (s, 1H), 6.72 (t, $J = 5.3$ Hz, 1H), 2.87 [apparent q (dt), $J = 6.3$ Hz, 2H], 1.92 (t, $J = 7.3$ Hz, 2H), 1.48–1.40 (m, 2H), 1.39–1.32 (m, 2H), 1.36 (s, 9H), 1.23–1.16 (m, 2H); ^{13}C NMR (125.6 MHz, $\text{DMSO}-d_6$) δ 169.1, 155.6, 77.3, 39.6, 32.3, 29.2, 28.3 (3C), 25.9, 24.9; HRMS (ESI) calcd for $\text{C}_{11}\text{H}_{22}\text{N}_2\text{O}_4\text{Na}$ [$\text{M} + \text{Na}$]⁺ 269.1477, found 269.1476.

7-(tert-Butoxycarbonylamino)-N-hydroxyheptanamide (10). Reaction of *N*-(tert-butoxycarbonyl)-7-aminoheptanoic acid (1.10 g, 4.48 mmol) with CDI (1.09 g, 6.72 mmol) and hydroxylamine hydrochloride (623 mg, 8.97 mmol) was performed under the same conditions that were used for **8** and afforded after purification hydroxamic acid **10** as a white solid (1.01 g, 86%): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.32 (s, 1H), 8.64 (s, 1H), 6.74 (t, $J = 5.3$ Hz, 1H), 2.86 [apparent q (dt), $J = 6.7$ Hz, 2H], 1.92 (t, $J = 7.5$ Hz, 2H), 1.49–1.43 (m, 2H), 1.36 (s, 9H), 1.36–1.31 (m, 2H), 1.23–1.19 (m, 4H); ^{13}C NMR (125.6 MHz, $\text{DMSO}-d_6$) δ 169.1, 155.6, 77.3, 39.7, 32.3, 29.4, 28.4, 28.3 (3C), 26.1, 25.1; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{24}\text{N}_2\text{O}_4\text{Na}$ [$\text{M} + \text{Na}$]⁺ 283.1364, found 283.1364.

8-(tert-Butoxycarbonylamino)-N-hydroxyoctanamide (11). Reaction of *N*-(tert-butoxycarbonyl)-8-aminooctanoic acid (1.10 g, 4.24 mmol) with CDI (1.03 g, 6.35 mmol) and hydroxylamine hydrochloride (589 mg, 8.48 mmol) was performed under the same conditions that were used for **8**

and afforded after purification hydroxamic acid **11** as a white solid (910 mg, 78%): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.32 (s, 1H), 8.65 (s, 1H), 6.74 (t, $J = 5.3$ Hz, 1H), 2.88 [apparent q (dt), $J = 6.7$ Hz, 2H], 1.92 (t, $J = 7.5$ Hz, 2H), 1.49–1.44 (m, 2H), 1.36 (s, 9H), 1.36–1.32 (m, 2H), 1.25–1.19 (m, 6H); ^{13}C NMR (125.6 MHz, $\text{DMSO}-d_6$) δ 169.2, 155.6, 77.3, 39.9, 32.3, 29.5, 28.6, 28.5, 28.3 (3C), 26.2, 25.1; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_4\text{Na}$ [$\text{M} + \text{Na}$]⁺ 297.1790, found 297.1790.

5-Amino-N-hydroxypentanamide Hydrochloride Salt (4). To a solution of compound **8** (270 mg, 1.16 mmol) in 10 mL of dry CH_2Cl_2 was added 30 mL of a 1 M anhydrous HCl solution in AcOEt . After being stirred overnight under argon at room temperature, the precipitate was filtered, washed with dry Et_2O , and dried *in vacuo* to afford hydroxamic acid **4** as an off-white powder (181 mg, 92%): ^1H NMR (500 MHz, D_2O) δ 2.99 (t, $J = 6.0$ Hz, 2H), 2.22 (t, $J = 6.8$ Hz, 2H), 1.68–1.64 (m, 4H); ^{13}C NMR (125.6 MHz, D_2O) δ 172.5, 39.0, 31.6, 26.0, 21.8; HRMS (ESI) calcd for $\text{C}_5\text{H}_{13}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$]⁺ 133.0977, found 133.0977.

6-Amino-N-hydroxyhexanamide Hydrochloride Salt (5). Compound **9** (200 mg, 812 μmol) was deprotected under the same conditions that were used for **4** to afford after filtration hydroxamic acid **5** as an off-white powder (140 mg, 95%): ^1H NMR (500 MHz, D_2O) δ 2.95 (t, $J = 7.5$ Hz, 2H), 2.15 (t, $J = 7.3$ Hz, 2H), 1.66–1.56 (m, 4H), 1.36–1.30 (m, 2H); ^{13}C NMR (125.6 MHz, D_2O) δ 173.1, 39.2, 32.0, 26.3, 24.9, 24.3; HRMS (ESI) calcd for $\text{C}_6\text{H}_{15}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$]⁺ 147.1138, found 147.1138.

7-Amino-N-hydroxyheptanamide Hydrochloride Salt (6). Compound **10** (300 mg, 1.15 mmol) was deprotected under the same conditions that were used for **4** to yield after filtration hydroxamic acid **6** as an off-white powder (207 mg, 91%): ^1H NMR (500 MHz, D_2O) δ 3.01 (t, $J = 7.5$ Hz, 2H), 2.20 (t, $J = 7.3$ Hz, 2H), 1.71–1.66 (m, 2H), 1.65–1.60 (m, 2H), 1.44–1.33 (m, 4H); ^{13}C NMR (125.6 MHz, D_2O) δ 173.4, 39.4, 32.2, 27.4, 26.5, 25.2, 24.6; HRMS (ESI) calcd for $\text{C}_7\text{H}_{17}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$]⁺ 161.1290, found 161.1298.

8-Amino-N-hydroxyoctanamide Hydrochloride Salt (7). Compound **11** (200 mg, 729 μmol) was deprotected under the same conditions that were used for **4** to yield after filtration hydroxamic acid **7** as an off-white powder (143 mg, 93%): ^1H NMR (500 MHz, D_2O) δ 2.95 (t, $J = 7.5$ Hz, 2H), 2.13 (t, $J = 7.5$ Hz, 2H), 1.64–1.58 (m, 2H), 1.58–1.53 (m, 2H), 1.37–1.26 (m, 6H); ^{13}C NMR (125.6 MHz, D_2O) δ 173.5, 39.4, 32.2, 27.6, 26.5, 25.3, 24.7; HRMS (ESI) calcd for $\text{C}_8\text{H}_{19}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$]⁺ 175.1447, found 175.1441.

Expression and Purification of APAH and HDAC8. APAH was expressed from a pET-21b plasmid in *Escherichia coli* BL21(DE3) cells and purified as previously described.³¹ HDAC8 was expressed from a pHD2-Xa-His plasmid (modified pET-20b plasmid) in *E. coli* BL21(DE3) cells and purified using previously described procedures.^{15,35}

Inhibitory Activity Measurements. The inhibition of APAH by the newly synthesized derivatives **4–7** was evaluated using a fluorimetric assay, as previously described.^{31,33} The IC_{50} values for compounds **1–3** were reported previously.³³ Activity was measured using the commercially available Fluor-de-Lys deacetylase fluorogenic substrate (BML-KI104, Enzo Life Sciences). Deacetylation of the acetyllysine–fluorophore substrate is followed by cleavage of the lysine–fluorophore amide bond by a protease developer, resulting in a fluorescence shift. In contrast with the shorter acetyllysine–fluorophore assay substrate used to assay APAH, the longer peptide

Table 1. Data Collection and Refinement Statistics

	APAH-1 complex	APAH-2 complex	APAH-3 complex	APAH-4 complex	APAH-5 complex	APAH-6 complex
Unit Cell						
space group symmetry	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
a, b, c (Å)	45.1, 121.4, 64.3	44.9, 121.0, 64.7	45.0, 121.1, 64.4	44.7, 120.9, 64.1	44.9, 120.5, 64.4	45.0, 120.7, 64.5
α, β, γ (deg)	90, 96.8, 90	90, 97.0, 90	90, 96.8, 90	90, 97.0, 90	90, 96.9, 90	90, 97.0, 90
Data Collection						
wavelength (Å)	1.075	1.075	1.069	1.075	1.075	0.979
resolution limits (Å)	38.9–1.42	41.8–1.40	38.9–1.33	38.7–1.42	41.8–1.22	22.8–1.13
no. of total/unique reflections	644824/128460	955700/134698	1075040/155090	913682/126724	1391188/199748	1643256/248014
$R_{\text{merge}}^{a,b}$	0.070 (0.328)	0.088 (0.795)	0.054 (0.272)	0.070 (0.398)	0.079 (0.525)	0.077 (0.455)
$I/\sigma(I)^c$	20.4 (4.8)	23.1 (2.4)	23.4 (6.8)	24.3 (5.0)	21.5 (3.7)	11.8 (3.9)
redundancy ^d	5.0 (4.8)	7.1 (5.7)	6.9 (6.6)	7.2 (6.8)	7.0 (7.0)	6.6 (6.1)
completeness (%) ^e	99.9 (99.7)	100 (100)	99.3 (98.4)	100 (100)	99.4 (100)	98.2 (94.4)
Refinement						
no. of reflections used in refinement/ test set	128411/6458	134649/6776	155046/7777	126680/6355	199693/10060	247921/12379
R_{cryst}^c	0.133	0.146	0.133	0.131	0.137	0.114
R_{free}^d	0.153	0.164	0.147	0.152	0.152	0.136
no. of protein atoms ^e	5557	5444	5514	5406	5534	5481
no. of water molecules ^e	953	856	1047	950	979	1094
no. of ligand molecules ^e	2	2	2	2	2	2
no. of Zn^{2+} ions ^e	2	4	2	4	4	2
no. of $\text{K}^+/\text{Na}^+/\text{NH}_4^+$ ions ^e	2/2/0	2/2/0	2/0/2	4/0/0	2/2/0	2/0/2
no. of nitrate ions ^e	4	—	—	4	—	—
no. of glycerol molecules ^e	2	3	5	4	4	3
rms deviation from ideal geometry						
bonds (Å)	0.009	0.009	0.009	0.009	0.008	0.010
angles (deg)	1.3	1.3	1.3	1.3	1.3	1.4
dihedral angles (deg)	13	12	13	13	13	12
Ramachandran plot (%) ^f						
allowed	92.6	91.2	92.4	90.8	92.4	92.0
additionally allowed	6.7	8.3	7.2	8.5	7.1	7.6
generously allowed	0.7	0.5	—	0.7	0.2	—
disallowed	—	—	0.4	—	0.4	0.4
PDB entry	4ZUM	4ZUN	4ZUO	4ZUP	4ZUQ	4ZUR

^aValues in parentheses refer to the data from the highest shell. ^b $R_{\text{merge}} = \sum |I_h - \langle I \rangle_h| / \sum I_h$, where $\langle I \rangle_h$ is the average intensity calculated from replicate reflections. ^c $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$ for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. ^d $R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum |F_o|$ for reflections contained in the test set held aside during refinement. ^ePer asymmetric unit. ^fCalculated with PROCHECK version 3.4.4.

fluorophore Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin is a poor substrate for APAH, presumably because of the constricted APAH active site, as previously reported.³¹ Activity assays were conducted at 25 °C and contained 250 nM APAH (~50% Zn^{2+} occupancy), 150 μM substrate, and 0–250 μM inhibitor in assay buffer [25 mM Tris (pH 8.2), 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl_2] in a final volume of 50 μL . Enzyme was first incubated with the inhibitor for 5 min before the reaction was initiated with substrate; the most potent inhibitor, compound 6, was also evaluated after incubation for 30 and 60 min to assess the possibility of time-dependent inhibition. After 30 min, reactions were quenched by adding 100 μM M344 (Sigma-Aldrich) and the appropriate Fluor-de-Lys developer (BML-KI105, Enzo Life Sciences, 50 μL). Because the developer is typically a serine protease, e.g., trypsin, we confirmed that trifluoromethylketone 1, even at millimolar concentrations, does not inhibit the developer enzyme (data not shown). Fluorescence was measured after 45 min using a Fluoroskan II plate reader (excitation at 355 nm, emission at 460 nm). Assays for each concentration of inhibitor were performed in triplicate in separate experiments. IC_{50} values for

each compound were determined using Graphpad Prism (2008).

The inhibition of HDAC8 by compounds 1–7 was evaluated using a similar fluorimetric assay, as previously described.³⁵ Activity assays were conducted at 25 °C and contained 500 nM HDAC8 enzyme, 150 μM Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin substrate (BML-KI178, Enzo Life Sciences), and 0–10 mM inhibitor in assay buffer [25 mM Tris (pH 8.2), 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl_2 ; 250 μM tris(2-carboxyethyl)phosphine was added for the assay of thiol compound 2] in a final volume of 50 μL . Enzyme was first incubated with inhibitor for 5 min before the reaction was initiated with substrate; the most potent inhibitor, compound 1, was also evaluated after incubation for 30 and 60 min to assess the possibility of time-dependent inhibition. After 30 min, reactions were quenched by adding 100 μM M344 (Sigma-Aldrich) and the appropriate Fluor-de-Lys developer (BML-KI176, Enzo Life Sciences, 50 μL). Fluorescence was measured after 45 min using a Fluoroskan II plate reader (excitation at 355 nm, emission at 460 nm). Assays for each concentration of inhibitor were performed in triplicate in separate experiments.

IC₅₀ values for each compound were determined using Graphpad Prism (2008).

Crystallization and Data Collection. Crystals of APAH–inhibitor complexes were prepared by cocrystallization at 4 °C (except for the complex with 4, 21 °C) in sitting drops using the vapor diffusion method. In general, a 500 nL drop containing 5 mg/mL (for 2 and 4) or 10 mg/mL (for 1, 3, 5, and 6) APAH, 20 mM Tris (pH 8.0), 50 mM NaCl, 0.01% sodium azide, ZnCl₂ (0.5 mM for 2 and 2 mM for 1 and 3–6), and 2.5 mM inhibitor was added to a 500 nL drop of precipitant solution and equilibrated against a 100 μ L reservoir of precipitant solution. Before the drops had been mixed, APAH was first incubated with ZnCl₂ for 15 min on ice before the inhibitor was added. Enzyme solutions were filtered before crystallization because of the formation of a slight precipitate after addition of ZnCl₂ and inhibitor. The precipitant solution for the cocrystallization of the APAH–1 and APAH–2 complexes consisted of 0.2 M LiNO₃ and 20% (w/v) PEG 3350. The precipitant solution for the cocrystallization of the APAH–3 complex consisted of 0.2 M ammonium chloride and 20% (w/v) PEG 3350; after being harvested, crystals were soaked for an additional 2 days in this precipitant solution supplemented with 2.5 mM 3. The precipitant solution for the cocrystallization of the APAH–4 complex consisted of 0.2 M KNO₃ and 20% (w/v) PEG 3350. The precipitant solution for the cocrystallization of the APAH–5 complex consisted of 0.2 M LiCl and 20% (w/v) PEG 3350. The precipitant solution for the cocrystallization of the APAH–6 complex consisted of 0.2 M ammonium chloride and 20% (w/v) PEG 3350; after being harvested, crystals were soaked for an additional 2 days in this precipitant solution supplemented with 2.5 mM 6. We were unable to prepare suitable crystals of the APAH–7 complex.

Typically, crystals appeared within 2–7 days. Crystals were flash-cooled in liquid nitrogen after being transferred to a cryoprotectant consisting of precipitant solution supplemented with 25–30% glycerol (v/v). X-ray diffraction data were collected on beamline X29 at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory, Upton, NY) for APAH complexes with inhibitors 1, 2, 4, and 5; on beamline BL14-1 at the Stanford Synchrotron Radiation Lightsource (SSRL, Stanford Linear Accelerator Center, Menlo Park, CA) for APAH complexed with 3; or on beamline NE-CAT 24-ID-E at the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, IL) for APAH in complex with 6. Data collection and reduction statistics are listed in Table 1. Diffraction data from crystals of all complexes except the APAH–6 complex were indexed, integrated, and scaled using HKL2000.³⁶ Data collected for the APAH–6 complex were indexed and integrated with MOSFLM³⁷ and scaled with SCALA (contained in the CCP4 suite of programs).³⁸

Phasing, Model Building, and Structure Refinement.

All structures belonged to space group *P*₂₁ with two molecules (APAH dimer) in the asymmetric unit. Structures were determined by molecular replacement using PHENIX³⁹ with the atomic coordinates of the APAH–M344 complex (PDB entry 3Q9B) less inhibitor, ion, and solvent used as a search probe for rotation and translation function calculations. Each model was refined with iterative cycles of refinement in PHENIX and manual model rebuilding in COOT.⁴⁰ Solvent molecules and inhibitors were added after several rounds of refinement for each structure. In the final stages, translation libration screw (TLS) refinement was performed.⁴¹ TLS groups were automatically determined using PHENIX. For the

APAH–6 complex (1.13 Å resolution), clear electron density was observed for many hydrogen atoms in late stages of refinement. Accordingly, hydrogen atoms were added to the model during the last stage of refinement, and anisotropic temperature factor refinement was performed for all atoms except hydrogens and water molecules. The protonation states of ionizable residues were determined by analysis of electron density as well as evaluation of their chemical environments. Refinement converged smoothly, and detailed refinement statistics are listed in Table 1.

In each structure, side chains of residues that were partially disordered were excluded from the final model as follows: APAH–1 complex, K187 (monomers A and B); APAH–2 complex, K84 and K187 (monomers A and B); APAH–3 complex, E106 (monomers A and B) and K187 (monomer A); APAH–4 complex, K84 (monomers A and B) and K187 (monomer B); APAH–5 complex, R2 (monomers A and B), K79 (monomer B), K84 (monomers A and B), K187 (monomers A and B), and K267 (monomer B); and APAH–6 complex, K15 (monomer B), K84 (monomer A), K187 (monomer B), K237 (monomer A), and K267 (monomer B). For the APAH–3, APAH–5, and APAH–6 complexes, H227 adopted a disallowed conformation that nonetheless was characterized by well-defined electron density.

Finally, in most of the reported structures, occasional ambiguous electron densities were observed either on the protein surface (e.g., near Y247 or F27) or in the protein interior (e.g., near M164). These electron densities were usually elongated and potentially corresponded to disordered PEG fragments or other molecules present in the buffer solution used for crystallization. However, because these electron density peaks were not confidently interpretable, they were left unmodeled.

RESULTS

Design, Synthesis, and Evaluation of New APAH Inhibitors. In our previous work, we designed analogues of N⁸-acetylspermidine as inhibitors of APAH.³³ This led us to identify the trifluoromethylketone, hydroxamic acid, and thiol functional groups as the best ligands for coordination to the active site Zn²⁺ ion (Table 2). Given that shorter substrates

Table 2. Inhibitory Potencies of Compounds 1–7 against *M. ramosa* APAH and Human HDAC8

compound	IC ₅₀ (μ M)	
	APAH	HDAC8
1	0.27 \pm 0.03, ^a 38 \pm 6 ^a	0.26 \pm 0.02
2	26 \pm 3 ^a	12 \pm 1
3	0.39 \pm 0.03 ^a	14 \pm 1
4	0.17 \pm 0.01	120 \pm 10
5	0.13 \pm 0.01	19 \pm 2
6	0.068 \pm 0.006	5.3 \pm 0.8
7	0.15 \pm 0.02	2.2 \pm 0.2

^aValues previously reported in ref 33.

such as acetylputrescine and acetylcadaverine bind to APAH with an affinity slightly higher than those of larger substrates such as N¹-acetylspermidine and N⁸-acetylspermidine (as reflected by *K_m* values),³⁰ we hypothesized that analogues of shorter acetylpolyamines might yield improved inhibitory potency. Consequently, we designed the four new hydroxamic

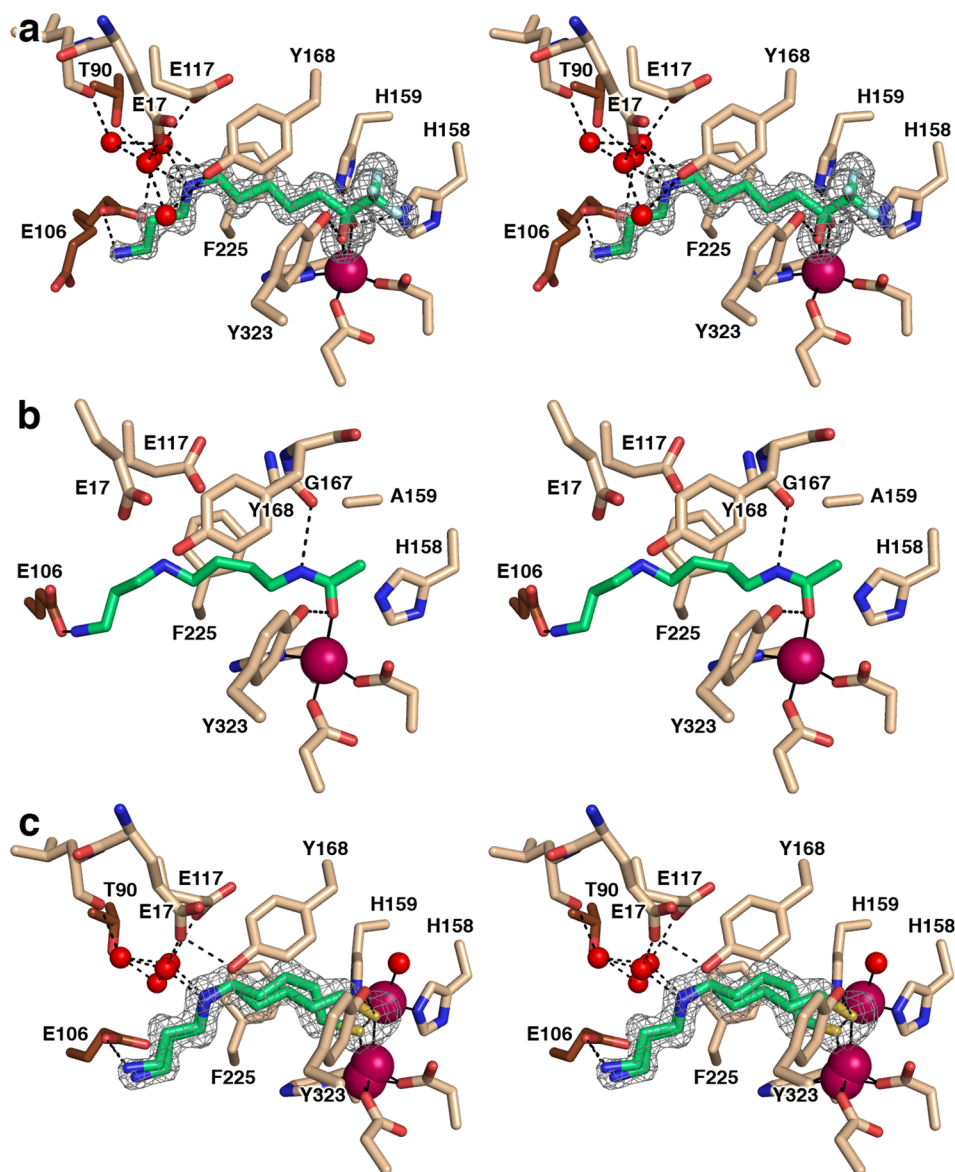


Figure 2. (a) Simulated annealing omit map showing trifluoromethylketone **1** bound as a gem-diol in the active site of APAH (monomer B, contoured at 3.5σ). Atomic color codes are as follows: C, wheat (protein, monomer B), brown (monomer A), or green (inhibitor); N, blue; O, red; F, light cyan; Zn²⁺, magenta sphere. Water molecules are represented as red spheres. Metal coordination and selected hydrogen bond interactions are shown as solid black and dashed black lines, respectively. (b) Stereoview of the N⁸-acetylspermidine substrate bound in the active site of the inactive mutant APAH H159A (PDB entry 3Q9C, monomer A). Atomic color codes are identical to those in panel a. Brown C atoms are those of adjacent monomer I of the APAH dimer. We note that in only monomer J of this structure does the secondary amine of N⁸-acetylspermidine donate a hydrogen bond to E117. Additionally, a water molecule makes a bridging interaction in some monomers between Y19 and the terminal amino group of the substrate. (c) Simulated annealing omit map of thiol **2** bound in the active site of APAH (monomer A, contoured at 3.0σ). Atomic color codes are identical to those in panel a, with S being colored gold.

acids **4–7** (Figure 1). These compounds were easily synthesized as hydrochloride salts in two steps from commercially available materials (Scheme 1; synthetic details are outlined in Methods).

Compounds **4–7** were assayed for inhibitory activity against APAH, and results are summarized in Table 2. The IC₅₀ plots are found in Figure S1 of the Supporting Information. Hydroxamate **6** (the hydroxamate analogue of acetylcadaverine) is the most potent APAH inhibitor of all compounds tested, with an IC₅₀ of 68 nM (time-dependent inhibition is not observed). The other short-chain hydroxamates exhibit slightly diminished inhibitor potency with IC₅₀ values of 130, 150, and 170 nM for compounds **5** (hydroxamate analogue of

acetylputrescine), **7**, and **4**, respectively. Even so, all of these compounds exhibit increased potency relative to that of the authentic hydroxamate analogue of N⁸-acetylspermidine, **3**, which has an IC₅₀ value of 390 nM.³³

Compounds **1–7** were additionally evaluated as inhibitors of the related deacetylase HDAC8 (Table 2). The IC₅₀ plots are found in Figure S2 of the Supporting Information. Hydroxamates **3–7** are modest inhibitors of human HDAC8 with IC₅₀ values in the mid to low micromolar range. In the short-chain analogue series, inhibitory potency increases with chain length, with IC₅₀ values of 120, 19, 5.3, and 2.2 μM for **4–7**, respectively. Thiol **2** is also a modest inhibitor of HDAC8 with an IC₅₀ of 12 μM, as is hydroxamate **3** (14 μM). Strikingly,

among the compounds listed in Table 2, trifluoromethylketone **1** is the most potent inhibitor of HDAC8 with an IC_{50} of 260 nM (time-dependent inhibition is not observed).

Structure of APAH in a New Crystal Form. We previously reported the structure of APAH in a triclinic crystal form, in which the $P1$ unit cell contained two trimers of dimers.³¹ These crystals were exceedingly difficult to work with because they contained multiple lattices that grew with different orientations. Suitable X-ray diffraction intensities could be measured from a single crystal at 2.2–2.5 Å resolution only through the use of a microfocus X-ray beam centered on a crystal corner. Importantly, the APAH cocrystallization studies reported herein yielded a new monoclinic crystal form with superior diffraction properties, consistently yielding crystals diffracting to 1.1–1.4 Å resolution.

Although the overall dimer architecture of APAH in the new monoclinic crystal form is identical to that previously reported in the triclinic crystal form, dimer–dimer interactions differ between these two crystal forms.³¹ These differences give rise to alternative crystal symmetries. The coordination geometry of the active site Zn^{2+} ion is also similar in each crystal form. However, the conformation of catalytically important residue Y323 exhibits structural differences between the two crystal forms: whereas Y323 adopts both “inward” and “outward” conformations in triclinic structures, Y323 exclusively adopts the “inward” conformation in all monoclinic structures except for the structure of the APAH–2 complex, in which Y323 adopts a low-occupancy (30%) intermediate conformation between the “inward” and “outward” conformations in monomer B only. As previously reported,³¹ the full-occupancy “outward” conformation of Y323 facilitates the binding of a third low-occupancy monovalent cation, but this additional cation is not observed in the structures reported herein. Additionally, the second monovalent cation site (comprised of F206, R209, V212, and T243) is observed to accommodate alternative cations in these structures: it is interpreted as Na^+ in the APAH–1, APAH–2, and APAH–5 complexes; K^+ in the APAH–4 complex; and NH_4^+ in the APAH–3 and APAH–6 complexes. The first monovalent cation site contains exclusively K^+ in all structures.

Structure of the APAH-1 Complex. Trifluoromethylketones exist predominantly in the gem-diol form in aqueous solution because of the extreme electrophilicity of the carbonyl group adjacent to the trifluoromethyl moiety, and as such, trifluoromethylketone derivatives are generally potent inhibitors of zinc metallohydrolases. The origin of tight binding is believed to be the resemblance of the gem-diol to the tetrahedral intermediate and flanking transition states in the hydrolytic reaction catalyzed by a zinc metallohydrolase, as first demonstrated for a trifluoromethylketone inhibitor of carboxypeptidase A ($K_i = 0.2 \mu M$).^{42,43} More recently, the crystal structure of human HDAC4 complexed with a trifluoromethylketone inhibitor ($IC_{50} = 0.367 \mu M$) bound in the gem-diol form has been reported.¹⁴

As previously reported, trifluoromethylketone **1** inhibits APAH with an IC_{50} of $0.27 \pm 0.03 \mu M$; a low-affinity binding species is also observed in solution with an IC_{50} of $38 \pm 6 \mu M$, which perhaps could correspond to the intact, substrate-like trifluoromethylketone instead of the tetrahedral gem-diol transition state mimic.³³ However, in the crystal structure of the APAH–1 complex reported here at 1.42 Å resolution, only a single binding mode for the tetrahedral gem-diol is observed: one inhibitor binds in the active site of each APAH monomer,

with the gem-diol moiety coordinating asymmetrically to the active site Zn^{2+} ion (Zn^{2+} –O1 and Zn^{2+} –O2 distances of 2.0 and 2.4 Å, respectively). Additionally, hydroxyl group O1 hydrogen bonds with Y323 (O–O separation of 2.6 Å), and hydroxyl group O2 hydrogen bonds with H158 and H159 (O–N separations of 2.7 Å). The overall metal coordination geometry is best described as trigonal bipyramidal, but distorted because of the bidentate coordination of the gem-diol moiety. This is generally comparable to Zn^{2+} coordination geometries observed in carboxypeptidase A complexes with inhibitory gem-diols.^{43,45} An electron density map of the HDAC8–1 complex is shown in Figure 2a.

The overall conformation of **1** is similar but not identical to that of N^8 -acetylsperridine in its complex with the inactive H159A APAH mutant (Figure 2b).³¹ As observed for N^8 -acetylsperridine, the primary amino group of **1** hydrogen bonds with E106 of the adjacent monomer of the dimer (N–O separation of 2.8 Å). However, in the structure presented here, E106 adopts two approximately equal-occupancy conformations, only one of which makes this hydrogen bond interaction. Although the secondary amino group of N^8 -acetylsperridine interacts with E117,³¹ E117 adopts an alternative conformation in the complex with **1** that precludes this interaction. Instead, the secondary amino group of **1** hydrogen bonds with water molecules that also interact with E17, E117, or T90 (from the adjacent monomer of the dimer). Additionally, the secondary amino group of N^8 -acetylsperridine makes an offset cation– π interaction with F225,³¹ and this interaction is conserved for the secondary amino group of **1**.

Structure of the APAH–2 Complex. The thiol moiety of **2** comprises a good ligand for Zn^{2+} coordination, and a favorable Zn^{2+} –S coordination interaction is proposed to account for moderate inhibitory potency ($IC_{50} = 26 \mu M$).³³ Indeed, a favorable Zn^{2+} –S coordination interaction accounts for the strong inhibitory potency of the marine depsipeptide inhibitor largazole against the histone deacetylases.^{46,47} The X-ray crystal structure of the HDAC8–largazole thiol complex ($IC_{50} = 0.102 \mu M$)⁴⁸ reveals that the geometry of thiolate– Zn^{2+} coordination is nearly ideal⁴⁹ with an average S– Zn^{2+} distance of 2.3 Å, a C–S– Zn^{2+} angle of 98°, and a C–C–S– Zn^{2+} dihedral angle of 92°; the Zn^{2+} ion is coordinated with nearly perfect tetrahedral geometry, with average ligand– Zn^{2+} –ligand angles ranging from 100° to 115°, and Y306 donates a hydrogen bond to the Zn^{2+} -bound thiolate anion.⁵⁰ Comparable binding modes are observed in HDAC8 complexes with largazole analogues.⁵¹

Surprisingly, the 1.40 Å resolution crystal structure of the APAH–2 complex reveals the presence of two Zn^{2+} ions in the active site, as well as two mutually exclusive positions for the catalytic Zn^{2+} ion separated by 0.7 Å. The catalytic Zn^{2+} ion is coordinated by D195, H197, and D284 in both positions, and the second Zn^{2+} ion (occupancy of 0.5) is coordinated by H158, H159, and a water molecule. The presence of this additional Zn^{2+} ion is due to the addition of $ZnCl_2$ in the crystallization buffer. As a consequence, the thiol inhibitor **2** adopts two conformations. When the second Zn^{2+} ion is presumably absent, the structure reveals that the thiolate– Zn^{2+} coordination geometry (Figure 2c) is characterized by an average S– Zn^{2+} distance of 2.3 Å, a C–S– Zn^{2+} angle of 115°, and a C–C–S– Zn^{2+} dihedral angle of 51°; the Zn^{2+} ion is coordinated with distorted tetrahedral geometry, with average ligand– Zn^{2+} –ligand angles ranging from 86° to 120°. The hydroxyl group of Y323 donates a hydrogen bond to the Zn^{2+} -

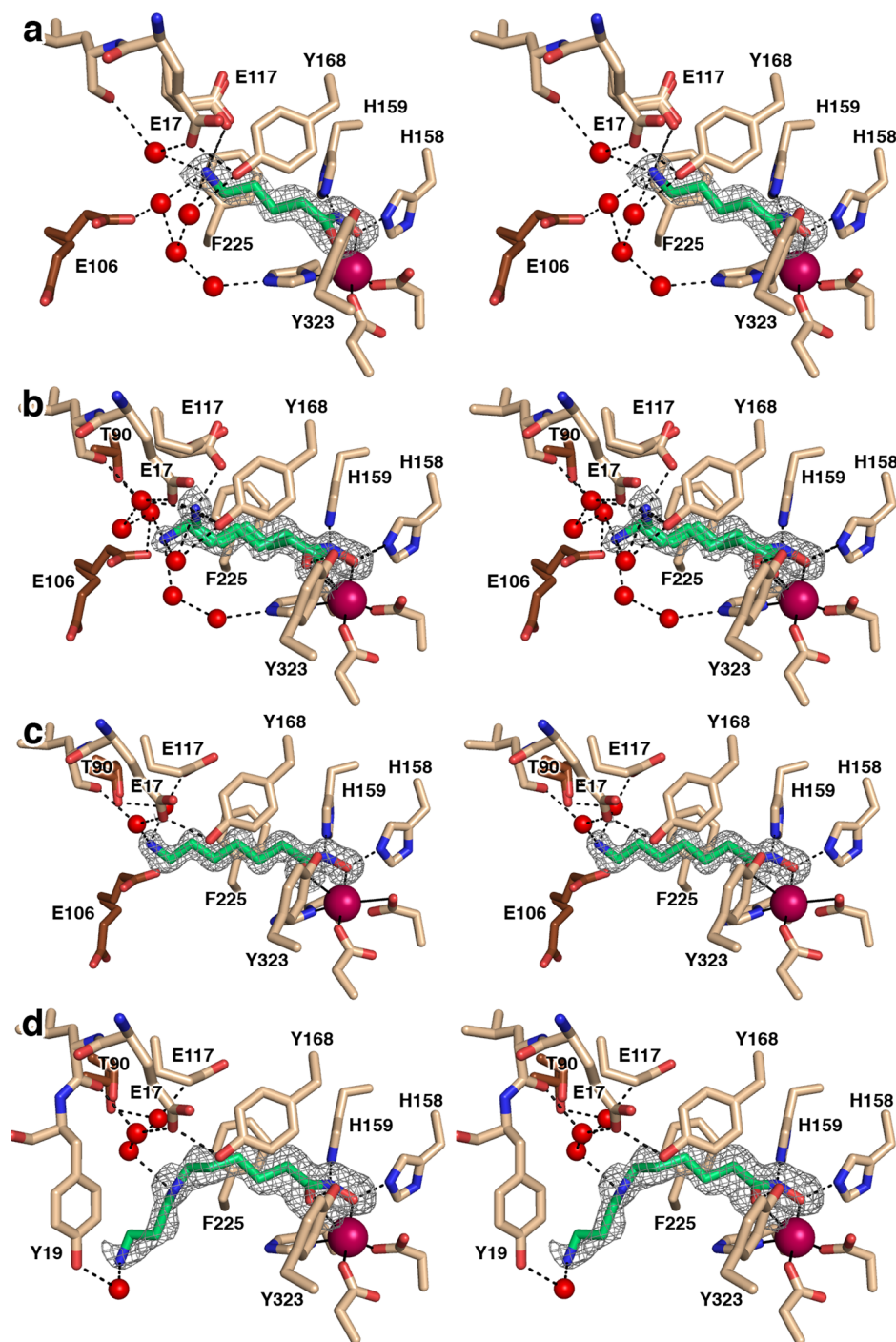


Figure 3. (a) Simulated annealing omit map of hydroxamate 4 bound in the active site of APAH (monomer A, contoured at 3.5σ). Atomic color codes are as follows: C, wheat (protein, monomer A), brown (monomer B), or green (inhibitor); N, blue; O, red; Zn^{2+} , magenta. Water molecules are represented as red spheres. Metal coordination and selected hydrogen bond interactions are shown as solid black and dashed black lines, respectively. (b) Simulated annealing omit map of hydroxamate 5 bound in two conformations in the active site of APAH (monomer B, contoured at 3.0σ). Atomic color codes are identical to those in panel a. (c) Simulated annealing omit map of hydroxamate 6 bound in the active site of APAH (monomer A, contoured at 4.0σ). Atomic color codes are identical to those in panel a. (d) Simulated annealing omit map of hydroxamate 3 bound in the active site of APAH (monomer B, contoured at 3.0σ). Atomic color codes are identical to those in panel a.

bound thiolate anion of 2. However, in monomer B, Y323 also partially adopts a conformation that is intermediate between the inward and outward conformations with 30% occupancy. When the second Zn^{2+} ion is present, the thiolate inhibitor adopts an alternative conformation in which the thiolate group bridges the two Zn^{2+} ions. For the catalytic Zn^{2+} ion, the average S– Zn^{2+} distance is 2.2 Å, the C–S– Zn^{2+} angle is 109° ,

and the C–C–S– Zn^{2+} dihedral angle is 173° ; the metal coordination geometry is distorted tetrahedral, with average ligand– Zn^{2+} –ligand angles ranging from 100° to 116° . Similarly, the coordination geometry of the second Zn^{2+} ion is distorted tetrahedral, with an average S– Zn^{2+} distance of 2.3 Å, a C–S– Zn^{2+} angle of 98° , and a C–C–S– Zn^{2+} dihedral

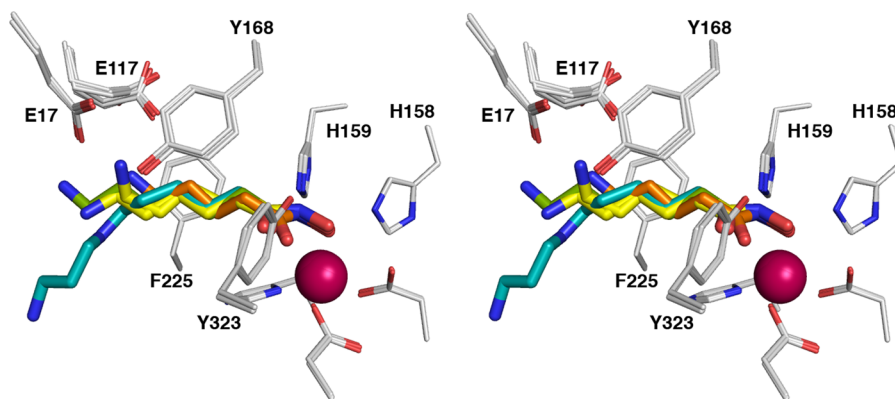


Figure 4. Superposition of APAH complexes with hydroxamate inhibitors 3–6 (monomer A for all). Atomic color codes are as follows: C, light gray (protein), teal (hydroxamate 3), orange (hydroxamate 4), yellow (hydroxamate 5), or green (hydroxamate 6); N, blue; O, red; F, light cyan; Zn²⁺, magenta.

angle of -73° ; average ligand–Zn²⁺–ligand angles range from 85° to 133° .

The rest of the inhibitor binds in the active site in a fashion similar to that observed for trifluoromethylketone **1**. As observed for **1**, the primary amino group of **2** hydrogen bonds with E106 of the adjacent monomer of the dimer (N–O separation of 2.7–3.1 Å); E106 adopts one and two conformations in monomers B and A, respectively. Similar to that observed for **1**, the secondary amino group of **2** hydrogen bonds with water molecules that also interact with E17, E117, or T90 (from the adjacent monomer of the dimer).

Structures of the Complexes of APAH with 3–6. The hydroxamate group generally comprises an excellent functional group for Zn²⁺ ion coordination in the active site of a metalloenzyme through the formation of a favorable five-membered ring chelate complex, as first observed in the crystal structure of thermolysin complexed with a hydroxamate inhibitor,⁵² although alternative binding modes are occasionally observed.⁵³ The hydroxamate moieties of compounds 3–6 tend to form five-membered ring chelate complexes with the active site Zn²⁺ ion of APAH, as revealed in X-ray crystal structures determined at resolutions of 1.33, 1.42, 1.22, and 1.13 Å, respectively. Electron density maps are shown in Figure 3 for complexes of APAH with 3–6. Although the coordination mode of the hydroxamate OH group is identical in each enzyme–inhibitor complex (Zn²⁺–O separation of 1.9–2.0 Å in each monomer of each APAH–hydroxamate complex), the coordination mode of the hydroxamate carbonyl group is more variable and tends toward outer sphere interactions, with Zn²⁺–O separations ranging from 2.3 to 3.1 Å for compounds 3–6. Additional interactions stabilize each bound hydroxamate moiety: Y323 donates a hydrogen bond to the hydroxamate carbonyl group; H158 donates a hydrogen bond to the anionic hydroxamate O; and H159 hydrogen bonds with the hydroxamate NH group. The Zn²⁺-bound hydroxamate is most likely ionized through the deprotonation of the hydroxamic acid OH group, as observed in other hydroxamate–metal ion complexes,⁵⁴ and as observed here in the ultra-high-resolution structure of the APAH–6 complex.

Compounds 4–6 differ by chain length between the hydroxamate moiety and the terminal amine, with four, five, and six methylene groups, respectively; compound 3 is an analogue of N⁸-acetylspermidine. As a result, each binds differently in the active site of APAH. The 1.42 Å resolution structure of the APAH–4 complex reveals that hydroxamate 4

has the ideal chain length so that its terminal amine directly interacts with the side chain of E117, which adopts two conformations in monomer B (with N–O separations of 2.5–3.1 Å in monomers A and B, respectively) (Figure 3a). The terminal amino group of 4 makes an offset cation– π interaction with F225. Additionally, the amino group of compound 4 hydrogen bonds with water molecules that also interact with the side chains of E17, Y168, or E106 (both from the adjacent monomer of the dimer), and various backbone amide groups of surrounding residues.

Compound 5 is one methylene longer than compound 4. The conformation of the inhibitor is bent such that the N–C–C–C torsion angle is synclinal (average value of -57°), whereas it is antiperiplanar for compound 4 (average value of 167°). This bent conformation allows the amino group of 5 to interact directly with one conformation of E117 (with an occupancy of 0.6) in monomers A and B (N–O separations of 2.6 and 2.7 Å in monomers A and B, respectively) (Figure 3b). Additionally, the amino group hydrogen bonds with the hydroxyl group of Y168 (the N–O separation is 3.0 and 3.1 Å in monomers A and B, respectively). Interestingly, an alternative conformation is evident for compound 5 (occupancies of 0.3 and 0.4 in monomers B and A, respectively) such that the N–C–C–C torsion angle is antiperiplanar (average value of -174°).

Compound 6 is one methylene group longer than compound 5. As such, even with a bent conformation, its terminal amine cannot interact with E117 (Figure 3c). As a result, the inhibitor conformation is extended such that the N–C–C–C torsion angle is antiperiplanar (average value of -177°). As observed for hydroxamate 4, hydroxamates 5 and 6 also hydrogen bond with a network of water molecules interacting with side chains and backbone amide groups of surrounding residues.

Finally, compound 3 is an analogue of N⁸-acetylspermidine. Compared with the other hydroxamate inhibitors, 3 has the same number of methylene groups as 5 but possesses an extra 1,3-diaminopropane “tail”. From the hydroxamate moiety out to the secondary amino group, 3 adopts a conformation that is similar to that of 5 in its alternative antiperiplanar conformation. Surprisingly, the electron density for the 1,3-diaminopropane group of 3 is weak, indicating a higher degree of disorder that is also reflected in higher thermal *B* factors (Figure 3d). Contrary to **1**, the 1,3-diaminopropane “tail” does not interact with E106, which is disordered in the structure presented here. Additionally, in contrast with N⁸-acetylspermi-

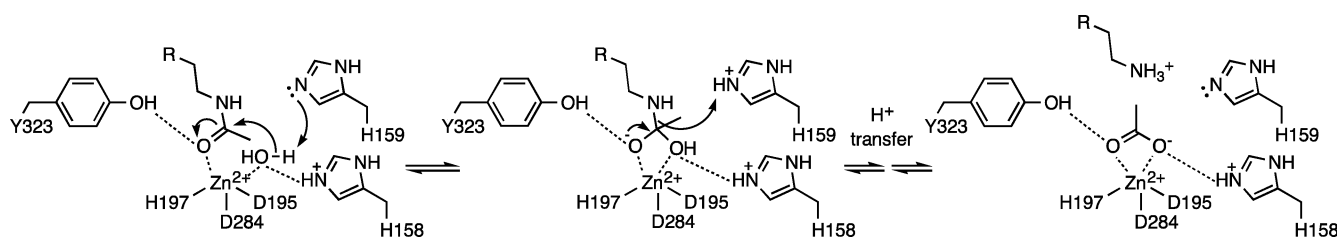


Figure 5. Proposed mechanism of APAH. The substrate binding mode is based on the complex of N^8 -acetylspermidine with the inactive mutant APAH H159A (PDB entry 3Q9C), and the binding of the tetrahedral intermediate is mimicked by the binding of trifluoromethylketone **1** (Figure 2a).

dine and analogues **1** and **2**, compound **3** makes no cation– π interaction with F225. The lack of this stabilizing interaction may account for the relative disorder of the 1,3-diaminopropane moiety. When the binding modes of compounds **3**–**6** are compared (Figure 4), it is clear that there are length-dependent effects that govern the conformation of the amino “tail” of each inhibitor while the hydroxamate– Zn^{2+} coordination geometry remains relatively constant.

DISCUSSION

Compounds **1**–**3** are analogues of N^8 -acetylspermidine bearing different zinc-binding groups. The IC_{50} values listed in Table 2 show that the trifluoromethylketone derivative **1** and the hydroxamate derivative **3** yield the most potent inhibition of APAH with IC_{50} values in the midnanomolar range.³³ The thiol derivative **2** is relatively modest in comparison, which is surprising in view of the potent inhibition afforded by thiol zinc-binding groups of macrocyclic histone deacetylase inhibitors^{46–48} (the eukaryotic histone deacetylases are related to prokaryotic APAH by divergent evolution). Presumably, macrocycle interactions in the greater active site enhance the inhibitory potency of the thiol moiety in these systems.

While thiol derivative **2** is the least active of the three N^8 -acetylspermidine analogues, the structure of its complex with APAH reveals an unusual binding mode caused by the presence of excess Zn^{2+} in the crystallization conditions. A second, half-occupancy Zn^{2+} ion binds in the APAH active site, and in the presence of two Zn^{2+} ions, the ionized thiolate group of **2** bridges the binuclear metal cluster. The second Zn^{2+} ion is coordinated by the imidazole side chains of H158 and H159 as well as a water molecule. Interestingly, this binuclear zinc cluster is reminiscent of that formed in the active site of the unrelated bacterial deacetylase LpxC in the presence of excess zinc.⁵⁵

Trifluoromethylketones are well-known to exist as gem-diols in aqueous solution and as such are usually excellent inhibitors of hydrolytic enzymes as first demonstrated by Gelb and colleagues.⁴² Accordingly, the gem-diol form of **1** binds to APAH as an analogue of the tetrahedral intermediate and its flanking transition states, and the structure of this complex provides critical inferences about the mechanism of deacetylation catalyzed by APAH. Specifically, this structure indicates that the developing negative charge of the tetrahedral oxyanion is stabilized by coordination to Zn^{2+} as well as a hydrogen bond with Y323. The imidazole side chains of H158 and H159 are presumed to hydrogen bond with the nucleophilic Zn^{2+} -bound water molecule; by analogy with enzymological studies of HDAC8,^{16,56} H159 of APAH likely serves as a general base in a promoted water mechanism in which both H159 and Zn^{2+} serve to activate the nucleophilic water molecule. Thus, the

observed binding modes of trifluoromethylketone **1** as well as the substrate N^8 -acetylspermidine strongly support the mechanistic proposal for APAH recently presented by Lombardi and colleagues⁸ as summarized in Figure 5.

Inhibitors **3**–**7** have in common a hydroxamate zinc-binding group that forms a favorable five-membered ring chelate complex with the catalytic Zn^{2+} ion in the APAH active site. Compounds **5** and **6** were designed as analogues of N -acetylputrescine and N -acetylcadaverine, respectively (Figure 1). These two acetylpolyamines are better substrates for APAH from *M. ramosa* than N^8 -acetylspermidine.³⁰ The inhibitory potencies of the corresponding hydroxamate analogues follow a similar trend, because compounds **5** and **6** are more potent than compound **3** (Table 2). Compounds **4** and **7** are hydroxamate analogues of the mono- N -acetylated derivatives of biological 1,3-diaminopropane and nonbiological 1,6-diaminohexane. Compounds **4** and **7** exhibit comparable activity in the midnanomolar range (IC_{50} values of 170 and 150 nM, respectively). The structures of APAH complexed with **4**–**6** reveal key interactions between the positively charged primary amino group of each inhibitor and E117 (for **4** and **5**) and/or interactions with various surrounding residues through networks of hydrogen-bonded water molecules. These results are consistent with enzymological measurements indicating that APAH is a promiscuous enzyme capable of accepting acetylpolyamines of various chain lengths as substrates.^{29,30}

Finally, while compounds **1**–**7** were first designed as APAH inhibitors, it is interesting that compounds **2**–**7** are generally modest inhibitors of the related zinc deacetylase, human HDAC8; indeed, compounds **3**–**7** exhibit inhibitory potency against HDAC8 that is weaker than that of APAH (Table 2). Effective HDAC8 inhibitors generally contain a “capping” group of some sort capable of interacting with the outer active site cleft, such as the macrocycle moiety of largazole.^{50,51} The lack of such a capping group in compounds **2**–**7** may account for the generally modest inhibitory potency against HDAC8.

Strikingly, however, trifluoromethylketone **1** retains essentially identical nanomolar inhibitory potency against both APAH and HDAC8, even though it does not contain a capping group to capture additional affinity interactions in the outer active site cleft. Although we limited this study to inhibitors that most closely resemble smaller polyamine substrates, it is interesting to consider that further derivatization of the primary amino group of **1** with a capping group might allow the capture of additional affinity interactions in the HDAC8 active site. Another capless inhibitor bearing a trifluoromethylketone zinc-binding group, a trifluoromethylketone analogue of L -arginine, was also reported to be a potent HDAC8 inhibitor.⁵⁷ These results may suggest that the trifluoromethylketone moiety is an ideal functional group for coordination to the active site Zn^{2+} ion in the active sites of metal-dependent deacetylases, as also

observed in an inhibitor complex with HDAC4.⁴⁴ These results may also suggest that one of the enzymes currently annotated as a human histone deacetylase may additionally serve as a polyamine deacetylase. However, enzymological assays of HDAC8 conclusively demonstrate that it does not catalyze the deacetylation of N⁸-acetylspermidine: overnight incubation of N⁸-acetylspermidine with HDAC8 does not yield any measurable spermidine product (data not shown). Further results in the development of APAH inhibitors and the identification of the human enzyme responsible for catalyzing polyamine deacetylation will be reported in due course.

■ ASSOCIATED CONTENT

■ Supporting Information

IC₅₀ plots showing inhibitory activity against APAH and HDAC8. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00536.

Accession Codes

The atomic coordinates and the crystallographic structure factors of APAH complexes with 1–6 have been deposited in the Protein Data Bank as entries 4ZUM, 4ZUN, 4ZUO, 4ZUP, 4ZUQ, and 4ZUR, respectively.

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■ ABBREVIATIONS

APAH, acetylpolyamine amidohydrolase; APS, Advanced Photon Source; CDI, carbonyldiimidazole; HDAC, histone deacetylase; HRMS (ESI), high-resolution mass spectrometry (electrospray ionization); M344, 4-(dimethylamino)-N-[7-(hydroxyamino)-7-oxoheptyl]benzamide; NSLS, National Synchrotron Light Source; PDB, Protein Data Bank; PEG, polyethylene glycol; SSRL, Stanford Synchrotron Radiation Lightsource; TLS, translation libration screw.

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