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Broad Spectrum Antiprotozoal Agents that Inhibit Histone Deacetylase: Structure–Activity Relationships of Apicidin. Part 1

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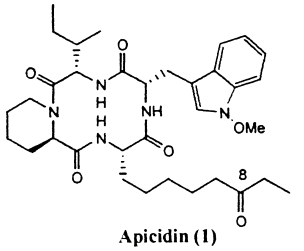
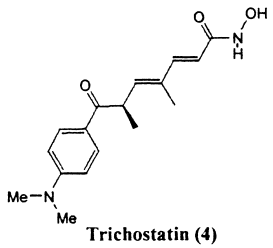
Abstract—Apicidin, a natural product recently isolated at Merck, inhibits both mammalian and protozoan histone deacetylases (HDACs). The conversion of apicidin, a nanomolar inhibitor of HDACs, into a series of side-chain analogues that display picomolar enzyme affinity is described within this structure–activity study.

The natural product apicidin (**1**) is a fungal metabolite recently isolated at Merck from endophytic fungi (*Fusarium pallidoroseum*) on twigs collected in Costa Rica.[1] The biochemical target of apicidin is a group of zinc metallopeptidases[2] termed histone deacetylases (HDACs).[3] These eukaryotic transcription factors are essential for chromatin remodeling and the functional regulation of gene transcription.[4] HDACs are integral nuclear isozymes that modulate the deacetylation of specific acetylated lysine residues (**2**) within the basic *N*-termini of core histones.[5] Deacetylation of these histones produces cationic lysine residues that facilitate histone binding to DNA through favorable ionic interactions with its anionic phosphodiester backbone. Thus, the reversible inhibition of a given HDAC by apicidin leads to histone hyperacetylation, subsequent mitotic arrest, and ultimately cell death. We have demonstrated that apicidin nonselectively induces both mammalian and parasite histone hyperacetylation,[6] and a functional consequence of this nonselective HDAC activity appears to be the reported toxicity observed in rats orally dosed with **1**.[7] Therefore, the identification of potent yet parasite-selective HDAC inhibitors represents a considerable challenge for the development of new antiprotozoal agents, and our effort in this area via the degradation and reconstitution of apicidin is described both within this, and the following letters.[8]



Apicidin (1)



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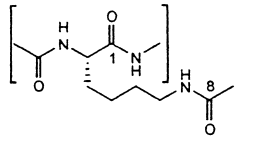
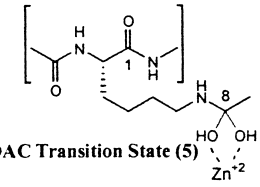
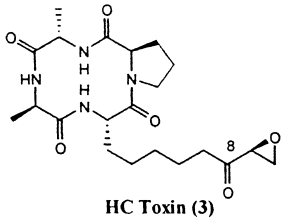


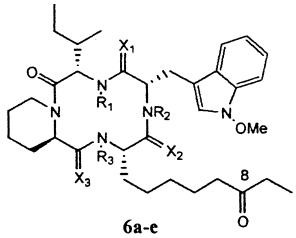
Acetylated Histone Lysine (2)



HDAC Transition State (5)



HC Toxin (3)



6a-e

Apicidin is structurally related to a family of α-keto-epoxide cyclic tetrapeptides which are known HDAC inhibitors that exhibit pronounced antiviral[9] and antineoplastic[10] activity. These cyclopeptide natural products, typified by HC-Toxin (**3**),[11] also include the extensively investigated trapoxins,[12] chlamydocin,[13] Cyl-1

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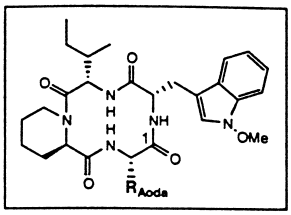
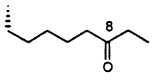
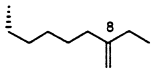
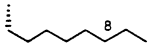
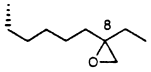
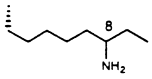
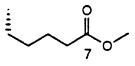
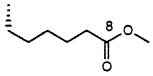
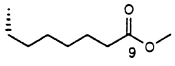
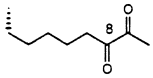
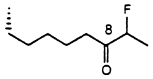
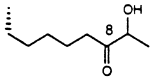
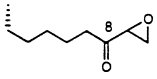
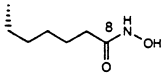
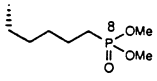
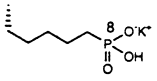
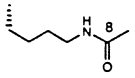
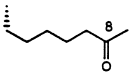
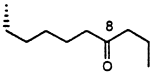
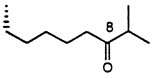
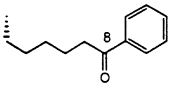
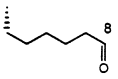
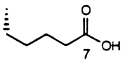
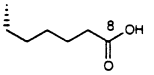
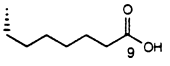
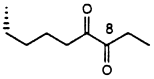
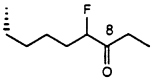
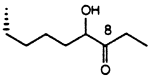
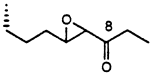
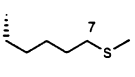
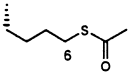
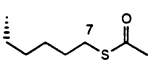
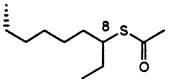
Table 1. Side-chain analogues of apicidin



Compound	R_{Aoda}	HDAC (nM)	*P. falciparum* (ng/mL)	*E. tenella* (ng/mL)
1		HeLa 1 *E. tenella* 1	58	94
7		HeLa 1450 *E. tenella* 1500	1000	1000
8		HeLa 61 *E. tenella* 300	610	1000
9		HeLa 60 *E. tenella* —	1000	>1000
10		HeLa 144 *E. tenella* —	>1000	>1000
11		HeLa 74 *E. tenella* —	>1000	>1000
12		HeLa 0.4 *E. tenella* 0.8	210	>1000
13		HeLa 7 *E. tenella* —	500	>1000
14		HeLa 30 *E. tenella* —	900	250
15		HeLa 1 *E. tenella* —	28	62
16		HeLa 0.3 *E. tenella* 4	22	8
17		HeLa < 0.1 *E. tenella* 1	< 15	3
18		HeLa 0.2 *E. tenella* 4	0.24	>500
19		HeLa 450 *E. tenella* —	850	>1000
20		HeLa 180 *E. tenella* —	>1000	>1000
21		HeLa 2000 *E. tenella* —	1000	>1000
22		HeLa 2 *E. tenella* —	55	125
23		HeLa 1 *E. tenella* —	150	125
24		HeLa 235 *E. tenella* —	500	750
25		HeLa 700 *E. tenella* —	350	1000
26		HeLa 6 *E. tenella* —	500	1000
27		HeLa 282 *E. tenella* —	—	>1000
28		HeLa 15 *E. tenella* 30	>1000	1000
29		HeLa 848 *E. tenella* —	>1000	>1000
30		HeLa 18 *E. tenella* —	700	313
31		HeLa 110 *E. tenella* —	700	313
32		HeLa 39 *E. tenella* —	—	1000
33		HeLa 5 *E. tenella* —	350	190
34		HeLa 326 *E. tenella* —	>1000	>1000
35		HeLa 110 *E. tenella* —	>1000	>1000
36		HeLa 3 *E. tenella* —	900	1000
37		HeLa 73 *E. tenella* 110	590	250

and Cyl-2,[14] WF-3161[15] and Tan-1746s[16] among others.[17] Unlike these related natural products, apicidin is unique in that it alone lacks substitution at the terminus of its 8-oxo-2-aminodecanoic acid (Aoda) side chain. In addition to these naturally occurring cyclic tetrapeptides, other known inhibitors of HDACs that display high affinity to these zinc metallopeptidases also exist. They are largely restricted to either hydroxamic acids, such as the natural product trichostatin A (**4**)[18] and related synthetic derivatives,[19] or cyclic tetrapeptides containing reactive α-ketoepoxide surrogates[20] whose presence is integral for biological efficacy. The structural homology of apicidin's side chain, which is approximately isosteric with **2**, suggests that **1** mimics an in-register acetylated histone lysine. Based upon this hypothesis, we prepared analogues incorporating side-chain registers from C6 to C9, providing access to mechanism-based HDAC inhibitors derived from transition state structure **5**.[21,22] These synthetic efforts led to the discovery that appropriate structural modifications of apicidin's Aoda significantly increased HDAC affinity and functional potency. Indeed, several picomolar HDAC inhibitors derived from apicidin were identified, delineating the contribution of the Aoda moiety to biological activity.

Chemistry

Synthetic modifications of **1** beginning with the macrocycle included the permethylation (3 equiv NaH, 8 equiv MeI, DMF, 23 °C, 20 h, 50%) of the ring nitrogens to form **6a** ($R_1=R_2=R_3=Me$ and $X_1=X_2=X_3=O$). When the macrocycle was treated with Lawesson's reagent (2.5 equiv, toluene, 80 °C, 20 min, 81% overall yield), a mixture of thioamides was isolated (8:6:1:1, **6b**:**6c**:**6d**:**6e**) and carefully separated to provide the mono-thioamide **6b** ($X_1=S$, $X_2=X_3=O$ and $R_1=R_2=R_3=H$), bis-thioamide **6c** ($X_1=X_2=S$, $X_3=O$ and $R_1=R_2=R_3=H$) and bis-thioamide **6d** ($X_1=X_3=S$, $X_2=O$ and $R_1=R_2=R_3=H$) along with the tris-thioamide **6e** ($X_1=X_2=X_3=S$ and $R_1=R_2=R_3=H$). The position of the thiocarbonyl within these compounds was established by ^1H NMR decoupling experiments.

The syntheses of side-chain analogues **7**–**9**, **11**–**13**, **16**–**18**, **21**–**29**, and **32**–**37** (Table 1) have been described.[21,22] Reductive amination of apicidin yielded amine **10** (HMDS, $ZnCl_2$, $NaBH_4$, EtOAc, 50 °C, 12 h, 55%). The α-diketones **14** and **30** were prepared from the respective α-hydroxyketones **16** and **32** via oxidation (TPAP, NMO, CH_2Cl_2, 4 Å sieves, 23 °C, 1 h, 70–80%). Likewise, the α-monofluoroketones **15** and **31** were derived from the respective α-hydroxyketones **16** and **32** via DAST fluorination (Et_2NSF_3, CH_2Cl_2, −78 °C→−10 °C, 1 h, 80–85%). The synthesis of phosphonic acid **20** proceeded via displacement of the C7 tosylate (not shown) with the sodium anion of dibenzyl phosphite (NaH, $(BnO)_2$-P(O)H, THF, 65 °C, 6 h, 40%). This dibenzyl phosphonate intermediate was then deprotected via hydrogenolysis (H_2, Pd–C, $KHCO_3$, *i*-PrOH–H_2O, 10 h, 85%) to provide the mono-potassium salt **20**. The dimethyl phosphonate **19** was prepared in 20% yield under analogous conditions using $(MeO)_2P(O)Na$, subsequent to failed Arbuzov conditions using trimethyl phosphite.

Biology[6]

The biological data presented in Table 1 was generated from three assays: (a) enzyme inhibition of HDAC derived from partially purified extracts of both human HeLa cells and *Eimeria tenella* protozoa reported as IC_{50} values; (b) an in vitro functional assay using whole red blood cells infected with *Plasmodium falciparum*, also reported as IC_{50} values; and (c) an in vitro functional assay using Mauden–Downing bovine kidney (MDBK) host cells infected with *Eimeria tenella* and reported as minimum inhibitory concentration (MIC).

Results and Discussion

The significance of the macrocyclic structure of **1** is immediately gleaned from the complete inactivity of both the linear tetrapeptide, [Ac-Pip-Aoda-Trp(*N*-OMe)-Ile-CO_2Me], and the linear tripeptide, [Ac-Pip-Aoda-Trp(*N*-OMe)-CO_2Me], prepared by acid-catalyzed methanolic solvolysis of **1**, followed by *N*-acetylation of the resultant linear peptides. Additionally, the permethylated macrocycle **6a** was completely inactive, lacking both binding affinity and functional activity. The equipotent thioamides **6b**–**d** displayed attenuated biological activity relative to **1**. These three thiono derivatives had an average binding IC_{50} to mammalian HDAC and *E. tenella* HDAC of 85 and 210 nM, respectively. Interestingly, **6b**–**d** also retained some of the functional activity of **1** with an average IC_{50} of 100 ng/mL for *P. falciparum* and an average MIC of 125 ng/mL for *E. tenella*. Thioamides **6b**–**d** also exhibited reduced toxicity to mammalian cells as indicated in an in vitro antiproliferative functional assay using either human HeLa cells or foreskin fibroblasts reported as the concentration required for 50% inhibition of cell growth (average $AP_{50}=1000$ ng/mL). In contrast, the tris-thioamide **6e** was virtually inactive relative to the natural product **1**. The significant loss or elimination of biological activity with these linear and macrocyclic analogues supports the requirement of a specific rigid conformation of **1** for HDAC affinity and functional activity. Analogues **6a**–**e** appear to disrupt the crucial transannular hydrogen bonds which influence the conformation of **1**, a structural feature elucidated from NMR studies[1] and supported by X-ray crystallography.[23]

Structure–activity relationships (SARs) of representative side-chain analogues of **1** are presented in Table 1. As noted previously, the design and synthesis of mechanism-based inhibitors derived from apicidin was based upon the zinc associated transition state model **5**.[24] Thus, the requirement of apicidin's ketone for zinc chelation (presumably as its hydrate) is apparent by the reduction in both HDAC affinity and functional potency of analogues **7**–**10** including the apicidin C8 alcohol (not shown). Ketones **22**–**25** explore the SAR with regard to the incorporation of sterically large groups interacting with the nonprime subsite of HDAC. Although the ethyl (**1**), methyl (**22**) and *n*-propyl (**23**) ketones were well tolerated, the larger isopropyl (**24**) and phenyl (**25**) ketones displayed diminished activity. As anticipated, the C8

aldehyde **26** (prone to hydration) retained most of the HDAC affinity associated with **1**, although its lack of functional activity may be ascribed to pharmacological deficiencies. Based upon mechanistic models **2** and **5**, the precise register of side-chain functionality should be integral to inhibitory efficacy, and the data in Table 1 supports this premise. For instance, the C8 methyl ester **12**, C9 methyl ester **13** and C7 methyl ester **11** displayed a range of HeLa HDAC affinity from 400 pM to 7 nM to 74 nM, respectively. This data suggests that while the higher C9 homologue retains some activity, the shorter C7 homologue is unable to access the catalytic active site as effectively. It also should be noted that **12** is approximately isosteric to **1**. Although the function of an ester as a metallopeptidase inhibitor is uncommon, related systems have precedent and may be explained by an enolization mechanism.[25] The relevance of side-chain register to biological activity is also exemplified with the C7, C8, and C9 carboxylic acids **27**–**29**, but the trend of HeLa HDAC affinity is altered from that observed for the corresponding esters. As expected, the most active analogue of this series was the C8 acid **28** (15 nM), whereas the least active derivative was now the higher homologue, C9 acid **29** (848 nM). Although carboxylic acids are known to be efficient zinc chelating moieties in other metallopeptidases, a 15- to 30-fold loss of HDAC affinity for **28** relative to **1** was observed. This result can be explained by the general lack of HDAC binding observed with analogues containing acidic functionality, conceivably because the endogenous substrates of HDACs are highly basic histones. From a survey of various putative metal chelating groups such as α-monofluoroketones **15** and **31** (to increase carbonyl hydration), α-hydroxyketones **16** and **32**, and α-ketoepoxides **17** and **33**, external C9 substitution was more tolerated than internal C7 substitution. This trend is consistent with the conventional metallopeptidase collected-product inhibitors which display weaker, less discriminate interactions with the nonprime subsite of the enzyme.[26] Conversely, the α-diketones **14** and **30** were equally inferior to **1**. Noteworthy, is α-hydroxyketone **16** (epimeric mixture at C9) which displayed an inhibition IC_{50} of 300 pM on HeLa HDAC, and functional activity of 22 and 8 ng/mL against *P. falciparum* and *E. tenella*, respectively, all of which surpass the biological activity of apicidin. As a point of reference, in our laboratories the IC_{50} value for chloroquine against *P. falciparum* is 92 ng/mL. More importantly, α-ketoepoxide **17** (epimeric mixture at C9) displayed <100 pM inhibition of HeLa HDAC, <15 ng/mL functional activity against *P. falciparum*, and 3 ng/mL functional activity against *E. tenella*. The basis for the remarkable potency of **17**, relative to both **1** and the structurally related α-ketoepoxide **3** (HeLa HDAC, $IC_{50} = 8$ nM), will be addressed in due course. Furthermore, introducing a C8-hydroxamate zinc-chelating moiety into apicidin produced **18**, which emerged as the most potent analogue within the *P. falciparum* assay (0.24 ng/mL) and displayed 200 pM inhibition of HeLa HDAC. The extraordinary in vitro functional activity of **18** against malaria may be derived from enhanced uptake into red blood cells due to iron chelation with the hydroxamic acid group.

Continuing with a survey of known zinc chelating moieties, thiols also were incorporated into **1**, but were inactive presumably due to their oxidative lability. In order to circumvent this problem, the thiolacetates of apicidin were tested for in situ deacetylation within the biological assays. This approach was clearly successful, and is exemplified by comparing the HDAC inhibition of C7-methylsulfide **34** (326 nM) with C7-thiolacetate **36** (3 nM). Homologues **35**–**37** nicely illustrate the significance of sulfur register to biological activity, with the in situ generated C8-thiol from **36** displaying comparable HDAC affinity relative to **1**, versus the less active in situ generated C9-thiol from **37** and the least active in situ generated C7-thiol from **35**. This trend follows a similar order of activity (HDAC inhibition, C8 >C9 >C7) seen with the methyl esters **11**–**13** above. Phosphonates were also incorporated into **1**, as these functionalities are known to bind zinc and represent tetrahedral transition state mimics of **5**. As expected, the neutral dimethyl phosphonate **19** was less active relative to the charged phosphonic acid mono-potassium salt **20**. The decrease in HDAC inhibition of **20** relative to **1** may be explained by the same reasoning applied to carboxylic acid **28** above. Lastly, the acetamide **21**, a hybrid of **1** and **2**, displayed greatly diminished HDAC binding affinity, consistent with the expectation that **21** would function as a substrate and be processed by the enzyme. In general, side-chain analogues of apicidin, although potent, did not exhibit parasite selectivity in either HDAC inhibition (Table 1) or competitive binding assays (data not shown). Accordingly, the AP_{50} values for compounds **7**–**37** (data not shown) were similar to the inhibitory concentrations against the protozoa given in Table 1. One exception to this trend was the phenyl ketone analogue **25**, which demonstrated 10-fold parasite selectivity in a competitive binding assay using extracts from chicken liver versus *E. tenella* (CL = 20,000 nM versus *E. tenella* = 2000 nM) and >16-fold parasite selectivity in the *P. falciparum* and *E. tenella* cell-based assays relative to the antiproliferative functional assay ($AP_{50} = >10{,}000$ ng/mL). This result suggests there is some heterogeneity in the nonprime subsite of protozoal HDAC relative to mammalian HDAC.

In summary, Part 1 of this SAR study describes a pharmacophore of apicidin consisting of the macrocycle and the side-chain region of the natural product. The biological data reported herein has elucidated the importance of the macrocyclic conformation of **1** to activity and has guided the development of picomolar side-chain mechanism-based transition state analogues of **1**. Other discoveries include an apparent heterogeneity in the nonprime subsites of mammalian and protozoal HDAC metallopeptidases, and the potency-reducing effect of acidic inhibitors in contrast to the highly basic histone endogenous substrates.

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