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Structure, Histone Deacetylase, and Antiprotozoal Activities of Apicidins B and C, Congeners of Apicidin with Proline and Valine Substitutions

Sheo B. Singh,* Deborah L. Zink, Jerrold M. Liesch, Anne W. Dombrowski, Sandra J. Darkin-Rattray, Dennis M. Schmatz, and Michael A. Goetz

Merck Research Laboratories, RY80Y-355, P.O. Box 2000, Rahway, New Jersey 07065

sheo_singh@merck.com

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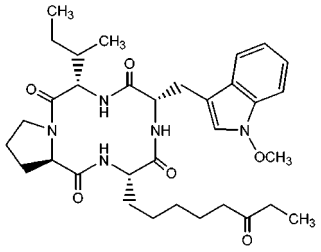
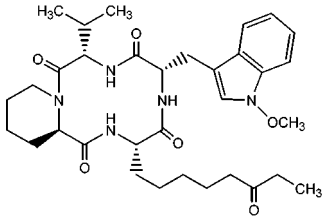
ABSTRACT



Apicidin B



Apicidin C

Isolation and structure elucidation of two novel cyclic tetrapeptides that show a variety of potent antiprotozoal activities by reversibly inhibiting HDAC have been reported. These are the new members of a unique family of cyclic tetrapeptides that do not require the electrophilic α-epoxyketone moiety of HC-toxin, trapoxin A, or chlamydocin for their potent activities against HDAC and the malarial parasite.

Protozoan parasites comprising the apicomplexan family are the causative agents of diverse diseases affecting the health of humans and animals alike. Some of these life threatening diseases include malaria, cryptosporidiosis, toxoplasmosis, sarcocystis, and coccidiosis, which are caused by *Plasmodium* sp., *Cryptosporidium parvum*, *Toxoplasma gondii*, *Sarcocystis neurona*, and *Eimeria* sp., respectively.[1] There are therapies to treat a number of these diseases, but developing resistance is cause for concern. Therefore, there may thus always be need for new therapies, particularly those based on new mechanisms of action, to stay ahead of infections caused by these parasites.

Recently we reported apicidin (**1**), a cyclic tetrapeptide isolated[2] from *Fusarium pallidoroseum*, an endophytic fungus collected from Costa Rica, as a potent and broad-spectrum antiprotozoal agent that exerts its biological activity by reversibly inhibiting histone deacetylase (HDAC) activity.[1] HDACs are nuclear isozymes that regulate gene transcription via a dynamic process of acetylation and deacetylation of lysine residues of histones. Blockade of the deacetylation process causes hyperacetylation of histones, and this leads to untimely cell death. Trapoxin A (**2**) is a natural cyclic tetrapeptide that is an irreversible inhibitor of HDAC.[3] The C-8 keto group of the 2-amino-8-oxodecanoic acid (Aoda) moiety of apicidin topologically registers to the C-8 keto group of the acetylated lysine residue (**3**) of histones and suggests that apicidin functionally mimics the substrate

* Tel: (732) 594-3222. Fax: (732) 594-6880.

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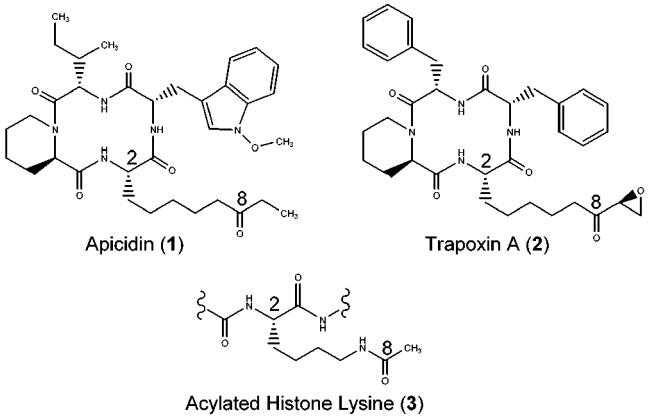
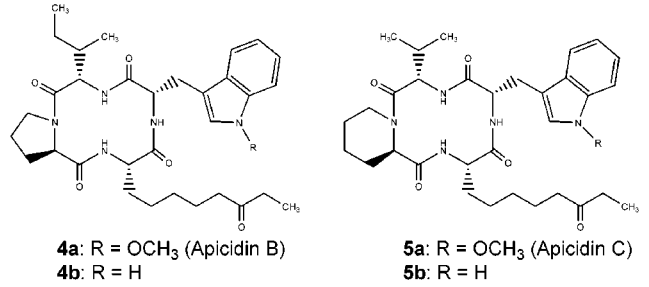
for HDAC, thus causing hyperacetylation.[4] Apicidin showed



in vivo efficacy against *P. berghei* malaria in mice.[1] While chemical modifications of Aoda and Trp residues of apicidin led to a limited SAR,[5] these modifications did not allow for amino acid substitutions in the absence of a total synthesis. Therefore, to study this aspect of the SAR, we decided to take advantage of the biosynthetic machinery of the producing organism for the discovery of novel apicidin analogues with amino acid substitutions by targeted analytical and biological screening of fermentation extracts. This process led to the discovery of two novel apicidin congeners containing proline (Pro) and valine (Val) substitutions, named herein apicidins B (**4a**) and C (**5a**). The isolation, structure elucidation, stereochemistry, and biological activities of the two compounds are described.



The fermentation broth of *Fusarium pallidoroseum* (ATCC 74289), grown on solid or liquid media, was extracted with methyl ethyl ketone, and the extract was chromatographed on Sephadex LH 20 in methanol. This was followed by chromatography on silica gel and reverse-phase HPLC to yield apicidin B {1.0 mg/L, $[\alpha]^{23}_D$ −65.1° (*c*, 0.40, CH_3-OH)} and apicidin C {2.5 mg/L, $[\alpha]^{23}_D$ −56° (*c*, 0.77, CH_3-OH)} both as amorphous powders.

Apicidin B (4a). EI mass spectral analysis of **4a** gave a parent ion at *m*/*z* 609. High-resolution measurement led to a molecular formula $C_{33}H_{47}N_5O_6$ (found 609.3525, calcd 609.3526) for apicidin B, which was supported by the ^{13}C NMR spectrum and suggested 13 degrees of unsaturation as in apicidin **1**. The infrared and UV spectra of **4a** were similar to those of apicidin and showed absorption bands for carbonyl, amide carbonyls, and indole moieties. The molecular formula of apicidin B indicated that it had one less methylene group compared to apicidin ($C_{34}H_{49}N_5O_6$), and

Table 1. ^1H and ^{13}C NMR Assignments of Apicidins B (**4a**) and C (**5a**) in C_5D_5N

	apicidin B (**4a**)		apicidin C (**5a**)	
no.	δC	δH	δC	δH
		Pro		Pip
1	172.04		171.86	
2	58.24	5.11, d, 7.6	50.74	5.52, brd, 5.2
3	25.40	2.43, m	25.55	2.07, m
		1.66, m		1.49, m
4	25.02	2.28, m	19.88	2.34, m
		1.78, m		1.45, m
5	46.90	4.11, dt, 10, 4	25.92	1.25, m
		3.59, appq, 8		1.50, m
6			44.03	4.36, brd, 13.6
				3.26, dt, 13.2, 2.4
		Ile		Val
1	174.07		174.24	
2	58.03	4.91, t, 10	55.73	5.16, t, 10.0
3	34.78	2.36, m	29.42	2.40, m
4	25.24	1.80, m	19.33	1.02, d, 7.6
		1.35, m		
5	10.84	0.92, t, 7.2	18.63	1.16, d, 6.8
6	15.84	0.98, d, 6.8		
	NH	8.33, d, 10.0	NH	8.27, d, 10
		Trp-*N*-OCH_3		Trp-*N*-OCH_3
1	175.18		174.67	
2	61.95	4.54, dt, 9.6, 6.8	61.49	4.61, td, 10.8, 6.0
3	25.95	4.23, dd, 14.8, 10.0	25.67	4.25, dd, 14.4, 10.4
		3.91, dd, 14.4, 7.2		3.82, dd, 14.8, 6.4
4	108.44		108.17	
5	124.32		124.00	
6	119.56	7.78, d, 8.0	119.29	7.80, d, 8.0
7	120.26	7.19, t, 8.0	120.07	7.19, dt, 8.0, 0.8
8	122.85	7.33, t, 8.0	122.74	7.34, dt, 8.0, 0.8
9	108.95	7.55, d, 8.0	108.74	7.55, d, 8.0
10	133.02		132.82	
11	122.94	7.59, s	122.74	7.58, s
12	65.74	3.94, s	65.54	3.94, s
	NH	9.98, d, 6.4	NH	10.03, d, 6.8
		Aoda		Aoda
1	176.06		176.44	
2	55.42	4.74, brq, 8.4	54.70	4.76, brq, 8.5
3	30.42	1.90, m; 1.65, m	30.12	1.89, m; 1.60, m
4	25.95	1.25, m	25.67	1.22, m
5	29.06	1.10, m	28.74	1.10, m
6	23.94	1.40, m	23.67	1.45, m
7	42.14	2.20, t, 7.6	41.86	2.18, t, 7.2
8	210.47		210.16	
9	35.72	2.29, q, 7.2	35.46	2.28, t, 7.6
10	8.09	1.01, t, 7.2	7.84	1.01, t, 7.2
	NH	7.92, d, 10.0	NH	7.32, d, 10.5

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this was supported by the methylene carbon count in the ^{13}C NMR spectrum (Table 1). The ^{1}H NMR spectra of the two compounds showed differences in the regions corresponding to the pipecolic (Pip) residue. This included upfield shifts of the two α-protons (δ 5.53 vs 5.11 and δ 5.18 vs 4.91); a downfield shift of one of the NH protons (δ 8.27 vs 8.33); and both up and downfield shifts (δ 4.37, 3.27 vs 4.11, 3.59) of a pair of methylene protons α to the ring nitrogen. This was supported by concomitant downfield shifts of the corresponding two methine carbons in the ^{13}C NMR spectrum of apicidin B. 2D COSY and HMQC experiments were used to decipher $^{1}H-^{1}H$ spin systems and distinguish proton-bearing carbons, respectively. From the analysis of the COSY spectrum it was quite clear that apicidin B contained a Pro residue instead of pipecolic acid, which would account for the lack of one methylene group. The COSY spectrum of **4a** showed unambiguous correlations of $\alpha-\beta-\gamma-\delta$ proline protons, and assignments were confirmed by the requisite HMBC correlations of the proline protons. Other HMBC correlations were identical to those observed in apicidin and were useful in deducing the amino acid sequence and the cyclic nature of the tetrapeptide. The structure of apicidin B was further corroborated by its mass spectral fragmentation (Figure 1). The Pro residue was recognized by a characteristic

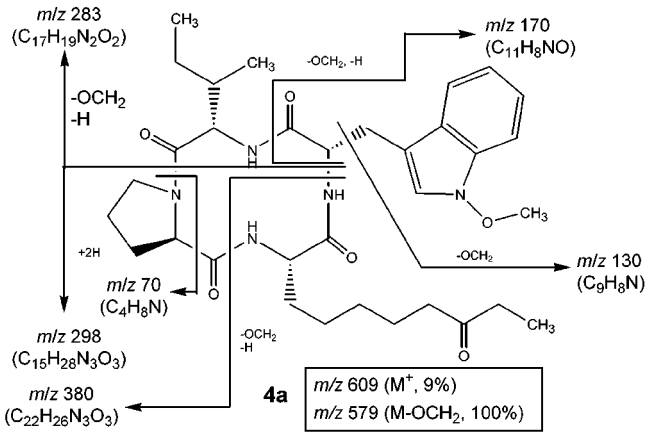

Figure 1. EIMS fragmentation of apicidin B (**4a**). Fragment ion intensities >5%, and all fragments were verified by high-resolution measurements.

fragment ion at *m*/*z* 70 (C_4H_8N). The corresponding fragment ion originating from the Pip residue appears at *m*/*z* 84 ($C_5H_{10}N$), as evidenced in the mass spectrum of apicidin. Fragment ions at *m*/*z* 283, 296/298, and 380 defined the connectivities Trp-Ile, Pro-Aoda, and Trp-Ile-Pro, respectively (Figure 1).

Like apicidin, the hydrogenation of apicidin B using 10% Pd/C produced exclusively des-methoxy apicidin B (**4b**). Hydrolysis[6] of **4b** with 6 N HCl containing 5% thioglycolic acid after degassing in a sealed tube at 110 °C produced the component amino acids. The hydrolysate was subdivided into three aliquots. A first aliquot of the mixture was reacted with α-methylbenzylisothiocyanate (AMBI);[7] two other aliquots were separately reacted with D- and L-amino acid oxidase[8] followed by reaction with AMBI. All three samples were analyzed by HPLC and compared with the corresponding derivatives of *R* (D) and *S* (L) amino acids. Both of these methods indicated that Ile and Trp residues were in the *S* (L)-configuration whereas Pro was in the *R* (D)-configuration. The stereochemistry of Aoda could not be determined by this method without an authentic sample but was deduced to be *S* on the basis of the *J* values of respective α and NH protons and analogy to apicidin.[2]

Apicidin C (5a). HREI mass spectral analysis of apicidin C (**5a**) gave a parent ion at *m*/*z* 609.3512, which afforded a molecular formula $C_{33}H_{47}N_5O_6$ that was identical to apicidin B. The ^{1}H and ^{13}C NMR spectra (Table 1) of apicidin C (**5a**) was virtually identical to the corresponding spectra of apicidin except for the absence of the methyl triplet of isoleucine (Ile) and the presence of a methyl doublet, suggesting substitution of Ile with a Val. The Val substitution was supported by the $^{1}H-^{1}H$ COSY and the HMQC spectra and was confirmed by HMBC spectrum. The mass spectral fragmentation of **5a** further corroborated this substitution, the sequence of the cyclic tetrapeptide, and the structure of apicidin C (Figure 2). Fragment ions at *m*/*z* 269 and 310/

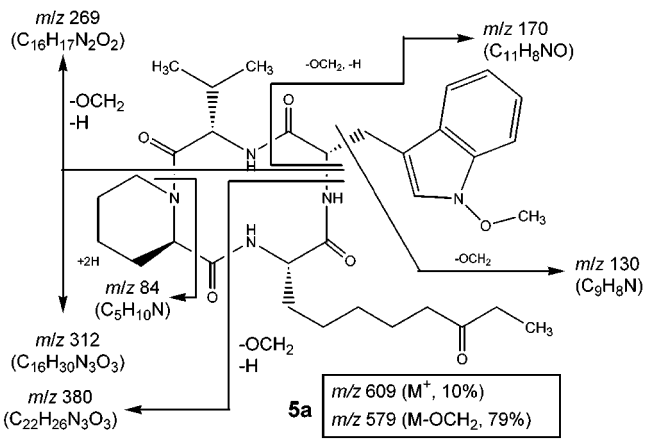

Figure 2. EIMS fragmentation of apicidin C (**5a**). Fragment ion intensities >15%, and all fragments were verified by high-resolution measurements.

312, originating from the splitting of the molecule into two halves, verify the Ile to Val and Pro to Pip substitution in apicidin C compared to apicidin B. Apicidin C has a negative sign of specific rotation like apicidin, and the identical *J* values of α-CH and the respective NH protons suggest the identical stereochemistry of constituent amino acids. Hydrogenation of **5a** using 10% Pd/C in a mixture of MeOH/EtOAc gave des-methoxy compound **5b** (*m*/*z* 579).

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(8) L- and D-Amino acid oxidase: Sigma A 9378 (Type IV isolated from *Crotalus adamanteus* venom) and Sigma A 5418 (Type X from *Porcine* kidney lyophilized powder) obtained from Sigma-Aldrich Chemical Co.

Apicidin B and apicidin C were evaluated in a variety of antiprotozoal assays. Compounds **4a** and **5a** inhibited the binding[9] of [^{3}H]-apicidin A to *Eimeria tenella* parasite HDAC with IC_{50} values of 10 and 6 nM, respectively. Apicidin B showed 5-fold selectivity for parasite HDAC over the host chick liver HDAC (IC_{50} 50 nM), but apicidin C did not show any selectivity. Apicidin B showed MIC values[9] of 12.8, 411, and 189 nM against *Besnoitia jellisoni*, *E. tenella*, and *P. falciparum*, respectively, in cell culture and was slightly less active than apicidin. However, apicidin C showed MIC values of 0.8, 101, and 69 nM in the above assays and was slightly more active than apicidin B and apicidin (6.4, 100, and 201 nM). The activities of the desmethoxy compounds **4b** and **5b** were comparable to their respective parents.

The substitution of *R*-Pip to *R*-Pro in apicidin B caused conformation changes as evidenced by the differences in the chemical shifts of the protons and carbons present in the immediate vicinity of Pro residue and may account for its somewhat lower activity.

In summary, we have discovered two novel cyclic tetrapeptides that show a variety of potent antiprotozoal activities by reversibly inhibiting HDAC. These are the new members of a unique family of cyclic tetrapeptides that do not require the electrophilic α-epoxyketone moiety of HC-toxin,[10] trapoxin A, chlamydocin,[11] cyl 1,[12] and WF3161[13] to be active against HDAC and the malaria parasite. Further studies on the apicidins may lead to better antimalarial and antiprotozoal agents.

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(9) For detailed description of assays, see ref 1.

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