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Apicidins: Novel Cyclic Tetrapeptides as Coccidiostats and Antimalarial Agents from *Fusarium pallidoroseum*

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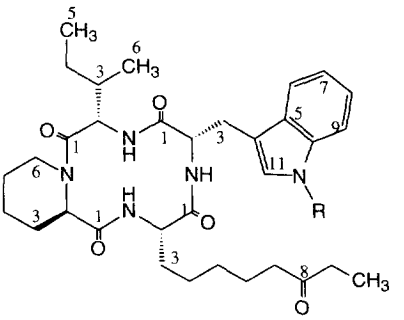
Abstract: Apicidin is a cyclic tetrapeptide [cyclo-(N-O-Methyl-L-Trp-L-Ile-D-Pip-L-2-amino-8-oxo-decanoyl)] isolated from *Fusarium pallidoroseum* by bioassay guided separation. It is a potent inhibitor of apicomplexan histone deacetylase (IC_{50} 1-2 nM), a broad spectrum antiparasitic agent *in vitro* against apicomplexan parasites and has shown *in vivo* efficacy against *Plasmodium berghei* malaria. Isolation, structure and stereochemistry are discussed.

Malaria, cryptosporidiosis, toxoplasmosis and coccidiosis are among the number of parasitic diseases caused by protozoa of sub-phylum Apicomplexa. These and other such diseases present significant threats to human and animal health worldwide. A number of medicines are available for treatment of some of these diseases but the rapid development of resistance is a serious problem. Medicinal agents based on new mechanisms of action are, therefore, needed to overcome emergence of resistance and to control an ever increasing number of epidemics caused by these parasites.

From our natural product screening, we have discovered a novel cyclic tetrapeptide that showed[1] *in vitro* MICs of 4 to 70 ng/mL against a broad range of apicomplexa. Apicidin showed *in vivo* efficacy against *Plasmodium berghei* malaria[1] in mice at less than 10 mg/kg (I. P.). Apicidin is a potent inhibitor (IC_{50} 1-2 nM)[1] of apicomplexan histone deacetylase (HDA), and this appears to be the mechanism of its apicomplexan activity.[1] We describe, herein, the isolation, structure elucidation and stereochemistry of apicidin and of its congener apicidin A.



1: R= OCH_3, Apicidin
2: R= H, Apicidin A

Size exclusion chromatography (Sephadex LH-20) of a methyl ethyl ketone extract of the fermentation broth of *F. pallidoroseum* [2] grown either on a solid or in a liquid nutrient medium- followed by silica gel and reverse phase HPLC afforded apicidin (25 mg/L) which was crystallized from methanol as colorless needles, mp. 195-97 °C, $[\alpha]^{22}_D$ -80.4° (c 1.2, $CHCl_3$).

STRUCTURE ELUCIDATION

Electron impact (EI) mass spectral analysis of apicidin gave a molecular ion at *m/z* 623. High resolution measurements gave the molecular formula $C_{34}H_{49}N_5O_6$ which was supported by the ^{13}C NMR spectrum. The formula suggested that apicidin has 13 degrees of unsaturation. The UV spectrum of **1** in $CHCl_3$ gave absorption bands at λ_{max} 242 (ε = 10729), 279 sh (ε = 4781) and 291 (ε = 5073) nm. The infra red spectrum showed absorption bands for amide NH (3283 cm^{-1}), ketone (1714 cm^{-1}), free amide (1694 cm^{-1}) and H-bonded amide (1662 cm^{-1}) and aromatic (1615 cm^{-1}) groups. ^{13}C NMR spectra (Table 1) of **1** in CD_2Cl_2 and C_5D_5N displayed 34 carbons. The APT/DEPT spectrum revealed the presence of four methyls (one being methoxy), thirteen methylenes, ten methines (four α-CH, an aliphatic, five olefinic/aromatic), three olefinic/aromatic quaternaries, four ester/amide carbonyls, and an acyclic ketone. The ^{13}C NMR shifts were assigned using an HMQC experiment. From the analysis of the $^{13}C/^1H$ NMR spectra it was apparent that apicidin was a tetrapeptide. The ^1H NMR (400 MHz) spectrum (Table 1) of **1** in C_5D_5N (also in CD_2Cl_2) exhibited a methyl doublet (δ 1.0) with J = 7.2 Hz connected to a methine (δ 2.42).The remaining two methyl groups (δ 0.94 and 1.00) appeared as triplets with a J = 7.2 Hz connected to two methylene groups. One of those methylenes was coupled only to a methyl group and appeared as a downfield quartet at δ 2.28 and therefore, must be next to a deshielding group such as a ketone. Analyses of the COSY, TOCSY and HMQC data (Figure 1) revealed several fragments which, in combination with ^{13}C shifts, were assigned to pipecolic acid (Pip), isoleucine (Ile), 2-amino-8-oxo-decanoic acid (Aoda) and tryptophan (Trp).

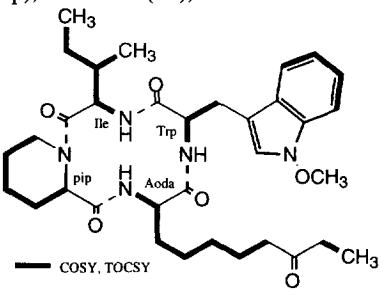


Figure 1: Apicidin Fragments Derived From COSY and TOCSY Correlations

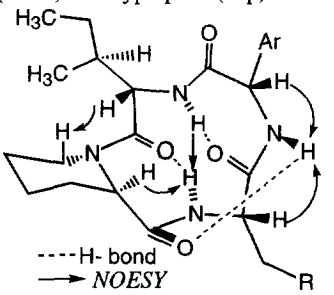


Figure 2: H-bonding and Selected NOESY Correlations of Apicidin.

Connectivities within each of these fragments and to one another were established by HMBC experiments in both solvents and the correlations are presented in Table 1. The correlations from each of the α-protons to two respective amide carbonyls established the cyclic tetrapeptide. The ^{13}C-NMR assignment of carbonyl groups were supported by the two bond HMBC correlations from the respective NH protons. The HMBC correlations from the ethyl group to the down field ketone established the placement of the ethyl ketone at C-7 of Aoda. Since the methoxy group did not show any HMBC correlations and was shifted downfield in the ^{13}C NMR spectrum, it was assigned as a N-methoxy and was placed at the indole nitrogen, the only available site which would lack HMBC correlations. The presence of the N-methoxy group was verified by hydrogenolysis of apicidin to apicidin A (**2**) (subsequently isolated from the fermentation broth in very low yield) using 10% Pd/C.

RELATIVE AND ABSOLUTE STEREOCHEMISTRY

Acid hydrolysis (6N HCl) of apicidin A (**2**) followed by GC-MS analysis of the silylated derivatives confirmed the identity of Pip, Ile and Aoda. Trp could not be detected in this hydrolysate but it was identified by using milder hydrolytic conditions[3] tailored for Trp residue. The stereochemistry of the amino acid(s) was determined by preparation of AMBI derivative[4] and amino-oxidase method.[5] Amino acid derivatives were analyzed by HPLC and compared with authentic samples of both D (*R*) and L (*S*) derivatives of the appropriate amino acids. This method was successful in determining the stereochemistry of Ile (*S*) and Trp (*S*). The unavailability of an authentic sample of the unusual amino acid, Aoda, precluded the determination of its

Table 1: NMR Assignment and HMBC Correlations of Apicidin in CD_2Cl_2 and C_5D_5N.

#	CD_2Cl_2			C_5D_5N		
	δC	mult	δH	δC	δH	HMBC
	Pip			**Pip**		J_{CH} = 7 Hz
1	171.80	C°		172.90		
2	51.10	CH	5.07, brd, J = 5.5 Hz	51.35	5.53, brd, J = 5.6 Hz	C1, 3, 4, 6, Ile-C1
3	24.55	CH_2	1.99, m	25.14	2.10, m	
			1.56, m		1.48, m	
4	19.78	CH_2	2.12, m	20.44	1.44, m	
			1.57, m		2.32, m	
5	25.69	CH_2	1.77, m	26.25	1.25, m	
			1.40, m		1.50, m	
6	44.33	CH_2	4.01,m	44.64	4.37,brd, J = 12.0 Hz	
			3.02, dt, J = 13, 3 Hz		3.27, dt, J = 13.2, 2.4 Hz	C4
	Ile			**Ile**		
1	174.71	C°		174.90		
2	54.40	CH	4.69, t, J = 10.5 Hz	54.87	5.18, t, J = 10 Hz	C1, 3, 4, 6, Trp-C1
3	35.24	CH	2.0, m	35.85	2.42, m	
4	25.11	CH_2	1.60, m	25.59	1.90, m	
			1.17, m		1.40, m	
5	10.87	CH_3	0.92, t, J = 7. 5 Hz	11.40	0.94, t, J = 7.2 Hz	C3, 4
6	15.72	CH_3	0.85, d, J = 7 Hz	16.26	1.00,d, J = 7.2 Hz	C2, 3, 4
		NH	7.01, d, J = 10 Hz	----	8.27, d, J = 10.4 Hz	Trp-C1
	Trp-N-OCH_3			**Trp-N-OCH_3**		
1	174.07	C°		175.25		
2	61.31	CH	4.00, m	62.08	4.61, td, J = 10.6, 6.4 Hz	C1, 3, 4, Aoda-C1
3	25.82	CH_2	3.68, dd, J = 15, 10 Hz	26.47	4.23, dd, J = 14.4, 10.4 Hz	C2, 3, 4, 5, 11
			3.48, dd, J = 15, 7.5 Hz		3.84, dd, J = 14.4, 6.4 Hz	C2, 3, 4, 5, 11
4	107.23	C°		108.72		
5	123.86	C°		124.60		
6	119.05	CH	7.58, d, J = 7.5 Hz	119.86	7.79, d, J = 8.0 Hz	C4, 5, 8, 10
7	120.12	CH	7.11, dt, J = 7.5, 1.0 Hz	120.63	7.18 dt, J = 8.0, 0.8 Hz	C5, 9
8	122.87	CH	7.24, dt, J = 7.5, 0.5 Hz	123.28	7.34, dt, J = 8.0, 0.6 Hz	C6, 9, 10
9	108.71	CH	7.41, d, J = 8.0 Hz	109.32	7.55, d, J = 8.0 Hz	C5, 7
10	132.70	C°		133.38		
11	122.41	CH	7.16, brs	123.31	7.58, s	C4, 5, 10
12	66.11	CH	4.04, s	66.10	3.94, s	
		NH	6.37, d, J = 6.8 Hz		10.00, d, J = 6.8 Hz	C2, 3, Aoda-C1
	Aoda			**Aoda**		
1	175.58	C°		177.00		
2	54.02	CH	4.20, dt, J = 10.0, 7.5 Hz	55.28	4.76, brq, J = 8.0 Hz	C1, 3, Pip-C1
3	29.60	CH_2	1.68, m	30.71	1.80, m	C1, 2, 4, 5
			1.50, m		1.60, m	C1, 2, 4, 5
4	25.53	CH_2	1.21, m	26.25	1.25, m	
5	29.07	CH_2	1.23, m	29.32	1.10, m	C3, 4, 7
6	23.86	CH_2	1.49, m	24.22	1.45, m	C4, 5, 7, 8
7	42.33	CH_2	2.35, t, J = 7 Hz	42.44	2.18, t, J = 7.2 Hz	C5, 6
8	211.55	C°		210.77		
9	36.06	CH_2	2.39, q, J = 7.5 Hz	36.04	2.28, q, J = 7.2 Hz	C8, 10
10	7.94	CH_3	1.00, t, J = 7.5 Hz	8.40	1.00, t, J = 7.6 Hz	C8, 9
		NH	6.40, d, J = 10.5 Hz		7.36, d, J = 10 Hz	Pip-C1

chirality. AMBI derivatives of L and D pip could not be resolved by HPLC using a number of conditions, therefore, its chirality could not be independently verified.

Once the stereochemistry of Ile and Trp was established, that of Pip and Aoda was determined by using NMR methods. Of particular use were the coupling constant of αH to respective NH and a number of NOESY correlations. αH of both Aoda and Ile were coupled with respective NH with a J value of ~10 Hz

thus indicating an *anti*-relationship (ϕ = 180°); the αH of Trp was coupled with its NH with a *J* value of ~7Hz indicating a *syn*-relationship. Once these relationships were put in place, interpretation of NOESY data resulted in assignment of the D (*R*) stereochemistry to Pip residue (a β-turn) and L (*S*) to Aoda. A prefered conformation (Figure 2) was deduced from the NOE data. Each of the amide NH's are subject to a strong intramolecular H-bond to a carbonyl and this results in a set of 7-membered rings, as shown in Figure 2. For example Ile NH is H-bonded with Aoda C=O, Trp NH is H-bonded with pip C=O and Aoda NH is H-bonded with Ile C=O. The NH chemical shifts in either C_5D_5N or CD_2Cl_2 were independent of concentration of sample, a characteristic of intramolecular H-bonding. This observation was further supported by a very small change in the chemical shift ($\Delta\delta$) of the amide NH's over a large range of temperature (ΔT, -10 to 70 °C). The H-bonds and overall conformation of apicidin depicted in Figure 2 are fully consistent with the energy minimized structure derived from molecular modeling. Ketone and amide NH's of Ile and Aoda point down-ward and Trp NH and pip C=O point up-ward in that Figure.

There are five other known naturally occuring cyclic tetrapeptides: HC-toxin,[6] Trapoxin A,[7] WF-3161,[8] Cly-2,[9] and chlamydocin.[10] These tetrapeptides all contain a terminal epoxide in the long chain amino acid and this feature is responsibe for their antiproliferative activity (IC_{50} 1-2 nM).[6b] The potent antiparasitic effects of apicidin are, therefore, remarkable.

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