PNEUMOCANDINS FROM *Zalerion arboricola*

III. STRUCTURE ELUCIDATION

OTTO D. HENSENS, JERROLD M. LIESCH, DEBORAH L. ZINK, JACK L. SMITH, CAROL F. WICHMANN and ROBERT E. SCHWARTZ

Department of Natural Products Chemistry, Merck Research Laboratories, P.O. Box 2000 (RY80Y-345), Rahway, N.J. 07065-0900, U.S.A.

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Pneumocandin B_0 (**6**) and six related lipopeptides are antifungal and anti-*Pneumocystis carinii* agents from mutants of *Zalerion arboricola*, whose structures were determined mainly on the basis of spectroscopic analysis. They belong, along with pneumocandin A_0 (L-671,329) previously isolated from these laboratories,[1] to the echinocandin class of antifungal agents. The product from base-catalyzed ring opening involving the hemiaminal position of the dihydroxyornithine residue of B_0, has been clearly defined as **6b**. Modifications were limited to the 3-hydroxy-4-methylproline, 3,4-dihydroxyhomotyrosine and 4,5-dihydroxyornithine residues of pneumocandin A_0.

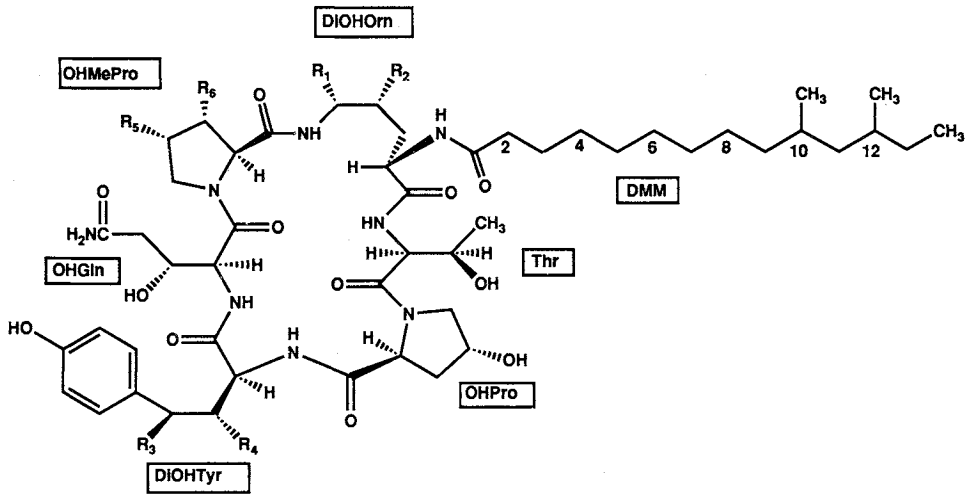
The recently reported lipopeptide antifungal agent pneumocandin A_0 (L-671,329) (**1**) from these laboratories,[1~3] is a new member of the echinocandin structural family and has been shown to be active against *Pneumocystis carinii*.[4] We now wish to report on the structure determination of pneumocandin B_0 (**6**) (Fig. 1), its base degradation product **6b** (Fig. 3) and six related lipopeptides, based primarily on spectroscopic evidence. Their discovery and isolation are reported in a companion paper.[5] Using the long-range ^1H-^{13}C ^1H-detected HMBC technique, the sequence of pneumocandin B_0 was rigorously established. Comparison of these data with that of pneumocandin A_0, required revision of some of the ^{13}C carbonyl resonance assignments previously made on the basis of limited long-range HETCOR data.[1] Biosynthetic studies with enriched [2-^{13}C]acetate necessitated the revision of the assignments of some of the dimethylmyristate carbons.[6] The peptide sequence, including the relative stereochemistry, has been independently confirmed on the basis of X-ray crystallographic studies.[7] The absolute stereochemistry, as depicted in **6**, followed from the L-configuration of threonine which was determined by HPLC methods. The structures of all pneumocandins described in this work are shown in Fig. 1.

Materials and Methods

Spectroscopic Methods

All NMR spectra were recorded on a Varian XL-400 or Unity 500 NMR spectrometer at ambient temperature in CD_3OD. ^1H NMR data listed in Table 2 were recorded at 400 MHz in CD_3OD using the solvent peak at δ 3.30 as internal reference. ^{13}C NMR data in Table 1 were recorded at 100 MHz in CD_3OD where chemical shifts are given in ppm downfield of TMS using the solvent peak at 49.0 ppm as internal reference. Coupled 'gated' ^{13}C NMR spectra with NOE were recorded using a one second acquisition time and 0.2 second delay. APT spectra were recorded in the usual manner.[8] ^1H-^1H correlation spectra (COSY) were recorded using the standard pulse sequence.[9] Long-range COSY (LR-COSY) spectra were optimized with delays in the 0.2~0.5 second range. RELAY spectra[10] were optimized for mixing periods of 0.02 and 0.05 seconds. Phase-sensitive NOESY experiments were performed using a repitition rate of 3 seconds and a mixing time of 0.5 second. ^1H-^{13}C chemical shift correlation spectra (HETCOR) were recorded using the standard pulse sequence.[11] The delay time

Fig. 1. Structures of pneumocandins A_0, A_1, A_2, A_3, A_4, B_0, B_2 and C_0.



	R_1	R_2	R_3	R_4	R_5	R_6
Pneumocandin A_0 (**1**)	OH	OH	OH	OH	CH_3	OH
Pneumocandin A_1 (**2**)	OH	OH	OH	H	CH_3	OH
Pneumocandin A_2 (**3**)	H	H	OH	OH	CH_3	OH
Pneumocandin A_3 (**4**)	OH	H	H	H	CH_3	OH
Pneumocandin A_4 (**5**)	H	H	H	H	CH_3	OH
Pneumocandin B_0 (**6**)	OH	OH	OH	OH	H	OH
Pneumocandin B_2 (**7**)	H	H	OH	OH	H	OH
Pneumocandin C_0 (**8**)	OH	OH	OH	OH	OH	H

between transients was 1 second and the experiment was optimized for $^1J_{CH}=130\sim140$ Hz. The corresponding long-range experiment (LR-HETCOR) was optimized for a multiple bond ^{13}C-^1H coupling constant of 10 Hz. HMQC spectra of pneumocandins A_0 and B_0 were recorded at 500 MHz, to establish and confirm the assignments of protonated carbons using the pulse sequence by BAX and SUBRAMANIAN.[12] Sequence determination of amino acid residues in pneumocandins A_0 and B_0 at 500 MHz were accomplished using the HMBC pulse sequence of BAX and SUMMERS.[13] The experiment was optimized for 4, 7 and 10 Hz.

Mass spectral data were obtained on a Finnigan-MAT 212 mass spectrometer at 90 eV or a Finnigan-MAT TSQ70 at 70 eV. Fast atom bombardment (FAB) spectra were recorded on a VG20-253 or Finnigan-MAT MAT90 instrument using dithioerythritol-dithioerythreitol (MB) or MB containing cesium iodide. Samples were hydrolyzed in 6 N HCl in tightly capped vials at 110°C for 18 hours and then evaporated to dryness under a stream of nitrogen. The hydrolysate residue was derivatized with a 1:1 mixture of BSTFA (or BSTFA-d_9) and pyridine at 50°C for 30 minutes. GC-MS analyses were carried out using a J & W DB-5 Durabond capillary column (15 m × 0.3 mm, 25 μm film). Components were identified by interpretation of their mass spectra and by comparison to library spectra.

Absolute Stereochemistry

To determine the absolute configuration of threonine in pneumocandin B_0, a sample was first hydrolyzed in 6 N aqueous HCl at 110°C for 18 hours. After evaporation of the hydrolysate, the (*R*)-α-methylbenzylisothiocyanate derivatives (AMBI) were prepared as previously described[14] and another portion silylated for GC-MS analysis. The AMBI derivatives were separated on an ABI 130-A HPLC system equipped with a Spheri-5RP-18 220 × 2.1 mm column and the components detected at 254 nm. The mobile phases consisted of: A, 60% aqueous ammonium acetate buffer (0.14 M, with 0.05% triethylamine adjusted to pH 6.40 with acetic acid) and 40% acetonitrile; B, 60% acetonitrile. The

following gradients were used: 10% B for 4 minutes, 15% B at 5 minutes, 40% B at 30 minutes, 100% B at 35 minutes and held for 5 minutes at a flow rate of 250 μl/minute and an oven temperature of 26°C. GC-MS analysis confirmed the presence of threonine, whereas, the HPLC analysis of the AMBI derivatives indicated its stereochemistry as L-threonine from its retention time by comparison with authentic samples.

Results and Discussion

For convenience, residue positions in the seven new pneumocandins will be referred to those assigned in pneumocandin A_0[1] namely 4,5-dihydroxyornithine (DiOHOrn), 3-hydroxy-4-methylproline (OHMePro), 3-hydroxyglutamine (OHGln), 3,4-dihydroxyhomotyrosine (DiOHTyr), 4-hydroxyproline (OHPro), threonine (Thr) and 10,12-dimethylmyristate (DMM). Modifications were limited to only the DiOHOrn, MeOHPro and DiOHTyr residues.

Structure of Pneumocandin B_0

Lithiated FAB-MS indicated the molecular weight 1,064 (observed $(M+Li)^+$ at *m/z* 1,071) which is 14 mass units less than pneumocandin A_0, $C_{51}H_{82}N_8O_{17}$ (MW 1,078).[1] The ^{13}C NMR spectrum in CD_3OD indicated the presence of 50 carbons, one less than for A_0, in which the methyl group of the 3-hydroxy-4-methylproline residue at 11.1 ppm was absent and the methine at 39.1 ppm was replaced by a methylene carbon at 34.8 ppm. ^{13}C NMR resonances of all other residues, assigned on the basis of HETCOR, HMQC and HMBC experiments, were found to be in agreement within 0.2 ppm (Table 1). GC-MS of the TMS derivatives of the total acid hydrolysate disclosed 5 major components. Threonine, 4-hydroxyproline, 3-hydroxyglutamic acid and a $C_{16:0}$ fatty acid were identified as in the case of pneumocandin A_0. The dihydroxyornithine and dihydroxyhomotyrosine residues do not survive the acid treatment.[1] The presence of the fatty acid was confirmed by preparation of the methyl ester after base hydrolysis. GC-MS afforded data identical to that obtained from the 10,12-dimethylmyristate liberated from component A_0. However, 3-hydroxy-4-methylproline was not observed. Instead an isomer of 4-hydroxyproline (*m/z* 287) was present, the retention times of the two hydroxyprolines differing by about one minute. Their TMS mass spectra are quite similar. Both exhibit ions of similar intensities at *m/z* 332 ($M-CH_3$), 304 (*m/z* 332$-$CO) and 230 (base peak, $[M-117T_1]$). The 4-hydroxy isomer also contains a fairly strong ion at *m/z* 140 (intensity 60% of base peak) which corresponds to *m/z* 230$-$TMSiOH. However, the *m/z* 140 ion in the other isomer is barely visible (intensity 2% of base peak). By comparison, the analogous ion in 3-hydroxy-4-methylproline (*m/z* 154) from pneumocandin A_0, is equally of very low intensity (2~4%). The cumulative ^{13}C NMR and MS evidence, therefore, suggested its assignment as 3-hydroxyproline. This was corroborated by detailed analysis of ^1H NMR data from COSY, LR-COSY, DQF-COSY and RELAY experiments. HETCOR and HMQC experiments allowed distinction between many of the overlapping methine and methylene proton resonances which facilitated the determination of ^1H-^1H connectivities of all amino acid residues, in particular that for 3-hydroxyproline (Table 2).

The sequence of the six amino acid residues and position of attachment of the fatty acid chain was unequivocally established at 500 MHz using the ^1H-^{13}C long-range HMBC technique.[13] Best results for the number of correlations involving the carbonyl resonances were obtained, when the experiment was optimized for 4 Hz (see Fig. 2). Four of the unambiguous amide carbonyl ^{13}C assignments thus obtained for pneumocandin B_0 (OHGln, DiOHTyr, Thr, DiOHOrn), necessitated revision by comparison with the

Table 1. ^{13}C NMR data for pneumocandins A_0, A_1, A_2, A_3, A_4, B_0, B_2 and C_0 and base conversion product **6b** of B_0 in CD_3OD (100 MHz)[a].

Carbon	A_0 (1)	A_1 (2)	A_2 (3)	A_3 (4)	A_4 (5)	B_0 (6)	6b	B_2 (7)	C_0 (8)
3,4-Dihydroxyhomotyrosine (DiOHTyr)									
C-1	172.5 s[b]	173.1 s	172.3 s[d]	173.4 s	173.6 s	172.5 s	172.0 s	172.6 s	172.5 s
C-2	56.4 d	52.7 d	56.7 d br	54.5 d	55.2 d	56.3 d	56.6 d	56.7 d	56.3 d
C-3	76.9 d	42.4 t	76.8 d	35.4 t	33.9 t[e]	76.9 d	75.5 d	76.8 d	76.9 d
C-4	75.8 d	72.6 d	75.9 d	33.1 t	32.9 t[e]	75.8 d	76.6 d	75.9 d	75.8 d
C-1′	133.0 s	136.1 s	133.1 s	133.0 s	133.0 s	133.0 s	133.0 s	133.1 s	133.0 s
C-2′/C-6′	129.6 d	128.5 d	129.6 d	130.5 d	130.5 d	129.6 d	129.7 d	129.6 d	129.6 d
C-3′/C-5′	116.2 d	116.2 d	116.2 d	116.2 d	116.3 d	116.2 d	116.2 d	116.2 d	116.2 d
C-4′	158.5 s	158.0 s	158.5 s	156.8 s	156.8 s	158.5 s	158.3 s	158.5 s	158.5 s
3-Hydroxyglutamine (OHGln)									
C-1	169.0 s[b]	169.4 s	169.7 s	169.0 s	170.2 s	169.1 s	170.7 s	169.6 s	169.2 s
C-2	55.6 d	55.5 d	55.7 d	55.3 d	55.7 d	55.6 d	55.6 d	55.6 d	56.1 d
C-3	70.7 d	70.9 d	70.6 d	71.0 d	70.7 d	70.7 d	69.7 d	70.7 d	70.9 d
C-4	39.5 t	39.9 t	39.9 t	39.8 t	40.1 t	39.4 t	39.8 t	39.9 t	39.4 t
C-5	177.2 s	176.8 s	176.8 s	176.9 s	176.4 s	177.2 s	176.6 s	176.8 s	177.3 s
3-Hydroxy-4-methylproline (OHMePro)									
C-1	172.7 s	172.6 s	172.4 s[d]	172.6 s	172.2 s	172.9 s	174.9 s[f]	172.4 s	172.9 s
C-2	70.2 d	70.1 d	70.3 d	70.1 d	70.3 d	69.8 d	70.2 d	69.9 d	60.7 d
C-3	75.9 d	75.8 d	76.1 d	76.1 d	76.0 d	74.3 d	74.8 d	74.3 d	38.5 t
C-4	39.1 d	39.1 d	39.0 d	39.1 d	39.0 d	34.8 t	34.9 t	34.5 t	71.0 d
C-5	53.0 t	53.0 t	52.9 t	53.0 t	52.8 t	47.0 t	46.7 t	46.9 t	57.9 t
4-CH_3	11.1 q	11.2 q	11.2 q	11.2 q	11.2 q	—	—	—	—
4-Hydroxyproline (OHPro)									
C-1	173.4 s	173.6 s	173.5 s	174.0 s	173.6 s	173.4 s	174.2 s	173.6 s	173.4 s
C-2	62.5 d	62.2 d	62.4 d	62.2 d	62.1 d	62.5 d	60.8 d	62.5 d	62.4 d
C-3	38.5 t	38.7 t	38.6 t	38.5 t	38.5 t	38.5 t	38.8 t	38.6 t	38.5 t
C-4	71.3 d	71.3 d	71.3 d	71.3 d	71.2 d	71.3 d	71.1 d	71.3 d	71.3 d
C-5	57.1 t	57.1 t	57.2 t	57.0 t	57.1 t	57.1 t	57.2 t	57.2 t	57.1 t
Threonine (Thr)									
C-1	172.7 s[b]	172.6 s	172.9 s	172.6 s	172.7 s	172.7 s	172.7 s	172.9 s	174.6 s
C-2	58.4 d	58.4 d	58.4 d	58.3 d	58.6 d	58.3 d	58.0 d	58.4 d	58.3 d
C-3	68.2 d	68.7 d	68.0 d	68.9 d	68.2 d	68.2 d	68.9 d	68.1 d	68.2 d
C-4	19.7 q	19.6 q	19.8 q	19.8 q	19.8 q	19.7 q	19.7 q	19.8 q	19.7 q
4,5-Dihydroxyornithine (DiOHOrn)									
C-1	174.6 s[b]	174.6 s	175.2 s	175.4 s	175.2 s	174.5 s	175.1 s[f]	175.3 s	174.5 s
C-2	51.4 d	51.6 d	52.9 d	52.2 d	53.0 d	51.4 d	61.3 d	52.9 d	51.2 d
C-3	34.8 t	34.9 t	27.7 t	26.8 t	28.06 t	34.5 t	35.9 t	27.6 t	34.9 t
C-4	70.6 d	70.6 d	24.3 t	30.7 t	24.6 t	70.6 d	77.1 d	24.3 t	70.6 d
C-5	74.0 d	74.3 d	37.9 t	71.9 t	38.3 t	73.9 d	88.8 d	37.9 t	73.8 d
10,12-Dimethylmyristate (DMM)									
C-1	175.8 s	175.9 s	176.1 s	176.0 s	176.3 s	175.8 s	176.1 s[f]	176.1 s	175.7 s
C-2	36.7 t	36.7 t	36.7 t	36.8 t	36.7 t	36.7 t	34.0 t	36.7 t	36.7 t
C-3	27.0 t[c]	27.0 t	27.0 t	27.1 t	27.0 t	27.0 t	26.2 t	27.0 t	27.0 t
C-4	30.29 t[c]	30.30 t	30.27 t	30.32 t	30.27 t	30.30 t	30.34 t	30.27 t	30.3 t
C-5	30.6 t[c]	30.6 t	30.5 t	30.6 t	30.5 t	30.6 t	30.6 t	30.5 t	30.6 t
C-6	30.7 t[c]	30.8 t	30.7 t	30.8 t	30.7 t	30.8 t	30.7 t	30.8 t	30.8 t
C-7	31.2 t[c]	31.3 t	31.1 t	31.2 t	31.1 t	31.18 t	31.09 t	31.11 t	31.2 t
C-8	28.0 t[c]	28.0 t	28.0 t	28.1 t	28.01 t	28.1 t	28.0 t	28.0 t	28.1 t
C-9	38.1 t	38.1 t	38.0 t	38.1 t	38.0 t	38.1 t	38.1 t	38.1 t	38.1 t
C-10	31.3 d	31.2 d	31.2 d	31.3 d	31.2 d	31.24 d	31.25 d	31.25 d	31.3 d
C-11	45.9 t	45.9 t	45.9 t	45.9 t	45.9 t	45.9 t	45.9 t	45.9 t	45.9 t
C-12	32.9 d	32.9 d	32.9 d	32.9 d	32.9 d	32.9 d	32.9 d	32.9 d	32.9 d
C-13	30.33 t	30.34 t	30.34 t	30.36 t	30.34 t	30.34 t	30.41 t	30.35 t	30.3 t
C-14	11.6 q	11.6 q	11.6 q	11.6 q	11.6 q	11.6 q	11.6 q	11.6 q	11.6 q
10-CH_3	20.7 q	20.7 q	20.7 q	20.7 q	20.7 q	20.7 q	20.7 q	20.7 q	20.7 q
12-CH_3	20.2 q	20.2 q	20.2 q	20.2 q	20.2 q	20.2 q	20.2 q	20.2 q	20.2 q

[a] Chemical shifts in ppm downfield of TMS. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; br, broad.

[b] Re-assigned on the basis of HMBC experiments on pneumocandins A_0 and B_0 at 125 MHz (see text).

[c] Re-assigned on the basis of [2-^{13}C] acetate incorporation[6] (see text).

[d~f] Assignments may be interchanged.

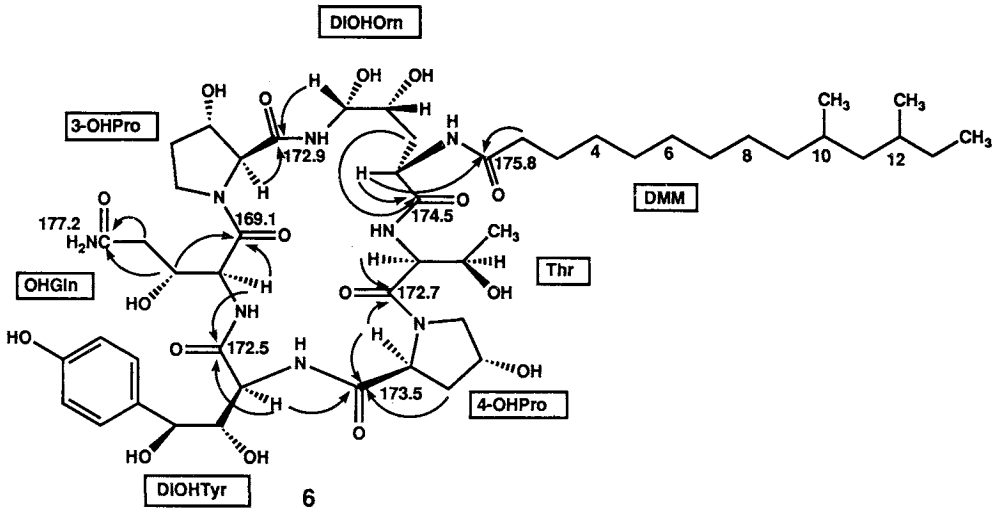
Table 2. ^{1}H NMR data of modified residues in pneumocandins A_0, A_1, A_2, A_3, A_4, B_0, B_2 and C_0 in CD_3OD (400 MHz)[a].

Assignment	A_0	A_1	A_2	A_3	A_4	B_0	B_2	C_0
3-Hydroxy-4-methylproline (OHMePro)								
2-H	4.36 d (2.2)	4.37 d (2.3)	4.32 d (2.2)	4.29 d (2.2)	4.36 d (2.1)	4.25 d (3.4)	4.21 d (3.2)	~4.54 m[b]
3-H	4.14 dd (2.2, 4.5)	4.17 dd (2.3, 4.5)	4.10 dd (2.2, 4.5)	4.13 dd (2.1, 4.6)	4.15 dd (2.1, 4.4)	~4.33 m[b]	4.30 m[b]	~2.10 m/~2.17 m
4-Ha	2.48 m[b]	2.51 m	~2.47 m[b]	~2.53 m[b]	~2.55 m[b]	~2.25 m[b]	~2.20 m[b]	4.44 m
4-Hb	—	—	—	—	—	1.95 m	1.94 m	—
4-CH_3	1.04 d (6.8)	1.06 d (6.9)	1.04 d (6.7)	1.06 d (6.8)	1.06 d (6.8)	—	—	—
5-Ha	3.34 t (9.6)	3.36 t (~10)	3.34 t (9.8)	3.37 t (9.5)	3.37 t (9.6)	~3.77 m[b]	3.76 m[b]	3.68 br d (~11)
5-Hb	4.09 dd (7.5, 9.4)	4.11 dd (7.4, 9.6)	4.02 dd (7.5, 9.6)	4.12 dd (7.5, 9.6)	4.04 dd (7.5, 9.5)	~4.01 m[b]	3.92 m[b]	3.91 dd (3.9, 11.3)
3,4-Dihydroxyhomotyrosine (DiOHTyr)								
2-H	4.31 d	4.39 dd (4.0, 8.5)	4.24 br s	4.42 dd (3.4, 11.0)	~4.15 m[b]	4.31 d (1.5)	4.25 br s	4.30 d (~1.5)
3-Ha	4.27 m[b]	~2.31 m[b]	4.29 s[b]	~2.24 m[b]	2.27 m[b]	4.27 m[b]	4.29 s	4.27 m[b]
3-Hb	—	2.13 m	—	1.94 m	2.14 m	—	—	—
4-Ha	4.28 m[b]	4.61 t (6.3)	4.29 s[b]	2.57 t (~7)	~2.55 m[b]	4.28 m[b]	4.29 s	4.27 m[b]
4-Hb	—	—	—	2.57 t (~7)	~2.55 m[b]	—	—	—
2′-H/6′-H	7.13 d (8.6)	7.16 d (8.6)	7.14 d (8.5)	7.00 d (8.5)	6.99 d (8.5)	7.13 d (8.6)	7.13 d (8.5)	7.13 d (8.5)
3′-H/5′-H	6.75 d (8.7)	6.74 d (8.7)	6.75 d (8.6)	6.68 d (8.5)	6.68 d (8.5)	6.75 d (8.5)	6.75 d (8.6)	6.74 d (8.5)
4,5-Dihydroxyornithine (DiOHOrn)								
2-H	4.43 dd (6.1, 11.1)	4.44 dd (6.2, 10.9)	4.41 dd (4.0, 12.6)	~4.42 m[b]	4.41 dd (3.5, 12.2)	4.45 dd (6.5, 10.9)	4.43 dd (3.7, 12.2)	4.52 dd (5.0, 11.6)
3-Ha	2.00 m[b]	~2.00 m[b]	~2.08 m[b]	~2.01 m[b]	2.02 m[b]	~2.03 m[b]	~2.10 m[b]	~1.99 m[b]
3-Hb	2.00 m[b]	~2.00 m[b]	~1.60 m[b]	~1.73 m[b]	~1.58 m[b]	~2.03 m[b]	~1.60 m[b]	~1.99 m[b]
4-Ha	4.00 ddd (2.6, 6.8, 8.9)	3.59 ddd (3.0, 7.2, 8.5)	1.73 m	~1.73 m[b]	1.69 m	~4.00 m[b]	~1.74 m	3.99 ddd (3.1, 6.3, 9.2)
4-Hb	—	—	1.73 m	~2.01 m[b]	1.69 m	—	~1.74 m	—
5-Ha	5.26 d (2.7)	5.24 d (3.0)	3.49 m	5.34 m	~3.43 m[b]	5.27 d (2.7)	3.53 dt (6.5, 13.9)	5.29 d (3.1)
5-Hb	—	—	2.94 dt (4.7, 13.8)	—	3.02 dt (~4.8, 14.0)	—	2.92 dt (4.5, 13.9)	—

[a] Chemical shifts in δ ppm downfield of TMS. Coupling constants in Hz in parentheses. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, br, broad.

[b] Overlapping or obscurred resonances.

Fig. 2. Sequence determination of pneumocandin B_0 using the ^1H detected long-range ^1H-^{13}C HMBC technique.



assignments in component A_0, based on limited ^1H-^{13}C long-range HETCOR data.[1] These were confirmed by separate HMBC experiments on A_0.

The structure of pneumocandin B_0 is therefore as depicted in **6** (Fig. 1), whose peptide sequence was independently established, including the relative stereochemistry, by X-ray crystallography.[7] The absolute stereochemistry was determined by HPLC analysis of the (*R*)-α-methylbenzylisothiocyanate derivatives of the total acid hydrolysate of B_0 indicating the presence of L-threonine. The threonine, 4-hydroxyproline, 3,4-dihydroxyhomotyrosine and 4,5-dihydroxyornithine residues have the same absolute configurations as previously determined for echinocandin B.[15] An in-depth conformational study of pneumocandin A_0 using vicinal ^1H-^1H coupling constants and NOE geometry constraints as input parameters for energy minimization and distance geometry calculations,[16] indicated the configuration of the 3-hydroxy-4-methylproline residue as the 2,3-*trans*-3,4-*cis* stereoisomer, the same as in echinocandin B.[17] The small coupling between 2-H and 3-H in 3-OHPro of pneumocandin B_0 (Table 2), suggested the same 2,3-*trans* relative stereochemistry as in pneumocandin A_0.

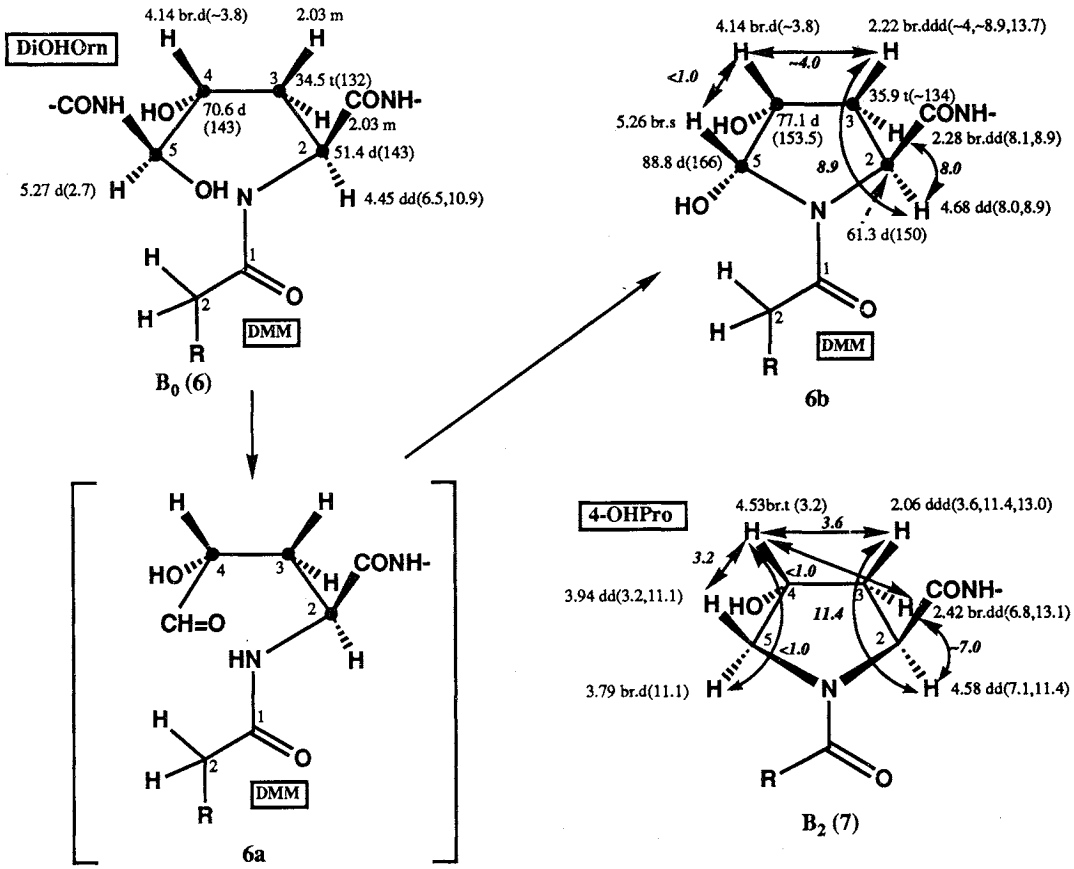
The biosynthetic origin of the modified 3-hydroxyproline as well as 4-hydroxyproline residue, was recently shown to derive from L-proline.[18] By contrast, the 3-hydroxy-4-methylproline residue in pneumocandin A_0 resulted from cyclization of L-leucine.[6] In the same study, [2-^{13}C]acetate was found to label all even-numbered carbon atoms (C-2 ~ C-14) of the myristic acid side chain which necessitated revision of the ^{13}C assignments for three closely spaced methylene resonances in A_0 (C-3, with C-8, C-4 with C-5 and C-6 with C-7). These could not be unequivocally assigned on the basis of ^1H-^{13}C HETCOR data because of the overlapping methylene ^1H NMR resonances.[1]

Structure of Base-conversion Product of Pneumocandin B_0

In all fermentations of pneumocandin B_0, an interfering substance with similar chromatographic properties was observed, whose formation could be slowed under acidic conditions and accelerated with base. A sufficient quantity of the predominant product was isolated for its structure to be investigated.[5] FAB-MS measurements gave the same molecular weight 1,064 as for B_0. Similarly, GC-MS of the

silylated derivatives of the total amino acid hydrolysate mixture gave the same results as for B_0. ^{13}C NMR spectra in CD_3OD showed appreciable differences between the two compounds however. In particular, the appearance of the diagnostic methine at 88.8 ppm correlated to the broad singlet at δ 5.23 in the HETCOR experiment, and exhibited a large single bond ^1H-^{13}C coupling constant of 166 Hz. ^1H-^1H connectivity experiments including COSY and 1D decoupling, showed that the latter proton resonance originated from 5-H of the DiOHOrn residue and suggested that the methine C-5 had moved from 73.9 ppm ($^1J_{CH}$ = 154 Hz) in B_0 to 88.8 ppm in the conversion product. In combination with other NMR evidence summarised in Scheme 1, it was evident that ring-opening had occurred at the C-5 hemiaminal position of the DiOHOrn residue. The formation of an incipient aldehyde **6a** was envisaged to undergo immediate ring-closure to the 4,5-dihydroxyproline derivative **6b** involving the α-amide NH group. The greater ring strain satisfactorily accounts for the ^{13}C NMR characteristics of the hemiaminal C-5 carbon as well as the downfield shifts observed for the other ring carbons C-1 ~ C-4 and

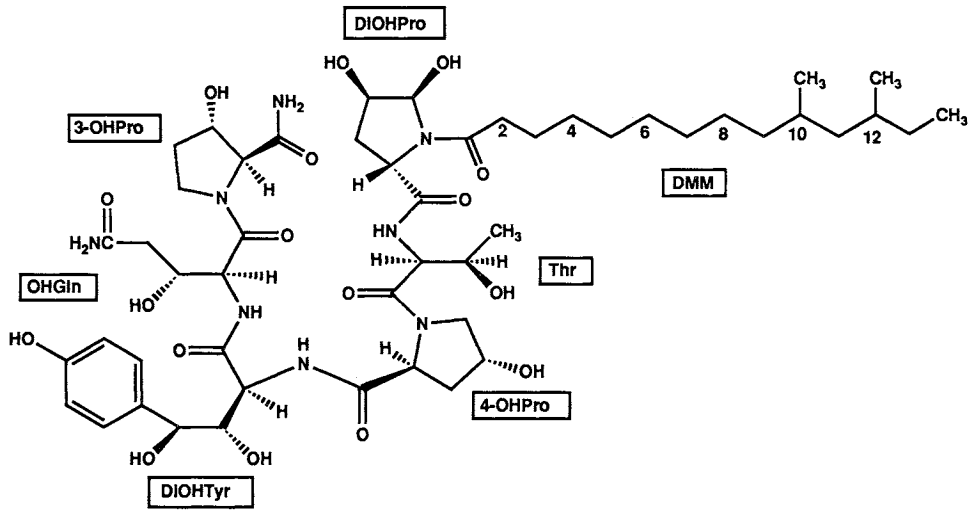
Scheme 1. Postulated mode of formation of base-conversion product **6b** from pneumocandin B_0 (**6**) through the intermediate aldehyde **6a**, involving the hemiaminal functionality of the DiOHOrn residue.



^1H and ^{13}C parameters are compared for the DiOHOrn residue of B_0 (**6**), the base conversion product **6b** and for the configurationally related 2,4-*trans*-4-hydroxyproline residue in pneumocandin B_2 (**7**). ^1H chemical shifts are given in δ_{ppm} downfield of TMS in CD_3OD with ^1H-^1H coupling constants (Hz) in parentheses and denoted by arrows (in italics). ^{13}C chemical shifts are shown in ppm downfield of TMS in CD_3OD with one-bond ^1H-^{13}C coupling constants (Hz) in parentheses.

Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad.

Fig. 3. Proposed structure of base-conversion product of pneumocandin B_0.



corresponding increases in $^1J_{CH}$ values (see Scheme 1).

Assuming no inversion at C-2 or C-4, the stereochemistry at these centers can be depicted as in **6b** (Fig. 3), the same as for the 4-hydroxyproline residue common to all pneumocandins described in this work. The resonances of this residue are well separated in pneumocandin B_2 (**7**) in CD_3OD (see below) and all the vicinal coupling constants could, therefore, be readily extracted and assigned in conjunction with previous conformational work in DMSO-d_6[16] (Scheme 1). Comparing the OHPro residue in **7** versus the newly formed ring in **6b**, reasonable correspondence between vicinal couplings exists between 2-H and 4-H with the C-3 methylene protons. The exact nature of the proline ring pucker may account for the small deviations observed. The coupling constant data do not allow a clear decision to be made in favor of either the 4,5-*cis* or *trans* configuration. In the absence of electronegativity effects upon introduction of a hydroxyl group at C-5, the less than one Hz coupling between 4-H and 5-H in **6b** suggests a *trans* configuration. However, it is well known that electronegativity effects on vicinal ^1H-^1H coupling constants are operative in five-membered rings but are poorly understood.[19~21] In the *cis* configuration, the 4-Hβ/5-Hβ coupling may therefore be reduced from 3.2 Hz in B_2 to <1.0 Hz in **6b**, similar in magnitude to the small 4,5-*trans* coupling in B_2. The moderate NOE observed between 4-H and 5-H and the *W* coupling between 5-H and 3-Hβ[22] lend support for such a proposal. Weak NOE's were also observed for the other *cis* pairs of protons namely 2-Hα/3-Hα and 3-Hβ/4-Hβ, thus suggesting the configuration as 2,4-*trans*-4,5-*cis*-4,5-dihydroxyproline. The strong NOE between 5-H and the C-2 methylene protons of the dimethylmyristate side chain indicated the *trans* configuration for the fatty acid amide bond.

Structure of Pneumocandin B_2

This component was shown to have a molecular weight of 1,032 by FAB-MS (observed $(M+H)^+$ at *m/z* 1,033; $(M+Cs)^+$ at *m/z* 1,165). The empirical formula $C_{50}H_{80}N_8O_{15}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated *m/z* 1,032.5743, found *m/z* 1,032.5805). This corresponds to two oxygens less than for pneumocandin B_0. GC-MS of the TMS derivative of the total acid hydrolysate

disclosed one equivalent each of threonine, 3-hydroxyproline, 4-hydroxyproline, 3-hydroxyglutamic acid, and the 10,12-dimethylmyristate fatty acid. The oxygens must therefore be lost from either the dihydroxyhomotyrosine or dihydroxyornithine residues. ^{13}C NMR comparison of the protonated carbons with B_0 (Table 1) shows that all assignments of residues agree to within 0.2 ppm with the exception of the ornithine residue. These assignments were confirmed from HETCOR data. ^1H-^1H connectivity data from COSY and RELAY experiments confirmed these findings and indicated the presence of ornithine instead of the dihydroxy analog in this component (Table 2). The structure is therefore represented by **7** (Fig. 1). The sequence and amide carbonyl ^{13}C assignments were found to be consistent with long-range ^1H-^{13}C HETCOR data.

Structure of Pneumocandin C_0

This component was difficult to separate from component B_0[5] and it was, therefore, not surprising to find it was isomeric with B_0 with a molecular weight of 1,064 by FAB-MS (observed $(M+Li)^+$ at *m/z* 1,071; $(M+Cs)^+$ at *m/z* 1,197). The empirical formula $C_{50}H_{80}N_8O_{17}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated *m/z* 1,064.5641, found *m/z* 1,064.5585). GC-MS of the TMS derivative of the total acid hydrolysate disclosed the presence of threonine, 3-hydroxyglutamic acid, 4-hydroxyproline, and the 10,12-dimethylmyristate fatty acid. ^{13}C NMR comparison with B_0 confirmed the carbon count of 50 and showed the absence of the 3-hydroxyproline resonances. Instead, the presence of five signals displayed chemical shifts very similar to those of 4-hydroxyproline (Table 1). ^1H-^1H connectivity data from COSY and 1D decoupling experiments confirmed the presence of a second 4-hydroxyproline residue in the molecule (Table 2). Internal comparison of the vicinal coupling constants between the two prolines and with those for components B_2 (**7**) (see Scheme 1) and A_0,[1] suggested the same 2,4-*trans* configuration as depicted in **8** (Fig. 1).

Structure of Pneumocandin A_1

The molecular weight of pneumocandin A_1 was determined to be 1,062 by FAB-MS (observed $(M+Na)^+$ at *m/z* 1,085). The empirical formula $C_{51}H_{82}N_8O_{16}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated *m/z* 1,062.5849, found *m/z* 1,062.5717) which contains one oxygen less than in component A_0.[1] ^{13}C NMR comparison with A_0 confirmed the same carbon count and showed differences only for the dihydroxyhomotyrosine residue with the appearance of a methylene resonance at 42.4 ppm replacing either the C-3 or C-4 signals at 76.9 and 75.8 ppm (Table 1). ^1H-^1H connectivities were determined by COSY, RELAY and 1D decoupling experiments which indicated that the methylene group was located at C-3 (Table 2). The structure can therefore be represented by **2** (Fig. 1). The GC-MS of the TMS derivative of the total acid hydrolysate was found to be identical to that of component A_0[1] as expected.

Structure of Pneumocandin A_2

The molecular weight of pneumocandin A_2 was determined to be 1,046 by FAB-MS (observed $(M+H)^+$ at *m/z* 1,047; $(M+Cs)^+$ at *m/z* 1,179). The empirical formula $C_{51}H_{82}N_8O_{15}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated *m/z* 1,046.5900, found *m/z* 1,046.5787) which contains two oxygens less than in component A_0[1] and one less than in A_1. The GC-MS of the TMS derivative of the total acid hydrolysate was found to be identical to that of component A_0[1] suggesting that modification had occurred in either or both of the dihydroxyornithine and dihydroxyhomotyrosine residues. ^{13}C

NMR comparison with A_0 confirmed the carbon count of 51. Only differences in the assignments for the dihydroxyornithine residue were noted with the appearance of two methylene resonance at 24.3 and 37.9 ppm replacing the C-4 and C-5 methines at 70.6 and 74.0 ppm (Table 1). Noteworthy was the absence of the diagnostic hemiaminal 5-H doublet at δ 5.26 as in component B_2. COSY and RELAY experiments confirmed the presence of the ornithine residue (Table 2) and indicated the structure to be as depicted in **3** (Fig. 1).[13]C assignments of protonated carbons were established by HETCOR, consistent with internal comparisons amongst the various components. Sequence information and ^{13}C amide carbonyl assignments followed from long-range HETCOR data.

Structure of Pneumocandin A_3

The molecular weight of pneumocandin A_3 was determined to be 1,030 by FAB-MS (observed $(M+Na)^+$ at m/z 1,053). The empirical formula $C_{51}H_{82}N_8O_{14}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated m/z 1,030.5950, found m/z 1,030.5829) which contains three oxygens less than in component A_0.[1] The GC-MS of the TMS derivative of the total acid hydrolysate was found to be identical to that of component A_0[1] suggesting modification of both the dihydroxyornithine and dihydroxyhomotyrosine residues. ^{13}C NMR comparison with A_0 confirmed the same carbon count and modification of both the dihydroxyornithine and dihydroxyhomotyrosine residues (Table 1). COSY and RELAY evidence indicated that the α-proton of the DiOHTyr residue was coupled to an ethylene unit indicating that A_3 was the first component to be isolated with the non-hydroxylated homotyrosine residue (Table 2). A noteworthy feature of the ^1H NMR spectrum was the change of the sharp doublet for the DiOHOrn hemiaminal proton at δ 5.26 in A_0 to a broad doublet of doublet multiplet, consistent with a methylene group at C-4. The connectivity data supported this modification (Table 2) as well as substitution of three methines in the 50~80 ppm region of the ^{13}C NMR spectrum with methylene resonances at 30.7, 33.1 and 35.4 ppm (Table 1). This allowed the structure to be represented by **4** (Fig. 1).

Structure of Pneumocandin A_4

The molecular weight of pneumocandin A_4 was determined to be 1,014 by FAB-MS (observed $(M+Na)^+$ at m/z 1,037). The empirical formula $C_{51}H_{82}N_8O_{13}$ was determined by HR-FAB from the $(M+Cs)^+$ ion (calculated m/z 1,014.6001, found m/z 1,014.5915) which contains four oxygen less than in component A_0. The GC-MS of the TMS derivative of the total acid hydrolysate was found to be identical to that of component A_0[1] suggesting modification of both the dihydroxyornithine and dihydroxyhomotyrosine residues. ^{13}C NMR comparison with A_0 confirmed the same carbon count and showed differences for both the dihydroxyornithine and dihydroxyhomotyrosine residues (Table 1). Comparison with A_3 indicated the presence of the nonhydroxylated homotyrosine residue whereas comparison with component A_2 indicated the presence of the ornithine residue. A noteworthy feature of the ^1H NMR spectrum was the absence of the doublet for the DiOHOrn hemiaminal proton at δ 5.26 as in components A_2 and B_2. The connectivity data (COSY, RELAY) (Table 2) as well as substitution of four methines in the 50~80 ppm region of the ^{13}C NMR spectrum with methylene resonances at 24.6, 32.9, 33.9 and 38.3 ppm (Table 1), adequately supported these modifications. The protonated ^{13}C resonances were rigorously assigned on the basis of HETCOR experiments. This allowed the structure to be represented by **5** (Fig. 1).

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