COCHINMICINS, NOVEL AND POTENT CYCLODEPSIPEPTIDE ENDOTHELIN ANTAGONISTS FROM A *Microbispora* sp.

II. STRUCTURE DETERMINATION

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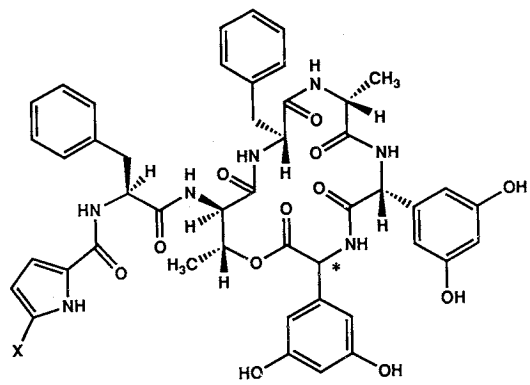
Cochinmicins I, II, and III are competitive endothelin antagonists produced by *Microbispora* sp. ATCC 55140. The cochinmicins are cyclic depsipeptides containing six α-amino acids and a pyrrolecarboxylic acid. Based upon MS, 1D and 2D NMR, and LC data, the structures and absolute stereochemistries of the cochinmicins have been assigned. All three components have the same basic sequence and contain one equivalent each of D-*allo*-threonine, D-alanine, L-phenylalanine, D-phenylalanine, 5-chloropyrrole-2-carboxylic acid (or pyrrole-2-carboxylic acid in cochinmicin I), plus two equivalents of 3,5-dihydroxyphenylglycine (DHPG). The phenylalanine residues were differentiated *via* a methanolysis product which contained only one of the phenylalanine residues. Both DHPG residues have the D configuration in the more active cochinmicins I and III. Cochinmicin II contains both D- and L-DHPG and these residues have been differentiated in the sequence based upon ^{1}H NMR data.

The cochinmicins are closely related cyclic depsipeptides produced by *Microbispora* sp. ATCC 55140. Cochinmicins II (**2**) and III (**3**) are stereoisomeric with each other and cochinmicin I (**1**) is the deschloro analog of **3**. Cochinmicin II appears to be identical to the previously reported compound 55185 RP,[1] whose stereochemistry was not assigned. In the preceding paper,[2] the production, isolation, and physico-chemical and biochemical properties of the cochinmicins have been described. In this paper, we report the structures of **1**, **2** and **3** including absolute stereochemistry.

Fig. 1. Structures of the cochinmicins.



	Cochinmicin	X	*
1	I	H	*R*
2	II	Cl	*S*
3	III	Cl	*R*

Results

The molecular weights of **2** and **3** were both found to be 923 by FAB-MS and their molecular ion clusters suggested the presence of one chlorine in each compound. The MW of **1** was similarly deduced to be 889 with no chlorine present. The molecular formulae were determined to be $C_{46}H_{47}N_7O_{12}$ for **1**, HR-LSIMS *m/z* 890.3380 $(M+H)^+$ (calcd for $C_{46}H_{48}N_7O_{12}$ 890.3361); $C_{46}H_{46}N_7O_{12}Cl$ for **2**, HRFAB-MS *m/z* 924.3005 $(M+H)^+$ (calcd for $C_{46}H_{47}N_7O_{12}Cl$ 924.2971);

and $C_{46}H_{46}N_7O_{12}Cl$ for **3**, HRFAB-MS *m/z* 924.2949 $(M+H)^+$ (calcd for $C_{46}H_{47}N_7O_{12}Cl$ 924.2971).

Analysis of the acid hydrolysates of the cochinmicins by GC-MS as the trimethylsilyl derivatives indicated that each component contained one equivalent of *allo*-threonine and alanine, and two equivalents of phenylalanine and dihydroxyphenylglycine. NMR data (COSY, RELAY, HETCOR, HMQC, HMBC) corroborated these results and allowed further assignment of the dihydroxyphenylglycine residues as 3,5-dihydroxyphenylglycine (DHPG). Additionally, NMR data indicated that **1** contained pyrrole-2-carboxylic acid and **2** and **3** contained 5-chloropyrrole-2-carboxylic acid. In each compound, the sum of the seven subunits minus 7 equivalents of water accounts for the molecular formula and suggests a cyclic structure. Preliminary examination of NMR data suggested that **2** and **3** were stereoisomeric, and that **1** was the deschloro analog of **3**.

NMR Analysis

Most of the structural work was performed on **2** which was the most abundant component. The sequence was established by HMBC (^1H-^{13}C long-range experiment) and 2D NOE studies (Fig. 2). The ^1H NMR proton assignments for **1**, **2**, and **3** are listed in Table 1 and the ^{13}C NMR carbon assignments are listed in Table 2.

FAB-MS-MS

The sequences of **1**~**3** were corroborated by FAB-MS-MS. The major component, **2**, will be used for discussion. Component **2** did not react when briefly treated with acetic anhydride in methanol which indicated that no free amino groups were present. Treatment of **2** with methanolic HCl afforded an increase in mass of 32 amu suggesting methanolysis of a lactone to its corresponding methyl ester, **4**. (Conversion of a free carboxylic acid moiety would have afforded an increase in mass of only 14 amu for each ester formed.) These data are consistent with a depsipeptide structure for **2** involving the hydroxyl group in the *allo*-threonine residue in lactone formation and having the 5-chloropyrrole-2-carboxylic acid residue at the *N*-terminal position.

Fig. 2. ^1H-^{13}C long range correlations of cochinmicin II.

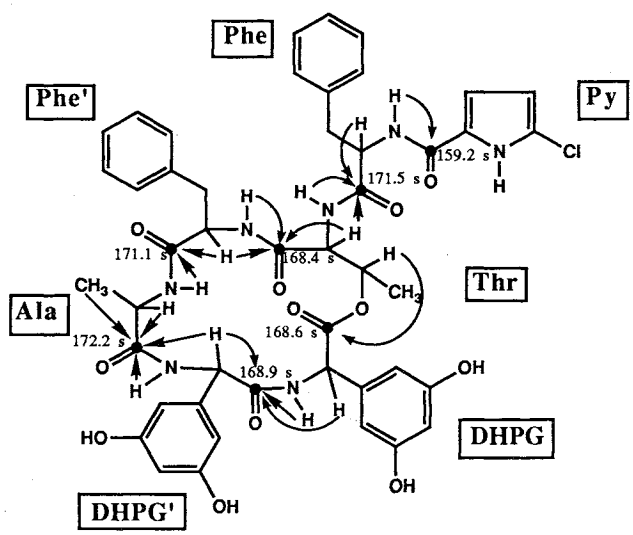


Table 1. ^{1}H NMR assignments of cochinmicins I (**1**), II (**2**) and III (**3**) in DMSO-d_6 at 30°C (400 MHz)[a].

Assignment	1	2	3
	D-Ala	D-Ala	D-Ala
2-H	4.22 dq (J=7.5)	3.94 dq (J=5, 7.5)	4.22 dq (J=7.5)
3-H	1.29 d (3H, J=7.5)	1.32 d (3H, J=7.5)	1.31 d (3H, J=7.5)
NH	7.80 d (J=7.5)	8.17 d (J=5)	7.81 d (J=7.5)
	D-*Allo*-Thr	D-*Allo*-Thr	D-*Allo*-Thr
2-H	4.62 t (J=~9.5)	4.45 t (J=~9.5)	4.62 t (J=~9.5)
3-H	5.18 dq (J=10, 6.5)	4.96 dq (J=10, 6.5)	5.18 dq (J=10, 6.5)
4-H	0.84 d (3H, J=6.5)	1.11 d (3H, J=6.5)	0.85 d (3H, J=6.5)
NH	8.81 d (J=9)	8.60 d (J=~8)	8.82 d (J=9.5)
	L-Phe	L-Phe	L-Phe
2-H	4.71 ddd (J=5, 8.5, 10.5)	4.79 ddd (J=5, 9, 10)	4.72 ddd (J=4.5, 8.5, 10.5)
3-Ha	3.06 dd (J=5, 13.5)	3.02 dd (J=5, 13.5)	3.07 ddd (J=4.5, 13.5)
3-Hb	2.92 dd (J=~10.5, ~14)	2.88 dd (J=10.5, 13.5)	2.89 dd (J=10.5, 13.5)
NH	8.10 d (J=8.5)	8.12 d (J=9)	8.15 d (J=8.5)
2′-H~6′-H	7.1~7.34 m (5H)	~7.15 m (5H)[c]	7.1~7.33 m (5H)
	D-Phe	D-Phe	D-Phe
2-H	4.33 dt (J=5.5, 8)	4.50 dt (J=6, 9)	4.33 dt (J=5.5, 8)
3-Ha	2.99 dd (J=~8, ~14)	3.04 dd (J=6, 13.5)	2.98 dd (J=8, 13.5)
3-Hb	2.90 dd (J=5.5, ~14)	2.94 dd (J=9, 13.5)	2.89 dd (J=5.5, 13.5)
NH	7.80 d (J=7.5)	8.62 d (J=~8.5)	7.79 d (J=8)
2′-H~6′-H	7.1~7.34 m (5H)	7.29 m (2H)[c], 7.22 m (2H)[c], 7.04 m (1H)[c]	7.1~7.33 m (5H)
	D-DHPG	L-DHPG	D-DHPG
2-H	5.22 d (J=8)	5.44 d (J=9)	5.23 d (J=~8)
NH	7.94 d (J=8)	7.78 d (J=9)	7.96 d (J=8)
2′-H/6′-H	6.07 s (2H, J=2)[b]	6.23 d (2H, J=2)	6.08 d (2H, J=2)[d]
4′-H	6.11 t (J=2)[b]	6.15 t (J=2)	6.12 t (J=2)[d]
	D-DHPG	D-DHPG	D-DHPG
2-H	5.22 d (J=~10)	5.25 d (J=8)	5.23 d (J=~10)
NH	8.39 d (J=9.5)	8.27 d (J=8)	8.39 d (J=10)
2′-H/6′-H	6.02 d (2H, J=2)[b]	6.17 d (2H, J=2)	6.03 d (2H, J=2)[d]
4′-H	6.09 t (J=2)[b]	6.20 t (J=2)	6.10 t (J=2)[d]
	Pyrrole	Cl-Pyrrole	Cl-Pyrrole
3-H	6.80 m	6.87 d (J=4)	6.87 d (J=4)
4-H	6.05 m	6.04 d (J=4)	6.02 d (J=4)
5-H	6.87 m	—	—
NH	11.32 br m	12.16 br s	~12.15 br s

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiple; br, broad.

[a] Coupling constants (±0.2 Hz) and proton intensities are given in parentheses. Except where otherwise noted, all chemical shift resonances correspond to one proton intensity and are given using the solvent peak at δ 2.49 as internal standard.

[b~d] Assignments may be interchanged.

FAB-MS-MS afforded strong spectra containing significant sequence information for both the naturally occurring depsipeptide and its simple methanolysis product, **4**. The linearized methyl ester derivatives obtained by mild methanolysis were used for MS-MS analysis to avoid potential ambiguities associated with the direct analysis of cyclic peptides. All the primary *C*-terminal fragmentation ions (Y series) were observed and those which contained the threonine residue exhibited Y-18 ions (Fig. 3, **4**). Only two of the B-series ions at m/z 197 and 361 were observed.

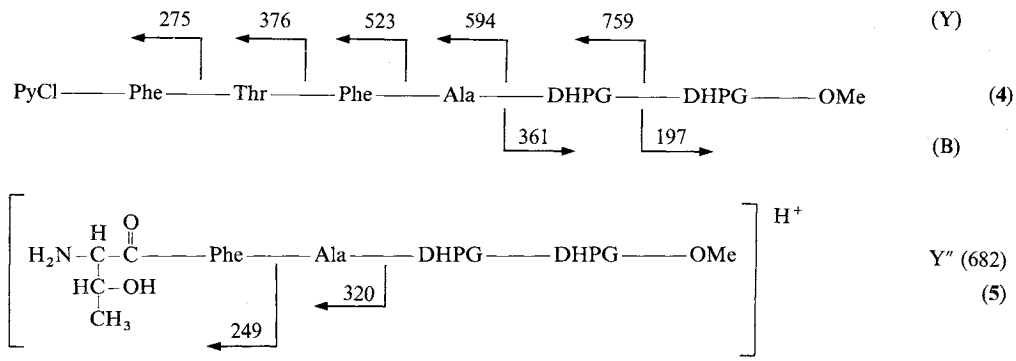
A facile Y″ cleavage at the *allo*-threonine α-amine position affords a pseudo molecular ion at m/z 682 which exhibits its own Y series ions. These ions include the loss of water to give m/z 664 (base peak) and the double elimination ions at m/z 249 (231, 249−18) and 320 (302, 320−18).

Table 2. ^{13}C NMR data for cochinmicins II (**2**) and III (**3**) in DMSO-d_6 at 30°C (100 MHz)[a].

Carbon No.	2	3	Carbon No.	2	3
	D-Ala	D-Ala		D-Phe	D-Phe
C-1	172.2 s	172.0 s	C-2′/C-6′	129.2 d[b]	129.1 d[f]
C-2	51.8 d	50.5 d	C-3′/C-5′	128.1 d[c]	128.3 d[g]
C-3	16.9 q	17.7 q	C-4′	126.3 d[d]	126.3 d[h]
	D-*Allo*-Thr	D-*Allo*-Thr		L-DHPG	D-DHPG
C-1	168.4 s	167.0 s	C-1	168.6 s	168.4 s
C-2	56.2 d	54.2 d	C-2	55.8 d	56.8 d
C-3	70.6 d	69.0 d	C-1′	138.3 s	138.8 s
C-4	16.6 q	15.7 q	C-2′/C-6′	106.6 d	104.2 d
	L-Phe	L-Phe	C-3′/C-5′	158.0 s[e]	158.3 s
C-1	171.5 s	171.7 s	C-4′	102.2 d	102.2 d
C-2	53.8 d	54.2 d		D-DHPG	D-DHPG
C-3	38.0 t	38.5 t	C-1	168.9 s	168.6 s
C-1′	136.9 s	136.8 s	C-2	56.2 d	56.8 d
C-2′/C-6′	129.0 d[b]	129.1 d[f]	C-1′	139.6 s	139.4 s
C-3′/C-5′	128.0 d[c]	128.0 d[g]	C-2′/C-6′	106.8 d	104.7 d
C-4′	126.2 d[d]	126.2 d[h]	C-3′/C-5′	158.2 s[e]	158.3 s
	D-Phe	D-Phe	C-4′	101.9 d	101.7 d
C-1	171.1 s	170.4 s		Pyrrole	Pyrrole
C-2	54.9 d	54.2 d	C-1	159.2 s	159.2 s
C-3	36.8 t	37.3 t	C-2	117.2 s	117.3 s
C-1′	137.8 s	137.8 s	C-3	111.8 d	111.7 d
			C-4	107.0 d	107.0 d
			C-5	125.7 s	125.6 d

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.
[a] Chemical shifts in ppm downfield of TMS.
[b~h] Assignments may be interchanged.

Fig. 3. MS-MS fragmentation data for **4** and **5**.



The MS-MS spectra for **2** and **3** were identical indicating the same sequence for both compounds. Component **1** exhibited the same basic sequence ions as **2** and **3** with those containing the pyrrole-2-carboxylic acid moiety appearing at 34 amu less (deschloro) than in **2** and **3**.

Absolute Stereochemistry

Amino acid enantiomers were differentiated by LC retention using the α-methylbenzyl isothiocyanate (AMBI) method[3] and the Marfey method.[4] The AMBI method afforded a useful derivative of the

Table 3. Approximate quantitation of AMBI derivatives.

Compound	D-*allo*-Thr	D-Ala	L-DHPG	D-DHPG	L-Phe	D-Phe
Standard	10.5	11.0	12.0	13.0	20.0	20.3
1	10.6 (0.9)	11.0 (1.0)*	11.9 (0.3)	13.0 (1.8)	20.0 (1.3)	20.3 (1.4)
2	10.7 (0.8)	11.1 (1.0)	12.0 (1.0)	13.0 (1.3)	20.1 (1.4)	20.3 (1.3)
3	10.8 (0.8)	11.2 (1.0)	12.1 (0.4)	13.2 (1.7)	20.1 (1.6)	20.4 (1.6)

Retention times are given in minutes.

* The values in parentheses are relative quantitations standardized to D-Ala = 1.0.

DHPG moieties, gave sharper peaks in general, and was cleaner, chromatographically, than the Marfey's derivative. It was, however, necessary to use Marfey's derivative for the quantitation of the D- and L-phenylalanine residues since it afforded better separation of these two isomers.

Using both methods it was difficult to differentiate all four isomers for *allo*-threonine and threonine. However, the mass spectra for the trimethylsilyl derivatives of threonine and *allo*-threonine differ clearly in the ratio of the 218/219 ions. MS allowed the unequivocal assignment of *allo*-threonine in these compounds thus simplifying the problem.

Relative quantitation and retention times for the six peaks observed in each case are listed in Table 3. The traces for **1** and **3** appear quite similar and contain two equivalents of D-DHPG. Compound **2** differs in that it contains approximately one equivalent of each enantiomer of DHPG. By $^{1}H/^{13}C$ NMR **2** is stereoisomeric with **3**, and **1** has the same stereochemistry as **3**. The DHPG residues have been differentiated in the sequence of **2** based upon ^{1}H NMR data. There is a major variation in the chemical shift of the D-*allo*-threonine C-4 methyl resonance between **2** and **3** whereas the D-alanine C-3 methyl resonance is similar in **2** and **3**. This suggests that the DHPG residue adjacent to the *allo*-threonine residue differs in stereochemistry in **2** and **3**.

To differentiate the two phenylalanine residues, **2** was subjected to more vigorous methanolysis and the mixture was monitored by FAB-MS. Among other components, the fragment *allo*-threonine-phenylalanine-alanine-DHPG-DHPG-methyl ester (**5**) was obtained. This same sequence was observed as a facile cleavage ion in the FAB-MS spectrum of the parent mild methanolysis product. The FAB-MS-MS spectrum of **5** exhibited the fragment ions expected based upon its sequence (Fig. 3, **5**). AMBI analysis of the acid hydrolysate of this peptide fragment isolated from methanolysis of both **2** and **3** disclosed that D-phenylalanine was present, thereby locating the D-phenylalanine residue in the position between *allo*-threonine and alanine. Insufficient amounts of **1** were available for direct determination in a similar manner. The phenylalanine residues are presumed to be identical in **1** and **3** due to the equivalence of their NMR spectra.

Early attempts to use enzymatic methods on the cochinmicin components failed which, in hindsight, is not too surprising due to the preponderance of D-amino acid residues, the blocked *N*-terminus, and the unusual DHPG residues at the *C*-terminus.

Experimental

MS data were obtained on a Finnigan-MAT TSQ 70. For FAB-MS-MS experiments, positive ion daughter spectra of the protonated molecules were recorded using Ar as the collision gas. For GC-MS analyses samples were hydrolyzed in 6N HCl at 110°C for 3 hours. The hydrolysate residue was

derivatized with bis(trimethylsilyl)trifluoroacetamide - pyridine, 1 : 1 (v/v) 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. High resolution LSIMS-Cs data were obtained on a JEOL HX110 at 10 kV using Ultramark 1621 as the internal standard. The ^{1}H NMR spectra were recorded at 400 MHz in DMSO-d_6 on a Varian XL400 NMR spectrometer. The ^{13}C NMR spectra were recorded on the same instrument in DMSO-d_6 and a Bruker AM-400 instrument both at 100 MHz. The HMBC experiment was optimized for 8 Hz. The 2D-NOESY experiment was recorded with a repetition rate of 3 seconds and mixing time of 0.5 second.

Amino acid stereochemistry was determined by the α-methylbenzyl isothiocyanate (AMBI)[3] and Marfey[4] methods. In both cases, hydrolysates were prepared as above. The AMBI derivatives were prepared as follows. Approximately 10 μg of sample was dried under vacuum. Residual amounts of water were removed by the addition of 10 μl of drying reagent (2 : 2 : 1 (v/v/v) water - ethanol - triethylamine) which was then removed under vacuum. The AMBI derivative was then formed by adding 20 μl of fresh derivatizing reagent (7 : 1 : 1 : 1 (v/v/v/v) ethanol - water - triethylamine - α-methylbenzyl isothiocyanate) and then allowing it to react at room temperature for 20 minutes in a closed vial. The reaction was stopped and the derivatizing reagents removed under vacuum for 60 minutes. The AMBI derivatives were separated on an ABI 130-A HPLC system with a Spheri-5RP-18 220 × 2.1 mm column and detected at 254 nm. The mobile phases consisted of; A, 60% ammonium acetate buffer (0.14 M, with 0.05% triethylamine, adjusted to pH 6.40 with glacial acetic acid) and 40% acetonitrile, B 60% acetonitrile and 40% water. The following gradient was used; 10% B for 4 minutes, 15% B at 5 minutes, 40% B at 30 minutes, 100% B at 35 minutes hold for 5 minutes at a flow rate of 250 μl/minutes and an oven temperature of 26°C. The Marfey's derivatives were separated using two Waters 510 pumps with an automated gradient controller and a 10 cm Brownlee OD-300 (C-18) 7 micron column with detection at 340 nm. The mobile phases consisted of A; 90% 50 mM triethylammonium phosphate pH 3.0 and 10% dichloromethane, B; 100% dichloromethane. A 40 minutes gradient from 0% B to 40% B at a flow rate of 1 ml/minutes was employed.

The methanolysis product was formed by adding 3 N methanolic HCl to the sample and heating at 50°C for 1 hour (**4**) or 18 hours (**5**).

Acknowledgments

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