CHLOROCARDICIN, A MONOCYCLIC β-LACTAM FROM A *STREPTOMYCES* SP.

II. ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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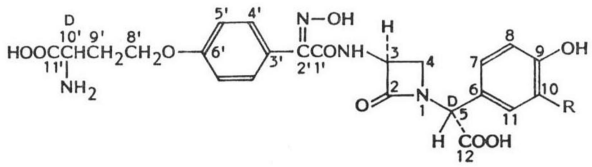
Chlorocardicin, a novel monocyclic β-lactam, was isolated from the fermentation broth of a *Streptomyces* sp. by the use of non-ionic porous resin and reverse phase chromatography. This chlorine-containing antibiotic is structurally related to nocardicin A. Its physico-chemical characteristics and detailed NMR analysis are described.

In the course of an investigation directed toward the discovery of cell-wall active antibiotics, a novel monocyclic β-lactam antibiotic related to nocardicin A (**1**) was isolated. A description of the producing organism and the fermentation conditions used are reported in the preceding paper[1]. The present paper describes the isolation and chemical characterization of this new product, chlorocardicin (**2**).



1 Nocardicin A R=H
2 Chlorocardicin R=Cl

Analytical and Isolation Procedures

Analytical Method

The titers of chlorocardicin in fermentation broths and various fractions obtained during the purification procedure were best determined by high performance liquid chromatography (HPLC). A preclean-up of the broth samples was needed before injecting onto the HPLC. This was achieved by acidification of 5 ml of clarified broth to pH 3 with phosphoric acid and passage onto a Water's Sep-Pak (C_{18}), followed by washing with 2×5 ml of phosphate buffer (0.01 M, pH 3) and then elution with 5 ml of a 1:1 mixture of methanol - water. Appropriate dilutions were made and samples injected onto the HPLC. These assays were performed using a Beckman Model 345/165 HPLC instrument equipped with an Altex Ultrasphere-ODS (5 μm) column (4.6 mm ID×15 cm) with UV detection at 275 nm. The mobile phase was a gradient of acetonitrile and 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/minute (15% acetonitrile for 5 minutes followed by a steep gradient to 25% acetonitrile

in one minute, held at 25% for 10 minutes and then returned to initial conditions).

Isolation and Purification

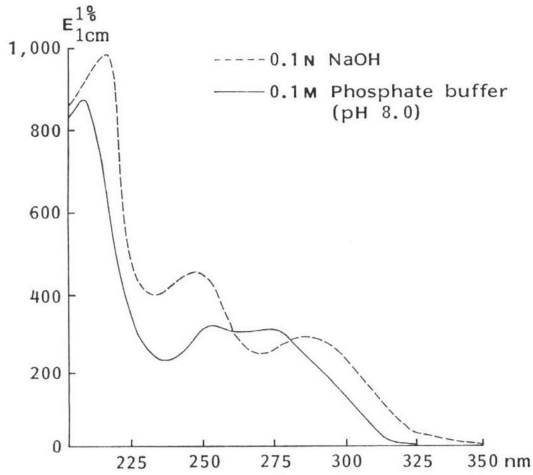
Fermentation broth from a 10-liter fermentor was filtered using 5% w/v Hyflo Supercel (Johns-Manville Corp.). The clarified broth (pH 8.3) which contained 40 μg/ml of chlorocardicin was adjusted to pH 4.0 with 1 N HCl and applied to Diaion HP-20 resin column (6.5×50 cm, Mitsubishi Corp.). The column was washed with 20 liters of deionized water and then eluted with 21 liters of 30% aqueous methanol, the active fractions were pooled and concentrated *in vacuo* on a rising film evaporator. The resulting concentrates were lyophilized to give 7.0 g of crude product which contained 220 mg of chlorocardicin (100% recovery). The crude lyophilized powder was dissolved in water and applied to a Whatman Partisil 40 ODS-3 reverse-phase column (2.5×50 cm). The column was washed with water and the active components were eluted using 5% aqueous methanol. The bioactive aqueous methanol fractions were concentrated *in vacuo* and then lyophilized to give 435 mg of crude powder (25% pure, 47% recovery). The crude powder was recrystallized from acidic water (pH 3) to give 84 mg of pure compound (77% recovery).

Physico-Chemical Properties

Chlorocardicin is a colorless powder which melts at 221~224°C. The sodium salt is freely soluble in water and has a specific rotation of $[\alpha]_D^{24}$ −135.7° (*c* 1.0, H_2O). Its molecular formula was determined to be $C_{23}H_{23}ClN_4O_9$ by high resolution FAB-MS in the positive ion mode. It gave a UV spectrum with absorption maxima at 210, 254 and 274 nm in pH 8 phosphate buffer; in 0.1 N NaOH the absorption maxima shifted to 223, 245 and 289 nm (Fig. 1). The IR spectrum of the sodium salt in KBr showed a β-lactam carbonyl at 1720 cm^{-1} and overall was very similar to the spectrum of nocardicin A run under similar conditions (Fig. 2). These properties are compared with nocardicin A and summarized in Table 1.

Anal Calcd for $C_{23}H_{23}ClN_4O_9$: C 51.64, H 4.30, N 10.48, Cl 6.64.
Found: C 51.69, H 4.54, N 10.30, Cl 6.78.
High resolution FAB-MS (MH^+) Calcd for $C_{23}H_{24}ClN_4O_9$: 535.123
Found: 535.125

Fig. 1. UV spectrum of chlorocardicin.



Structure Elucidation of Chlorocardicin

The physico-chemical characteristics of chlorocardicin are closely related to nocardicin A except for the presence of chlorine as described in Table 1. The presence of chlorine was first detected in the FAB-MS which exhibited an isotopic cluster both in the positive mode and negative mode for the molecular ion characteristic of a single chlorine. This was further confirmed by high resolution FAB-MS and elemental analysis.

Analysis of the ^1H NMR and ^{13}C NMR spectra of chlorocardicin also indicated close resemblances to those of nocardicin A[2] except for the proton and carbon resonances due to their phenolic moieties as shown in Tables 2 and 3. It is worth noting that, in accord with TOWNSEND

Fig. 2. IR spectrum of chlorocardicin (KBr pellet).

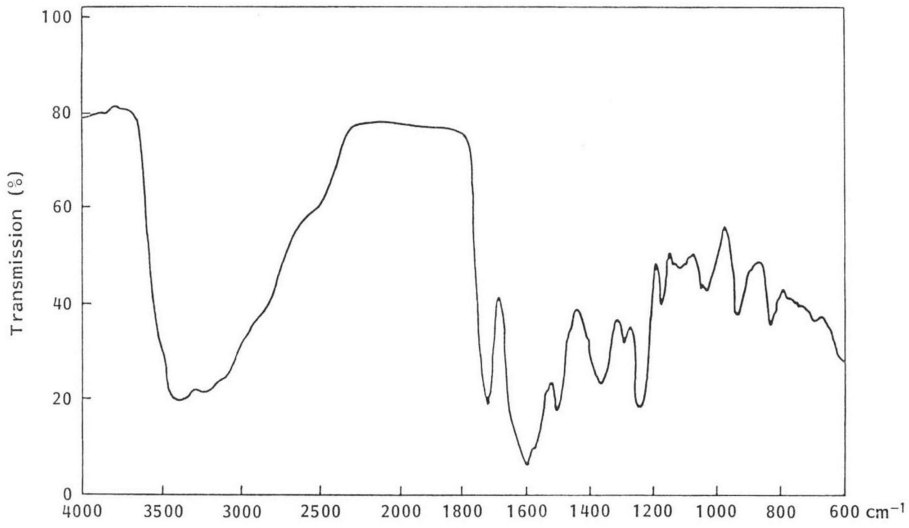


Table 1. Physico-chemical characteristics of chlorocardicin and nocardicin A.

	Chlorocardicin	Nocardicin A[2]
Molecular formula	$C_{23}H_{23}ClN_4O_9$ (FAB-MS)	$C_{23}H_{24}N_4O_9$ (Elemental Analysis)
$[\alpha]_D^{24}$ (c 1.0, H_2O)	−135.7°	−135.0°
CD (H_2O) Extrema [θ]	205 nm (-5.0×10^4), 225 nm (-3.5×10^4), 275 nm ($-1.7\times\times10^4$)	200 nm (-7.8×10^4), 220 nm (-3.6×10^4), 275 nm (-0.5×10^4)
UV absorption max (nm) ($E_{1cm}^{1\%}$)	210 (727), 254 (294), 274 (304) in phosphate buffer (pH 8) 223 (417), 245 (415), 289 (277) in 0.1 N NaOH	220 (sh), 272 (310) in phosphate buffer (pH 8) 244 (460), 283 (270) in 0.1 N NaOH
MP	222~224°C	214~216°C (dec)
IR (KBr), sodium salts	1720 cm^{-1} for β-lactam carbonyl	1715 cm^{-1} for β-lactam carbonyl
TLC*		
Analtech Avicel (BuOH - AcOH - H_2O, 4:1:2)	Rf 0.55	Rf 0.45
Analtech Silica Gel F (0.2 M NH_4Cl (pH 5) - MeOH, 5:95)	Rf 0.50	Rf 0.45
HPLC	9.2 minutes	4.8 minutes

* Detected by UV, ninhydrin and bioautography against *E. coli* KN102 using 1/4 MIC of D-cycloserine in the test agar.

et al.[3], the assignments of H-4α and H-10′ due to HASHIMOTO[2] in nocardicin A should be reversed, to δ 3.72 and δ 3.97, respectively. We have confirmed these assignments in chlorocardicin *via* spin decoupling, and carbon-hydrogen coupling correlation of H-10′ with C-10′ (*vide infra*).

Substitution Pattern in the Aromatic Rings

The aromatic proton signals were assigned based on integration and spin-decoupling. Although the H-8 and H-5′ signals overlap at δ 6.85, the decoupling experiments readily permit them to be distinguished. The shifts are tabulated in Table 2. The aromatic signals are divided between two

Table 2. Proton assignments of chlorocardicin and nocardicin A.

H	Chlorocardicin (Na salt)*			Nocardicin A (Na salt)[2]		
	ppm	Multiplicity	J(Hz) (Apparent first order)	ppm	Multiplicity	J(Hz) (Apparent first order)
4′	7.37(2H)	d	8.8	7.42 (2H)	d	9.0
11	7.28	d	2.1	Equivalent to H7		
7	7.06	dd	2.1, 8.2	7.23 (2H)	d	9.0
8	6.92	d	8.2	6.91 (2H)	d	9.0
10	—	—	—	Equivalent to H8	—	—
5′	6.90(2H)	d	8.8	6.99 (2H)	d	9.0
5	5.24	s	—	5.33	s	—
3	4.96	dd	2.4, 5.2	5.01	dd	5.0, 2.0
8′	4.16(2H)	br t	second order	4.22	t	6.0
10′	3.93	dd	7.6, 4.6	3.81**	dd	6.0, 5.0
4α	3.72	dd	second order	3.97**	t	6.0
4β	2.99	dd	6.1, 2.4	3.14	dd	6.0, 2.0
9′	2.35(2H)	complex	second order	2.39	m	—

Solvent: D_2O.

* Bruker WM 360. HOD at 4.8 ppm as reference. HOD suppressed by pre-irradiation.

** Assignment between H-4α and H-10′ should be reversed (see text).

Table 3. Carbon assignments of chlorocardicin and nocardicin A.

C	Chlorocardicin* (Na salt) (ppm)	Nocardicin A[2] (Na salt) (ppm)
9′	30.60	30.63
4	46.87	47.02
10′	54.12	54.17
5	54.97	54.90
3	61.07	61.58
8′	65.98	66.01
5′	121.48	115.88
8	118.14	116.54
10	121.48	Equivalent to 8C
3′	123.91	123.95
6	127.73	127.46
4′	128.65	128.68
11	129.20	Equivalent to 7C
7	130.92	131.04
9	153.66	156.41
2′	153.77	153.74
6′	160.51	160.53
1′	166.84	166.84
2	168.51	168.54
11′	174.85	174.73
12	176.02	176.61

Solvent: H_2O.

* Bruker WM 360.

rings, one *para*-disubstituted (AM) and one 1,2,4-trisubstituted (AMX) spin pattern. The latter corresponds to the chlorophenol ring, whose subspectrum is given schematically in Fig. 3. There are six possible substitution patterns for the 1,2,4-trisubstituted chlorophenol moiety (Fig. 4). Since the "*meta*-only" coupled proton is furthest downfield, only structures **A** and **B** need be considered, because in the other four possible arrangements of substituents, the "*meta*-coupled" spin would be proximal to oxygen, and expected to be further upfield. Even though the "*ortho*-coupled" proton is furthest upfield, the proton patterns of **A** and **B** would not be expected to be dissimilar. The similar chemical shifts of H-5 in chlorocardicin and nocardicin A suggest that **A** is the correct pattern. However, conclusive proof of the substitution pattern rests on the long-range proton couplings in the ^{13}C NMR spectrum.

The ^{13}C "GASPE" edited spectrum[4] confirms that in the chlorophenol ring there is only one protonated carbon (thus, one proton) adjacent to oxygen. This does not distinguish **A** from **B**, indeed we find that a crude prediction of ^{13}C shifts based on substituent contributions does not argue in favor of either, due to the similar effects on other ring carbons shifts by both chlorine and the aminoacyl group* (Fig. 5).

* The substituent effects on carbon due to the aminoacyl group were extracted by comparison of shifts in nocardicin A with those in phenol. Chlorine substituent effects are those tabulated from G. LEVY (see Ref 5).

Fig. 3. Subspectrum of the chlorophenol moiety.

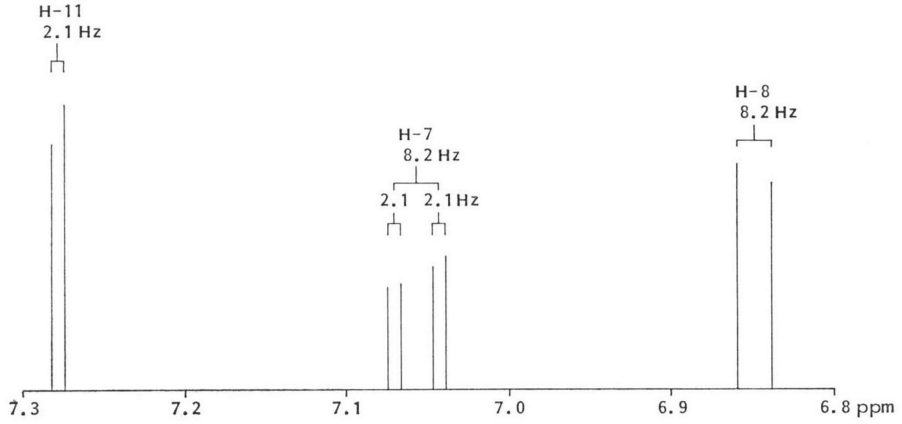
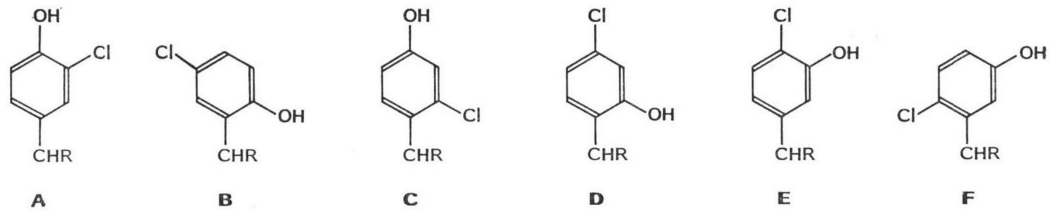
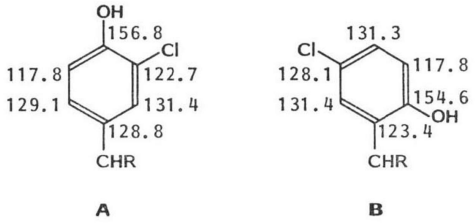


Fig. 4. Six possible substitution patterns of the chlorophenol moiety.



We see that the quarternary carbons at δ 121.5 and δ 127.7 would facilitate distinguishing **A** from **B**, that is, if the higher field (121.5) of these bears chlorine, the structure is **A**, if it bears CHα (H-5), **B** is the structure. To make these assignments, we performed a "COLOC" (Correlation of long-range $^{13}C/^{1}H$ couplings)[6] 2D NMR experiment. This experiment maps out the connectivities of ^{13}C shifts to the shifts of protons to which those carbons are long-range (3~7 Hz) coupled. This method suppresses proton-bearing carbons. We found that the δ 121.5 carbon is coupled to the upfield H-8 (*ortho*-only at 6.85) and less strongly to the downfield H-11 (*meta*-only at δ 7.28) aromatic protons. Similarly the δ 127.7 carbon is coupled to the H-5 (benzylic proton, at δ 5.22) and to the upfield H-8 (δ 6.85) aromatic doublet. These two sets of observations clearly argue for **A** as the correct substitution pattern.

Fig. 5. Predicted ^{13}C shifts of structure **A** and **B**.



The ^{1}H NMR spectrum of chlorocardicin in DMSO-d_6 showed an amide proton at 9.15 ppm (d, J=8 Hz) which is identical with that of nocardicin A therefore establishing the stereochemistry of the oxime group to be syn-*Z* to the acylamino group. This is in contrast to nocardicin B, an *anti* (*E*)-isomer which showed an amide proton at 8.81 ppm[2]. The absolute stereochemistry of the chiral centers in chlorocardicin were assigned to be identical to those of nocardicin A based on specific optical rotation and CD spectra (see Table 1).

In conclusion, we propose structure **2** for chlorocardicin, a novel monocyclic β-lactam antibiotic

related to nocardicin A, as determined by its physico-chemical characteristics and detailed NMR analysis. This compound is different from the only other reported chlorine-containing nocardicin, a synthetic isomer with chlorine substitution in the 5′ carbon position reported in a Fujisawa patent[7].

Acknowledgments

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