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

I. DISCOVERY, PRODUCTION AND BIOLOGICAL ACTIVITIES

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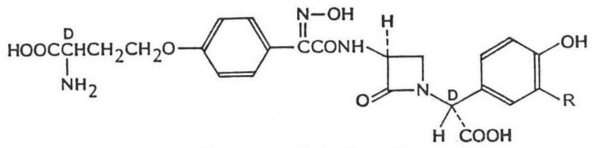
Chlorocardicin is a new monocyclic β-lactam produced by a *Streptomyces* sp. It is structurally related to nocardicin A but differs in having a *m*-chloro substituent on the *p*-hydroxyphenylglycine unit. The biological activity of chlorocardicin was similar to nocardicin A but the former showed less antagonism in complex media. Moderate *in vitro* activity was observed against Enterobacteriaceae and *Pseudomonas aeruginosa*. Chlorocardicin showed low activity against *Staphylococcus aureus* whereas nocardicin A was inactive. Both compounds were shown to be strongly potentiated by antibiotics that inhibit peptidoglycan biosynthesis and were antagonized by selected L- and D-amino acids.

Antibacterial monocyclic β-lactams were first described by AOKI *et al.*[1]. They discovered nocardicins A~G[2] as products of *Nocardia uniformis* subsp. *tsuyamanensis* ATCC 21806. Subsequently the sulfazecins[3], or monobactams[4], were reported from Gram-negative bacteria. A feature of these antibiotics is the preferential inhibition of clinically important Gram-negative bacteria, including *Pseudomonas* sp. and *Proteus* sp. We report the first discovery of a naturally occurring halogenated monocyclic β-lactam closely related to nocardicin A (Fig. 1).

Fig. 1. Structure of chlorocardicin and nocardicin A.



Chlorocardicin R=Cl
Nocardicin A R=H

Materials and Methods

Reference Antibiotics

Nocardicin A was supplied by Fujisawa Pharm., Japan and D-cycloserine was obtained from Calbiochem.

Bacteria

The strains used in sensitivity testing were recently obtained clinical isolates from the SK&F clinical collection.

Determination of Minimum Inhibitory Concentrations (MIC's)

The MIC was determined by the microtiter serial dilution method. The defined medium was M-9

broth with vitamin B_1 supplement and the complex medium was Mueller-Hinton broth.

Amino Acid Antagonism Tests

Antagonism was determined by incorporating 200 μg of the amino acid in dry discs containing 10 μg, 5 μg or 2.5 μg of the β-lactams. The discs were placed on medium M-9 agar plates (20 ml/15 cm plate) seeded with 10^5 cells of *Escherichia coli* K802N per ml of agar. The discs containing amino acids were compared with control discs impregnated with β-lactam only.

Studies of Antibiotic Interactions

MIC of single antibiotics and combinations of antibiotics were determined against *E. coli* K802N (10^5/ml) by the microtiter serial dilution method using M-9 broth with vitamin B_1 supplement. Combinations of antibiotics were prepared by the microtiter cross-dilution procedure and the MIC of combinations was determined as for individual antibiotics. The fractional inhibitory concentration (FIC) is the concentration of one antibiotic in the combination at the MIC of the combination divided by the MIC of the antibiotic alone[8]. Fractional inhibitory concentration index (FICI)=FIC of D-cycloserine of β-lactam[8]. An FICI of 1 indicates an additive effect; >1 indicates antagonism; <1 indicates synergy; ≤ 0.5 indicates marked synergy.

Results

Discovery of Chlorocardicin

The producing organism, a *Streptomyces* sp. (SK&F-AAH-873), was isolated from a soil sample collected from the root zone of a cactus in Pima County, Arizona. Whole-cell hydrolysates of AAH-873 contain L-diaminopimelic acid while whole-cell hydrolysates of the nocardicin A producer, *N. uniformis* subsp. *tsuyamanensis* (ATCC 21806) contain *meso*-diaminopimelic acid with galactose, glucose, mannose, ribose and rhamnose. The nocardicin A producer, having motile spores and type C whole-cell sugar pattern[5], would be more correctly placed in the genus *Actinosynnema*[6]. Activity was detected in microbial broth cultures using a variety of tests developed to indicate "cell wall active" antibiotics. These were based on morphological effects on *Bacillus subtilis* ATCC 6633 and potentiation by other antibiotics.

Production of Chlorocardicin

The first seed culture was prepared by inoculating the growth from an agar slant (14 days, 28°C) of AAH-873 into 500 ml of medium 13H (Table 1) in a 4-liter aspirator bottle. This was incubated at 28°C for 4 days on a rotary shaker (New Brunswick Model G53) at 250 rpm and 5-cm throw and then transferred to a 14-liter fermentor (New Brunswick Model 19) containing 10 liters of the same medium. The second seed was grown at 28°C for 3 days with aeration and agitation at 4 liters/minute and 400 rpm respectively and transferred (10% v/v) into 80 liters of medium 19 (Table 1) in a 130-liter fermentor (New Brunswick Fermacell Model F130). The production process was carried out at 28°C, aerated at 40 liters/minute and agitated at 250 rpm.

The biomass (packed cell volume, % v/v), pH and antibiotic production (μg/ml) were monitored daily (Fig. 2) and antibiotic concentration was assayed using a HPLC procedure[7]. The maximum volumetric titer of chlorocardicin, of 165 μg/ml, was obtained after 6.5 days in the production medium. Production of chlorocardicin began only after the first 18 hours, by which time the growth rate of the strain was markedly decreased. The production phase continued during the period of slow growth and throughout the stationary phase.

Antimicrobial Activity of Chlorocardicin

Chlorocardicin caused morphological alterations of *Bacillus subtilis* at concentrations much lower than nocardicin A. At 30 μg/disc, chlorocardicin largely produced spheroplasts with some filaments, indicating activity against cell wall synthesis (Fig. 3). The same concentration of nocardicin A gave filaments with a few spheroplasts.

The antibacterial spectrum of chlorocardicin was similar to nocardicin A but the former was generally more active in complex media. Chlorocardicin showed moderate activity against a wide range of Gram-negative bacteria. Low activity was observed against *Staphylococcus aureus* whereas nocardicin A was inactive (Table 2).

Table 1. Media used for production of chlorocardicin.

Seed medium 13H (g/liter)		Fermentation medium 19 (g/liter)	
Starch	15.0	Soy-peptone	20.0
Sucrose	5.0	Cerelose	30.0
Dextrose	5.0	Starch	10.0
Corn steep liquor	5.0	$CaCO_3$	1.0
HY-SOY	7.5	$CoCl_2$	0.001
K_2HPO_4	1.5	pH 7.0	
NaCl	0.5		
$CaCO_3$	1.5		
Mineral "S"	5.0		
pH 7.0			
Mineral "S"			
$ZnSO_4 \cdot 7H_2O$	2.8	$CoCl_2 \cdot 6H_2O$	0.1
$Fe(NH_4)_2HC_6H_5O_7$	2.7	$NaB_4O_7 \cdot H_2O$	0.09
$CuSO_4 \cdot 5H_2O$	0.125	$Na_2MoO_4 \cdot 2H_2O$	0.05
$MnSO_4 \cdot H_2O$	1.0		

No *in vivo* activity was observed at 50 mg/kg with chlorocardicin or nocardicin A against infections with *E. coli* 12140 in mice.

Fig. 2. Fermentation profile of chlorocardicin.
● Chlorocardicin, △ biomass, ■ pH.

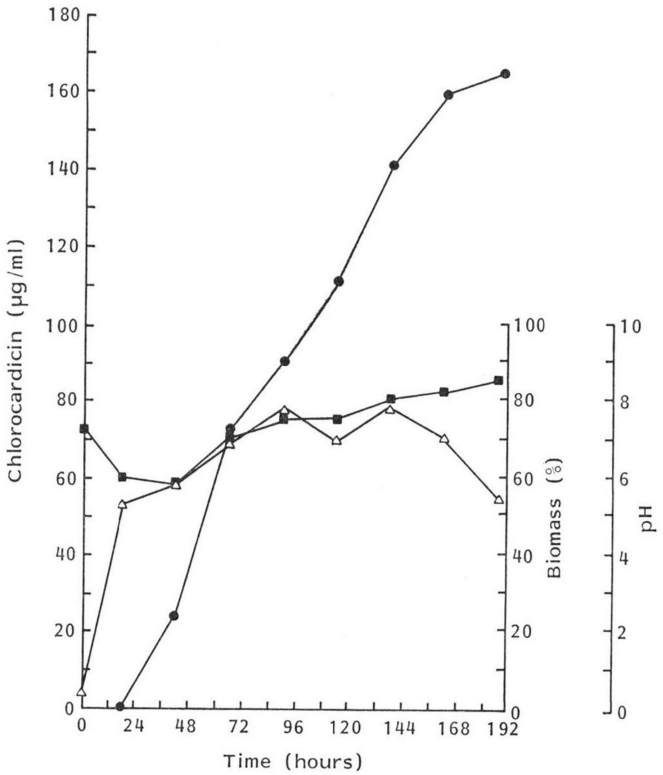


Fig. 3. Morphological effects of chlorocardicin on *B. subtilis* ATCC 6633.

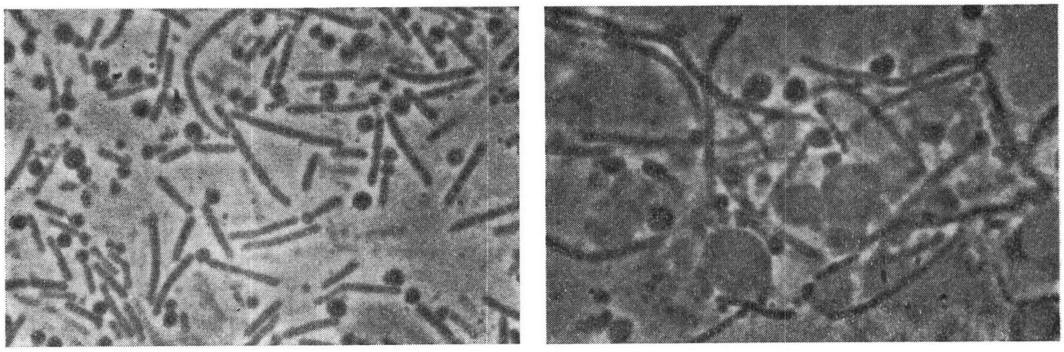


Table 2. Antimicrobial spectrum of chlorocardicin.

Test microorganism	MIC (μg/ml)			
	Chlorocardicin		Nocardicin A	
	Defined medium	Complex medium	Defined medium	Complex medium
Escherichia coli K802N	3.1	50	12.5	100
E. coli 2631	12.5	200	6.3	200
E. coli 12140	12.5	50	12.5	200
Klebsiella pneumoniae 4200	12.5	25	1.6	200
Salmonella gallinarum 595		50		100
Enterobacter cloacae 3125	200	200	200	200
Serratia marcescens ATCC 13880	6.3	50	6.3	50
Proteus mirabilis PM-444		25		25
Morganella morganii 179		100		200
Providenicia sp. PR276	3.1	50	1.6	25
Pseudomonas aeruginosa HH63	12.5	200	6.3	>200
Staphylococcus aureus 910		200		>200
S. aureus HH127		100		>200

Amino Acid Antagonism

Since chlorocardicin and nocardicin A were antagonized by complex media, we examined specific antagonism by a variety of L- and D-amino acids (Table 3). Both compounds were markedly antagonized by a number of L-amino acids including alanine, serine, homoserine and leucine. The activity of the compounds differed in the presence of glycine, which showed marked antagonism of nocardicin A but only weak antagonism of chlorocardicin. Of the D-amino acids, glutamic acid and cysteine showed marked antagonism whereas the L-isomers showed little or no effect.

Potentiation of Chlorocardicin and Nocardicin A by D-Cycloserine (DCS)

Significant potentiation of the activity of the monocyclic β-lactams against *E. coli* K802N was obtained in combination with DCS, an inhibitor of alanine racemase (E.C. 5.1.1.1) and D-alanyl alanine synthetase (E.C. 6.3.2.4). Fractional inhibitory concentration indices[8] of 0.25 were observed with both compounds, although with chlorocardicin only a quarter of the concentration was required compared with nocardicin A (Table 4).

Discussion

Semi-synthetic and synthetic studies on β-lactams and monobactams have yielded a variety of therapeutically valuable antibiotics. The nocardicins have also been subjects of extensive modification but as yet have not formed the nucleus of a useful agent. This paper describes the discovery of a new naturally occurring β-lactam related to nocardicin A that showed little promise *per se* as a therapeutic agent. However, the strong synergy observed with DCS suggests that the activity of these compounds might be markedly improved in combination with other "cell wall active" antibiotics.

Previously DCS has been shown to potentiate agents that inhibit cell wall synthesis, including alafosfalin[8] and FR-31564 (MEHTA, R. J.; L. J. NISBET & C. G. PHELEN, unpublished observations). This is the first report of DCS potentiation by monocyclic β-lactams and it is a subject for further investigation.

Antagonism of nocardicins by amino acids has not previously been studied extensively. KOJO *et al.*[9] demonstrated that a mixture of amino acids (Gly, Hom, Met, Thr, Val) slightly decreased the *in vitro* activity of nocardicin A against *Pseudomonas aeruginosa* and *Proteus vulgaris*. Our results showed that strong antagonism of nocardicin A activity against *E. coli* was observed with alanine, serine, and leucine, as well as with homoserine and glycine. Most of these amino acids also antagonized chlorocardicin. No antagonism was found with methionine, threonine or valine. The strong antagonism found with certain D-amino acids, particularly glutamic acid and cysteine, leads us to postulate that these monocyclic β-lactams may inhibit enzymes other than the penicillin binding proteins of sensitive bacteria. Potent synergy with D-cycloserine indicates that the steps involved in the biosynthesis of peptidoglycan may be worth considering as alternative targets for monocyclic β-lactams.

Table 3. Effects of amino acids on the activity of chlorocardicin and nocardicin A against *E. coli*.

Amino acid	Isomer	Antagonism[a]	
		Chlorocardicin	Nocardicin A
Alanine	L	##	##
	D	#	##
Glycine	—	+	##
Glutamic acid	L	—	—
	D	#	##
Cysteine	L	+	+
	D	##	##
Serine	L	#	##
	D	+	—
Homoserine	L, D	##	##
Leucine	L	##	##
	D	+	+
Histidine	L	—	+
	D	#	+

[a] Interpretation of scores: — No effect, + partial antagonism at low concentrations of antibiotic, # partial antagonism at all concentrations of antibiotic, ## marked antagonism at all concentrations of antibiotic.

Table 4. Potentiation of D-cycloserine (DCS) by chlorocardicin and nocardicin A against *E. coli* K802N.

Antibiotics	MIC (μg/ml)	FIC		FICI
		DCS	β-Lactam	
DCS	3.2	—	—	
Chlorocardicin	6.3	—	—	
Nocardicin A	25.0	—	—	
DCS+chlorocardicin	0.4+0.8	$0.125\left\{\frac{0.4}{3.2}\right\}$	$0.125\left\{\frac{0.8}{6.3}\right\}$	0.25
DSC+nocardicin A	0.4+3.2	$0.125\left\{\frac{0.4}{3.2}\right\}$	$0.125\left\{\frac{3.2}{25.0}\right\}$	0.25

Acknowledgments

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