JANTHINOCINS A, B AND C, NOVEL PEPTIDE LACTONE ANTIBIOTICS PRODUCED BY *JANTHINOBACTERIUM LIVIDUM*

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION

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Janthinocins A, B and C are novel antibacterial agents produced by *Janthinobacterium lividum*. They were isolated from fermentation broths and characterized by UV, IR, NMR and mass spectroscopy. They are cyclic decapeptide lactones with marked activity against aerobic and anaerobic Gram-positive bacteria and are 2 to 4 times more potent *in vitro* than vancomycin. Janthinocins A and B were also found to be effective in a *Staphylococcus aureus* systemic infection in mice.

In view of the increased incidence of infections due to Streptococci, Staphylococci, Enterococci and other organisms resistant to β-lactams, macrolides and tetracyclines, there has been an increased use of vancomycin in the clinic. This has stimulated the search for agents with a spectrum of activity similar to that of vancomycin. However, there has also been a gradual increase in the number of organisms resistant to vancomycin and very recently reports of plasmid determined resistance in *Enterococcus faecium* have appeared[1]. This has led to an interest in compounds with a spectrum of activity similar to vancomycin but with an altered mode of action.

In the course of our screening program, we recently reported the discovery of lysobactin[2], a novel compound with potent activity against aerobic and anaerobic Gram-positive bacteria. Lysobactin inhibited peptidoglycan biosynthesis but did not affect RNA or DNA biosynthesis or cause membrane damage at the MIC[3]. We now report on a new family of compounds, the janthinocins, with activities similar to that of lysobactin. The structures of the janthinocins are presented in the accompanying paper[4].

Description of the Producing Organism

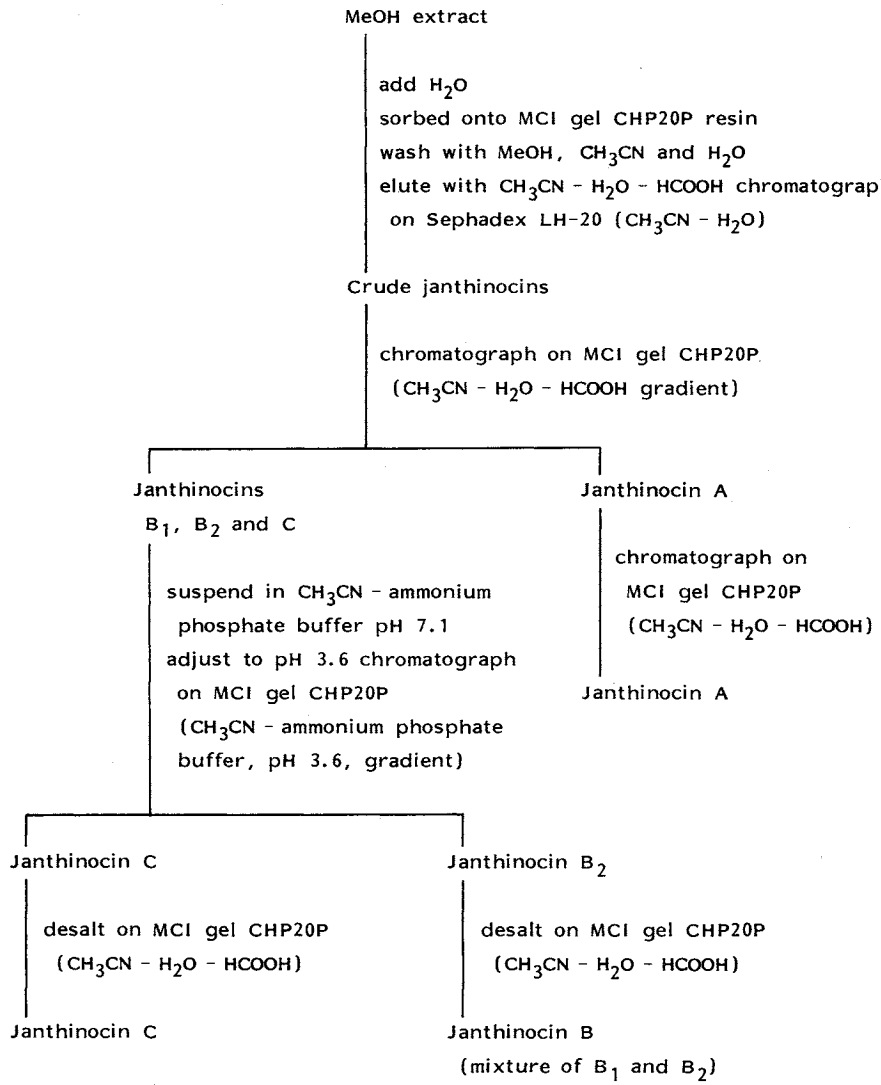
A strain of *Janthinobacterium lividum* was isolated from stagnant water collected in Tyler State Park, Newtown, Pennsylvania. The organism is a motile Gram-negative bacterium that is rod-shaped, 0.8 to 1 μm wide and 2.5 to 3 μm long with sub-polar to lateral flagella. It grows optimally at 25°C (4°C minimum, 30°C maximum) and pH 7 to 8. Colonies on nutrient agar are gelatinous and dark purplish-black in color. The gelatinous material is extracellular polysaccharide and the pigment produced is violacein. Glucose is utilized oxidatively and acid is produced from trehalose but not from L-arabinose or D-xylose. The organism is negative for arginine dihydrolase, the indole and Voges-Proskauer reactions, nitrate and nitrite reduction, citrate utilization, production of HCN and esculin hydrolysis. The bacterium is identified as an aberrant strain of *J. lividum*.

Fermentation

Seed cultures of *J. lividum* (ATCC 53857) were prepared by transferring a loopful of surface growth

from an agar slant into three 500-ml Erlenmeyer flasks each containing 100 ml of a medium consisting of yeast extract 0.5%, glucose 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.1%, soil extract filtrate 20% (prepared by boiling 1 vol soil and 2 vol water for 1 hour and filtering) and tap water. The flasks were incubated on a rotary shaker for approximately 96 hours with a resulting broth pH of 8.0~8.5. A 1%-transfer was made from the grown culture flasks to Erlenmeyer flasks containing sterilized medium consisting of yeast extract 0.5%, glucose 0.5%, $MgSO_4 \cdot 7H_2O$ 0.01%, $FeSO_4 \cdot 7H_2O$ 0.01% and tap water. After inoculation, the flasks were once again incubated at 25°C on a rotary shaker for 24~28 hours with a resulting broth pH of 7.1~7.5. The contents of the flasks were then pooled, $(NH_4)_2SO_4$ (25% w/v) was added to the pooled broth and the mixture was stirred for 1 hour. The broth-$(NH_4)_2SO_4$ mixture was then centrifuged and the resulting pellet extracted with methanol. The methanol-pellet mixture was centrifuged, and the resulting methanol extract made approximately 10% aqueous by the addition of water and then extracted with carbon tetrachloride. The layers were separated and the methanol extract used

Fig. 1. The isolation and purification of janthinocins A, B and C.



for isolation.

Isolation

The janthinocins were isolated from the methanol extract as outlined in Fig. 1 and activity was monitored using *Staphylococcus aureus* FDA 209P as the test organism. The methanol extract was added to MCI gel CHP20P in water and the mixture was stirred for 1 hour. The charged resin was collected by vacuum filtration and washed with methanol, water, and acetonitrile. The charged resin was then packed in a column and the antibiotics eluted with acetonitrile - water - formic acid (70 : 30 : 1). Further purification was achieved by chromatography on Sephadex LH-20 in acetonitrile - water (8 : 2).

In both of the preceding chromatographic steps, janthinocins A, B and C coelute. Partial resolution of the three antibiotics was effected by chromatography on MCI gel CHP20P, eluting with a gradient of acetonitrile - water - formic acid (20 : 80 : 0 to 60 : 40 : 1). Final purification of janthinocin A was achieved by one repetition of the MCI gel CHP20P chromatography on the partially purified, pooled A fractions.

Final purification of janthinocins B and C was complicated by the fact that janthinocin B exists in solution as a mixture of two isomers (see Table 1). These two isomers, janthinocins B_1 and B_2, are separable by reverse phase chromatography, but slowly interconvert to give a pH and solvent dependent equilibrium mixture. Unfortunately, janthinocins B_1 and C coelute in each of the solvent systems explored, as well as in numerous TLC systems. Therefore, purification of these two antibiotics was achieved by conversion of the janthinocins B_1 and B_2 mixture to predominantly the B_2 isomer before chromatography on MCI gel CHP20P resin. This equilibrium was shifted to favor

Table 1. pH and solvent effects on the B_1 and B_2 equilibrium.

Solvent	24 hours[a]	
	B_1 (%)	B_2 (%)
H_2O[b]	73	23
0.01 N HCl - CH_3CN (4 : 1)	69	29
0.1 N HCl - CH_3CN (4 : 1)	61	37
H_2O - CH_3CN (4 : 1)	50	43
H_2O - CH_3CN (1 : 4)	21	76
1% $(NH_4)_2HPO_4$ - CH_3CN, 1 : 1 (pH 7.1)	15	80

[a] Analysis of 1 mg/ml solutions by integration of peak area on HPLC chromatograms; equilibrium is established by 24 hours.

[b] The pH of a 1 mg/ml aqueous solution of janthinocin B is 4.8.

Table 2. Physico-chemical characteristics of the janthinocins.

Property	A	B	C
Formula	$C_{57}H_{84}N_{12}O_{16}$	$C_{57}H_{82}N_{12}O_{16}$	$C_{57}H_{82}N_{12}O_{15}$
$[\alpha]_D^{25}$	+28.7° (1.0, H_2O)	+37.9° (1.0, H_2O)	+17.3° (0.37, H_2O)
MW[a]	1,192	1,190	1,174
UV $\lambda_{max}^{H_2O}$ nm ($E^{1\%}$)[b]	204 (270), 276 (36), 287 (30), 337 (8)	204 (350), 260 (56), 262 (120), 312 (82)	195 (400), 220 (250), 339 (100)
IR (KBr) cm^{-1}	3342, 2962, 2927, 1743, 1659, 1602, 1525, 1383, 1126	3322, 3066, 2967, 1742, 1655, 1522, 1384, 1116	3342, 3066, 2968, 1744, 1654, 1602, 1526, 1384
Electrophoresis[c] pH 2.0	−0.48	−0.48	−0.48
pH 4.0	−0.12	−0.12	−0.12
pH 7.0 and 9.2	0	0	0
HPLC[d] Rt (minutes)	7.39	2.17/4.43[e]	2.68

[a] Determined by FAB-MS.

[b] All UV spectra were unchanged by treatment with 10 mM HCl or 10 mM NaOH.

[c] Mobility relative to *p*-nitrobezenesulfonate anion (+1.00) and vitamin B_{12} (0).

[d] Hamilton PRP-1, 150 × 4.1 mm, CH_3CN - H_2O (34 : 66) 1% in $NH_4H_2PO_4$, 1 ml/minute, 220 nm.

[e] Janthinocin B exists in two isomeric forms in solution; see text.

the B_2 isomer by suspending the janthinocins B and C mixtures (from the above MCI gel CHP20P chromatography) in acetonitrile-aqueous ammonium phosphate buffer pH 7.1 for several hours. The sample was then adjusted to pH 3.6 with 85% H_3PO_4 immediately before application to another MCI gel CHP20P column and the antibiotics were eluted with a gradient of acetonitrile-aqueous ammonium phosphate buffer pH 3.6, (33:67 to 60:40). Finally, the combined fractions containing janthinocin B or C were desalted on MCI gel CHP20P, eluting with acetonitrile-water-formic acid (70:30:1), to give the pure antibiotics as white powders.

Physico-chemical Characteristics

Selected physico-chemical characteristic of the janthinocins are presented in Table 2. The antibiotics are obtained as weakly basic, amorphous white solids that are soluble in water and mixed aqueous-organic solvents. They are also slightly soluble in methanol, dimethyl sulfoxide and pyridine. The janthinocins are detected on TLC plates by fluorescence quenching and with ninhydrin and Rydon-Smith reagents, but not with phosphomolybdic acid, vanillin-sulfuric acid or iodine vapor. The IR spectra of janthinocins A, B and C are similar and have absorbance at 1743 and 1650 cm^{-1} consistent with peptide lactones. Each of the antibiotics has a distinctive UV spectrum that is unchanged by addition of 0.01 M acid or base.

Biological Characteristics

The antibacterial spectrum of the janthinocins was very similar to that for vancomycin (Table 3) since

Table 3. Antibacterial activity *in vitro*.

Organism	SC No.[a]	MIC (μg/ml)[b] 10^4 cfu			
		Janthinocin A	Janthinocin B	Janthinocin C	Vancomycin
Staphylococcus aureus	1276	0.8	0.4	1.6	0.8
S. aureus	2399	1.6	1.6	3.1	1.6
S. aureus	2400	1.6	1.6	3.1	1.6
S. aureus	10165	1.6	0.8	1.6	1.6
Enterococcus faecalis	9011	1.6	1.6	12.5	0.8
Streptococcus agalactiae	9287	0.2	<0.05	0.8	0.4
Micrococcus luteus	2495	0.2	0.2	1.6	0.4
Escherichia coli	8294	25	25	100	100
E. coli	10857	12.5	12.5	50	25
E. coli	10896	6.3	6.3	50	25
E. coli	10909	6.3	6.3	25	25
Klebsiella pneumoniae	10440	25	50	100	>100
K. pneumoniae	9527	25	25	>100	>100
Proteus mirabilis	3855	100	100	>100	>100
P. vulgaris	9416	12.5	12.5	>100	12.5
Providencia rettgeri	8479	50	100	>100	>100
Salmonella typhosa	1195	12.5	12.5	100	100
Shigella sonnei	8449	12.5	12.5	100	50
Enterobacter cloacae	8236	25	50	>100	100
E. aerogenes	10078	25	50	>100	>100
Citrobacter freundii	9518	25	25	100	100
Serratia marcescens	9783	25	50	>100	>100
Pseudomonas aeruginosa	9545	50	50	>100	>100
P. aeruginosa	8329	100	100	>100	>100
Acinetobacter calcoaceticus	8333	12.5	25	25	>100

[a] SC No. is the microorganism number in the Squibb Culture Collection.
[b] MICs were determined by the agar dilution method using yeast-beef agar (BBL).

Table 4. Antibacterial activity *in vitro* (secondary screen).

Organism	SC No.[a]	MIC[b](μg/ml) 10^4cfu		
		Janthinocin A	Janthinocin B	Vancomycin
Bacillus subtilis	3777	0.4	0.1	0.4
Staphylococcus epidermidis (Penicillin S)[c]	9052	0.8	0.4	1.6
S. epidermidis (Penicillin R)[d]	9083	0.8	0.4	1.6
S. epidermidis (Penicillin R)	9087	0.8	0.4	3.1
S. epidermidis (Penicillin R)	9607	0.4	0.4	1.6
S. epidermidis (Penicillin R)	10547	0.8	0.4	3.1
S. saprophyticus	12875	0.8	0.4	3.1
S. aureus (Penicillin S)	2399	0.8	0.4	1.6
S. aureus (Tetracycline R)	10016	0.2	0.4	1.6
S. aureus (Penicillin R)	2400	0.4	0.8	1.6
S. aureus (Penicillin R)	9593	0.8	0.4	1.6
S. aureus (Penicillin R)	9998	0.8	0.1	1.6
S. aureus (Methicillin R)	3184	1.6	0.4	3.1
S. aureus (Methicillin R)	10014	0.4	0.4	1.6
S. aureus (Methicillin R)	10020	0.4	0.4	1.6
S. aureus (Gentamicin R)	11239	0.8	0.2	1.6
S. aureus (Erythromycin R)	10820	0.8	0.2	1.6
S. aureus (Erythromycin R)	12691	0.4	0.4	1.6
Enterococcus faecalis	9011	0.8	1.6	1.6
E. faecalis	9376	0.8	1.6	3.1
E. faecalis	10938	1.6	0.8	1.6
Streptococcus agalactiae	9593	0.8	0.4	1.6
S. agalactiae	9998	0.8	0.1	1.6
Nocardia asteroides	3184	1.6	0.4	3.1
Listeria monocytogenes	10014	0.4	0.4	1.6

[a] SC No. is the microorganism number in the Squibb Culture Collection.
[b] MICs were determined by the agar dilution test using Diagnostic Sensitivity Test Agar (Oxoid). The agar was supplemented with 5% NaCl for methicillin-resistant bacteria.
[c] S indicates sensitivity to the antibiotic named.
[d] R indicates resistance to the antibiotic named.

both were predominantly active against Gram-positive bacteria. However, janthinocins A and B were also moderately active against Gram-negative bacteria.

When tested in a secondary screen consisting of several Gram-positive bacteria resistant to various antibiotics, janthinocins A and B were 2- to 4-fold more active than vancomycin (Table 4). For testing

Table 5. Antianaerobic activity.

Organism	SC No.[a]	MIC (μg/ml)[b] 10^5cfu	
		Mixture of janthinocins A and B	Vancomycin
Bacteroides thetaiotaomicron	9005	6.3	100
B. fragilis	9844	25	50
B. fragilis	10277	50	50
B. thetaiotaomicron	10278	25	100
B. fragilis	10279	25	50
B. fragilis	10280	50	50
B. fragilis	11085	50	50
Clostridium histolyticum	8572	0.4	3.1
C. perfringens	11256	0.4	1.6
C. septicum	1780	0.2	3.1
C. sporogenes	2372	0.05	12.5
C. difficile	11251	50	1.6
Hemophilus vaginalis	8568	0.4	0.8
H. vaginalis	9640	0.1	0.8
Bifidobacterium dentium	11260	0.2	0.8
Peptococcus variabilis	11264	0.8	0.4
Peptostreptococcus anaerobius	11263	0.8	0.8
Propionibacterium acnes	4020	0.4	0.8

[a] SC No. is the microorganism number in the Squibb Culture Collection.
[b] MICs were determined by the agar dilution method using Diagnostic Sensitivity Test Agar (Oxoid).

against anaerobes, a mixture of janthinocins A and B was used and from Table 5 it can be seen that the mixture was significantly more active than vancomycin. The activity of the janthinocins was directed predominantly, but not exclusively, against Gram-positive bacteria.

In a tube dilution test against *E. faecium*, which is vancomycin-resistant (MIC $>100\,\mu$g/ml) as a consequence of a plasmid borne resistance determinant, the MIC for janthinocins A and B was 0.06 and $0.5\,\mu$g/ml for janthinocin C.

When administered intravenously to mice, LD_{50} values of 90, 120 and 430 mg/kg were found for janthinocins A, B and vancomycin, respectively. In an efficacy study with a *Staphylococcus aureus* systemic infection model, janthinocin B had an ED_{50} of <1.6 mg/kg compared to 2.0 mg/kg for vancomycin and 3.1 mg/kg for cephalothin.

The janthinocins, together with daptomycin[5] and lysobactin, represent a group of antibiotics which are not cross-resistant with vancomycin. It was shown that lysobactin inhibited the incorporation of diaminopimelic acid into cell-wall material in *Bacillus megaterium*, but had no effect on RNA or DNA biosynthesis at MIC concentrations; lysobactin also did not cause significant membrane damage until concentrations greater than ten times the MIC were reached. The janthinocins were discovered using the same screening method as was used for lysobactin and were found not to be active against *Acholeplasma laidlawii* or cause membrane damage at the MIC. It is likely, therefore, that they have a mode of action like lysobactin directed, in part, against the cell wall.

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