LYSOBACTIN, A NOVEL ANTIBACTERIAL AGENT PRODUCED BY *LYSOBACTER* SP.

I. TAXONOMY, ISOLATION AND PARTIAL CHARACTERIZATION

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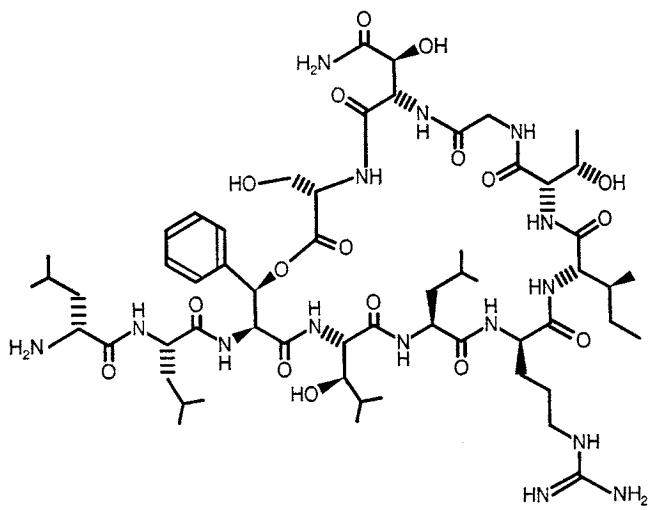
A new antibacterial agent, lysobactin, has been isolated from a species of *Lysobacter* (ATCC 53042). The antibiotic was recovered from the *Lysobacter* cell mass by extraction and reversed phase chromatography. Lysobactin is a dibasic peptide with marked activity against Gram-positive aerobic and anaerobic bacteria.

In the course of screening for agents with activity against Gram-positive bacteria, we discovered a novel compound produced by a species of *Lysobacter* (ATCC 53042) which we called lysobactin (Fig. 1). The antibiotic was isolated from cells and broth grown for 1~2 days at 25°C. We describe here the taxonomy of the producing organism, fermentation, isolation and some characteristics of lysobactin.

Taxonomy

Lysobacter sp. SC 14067 (ATCC 53042) was isolated from a leaf litter sample collected in Washington Crossing State Park, New Jersey. The sample was plated onto LITTMAN's agar containing 30 μg/ml streptomycin and after 6-day incubation at 25°C, colonies of *Lysobacter* were isolated from the plate. *Lysobacter* is a Gram-negative rod that exhibits varying morphology depending on the medium. On Trypticase soy agar the cells are short rods with rounded ends and fairly uniform in

Fig. 1. Structure of lysobactin.



length. On 0.2% Tryptone agar, the rods are thin, slightly flexous and range in length from about 0.4~40 μm. On this medium, they exhibit characteristic gliding motility. No fruiting bodies are formed. Colonies are slimy or mucoid and a dirty yellow in color.

In the Hugh-Leifson glucose test,[1] *Lysobacter* sp. ATCC 53042 grows oxidatively, but no acid production is evident since it is masked by ammonia from the peptone in the medium. On BOARD and HOLDING's peptone-free medium[2] with monobasic ammonium phosphate at 0.05% as nitrogen source and 0.5% glucose as the sole carbon source, detectable acid is produced. The organism is cytochrome oxidase, catalase and phosphatase positive.

Lysobacter sp. ATCC 53042 is chitinolytic and lyses cells of yeasts, *e.g.*, *Saccharomyces*. It is strongly proteolytic on gelatin and casein. No cellulolytic or agarolytic activity is evident. Tests for indole, methyl red and the Voges-Proskauer reaction are all negative. The mol % guanine plus cytosine of the *Lysobacter* sp. DNA is 64.9.

The above characteristics are all consistent with those of members of the genus *Lysobacter* as described by CHRISTENSEN and COOK.[3]

Assay

Vancomycin inhibits the growth of Gram-positive bacteria by preventing the polymerization of disaccharide-pentapeptide units necessary for cell wall peptidoglycan formation. It binds to the acyl-D-alanyl-D-alanine (D-ala-D-ala) terminus of uridine diphosphate-*N*-acetylmuramyl pentapeptide thereby preventing cell wall growth.[4]

Screens for vancomycin-like antibiotics have been described in which diacetyl-lysyl-D-ala-D-ala has been used to reverse the activity of the antibiotic.[5]

We wished to screen for agents with a mode of action similar to but not necessarily identical with vancomycin and we therefore devised a two plate assay using *Bacillus subtilis* as test organism. One plate contains a complex cell wall preparation which should contain a variety of potential binding determinants in addition to the diacetyl-lysyl-D-ala-D-ala; agents of interest would therefore give smaller zones of inhibition on this plate than on a plate without cell wall material.

The cell wall preparation was prepared by growing *Staphylococcus aureus* SC 12691 overnight at 37°C in antibiotic assay broth (Difco) and harvesting by centrifugation. The cell pellet was resuspended in one fifth the original volume and an equal volume of 20% trichloroacetic acid was added. The mixture was boiled for 20 minutes and then centrifuged. The pellet was washed and resuspended in 0.1 M phosphate buffer pH 7.0 containing 0.2 mg/ml trypsin. This suspension was incubated for 2 hours at 37°C, centrifuged and the pellet washed twice with distilled water. The cell wall pellet was taken up in one fiftieth the original volume and this suspension was used as a stock solution for addition (2%) to agar plates.

Fermentation

Seed cultures of *Lysobacter* sp. ATCC 53042 were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following; yeast extract 0.5%, peptone 0.3%, mannitol 0.5% in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm, 5 cm stroke) for approximately 48 hours. A 1% transfer was made from the seed culture to fifty 500-ml Erlenmeyer flasks each containing 100 ml of the sterilized medium described above. After inoculation, the flasks were once again incubated at 25°C on a rotary shaker

(as previously described) for approximately 24~28 hours. At this time, the contents of the flasks were combined and the broth was centrifuged yielding approximately 4.8 liters of supernatant broth and 33 g of cells (wet weight).

Isolation

Lysobactin was isolated from cells and broth as outlined in Fig. 2. The cell mass (33 g) was extracted with three 200-ml portions of acetone - water (4:1). The filtered extracts were combined and concentrated to dryness. The residue was triturated with methanol and the soluble portion was concentrated to a yellow glass (705 mg). Chromatography of the methanol soluble material was performed on a 2.5×43 cm column of MCI gel CHP20P (Mitsubishi Chemical Industries Limited, Japan), with a linear gradient prepared from 1 liter of 0.1% trifluoroacetic acid in water and 1 liter of 0.1% trifluoroacetic acid in acetonitrile. Fractions giving a single Rydon-positive component by TLC (Merck silica gel-60; butanol - acetic acid - water, 4:1:1; Rf 0.42) were combined and dried to yield an off-white solid (173 mg). The solid was dissolved in acetonitrile - water - trifluoroacetic acid (50:50:0.1) and left to evaporate under a stream of nitrogen to afford 73 mg of crystalline lysobactin as its trifluoroacetate salt.

Lysobactin present in the fermentation broth was concentrated by extracting the broth supernatant (adjusted to pH 7.0) with butanol. The butanol extract was concentrated *in vacuo* and then triturated and chromatographed in the same manner as described for the cell extract to provide lysobactin as its trifluoroacetate salt.

Elemental analysis was performed on a sample after drying *in vacuo* at 70°C for 3 hours. The measured values were C 50.01, H 6.92, N 13.98, F 7.4 and based on this and fast atom bombardment MS data, the molecular formula $C_{58}H_{97}N_{15}O_{17}$ was assigned.

The UV spectrum of the trifluoroacetate salt of lysobactin is presented in Fig. 3 and shows: UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 250 (sh, 1.4), 257 (1.5), 262 (1.4), 268 (0.9) in addition to end absorption.

The IR spectrum of the trifluoroacetate salt of lysobactin in potassium bromide is shown in Fig. 4. The following peaks are evident: 3345 (br), 2965, 2937, 2878, 1745 (sh), 1666 (s), 1530 (s), 1203, 1138, 838, 800, 722, 702 cm^{-1}.

Details of the structure determination and physico-chemical properties of lysobactin will be reported elsewhere.[6]

Fig. 2. Isolation of lysobactin.

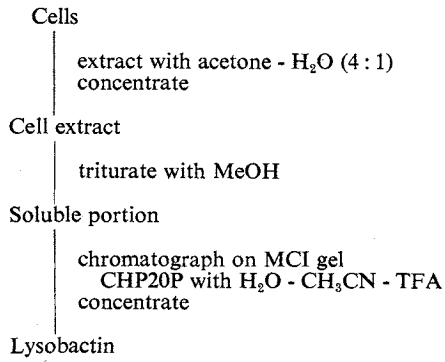


Fig. 3. UV spectrum of lysobactin.

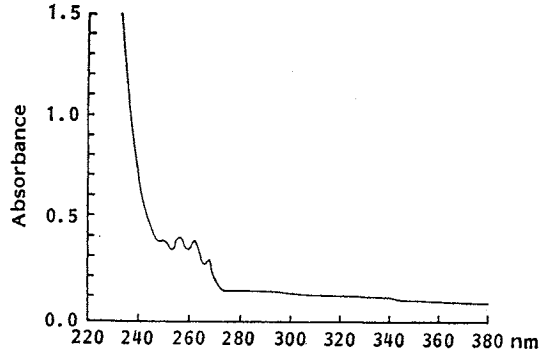


Fig. 4. IR spectrum of lysobactin.

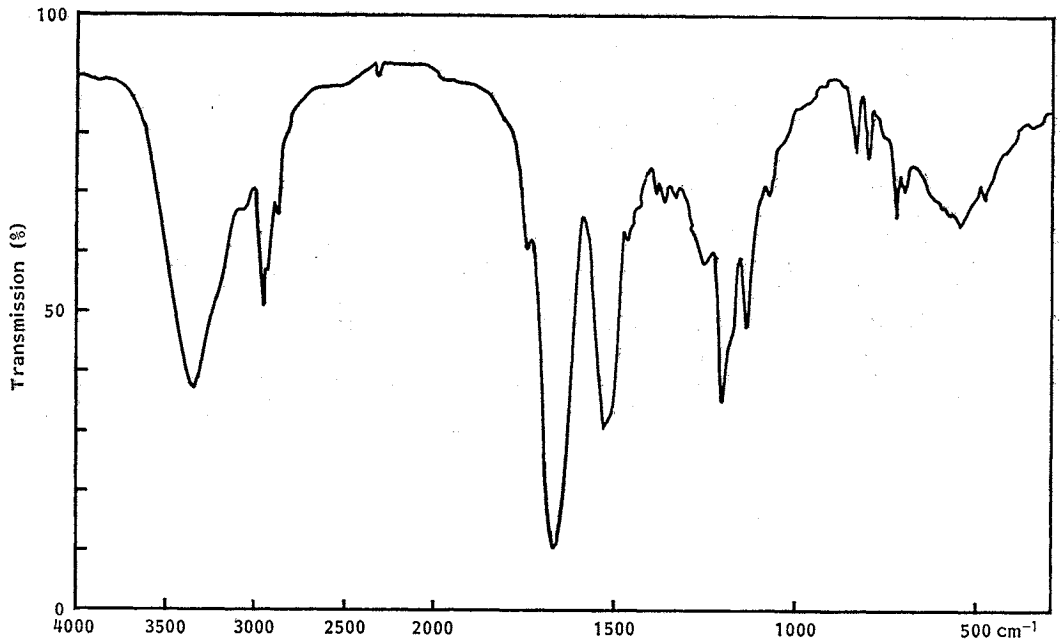


Table 1. Antibacterial activity of lysobactin.

Organism	SC No.	MIC[a] (μg/ml)	
		Lysobactin	Vancomycin
Staphylococcus aureus	1276	0.1	0.4
S. aureus	2400	0.4	0.8
Micrococcus luteus	2495	0.1	0.4
Clostridium difficile	11251	0.2	1.6
Escherichia coli	10857	6.3	12.5
Pseudomonas aeruginosa	9545	25	>100
Bacteroides fragilis	10279	50	50

[a] MICs were determined by the agar dilution method using yeast - beef agar (BBL) 10^4 cfu per plate.

Biological Properties

Lysobactin was found to be 2 to 4-fold more active than vancomycin against aerobic and anaerobic Gram-positive bacteria. Table 1 shows that there is little activity against Gram-negative aerobic and anaerobic bacteria. The compound showed efficacy in animal model infections involving susceptible microorganisms. Further details of its biological activity and mode of action studies are reported in the following paper.[7]

Addendum in Proof

Since this manuscript was prepared, a report from workers at Shionogi appeared (J. Antibiotics 41: 713~718 and 719~725, 1988) describing structures called katanosins A and B. Katanosin B is very similar to lysobactin but is not identical to it. Lysobactin contains L-*allo*-threonine whereas katanosin B was reported to have a D-*allo*-threonine moiety. The katanosins were produced by a *Cytophaga* species.

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