STUDIES ON BACTERIAL CELL WALL INHIBITORS

VII. AZUREOMYCINS A AND B, NEW ANTIBIOTICS PRODUCED BY *PSEUDONOCARDIA AZUREA* NOV. SP. TAXONOMY OF THE PRODUCING ORGANISM, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES*

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Two new basic water-soluble antibiotics, azureomycins A and B, were isolated from the culture broth of an actinomycete, strain AM-3696, designated as *Pseudonocardia azurea* nov. sp. The antibiotics exhibit moderate antimicrobial activities against Gram-positive bacteria including penicillin-resistant *Staphylococcus, Mycobacterium* and *Clostridium*. They inhibit the synthesis of bacterial cell wall peptidoglycan.

In the preceding paper[1] we have reported a method of screening for new inhibitors of bacterial cell wall synthesis. This method is based on the insensitivity of *Mycoplasma* to cell wall synthesis inhibitors and the selective inhibition of the incorporation of labeled diaminopimelic acid by these compounds into the acid-insoluble fraction of a diaminopimelic acid-requiring *Bacillus*.

In the course of our search for new inhibitors of cell wall synthesis by this method, new antibiotics were obtained from the culture broth of an actinomycete, strain AM-3696, isolated from a soil sample collected in Yamagata Prefecture, Japan. The producing strain was designated as *Pseudonocardia azurea* nov. sp., while the antibiotics were named azureomycins A and B.

The present paper deals with the taxonomy of the producing organism, as well as the isolation, characterization and biological properties of azureomycins A and B.

Taxonomic Studies of the Producing Organism

The antibiotic-producing actinomycete, strain AM-3696, was isolated from a soil sample collected in Kahoku-cho, Nishimurayama-gun, Yamagata Prefecture, Japan. Taxonomic studies of the strain were carried out by the methods of SHIRLING & GOTTLIEB[2] and WAKSMAN[3].

Morphological Characteristics

The morphology of the strain was observed microscopically. The substrate mycelium is zig-zag in shape (Plate 1). The aerial mycelia form a white abundant mass with long spore chains (Plate 2). Matured spore chains are straight with a bamboo-like appearance, and consist of more than twenty spores with smooth surfaces (Plate 3). The spores are cylindrical, of variable length with a spore size

* Part VI of this series appears in Ref. 1.

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Plate 1. Substrate mycelium of strain AM-3696. Yeast extract-malt extract agar, 4 days at 27°C.

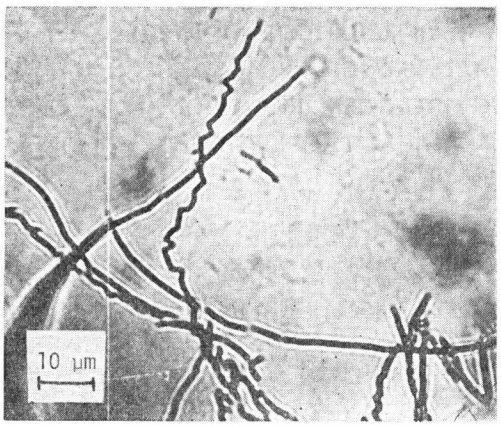


Plate 2. Sporophore of strain AM-3696. Oatmeal agar, 9 days at 27°C.

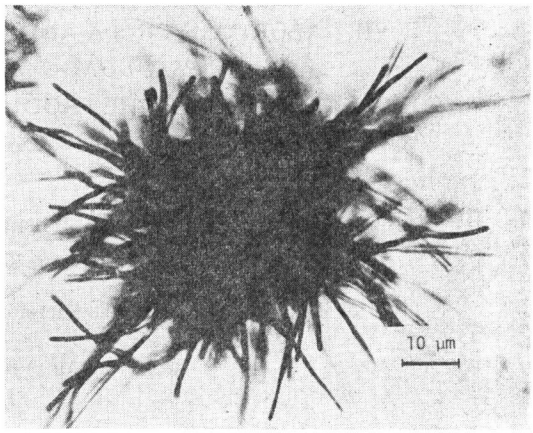


Plate 3. Spore of strain AM-3696. Yeast extract-malt extract agar, 14 days at 27°C.

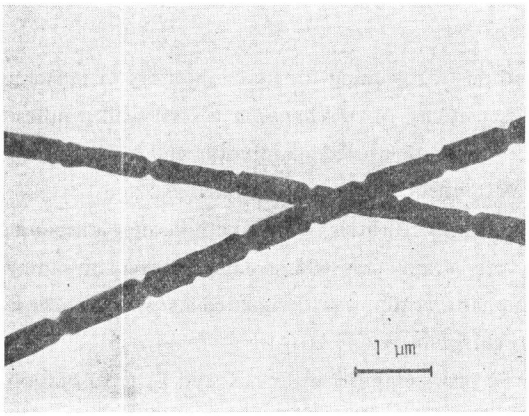
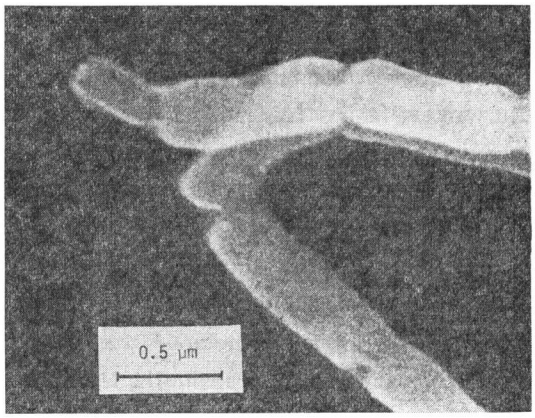


Plate 4. Blastospore of strain AM-3696. Inorganic salts-starch agar, 4 days at 27°C. Acropetal budding is observed at the top of the spore chain.



of $1.1 \sim 3.7 \times 0.4$ μm. Acropetal buddings are observed in the course of blastospore formation (Plate 4). Sclerotic granules, sporangia or zoospores are not observed.

Cultural and Physiological Characteristics

Table 1 summarizes the cultural characteristics of strain AM-3696 observed after incubation for two weeks at 27°C on various media. Color names are used according to the Color Harmony Mannual (4th edition)[4]. Growth is moderate to good. The substrate mycelium has no characteristic color. The aerial mass is moderate to abundant, and is ordinarily white, but blue on sucrose-nitrate, glucose-nitrate and tyrosine agar, or pink on glucose-peptone agar. Soluble pigment is not generally produced except for the blue pigment on glucose-nitrate agar.

Strain AM-3696 is non-chromogenic (Table 2). The optimum temperature range for growth is 20~36°C with only faint growth occurring at 37°C or higher. The strain utilizes various carbon sources (Table 3), as determined by the method of PRIDHAM & GOTTLIEB[2].

Table 1. Cultural characteristics of strain AM-3696

Medium	Cultural characteristics
Sucrose-nitrate agar	Growth (G): good, penetrating, colorless Reverse (R): colorless to dawn blue (ngs. 15dc) Aerial Mycelium (AM): moderate, velvety, white (gs.a) to 1t. sky blue (13½ea) Soluble Pigment (SP): none
Glucose-nitrate agar	G: good, penetrating, shadow blue (14ie) R: 14pl AM: abundant, velvety, moonstone blue (13½ec) SP: 14pi
Glycerol-asparagine agar (ISP)	G: good, penetrating, colorless R: colorless AM: poor, velvety, alabaster tint (ngs.13ba) SP: none
Glucose-asparagine agar	G: good, penetrating, colorless R: pearl pink (3ca) to beige (3ge) AM: abundant, velvety, white (gs.a) SP: none
Glycerol-calcium malate agar	G: good, penetrating, colorless R: colorless AM: abundant, velvety, oyster white (gs.b) to bisque (4ec) SP: none
Inorganic salts-starch agar (ISP)	G: good, penetrating, colorless R: colorless AM: abundant, velvety, white (gs.a) to oyster white (gs.b) SP: none
Tyrosine agar (ISP)	G: good, penetrating, ashes (ngs.5fe) R: colorless to dawn blue (ngs.15dc) AM: moderate, velvety, white (gs.a) to 1t. sky blue (13½ea) SP: none
Glucose-peptone agar	G: good, raised, 1t. ivory (2ca) R: 1t. ivory (2ca) to 1t. wheat (3ea) AM: abundant, velvety, center: flesh pink (5ca), outer: white (gs.a) SP: none
Yeast extract-malt extract agar (ISP)	G: good, penetrating, ivory tint (ngs.2cb) R: mustard tan (21g) AM: abundant, velvety, oyster white (gs.b) to shell pink (ngs.5ba) SP: none
Oatmeal agar (ISP)	G: moderate, penetrating, colorless R: colorless AM: moderate, velvety, white (gs.a) SP: none
Peptone-yeast extract-iron agar (ISP)	G: moderate, raised, 1t. ivory (2ca) partially dusty blue (14ge) R: 1t. ivory (2ca) partially dusty blue (14ge) AM: poor, oyster white (gs.b) SP: none
Nutrient agar	G: good, penetrating, colorless R: pearl pink (3ca) AM: moderate, velvety, white (gs.a) SP: none

Chemical Analysis of Cell Constituents

Following the procedures of BECKER *et al.*[5] and YAMAGUCHI[6] it was determined that the whole cell hydrolysate contains the *meso*-isomer of diaminopimelic acid with arabinose and galactose as the major constituents. Glycine was detected in only a small amount. Consequently, strain AM-3696 is assigned to cell wall type IV and to whole cell sugar pattern A[7].

Comparison with Related Organisms

The morphological and cultural studies and the cell wall constituents of strain AM-3696 described above indicate that this strain resembles members of the genera *Pseudonocardia*[8~11], *Nocardia*[12], *Micropolyspora*[13] and *Saccharopolyspora*[14]. However, the new strain differs from the latter three genera as follows: In the genus *Nocardia* fragmentation of substrate mycelium is prompt, and poor aerial mycelium is formed. In the genus *Micropolyspora* short spore chains (less than twenty spores) are formed on both aerial and substrate mycelia. In the genus *Saccharopolyspora* bead-like spore chains with empty hyphae are produced.

Strain AM-3696 shares several key morphological characteristics including acropetal buddings with members of the genus *Pseudonocardia*. Minor differences are observed, however, between the abilities of strain AM-3696 and members of *Pseudonocardia*[12] to hydrolyze starch, to peptonize and to coagulate milk (Table 2). These discrepancies may not be accepted as determinative, because only limited information is availabe for characterizing the genus *Pseudonocardia*. Consequently, strain AM-3696 is assigned to the genus *Pseudonocardia*.

Strain AM-3696 was compared with the three known *Pseudonocardia* species: *P. thermophila*[8~10], *P. spinosa*[5] and *P. fastidiosa*[11]. Strain AM-3696 differs from these three species either in optimum growth temperature (28~60°C for *P. thermophila* but 20~36°C for strain AM-3696), in spore surface (spiny in *P. spinosa* but smooth in strain AM-3696), or in cultural characteristics observed on direct comparison with *P. fastidiosa* (Table 4). Furthermore, moderate to good growth of strain AM-3696 occurred on ISP No. 9 medium with glucose and several other carbon sources, whereas only slight or no growth of *P. fastidiosa* was observed in the same conditions (unpublished observation).

From the results of the above comparison, strain AM-3696 is considered to be a new species of the genus *Pseudonocardia*, and therefore, the name *Pseudonocardia azurea* TAKAHASHI and ŌMURA strain AM-3696 is proposed. The name "azurea" was chosen because of the characteristic blue pigment produced on glucose-nitrate agar (Table 1).

Table 2. Physiological properties of strain AM-3696.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	+
Hydrolysis of starch	+
Liquefaction of gelatin	+
Peptonization of milk	+*
Coagulation of milk	+
Cellulolytic activity	−
Temp. range for growth	20~36°C

* Incubation at 27°C

Table 3. Utilization of carbon sources by strain AM-3696.

Response	Carbon source
Positive	D-Glucose, D-fructose, raffinose, *i*-inositol, D-mannitol, L-arabinose, sucrose, D-xylose, melibiose, salicin
Ambiguous	L-Rhamnose

Table 4. Comparison of strain AM-3696 with *P. fastidiosa* in the cultural characteristics.

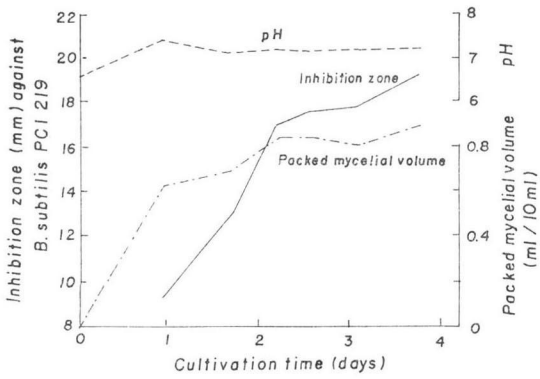
Medium	Strain AM-3696	*P. fastidiosa* ATCC 31181
Yeast extract-malt extract agar (ISP)	G: good, penetrating, ivory tint (ngs.2cb) R: mustard tan (21g) AM: abundant, velvety, oyster white (gs.b) to shell pink (ngs.5ba) SP: none	G: good, raised, 1t. ivory (2ca) R: 1t. wheat (2ea) AM: poor, white (gs.a) SP: none
Inorganic salts-starch agar (ISP)	G: good, penetrating, colorless R: colorless AM: abundant, velvety, white (gs.a) to oyster white (gs.b) SP: none	G: good, raised, colorless R: 1t. ivory (2ca) AM: poor, white (gs.a) SP: none
Glycerol-asparagine agar (ISP)	G: good, penetrating, colorless R: colorless AM: poor, velvety, alabaster tint (ngs.13ba) SP: none	G: moderate, penetrating, colorless R: colorless AM: poor, white (gs.a) SP: none
Glycerol-calcium malate agar	G: good, penetrating, colorless R: colorless AM: abundant, velvety, oyster white (gs.b) to bisque (4ec) SP: none	G: poor, penetrating, colorless R: colorless AM: poor, white SP: none
Nutrient agar	G: good, penetrating, colorless R: pearl pink (3ca) AM: moderate, velvety, white (gs.a) SP: none	G: moderate, raised, 1t. wheat (2ea) R: 1t. wheat (2ea) AM: none SP: none

This strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan, with the accession number of FERM-P No. 4738, and at the United States Department of Agriculture, Agricultural Research Service, Northern Regional Research Center (Peoria, Illinois 61604) with the accession number of NRRL 11412.

Fermentation

Pseudonocardia azurea strain No. 16 isolated from the original strain AM-3696 by a conventional monospore isolation technique was used for the production of the azureomycins. Spores and mycelia of strain No. 16 grown on an agar slant (1% glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl and 1.5% agar, pH 7.0) were transferred into a 500-ml SAKAGUCHI's flask containing 100 ml of a seed medium (2% glycerol, 2% soybean meal, 0.3% NaCl, pH 7.0 prior to sterilization), and cultivated for 2 days at 27°C. The seed culture (600 ml) was transferred into a 50-liter jar fermentor containing 30 liters of a fermentation medium composed of 2% dextrin, 1% soybean meal, 0.3% yeast extract, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ (pH 7.0 prior to sterilization), and was cultivated for 4 days at 27°C with agitation of 250 rpm and with aeration of 15 liters/min. The antibiotic activity in the culture filtrate was monitored by a paper disc method using *Bacillus subtilis* PCI 219 as test organism. As is shown in Fig. 1, the antibiotic production started one day after inoculation and reached a maximum level (*ca.* 10 μg/ml) after incubation for 4~5 days. The antibiotic activity did not decrease

Fig. 1. Time course of azureomycin production.



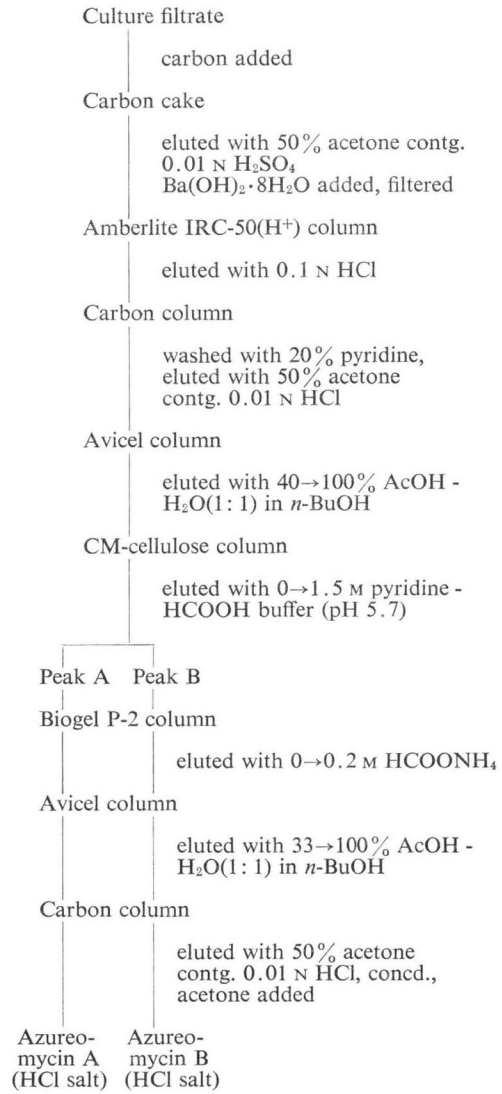
until the 6th day.

A large-scale fermentation was carried out using a 1,000-liter fermentor containing 500-liters of the fermentation medium described above.

Isolation and Characterization

The isolation procedure of azureomycins A and B is outlined in Fig. 2. The antibiotics in the culture filtrate (1,000 liters) were adsorbed on carbon (50 liters), and eluted from the carbon cake with a 50% acetone solution containing 0.01 N sulfuric acid. The active eluate was concentrated, and the concentrate was desalted by a batchwise treatment with barium hydroxide and filtration. The filtrate was passed through a column of Amberlite IRC-50 (H^+ form, 50 liters), eluted with 0.1 N hydrochloric acid. The active eluate (150 liters) was neutralized with 6 N sodium hydroxide. The solution was passed through a carbon column (10 liters) and the active substances were eluted with the acidic acetone solution. The active eluate (50 liters) from the column was neutralized with a slurry of Amberlite IR-45 (OH^- form), filtered, and then lyophilized to give a crude powder (170 g, *ca.* 1% pure).

Fig. 2. Isolation procedure of azureomycins A and B.



The crude powder (160 g) was purified by successive column chromatography on carbon (1.1 liters), cellulose (Avicel, 800 ml) and then carboxymethylcellulose (CM-cellulose, H^+ form, 370 ml). On CM-cellulose column chromatography performed with gradient elution from 0~1.5 M pyridine buffer (Fig. 2), two major components were eluted with 0.8 M and 1.2 M pyridine, containing azureomycins B and A, respectively. The fractions containing each component were pooled separately, and concentrated *in vacuo*. They were further purified by column chromatography on Biogel P-2 (200 ml). Azureomycins A and B were eluted from the column with about 0.1 and 0.03 M ammonium formate, respectively. Finally each component was applied on an Avicel column (150 ml), and eluted with a

solvent system of *n*-butanol - acetic acid - water in a ratio of 5 : 3 : 3. The active eluate was concentrated, neutralized with 6 N sodium hydroxide, and desalted with a carbon column. The active eluate from the carbon column was concentrated, and to the concentrate ten volumes of acetone were added. The precipitate was collected by centrifugation, dissolved in a small volume of water, and then lyophilized to give the purified powders of azureomycin A hydrochloride (100 mg) and azureomycin B hydrochloride (200 mg). Both azureomycins A and B, thus obtained, gave a single spot on thin-layer chromatography (TLC, Table 5).

Table 5. Rf values of azureomycins A and B on thin-layer chromatography.

Solvent system	Rf value	
	A	B
(Avicel)		
n-Butanol - benzene - pyridine - water (5: 1: 5: 3, pH 5.6*)	0.34	0.15
Ethyl acetate - pyridine - water (4: 3: 2, pH 5.6*)	0.38	0.25
n-Butanol - acetic acid - water (1: 1: 1)	0.60	0.55
(Silica gel)		
Acetone - methanol - formic acid - water (3: 1: 1: 1)	0.90	0.85
Ethyl acetate - pyridine - water (4: 3: 2)	0.45	0.48

* pH was adjusted with formic acid.

The hydrochlorides of azureomycins A and B are colorless amorphous powders. Both antibiotics are soluble in water, and slightly soluble in dimethylsulfoxide, methyl Cellosolve, acidic methanol, but insoluble in methanol, ethanol, ethyl acetate, acetic acid, dioxan, dimethylformamide, pyridine, chloroform, and benzene. They exhibit positive reactions to RYDON-SMITH's, anisaldehyde-H_2SO_4, and $KMnO_4$-bromophenol blue reagents, but were inert to ninhydrin, ELSON-MORGAN's, triphenyltetrazolium chloride (TTC), *p*-anisidine, SAKAGUCHI's, DRAGENDORFF's, ferrichydroxamate, or platinum chloride reagent. Physico-chemical properties of azureomycins A and B are summarized in Table 6. These results suggest that azureomycins A and B closely resemble each other.

Table 6. Physico-chemical properties of azureomycins A and B (HCl salts).

	Azureomycin A	Azureomycin B
Anal. Found	C: 43.5% H: 4.46 N: 4.97 Cl: 4.33	C: 47.6% H: 5.27 N: 4.74 Cl: 3.70
MP	231°C (decomp.)	220°C (decomp.)
$[\alpha]_D^{22}$	−124° (*c* 0.5, water)	−116° (*c* 2, water)
Mol. Wt.	850±40	870±20
Mol. Formula	$C_{30\sim34}H_{36\sim44}N_3O_{21\sim24}$·HCl	$C_{35\sim36}H_{46\sim48}N_3O_{19\sim21}$·HCl
UV λ_{max} ($E_{1cm}^{1\%}$)		
in 0.05 N HCl	282 (54)	279 (50)
in 0.05 N NaOH	301 (81)	286 (77 sh.)
Nature	basic, colorless, water-soluble	
Color reaction		
positive to	RYDON-SMITH, anisaldehyde-H_2SO_4, $KMnO_4$	
negative to	ELSON-MORGAN, EHRLICH, TTC, SAKAGUCHI	

Attempts were made to determine the molecular weights by various methods including mass spectrometry, but none gave satisfactory results. Then, on the basis of the elementary analysis and the fact that 20~30% of the antibiotic activity passed through a membrane filter Diaflo UM-2 (Amicon, molecular weight allowance < 1,000) and less than 1% of the activity could pass through a Diaflo UM-05 membrane (molecular weight allowance < 500), they were estimated to be around 850 for azureomycin A hydrochloride, and 870 for azureomycin B hydrochloride. The ultraviolet absorption spectrum

Fig. 3. Ultraviolet absorption spectra of azureomycins A and B (HCl salts).

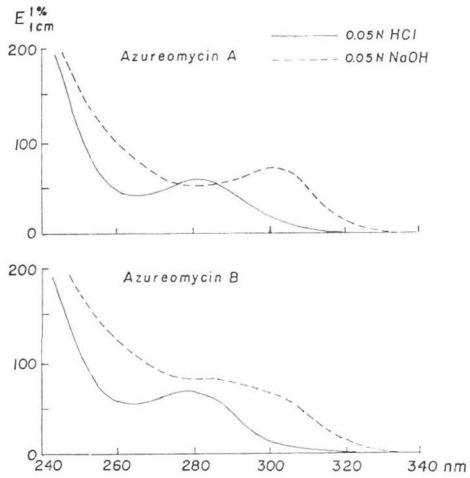


Fig. 4. Infrared absorption spectra of azureomycins A and B (HCl salts).

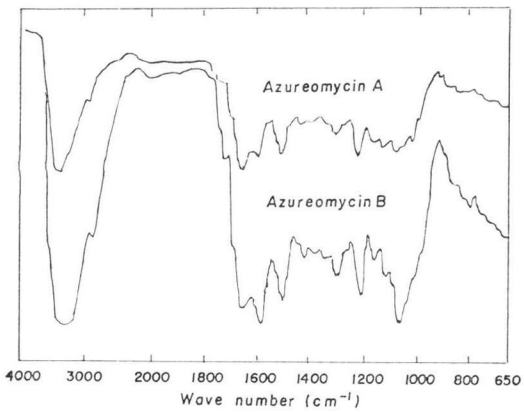


Table 7. Antimicrobial spectra of azureomycins A and B (HCl salts).

Microorganism	MIC(μg/ml)*	
	A	B
Staphylococcus aureus FDA 209P	6.25	12.5
S. aureus FS 1277 (penicillin-resistant)	6.25	12.5
Bacillus subtilis PCI 219	1.56	1.56
B. cereus T	6.25	6.25
B. megaterium KM	0.39	0.10
Sarcina lutea PCI 1001	0.78	0.78
Mycobacterium smegmatis ATCC 607	12.5	12.5
Nocardia asteroides KB 54	3.13	3.13
Streptococcus pneumoniae III	3.13	1.56
Streptococcus pyogenes C203	3.13	1.56
Clostridium perfringens PB6KN5	3.13	6.25
Cl. perfringens MB 2237	1.56	6.25
Cl. botulinum IFO 3733	1.56	0.39
Cl. kainantoi IFO 3353	—	0.39
Cl. sporogenes IFO 3987	—	0.39
Escherichia coli NIHJ	>100	>100
Salmonella typhimurium KB 20	>100	>100
Pseudomonas aeruginosa P3	>100	>100
Proteus vulgaris IFO 3167	>100	>100
Shigella sonnei E33	>100	>100

* Heart infusion agar, 20 hours at 37°C. *Clostridium* strains were incubated for 48 hours at 37°C on Gifu Anaerobic Medium (Nissui).

of azureomycin A hydrochloride (Fig. 3) shows a peak at 282 nm ($E^{1\%}_{1cm}$ 54) in 0.05 N hydrochloric acid, which shifts to 301 nm ($E^{1\%}_{1cm}$ 81) in 0.05 N sodium hydroxide, suggesting the presence of a phenolic chromophore in the molecule. A similar shift was observed with azureomycin B (Fig. 3). The infrared absorption spectrum of azureomycin A hydrochloride in KBr method (Fig. 4) exhibits characteristic bands at 3400 cm^{-1} (amine or hydroxyl group), 1740 and 1650 cm^{-1} (carbonyl group of a peptide bond) and 1060 cm^{-1} (C-O-C stretching). Similar bands with slightly different transmission were observed with azureomycin B hydrochloride (Fig. 4). The proton nuclear magnetic resonance spectrum of azureomycin A exhibits characteristic signals of aromatic protons at δ 6~8 ppm, anomeric or olefinic protons at δ 5~6 ppm, and methine protons at δ 3~4 ppm (unpublished observation). Unidentified amino acids and sugars were detected by TLC and amino acid analysis of the acid hydrolysate (6 N hydrochloric acid, 20 hours at 105°C) of azureomycin B.

Biological Properties

The antimicrobial spectra of azureomycins A and B were determined by a conventional agar dilution method using heart infusion agar for aerobic bacteria (37°C, 20 hours), Gifu Anaerobic Medium (Nissui) for anaerobic bacteria (37°C, 48 hours), and potato-glucose agar for fungi (27°C, 48 hours). As shown in Table 7, azureomycins A and B inhibit the growth of Gram-positive bacteria including penicillin-resistant *Staphylococcus aureus, Mycobacterium smegmatis* and *Clostridium* in a range of 0.1~12.5 μg/ml. But they are inactive against Gram-negative bacteria and fungi.

Azureomycin B had no effect when administered intraperitoneally in mice at 500 mg/kg. Higher concentrations have not been tested. The antibiotics induce lysis of the growing bacterial cells and inhibit the synthesis of cell wall peptidoglycan in bacteria[15].

Discussion

Azureomycins A and B are basic water-soluble compounds with an ultraviolet absorption maximum around 280 nm, and are active against Gram-positive bacteria. Among known antibiotics, avoporcin (LL-AV 290)[16], vancomycin[17], enduracidin[18], triculamin[19], antibiotic A-4696[20], antibiotic A-477[21], and antibiotic AM-374[22] show some resemblance to azureomycins A and B. But all of these compounds are differentiated from azureomycins A and B by their UV spectra in acidic and alkaline solutions, elementary analysis values, and molecular weights. Consequently, azureomycins A and B are concluded to be new antibiotics.

From the information obtained by UV, IR and PMR spectra as well as their color reactions, azureomycins A and B are sugar peptide compounds with a phenolic chromophore.

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