# IZUPEPTINS A AND B, NEW GLYCOPEPTIDE ANTIBIOTICS PRODUCED BY AN ACTINOMYCETE

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In the course of screening for new high molecular weight peptidoglycan synthesis inhibitors, izupeptins A and B, antibiotics active against Gram-positive bacteria including methicillin-resistant Staphylococci and *Clostridium* were discovered. The antibiotics were found to be new members of the glycopeptide antibiotic family, namely of the vancomycin type, based on chemical and spectroscopic data. The molecular weight of izupeptin A was determined to be 1,475 by SI (secondary ion)-MS (integer molecular weight, 1,473), and it contains two chlorine atoms.

In our earlier screening for specific inhibitors of cell wall peptidoglycan synthesis, which was based on lack of activity against *Mycoplasma* and selective inhibition of incorporation of [$^3$H]diaminopimeric acid (DAP) into acid-insoluble macromolecular fraction of growing *Bacillus* sp. ATCC 21206 (DAP$^-$) cells,[1,2] we found, among others, 15 strains producing high molecular weight inhibitors as estimated from the percentage of passage through an Amicon UM-2 membrane. After partial purification and comparison by HPLC of the active components of these 15 strains with known antibiotics mainly of the glycopeptide family, we found one strain (AM-5289) producing a new member of this group of antibiotics. Spectral and chemical of the purified substances, named izupeptins A and B, revealed a close relationship to the vancomycin-type.

The present paper deals with taxonomy of the izupeptin-producing strain, production, isolation and chemical and biological characterization of the antibiotics.

## Taxonomy of Izupeptin-producing Strain AM-5289

Morphological Characteristics: The vegetative mycelia grow well on both synthetic and organic agar media. Fragmentation of the vegetative mycelia was observed on some organic agar media. Mycelial fragmentation into a coccoid form was observed in submerged culture. Aerial mycelia were moderately produced on yeast extract - malt extract agar and inorganic salts - starch agar. However, sporulation of the aerial mycelia was immature even after three weeks of cultivation (Plate 1).

Whole Cell Analysis: Hydrolyzed whole cells contain *meso*-DAP, arabinose and galactose. This indicates that strain AM-5289 is an actinomycete of cell-wall type IV-A according to the classification of LECHEVALIER and LECHEVALIER.[3]

Based on the taxonomic properties described above, strain AM-5289 is considered to be related to genus *Nocardia*. Strain AM-5289 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name strain AM-5289 and the accession No. FERM P-8656.

Plate 1. Scanning electron micrograph of aerial mycelia (a) and vegetative mycelia (b) of strain AM-5289 grown on sucrose-nitrate agar for 3 weeks.
Bar marker represents 1 $\mu$m.

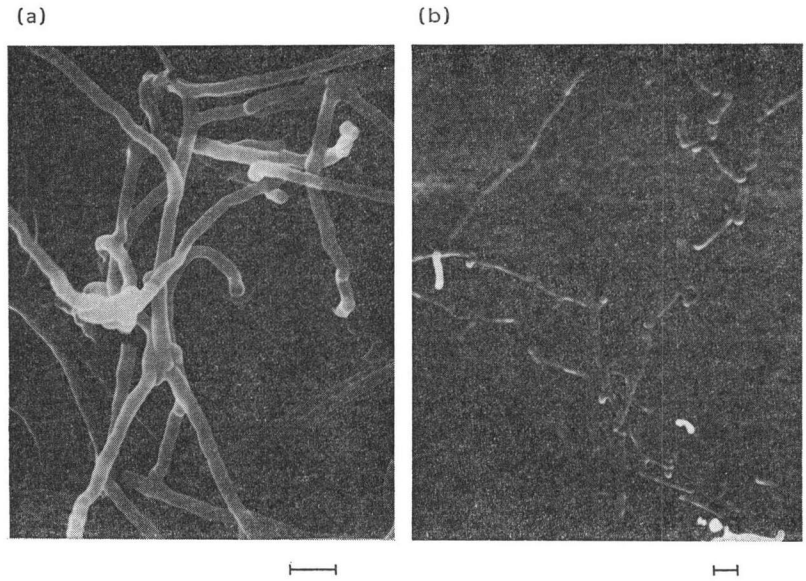
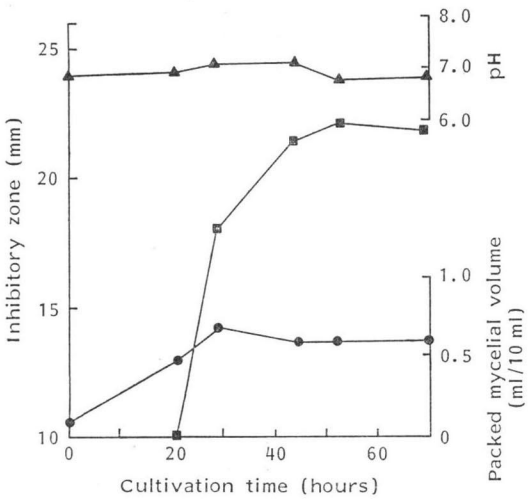



Fig. 1. A typical time course of izupeptin production.
▲; pH, ■; production, ●; mycelium.




## Production of Izupeptins A and B

A loopful of spores and mycelia of strain AM-5289 grown on an agar slant was inoculated in a 500-ml Sakaguchi flask containing 100 ml of a seed medium (glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0) and cultivated for 3 days at 27°C on a rotary shaker to give a seed culture. The seed culture (900 ml) was transferred into a 50-liter jar fermentor containing 30 liters of a production medium composed of starch 2%, soybean meal 1%, NaCl 0.3%, $CaCO_3$ 0.3% (pH 7.0) and was cultivated for 68 hours at 27°C with aeration of 15 liters/minute and with agitation of 250 rpm. The growth was measured as packed mycelial volume obtained by centrifuging 10 ml of culture broth at 2,000 rpm for 10 minutes. The activity of the culture broth was monitored by a paper disc method using *Bacillus subtilis* KB 27 (PCI 219) grown on DAVIS' minimal medium as test organism.

A typical time course of the production of izupeptins is given in Fig. 1. The growth gradually increased in the first 30 hours of fermentation and remained constant. Antibiotic production started 30 hours after inoculation and reached a maximum at 54 hours.

Table 1. Physico-chemical properties of izupeptins A and B.

| | Izupeptin A | Izupeptin B |
|---|---|---|
| MP (°C, dec) | 260~280 | 280~300 |
| $[\alpha]_D$ | −26° (25°C, *c* 0.1, $H_2O$) | −16° (27°C, *c* 0.1, $H_2O$) |
| Elementary analysis (%) | C 51.68, H 5.23, N 8.22, Cl 5.23 | — |
| SI-MS (*m/z*) | 1,474 $(M+H)^+$ | — |
| MW | 1,475 (integer MW: 1,473) | — |
| UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$) | 280 (38.0) | 280 (36.2) |
| IR $\nu_{max}^{KBr}$ $cm^{-1}$ | 3450, 2950, 1640, 1600, 1490, 1380, 1220, 1120, 1060, 1020 | 3450, 2980, 1660, 1600, 1480, 1410, 1240, 1140, 1070, 1040 |
| Amino acid analysis | Aspartic acid, unidentified amino acid | Aspartic acid, unidentified amino acid |

## Isolation of Izupeptins A and B

The fermented broth (30 liters) was centrifuged, and the supernatant (24 liters) was filtered using Celite. The filtrate was passed through a Diaion PA-416 ($OH^-$) column (4.5 liters). After washing with deionized water, the active principle was eluted with 0.5 N acetic acid. The active eluate was concentrated and subsequently lyophilized. The resulting crude powder was added to MeOH (200 ml) with vigorous stirring, and then centrifuged at 2,000 rpm for 10 minutes to give a precipitate (5.7 g). A portion (50 mg) of it was dissolved in 2 ml DMSO, loaded on a polyamide C-200 (180 ml) column, and developed with deionized water. The elution was monitored by TLC and detected by UV and bioautography against *Bacillus subtilis*. Two active components (A and B) were obtained. Further purification was performed by preparative HPLC on ODS column (YMC-324; 1.0×30 cm), using a 10~30% acetonitrile gradient system in 0.1 M ammonium formate (pH 7.3) at 254 nm. Two active peaks were obtained. The active eluates were concentrated and lyophilized to give white powders of izupeptins A and B, respectively. The purified izupeptins are amorphous solid substances which are slightly soluble in water or DMSO, but insoluble in common organic solvents.

## Physico-chemical Properties

The physico-chemical properties of izupeptins A and B are shown in Table 1. Izupeptins A and B displayed similar physico-chemical properties. Their UV spectra showed a maximum at 280 nm in neutral or acidic solution, which shifts to 304 nm under basic conditions. These results indicate the presence of phenolic groups in both compounds. The IR spectra demonstrate the existence of hydroxyl (3450 $cm^{-1}$), amide (A: 1640, B: 1660 $cm^{-1}$) and aromatic (A: 1600, 1490, 1380, B: 1600, 1480, 1410 $cm^{-1}$) functions.

The above spectral data indicate similarities to antibiotics of the glycopeptide-class exemplified by vancomycin,[4] ristocetin[5] and teicoplanin.[6]

In their field desorption (FD) or electron impact (EI) mass spectra, the molecular ion peak could not be observed because of their high molecular weights and/or high polarities, while the secondary ionization mass spectrometry (SI-MS) gave the $(M+H)^+$ ion peak of izupeptin A at *m/z* 1,474. That of B, however, could not be obtained, as the component contained some impurity. In the SI-MS spectrum of A, two characteristic fragment ion peaks, *m/z* 1,332 and 1,171, reflecting the structural feature were observed. It suggests that A contains two carbohydrate components. The ion peak pattern of the $(M+H)^+$ region clearly indicated that the antibiotics contain two chlorine atoms.[7]

Table 2. Antimicrobial spectra of izupeptins A and B.

| Test organism | MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | Izupeptins | | Vancomycin | Teicoplanin |
| | A | B | | |
| *Staphylococcus aureus* KB34 (FDA 209P) | 3.12 | 1.56 | 0.78 | 1.56 |
| *S. aureus* KB199 (MC, TC$^r$) | 3.12 | 1.56 | 1.56 | 0.78 |
| *S. aureus* KB238 (MRSA No. 1)* | 3.12 | 1.56 | 1.56 | 1.56 |
| *S. aureus* KB285 (MRSA No. 108)* | 3.12 | 1.56 | 1.56 | 0.78 |
| *Micrococcus luteus* KB212 (ATCC 9341) | 1.56 | 0.78 | 0.78 | 0.4 |
| *Bacillus subtilis* KB211 (ATCC 6633) | 0.78 | 0.78 | 0.4 | 0.4 |
| *B. megaterium* KB144 (IFO 12108) | 0.78 | 0.4 | 0.2 | 0.2 |
| *Mycobacterium smegmatis* KB42 (ATCC 607) | >100 | >100 | >100 | >100 |
| *Escherichia coli* KB213 (NIHJ) | >100 | >100 | >100 | >100 |
| *Pseudomonas aeruginosa* KB115 (IFO 3080) | >100 | >100 | >100 | >100 |
| *Clostridium perfringens* KB129 (ATCC 3624) | 12.5 | 6.25 | 3.12 | 0.4 |
| *C. difficile* KB258 (ATCC 9689) | 6.25 | 6.25 | 3.12 | 0.4 |
| *Bacteroides fragilis* KB169 (ATCC 23745) | 100 | 100 | 50 | 100 |
| *Peptococcus variabilis* KB235 (ATCC 14955) | 3.12 | 3.12 | 0.78 | 0.78 |
| *Fusobacterium varium* KB234 (ATCC 8501) | >100 | >100 | >100 | >100 |

* Methicillin-resistant strain.

### Biological Properties

The antimicrobial spectra of izupeptins A and B were determined by a conventional agar dilution method using Sensitivity Test Agar (Nissui) for aerobic bacteria and GAM Agar (Nissui) for anaerobic bacteria. The plates were incubated at 37°C for 20 hours. The antimicrobial activities of izupeptins A and B were compared with those of the known glycopeptide antibiotics vancomycin and teicoplanin.

As shown in Table 2, izupeptins A and B showed potent inhibitory activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* strains and some anaerobes, but slightly weaker than vancomycin and teicoplanin.

Izupeptins A and B showed no adverse effect when administered intraperitoneally to mice at 200 mg/kg.

## Discussion

In the course of our screening for new high molecular weight peptidoglycan synthesis inhibitors, we discovered new members of the glycopeptide family, named izupeptins A and B.

The SI-MS spectrum of component A revealed a molecular weight of 1,475 (integer molecular weight, 1,473), suggests that the antibiotics are related to vancomycin. Recently further vancomycin-related antibiotics, demethylvancomycin[8] and M43 series,[9] have been reported. However, izupeptin A can be differentiated from these antibiotics in molecular weight. The difference in structure is under investigation.

### Acknowledgments

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