PRODUCTION OF *N*-ACETYLISOPENICILLIN N AND ISOPENICILLIN N FROM *STREPTOMYCES TOKUNONENSIS* SP. NOV.

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N-Acetylisopenicillin N was isolated from the culture broth of *Streptomyces tokunonensis* sp. nov., and identified from ^{1}H NMR spectroscopy and release of L-α-aminoadipic acid by acid hydrolysis. In the course of this study, the specimen of penicillin N isolated from this strain was proved to contain a small amount of isopenicillin N by HPLC.

In the tripeptide theory[1] for the biosynthesis of penicillins and cephalosporins, isopenicillin N is considered to be an important intermediate. The production of isopenicillin N from *Penicillium chrysogenum* has been shown earlier[2]. The biosynthetic conversion of L-α-aminoadipyl-L-cysteinyl-D-valine into isopenicillin N has been shown in the cell-free system of *Cephalosporium acremonium*[3,4] and very recently the product of the cell-free reaction was isolated and directly identified by HPLC[5]. However, the production of isopenicillin N from *Streptomyces* species has not been reported.

We have reported the isolation of novel carbapenem antibiotics, asparenomycins A, B and C, from *Streptomyces tokunonensis* sp. nov. whose main product is penicillin N[6]. During the course of our studies for searching minor products as new active compounds from the fermentation products of this strain, we isolated *N*-acetylisopenicillin N and proved the occurrence of isopenicillin N by HPLC as will be described in this paper.

The majority of the active compounds in the fermentation products of *Streptomyces tokunonensis* sp. nov. were removed by successive adsorption and elution procedures using (1st) Amberlite IRA-68 (Rhom and Haas), (2nd) Diaion HP-20 (Mitsubishi Kasei) and then (3rd) an activated carbon, as described in the preceding paper[6]. Penicillin N was recovered from the non-adsorbed fraction in the 1st step (adsorption on IRA-68) by adsorption on a Dowex 1 column (Dow Chemical) and finally purified by HPLC on a Nucleosil 10 C_{18} column (Macherey-Nagel). The active compounds contained in the non-adsorbed fraction in the 3rd step (adsorption with an activated carbon) were recovered by adsorption on an HP-20 column. The crude powder obtained was fractionated by column chromatography with QAE Sephadex A-25 (Pharmacia Fine Chemicals) and then with HP-20 AG. When the active fraction obtained by the above was analyzed by the HPLC method described in the preceding paper[6], two unknown hydroxylamine-sensitive peaks (extinctive peaks by treatment with hydroxylamine) were observed. These were isolated by HPLC on a Nucleosil 10 C_{18} column. One was identified with *N*-acetylisopenicillin N as described below and the other was elucidated to be a new carbapenem antibiotic, desulfo-MM 4550*.

The sodium salt of *N*-acetylisopenicillin N is a colorless powder, soluble in water. It gives negative reaction to ninhydrin reagent. On paper electrophoresis with 50 mM phosphate buffer, pH 7.0 at 10 volt/cm for 2 hours, it migrated to the anode with Rm (relative mobility to penicillin N) 1.9. It shows

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Fig. 1. IR spectrum of *N*-acetylisopenicillin N (KBr).

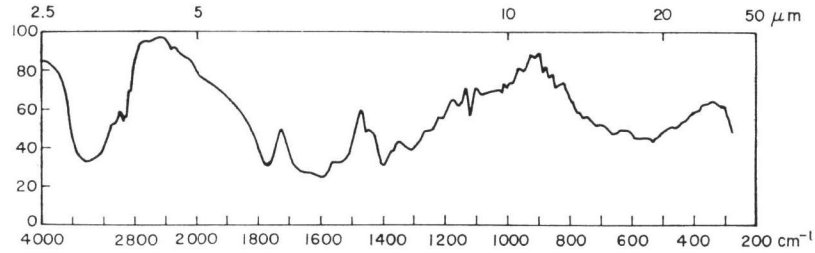
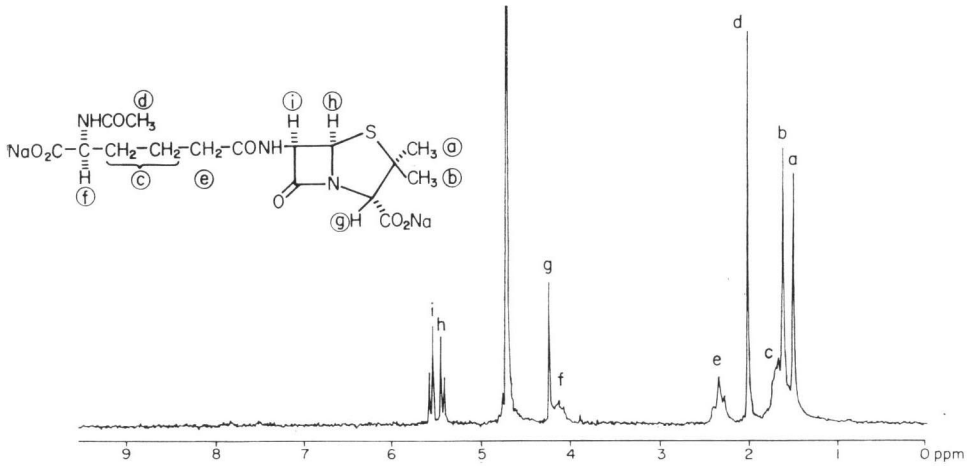


Fig. 2. ^{1}H NMR spectrum of *N*-acetylisopenicillin N (D_2O, internal reference DSS).



an end absorption in the UV spectrum and an absorption at 1772 cm^{-1} attributable to β-lactam in the IR spectrum (Fig. 1). The ^{1}H NMR spectrum was measured in D_2O and compared with that of penicillin N. Difference was observed in the appearance of signal d (*N*-acetyl group) and the shift of signal f to lower field (Fig. 2). The compound released approximately one mole of α-aminoadipic acid by acid hydrolysis. When the amino acid was L-leucylated and analyzed by HPLC[7], only the peak of L-leucyl-L-α-aminoadipic acid (LL) was observed (Fig. 4).

The main product of the strain, penicillin N, was isolated and purified to show a single peak in HPLC (Fig. 3). The specimen was *N*-acetylated with *p*-nitrophenyl acetate which has been known to produce negligible racemization. When the acetyl derivative was analyzed by HPLC, a minor peak identical with that of *N*-acetylisopenicillin N (natural product) was shown beside the peak of *N*-acetylpenicillin N (Fig. 3). The content of the minor peak was estimated to be approximately 5%. Furthermore, when the specimen was hydrolyzed and the chirality of the released α-aminoadipic acid was examined by the above method, a small peak of (LL) was shown beside the peak of (LD) (Fig. 4). These facts clearly indicated that the specimen of penicillin N isolated from the *Streptomyces* strain contained a small amount of isopenicillin N.

NEUSS *et al.* reported a method for separation of penicillin N and isopenicillin N by HPLC[5]. In the method these epimers were derivatized with a chiral compound, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate, before injection. In our case, only *N*-acetylation made it possible to separate these epimers by HPLC. It would be considered that the bulkiness of the acetyl group enhanced the

Fig. 3. HPLC of penicillin N, *N*-acetylpenicillin N and *N*-acetylisopenicillin N.

Column: Nucleosil 10 C_{18} (4×300 mm). Mobile phase: 20 mM Phosphate buffer, pH 7.0. Flow rate: 2.0 ml/minute. Chart speed: 5 mm/minute. Monitored at 220 nm.

1) The penicillin N specimen from *S. tokunonensis* sp. nov. 2) *N*-Acetylisopenicillin N (natural product). 3) *N*-Acetyl derivative of the penicillin N specimen from *S. tokunonensis* sp. nov.

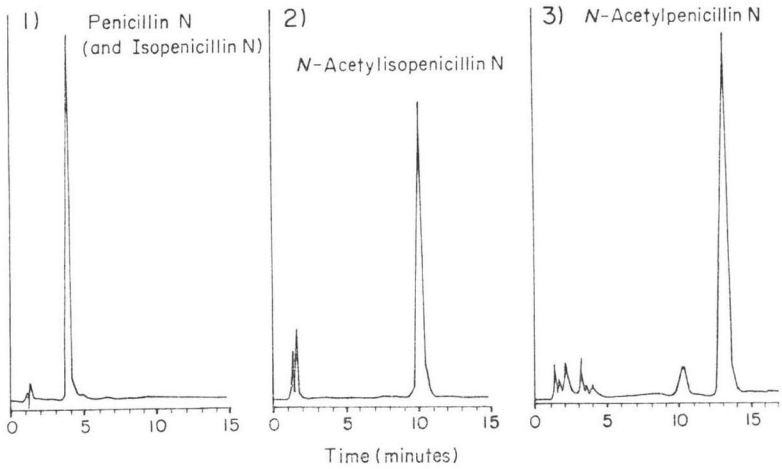
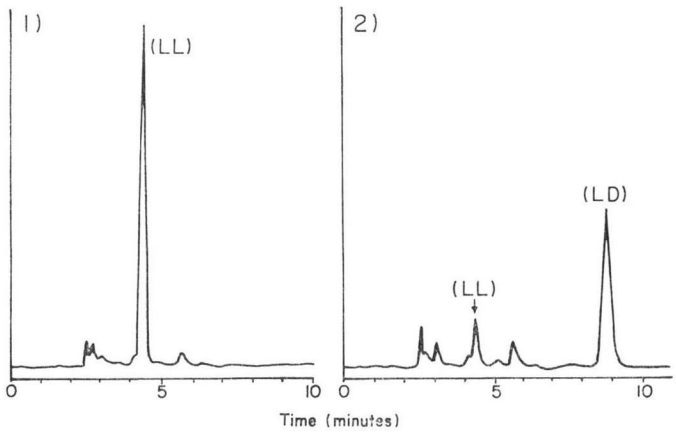


Fig. 4. HPLC of L-leucyl-α-aminoadipic acid (LL and LD).

Column: Nucleosil 10 C_{18} (4×300 mm). Mobile phase: 20 mM Phosphate buffer, pH 7.0. Flow rate: 1.0 ml/minute. Chart speed: 10 mm/minute. Monitored at 220 nm.

1) Preparation from *N*-acetylpenicillin N (natural product). 2) Preparation from the penicillin N specimen from *S. tokunonensis* sp. nov.



effect of the chirality of the molecules. Moreover, R. KONAKA of our laboratories deviced an improved HPLC method, by which penicillin N and isopenicillin N could be separated without any derivatization.*

Conclusively, we proved the presence of *N*-acetylisopenicillin N and isopenicillin N in the products of *S. tokunonensis* sp. nov. *N*-Acetylisopenicillin N is considered to be a shunt metabolite and isopenicillin N to be an intermediate compound before epimerization to penicillin N in the biosynthetic route in the *Streptomyces* strain. This may provide new evidence that the tripeptide theory also applies to *Streptomyces* species, and not only to fungal strains.

* Details will be published elsewhere by R. KONAKA.

Experimental

HPLC were obtained using a Waters 6000A pump, a Rheodyne model 7125 injector and a Japan Spectrooptics UVIDEC-100-II variable wave length UV spectrometer. ^{1}H NMR spectrum was recorded with a Varian XL-100-12A spectrometer.

Isolation of Penicillin N

Some 17 liters of the effluent in the adsorption procedure on an IRA-68 column with the culture filtrate of *S. tokunonensis* sp. nov. was passed through a Dowex 1×2 (Cl^-) column (1.3 liters) at pH 8.0. The column was eluted with 5% NaCl. Active eluate fractions by the pulp disk diffusion method on an assay plate of *E. coli* LS-1 (a super sensitive mutant to β-lactams) were passed through an HP-20 column (1.0 liter) at pH 5.0. The column was eluted with water and the active eluate was freeze-dried to give a crude powder (3.0 g) of penicillin N. The crude powder was chromatographed on a Pre PAK-500/C_{18} column of a High Speed Liquid Chromatograph System 500 (Waters Co., Ltd.) with 50 mM phosphate buffer, pH 7.0. The active fraction was desalted on an HP-20 column and freeze-dried to give a substantially pure preparation of penicillin N (250 mg).

A portion was further purified by HPLC on a Nucleosil 10 C_{18} column (10×300 mm) with 20 mM phosphate buffer, pH 7.0 to afford a preparation which showed a single peak on HPLC.

Isolation of *N*-Acetylisopenicillin N

To the non-adsorbed fraction in the adsorption procedure on an activated carbon (see text) from 100 liters of culture filtrate, NaCl was added to 5% concentration. The solution was passed through an HP-20 column (2.0 liters), and the column was eluted with water. The active eluate was freeze-dried to give a crude powder (*ca.* 10 g). The crude powder was chromatographed on a QAE Sephadex A-25 column (5.0×36.0 cm) with 0.5 M NaCl in 50 mM phosphate buffer, pH 7.0. Some 2.0 g portion was charged for a run. Two active fractions appeared. The former fraction was desalted on an HP-20 column and freeze-dried to give a powder (1.6 g), which gave two unknown hydroxylamine-sensitive peaks by the HPLC method (details of the method have been described in the preceding paper[6]). When the powder was chromatographed on a Sepacoal column (2.2×90 cm) packed with HP-20 AG (200~400 mesh) with 30 mM phosphate buffer, pH 7.0 (flow rate: 10 ml/minute, sample charge: 200 mg) monitored by a UV ditector at 220 nm, two fractions each of which caused the hydroxylamine-sensitive peak, were separated. The fast eluting fraction was desalted and freeze-dried to give a crude preparation of *N*-acetylisopenicillin N (40 mg). Finally it was purified by HPLC on a Nucleosil 10 C_{18} column (10×300 mm) with 20 mM phosphate buffer, pH 7.0. Desalination and freeze-drying of the peak fraction gave the sodium salt of *N*-acetylisopenicillin N (16 mg).

N-Acetylation

Some 2.0 mg of the specimen of penicillin N isolated and purified as in the preceding section was dissolved in dimethylformamide (100 μl) and water (40 μl). *p*-Nitrophenyl acetate (1.0 mg) and triethylamine (1.25 μl) were added, and the solution was stirred for 16 hours at 4°C. The reaction mixture was evaporated under reduced pressure to a residue, which was dissolved in a small amount of water and applied to a short column of a Diaion HP-20 AG. The column was eluted with water and the active eluate on an assay plate of *E. coli* LS-1 was freeze-dried to give a colorless powder, which was found to be a mixture of *N*-acetylpenicillin N (main) and *N*-acetylisopenicillin N (minor) by HPLC (Fig. 3).

Determination of Chirality of α-Aminoadipic Acid by HPLC

Some 2 mg of *N*-acetylisopenicillin N (natural product) or the penicillin N specimen isolated from the *Streptomyces* strain was hydrolyzed with constant boiling hydrochloric acid at 110°C for 16 hours. The hydrolysate was applied to a cellulose plate (Eastman Chromatogram Sheet) and developed with *n*-butanol - acetic acid - water (4: 1: 2). The zone of α-aminoadipic acid was extracted with water and was adsorbed on a short column of Dowex 50×8 (NH_4^+) at pH 2.0. Elution with 0.3 N NH_4OH followed by evaporation of the eluate gave a partially purified preparation of the amino acid, whose amount was estimated by automatic amino acid analysis.

L-Leucylation was carried out in essentially the same manner as in our previous publication[7]. The resulting preparations of L-leucyl-α-aminoadipic acid were subjected to HPLC and compared with the

authentic specimens of L-leucyl-L-α-aminoadipic acid (LL) and L-leucyl-D-α-aminoadipic acid (LD). The preparation derived from *N*-acetylisopenicillin N (natural product) exhibited a peak corresponding to (LL) and no peak of (LD). Whereas the preparation from the penicillin N specimen isolated from the *Streptomyces* strain showed a main peak corresponding to (LD) and a small peak of (LL) (Fig. 4).

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