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

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(Received for publication August 20, 1982)

N-Acetylisopenicillin N was isolated from the culture broth of Streptomyces tokunonensis sp. nov., and identified from 1H 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 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. 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 and very recently the product of the cell-free reaction was isolated and directly identified by HPLC. 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. 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. 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, 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

* Presented as a name, PA-31088-II, in Japan Kokai (Patent) 57-102,890, June 26, 1982
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 ¹H NMR spectrum was measured in D₂O 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-amino adipic acid by acid hydrolysis. When the amino acid was L-leucylated and analyzed by HPLC⁷, only the peak of L-leucyl-L-α-amino adipic 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-amino adipic 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 HPLC⁸. 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
effect of the chirality of the molecules. Moreover, R. Konaka of our laboratories devised 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. 1H 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 x 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/C18 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 C18 column (10 x 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 x 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 paper6)). When the powder was chromatographed on a Sepacoal column (2.2 x 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 detector 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 C18 column (10 x 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-aminoacidic acid was extracted with water and was adsorbed on a short column of Dowex 50 x 8 (NH4+) at pH 2.0. Elution with 0.3 N NH4OH followed by evaporation of the eluate gave a partially purified preparation of the amino acid, whose amount was estimated by automatic amino acid analysis.

1-Leucylation was carried out in essentially the same manner as in our previous publication7). The resulting preparations of 1-leucyl-α-aminoacidic acid were subjected to HPLC and compared with the
authentic specimens of L-leucyl-L-α-amino adipic acid (LL) and L-leucyl-D-α-amino adipic 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).

References