DEACETYLATION OF PS-5, A NEW β-LACTAM COMPOUND*

I. MICROBIAL DEACETYLATION OF PS-5

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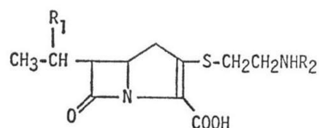
PS-5 was deacetylated to NS-5 (deacetylated PS-5) by L-amino acid acylase from porcine kidney and D-amino acid acylase from *Streptomyces olivaceus* but not by L-amino acid acylase from *Aspergillus* sp. Using PS-5, N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine as substrates, acylase producers were screened among facultative methanol-assimilating bacteria. Most of the microbes tested were active and could be classified into two groups of L-acylase producers and L- & D-acylase producers. *Pseudomonas* sp. 1158 which deacetylated the three substrates was chosen for further study. Cells of the bacterium entrapped in polyacrylamide gel and its acylase activities immobilized on DEAE-Sephadex were found to be useful for conversion of PS-5 to NS-5.

Compound PS-5, 3-(2-acetamidoethyl) thio-6-ethyl-7-oxo-1-azabicyclo [3. 2. 0] hept-2-ene-2-carboxylate (Fig. 1) is a new β-lactam antibiotic of the olivanic acid family with potent β-lactamase inhibitory properties[1,2]. As thienamycin[3] (Fig. 1) was antimicrobially more active against some bacteria than acetylthienamycin[4], deacetylated PS-5, 3-(2-aminoethyl) thio-6-ethyl-7-oxo-1-azabicyclo [3. 2. 0] hept-2-ene-2-carboxylate (abbreviated hereafter as NS-5; Fig. 1) seemed to be an interesting therapeutic agent as well as a useful intermediate compound for biological and chemical modification.

So-called "facultative methanol-assimilating bacteria" are a group of Gram-negative organisms which have been isolated for utilization of methanol to produce single-cell protein[5]. In spite of decisive difference from *Pseudomonas* in the utilization pattern of carbon sources, they are tentatively included in *Pseudomonas*[6]. KAMEDA *et al.* reported that some pseudomonads which, although taxonomically unidentified, apparently belonged to *Pseudomonas aeruginosa* deacylated various N-acyl-L- and D-amino acids by action of L- and D-amino acid acylase activities[7].

The present paper describes deacetylation of PS-5 to NS-5 by L-amino acid acylase from porcine kidney and D-amino acid acylase from *Streptomyces olivaceus*[8] but not by L-amino acid acylase from *Aspergillus* sp.[10] Based on these findings, PS-5-decetylating microbes were screened with PS-5 and N-chloroacetylamino acids among facultative methanol-assimilating bacteria. *Pseudomonas* sp. 1158 which deacetylated PS-5, N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine was chosen for further study. Lyophilized cells and the crude extract of the organism were immobilized for conversion of PS-5 to NS-5.

Fig. 1. Structures of PS-5, NS-5 and thienamycin.



PS-5	R_1=H	R_2=$COCH_3$
NS-5	R_1=H	R_2=H
Thienamycin	R_1=OH	R_2=H

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Materials and Methods

Materials

D-Amino acid acylase from *Streptomyces olivaceus*[8] (a preparation obtained from sonicate by ammonium sulfate fractionation) was a kind gift of Drs. SUGIE and SUZUKI, Fermentation Research Institute, Chiba, Japan. L-Amino acid acylases from porcine kidney (acylase I; catalogue No. A3010)[9] and from *Aspergillus* sp. (Amano 1500)[10] were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. and Amano Pharmaceutical Co., Nagoya, Japan, respectively.

PS-5 sodium salt was prepared in our laboratories and was checked for purity by u.v. spectrophotometry (ε at 301 nm, pH 7.0=8687; $A_{301}/A_{245}=3.6$; $\Delta\varepsilon$ at 301 nm, pH 7.0, before and after hydroxylamine treatment=8120)[2], paper chromatography and high voltage paper electrophoresis (h.v.p.e.)[1].

N-Chloroacetyl-L-phenylalanine (catalogue No. C1503), N-chloroacetyl-D-valine (catalogue No. C2003) and N-acetyl-DL-methionine (catalogue No. A1626) were obtained from Sigma Chemical Co. Other reagents were of analytical grade.

Deacetylation of PS-5 to NS-5 by L- and D-amino acid acylases

Reaction mixture containing 5 μl of *ca.* 100 mM PS-5 (35 mg/ml), 35 μl of enzyme and 10 μl of 0.25 M potassium phosphate buffer, pH 7.4, in a total volume of 50 μl was incubated at 30°C for 1 ~ 6 hours and rapidly cooled to a temperature of 0 ~ −5°C in ice-methanol. Two microliters of the solution was applied on Whatman filter paper No. 1 and was subjected to h.v.p.e. at pH 8.6 for 40 minutes. NS-5 and PS-5 were qualitatively assayed by bioautography with *Staphylococcus aureus* FDA 209P.

Cultivation of bacteria

Original medium for facultative methanol-assimilating bacteria consisted of 20 g glucose, 8 g Pharmamedia and 5 ml of corn steep liquor in 1 liter of tap water (pH 7.0). As insoluble particles in the medium were inconvenient for cell collection, a hot decoction (100°C for 30 minutes) of the medium was employed throughout the present study.

Facultative methanol-assimilating bacteria employed in the screening were stock cultures of our laboratories[5] and were grown at 28°C for 90 hours under shaking (throw 7 cm; 220 r.p.m.) in 100 ml Erlenmeyer flasks containing 20 ml of the decoction medium. Cell growth was densitometrically measured at 610 nm.

Acylase screening

Cells were collected from one flask of culture by centrifugation (10,000 r.p.m. for 10 minutes), washed in a small volume of saline by centrifugation and suspended in 1.5 ml of 0.02 M potassium phosphate buffer, pH 7.4. The cell suspension was sonicated with a 20 KHz ultrasonic oscillator (60 W; Tomy Seiko Co., Ltd., Tokyo, Japan) at 0°C for a total period of 1 minute (4 × 15-second oscillations with 30-second cooling periods interposed) and centrifuged at 10,000 r.p.m. for 30 minutes to give a clear supernatant solution. The content of protein was spectrophotometrically measured by the method of KALB, Jr. *et al.*[11]

PS-5, N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine were dissolved in distilled water and adjusted to pH 6.5 ~ 7.5. Reaction mixture containing 0.1 ml of the enzyme solution, 0.1 ml of the substrate solution and 0.05 ml of 0.25 M potassium phosphate buffer, pH 7.4, in a total volume of 0.25 ml was incubated under shaking at 30°C for 1 hour. Final concentrations of PS-5, N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine were 4, 20 and 20 μmoles/ml respectively.

Assay procedures

(1) NS-5 and PS-5

NS-5 and PS-5 in assay samples were separated by h.v.p.e. and paper chromatography before bioautography under the following conditions:

H.v.p.e.: 1,500 V/30 cm for 40 minutes on Whatman filter paper No. 1 at I 0.027 in Veronal buffer, pH 8.6 (sodium Barbital 5 g, Barbital 1.2 g, distilled water 1,000 ml)

NS-5 moved 0.5 cm toward the cathode and PS-5, 4.0 cm toward the anode.

Paper chromatography: on Toyo filter paper No. 50 in a solvent system of acetonitrile - 0.1 M Tris-HCl buffer, pH 7.5 - 0.1 M ethylenediamine tetraacetate, pH 7.5 (120: 30: 1)

Rf was 0.22 for NS-5 and 0.38 for PS-5. The two β-lactam compounds were located by bioautography with *Comamonas terrigena* IFO 12685 and *Staphylococcus aureus* FDA 209P. The latter detector organism was useful for preferential bioassay of NS-5, as NS-5 was about ten times more active against it than PS-5.

(2) L-Phenylalanine, D-valine and DL-methionine

An aliquot (2~5 μl) of a sample solution to be assayed was spotted on a silica gel thin-layer chromatographic (t.l.c.) plate (Pre-Coated TLC Plate Silica Gel 60 F-254; E. Merck, Darmstadt) and developed for 2 hours in a solvent system of *n*-butanol - acetic acid - water (4: 1: 2). After the solvent was removed by evaporation, the t.l.c. plate was treated with 0.02 M ninhydrin in water-saturated *n*-butanol and then heated at 80°C for 10 minutes. The color intensity of the amino acid spots was read with a Shimadzu chromatoscanner CS-900 (assay wavelength 500 nm, reference wavelength 700 nm; slit width and height 1.25 mm; scan speed 10 mm/minute by zig-zag scanning; Shimadzu Seisakusho Ltd., Kyoto, Japan), and compared with the known amounts of the authentic amino acids (0.2~2 nmoles/spot).

Immobilization on DEAE-Sephadex of L- and D-amino acid acylase activities from *Pseudomonas* sp. 1158

Wet cells (about 1.6 g) of the organism were sonicated under the above-described conditions for 10 minutes and then centrifuged for 30 minutes to give 80 ml of crude enzyme solution. The enzyme solution was mixed with about 50 g (wet weight) of DEAE-Sephadex A-50 which had been equilibrated in 0.01 M potassium phosphate buffer, pH 7.4, and the mixture was gently agitated at 5°C for 5 hours and allowed to stand overnight at 5°C[12]. The DEAE-Sephadex was collected by filtration and washed with 1 liter of distilled water.

The acylase-DEAE-Sephadex gel (1 g, wet weight) was mixed with 0.1 ml of 100 mM PS-5 in 0.05 M potassium phosphate buffer, pH 7.4, and 0.2 ml of 0.25 M potassium phosphate buffer, pH 7.4. The mixture was incubated at 30°C under mild agitation for the indicated periods of time and the amounts of NS-5 and PS-5 were assayed by h.v.p.e. followed by bioautography.

Entrapment of *Pseudomonas* sp. 1158 cells

Cells of the bacterium were harvested from 2 liters of the broth and air-dried on a Petri dish at room temperature. The cell cake was ground to powder in a mortar with a pestle and the powder was completely dehydrated over P_2O_5 in a desiccator to give about 50 mg of the dry cells.

The method of MORI *et al.*[13] was employed for entrapping. The dry cells were suspended in 4 ml of 0.1 M potassium phosphate buffer, pH 7.4, and mixed with 750 mg of acrylamide monomer and 40 mg of N,N′-methylenebisacrylamide. After 0.5 ml of 5% β-dimethylaminopropionitrile in 5% potassium persulfate was added, the mixture was covered with distilled water and incubated at 37°C for 30 minutes. After polymerization, the gel block was cut into small pieces in ice water, washed in distilled water and in 0.01 M potassium phosphate buffer, pH 7.4, and then treated for 30 seconds in 10 ml of the said buffer with a Waring blender. The gel particles were recovered by filtration and washed in the same buffer to yield about 9 g (wet weight) of the entrapped cells of *Pseudomonas* sp. 1158.

The entrapped cells were suspended in 0.05 M potassium phosphate buffer, pH 7.4, and packed in a jacketed column (inner diameter 15 mm, length 100 mm) which was kept at 30°C by circulating warm water through the jacket. PS-5 solution (10 mM) in the said buffer was passed through the column at a flow rate of 1.8 ml/hour for 24 hours, while the effluent was collected in 0.6 ml fractions under cooling with ice-water. The conversion rate of PS-5 to NS-5 was estimated by h.v.p.e.-bioautography with *Staphylococcus aureus* FDA 209P and *Comamonas terrigena* IFO 12685.

Results

Deacetylation of PS-5 to NS-5 by Known L- and D-Amino Acid Acylases

Three available types of amino acid acylase were tested for conversion of PS-5 to NS-5. Fig. 2 shows the results of h.v.p.e.-bioautography assay.

D-Amino acid acylase from *Streptomyces olivaceus* and L-amino acid acylase from porcine kidney catalysed the PS-5 deacetylation to NS-5, whereas L-amino acid acylase from *Aspergillus* sp. could not convert PS-5 to NS-5 even at a high concentration of 130 mg/ml.

Screening for PS-5-Deacetylating Bacteria

Since PS-5 and other olivanate family β-lactam compounds are fairly labile under physiological conditions[14] and susceptible to some types of β-lactamase[2], their quantitative assay is very difficult particularly in screening. In addition, NS-5 is more unstable than PS-5 because of the presence of the free amino group,

Fig. 2. Deacetylation of PS-5 to NS-5 by known L- and D- amino acid acylases.

PS-5 (10 mM) and amino acid acylases were incubated at pH 7.4 and 30°C for 3 hours and 2 μl of the reaction mixtures were analysed for NS-5 by h.v.p.e. followed by bioautography with *Staphylococcus aureus* FDA 209P.

A: PS-5; B: NS-5; C: L-amino acid acylase from porcine kidney; D: L-amino acid acylase from *Aspergillus* sp.; E: D-amino acid acylase from *Streptomyces olivaceus*.

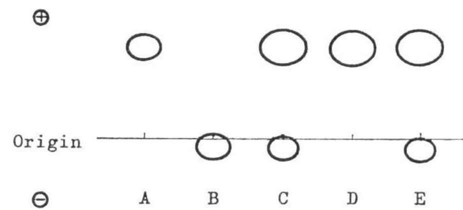


Table 1. Screening for acylase producers.

Strain	Substrate			Strain	Substrate		
	PS-5	N-ClAc-L-Phe*	N-ClAc-D-Val**		PS-5	N-ClAc-L-Phe*	N-ClAc-D-Val**
Pseudomonas sp. 2	−	−	−	724	−	+	−
35	++	+++	++	740	±	+	±
37	++	+++	++	767	+++	+++	+++
45	++	+++	+++	789	+	++	−
58	+	++	+	823	+	++	±
205	−	+	−	1158	+++	++	++
291	±	+	−	N-3	−	±	−
317	±	+	−	N-4	−	+	−
434	+	++	−	N-10	±	++	−
437	+	++	++	N-12	−	±	−
465	−	+	−	O-7	++	+++	++
470	++	+++	++	*Pseudomonas aminovorans* NCIB 9039	++	+++	++
476	++	+++	+++	*Pseudomonas extorquens* NCIB 9399	±	+	−
488	++	+++	++	*Pseudomonas* sp. AM1 NCIB 9133	±	+	−
530	+	+	−	*Pseudomonas* sp. M-27 NCIB 9686	±	+	−
532	+	++	±	*Protaminobacter ruber* ATCC 8457	±	++	−
539	+	++	−	M159-1	±	++	−
617	++	+++	++				
670	++	+++	++				
674	−	−	−				
679	+	++	±				
696	+	++	++				

Activity was visually determined on bioautograms and t.l.c. plates relative to the highest activity observed (*Pseudomonas* sp. 1158 for PS-5-deacetylase activity; *Pseudomonas aminovorans* for L-amino acid acylase activity; *Pseudomonas* sp. 45 for D-amino acid acylase activity).

− no activity; ± less than 10%; + 10∼25%; ++ 25∼50%; +++ 50∼100%

* N-Chloroacetyl-L-phenylalanine

** N-Chloroacetyl-D-valine

as is the case in thienamycin and acetylthienamycin. Thus the sole use of PS-5 as the assay substrate was assumed to lead to false assay results. To overcome this disadvantage, N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine were also employed as presumably more sensitive and stable substrates for deacetylation.[9] The results of screening among facultative methanol-assimilating bacteria are summarized in Table 1.

Among 39 strains of facultative methanol-assimilating bacteria tested, 22 showed apparent conversion of PS-5 to NS-5. This table clearly indicates the presence of a significant relationship between the PS-5-deacetylating activity and the amino acid acylase activities. It is noteworthy that all the potent microbes (37 strains) had the L-amino acid acylase activity, whereas the D-amino acid acylase activity was detected in 18 out of 37.

As a relatively large amount of information was available on *Pseudomonas* sp. 1158 in our laboratories, the strain was chosen for further study as a representative organism for deacetylation of PS-5 to NS-5.

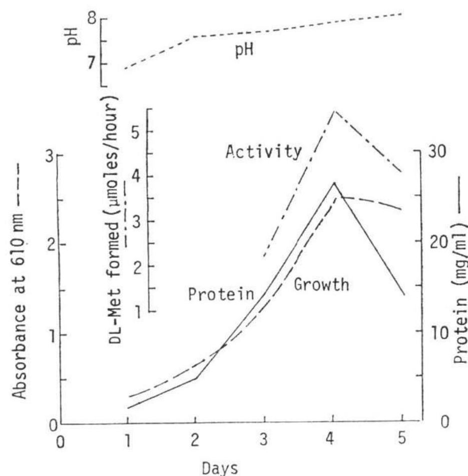
Time Course of Amino Acid Acylase Production by *Pseudomonas* sp. 1158

Pseudomonas sp. 1158 was cultured in 20 ml of the decoction medium and the cells were harvested at the indicated times. The activities of L- and D-amino acid acylase in the sonicates were collectively assayed with N-acetyl-DL-methionine. Time course of DL-amino acid acylase formation in cells of *Pseudomonas* sp. 1158 is shown in Fig. 3.

The bacterium grew relatively slowly to reach a maximum at 4 days of cultivation where the peaks of protein and amino acid acylase activity were observed.

Fig. 3. Time course of the growth and the amino acid acylase activity of *Pseudomonas* sp. 1158.

Pseudomonas sp. 1158 was cultivated in a 100-ml flask containing 20 ml of the decoction medium. At the indicated times of incubation, the cell density was measured at 610 nm and the protein content in the sonicate by the method of KALB, Jr. and BERNLOHR. The amino acid acylase activity was collectively determined with N-acetyl-DL-methionine.

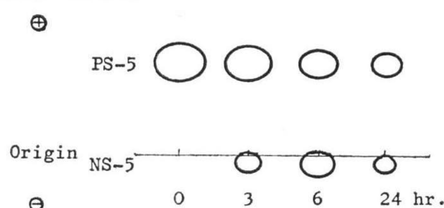


Conversion of PS-5 to NS-5 by the DEAE-Sephadex-Immobilized Amino Acid Acylase Activities and the Gel-Entrapped Cells of *Pseudomonas* sp. 1158

For future large-scale preparation of NS-5, adaptability of the L- and D-amino acid acylase activities from *Pseudomonas* sp. 1158 was ex-

Fig. 4. Conversion of PS-5 to NS-5 by the L- and D-amino acid acylase activities immobilized on DEAE-Sephadex.

PS-5 and the L- and D-amino acid acylase-DEAE-Sephadex complex were incubated at pH 7.4 and 30°C for the indicated periods of time. NS-5 and PS-5 were analysed by h.v.p.e.-bioautography with *Staphylococcus aureus* FDA 209P. NS-5 was about 10-fold more active than PS-5 against this detector microbe.



amined by using the L- and D-amino acid acylase activities adsorbed on DEAE-Sephadex and the cells entrapped in polyacrylamide gel.

Fig. 4 presents the time course of NS-5 formation by the DEAE-Sephadex-immobilized amino acid acylase. Qualitative h.v.p.e.-bioautography assay indicated that the amount of NS-5 increased with the period of reaction time upto 6 hours. However it did not seem to correspond with the amount of PS-5 consumed, probably because NS-5 was unstable under the specified reaction conditions. At 24 hours when a substantial amount of PS-5 still remained, the quantity of NS-5 was found to be less than at 6 hours, which might suggest low affinity of the amino acid acylase to PS-5.

When PS-5 was passed through a column of the gel-entrapped cells at a flow speed of 1.8 ml/hour, the approximate conversion rate of NS-5 from PS-5 was estimated to be 10% in fractions from 15 to 60 by h.v.p.e. followed by bioautography.

Discussion

A number of thienamycin-related β-lactam antibiotics have been reported from several laboratories.[1,3,14~18] Although their biosynthetic relationship is little known at present[18], the possible involvement of amino acid acylase might be expected, particularly because some of them are produced by *Streptomyces olivaceus*[19]. PS-5 and other olivanate derivatives, on the other hand, seem to be an interesting biochemical tool for the study of the molecular mechanism of action of amino acid acylase, since the N-acetylamino group of PS-5 is optically inactive and N-acetylamines are reported to be insusceptible to the enzyme.[9]

Facultative methanol-assimilating bacteria which are proved to be economically important for production of single-cell protein have so poorly been characterized that their taxonomical position is not yet determined.[5,6] It is very apparent that this group of bacteria should be distinguished from *Pseudomonas*, since the latter is defined to be "able to use other than single-carbon compounds as sole source of carbon"[6]. In spite of this fact, they are tentatively included in *Pseudomonas* (Addenda to the genus *Pseudomonas*, Addendum I)[6]. In this paper, about a half of the bacterial strains tested except two negative cultures showed the L- and D-amino acid acylase activities, while the rest showed the L-amino acid acylase activity only (Table 1). Together with the enzymological studies of KAMEDA *et al.* on acylase from *Pseudomonas*[7], this might be one of the important criteria for the future taxonomical definition of facultative methanol-assimilating bacteria.

As NS-5 is more labile than PS-5, an enzymological method was assumed to be superior to a chemical process for the preparation of NS-5. Similar to known amino acid acylases, the amino acid acylase activities from *Pseudomonas* sp. 1158 could successfully be immobilized on DEAE-Sephadex and entrapped in polyacrylamide gel. Contrary to our expectation, the conversion of PS-5 to NS-5 was found to be small and very slow (Fig. 4). Moreover the decomposition of NS-5 and, less significantly, PS-5 seemed far more than what was assumed from non-specific breakdown. As will be detailed in a subsequent paper, the L- and D-amino acid acylase activities from *Pseudomonas* sp. 1158 are accompanied by peptidase activities which might be involved in the rapid breakdown of NS-5 and PS-5 during incubation.

In the following papers, the L- and D-amino acid acylase activities from *Pseudomonas* sp. 1158 will be separated and enzymologically characterized.

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