STUDIES ON THE BIOSYNTHESIS OF CARBAPENEM ANTIBIOTICS
II. ISOLATION AND FUNCTIONS OF A SPECIFIC ACYLASE INVOLVED IN
THE DEPANTOTHENYLATION OF THE OA-6129 COMPOUNDS

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A specific acylase designated A933 acylase was isolated and purified to 90% protein
homogeneity from Streptomyces fulvoviridis A933 17M9 which produces PS-5, epithienamycins A and C and MM 17880 together with minor carbapenem analogs, penicillin N and cephamycin C. This enzyme was found to catalyze the depantothenylation of OA-6129 carbapenems; the acyl exchange of OA-6129 carbapenems with acyl CoA's; the deacetylation of N-acetyl-L-amino acids; and the acylation of NS-5 and 6-aminopenicillanate with acyl CoA's, whereas the deacetylation of PS-5 and N-acetyl-α-amino acids; and the decylation of benzylpenicillin and cephalosporin C were not observed. Similar enzyme activities were also detected in Streptomyces cattleya, Streptomyces cremeus subsp. auratilis and Streptomyces argenteolus which are all carbapenem producers.

In the previous paper, the successful isolation of a blocked mutant (mutant 1501) from Streptomyces fulvoviridis A933 17M9 indicated that OA-6129A, B1, B2 and C are the direct precursors for PS-5, epithienamycins A and C and MM 17880, respectively, and that a specific acylase tentatively designated A933 acylase is responsible for the depantothenylation of the OA-6129 group of carbapenems. The further characterization of this acylase was expected to reveal its central role in the biosynthesis of carbapenems.

This paper describes the isolation and purification of A933 acylase from mycelia of S. fulvoviridis A933 17M9, and the enzymological characterization of functions of the acylase. The detection of the OA-6129A-depantothenylating activity and the L-amino acid acylase activity in Streptomyces cattleya, Streptomyces cremeus subsp. auratilis and Streptomyces argenteolus suggests that some enzymes similar to A933 acylase are probably involved in the biosynthesis of carbapenems by these streptomycetes.

Materials and Methods

Streptomycetes
The following carbapenems-producing streptomycetes were employed in the present study: S. fulvoviridis A933 17M9, S. cattleya NRRL 8057, S. cremeus subsp. auratilis ATCC 31358 and S. argenteolus ATCC 11009.

Materials
PS-5, OA-6129A, OA-6129B1, OA-6129B2 and OA-6129C (all as sodium salts) were prepared by fermentation as detailed in previous papers. NS-5 was obtained from PS-5 by enzymological deacetylation with pseudomonal amino acid acylases. Other antibiotics such as benzylpenicillin, cephalosporin C, 6-aminopenicillanate and 7-aminopenicillosporanate were from commercial sources. Acyl CoA's, N-acetyl-L-amino acids and N-acetyl-α-amino acids were purchased from Sigma Chemical Co.
Cultivation of *S. fulvoviridis* A933 17M9 and the Purification of A933 Acylase

Mature spores of *S. fulvoviridis* A933 17M9 from ISP-2 agar medium were inoculated in a 250-ml Erlenmeyer flask containing 15 ml of medium composed of glycerol 8%, soya bean meal 3%, fish meal 1%, K₂HPO₄ 0.2%, MgSO₄·7H₂O 0.2% and CaCO₃ 0.3%. Thirty flasks were cultured at 28°C for 3 days on a rotary shaker. The mycelia were harvested by centrifugation, rinsed twice in physiological saline and suspended in 80 ml of 0.01 M potassium phosphate, pH 7.1. The mycelium suspension was kept frozen until use.

Unless stated otherwise, the following purification procedure for A933 acylase was carried out at 0°C, using 0.01 M potassium phosphate buffer, pH 7.1. The frozen mycelium suspension was thawed and subjected to intermittent sonication for 5 minutes at 20 KHz (60 watt). After centrifugation for 15 minutes at 10,000 rpm, powdered ammonium sulfate was added in the supernate to 60% saturation under agitation and stirred for a further 30 minutes. The precipitate was collected by centrifugation at 10,000 rpm for 20 minutes and dissolved in 5 ml of the buffer. The protein solution was dialyzed twice against 8 liters each of the buffer and then charged on a DEAE-Sephacel column (OH⁻ form; 20 × 400 mm; previously equilibrated with the buffer). After washing with 250 ml of the buffer, the column was eluted with a linear concentration gradient of sodium chloride from 0.1 M (400 ml) to 0.4 M (400 ml) in the buffer. The fraction weight was 5 g. After the L-amino acid acylase assay [see assay (4)], fraction Nos. 80 to 120 were combined and concentrated to 5 ml in a Collodion bag 25 (Sartorius GmbH). The concentrate was dialyzed against 4 liters of the buffer and applied on a DEAE-Sephadex A-50 column (15 × 150 mm) which had been equilibrated with the buffer. Following thorough washing, a linearly-increasing concentration gradient of sodium chloride from 0.1 M (150 ml) to 0.4 M (150 ml) in the buffer was applied to the column. The enzyme activity from fraction No. 62 to No. 85 (3 g/fraction) was again concentrated by treatment with a Collodion bag 25, dialyzed against 4 liters of the buffer and then subjected to preparative isoelectric focusing. The gel electrophoretogram was sliced at 8 mm-intervals transversely to the running direction and numbered from cathode to anode. Each slice was macerated in 2 ml of 1 M phosphate, pH 7.1, and poured into a small glass column. The column was eluted with the buffer and assayed for the L-amino acid acylase activity. Slice Nos. 11 to 13 contained the enzyme activity. The active eluates were combined, concentrated with a Collodion bag 25 and then passed through a Sephadex G-150 column (19 × 600 mm; equilibrated with the buffer). Fraction Nos. 24 to 29 (3 g/fraction) were collected, concentrated in a Collodion bag 25 and again passed through the same Sephadex G-150 column. Fraction No. 26 was concentrated in a Collodion bag 25 to give a final preparation of A933 acylase (1 ml).

Preparation of Cell-free Homogenates of *S. cattleya*, *S. cremese subsp. auratilis* and *S. argenteolus*

These streptomycetes were grown as described in previous papers and the mycelia which were harvested by refrigerated centrifugation were sonicated as specified above. After centrifugation, the supernates were used for enzymological studies without further purification.

Gel Electrophoreses

1. Preparative Isoelectric Focusing: According to the manufacturer's recommendation, a granulated polyacrylamide gel plate containing 2% Ampholine (pH 4~6) (LKB-Produkter AB) was prepared and the enzyme was electrophoresed for 20 hours at 200 V/30 cm on a Bio-Rad Flat Bed Electrophorator 1405.

2. Disc Gel Electrophoresis: The disc gel electrophoresis was carried out by the method of Davis, except that the spacer gel was replaced by 40% sucrose containing the enzyme which was applied directly on the coarse gel. Bromophenol blue was used as marker dye. The electrophoretic conditions were as follows: electric current 2 mA/5 × 50 mm at pH 8.3; 30 minutes at 4°C. After electrophoresis, the disc was longitudinally sliced into halves. One half of the disc was stained with Coomassie Brilliant Blue R for visualization of protein bands, and the other half was sliced into 1 mm sections which were used for location of the L-amino acid acylase activity. Each gel slice was incubated for 60 minutes at 37°C with 50 μl of 0.2 M potassium phosphate, pH 7.1, and 50 μl of 100 mM N-chloroacetyl-l-phenylalanine. After boiling for 5 minutes, 5 μl of the reaction solution was spotted on a silica gel TLC plate for amino acid analysis.
Assays

(1) Bio-autography for the Antimicrobial Activity and the Pantothenate Content: The methods and procedures as detailed in the previous paper1 were employed.

(2) Protein Content: The protein concentration was measured spectrophotometrically by the method of KALB, Jr. and BERNFLOHR.8

(3) Depantothenylation Activity: For the routine assay of the depantothenylation activity, OA-6129A was used as substrate. A reaction mixture containing 20 µl of 0.2 M phosphate, pH 7.4, 20 µl of 500 µg/ml OA-6129A and 20~60 µl of an enzyme solution was incubated at 37°C for 1~4 hours. An aliquot (5~10 µl) of the reaction mixture was applied on Whatman No. 1 filter paper and subjected to high voltage paper electrophoresis (1,500 V/30 cm; pH 8.6) for 40 minutes at 0~4°C. Carbapenems on the electrophoretogram were located by bio-autography using Comamonas terrigena B-996 or Staphylococcus aureus FDA 209P.

(4) N-Acetylamino Acid-deacetylating Activity: N-Chloroacetyl-L-phenylalanine was the assay substrate. A reaction mixture containing 10 µl of 0.2 M phosphate, pH 7.4, 20 µl of 100 mM N-chloroacetyl-L-phenylalanine (previously adjusted to pH 7.5) and 20 µl of an enzyme solution was incubated at 37°C for 15 minutes. After boiling for 1 minute, an aliquot (1~5 µl) of the reaction mixture was spotted on a silica gel TLC plate and developed in a solvent system of 1-BuOH - AcOH - H2O (4:1:1). The L-phenylalanine formed was visualized with ninhydrin and quantified by densitometric scanning.

N-Acetyl-L- and D-methionines, N-acetyl-L- and D-alanines, N-acetyl-L- and D-leucines, N-acetyl-L- and D-valines and N-acetyl-L- and D-phenylalanine were tested under the same assay conditions as described above for N-chloroacetyl-L-phenylalanine.

(5) Acyl Exchange Activity: A reaction mixture composed of 20 µl of 500 µg/ml OA-6129A, 20 µl of 5 µmol/ml acyl CoA, 10 µl of 0.2 M phosphate, pH 7.4, and 10 µl of A933 acylase was incubated at 37°C for 3 hours. An aliquot (10 µl) of the reaction mixture was spotted on Whatman No. 1 filter paper and electrophoresed for 40~60 minutes at 1,500 V/30 cm and pH 8.6. The electrophoretogram was visualized by bio-autography using Comamonas terrigena B-996. The assay results were also confirmed by descending paper chromatography.8

(6) PS-5-deacetylating Activity: Forty microliters of 1 mg/ml PS-5, 20 µl of 0.2 M phosphate, pH 7.4, and 40 µl of A933 acylase were mixed and incubated at 37°C for 3 hours. The production of NS-5 was detected by high voltage paper electrophoresis at pH 8.6 followed by bio-autography using Staphylococcus aureus FDA 209P.

(7) Penicillin and Cephalosporin-deacetylating Activity: A reaction mixture contained 20 µl of 20 mg/ml benzylpenicillin or cephalosporin C, 20 µl of 0.2 M phosphate, pH 7.4, and 60 µl of A933 acylase. After 3 hours of incubation at 37°C, the reaction mixture was analyzed for the production of 6-aminopenicillanate or 7-aminocephalosporanate at pH 1.8, followed by bio-autography with C. terrigena B-996.

(8) 6-Aminopenicillanate and 7-Aminocephalosporanate-acylating Activity: A mixture composed of 10 µl of 2 mg/ml sodium 6-aminopenicillanate or 7-aminocephalosporanate, 20 µl of 5 µmol/ml acyl CoA, 10 µl of 0.2 M phosphate, pH 7.4, and 20 µl of A933 acylase was allowed to react for 3 hours at 37°C. The reaction products were analyzed by high voltage paper electrophoresis at pH 1.8 or pH 4.5, followed by bio-autography.

(9) NS-5-acylating Activity: Fifty microliters of 100 µg/ml NS-5, 20 µl of 0.2 M phosphate, pH 7.4, 20 µl of 10 µmol/ml acetyl CoA and 10 µl of A933 acylase were mixed and incubated for 3 hours at 37°C. The formation of PS-5 was detected by high voltage paper electrophoresis and bio-autography using C. terrigena B-996.

Results and Discussion

Isolation and Purification of A933 Acylase

A preliminary characterization test revealed that A933 acylase possessed an L-amino acid acylase
activity which seemed to be parallel with the OA-6129A-depantothenylating activity. As the OA-6129 group of carbapenem antibiotics were not available in sufficient quantity and are fairly labile under usual reaction conditions, N-chloroacetyl-L-phenylalanine was employed as assay substrate for quick and sensitive analysis of the enzyme activity throughout the purification procedure. The validity of activity fractionation depending on the L-amino acid acylase activity was doubly checked one day later by assay of the OA-6129A-depantothenylating activity.

Purity of the A933 acylase preparation was examined by disc gel electrophoresis. The gel electrophoretogram was longitudinally sliced into halves which were used for the location of the enzyme activity and for the protein staining with Coomassie Brilliant Blue R, respectively. Densitometry indicated that the enzyme preparation was more than 90% homogeneous (Rm against bromophenol blue=0.57); and that a minor band at Rm 0.74 had no enzyme activity as far as the L-amino acid acylase activity and the OA-6129A-depantothenylating activity were concerned. This enzyme prepara-
Enzymological Functions of A933 Acylase

OA-6129 Carbapenems-depantetheinylating Activity

To ascertain that A933 acylase is the sole enzyme responsible for the depantetheinylating step, we have treated OA-6129A, OA-6129B1, OA-6129B2 and OA-6129C with the A933 acylase preparation and analyzed the reaction products by high voltage paper electrophoresis followed by bio-autography. The results of analysis are presented in Fig. 1. Because of the hydroxysulfonyloxy group at C-8, the depantetheinylated OA-6129C moves to the anode, while the depantetheinylated OA-6129A, B1 and B2 do to the cathode.

It is apparent from Fig. 1 that depantetheinylation by A933 acylase occurs independent of the structural difference in the C-6 side chain.

Amino Acid Acylase Activity

L-Amino acid acylase from porcine kidney (acylase I) catalyzes the depantetheinylation of OA-6129A.1 The stereospecific selectivity of A933 acylase was examined with several N-acetyl-L- and D-amino acids by silica gel TLC analysis. Fig. 2 shows that the amino acid acylase activity of A933 acylase is unambiguously specific to the L-type amino acids.

As is observed in the deacetylation of PS-5 by L-amino acid acylases from porcine kidney and Pseudomonas sp. 1158 and by D-amino acid acylases from Streptomyces olivaceus and Pseudomonas sp. 1158,6 the stereo-specificity of the L-amino acid acylase activity of A933 acylase seems not to be related to the stereo-chemical structure of the C-3 pantetheinyl side chain.
Acyl Exchange Activity with Acyl CoA's

It is well known that penicillin acylase produces 6-aminopenicillanic acid from penicillins in the absence of acyl CoA's, whereas the coexistence of acyl CoA's with penicillins results in the acyl exchange, giving new penicillin derivatives. Thus OA-6129A was incubated with A933 acylase in the presence of acyl CoA's. Fig. 3 illustrates the results of high voltage paper electrophoresis followed by bio-autography.

A933 acylase converts OA-6129A to PS-5 in the presence of acetyl CoA. Furthermore, glutaryl CoA, for example, provides a new NS-5 derivative which has glutaryl at the terminal amino group of the C-3 aminoethylthio side chain. Accordingly the formation of MM 27696 which has an n-propyl-aminoethenylthio side chain at C-3\(^\text{39}\) can be ascribed to the availability of a substantial amount of n-propionyl CoA instead of acetyl CoA during the acyl exchange reaction. In this context, the biogenesis of non-acylated carbapenems such as thienamycin\(^\text{3}\) NS-5\(^\text{10}\) 8U-207\(^\text{12}\) northienamycin\(^\text{11}\) and 8-epi-thienamycin\(^\text{11}\) may be explained by an insufficient supply of acyl CoA's and/or the presence of a different type of acylase.

Deacetylation of PS-5 to NS-5

The reaction mechanism of deacetylation of PS-5 to NS-5 by L- and D-amino acid acylases\(^\text{8}\) is
still inexplicable, but seems to depend on the molecular peculiarity of carbapenems. In spite of its N-acetyl-l-amino acid-deacetylating activity, A933 acylase could not deacetylate PS-5 to NS-5 (Fig. 4).

Considered from the finding that S. fulvoviridis A93317Mg normally produces acetylated carbapenems such as PS-5, epithienamycins A and C and MM 17880 (but not deacetylated counterparts), the inability of A933 acylase to deacetylate PS-5 to NS-5 is understandable. If A933 acylase had a PS-5-deacetylating activity, NS-5 would usually be detected in fermentation broths of S. fulvoviridis A933 17M9 even with a sufficient supply of acetyl CoA.

Deacylation of Penicillin and Cephalosporin
A933 acylase showed no so-called penicillin acylase activity on benzylpenicillin and cephalexin C.

Acylation of 6-Aminopenicillanate and 7-Aminocephalosporanate with Acyl CoA's
In the presence of A933 acylase and acyl CoA's, 6-aminopenicillanate was converted to new penicillin derivatives, whereas 7-aminocephalosporanate was not found to be an acyl acceptor (Fig. 5). Thus the acylation of the amino group seems to be limited to 6-aminopenicillanate and carbapenems having the C-3 aminoethylthio side chain.

Acylation of Deacylated Carbapenems with Acyl CoA's
As is anticipated from the acyl exchange of OA-6129 carbapenems and the acylation of 6-aminopenicillanate with acyl CoA's, A933 acylase also catalyzed the acylation of deacylated carbapenems with acyl CoA's (Fig. 6). As 6-aminopenicillanate and deacylated carbapenems are good substrates, the structural requirement for acyl receptors might be seen in $\text{H}_2\text{N} - \text{C} - \text{C} - \text{S} - \text{C} - \text{C} - \text{COOH}$. 

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Fig. 4. No deacetylation of PS-5 by A933 acylase.

![Diagram of deacylation reaction](image)

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Fig. 5. Conversion of 6-aminopenicillanate to new penicillin derivatives.

![Diagram of acylation reaction](image)

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Fig. 6. Catalysis of acylation of deacylated carbapenems with acyl CoA's.
Similar Acylase Activities in Other Carbapenems-Producing Streptomycetes

For further confirmation of the key role of the depantothenylation activity in the biosynthesis of carbapenems, the mycelia of *S. cattleya*, *S. cremeus* subsp. *auratilis* and *S. argenteolus*, which are all carbapenems-producing streptomycetes were sonicated and centrifuged to give their cell-free homogenates. The homogenates were examined for the OA-6129A-depantothenylation, the acyl exchange of OA-6129A, the deacetylation of N-acetylamino acids, the deacetylation of PS-5 and the acylation of 6-aminopenicillanate. Table 1 summarizes the results.

![Diagram](image)

**Table 1.** Functions of acylases from carbapenems-producing streptomycetes.

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<th><em>S. ful.</em></th>
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<td>Depantothenylation of OA-6129A</td>
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<td>Acyl exchange of OA-6129A</td>
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<td>Deacetylation of PS-5</td>
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<td>Acylation of 6-aminopenicillanate</td>
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It is noteworthy that all the carbapenems-producing streptomycetes exhibit the OA-6129A-depantothenylating activity, although the acyl exchange of OA-6129A and the acylation of 6-amino-penicillicinate were not observed in the last two species, presumably because of insufficient purity of the enzyme preparations employed. Despite repeated trials, we have not yet succeeded in purification of the corresponding acylase from S. cattleya. Compared with A933 acylase, the S. cattleya enzyme appears very labile and did not survive the purification procedure used for A933 acylase. After ammonium sulfate fractionation, the OA-6129A-depantothenylation activity became very labile and was lost quickly.

References


