STUDIES ON THE BIOSYNTHESIS OF CARBAPENEM ANTIBIOTICS

I. BIOSYNTHETIC SIGNIFICANCE OF THE OA-6129 GROUP OF CARBAPENEM COMPOUNDS AS THE DIRECT PRECURSORS FOR PS-5, EPITHIENAMYCINS A AND C AND MM 17880

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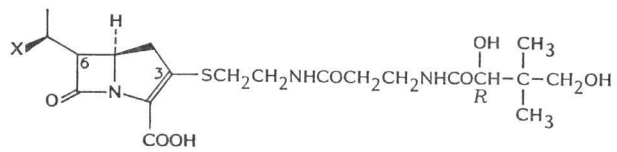
Based on the working hypothesis that the OA-6129 group of carbapenem compounds might be the direct precursors for PS-5, epithienamycins A and C and MM 17880, *Streptomyces fulvoviridis* A933 17M9, a producer of PS-5, PS-6, PS-7, PS-8, epithienamycins A, B, C and D, MM 17880, MM 13902 and MM 4550, was subjected to NTG-mutation to provide a blocked mutant numbered 1501 which was found to produce OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C instead of PS-5, epithienamycins A and C and MM 17880, respectively. In a cell-free system, the parent strain demonstrated an ability to convert OA-6129A to NS-5, whereas the mutant did not. The L- and D-amino acid acylases were also shown to depantothenylate the OA-6129 group of carbapenems.

Carbapenems are a recently-discovered group of β-lactam antibiotics which are characterized by an exceptionally broad spectrum of potent antimicrobial activity and a strong β-lactamase-inhibitory property in comparison with conventional penicillins and cephalosporins.

There are 46 naturally-occurring carbapenem and carbapenam compounds reported in the literature, but their biosynthetic relationship is obscure.[1] The OA-6129 group of carbapenem and carbapenam antibiotics are distinct from other carbapenem and carbapenam compounds in the C-3 pantetheinyl side chain (Fig. 1).[2] When the chemical structures of the OA-6129 carbapenem compounds were elucidated by YOSHIOKA *et al.*,[3] we considered it useful to examine the possibility that they might be the biosynthetically-direct precursors for PS-5, epithienamycins A and C and MM 17880, as the chemical difference between the OA-6129 group and the other compounds is seen only in the C-3 side chain; and the involvement of pantothenate in the biogenesis of gramicidins was well

Fig. 1. Chemical structures of OA-6129 carbapenems.



X	C_6	Compound
H	*R*	OA-6129A
OH	*R*	OA-6129B_1
OH	*S*	OA-6129B_2
OSO_3H	*R*	OA-6129C

documented by LIPMANN.[4] To prove this working hypothesis, we have attempted to obtain an OA-6129 carbapenems-producing mutant from a known carbapenems-forming streptomycete.

The present paper reports the isolation of a blocked mutant (mutant 1501) which yields OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C, from *Streptomyces fulvoviridis* A933 17M9[5], the parent strain which produces PS-5, PS-6, PS-7, PS-8, epithienamycins A, B, C and D, MM 17880, MM 13902 and MM 4550 together with penicillin N and cephamycin C. The site of metabolic blockage in mutant 1501 was located in the step of depantothenylation of the OA-6129 compounds. The depantothenylation of the OA-6129 group of carbapenems was also found to be catalysed by L- and D-amino acid acylases.

Materials and Methods

Antibiotics and Enzymes

Antibiotics: OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C were prepared as sodium salts by fermentation of *Streptomyces* sp. OA-6129 followed by column chromatography as detailed previously.[2] PS-5, PS-6, PS-7, PS-8, epithienamycins A, B, C and D, MM 17880, MM 13902 and MM 4550 were produced by fermentation of *S. fulvoviridis* A933 17M9 as described in previous papers.[6~8] NS-5 was obtained from PS-5 by deacetylation with a mixture of L- and D-amino acid acylases of *Pseudomonas* sp. 1158.[9]

Enzymes: The L- and D-amino acid acylases of *Pseudomonas* sp. 1158 were prepared as reported in previous papers.[9,10] The following enzymes were obtained from commercial sources: Acylase I (Sigma A3010, porcine kidney); peptidase (Sigma P7625, porcine intestinal mucosa); kidney acetone powder (Sigma K7250, porcine, type II); L-amino acid acylase (Amano acylase 15000 from *Aspergillus* sp.).

Physico-chemical Analyses

The routine product analysis of the OA-6129 and other groups of carbapenems in fermentation broths was carried out by paper chromatography and silica gel thin-layer chromatography (TLC).[11,12] For resolution of NS-5, non-sulfated carbapenems and sulfated carbapenems, high voltage paper electrophoresis described in a previous paper[9] was employed. The identity of a detected component with a reported carbapenem was confirmed by high performance liquid chromatography.[7]

Biological Analyses

Bio-autography: The bio-assay procedure using *Comamonas terrigena* B-996[5] was employed for detection of antimicrobially-active carbapenem components.

Pantothenate Assay: *Saccharomyces carlsbergensis* ATCC 9080 was employed for bio-assay of pantothenate according to the method of BIRD *et al.*[13] The assay agar plate with or without L-amino acid acylase was contacted for 10~30 minutes with a chromatogram or an electrophoretogram and incubated overnight at 28°C. The presence of pantothenate was visualized as a growth zone.

Mutagenesis and Fermentations

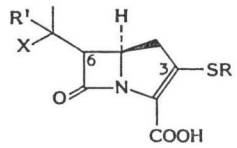
Mature spores of *S. fulvoviridis* A933 17M9 were suspended in 0.1 M Tris-maleate buffer (pH 8.5) and passed through a No. 3 glass filter. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to a final concentration of 1 mg/ml and the spore suspension was incubated at 28°C for 60 minutes under gentle shaking. The spores were recovered by centrifugation, washed in physiological saline and spread on ISP-2 agar. After incubation at 28°C for 4~5 days, colonies were transferred on ISP-2 agar slants and cultured for 2 weeks.

Fermentation media and conditions for production of the OA-6129 and other groups of carbapenems were largely identical with those detailed in previous papers.[2,5~8]

OA-6129A-depantothenylating Reaction

Forty microliters of 1,000 μg/ml OA-6129A sodium salt, 20 μl of 0.2 M phosphate buffer, pH 7.5, and 40 μl of an enzyme solution were mixed and incubated at 30°C for 120 minutes. The reaction mixture was subjected to high voltage paper electrophoresis followed by bio-autography.

Fig. 2. Carbapenem compounds produced by *S. fulvoviridis* A933 17M9.



R′	X	C_6	SR	Compound
H	H	*R*	$SCH_2CH_2NHCOCH_3$	PS-5
CH_3	H	*R*	$SCH_2CH_2NHCOCH_3$	PS-6
H	H	*R*	$SCH{=}CHNHCOCH_3$	PS-7
CH_3	H	*R*	$SCH{=}CHNHCOCH_3$	PS-8
H	OH	*S*	$SCH_2CH_2NHCOCH_3$	Epithienamycin C
H	OH	*S*	$SCH{=}CHNHCOCH_3$	Epithienamycin D
H	OH	*R*	$SCH_2CH_2NHCOCH_3$	Epithienamycin A
H	OH	*R*	$SCH{=}CHNHCOCH_3$	Epithienamycin B
H	OSO_3H	*R*	$SCH_2CH_2NHCOCH_3$	MM 17880
H	OSO_3H	*R*	$SCH{=}CHNHCOCH_3$	MM 13902
H	OSO_3H	*R*	$S(O)CH{=}CHNHCOCH_3$	MM 4550

Strains 17M9 (parent) and 1501 (blocked mutant) were grown at 28°C for 3 days in 15 ml of medium composed of glycerol 8%, soybean meal 3%, fish meal 1%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.2% and $CaCO_3$ 0.3% (pH 7.1). The mycelia were collected by centrifugation and washed in physiological saline. The cells were suspended in 5 ml of 0.01 M phosphate buffer, pH 7.5; frozen; thawed; and then sonicated intermittently for 5 minutes at 20 KHz (60 watt) under cooling in ice water. The supernate was used as an enzyme solution.

Results and Discussion

Product Analysis of *S. fulvoviridis* A933

Except for *Streptomyces cattleya* which is the producer of the thienamycin group of carbapenems,[14] it seems likely that carbapenems-producing streptomycetes reported in the literature have an ability to produce all the other groups of carbapenems and carbapenams, and that the final composition of carbapenem and carbapenam products in fermentation broths depends largely on the fermentation conditions. As the number of carbapenems which had to be considered was more than 30, it was necessary to first examine the carbapenems in the fermentation broth of *S. fulvoviridis* A933 17M9, a parent strain for this mutation study. The detailed examination of fermentation broths of *S. fulvoviridis* A933 17M9 by paper chromatography, silica gel TLC, high voltage paper electrophoresis and high performance liquid chromatography showed the production of substantial amounts of PS-5, epithienamycins A and C and MM 17880; and small or trace amounts of PS-6, PS-7, PS-8, epithienamycins B and D, MM 13902 and MM 4550 (Fig. 2) together with considerable amounts of penicillin N and cephamycin C.

Although other carbapenems such as pluracidomycins, carpetimycins and asparenomycins[1] could not be detected, further alteration of the fermentation conditions (for example, fermentation pH, temperature, aeration and medium composition) results in production of such carbapenems by *S. fulvoviridis* A933 17M9.

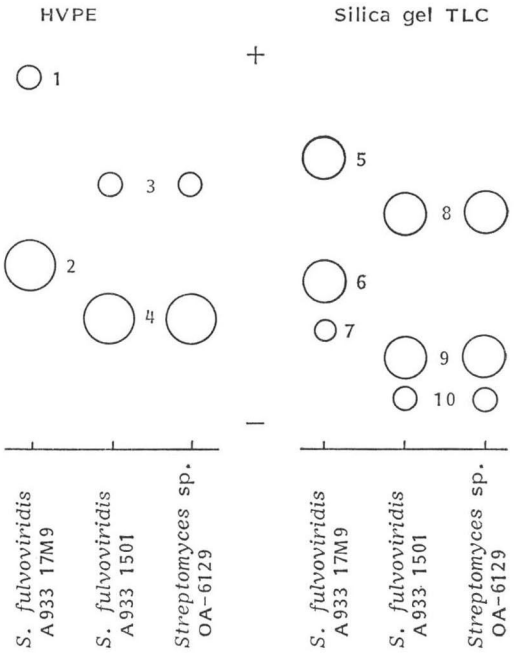
NTG-Mutation of *S. fulvoviridis* A933 17M9

To prove our working hypothesis that OA-6129 A, B_1, B_2 and C are direct precursors for PS-5, epithienamycins A and C and MM 17880, respectively, *S. fulvoviridis* A933 17M9 was subjected to NTG-mutagenesis and the isolated colonies were fermented and analyzed by silica gel TLC. From about ten thousand colonies, a mutant numbered 1501 was obtained. This mutant, the parent strain and *Streptomyces* sp. OA-6129 were fermented in various media and analyzed by paper chromatography, silica gel TLC, high voltage paper electrophoresis and high performance liquid chromatography, followed by bio-autography for antimicrobial activity and pantothenate. Fig. 3 presents the results of product analysis by high voltage paper electrophoresis and silica gel TLC.

Fig. 3. Comparison of carbapenem components produced by *S. fulvoviridis* A933 17M9 and 1501 and *Streptomyces* sp. OA-6129.

HVPE: High voltage paper electrophoresis.

1 MM 17880, MM 13902, MM 4550; 2 PS-5, 6, 7 & 8, epithienamycins A, B, C & D; 3 OA-6129C; 4 OA-6129A, B_1 & B_2; 5 PS-5, 6, 7 & 8; 6 epithienamycins A, B, C & D; 7 MM 17880, MM 13902, MM 4550; 8 OA-6129A; 9 OA-6129B_1 & B_2; 10 OA-6129C.



As the bio-autographic pattern of mutant 1501 was identical with that of *Streptomyces* sp. OA-6129, the fermentation products of mutant 1501 were critically analyzed, showing that the mutant produces OA-6129A, B_1, B_2 and C instead of PS-5, epithienamycins A and C and MM 17880, respectively. No minor carbapenem compounds such as PS-6, PS-7, PS-8, epithienamycins C and D, MM 13902 and MM 4550 could be detected. It is noteworthy that not even a trace of PS-5, epithienamycin A, epithienamycin C or MM 17880 was detected in the fermentation broths of mutant 1501, indicating that, as expected from the proposed map of metabolic conversion,[1] the biosynthesis of carbapenems proceeds from the OA-6129 group to PS-5, epithienamycins A and C and MM 17880; that the same reaction is involved in the conversion from the OA-6129 group to PS-5, epithienamycins A and C and MM 17880; and that the supposed genetic blockage ascribable to the accumulation of OA-6129 carbapenems is not leaky in the mutant. If more than one pathways were operative in this step of conversion, any one of the said then-known carbapenems would be found in the mutant.

Depantothenylation of OA-6129A by *S. fulvoviridis* A933 17M9 and Known L- and D-Amino Acid Acylases

Although no plausible interpretation has yet been presented for the susceptibility of PS-5 to various L- and D-amino acid acylases,[10] carbapenem compounds seem to behave as unusual substrates for various enzymes.[10,15,16] In this context, OA-6129A was incubated with L- and D-amino acid acylases, peptidase and kidney acetone powder (Fig. 4).

OA-6129A is not affected by porcine peptidase, but is susceptible to L- and D-amino acid acylases

Fig. 4. Depantothenylation of OA-6129A by various enzyme preparations.

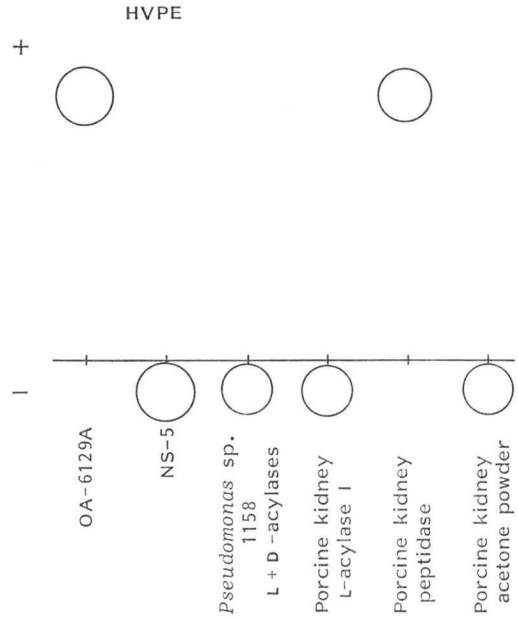
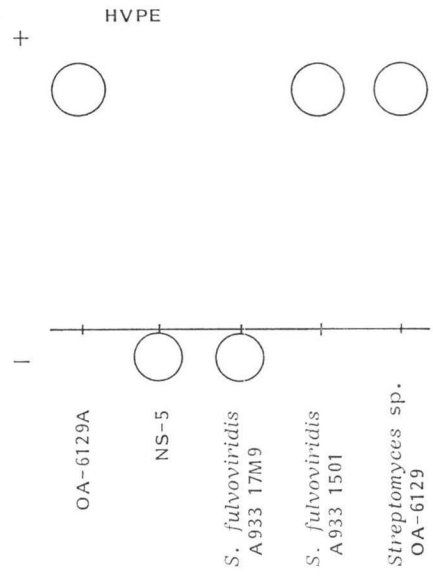


Fig. 5. Depantothenylation of OA-6129A with cell homogenates of *S. fulvoviridis* A933 17M9 and 1501 and *Streptomyces* sp. OA-6129.



and kidney acetone powder, providing NS-5 and pantothenate. As was discussed in a previous paper,[17] PS-5 is deacetylated to NS-5 by L- and D-amino acid acylases; and the characteristic deacetylation activity and stereospecificity of these enzymes are valid for *N*-acyl-amino acids only. They can also attack dipeptides and tripeptides without apparent stereospecific selectivity, suggesting that their deacetylating ability can be regarded as a distorted form of peptidase activity. Thus it is not surprising that OA-6129A is converted to NS-5 and pantothenate by these acylases.

Amidases from rat, horse and pig kidneys are known to attack the amide bond of pantetheine, giving cysteamine and pantothenate.[18~21] According to DUPRE and CAVALLINI,[19] the reduced form of pantetheine is the best substrate for horse kidney amidase (or "pantetheinase"), while WITTWER *et al.*[21] suggest that acyl-*S*-pantetheine might be an active or better substrate for pig kidney amidase. The susceptibility of OA-6129A to hog kidney acetone powder suggests the possibility that the responsible activity of the amidase (or pantetheinase) is identical with the *N*-deacylation activity of L-amino acid acylase (or acylase I).

Based on the above findings with amino acid acylases, the cell homogenates of *S. fulvoviridis* A933 17M9, mutant 1501 and *Streptomyces* sp. OA-6129 were incubated with OA-6129A and the reaction mixtures were subjected to high voltage paper electrophoresis. Bio-autographic analyses for antimicrobial activity and pantothenate show that only the parent strain can depantothenylate OA-6129A to NS-5, whereas the mutant and *Streptomyces* sp. OA-6129 have no such activity (Fig. 5). In other words, the NTG-mutation of *S. fulvoviridis* A933 17M9 yielded the blocked mutant 1501 which is defective in OA-6129A-depantothenylating activity.

For convenience, the OA-6129A-depantothenylating activity of *S. fulvoviridis* A933 17M9 has tentatively been designated A933 acylase. In the following paper, the functions of A933 acylase will be described.

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