MUTAGENESIS OF OA-6129 CARBAPENEM-PRODUCING BLOCKED MUTANTS AND THE BIOSYNTHESIS OF CARBAPENEMS

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Streptomyces fulvoviridis A933 17M9 1501 is an A933 acylase-defective mutant derived from *S. fulvoviridis* A933 17M9 and thus produces the OA-6129 group of carbapenems and carbapenams. By further mutation of mutant 1501, 4 types of mutants (OA-6129 A+B1+B2 producers; OA-6129 A+B2 producers; an OA-6129 A producer; non-producers) were obtained. The second type of mutant strains 4N 3607, 5NA 3949-40 and 5NE 252 proved useful for the fermentative production of carbapenem OA-6129 B2. These results of mutagenesis demonstrated that the sequence of carbapenem bioconversion in the horizontal route was hydroxylation at C-8→isomerization at C-6→sulfation at C-8 hydroxyl.

Carbapenems are a novel class of β-lactam compounds that possess an extremely wide spectrum of potent antimicrobial activity against Gram-positive and Gram-negative bacteria. Many carbapenem-producing microbes have been reported to date which can be classified into three groups of microorganisms (unicellular bacteria, *Streptomyces cattleya*, and other streptomycetes), based on their microbiological characteristics and product analyses. This paper is concerned with the last group of carbapenem-producing streptomycetes, and more particularly, with *Streptomyces fulvoviridis*.[1~3]

As carbapenems have more chiral centers than penicillins and cephalosporins, the production of a clinically useful carbapenem derivative will probably be carried out more economically by employing a naturally occurring carbapenem compound as a starting material rather than by total synthesis. OA-6129 B2 was chosen as a carbapenem intermediate useful for chemical derivation.[4] Presently available information of carbapenem biosynthesis, however, indicates that carbapenem-producing streptomycetes other than *S. cattleya* are capable of forming 42 carbapenem and carbapenam compounds.[3,4] It is not practical to attempt to preferentially produce one carbapenem component, such as PS-5, among the 42 known compounds simply by manipulating fermentation conditions. A promising approach to production of OA-6129 B2 is to utilize specifically-blocked mutants which accumulate OA-6129 B2 as a single major product. As *S. fulvoviridis* A933 17M9 1501 produces only the OA-6129 group of carbapenems and carbapenams among the 42 known compounds,[5] it was chosen as the parent strain for mutagenic preparation of OA-6129 carbapenem-producing blocked mutants in the present study.

This paper describes isolation of 4 types of blocked mutants obtained by mutagenesis of *S. fulvoviridis* A933 17M9 1501. Results of product analysis in these mutants allowed us to conclude that the horizontal sequence of bioconversion among the OA-6129 group of carbapenems is as follows: OA-6129 A is hydroxylated at C-8 to form OA-6129 B2 (type III mutant); OA-6129 B2 is isomerized at C-6 to give OA-6129 B1 (type II mutants); and OA-6129 B1 is sulfated at the C-8 hydroxyl group

to reach OA-6129 C (type I mutants). Also obtained were mutants producing none of the above compounds (non-producers). Type II blocked mutants which produce OA-6129 A and B2 were found useful for preferential fermentative production of OA-6129 B2 under highly aerated conditions in jar and tank fermentors.

Materials and Methods

Streptomycetes

S. fulvoviridis A933 17M9 1501 is an A933 acylase-defective mutant derived from *S. fulvoviridis* A933 17M9 and was employed as the parent strain.[5] All other strains used in this study were produced from *S. fulvoviridis* A933 17M9 1501 by mutation; and had no A933 acylase activity.

Growth Conditions and Culture Media

Liquid Cultivation: Spores grown on complete agar medium (CAM) were inoculated into 30 ml of seed medium (SE-4) in a 250-ml Erlenmeyer flask and grown at 28°C for 2 days on a rotary shaker (7-cm throw, 220 rpm). One ml of seed culture was transferred into 10 ml of liquid production medium (LPM) in a 250-ml Erlenmeyer flask and cultivated at 28°C for 4 days on a rotary shaker.

Solid Cultivation: Colonies grown on CAM were transferred onto plugs (5~10 mm in diameter ×5 mm in height) of solid production medium (SPM) and incubated at 28°C for 4~5 days under humid conditions.

CAM contained (%) glycerol 0.4, malt extract 1.0, yeast extract 0.4 and agar 2.0 in tap water (pH 7.0). SE-4 was composed of (%) beef extract 0.3, Bacto-Tryptone 0.5, glucose 0.1, soluble starch 2.4, yeast extract 0.5, calcium carbonate 0.5 and soybean meal 0.5 in tap water (pH 7.0). LPM had the following composition (%) glycerol 8.0, soybean meal 3.0, Formosan dry yeast 1.0, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, calcium carbonate 0.3, $CoCl_2 \cdot 6H_2O$ 0.0005 and antifoam KM-75 (Shin-Etsu Chemical Co., Ltd.) 0.1 in tap water (pH 7.0). SPM included (%) glycerol 2.0, soybean meal 1.5, Formosan dry yeast 0.5, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, calcium carbonate 0.15, $CoCl_2 \cdot 6H_2O$ 0.0005 and agar 2.0 in tap water (pH 7.0).

Assay Methods

Bioassay: Total carbapenem activity in broth filtrates or agar plugs was assayed by the agar diffusion method using *Comamonas terrigena* B-996 and cephaloridine as test organism and reference antibiotic, respectively.[6]

TLC Assay: After solids were removed from culture broths by centrifugation, an aliquot of the supernatant was spotted on a silica gel TLC plate (pre-coated Silica gel 60 F254, E. Merck, Darmstadt). The plate was developed in the cold in a solvent system of $CHCl_3$, MeOH and 0.01 M phosphate buffer, pH 7.5 (8:6:1) and then visualized with Ehrlich reagent.[7,8] By this method, OA-6129 A, B (B1+B2) and C can be estimated quantitatively and qualitatively. HPLC is employed for analytical separation of OA-6129 B1 and B2.

HPLC Assay: The Hitachi 638-50 system was employed under the following conditions; column, Waters μBondapak C18; mobile phase, 0.1 M ammonium acetate, pH 4.7 and acetonitrile (91.25:8.75); flow rate, 1.0 ml/minute (80 kg/cm² G); detection, UV absorption at 300 nm. The broth supernatant was diluted with 0.01 M phosphate buffer, pH 8.0, and an aliquot of the dilution was injected into the column. OA-6129 A, B1, B2 and C were detected at 46, 11, 14 and 8 minutes, respectively.

Mutagenesis

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) Treatment: A fresh spore suspension in 0.05 M Tris-malate buffer, pH 9.0, was filtered through cotton, incubated at 30°C for 1 hour with 3 mg/ml (final concentration) of NTG and then subjected to centrifugation. The spores were washed twice with MS solution (0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$) and cultured on CAM at 28°C for 7~10 days.

UV Irradiation: A fresh spore suspension was filtered through cotton, and irradiated for 80 seconds with a 15-W UV-lamp (survival rate 0.6%). The treated spores were grown on CAM at

28°C in the dark for 7~10 days.

Isolation of Carbapenem-producing Blocked Mutants

Colonies after NTG-treatment on CAM were replicated on CAM supplemented with sodium selenate (final concentration 1~4 mg/ml), *S*-2-amino-L-cysteine (0.5~1 mg/ml), ethionine (0.5~1.0 mg/ml) or norleucine (2~4 mg/ml), and grown at 28°C for a further 7~10 days. Colonies showing normal growth were purified on CAM and subjected to carbapenem product analyses after liquid cultivation, as described above.

Isolation of Carbapenem Non-producing Strains

After NTG or UV mutation, carbapenem non-producers were screened by the liquid and solid cultivation methods.

Chemicals

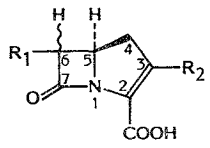
Sodium selenate (sulfate inhibitor, Wako Pure Chemical Industries, Ltd., Japan); *S*-2-aminoethyl-L-cysteine (lysine inhibitor, Sigma Chemical Co., U.S.A.); and DL-ethionine (methionine inhibitor, Tokyo Chemical Industry Co., Ltd., Japan) and DL-norleucine (methionine inhibitor, Tokyo Chemical Industry Co., Ltd., Japan) were employed as metabolic inhibitors in the present study.

Results

S. fulvoviridis A933 17M9 is considered to have an ability to produce the 42 known carbapenem and carbapenam compounds. Biosynthetically speaking, both the horizontal route that governs the bioconversion of the C-6 side chain of carbapenem (R_1 in Fig. 1); and the vertical route that governs the catabolism of the C-3 side chain of carbapenem (R_2 in Fig. 1) are active in this streptomycete, which is responsible for a high diversity of carbapenem fermentation products.[1~4]

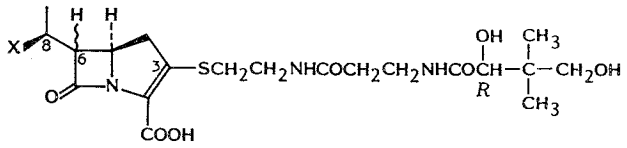
In the course of biosynthetic studies of carbapenems in *S. fulvoviridis* A933 17M9, a mutant numbered 1501 that produces only the OA-6129 group of carbapenems (Fig. 2) and carbapenams was found to be defective in A933 acylase, an enzyme that plays a key role in the vertical route of bioconversion of carbapenems.[5] Since only the horizontal route of bioconversion is active in mutant 1501, the chance of obtaining various types of OA-6129 carbapenem-producing mutants was assumed to be much higher with mutant 1501 than with other strains.

Fig. 1. Basic structure of carbapenem antibiotics.



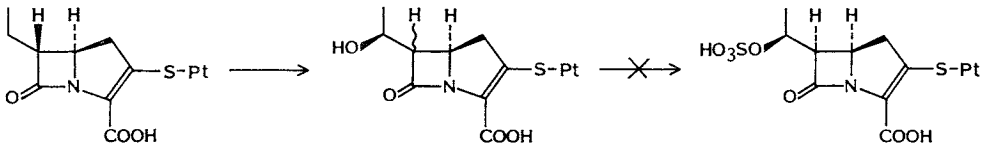
The parent strain 1501 was subjected to NTG-mutation for higher carbapenem productivity, yielding mutant N2-102. Mutant N3-783 is selenate-resistant and showed a higher fermen-

Fig. 2. Chemical structures of the OA-6129 group of carbapenems.

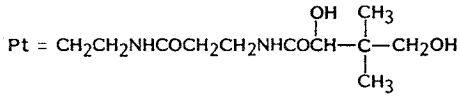


Compound	X	C-6 configuration
OA-6129 A	H	*R*
OA-6129 B1	OH	*R*
OA-6129 B2	OH	*S*
OA-6129 C	OSO_3H	*R*

Fig. 3. Type I blocked mutants.

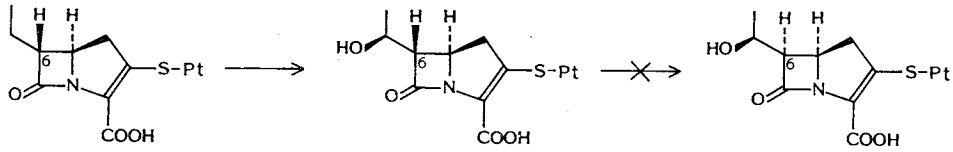


OA-6129 A OA-6129 B1 OA-6129 C
OA-6129 B2



Mutant strains: N3-504, 4N 1181.
Blocked step: Sulfation.

Fig. 4. Type II blocked mutants.



OA-6129 A OA-6129 B2 OA-6129 B1

Mutant strains: 4N 3607, 5NA 3949-40, 5NE 252.
Blocked step: Isomerization at C-6.

tation titer of carbapenem than mutants N2-102 and N3-504 (Fig. 6).

Isolation and Characterization of Blocked Mutants

Type I Blocked Mutants that Accumulate OA-6129 A, B1 and B2, but no OA-6129 C (Blockage in Sulfation of the C-8 Hydroxyl Group)

Two mutants (N3-504 and 4N 1181) that produce non-sulfated OA-6129 carbapenems (OA-6129 A, B1 and B2) but not the sulfated one (OA-6129 C) (Fig. 3) were derived from strains N2-102 and N3-783, respectively, as highly selenate-resistant strains.

Radiolabel experiments with $^{35}SO_4^{2-}$ demonstrated that the parent strain (mutant 1501) incorporated the radiolabel into mycelia, whereas no radioactivity was taken into mycelia of mutants N3-504 and 4N 1181 (data not shown).[9]

Type II Blocked Mutants that Accumulate OA-6129 A and B2, but no OA-6129 B1 and C (Blockage in Isomerization at C-6)

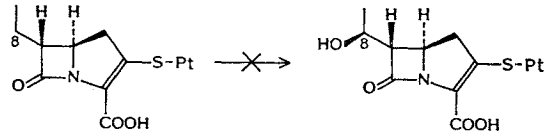
Mutants 4N 3607, 5NA 3949-40 and 5NE 252 that produce OA-6129 A and B2 without OA-6129 B1 and C were obtained (Fig. 4).

Mutant 4N 3607 was obtained as a selenate-resistant strain from strain N3-783. Mutants 5NA 3949-40 and 5NE 252 were derived from mutant 4N 3607, and exhibited high levels of resistance to *S*-2-aminoethyl-L-cysteine and ethionine, respectively.

From the viewpoint of carbapenem biosynthesis, it is worth noting that no strain which produces

OA-6129 A and B1 without OA-6129 B2 and C has been observed throughout the present study, indicating that the valid sequence of bioconversion between OA-6129 B1 and B2 is OA-6129 B2→B1, and not OA-6129 B1→B2.

Fig. 5. Type III blocked mutant.



Mutant strain: 6N(NL) 144.
Blocked step: Hydroxylation at C-8.

Type III Blocked Mutant that Produces OA-6129 A, but no OA-6129 B1, B2 and C (Blockage in Hydroxylation at C-8)

A highly norleucine-resistant mutant [6N(NL) 144] was obtained from 5NA 3949-40 and found to form only OA-6129 A without OA-6129 B1, B2 and C by silica gel TLC and HPLC (Fig. 5).

Type IV Mutants that Accumulate no Carbapenem Compound (Non-producers)

In the initial stage of this study, when the first screen for mutation analysis depended only on the silica gel TLC pattern, *i.e.*, (a) the detection of carbapenems by coloration; and (b) the percent composition of OA-6129 A, (B1+B2) and C was examined, many putative non-producers were observed in every run of mutation. In the confirmation step using the more sensitive bioassay, however, none was confirmed to be a non-producer. In brief, the results of the detailed product analysis by the combination of bioassay, silica gel TLC and HPLC yielded the following conclusions:

(1) Some were falsely determined to be non-producers because of low sensitivity of the silica gel TLC assay.

(2) Some of the rest produced no carbapenem on liquid cultivation but a small amount of carbapenem on solid cultivation; and others were found to be non-producers on solid cultivation and very poor producers on liquid cultivation.

Since complete carbapenem non-producers are needed for future studies such as cosynthesis tests and gene engineering experiments, the first screen for product analysis was switched from the silica gel TLC method to the bioassay. After more severe mutagenesis followed by repeated purification and confirmation, 41 and 61 complete non-producer strains which produce no carbapenem on both solid and liquid cultivation modes were obtained from 36,000 UV-treated and 23,000 NTG-treated colonies, respectively. Among these non-producers, 22 were sporogenous, while the rest were asporogenous. Cosynthesis tests with possible combinations of the 22 sporogenous non-producers were negative by the solid and liquid cultivation methods.

Pedigree of *S. fulvoviridis* A933 17M9 1501 and Related Mutants

Table 1 summarizes the results of carbapenem product and enzyme analyses in *S. fulvoviridis* A933 17M9 1501 and progenies; and the sequence of their mutagenesis is presented in the pedigree in Fig. 6.

Preferential Large-scale Production of OA-6129 B2 Using Type II Blocked Mutants

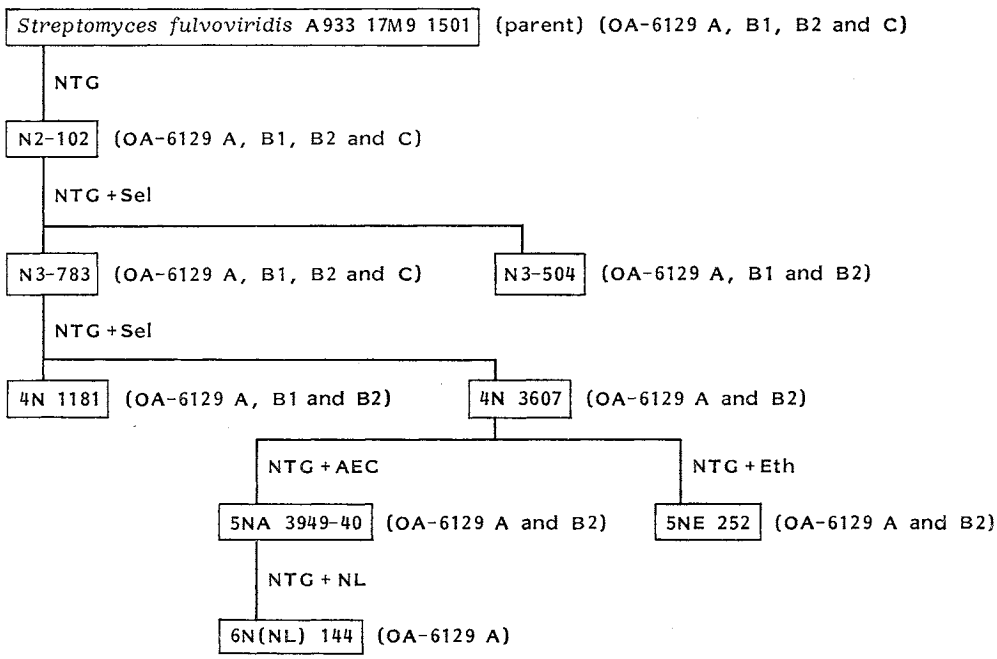
As the isomerization at C-6 is blocked, type II mutants such as 4N 3607, 5NA 3949-40 and 5NE 252 form only OA-6129 A and B2. Previous fermentation experience with *S. fulvoviridis* A933 17M9 1501 showed that, if the dissolved oxygen was carefully controlled under a certain limit during fermentation, the hydroxylation at C-8 was largely prevented, giving OA-6129 A as a major carbapenem. Conversely, a high level of dissolved oxygen was found to promote the hydroxylation at C-8, providing

Table 1. Product analysis of *Streptomyces fulvoviridis* A933 17M9, *S. fulvoviridis* A933 17M9 1501 (parent) and mutants.

Strain No.	MM 4550, 13902 & 17880	ETHM A & B	ETHM C & D	PS-5 & -7	A933 acylase	OA-6129			
						C	B1	B2	A
A933 17M9	+	+	+	+	+	−	−	−	−
1501 (parent)	−	−	−	−	−	+	+	+	+
N3-504 & 4N 1181	−	−	−	−	−	−	+	+	+
4N 3607, 5NA 3949-40 & 5NE 252	−	−	−	−	−	−	−	+	+
6N (NL) 144	−	−	−	−	−	−	−	−	+

+: Detected, −: not detected.
ETHM: Epithienamycin.

Fig. 6. Pedigree of *Streptomyces fulvoviridis* A933 17M9 1501 and mutants.



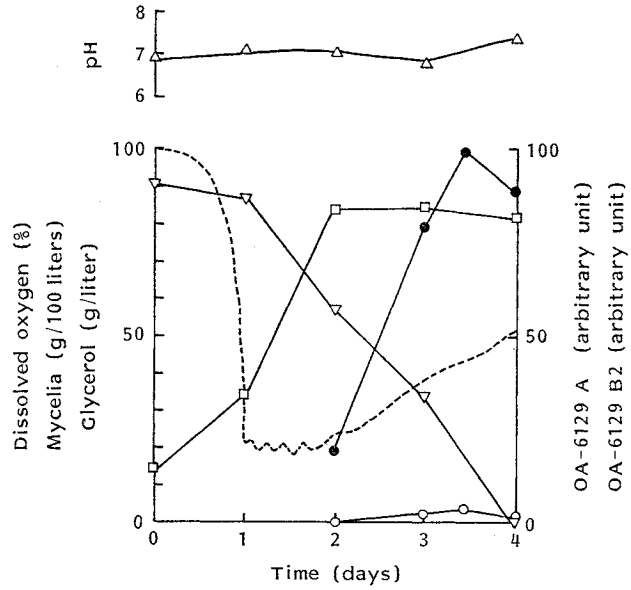
Abbreviations: Sel, sodium selenate; AEC, *S*-2-aminoethyl-L-cysteine; Eth, ethionine; NL, DL-norleucine.

OA-6129 B1 and B2 as major components. Finally, if the amount of assimilable sulfur was above a certain level in the presence of sufficient amounts of OA-6129 B1 and B2, the major product was OA-6129 C.

Accordingly, for preferential fermentation of OA-6129 B2, mutant 4N 3607 was cultivated under highly aerated conditions in a 15-liter jar fermentor. The time course of fermentation of OA-6129 B2 and A is presented in Fig. 7, showing the practical usefulness of type II mutants for preferential production of OA-6129 B2.

Fig. 7. Time course of fermentation of mutant 4N 3607 for preferential production of OA-6129 B2 in a jar fermentor.

--- Dissolved oxygen, □ mycelia, ▽ glycerol, ○ OA-6129 A, ● OA-6129 B2.



Discussion

S. fulvoviridis A933 17M9 is a high-titer producer of PS-5, epithienamycins A and C, MM 17880 and other minor carbapenems, the final composition of which depends on fermentation conditions. Although the OA-6129 group of carbapenems are also produced, active A933 acylase that is the key enzyme in the vertical route of carbapenem bioconversion rapidly converts them by acyl exchange, leading to the absence of OA-6129 carbapenems in the broth of *S. fulvoviridis* A933 17M9. In *S. fulvoviridis* A933 17M9 1501 (the parent strain in this study), as no A933 acylase activity is available (or as the vertical route of carbapenem bioconversion is inactive), bioconversion is limited to the horizontal route, resulting in the accumulation of the OA-6129 group of carbapenems.

No A933 acylase activity reappeared in any of the new mutants by reverse mutation, which means that the bioconversion of OA-6129 carbapenems was limited to the horizontal route (Table 1).

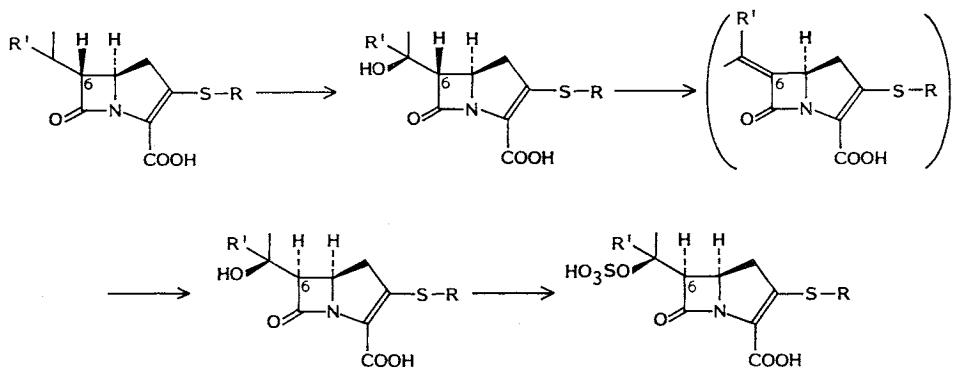
OA-6129 A is the initial member of the OA-6129 group of four carbapenems. This conclusion is supported by the isolation of type III mutant, the lowest oxidation level of the C-6 ethyl side chain and the above-described fermentation findings.

OA-6129 A is then hydroxylated at C-8 to give OA-6129 B (B1+B2), which is clearly indicated by the isolation of types I and II mutants, the fermentation findings and the jar fermentation data described above. More particularly, as noted earlier, OA-6129 A is first hydroxylated at C-8 to OA-6129 B2 with the 5,6-*trans* conformation retained. The subsequent isomerization of OA-6129 B2 at C-6 yields OA-6129 B1 that has the 5,6-*cis* conformation. This sequence of conversion without branching is favored, because no mutant producing OA-6129 A and B1 was obtained, carbapenem compounds having alkylidene groups at C-6 which will serve as biosynthetic intermediates in the isomerization at C-6 are known in nature, and OA-6129 C possesses the 5,6-*cis* conformation.

OA-6129 C is the final product in the horizontal route of conversion among the OA-6129 carbapenem series. This is apparent from the isolation of type I mutants, the highest level of oxidation of the C-8 hydroxysulfonyloxyethyl side chain and the above-explained fermentation findings.

The present results of mutagenesis have thus served to substantiate the proposed sequence of the horizontal carbapenem bioconversion (Fig. 2 in ref 3). At present, the general horizontal route of

Fig. 8. Proposed sequence of biosynthesis of the C-6 side chain of carbapenems (horizontal route).



carbapenem bioconversion will be illustrated as shown in Fig. 8.

OA-6129 B2 was selected as a promising intermediate for chemi al derivation such as 6-aminopenicillanate among the OA-6129 group of carbapenems, because it is the least antimicrobial against streptomycetes;[2] its physico-chemical stability is the best among the known carbapenems;[2] and the stereochemistry of the 8-hydroxy group is (*S*) that is suitable for chemical derivation at C-8.[4]

As described in the Results section, the cosynthesis tests with the non-producer strains have so far been negative. Furthermore, the progress of the strain improvement is very slow. As a future approach to industrial fermentation of carbapenems, genetic engineering studies on this streptomycete will be interesting. *S. fulvoviridis* A933 17M9 bears no plasmids, but was found to be transformable by pIJ702 (a pIJ101 derivative), pIJ922 (a SCP2* derivative), pSK21-K2 (a pSK2 derivative from *Streptomyces kasugaensis*)[10] and 3BM46 (a pSCY3 derivative from *Streptomyces noursei*)[11] (unpublished work). Such work will also be useful in further elucidation of carbapenem biosynthesis including responsible enzymes such as A933 acylase.

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