STUDIES ON THE BIOSYNTHESIS OF CARBAPENEM ANTIBIOTICS

IV. β-ALANINE IN THE C-3 PANTETHEINYL SIDE CHAIN OF THE OA-6129 GROUP OF CARBAPENEMS

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Radioactive β-alanine was specifically incorporated into the β-alanine moiety of the C-3 pantetheinyl side chain of the OA-6129 group of carbapenems by *Streptomyces* sp. OA-6129, while no added radioactive pantothenate was taken up into the mycelia. The extracellular concentrations of β-alanine and pantothenate in the broth of *Streptomyces* sp. OA-6129 increased with the production of the carbapenems. The production levels of β-alanine and pantothenate markedly differed between carbapenem-producing streptomycetes and non-producers, with the exception of *Streptomyces cattleya*, a thienamycin producer, suggesting a possible relation of carbapenem biosynthesis with the levels of β-alanine and pantothenate.

In previous papers,[1~4] we have reported that the C-3 pantetheinyl side chain of the OA-6129 group of carbapenems is converted to the C-3 acetylaminoethylthio side chain of PS-5, epithienamycins A and C and MM 17880 in the presence of acetyl CoA, or to the C-3 aminoethylthio side chain of NS-5 and deacylated carbapenems in the absence of acyl CoA, by action of a specific acylase tentatively designated A933 acylase. The terminal phosphorylation of the C-3 pantetheinyl group of the OA-6129 group of carbapenems, however, leads to insensitivity to A933 acylase.

In the biosynthesis of carbapenems by streptomycetes, pantetheine can be considered to have three possible roles. First, it could be the precursor of the C-3 pantetheinyl side chain. If so, the intracellular pool of pantetheine or its related compounds might be critically important for production of high titers of carbapenem compounds. Second, CoA (which has a pantetheinyl moiety as acyl carrier) is involved in cell growth and is also used for derivation of the C-3 acetylaminoethylthio side chain by an acyl exchange reaction.[2,3] Third, the thio-template system of peptide elongation for peptide antibiotics which was elucidated for biosynthesis of gramicidin S and tyrocidine by LIPMANN[5] might be involved in the biosynthesis of carbapenems. During activation and transfer of constitutive amino acids, pantetheine in the thio-template system might work as amino acyl carrier.

This paper describes studies on the biosynthesis of the C-3 pantetheinyl side chain with radiolabeled β-alanine.

Materials and Methods

Materials

β-[1-^{14}C]Alanine (54.7 mCi/mmol), β-[3-^{3}H]alanine (40.0 Ci/mmol), D-[1-^{14}C]pantothenate (sodium salt; 57.6 mCi/mmol) and Atomlight (scintillation fluid) were obtained from New England Nuclear. Type II β-lactamase of *Bacillus cereus* was prepared from *B. cereus* 569/H.[6] Renal dipeptidase was isolated from rat kidneys according to the method of SHIBAMOTO *et al.*[7] Acylase I (hog kidney) was purchased from Sigma Chemical Co.

Microorganisms and Cultivation

Streptomyces fulvoviridis A933 17M9,[8] *Streptomyces* sp. OA-6129[9] and other streptomycetes listed in Table 1 are from our culture collection and were cultivated at 28°C on a rotary shaker for 4~5 days in the following media:

1) CD medium (chemically defined medium); maltose 4% (sterilized separately), diammonium citrate 0.5%, NaCl 0.1%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 200 ppm, $ZnSO_4 \cdot 7H_2O$ 30 ppm, $FeSO_4 \cdot 7H_2O$ 10 ppm, $CoCl_2 \cdot 6H_2O$ 1 ppm; pH 7.0.

2) NZ medium; glycerol 2%, NZ-amine (type A) 1%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%; pH 7.0.

3) TP medium; glycerol 10%, dry yeast 1%, soybean meal 3%, $MgSO_4 \cdot 7H_2O$ 0.2%, K_2HPO_4 0.2%, $CaCO_3$ 0.3%, vitamin B_{12} (separately sterilized) 10 ppm; pH 7.2.

After centrifugation, the broth supernates were used for assays.

Analyses

The content of pantothenate was measured by bioassay using *Saccharomyces carlsbergensis* ATCC 9080 as test organism.[10] Extracellular amino acids were quantified with a Hitachi 835 automatic amino acid analyzer. The total titer of carbapenems is expressed in antimicrobial activity (CCU=cephaloridine *Comamonas terrigena* unit) as determined by disc-assay using *C. terrigena* B-996.[11] A semi-quantitative assay of carbapenem components was carried out by high voltage paper electrophoresis (HVPE; pH 8.6, 1,500 V/30 cm for 40 minutes at 0~5°C) followed by bioautography using *C. terrigena* B-996.[11]

Incorporation Experiments with Radiolabeled Compounds

Streptomyces sp. OA-6129 was cultivated at 28°C for 48 hours in a 500-ml Erlenmeyer flask containing 20 ml of TP medium. A radioactive compound (5 μCi) was added to the culture, and 1 ml of the culture was withdrawn at indicated times of incubation and centrifuged for 10 minutes at 3,000 rpm.

For determination of radioactivity, an aliquot of a sample solution was dissolved in 10 ml of Atomlight and counted in an ALOKA LSC-900 liquid scintillation counter.

For separation of the sulfated carbapenem (OA-6129C) and the non-sulfated carbapenems (OA-6129A, B_1 and B_2) from β-alanine and pantothenate, an aliquot of a sample solution was spotted on a sheet of Whatman No. 1 filter paper and subjected to HVPE. A 1.8 cm-wide paper strip was cut from the electrophoretogram along the current flow and then sectioned into 1.0×1.8 cm rectangular pieces which were dropped into 10 ml Atomlight for radioactivity determination.

Silica Gel TLC was also employed for separation of β-alanine and pantothenate (pre-coated TLC plates Silica Gel 60 F-254; E. Merck, Darmstadt). A TLC plate was developed in a solvent system of BuOH - AcOH - H_2O (5: 2: 3) and the air-dried plate was scanned in an Aloka TLC-101 radio-autoscanner.

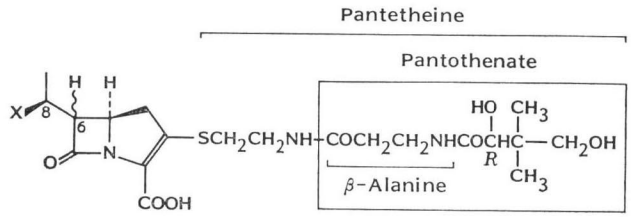
Isolation of the OA-6129 Group of Carbapenems from Broth

For better recovery of carbapenems, sodium chloride was added at 3% to the broth filtrate. The radioactive filtrate (10 ml) was charged on a Diaion HP-20 column (10 ml). After the chromatographic column was rinsed with 3% NaCl and then with H_2O, radioactive carbapenems were eluted with 20 ml of 50% acetone. The acetone was removed by evaporation under reduced pressure and the aqueous solution was lyophilized to give a crude powder of OA-6129 carbapenems.

Treatment of Carbapenems by Enzymes

A crude powder of OA-6129 carbapenems was dissolved in neutral water at a concentration of 5 mg/ml. The OA-6129 solution (50 μl) was mixed with 50 μl of an enzyme solution in a suitable buffer and incubated for 30 minutes at 37°C. The reaction mixture was applied on Whatman No. 1 filter paper and subjected to HVPE.

Fig. 1. Structure of the OA-6129 group of carbapenem compounds.



Results and Discussion

Lack of Uptake of D-[1-^{14}C]Pantothenate by *Streptomyces* sp. OA-6129

Based on the established route of bacterial CoA biosynthesis,[12] pantothenate was first assumed to be incorporated into the C-3 pantetheinyl side chain of the OA-6129 group of carbapenems (Fig. 1). D-[1-^{14}C]Pantothenate was thus added to the 48 hour-old culture of *Streptomyces* sp. OA-6129 and was incubated under rotary shaking for a further 72 hours. Contrary to our expectation, no radioactivity of the added pantothenate was taken into the mycelia even after 72 hours of incubation, and the total radioactivity added was recovered as pantothenate in the broth filtrate, suggesting the existence of a permeability barrier to pantothenate.

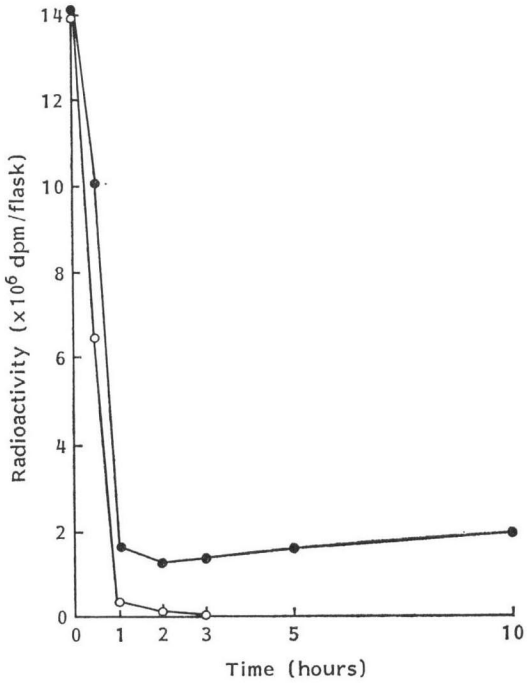
Incorporation of β-[1-^{14}C]Alanine into the Mycelia of *Streptomyces* sp. OA-6129

As β-alanine is known to be a precursor of pantothenate, β-[1-^{14}C]alanine was supplied to the 48 hour-old broth of *Streptomyces* sp. OA-6129 and the radioactivity was followed for 24 hours in the broth supernate. The time course of β-alanine incorporation presented in Fig. 2 indicates that, under the above-specified incorporation conditions, the radioactive β-alanine rapidly disappears from the broth supernate in 60 minutes, although about 10% of the added radioactivity remains in the broth supernate even after 24 hours of incubation.

Fig. 2. Incorporation of β-[1-^{14}C]alanine by *Streptomyces* sp. OA-6129.

An aliquot of the broth supernate was subjected to HVPE for separation of radioactive β-alanine, followed by liquid scintillation counting.

● Broth supernate, ○ β-Ala spot.



Subsequently the radioactivity of the broth supernate collected after 40 minutes of incorporation was analyzed by silica gel TLC followed by autoradiography (Fig. 3). A large peak of pantothenate is seen, indicating that the radiolabeled β-alanine is incorporated into pantothenate.

Extracellular Amino Acids of *Streptomyces* sp. OA-6129 during Carbapenem Production

Since carbapenems belong to the group of peptide antibiotics, the extra- and intra-cellular con-

Fig. 3. TLC analysis of the broth supernate at 0 and 40 minutes of β-[1-^{14}C]alanine incorporation by *Streptomyces* sp. OA-6129.

For easy comparison, the sensitivity of the radio-auto-scanner was increased about 3-fold for the 40 minutes sample.

PaA: Pantothenate.

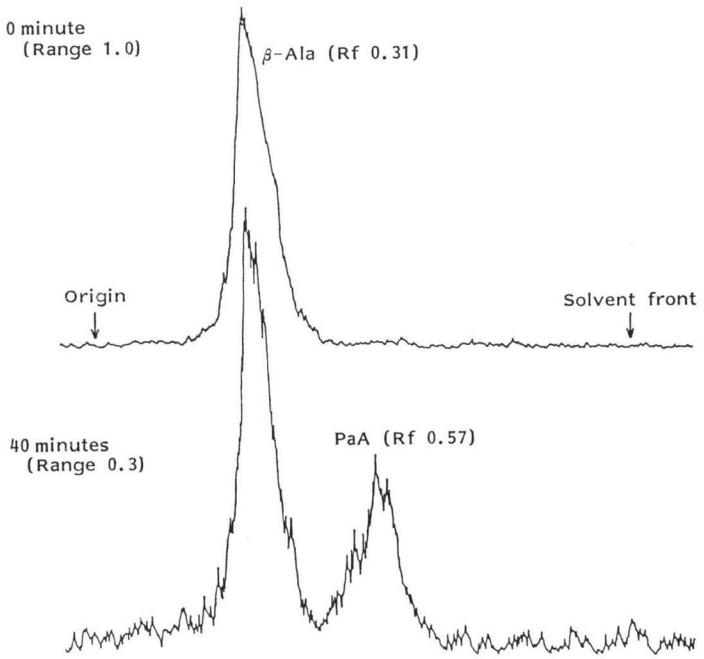
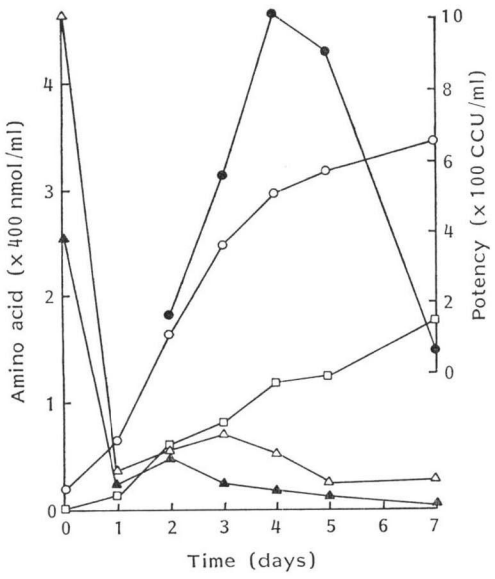


Fig. 4. Time course of change in extracellular amino acid concentrations during OA-6129 carbapenem production in TP medium by *Streptomyces* sp. OA-6129.

● Potency, ○ β-Ala, □ Met, ▲ Ala, △ Glu.



centrations of amino acids should be important factors for the amount and composition of final carbapenem compounds produced by the streptomycete. Thus *Streptomyces* sp. OA-6129 was grown in CD and TP media, and the time courses of change in the extracellular amino acids were analyzed with an automatic amino acid analyzer. In CD medium, the fermentation showed only a high peak of β-alanine together with ammonia. The amino acid analysis of the culture in TP medium is summarized in Fig. 4.

Glutamate and alanine derived from the nutrient components of TP medium are rapidly assimilated by *Streptomyces* sp. OA-6129, while the levels of β-alanine and methionine rise with the fermentation period. Taken together with the rapid incorporation of radiolabeled β-alanine described above, the high extracellular level of β-alanine seemed to have some relation to the biosynthesis of carbapenems.

Table 1. Levels of β-alanine and pantothenate production by streptomycetes.

Strain	Antibiotic	β-Ala (μg/ml)		Panthothenate (μg/ml)	
Streptomyces sp. P2714	LM	0.57		19.5	
〃 sp. P3335	PCM	1.11		3.7	
〃 sp. P3814	AGM	0.70		0.6	
〃 sp. P4117	XTM	0.69		8.1	
〃 sp. P4259	LM	0.47	Average	1.1	Average
〃 sp. P4448	STH	1.61	0.74	3.0	5.7
〃 sp. P5359	CRM	0.23		13.2	
〃 sp. P8836	ACM	0.59		2.7	
〃 sp. P8993	NM	1.74		1.7	
〃 sp. P9151	AGM	0.14		4.5	
〃 sp. P9618	PRM	0.25		4.5	
〃 sp. P9306	Carbapenems*	8.45		11.9	
〃 sp. A170	〃	9.49		26.1	
〃 *cremeus* subsp. *auratilis* A271	〃	13.69	Average 10.09	21.5	Average 17.1
〃 sp. A704	〃	7.31		16.0	
〃 *fulvoviridis* A933	〃	11.50		9.8	
〃 *cattleya* NRRL 8057	THM	0.73		9.8	
〃 sp. OA-6129	OA-6129's	6.03		4.9	

LM=Leucomycin, PCM=picromycin, AGM=angolamycin, XTM=xanthomycin, STH=streptothricin, CRM=cirramycin, ACM=actinomycin, NM=neomycin, PRM=paromomycin, THM=thienamycin.

* Carbapenem compounds other than the OA-6129 and thienamycin groups.

Relation of the High Levels of β-Alanine and Pantothenate with Carbapenem-producing Ability

As the unexpectedly high level of β-alanine in the broth of *Streptomyces* sp. OA-6129 suggested the potential classification of carbapenem-producing streptomycetes from non-producers based on their production levels of β-alanine, various types of antibiotic-forming streptomycetes were chosen from our culture collection and were cultivated in NZ medium. The extracellular production of β-alanine and pantothenate was assayed with an automatic amino acid analyzer and with *S. carlsbergensis*, respectively (Table 1).

It is obvious that the extracellular concentrations of β-alanine produced by carbapenem producers excluding *Streptomyces cattleya* (thienamycin producer) are about 10 times higher than those by carbapenem non-producers. This high level of β-alanine seems to lead to higher extracellular concentrations of pantothenate observed in carbapenem-producing streptomycetes. As described in a previous paper,[3] *S. cattleya* which produces thienamycin, a non-acylated carbapenem, as a major product, possesses an acylase activity different from A933 acylase, which might have some relevance to the low production of β-alanine. The significantly high level of pantothenate in the two macrolide producers (P2714 and P5359) may suggest the possible involvement of pantothenate or related compounds in the biosynthesis of macrolide antibiotics.

Incorporation of β-[3-^{3}H]Alanine into the OA-6129 Group of Carbapenems by *Streptomyces* sp. OA-6129

To accomplish a maximum rate of incorporation of β-alanine into the OA-6129 group of car-

Fig. 5. Analysis of the radioactive OA-6129 carbapenems by chemical or enzymatic degradation.

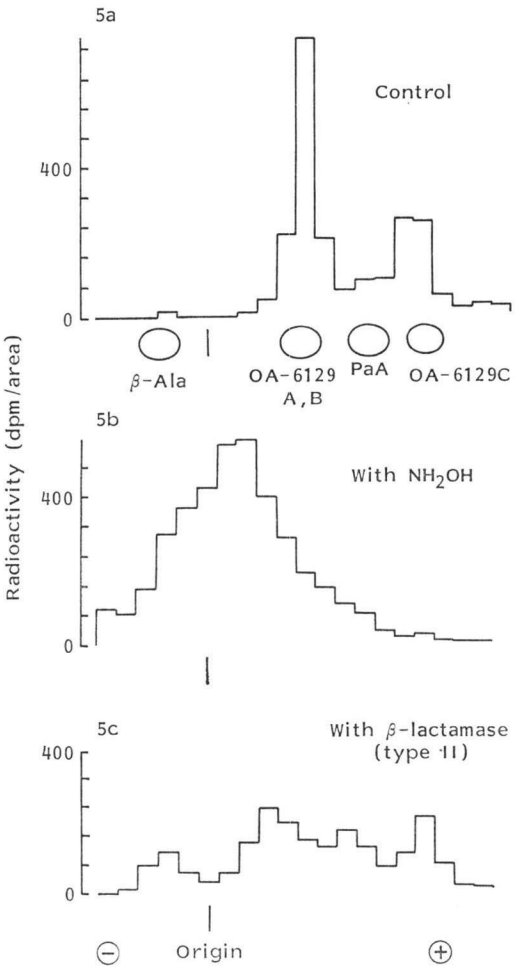
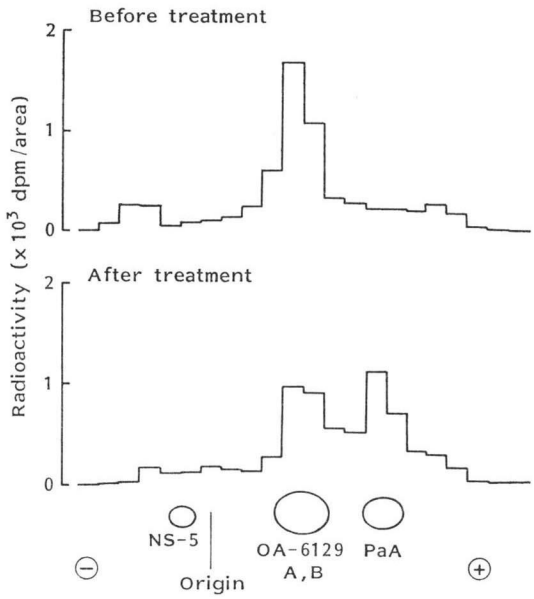


Fig. 6. HVPE analysis of the radioactive OA-6129 carbapenems before and after treatment with L-amino acid acylase.

Depantothenylated OA-6129 B_1 and B_2 (or deacetylated epithienamycins A and C) were located at the same site as NS-5.



bapenems, the eventual loss of radioactivity by decarboxylation was avoided by replacing β-[1-^{14}C]alanine by β-[3-^{3}H]alanine. To the 48 hour-old culture of *Streptomyces* sp. OA-6129 in TP medium, 50 μCi of β-[3-^{3}H]alanine was added and incubated for a further 24 hours. As described above for β-[1-^{14}C]alanine, silica gel TLC detected no radioactivity in the extracellular β-alanine spot, whereas the extracellular pantothenate area contained about 2% of the initial radioactivity, showing that both [1-^{14}C]- and [3-^{3}H]-β-alanines behave similarly in incorporation experiments using *Streptomyces* sp. OA-6129.

The fraction containing the OA-6129 group of radioactive carbapenems was separated from the broth supernate by Diaion HP-20 column chromatography. The absence of radioactive free pantothenate in the antimicrobially active fraction was evidenced by HVPE followed by scintillation counting. The radioisotopic purity of the carbapenems in the antimicrobial fraction was further confirmed by treating the carbapenem fraction with hydroxylamine and type II β-lactamase, followed by HVPE (Fig. 5).

It is apparent from Fig. 5b that the radioactive OA-6129A, B_1, B_2 and C are non-specifically broken down with hydroxylamine. Type II β-lactamase of *B. cereus* opens the β-lactam ring of the OA-6129 carbapenems (Fig. 5c), giving more acidic breakdown products. The treatment of the carbapenem fraction by renal dipeptidase yielded the substantially same results (data not shown). All these findings allowed us to conclude that the OA-6129 carbapenem fraction was practically pure in

radioactivity, and consequently the percent incorporation of [1-^{14}C]- or [3-^{3}H]-β-alanine was calculated to be about 4% of the added radioactivity under the above-specified conditions. This incorporation rate was significantly higher than those of putative amino acid precursors such as glutamate and cysteine (less than 0.1%; unpublished data), probably because β-alanine is less widely and less rapidly utilized in the streptomycete mycelia than other amino acids.

Location of the β-Alanine Radioactivity in the OA-6129 Carbapenems

Since hog kidney acylase I (=L-amino acid acylase) hydrolyzes the OA-6129 carbapenems to give deacylated carbapenems and pantothenate,[1] the distribution of the radioactivity in the molecules of OA-6129 carbapenems can be determined using the L-amino acid acylase.

As the different mobilities of the sulfated (OA-6129C) and non-sulfated (OA-6129A, B_1 and B_2) carbapenems on HVPE and TLC might interfere with the subsequent product analysis, OA-6129C, a minor component, was deleted from the OA-6129 carbapenem fraction by Diaion PA-306S column chromatography.[9] Removal of OA-6129C was checked by HVPE and scintillation counting. The new OA-6129 carbapenem fraction contained OA-6129A as major component together with minor components of OA-6129B_1 and B_2 (data not shown). The new fraction was treated with acylase I and then subjected to HVPE followed by radioactivity counting. Fig. 6 reveals that all the radioactivity is present in the pantothenyl moiety of the OA-6129 carbapenems, while the deacylated carbapenems (NS-5 and deacetylated epithienamycins A and C) possess no radioactivity. Furthermore, the radioactivity of the pantothenate was finally localized in the β-alanine moiety by acid hydrolysis (data not given).

Although the possibility that the C-3 aminoethylthio side chain of carbapenems such as NS-5 and thienamycin is directly acylated with pantothenate has not yet been ruled out, we assume it more likely that radioactive β-alanine is incorporated into the mycelia of *Streptomyces* sp. OA-6129 where the amino acid is condensed with pantoate to produce pantothenate, which is further bound to cysteine and subsequently decarboxylated to pantetheine before incorporation into the C-3 pantetheinyl side chain. The behavior of β-alanine in the biosynthesis of CoA and the thio-template system remains to be studied in *Streptomyces* sp. OA-6129 and other carbapenem producers.

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