# MICROBIAL PHOSPHORYLATION OF THE OA-6129 GROUP OF CARBAPENEM COMPOUNDS

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The OA-6129 group of carbapenem antibiotics were phosphorylated with ATP by *Brevibacterium ammoniagenes* at the primary hydroxyl group of the C-3 pantetheinyl side chain. The phosphorylation resulted in the reduced antimicrobial activity against some Gram-positive bacteria, and the improved activity against some Gram-negative microbes. The increased resistance of the OA-6129 carbapenems due to phosphorylation was significant to mouse renal dehydropeptidase and moderate to the human enzyme. OA-6129A and $B_2$ phosphates were found to be unsusceptible to A933 acylase, while OA-6129A and $B_2$ were depantothenylated.

The OA-6129 group of carbapenems[1,2] are structurally distinct from other carbapenem compounds in a pantetheinyl side chain at the C-3 position; and have been proved to be biosynthetic precursors for PS-5, epithienamycins A and C and MM 17880[3,4]. The presence of pantetheinyl at C-3 has provided us with an opportunity to study the possible effects of phosphorylation on biological properties of carbapenem compounds, particularly on their antimicrobial activity and resistance to renal dehydropeptidase[5].

Pantothenate kinase (EC 2.7.1.33) which catalyzes the phosphorylation of pantothenic acid in the first step of CoA biosynthesis is also known to phosphorylate pantetheine[6]. SHIMIZU *et al.*[7] reported that *Brevibacterium ammoniagenes* converted pantetheine to 4′-phosphopantetheine in the presence of ATP. In the light of seemingly broad specificity of the bacterial enzyme, we have successfully attempted to phosphorylate the OA-6129 group of carbapenems with ATP by *B. ammoniagenes*.

The present paper describes the microbial phosphorylation of the OA-6129 group of carbapenem antibiotics and the physico-chemical, antimicrobial and enzymological characterization of the phosphorylated carbapenems.

## Materials and Methods

### Materials

Sodium salts of OA-6129A, OA-6129$B_2$ and OA-6129C were prepared in our laboratories by fermentation of *Streptomyces* sp. OA-6129 as detailed in a previous paper[1]. A933 acylase was the same preparation as previously described[4]. Alkaline phosphatase (P3877) and ATP were purchased from Sigma Chemical Co.

### Microorganisms

*B. ammoniagenes* ATCC 6871 was cultivated as described by SHIMIZU *et al.*[7]. Microorganisms employed for the evaluation of the antimicrobial spectrum were from our culture collection.

### Microbial Assays

The concentrations of carbapenem compounds were assayed by the disc-agar diffusion method using *Comamonas terrigena* B-996[8]. This detector organism was also used for bioautography.

The antimicrobial spectrum of a carbapenem compound was determined as reported in a previous

paper[9].

Dipeptidase Assay

Apparently healthy tissues of mouse, dog and human kidneys were homogenized for 3 minutes at 0°C in 5 volumes of 0.02 M phosphate buffer, pH 7.0, with a Polytron homogenizer. The homogenates were centrifuged at 0°C for 20 minutes at 8,000×*g* to give crude dipeptidase solutions. A reaction mixture containing 100 μl of a carbapenem solution (100~300 μg/ml) and 100 μl of a dipeptidase solution was incubated at 37°C for 60 minutes. After inactivation for 15 seconds at 100°C, the reaction mixture was centrifuged, and the concentration of the carbapenem remaining in the supernate was bio-assayed by *C. terrigena* B-996. In the control test, the dipeptidase solution was replaced by 0.01 M phosphate buffer, pH 7.0.

Paper Chromatography and High Voltage Paper Electrophoresis

The solvent system for descending paper chromatography (PC) was $CH_3CN$ - 0.1 M Tris-HCl, pH 7.5 - 0.1 M EDTA, pH 7.5 (120: 30: 1). High voltage paper electrophoresis (HVPE) was carried out at 0°C and 1,500 V for 40 minutes in Veronal buffer, pH 8.6.[8] Carbapenem compounds on a paper chromatogram or an electrophoretogram were located by bioautography using *C. terrigena* B-996.

Physico-chemical Determinations

The UV spectra of OA-6129A and $B_2$ phosphates were recorded in 0.01 M phosphate buffer, pH 7.4. $^1H$ NMR measurements were carried out with a 90 MHz NMR spectrometer (Varian EM390), using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard in deuterium oxide.

Susceptibility of OA-6129A Phosphate and OA-6129$B_2$ Phosphate to Alkaline Phosphatase and A933 Acylase

The susceptibility to alkaline phosphatase was checked by incubation at pH 8.5 and 37°C for 30 minutes.

For the test of A933 acylase susceptibility, a reaction mixture composed of 20 μl of 380 μg/ml OA-6129A phosphate or 300 μg/ml OA-6129A, 10 μl of 5 mM acetyl CoA, 10 μl of A933 acylase and 10 μl of 0.2 M phosphate buffer, pH 7.4, was incubated at 37°C for 2 hours and then subjected to HVPE and PC, followed by bioautography. OA-6129$B_2$ phosphate and OA-6129$B_2$ were similarly treated.

Microbial Phosphorylation

Small-scale tests were carried out under reaction conditions similar to those reported by SHIMIZU *et al.*[7].

For preparation of OA-6129A and $B_2$ phosphates, a reaction mixture containing 40 mg OA-6129A or OA-6129$B_2$, 300 mg ATP (pre-adjusted to pH 7), 148 mg $MgSO_4 \cdot 7H_2O$, 100 mg sodium dodecylsulfate, 20 ml of 0.5 M phosphate buffer, pH 7.1, and 100 ml of the cell suspension of *B. ammoniagenes* was incubated at 37°C for 3 hours. The phosphorylated carbapenem was isolated by column chromatography on QAE-Sephadex A-25 ($Cl^-$), Diaion HP-20AG and Bio-Gel P-2.

## Results and Discussion

### Phosphorylation of the OA-6129 Group of Carbapenems by *B. ammoniagenes*

SHIMIZU *et al.* reported that the intact or air-dried cells of *B. ammoniagenes* convert pantetheine to 4′-phosphopantetheine with ATP in the presence of a surfactant[7]. The phosphorylation of OA-6129A by this microorganism was investigated in several reaction systems (Fig. 1). As the bacterium has an acylase activity, the air-dried cells or the cell-free extract convert OA-6129A to NS-5, while a very small amount of OA-6129A phosphate is formed. Interestingly, however, when the intact cells are employed together with a surfactant, the acylase activity is not expressed and accordingly OA-6129A is completely phosphorylated in one hour of incubation. One plausible conjecture is that sodium dodecylsulfate, a

Fig. 1. Formation of OA-6129A phosphate by *B. ammoniagenes* ATCC 6871.

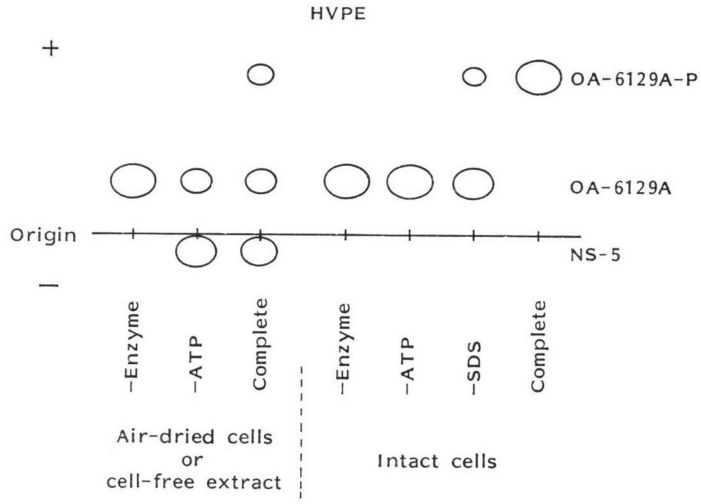
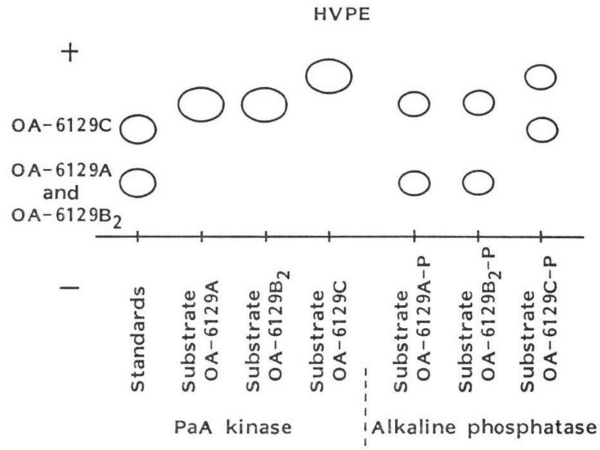



Fig. 2. Phosphorylation of OA-6129 carbapenems and dephosphorylation of OA-6129 carbapenem phosphates.




surfactant, might have some selective effects on leakage of enzymes from cells, as SHIMIZU *et al.* reported the necessity of a surfactant for phosphorylation of pantetheine by the intact cells[7].

Fig. 2 illustrates the phosphorylation of OA-6129A, $B_2$ and C by *B. ammoniagenes* and the dephosphorylation of OA-6129A, $B_2$ and C phosphates by alkaline phosphatase. Although OA-6129$B_1$, OA-6129D and OA-6129E[10] have not yet been tested, it is very likely that they are also phosphorylated under similar conditions.

## Physico-chemical Properties of OA-6129A and $B_2$ Phosphates

Table 1 lists the physico-chemical properties of OA-6129A and $B_2$ phosphates.

The phosphoryl group was located by $^1$H NMR at the primary hydroxyl of the C-3 pantetheinyl side chain (Fig. 3).

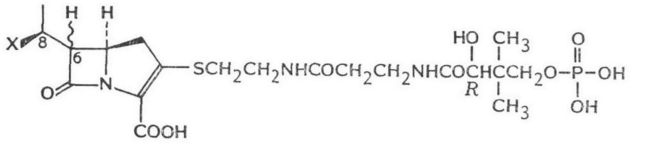
## Antimicrobial Activity of OA-6129A and $B_2$ Phosphates

Table 2 includes test microorganisms which showed significant differences in antimicrobial sensiti-

Table 1. Physico-chemical properties of OA-6129A and $B_2$ phosphates.

| | OA-6129A phosphate | OA-6129$B_2$ phosphate |
|---|---|---|
| UV $\lambda_{max}$ nm ($E^{1\%}_{1cm}$) | 302 (87.9) | 302 (82.4) |
| $^1$H NMR in $D_2O$ (internal standard DSS) $\delta$ (ppm) | 0.89 (3H, s, $CH_3C(CH_3)$–)<br>0.97 (3H, s, $CH_3C(CH_3)$–)<br>0.98 (3H, t, $J$=7.0 Hz, $CH_2CH_3$)<br>1.50~2.00 (2H, m, $CH_2CH_3$)<br>2.47 (2H, t, $J$=6.5 Hz, $NHCH_2CH_2CO$)<br>2.70~3.83 (11H, m, C-4$H_2$, C-6H, $SCH_2CH_2NH$, $NHCH_2CH_2CO$, $-CCH_2OPONa$)<br>3.85~4.20 (2H, m, C-5H, HOCHCO) | 0.87 (3H, s, $CH_3C(CH_3)$–)<br>0.95 (3H, s, $CH_3C(CH_3)$–)<br>1.27 (3H, d, $J$=7.0 Hz, $CH_3CH(OH)$–)<br>2.47 (2H, t, $J$=6.5 Hz, $NHCH_2CH_2CO$)<br>2.80~4.50 (14H, m, C-4$H_2$, C-5H, C-6H, $CH_3CH(OH)$–, $SCH_2CH_2NH$, $NHCH_2CH_2CO$, $-CCH_2OPONa$, HOCHCO) |
| PC (Rf) | 0.03 | 0.01 |
| HVPE | 11~12 cm to anode | 11~12 cm to anode |
| Color reaction | | |
| Positive | Ehrlich reagent, chloroplatinate, Hanes-Ischerwood reagent | Ehrlich reagent, chloroplatinate, Hanes-Ischerwood reagent |
| Negative | Ninhydrin | Ninhydrin |

Fig. 3. Structures of the OA-6129 group of carbapenem phosphates.




| | X | C-6 | C-8 |
|---|---|---|---|
| OA-6129A phosphate | H | *R* | — |
| [OA-6129$B_1$ phosphate | OH | *R* | *S* (estimated)] |
| OA-6129$B_2$ phosphate | OH | *S* | *S* |
| OA-6129C phosphate | $OSO_3H$ | *R* | *S* (estimated) |

vity to the carbapenems before and after phosphorylation. The following bacteria possessed similar sensitivities in the assay limit of 0.5~2.0×MIC before and after phosphorylation: *Micrococcus luteus* S19, *Staphylococcus aureus* FDA 209P, *S. aureus* Smith, *Alcaligenes faecalis* A1, *Citrobacter freundii* GN346*, *C. terrigena* B-996, *Enterobacter aerogenes* E19*, *E. cloacae* 45*, *Klebsiella pneumoniae* K13, *Proteus mirabilis* P6, *P. rettgeri* P7, *Providencia* sp. P8, *Pseudomonas aeruginosa* IFO 3445*, *P. aeruginosa* NCTC 10490*, and *Serratia marcescens* T55* (* means the production of β-lactamase). In general, the phosphorylation has a moderate tendency to increase the antimicrobial activity of the carbapenems against Gram-negative bacteria and to decrease the activity against Gram-positive bacteria.

Table 2. Antimicrobial activities of OA-6129A phosphate, OA-6129$B_2$ phosphate, OA-6129A, OA-6129$B_2$ and cephaloridine (MIC in $\mu$g/ml).

| Test microorganism | OA-A-P | OA-$B_2$-P | OA-A | OA-$B_2$ | CER |
|---|---|---|---|---|---|
| *Bacillus subtilis* ATCC 6633 | 3.13 | 25 | 0.39 | 1.56 | 0.10 |
| *Micrococcus luteus* S19 | 6.25 | >25 | 6.25 | 6.25 | 0.20 |
| *Staphylococcus aureus* FDA209P | 1.56 | 12.5 | 0.78 | 6.25 | 0.10 |
| 〃 Smith | 3.13 | >25 | 1.56 | 12.5 | 0.10 |
| 〃 Russell | 6.25 | >25 | 0.78 | 12.5 | 0.10 |
| *S. epidermidis* | 6.25 | >25 | 1.56 | 12.5 | 0.10 |
| *Alcaligenes faecalis* A1 | 1.56 | 6.25 | 1.56 | 6.25 | 3.13 |
| *Citrobacter freundii* GN346* | >25 | >25 | 50 | 25 | >400 |
| *Comamonas terrigena* B-996 | 0.05 | 0.39 | 0.05 | 0.39 | 0.10 |
| *Enterobacter aerogenes* E19* | 12.5 | 25 | 25 | 25 | >400 |
| *E. cloacae* 45* | 25 | >25 | 50 | 50 | >400 |
| *Enterobacter* sp. E8 | 3.13 | 12.5 | 12.5 | 25 | 1.56 |
| *Escherichia coli* K-12 | 3.13 | 12.5 | 12.5 | 12.5 | 1.56 |
| 〃 RGN823* | 3.13 | 12.5 | 12.5 | 12.5 | 1.56 |
| *Klebsiella pneumoniae* K13 | 25 | 12.5 | 50 | 12.5 | 25 |
| *Proteus mirabilis* P6 | 25 | 25 | 50 | 50 | 6.25 |
| *P. rettgeri* P7 | 12.5 | 12.5 | 25 | 25 | 1.56 |
| *P. vulgaris* GN76* | 25 | 25 | 100 | 50 | >400 |
| *Proteus* sp. P22* | 25 | 25 | 100 | 50 | >400 |
| *Providencia* sp. P8 | 6.25 | 6.25 | 12.5 | 6.25 | 3.13 |
| *Pseudomonas aeruginosa* IFO 3445* | >25 | >25 | >100 | >100 | >400 |
| 〃 NCTC 10490* | >25 | >25 | >100 | >100 | >400 |
| *Serratia marcescens* S18* | 25 | 12.5 | 100 | 25 | >400 |
| 〃 T55* | >25 | 25 | 100 | 50 | >400 |

Medium: Heart infusion agar (Difco); inoculum size, $10^6$ cells/ml.

* $\beta$-Lactamase producer.

Abbreviations: OA-A-P=OA-6129A phosphate, OA-$B_2$-P=OA-6129$B_2$ phosphate, OA-A=OA-6129A, OA-$B_2$=OA-6129$B_2$, CER=cephaloridine.

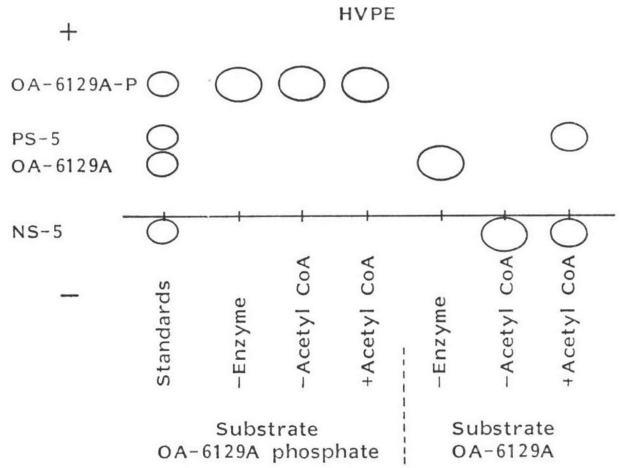
Table 3. Comparative dipeptidase stabilities of OA-6129A phosphate, OA-6129$B_2$ phosphate, OA-6129A, OA-6129$B_2$ and PS-5.

| Kidney homogenate | OA-6129A-P | OA-6129$B_2$-P | OA-6129A | OA-6129$B_2$ | PS-5 |
|---|---|---|---|---|---|
| Control (PBS) | 114% | 100% | 100% | 98% | 100% |
| Mouse | 47 | 42 | 7 | 20 | 10 |
| Dog | 18 | 29 | 25 | 20 | 10 |
| Human | 96 | 72 | 57 | 47 | 28 |

## Stability of OA-6129A and $B_2$ Phosphates to Renal Dipeptidases

It is well acknowledged that naturally-occurring carbapenem compounds are labile *in vivo*, and, more particularly, are hydrolyzed by renal dipeptidase[5]. CAMPBELL[11] describes that phosphates such as ATP are potent inhibitors of dipeptidase. As we have not yet succeeded in establishing enzymologically sound reaction conditions which allow us to objectively evaluate the susceptibility or resistance of a carbapenem compound to renal dipeptidase, a very primitive comparison method was employed here. The results in Table 3 show that the improved resistances of OA-6129A and $B_2$ phosphates are moderate to the human enzyme and significant to the mouse enzyme, whereas the dog dipeptidase is similarly active before and after phosphorylation.

Fig. 4. Action of A933 acylase on OA-6129A phosphate and OA-6129A.




Susceptibility of OA-6129A and $B_2$ Phosphates to A933 Acylase

In previous papers[3,4], we have reported the isolation and enzymological characterization of A933 acylase which governs the depantothenylation and acyl exchange of the OA-6129 group of carbapenems in the absence and presence of acyl CoA. In the acyl carrier protein, CoA is linked *via* phosphate to the hydroxyl group of the serine moiety. Therefore the susceptibility of OA-6129A phosphate to A933 acylase was examined in the presence and absence of acetyl CoA (Fig. 4).

Contrary to our expectation, the phosphorylation of the C-3 pantetheinyl side chain makes OA-6129A insusceptible to A933 acylase. Similar results were obtained with OA-6129$B_2$ phosphate as substrate.

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