STUDIES ON THE OA-6129 GROUP OF ANTIBIOTICS, NEW CARBAPENEM COMPOUNDS

I. TAXONOMY, ISOLATION AND PHYSICAL PROPERTIES

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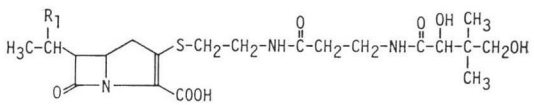
Streptomyces sp. OA-6129, a soil actinomycete, was found to produce a new group of carbapenem antibiotics named OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C. The taxonomy of the producer and the isolation and physicochemical properties of these new carbapenem compounds are described.

In previous papers, we have described the production of the PS group of antibiotics (PS-5[1], PS-6[2], PS-7[2] and PS-8[3]) by streptomycetes. In the continued screening for new β-lactam antibiotics, we have discovered new carbapenem antibiotics in the fermentation broth of a soil actinomycete.

For convenience of explanation, the chemical structures of these new compounds are shown in Fig. 1. The detailed chemical studies on the OA-6129 group of carbapenem compounds will be published elsewhere[4]. It is apparent from Fig. 1 that the OA-6129 group of antibiotics differ in the C-3 pantetheinyl side chain not only from the PS group of antibiotics but also from the other hitherto-known carbapenem compounds[5~10].

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



OA-6129A	R_1=–H
OA-6129B_1	R_1=–OH
OA-6129B_2	R_1=–OH
OA-6129C	R_1=–OSO_3H

This paper describes the taxonomy of the producing organism and the isolation and physicochemical properties of OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C.

Materials and Methods

Chemicals

PS-5 (sodium salt) was prepared in our laboratories as described in a previous report[1].

Disk-agar Diffusion Assay

Overnight culture of *Comamonas terrigena* B-996 on a nutrient agar slant was suspended in nutrient broth to give a seed cell suspension which had an optical density of 0.040 at 610 nm[1]. One percent of the seed cell suspension was inoculated in molten agar medium consisting of 0.8% Kyokuto Nutrient Broth Powder (Kyokuto Seiyaku Kogyo Co., Ltd., Japan) and 1.0% Bacto-agar (Difco Laboratories, U.S.A.). Seven milliliters of the inoculated molten agar medium was poured into a 9-cm Petri dish and allowed to solidify to provide a *Comamonas*-disk assay plate.

Bioautography

Instead of a 9-cm Petri dish, a 32×24 cm tray was filled with 100 ml of the inoculated molten agar medium. A sheet of chromatographic paper on which antimicrobial compounds had been developed

in a suitable solvent system was air-dried for removal of the solvent and was then kept for 15 minutes in contact with the surface of the agar medium. After the chromatogram was removed, the agar assay tray was incubated at 30°C for 20 hours.

Thin-layer Chromatography

A new thin-layer chromatographic method was devised for qualitative and semi-quantitative analysis of the OA-6129 group of carbapenem compounds. Assay samples were spotted on a precoated silica gel thin-layer plate (silica gel 60 F_{254}; E. Merck, Darmstadt) and developed in an appropriate solvent system. The OA-6129 group of carbapenem compounds were visualized with the Ehrlich reagent. This thin-layer chromatographic method will be detailed in a separate report[11].

pH Stability Test

Monopotassium phosphate (0.05 M) solution was adjusted to the indicated pH's by adding 2% sodium hydroxide or 10% hydrochloric acid, and distributed in 3 ml volumes into test tubes. 0.05 ml each of OA-6129A (sodium salt) or PS-5 (sodium salt) solution (2 mg/ml) was added to the buffer solutions and incubated at 28°C. The concentration of the unchanged antibiotic was measured at 300 nm by spectrometry.

Results and Discussion

Taxonomical Study of Strain OA-6129

By a modification of the previously-described screening system for β-lactam compounds[1] in which the thin-layer chromatographic analysis was included for selective detection of carbapenem compounds in fermentation broths, a soil microorganism which was found to produce seemingly new carbapenem antibiotics was isolated from a soil sample collected near the Sumiyoshi Shrine in Fukuoka-shi, Japan. The soil isolate was taxonomically studied by the methods of SHIRLING and GOTTLIEB[12~16]; PRIDHAM and TRESNER[17]; WAKSMAN[18].

Morphological Characteristics

On slide glass cultivation, straight or flexuous aerial mycelia without verticillate branchings grow from well-branched substrate mycelia. A mature spore chain usually consists of more than ten elliptical or cylindrical spores. Spores are non-flagellated; 0.6~1.0×0.7~2.5 microns in size; and have smooth surfaces. No sporangium is observed.

Cultural Characteristics

Table 1 summarizes the cultural characteristics of strain OA-6129. Unless specifically stated, the microorganism was cultured at a temperature of 28~30°C. According to the method of TRESNER and BACKUS[20], the color symbols in parentheses refer to the Color Harmony Manual of Container Corporation of America[19].

Physiological Characteristics

Table 2 shows the physiological properties of strain OA-6129. Gelatin liquefaction, starch hydrolysis and milk peptonization are positive, whereas milk coagulation and melanoid formation are negative.

The carbohydrate utilization pattern of strain OA-6129 is presented in Table 3.

Based on the above-described taxonomical findings, strain OA-6129 was concluded to belong to *Streptomyces*. A culture of *Streptomyces* sp. OA-6129 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan with the deposit number of FERM BP-11.

Table 1. Cultural characteristics of strain OA-6129.

Nutrient agar	G*:	abundant	Sucrose - nitrate agar	G:	moderate
	AM:	light grayish reddish brown [5fe]		AM:	yellowish gray [2dc]~grayish pink [5dc]
	SM:	pale yellow [2db] or light yellow [2fb]~light olive brown [2ge]		SM:	yellowish gray [2dc]~light grayish yellow brown [3ge]
	SP:	none		SP:	none
Yeast extract - malt extract agar (ISP-2 medium)	G:	abundant	Glucose - asparagine agar	G:	abundant
	AM:	grayish yellow pink [5dc] or at a somewhat later stage, light gray [d]		AM:	light gray [d]
	SM:	moderately yellowish pink [4gc]~light brown [4ie]		SM:	pale yellow [2db]~light olive brown [2ge], later turning to be yellow pink [5dc]
	SP:	none		SP:	none
Oatmeal agar (ISP-3 medium)	G:	good	Glycerol - asparagine agar	G:	moderate
	AM:	light brownish gray [3fe]~light grayish reddish brown [5fe]		AM:	light gray [d]~light grayish reddish brown [5fe]
	SM:	grayish yellow [3ec]~light orange yellow [3ea]		SM:	yellowish pink [4gc]~light brown [4ie]
	SP:	brown (slightly)		SP:	none
Calcium malate agar	G:	moderate	Starch - inorganic salt agar (ISP-4 medium)	G:	good
	AM:	light gray [d]~light grayish reddish brown [5fe]		AM:	light gray [d]
	SM:	colorless~pale yellow [2db] or yellowish gray [2dc]		SM:	pale yellow [2db]~gray [2fe]
	SP:	none		SP:	none
Glucose - peptone - gelatin (cultivated at 20°C)	G:	good	Tyrosine agar (ISP-7 medium)	G:	moderate
	AM:	white [b]~grayish yellow pink [5cb]		AM:	light gray [d]~light brownish gray [3fe]
	SM:	pale yellow [2bd]~brown		SM:	grayish yellow [3ec]~light brown [4ie]
	SP:	brown		SP:	none

* G: growth, AM: aerial mycelium, SM: substrate mycelium, SP: soluble pigment. [ ]: color code of the Color Harmony Manual[10].

Table 2. Physiological properties of strain OA-6129.

Growth temperature	growth occurs at 10~40°C better growth at 20~30°C
Liquefaction of gelatin	positive
Hydrolysis of starch	positve
Coagulation of skimmed milk	peptonization without coagulation
Formation of melanoid pigment	no melanoid pigment is formed in peptone-yeast extract-iron agar (ISP-6 medium) and Tryptone-yeast extract broth (ISP-11 medium). In tyrosine agar, slightly brown color is observed with a trace amount of melanin.

Table 3. Utilization of carbon sources by strain OA-6129.

Carbon source	Growth
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	±
Inositol	−
L-Rhamnose	+
Raffinose	−
D-Mannitol	+

Symbols: +; positive, ±; doubtful, −; negative

Fermentation

One hundred milliliters of the 48 hour-old culture of *Streptomyces* sp. OA-6129 in a 500-ml Erlenmeyer flask was inoculated into a 30-liter jar fermentor containing 15 liters of seed medium SE-4 (Table 4) and was cultivated at 28°C for 24 hours, using an agitation rate of 200 rpm and an aeration rate of 0.5 volume/volume/minute. One liter of the seed culture was transferred into a 200-liter stainless steel fermentor containing 100 liters of medium GM-1 (Table 4). The fermentor was operated at 28°C under forced aeration (agitation 200 rpm; aeration 0.5 volume/volume/minute).

The production of the OA-6129 group of antibiotics was traced by the disk-agar diffusion assay with *Comamonas terrigena* B-996 using PS-5 sodium salt as a tentative bioassay standard. Growth of the microorganism was measured in the packed volume of sediment by centrifuging 3 ml of a broth sample at 1,500 rpm for 15 minutes. The concentration of glycerol was determined by the method of IWAI *et al.*[21]. A typical time course of fermentation of *Streptomyces* sp. OA-6129 is illustrated in Fig. 2.

Thin-layer Chromatographic Analysis of the Fermentation Broth

By the newly-developed thin-layer chromatographic method, the fermentation broths of *Strepto-*

Table 4. Seed medium and fermentation medium.

Seed medium (SE-4)		Production medium (GM-1)	
Beef extract (Difco)	0.3%	Glycerol	8.0%
Bacto - Tryptone (Difco)	0.3%	Fish meal	1.0%
Defatted soybean meal	0.5%	Defatted soybean meal	3.0%
Glucose	0.1%	$CaCO_3$	0.3%
Soluble starch	2.4%	K_2HPO_4	0.2%
Yeast extract	0.4%	$MgSO_4$	0.2%
$CaCO_3$	0.4%	pH (adjusted with NaOH	
pH (before sterilization)	7.5	before sterilization)	7.5
		Vitamin B_{12} in 0.01 M phosphate buffer, pH 5.5, was separately autoclaved for 5 minutes at a pressure of 1 kg/cm^2·G and added in an amount of 0.0005% (w/v).	

Fig. 2. A typical time course of the fermentation by strain OA-6129 in a 200-liter fermentor.

Medium: GM-1. Temperature: 28°C. Agitation and aeration: 200 rpm, 0.5 vol./vol./minute.

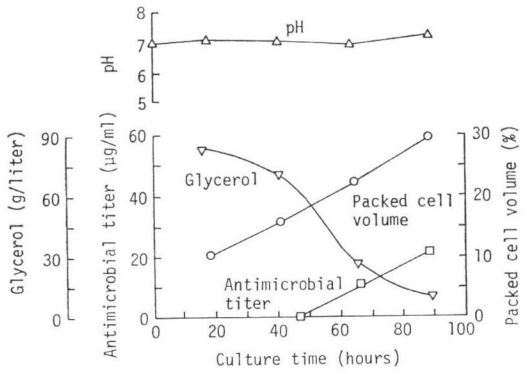
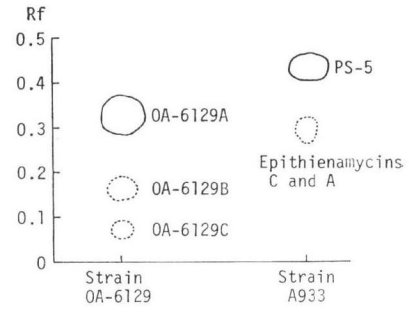


Fig. 3. Thin-layer chromatograms of the fermentation broths of *Streptomyces* sp. OA-6129 and *Streptomyces fulvoviridis* A933.

Plate: Silica gel 60 F_{254} (Merck)
Solvent system: CH_3CN - 0.75% CH_3COOH, 9:2
Detection: Ehrlich reagent



myces sp. OA-6129 and *Streptomyces fulvoviridis* A933[1] were compared (Fig. 3).

It is apparent from Fig. 3 that, in this run of fermentation, OA-6129A was a major component together with small amounts of OA-6129B and OA-6129C. The thin-layer chromatographic analysis which will be reported elsewhere[11] was found to be very convenient for component analysis of fermentation broths, as it was rapid, semi-quantitative, less expensive and easy to run in comparison with the conventional methods such as paper chromatography, high voltage paper electrophoresis and high performance liquid chromatography.

Isolation and Purification

Procedures for isolation and purification of OA-6129A, OA-6129B_1, OA-6129B_2 and OA-6129C are schematically presented in Figs. 4-a, 4-b and 4-c.

The fermentation broth (100 liters) of *Streptomyces* sp. OA-6129 was mixed with 5% of Topco Perlite No. 34 (a filter aid produced by Toko Perlite Co., Ltd., Japan) and then filtered with a filter press. The clear filtrate (90 liters) was adsorbed on a chromatographic column (15 ×100cm) of Diaion HP 20 (Mitsubishi Chemical Industries Ltd., Japan) and the column was eluted with 30% aqueous acetone. Antimicrobially active fractions were collected; combined

Fig. 4-a. Recovery process for the OA-6129 group of antibiotics from the fermentation broth.

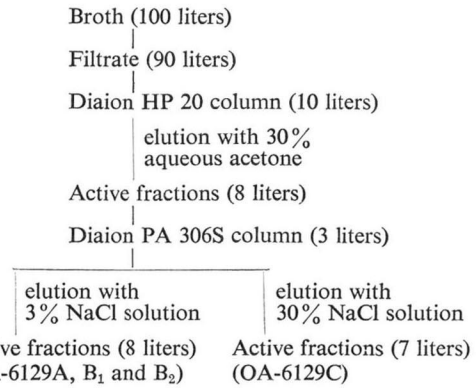


Fig. 4-b. Purification process for compounds OA-6129A, B_1 and B_2.

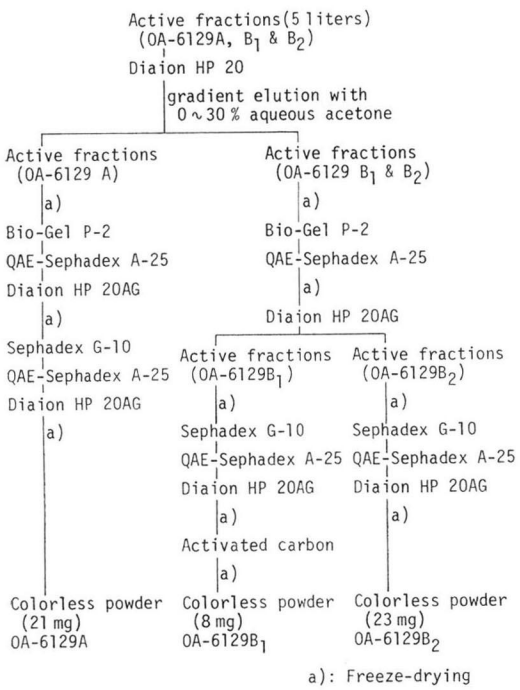
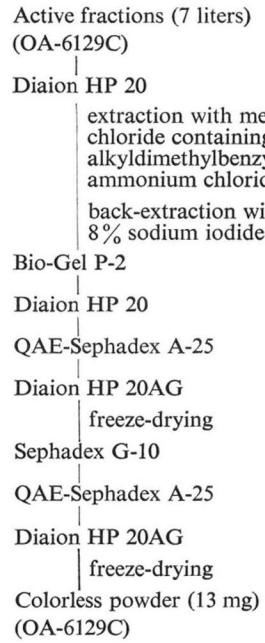


Fig. 4-c. Purification process for compound OA-6129C.



and charged on a Diaion PA 306S column (8×60 cm; Mitsubishi Chemical Industries Ltd., Japan). With 3.0% sodium chloride as eluant, fractions containing OA-6129A, OA-6129B_1 and OA-6129B_2 were first collected from the column. Subsequently the concentration of sodium chloride in the eluant was increased to 30% for elution of OA-6129C (Fig. 4-a).

The solution containning OA-6129A, OA-6129B_1 and OA-6129B_2 was subjected to successive column chromatographies using Diaion HP 20, Bio-Gel P-2 (Bio-Rad Laboratories, U.S.A.), QAE-Sephadex A-25 (Pharmacia Fine Chemicals AB, Sweden) and Diaion HP 20AG (Mitsubishi Chemical Industries Ltd., Japan), as is illustrated in Fig. 4-b. The final yields of OA-6129A, OA-6129B_1 and OA-6129B_2 were 21 mg, 8 mg and 23 mg respectively.

OA-6129C was assumed to have a sulfuryl function on the basis of its high voltage electrophretic data. The extraction process with a quarternary ammonium salt, which was described for isolation

Fig. 5-a. IR spectrum of compound OA-6129A in KBr.

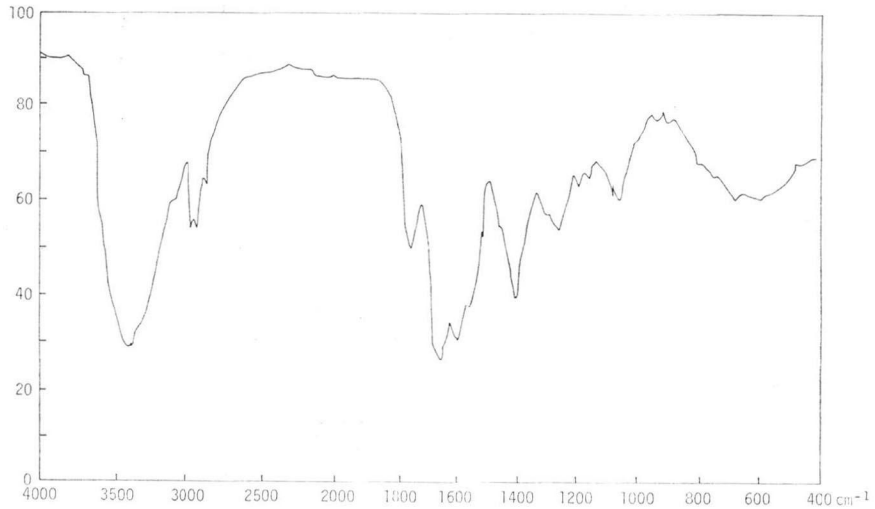
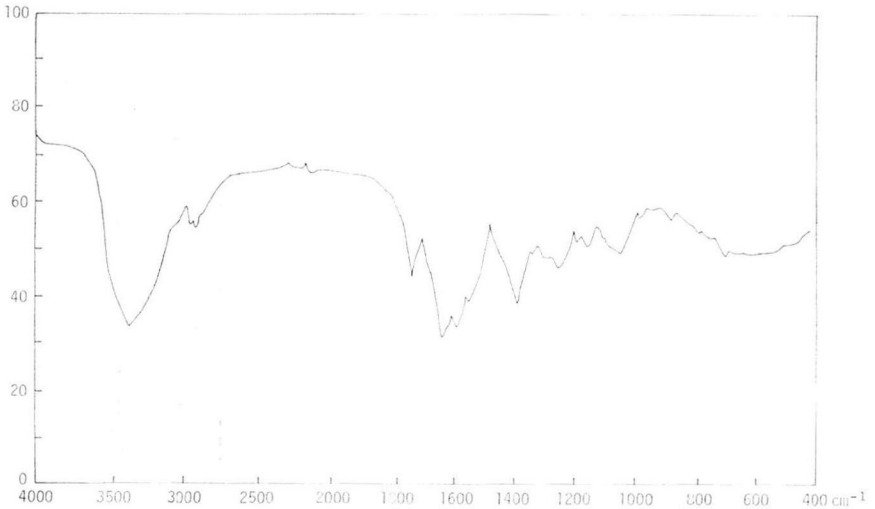


Fig. 5-b. IR spectrum of compound OA-6129B_1 in KBr.



and purification of the olivanates[5], was effectively employed for preparation of OA-6129C. After desalting with Diaion HP 20, the OA-6129C solution from Fig. 4-a was extracted with methylene chloride containing 3.0% alkyldimethylbenzylammonium chloride (Tokyo Chemical Industry Co., Ltd., Japan). The organic layer was separated and back-extracted with 8.0% sodium iodide. According to the basically same processes as for OA-6129A, OA-6129B_1 and OA-6129B_2, the aqueous solution of OA-6129C was purified to give 13 mg of colorless powder of OA-6129C (Fig. 4-c).

Physicochemical Properties

The four carbapenem compounds were isolated as the substantially pure sodium salts. They are freely soluble in water, methanol and ethanol, but practically insoluble in other common organic solvents. Their IR absorption spectra are reproduced in Figs. 5-a, 5-b, 5-c and 5-d.

Fig. 5-c. IR spectrum of compound of OA-6129B_2 in KBr.

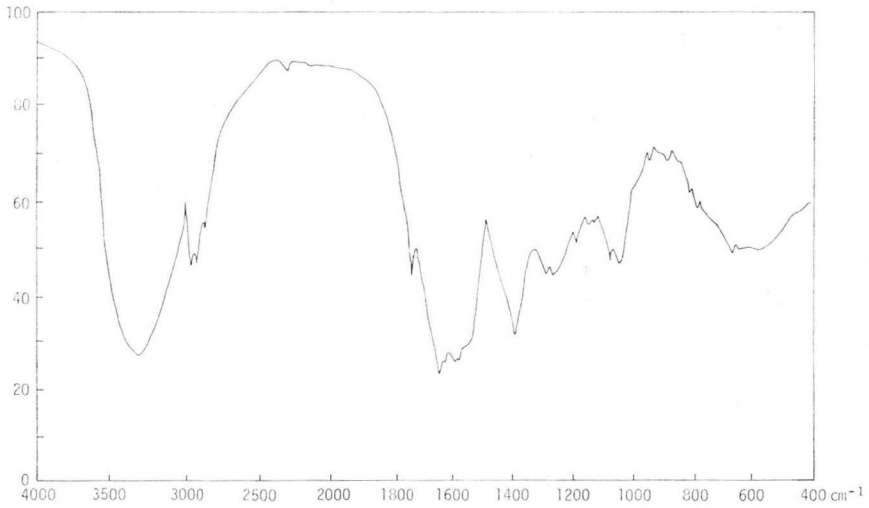


Fig. 5-d. IR spectrum of compound OA-6129C in KBr.

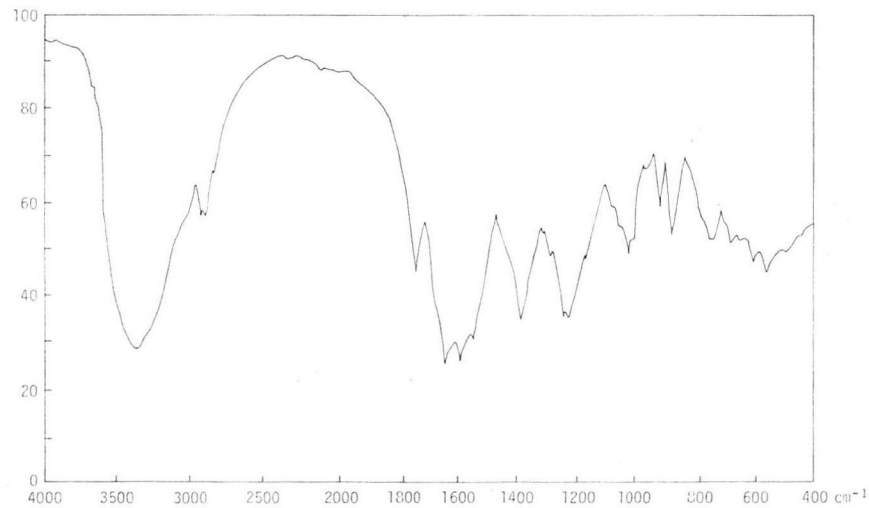


Table 5. Physicochemical properties of the OA-6129 group of antibiotics.

	OA-6129A	OA-6129B$_1$	OA-6129B$_2$	OA-6129C
Appearance	Colorless	Colorless	Colorless	Colorless
IR ν_{max}^{KBr} cm^{-1}	1760, 1660, 1600	1750, 1650, 1590	1760, 1660, 1600	1750, 1660~1595, 1250~1220
UV $\lambda_{max}^{H_2O}$ nm(ε)	300 (5,600)	300 (6,400)	300 (5,400)	300.5 (7,600)
Optical rotation $[\alpha]_D^{24}$	11.6° (c 1.0, 0.01 M PBS*, pH 8.4)	24.2° (c 0.5, H_2O)	14.7° (c 1.0, 0.01 M PBS*, pH 8.4)	17.4° (c 0.55, 0.01 M PBS*, pH 8.2)
HVPE Rm**	0.67	0.67	0.67	1.69
PC Rf***	0.29	0.17	0.17	0.09

* PBS =phosphate buffer.

** HVPE=high voltage paper electrophoresis; 1,500 V/35 cm in Veronal buffer, pH 8.6 (I 0.027); Rm=relative to PS-5 sodium salt.

*** PC=paper chromatography (descending); acetonitrile - 0.1 M tris-HCl (pH 7.5) - 0.1 M EDTA (pH 7.5), 120: 30: 1.

Table 5 summarizes the physicochemical data, relative HVPE mobilities and Rf values of the OA-6129 group of carbapenem compounds.

The *in vitro* evaluation of the antimicrobial activities of OA-6129A, OA-6129B$_1$, OA-6129B$_2$ and OA-6129C will be reported in the subsequent paper[23].

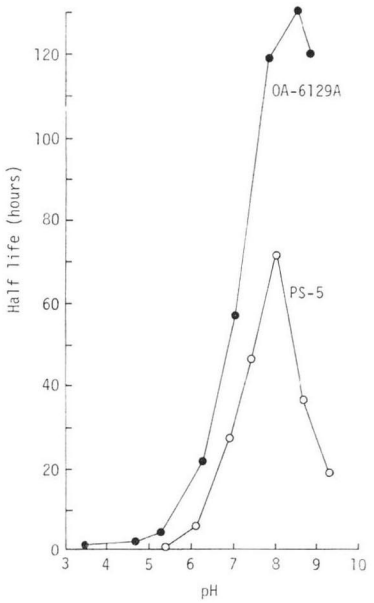
Comparative pH Stabilities of OA-6129A and PS-5

Chemical stabilities of OA-6129A and PS-5 in aqueous solution were compared at the indicated pH's by UV spectrometry.

Fig. 6 shows that OA-6129A is significantly more stable than PS-5 over the pH range tested, although both compounds are practically stable only in a narrow range of pH close to neutrality. The other members of the OA-6129 group seem to have the substantially same pH stabilities as OA-6129A, indicating that the 3-pantetheinyl side chain serves to stabilize the carbapenem nucleus.

Fig. 6. Comparative pH stabilities of OA-6129A and PS-5.

The amount of unchanged carbapenem in 0.05M phosphate buffer was followed at 28°C by spectrometry.



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