PS-6 AND PS-7, NEW β-LACTAM ANTIBIOTICS

ISOLATION, PHYSICOCHEMICAL PROPERTIES AND STRUCTURES

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Antibiotics PS-6 and PS-7 which are shown to be 5*R*,6*R*-3-(2-acetamido)ethylthio-6-isopropyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid and 5*R*,6*R*-3-(*E*)-(2-acetamido) vinylthio-6-ethyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid respectively, are new β-lactam compounds isolated from fermentation broths of *Streptomyces cremeus* subsp. *auratilis* A271, *S. fulvoviridis* A933, *S. olivaceus* ATCC 31126 and *S. flavogriseus* NRRL 8139. Fermentation, isolation, physicochemical properties and structures of antibiotics PS-6 and PS-7 are described.

In a previous paper,[1] we have reported the isolation of a new β-lactam antibiotic called PS-5 from fermentation broth of *Streptomyces cremeus* subsp. *auratilis* A271 (ATCC 31318). PS-5 has a good antimicrobial activity against Gram-positive and Gram-negative bacteria[2] and exhibits a potent β-lactamase-inhibitory activity.[3,4] The absolute structure of PS-5 has been determined[5]. L- and D-Amino acid acylases deacylate PS-5 to give a compound called NS-5{3-(2-amino)ethylthio-6-ethyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid}[6].

During the fermentation study of PS-5, two minor components named PS-6 and PS-7 were discovered together with PS-5. Like thienamycin[7], epithienamycins[8], PS-5[1] and olivanic acids[9], they were found to have the carbapenem nucleus.

This paper reports the fermentation, isolation, physicochemical characteristics and structures of PS-6 and PS-7.

Materials and Methods

Fermentation

S. cremeus subsp. *auratilis* A271 was grown on an ISP-2 agar slant at 28°C for 2 weeks. Spores were collected from a slant culture with 10 ml of physiological saline.

One milliliter (*ca.* 10^8 cells/ml) of the spore suspension was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium SE-4. The seed medium SE-4 consisted of 0.3% beef extract (Difco), 0.5% Bacto-tryptone(Difco), 0.5% defatted soybean meal, 0.1% glucose, 2.4% soluble starch, 0.5% yeast extract and 0.4% $CaCO_3$ in tap water, the pH being adjusted to 7.5 before sterilization. After 48 hours cultivation with shaking, 2 ml each of the seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml each of production medium AGB-7(3.0% maltose, 2.0% dry yeast, 0.3% defatted soybean meal, 0.1% peptone, 0.5% NaCl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.4% $CaCO_3$ and 0.00013% $CoCl_2 \cdot 6H_2O$ in tap water, pH 7.0). The flasks were shake-cultured at 28°C for

96 hours on a rotary shaker (throw 70 mm; 220 rpm).

Assay methods

(1) Total potency: The production of the PS-group of antibiotics (PS-5, PS-6, PS-7 and other components) was followed as the total antibacterial activity by the disc plate assay method using *Comamonas terrigena* IFO 12685[1]. The potency was expressed in ccu (*Comamonas*-cephaloridine units) with cephaloridine as a reference β-lactam compound.

(2) Differentiation of PS-6 and PS-7 from PS-5 and other components: For quantitative assay of PS-6 and PS-7, broth samples were subjected to column chromatography on an anion-exchange resin. The broth filtrate (20 ml) was applied on a Diaion PA306S ion exchange column (Cl^- form; 0.5 × 20 cm). PS-6 was eluted first with 20 ml of 30% NaCl and then PS-7 with 20 ml of 50% methanol containing 4% NaCl. The methanol in the PS-7 eluate was removed by evaporation *in vacuo* at 10°C. Each of the eluates was then adsorbed onto a Diaion HP-20 column (0.5 × 20 cm) and PS-6 or PS-7 was eluted with 10 ml of 50% acetone. The most active fraction was assayed by high performance liquid chromatography and/or paper or thin-layer chromatography[1] followed by bioautography using *Comamonas terrigena* IFO 12685.

(3) Time course of PS-6 and PS-7 production: Cell growth was measured using the packed volume of sediment from 10 ml broth after centrifugation at 1,500 *g* for 10 minutes. Sugar was measured by the anthrone method[10].

(4) High performance liquid chromatography: An Atto 711 liquid chromatograph was equipped with a solvent delivery system and a sampling loop injector (100 μl). The column employed in this study was μBondapak C_{18} (reversed phase, 4 × 300 mm; Waters Associates Ltd.). A Shimadzu SPD-1 spectrophotometer with a flow cell of 6.4 μl (8 mm path length) and an Atto SF-1205 spectrophotometer with a flow cell of 8 μl (10 mm path length) were connected in series to the column for monitoring at 300 nm and 254 nm respectively. The routine injection volume was 100 μl. A solvent system consisting of ammonium phosphate buffer (0.01 M, pH 7.6) containing 20% methanol was found useful for assay of PS-5, PS-6 and PS-7 at an elution velocity of 0.84 ml/minute. Retention times of PS-5, PS-6 and PS-7 are presented in Table 1.

General methodology

UV absorption spectra were taken with a Hitachi 200–20 spectrophotometer; IR spectra with a Hitachi 260–30 spectrophotometer; optical rotations with a JASCO DIP-181 digital polarimeter; high resolution mass spectra with a Hitachi RMU-7 mass spectrometer; and NMR spectra with a JEOL PS-100 spectrometer.

Preparation of the methyl esters of PS-6 and PS-7

Twenty milligram of triethylamine and 0.1 ml of methyl iodide were added to a suspension of PS-6 sodium salt (15 mg) in 2 ml of dried dimethylformamide. After stirring for 2.5 hours at room temperature, the suspension was diluted with benzene to make a final volume of 50 ml. The benzene solution was washed with 50 ml of 0.1 M sodium phosphate buffer, pH 7.0; dried over anhydrous sodium sulfate and then concentrated to a small volume (*ca.* 2 ml) under reduced pressure. The concentrate was applied on a Bio-Beads S × 3 column (1.1 × 90 cm) and eluted with benzene. Fractions were checked by silica gel thin-layer chromatography (Merck TLC plates silica gel 60 F_{254}; solvent system benzene-acetone (2: 1)). The PS-6 methyl ester was detected under a UV lamp (254 nm), and active fractions were collected and evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of acetone and chromatographed on a Sephadex LH-20 column (1.1 × 90 cm) with acetone. Fractions were assayed by t.l.c. and the evaporation of acetone under reduced pressure yielded 6.2 mg of PS-6 methyl ester.

Table 1. High performance liquid chromatography of PS-5, PS-6 and PS-7.

Compound	Retention time (minutes)
PS-5	5.95
PS-6	10.2
PS-7	8.5

Chromatographic conditions: μBondapak C_{18} column (reversed phase); eluant 0.01 M ammonium phosphate buffer (pH 7.6) containing 20% methanol; flow rate 0.84 ml/min: monitored at 300 nm.

Methylation of PS-7 was also carried out under the same reaction conditions as for PS-6 and 5.0 mg of PS-7 methyl ester was obtained.

Results

Fermentation

The time course of PS-group antibiotics production is shown in Fig. 1. The production of the antibiotics closely paralleled cell growth and reached a maximum at day 5. As the sugar content decreased, the pH of the broth gradually rose. After the sugar was exhausted, the pH of the broth increased rapidly with a concomitant fall in the antibacterial potency.

For the preferential production of PS-6 and PS-7, various medium components were investigated. As the differential assay of PS-6 and PS-7 was not accurate enough to determine the amounts of all antibacterial components in broths, the bioautographic analysis was employed, after MM 13902 and MM 17880 were largely removed by pretreatment with Diaion HP20. As is apparent from Fig. 2, when peptone and defatted soybean meal were deleted from AGB-7, the relative amounts of PS-6 and PS-7 were reduced to about a fourth of those in AGB-7, whereas the total potency was increased by 50%. The similar stimulatory effects on the production of PS-6 and PS-7 were also observed with 0.1% of valine, leucine and isoleucine. Coexistence of epithienamycins A, B, C and D, MM 13902 and MM 17880 was confirmed by paper chromatographic, high performance liquid chromatographic and physicochemical analyses.

Isolation and Purification

The procedure for the isolation and purification of PS-6 and PS-7 is summarized in Fig. 3. The sodium salts of PS-6 (3 mg) and PS-7(4 mg) were obtained from 22-liter broth.

Physicochemical Properties and Structure of PS-6

The physicochemical properties of PS-6 are presented in Table 2. PS-6 sodium salt is soluble in water and substantially insoluble in acetone, ethyl acetate and benzene. When measured in a Kofler apparatus BY-1 (Yazawa Scientific Mfg.

Fig. 1. Time course of PS-group antibiotics fermentation.

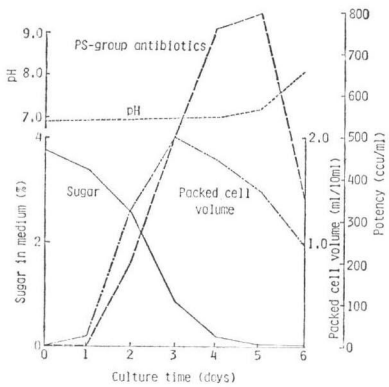
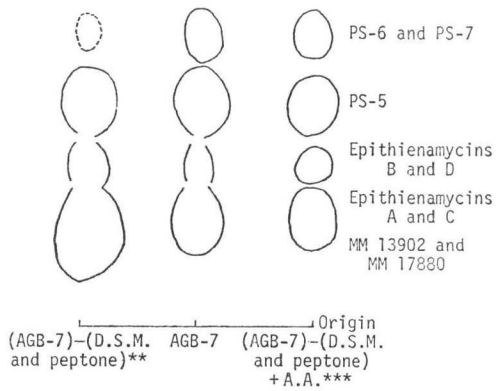


Fig. 2. Paper chromatography of culture broths after HP20 treatment.
Solvent system: acetonitrile-Tris buffer-EDTA*
Detection: bioautography with *C. terrigena*

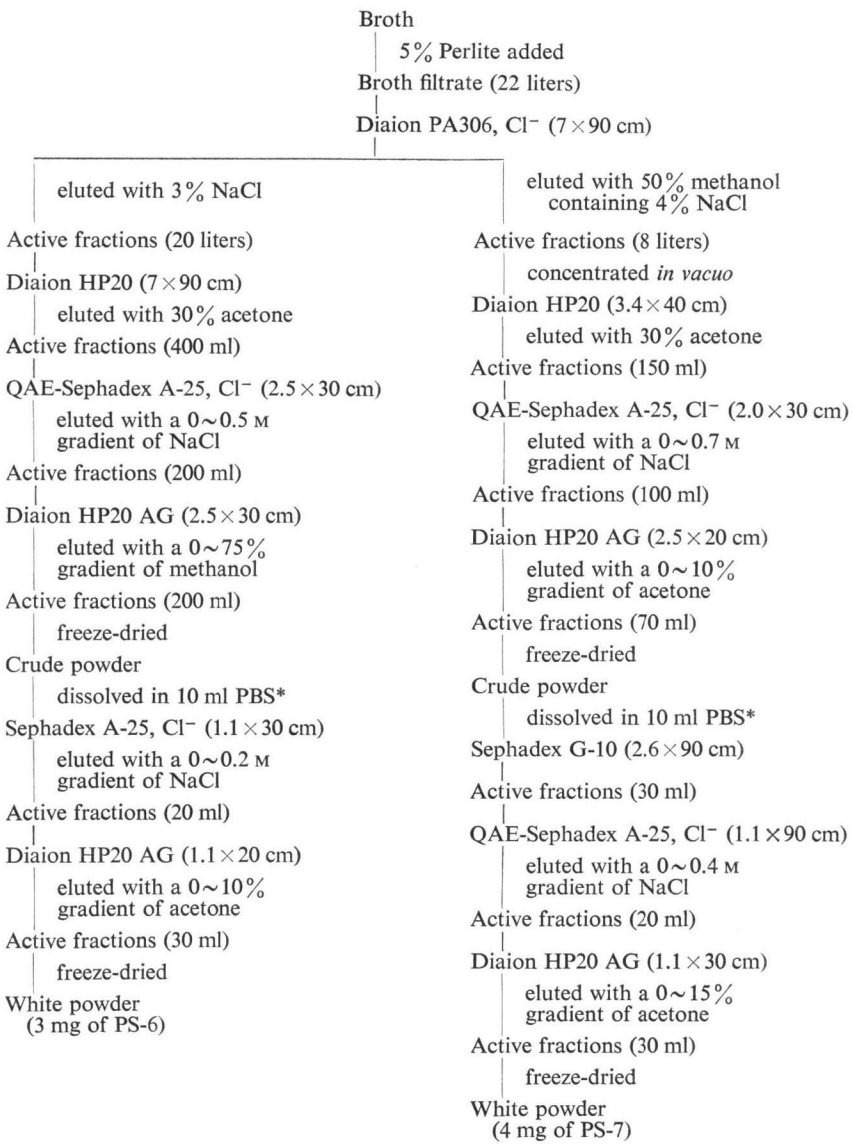


* acetonitrile 120 ml: 0.1 M Tris buffer, pH 7.5 30 ml: 0.1 M EDTA, pH 7.5, 1 ml.

** 0.3% defatted soybean meal and 0.1% peptone.

*** 0.1% amino acid (valine, leucine or isoleucine).

Fig. 3. Isolation and purification of PS-6 and PS-7.



* 0.01 M Phosphate buffer, pH 8.0

Co., Ltd.), the compound did not show a clear melting point, although it turned yellow around 148°C and gradually decomposed above 160°C. It has a UV absorption maximum at 300 nm and a minimum at 246 nm in water. The IR spectrum of PS-6 sodium salt in a KBr disc is reproduced in Fig. 5. Characteristic absorption bands attributable to β-lactam, amide and carboxylate are seen at 1750, 1550, (1650) and 1600 cm^{-1}, respectively.

The NMR spectrum of PS-6 in D_2O was recorded at 100 MHz using 3-(trimethylsilyl)propionic acid-d^4 sodium salt as the internal reference (Fig. 6 and Table 2).

In a high resolution mass spectrum of the methyl ester of PS-6, the molecular ion peak was observed at m/z 326.1306 (M^+ 326.1300 calculated for $C_{15}H_{22}N_2O_4S$). This value was 14 mass units larger than

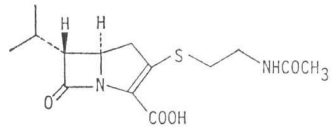
Table 2. Properties of PS-6 and PS-7.

	PS-6	PS-7
Appearance	White powder	White powder
Solubility	Soluble in water; insoluble in acetone, ethyl acetate and benzene	
m. p.	Turns yellow around 148°C and decomposes above 160°C	
UV (in H_2O)	300 nm (ε 9003)	226 nm (ε 14300), 308 nm (ε 13900)
IR (KBr)cm^{-1}	1750, 1650, 1600, 1550, 1390	1755, 1675, 1620, 1550, 1400
NMR ppm (100 MHz) D_2O	0.94 (3H, d, J=7.0), 0.98 (3H, d, J=7.0), 1.96~2.2 (1H, m), 1.95 (3H, s), 2.80~3.60 (3H), 4.00 (1H, dt, J=3.0, 9.0)	0.91 (3H, t, J=7.0), 1.54~1.84 (2H, m), 2.01 (3H, s), 2.45~3.30 (3H), 3.90 (1H, dt, J=3.0, 9.0), 5.97 (1H, d, J=14), 7.14 (1H, d, J=14)
$[\alpha]_D^{25}$	+55° (c 0.22, H_2O)	+62° (c 0.23, H_2O)
Mass spectrum	326.1306 (M^+ of methyl ester) 326.1300 (calcd. for $C_{15}H_{22}N_2O_4S$)	310.0976 (M^+ of methyl ester) 310.0987 (calcd. for $C_{14}H_{18}N_2O_4S$)
Mol. formula (Sodium salt)	$C_{14}H_{19}N_2O_4S\,Na$	$C_{13}H_{15}N_2O_4S\,Na$
Color reaction	positive : EHRLICH reagent, iodine-chloroplatinic acid negative : ninhydrin	
Paper chromatography		
Acetonitrile - Tris buffer - EDTA*	Rf 0.41	Rf 0.41
Ethanol - water (7 : 3)	Rf 0.65	Rf 0.68
T. L. C. (Avicel cellulose)		
n-Butanol - ethanol - water (4 : 1 : 5)	Rf 0.67	Rf 0.60
n-Propanol - water (7 : 3)	Rf 0.69	Rf 0.81
Acetonitrile - water (8 : 2)	Rf 0.63	Rf 0.65
Paper electrophoresis***	Rm (BTB)** : 0.82	Rm (BTB) : 0.82

* Acetonitrile, 120 ml : 0.1 M Tris buffer pH 7.5, 30 ml : 0.1 M EDTA pH 7.5, 1 ml.
** Bromthymol blue
*** 75 V/cm, 20 min., pH 8.6 Veronal buffer, I=0.05

that of PS-5 methyl ester (m/z 312.1131). The mass fragmentation pattern of PS-6 methyl ester is given in Fig. 7 and seems highly reasonable in comparison with that of PS-5 methyl ester. The presence of the isopropyl group at the C-6 position of PS-6 instead of the ethyl group in PS-5 is indicated by the detection of a fragment peak of m/z 84($(CH_3)_2CHCH{=}C{=}O$).

Fig. 4. Structure of PS-6.



PS-6

Fig. 5. IR spectrum of PS-6 sodium salt (KBr).

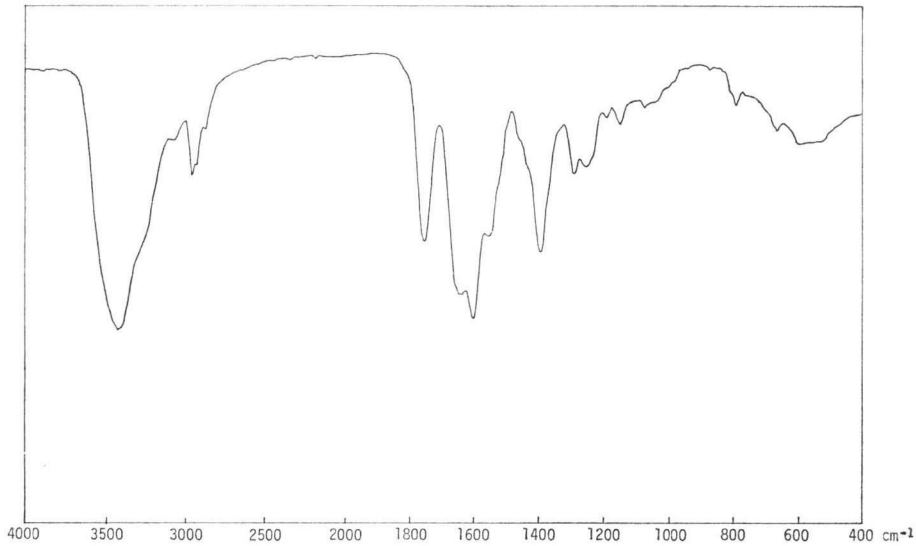
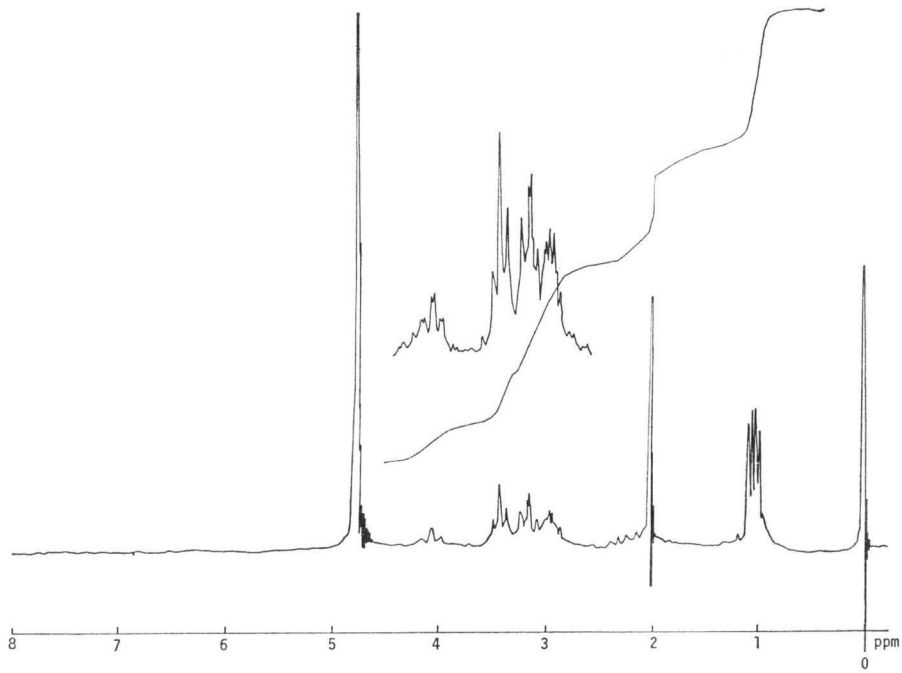


Fig. 6. ^{1}H-NMR spectrum of PS-6 sodium salt (D_2O, 100 MHz).



In the NMR spectrum of PS-6 sodium salt (Fig. 6), two doublets, one centering at δ 0.94 and the other at δ 0.98, were observed, which supports the presence of an isopropyl group at the C-6 position of PS-6.

Physicochemical Properties and Structure of PS-7

The solubility and the melting point of PS-7 are almost the same as those of PS-6. PS-7 has UV absorption maxima at 226 nm and 308 nm and a minimum at 262 nm in water (Table 2). The IR spec-

trum of PS-7 sodium salt in a KBr disc is presented in Fig. 9. The characteristic β-lactam carbonyl absorption band is located at 1755 cm^{-1}.

The NMR spectrum of PS-7 was recorded under the same conditions as that of PS-6 (Fig. 10 and Table 2).

The molecular ion peak of PS-7 methyl ester was detected at m/z 310.0976 (M^+ 310.0987 calculated for $C_{14}H_{18}N_2O_4S$) in a high resolution mass spectrum. On the basis of the difference of 2 mass units from PS-5 methyl ester, PS-7 was assumed to contain a double bond. Fig. 11 shows the fragmentation pattern of PS-7 methyl ester which was deduced from data of PS-5 and PS-6 methyl esters and relevant thienamycin compounds reported in the literature. The occurence of a fragment ion of m/z 84

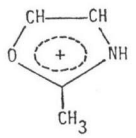
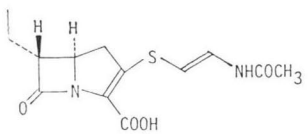


Fig. 8. Structure of PS-7.



PS-7

Fig. 7. Mass-spectral fragmentation pattern of PS-6 methyl ester.

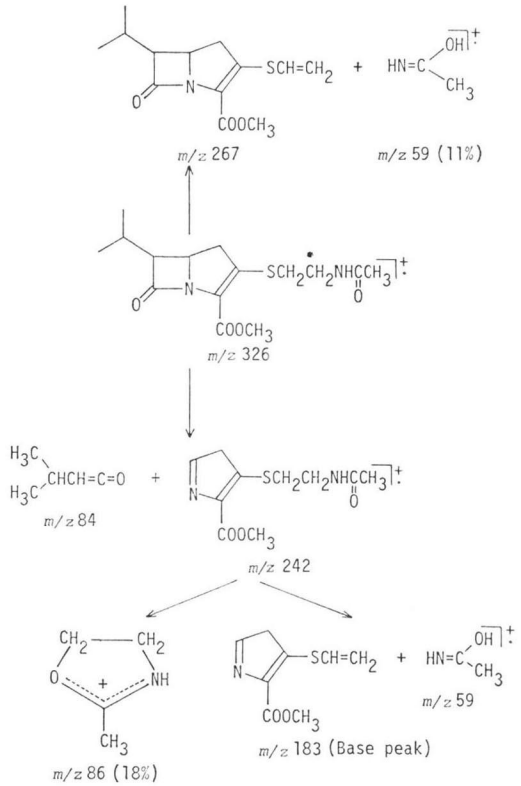


Fig. 9. IR spectrum of PS-7 sodium salt (KBr).

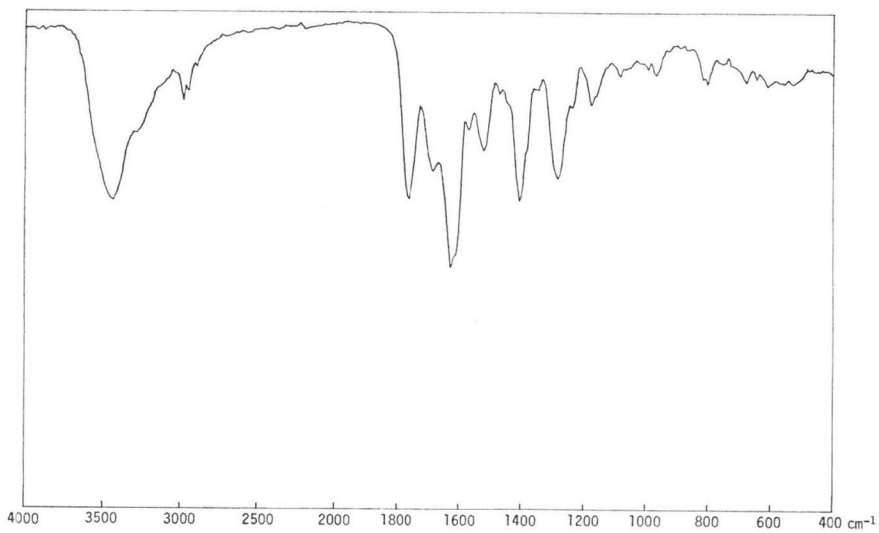


Fig. 10. ^{1}H-NMR spectrum of PS-7 sodium salt (D_2O, 100 MHz).

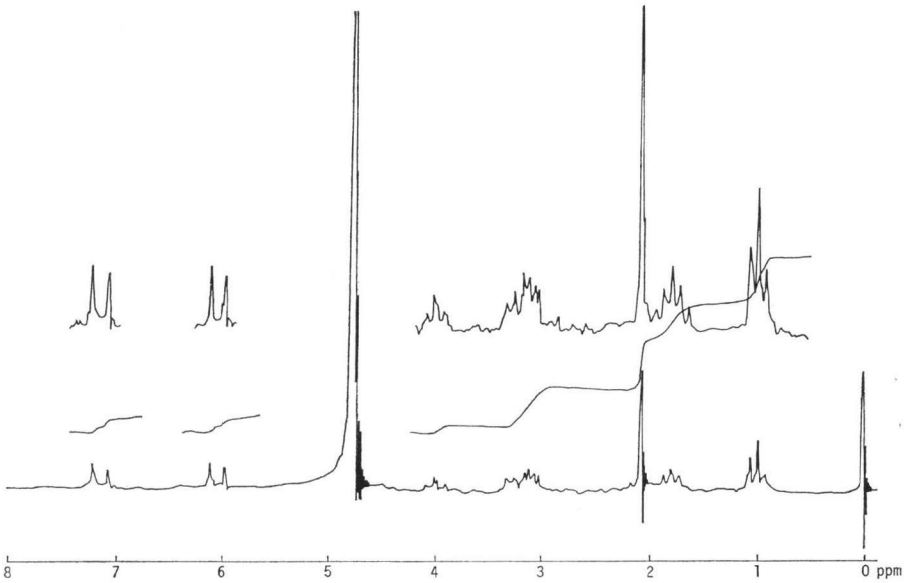
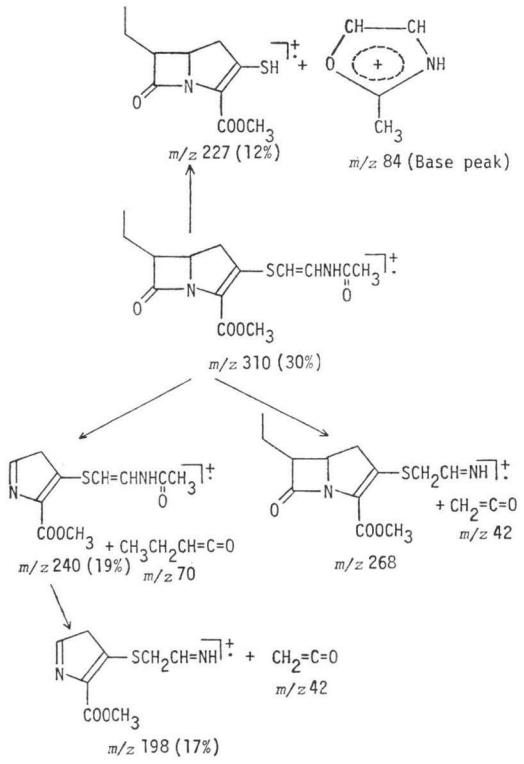


Fig. 11. Mass-spectral fragmentation pattern of PS-7 methyl ester.



in the mass spectrum (Fig. 11) and of a pair of doublets (δ 5.97 and 7.14) in the low field region of the NMR spectrum (Fig. 10) suggested a partial structure of $-SCH=CHNHCOCH_3$ as the C-3 side chain.

The presence of the ethyl group at the C-6 position was confirmed by a fragment ion peak of m/z 70 ($CH_3CH_2CH=C=O$) (Fig. 11) and by NMR signals at δ 0.91 and δ 1.54~1.84 (Fig. 10).

Discussion

As PS-6 and PS-7 were minor components in the fermentation broth in comparison with other co-products such as PS-5 and epithienamycins, it was difficult to analyse the accurate contents of PS-6 and PS-7 in mixtures of PS-group antibiotics (Fig. 1). These new β-lactam compounds were produced in the AGB-7 medium not only by *S. cremeus* subsp. *auratilis* A 271[1], but also by *S. fulvoviridis* A933[11], *S. flavogriseus* NRRL 8139 (epithienamycins-producer)[8] and *S. olivaceus* ATCC 31126 (olivanic acids-producer)[9] (data not shown). Thus the formation of PS-6 and PS-7 is considered not to be strain-specific, but biosynthetically controllable.

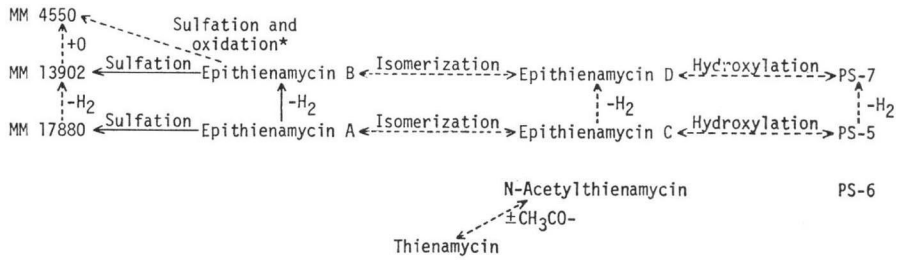
The relative configuration of the C-5 and C-6 hydrogen atoms in the β-lactam skeleton of PS-6 and PS-7 is estimated to be *trans* on the basis of their coupling constants ($J_{5,6}$=3.0 Hz) for the signal of dou-

blet-triplet centering at approximately 4 ppm (Figs. 6 and 10). In addition, the absolute configuration of C-5 is assumed to be *R* as is the case in all the known naturally occurring β-lactam antibiotics.[5,9] Consequently C-6 is also considered to have the *R* configuration. The disubstituted double bond in the C-3 side chain of PS-7 was determined to have the *trans* configuration, because its coupling constant was 14 Hz in D_2O (Fig. 10).

Although a biosynthetic relationship of the *cis*-olivanic acids has briefly been discussed based on experimental results with a blocked mutant of *S. olivaceus*[12], no information is yet available on the conversion of *trans*-olivanates to the corresponding *cis*-compounds. As PS-7 is structurally different from PS-5 only in the presence of a double bond in the S-side chain, the former may be derived from PS-5 presumably by the same dehydrogenation step as is operative in the formation of epithienamycin D and epithienamycin B from epithienamycin C and epithienamycin A respectively.

Of the thienamycin family of β-lactam compounds, to the best of our knowledge, PS-6 is a sole compound having the isopropyl group at C-6, while the rest have the two carbon side chain. Although no clear data have been obtained indicating which precursor is specific for the preferential production of PS-6 over PS-5, some amino acids such as leucine, valine and isoleucine seem to promote the biosynthesis of PS-6.

PS-5, PS-6 and PS-7 were found to have the 5,6-*trans* configuration[5], and no *cis*-counterparts of them have been reported. In contrast, epithienamycins were described to be produced not only in the 5,6-*trans* configuration (epithienamycins C and D) but also in the 5,6-*cis* configuration (epithienamycins A and B)[8,12]. Based on these findings together with the bioconversion data of the olivanate family[12], the following scheme can tentatively be proposed for the biosynthetic relationship among the relevant compounds:



* Reference 12)

Acknowledgements

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