ISOSULFAZECIN, A NEW β-LACTAM ANTIBIOTIC, PRODUCED BY AN ACIDOPHILIC PSEUDOMONAD

FERMENTATION, ISOLATION AND CHARACTERIZATION

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A novel β-lactam antibiotic, isosulfazecin (iSZ), was found to be produced by an acidophilic pseudomonad, *Pseudomonas mesoacidophila* sp. nov. iSZ was produced in parallel with bacterial growth in nutrient broth containing glycerol and sodium thiosulfate under aerated conditions. iSZ was isolated by chromatography on activated charcoal and anion-exchangers and crystallized from 70% aqueous methanol. The molecular formula was determined to be $C_{12}H_{20}N_4O_9S$ from physicochemical data. The IR and NMR spectra suggested that iSZ has a β-lactam ring, methoxyl and sulfonate groups. On acid hydrolysis, it gave L-alanine and D-glutamic acid. iSZ is an epimeric isomer of sulfazecin. iSZ was weakly active against Gram-positive and -negative bacteria, and was strongly active against mutants hypersensitive to β-lactam antibiotics.

In the course of screening for β-lactam antibiotics, we previously found that a new β-lactam antibiotic, sulfazecin (SZ), was produced by an acidophilic pseudomonad named *Pseudomonas acidophila*[1,2,3]. This finding led us to examine the production of β-lactam antibiotics by other acidophilic bacteria growing at pH 4.5. Five strains were isolated as the producers. Two of them were similar to *P. acidophila* and produced SZ. The other three strains were distinguishable from *P. acidophila* in cultural, morphological and physiological characteristics, and produced a new antibiotic, isosulfazecin (iSZ) which is an epimeric isomer of SZ.

This paper describes isolation of iSZ producers, brief taxonomical characterization of them, and production, isolation, chemical and biological characterization of iSZ. A preliminary account of part of this work has appeared previously[1].

Materials and Methods

Microorganisms

The β-lactam antibiotic-producing organisms were isolated from soil samples on nutrient agar plates adjusted to pH 4.5. Mutants hypersensitive to β-lactam antibiotics, *Pseudomonas aeruginosa* PsC^{ss} [4] and *Escherichia coli* PG-8[5], were derived from *P. aeruginosa* Ps (IFO 3080) and *E. coli* LD-2, respectively.

Media

Isolation medium contained (g/liter): Polypepton (Daigo Nutritive Chem.) 2, meat extract (from bonito, Wako Pure Chem. Ind.) 2, NaCl 1, KH_2PO_4 6.8 and Bacto agar 20. It was adjusted to pH 4.3 before autoclaving; pH became 4.5 after sterilization. The medium for examination of assimilation of carbon compounds contained (g/liter): carbon compound 10 or 3 (see Table 3), K_2HPO_4 7, KH_2PO_4 3, $(NH_4)_2SO_4$ 1, $MgSO_4 \cdot 7H_2O$ 0.5 and Bacto agar 20 (pH 7.0). Carbon compounds were sterilized separately. Medium A used for seed culture contained (g/liter): glycerol 30, Polypepton 5, meat extract

5 and NaCl 5 (pH 6.3). Fermentation medium contained 1 g/liter each of glucose and $Na_2S_2O_3$ in A medium. Medium D-NB used for the assay of antibiotic activity contained (g/liter): Polypepton 10, meat extract 10, NaCl 1, *meso*-diaminopimelic acid (Sigma) 0.02 and Bacto agar 20 (pH 7.0).

Assay of iSZ

The titre of iSZ in the culture broth was determined by a paper disc method using *E. coli* PG-8 as a test organism. In a typical assay, solutions containing 12.5 and 100 μg/ml of authentic iSZ gave 14 and 25.5 mm inhibition zones, respectively. The monitoring of iSZ during the isolation was performed with *E. coli* PG-8 and *P. aeruginosa* PsC^{ss}.

Antibacterial Activity

The minimum inhibitory concentration (MIC) was determined by the serial agar dilution method. The lowest concentration of drug that inhibited macroscopic growth of bacteria was regarded as the MIC.

Sensitivity to β-Lactamases

The sensitivity to β-lactamases was determined by using the paper disc method described previously[2].

Chemicals

Benzylpenicillin is a product of our company. Cephalosporin C and cephamycin C were prepared in our laboratories. Penicillinase was purchased from Schwarz-Mann Co. Cephalosporinase was prepared as described previously[4].

Results

Isolation of β-Lactam Antibiotic-producing Bacteria

Pseudomonas acidophila G-6302[2], a SZ-producing bacterium, grows at pH 4.5 but not at pH 8.5. Such an acidophilic characteristic of growth is unique among bacteria other than those of *Gluconobacter* and *Acetobacter*. Additional bacteria, which grow at pH 4.5, have been isolated and tested for their productivity of β-lactam antibiotics. Of 6,214 isolates from 926 soil samples, five strains (SB-72310, SB-84204, SB-84517, SB-87411 and SB-91502) were selected as candidates for β-lactam antibiotic producers. Culture filtrates of the five strains contained antibacterial substance which 1) selectively inhibited mutants hypersensitive to β-lactam antibiotics, 2) was partially inactivated by β-lactamases, and 3) induced the formation of spheroplasts from the sensitive mutants. Two of them, SB-84204 and SB-87411, resembled *P. acidophila* and produced SZ. The other three strains, SB-72310, SB-84517 and SB-91502, were taxonomically different from SZ producers, and biological activities of their products seemed to be different from SZ. Further experiments were carried out with strain SB-72310.

Characterization of Strain SB-72310

Strain SB-72310 is a Gram-negative rod motile by means of multitrichous polar flagella. Its cultural and physiological characteristics are listed in Tables 1, 2 and 3, and the pH-dependence pattern for growth in Fig. 1. Strain SB-72310

Table 1. Cultural characteristics of strain SB-72310.

Medium	Characteristic
Nutrient agar plate	Circular, raised colonies of 1~2 mm diameter with entire margins. Smooth surface, opaque and grayish white. No diffusible pigment.
Nutrient agar slant	Moderate growth, filiform, opaque and grayish white.
Nutrient broth	Turbid growth with a small amount of sediment. Pellicle formation.
Nutrient gelatin stab	Liquefaction.
Litmus milk	Peptonization.

Table 2. Physiological characteristics of strain SB-72310.

Reduction of nitrates	+	Oxidase	−
Denitrification	−	Catalase	+
Methyl red test	−	Range of growth	
Voges-Proskauer test	−	pH	4~8.85 (opt. 4.5~7.0)
Production of indole	−	Temperature	8~42°C (opt. 24~36°C)
Production of hydrogen sulfide	−	Oxygen demand	Aerobic
Hydrolysis of starch	−	O-F test	Oxidative
Utilization of citrate	+	Arginine dihydrolase	+
Utilization of inorganic nitrogen sources		Hydrolysis of Tween 80	+
Potassium nitrate	+		
Ammonium sulfate	+		
Production of pigments	−		

Table 3. Assimilation of carbon compounds by strain SB-72310.

Carbon compound	Final concentration (w/v %)	Growth	Carbon compound	Final concentration (w/v %)	Growth
L-Arabinose	1	+	Inositol	1	+
D-Xylose	1	+	Glycerol	1	+
D-Glucose	1	+	Starch	1	−
D-Mannose	1	+	Raffinose	1	+
D-Fructose	1	+	Citrate	0.3	+
D-Galactose	1	+	Acetate	0.3	+
Maltose	1	+	L-Alanine	0.3	+
Sucrose	1	+	β-Alanine	0.3	+
Lactose	1	−	Succinate	0.3	+
Trehalose	1	+	2-Ketogluconate	0.3	+
D-Sorbitol	1	+	L-Arginine	0.3	+
D-Mannitol	1	+	Betaine	0.3	+

Notes: +: Growth, −: No growth

is oxidative, strictly aerobic and catalase-positive, accumulates poly-β-hydroxybutyrate and shows arginine dihydrolase activity. Neither fluorescent nor non-fluorescent pigments were formed. It does not require growth factors and utilizes a wide range of carbon compounds for growth.

Comparing these characteristics of strain SB-72310 with those of bacteria described in the 8th edition of BERGEY's Manual of Determinative Bacteriology, 1974, it is obvious that strain SB-72310 is a member of the genus *Pseudomonas*. However, it is identical with none of the defined species of *Pseudomonas*. SB-72310 is thus thought to be a new *Pseudomonas* species for which was chosen the name *Pseudomonas mesoacidophila* to denote the moderate acidophilicity of the strain.

Fermentation of iSZ

The time course of iSZ fermentation by strain SB-72310 is shown in Fig. 2. The production was associated with bacterial growth. The amount of iSZ reached a maximum at hour 42, at which time the amount of iSZ accumulated was about 150 μg/ml.

Fig. 1. Effect of pH on growth of strain SB-72310.

Freshly grown cells on nutrient agar were suspended in sterile water at 10^9 viable cells/ml, and 0.1 ml of the suspension was inoculated into a test tube containing the following medium with the indicated pH values. The growth medium contained (g/liter): $(NH_4)_2SO_4$ 1, glucose 1, NaCl 1, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 0.1 and H_3PO_4 4.9. pH was adjusted with KOH.

The inoculated test tube was incubated at 28°C for 2 days, and the absorbance of the culture broth was measured at 600 nm by a Spectronic 20 colorimeter (Shimadzu, Bausch & Lomb).

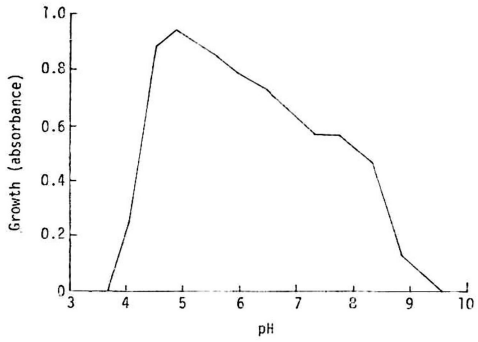
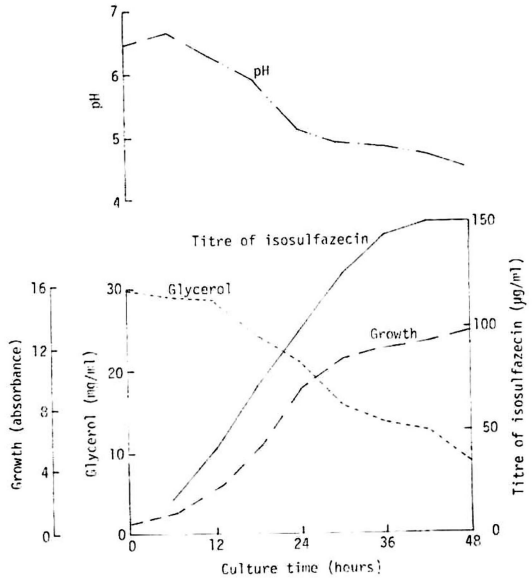


Fig. 2. Time-course of iSZ production.

Strain SB-72310 was cultivated in a 2-liter Sakaguchi shaking flask containing 500ml of seed medium (medium A) at 28°C for 2 days. The seed culture was transferred to 120 liters of fermentation medium in a 200-liter fermentor. The cultivation was carried out at 28°C for 48 hours under agitation (180 rpm) and aeration (90 liters/min.).



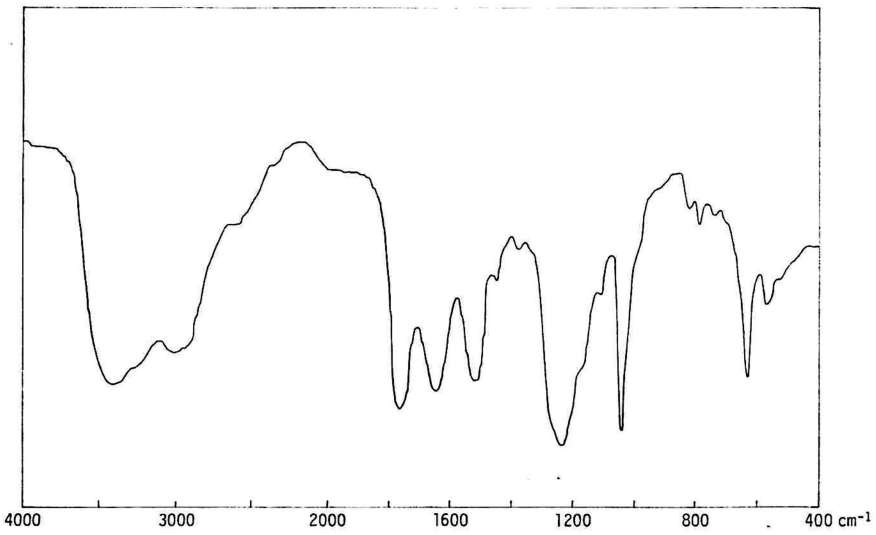
Isolation of iSZ

The fermentation broth (120 liters, 48-hour culture) was centrifuged with a Sharpless centrifuge to remove cells. The supernatant (110 liters) was adjusted to pH 4.2 and passed through a column of activated charcoal (15 liters). After washing the column with 45 liters of water, the active substance was eluted with 50% aqueous acetone. The eluate was diluted twice with water and passed through a column of Dowex 1×2 (Cl^-, 10 liters). The adsorbed antibiotic was eluted with 5% NaCl and recovered with activated charcoal (8 liters) as above by elution with 20% aqueous methanol. The active fractions were combined and concentrated *in vacuo* to 50 ml, and 200 ml of acetone was added. The resulting precipitate was recovered by filtration, washed with 50 ml of acetone and then with 100 ml of ether and dried under reduced pressure. Twelve grams of the crude powder obtained was dissolved in 600 ml of 0.01 M phosphate buffer, pH 6.6 (PB), and the solution was passed through a column of DEAE Sephadex A-25 (240 ml) which had been equilibrated with PB. The adsorbed antibiotic was eluted with PB containing 0.5% NaCl. The active fractions were combined, adjusted to pH 3.2 with 1 N HCl and passed through a column of activated charcoal (60 ml). The column was washed with 200 ml of water, 100 ml of 20% aqueous methanol and finally with 50% aqueous acetone. The active fractions were pooled, evaporated under reduced pressure to remove acetone and then freeze-dried to obtain 4.5 g of purified powder (iSZ). iSZ (2.0 g) was dissolved in 45 ml of water and the pH was adjusted to 6.5 by the addition of 1 N NaOH. The solution was freeze-dried to obtain 2.1 g of iSZ monosodium salt (iSZ Na). Similarly, iSZ monopotassium salt was prepared by using KOH instead of NaOH. The potassium salt was crystallized from

Table 4. Physico-chemical properties of iSZ, iSZ K and iSZ Na.

	iSZ	iSZ K	iSZ Na
Appearance	Colorless powder	Colorless needles	Colorless powder
Melting point		>250°C	
Optical rotation (in H_2O)	$[\alpha]_D^{25}$+4.5° (*c* 1.01)	$[\alpha]_D^{25}$+10° (*c* 1.01)	$[\alpha]_D^{23}$+8.5° (*c* 0.91)
Elemental analysis			
Found	C 34.40	C 32.14	C 31.75
	H 5.56	H 5.29	H 5.19
	N 13.30	N 12.41	N 12.57
	S 7.56	S 6.68	S 7.10
		K 7.80	Na 5.10
Calcd. for	$C_{12}H_{20}N_4O_9S \cdot H_2O$	$C_{12}H_{19}N_4O_9SK \cdot H_2O$	$C_{12}H_{19}N_4O_9SNa \cdot 2H_2O$
	C 34.78	C 31.85	C 31.72
	H 5.35	H 4.67	H 5.10
	N 13.52	N 12.38	N 12.33
	S 7.74	S 7.08	S 7.06
		K 8.64	Na 5.06
UV spectrum	End absorption		

Fig. 3. Infrared absorption spectrum of iSZ (free acid) (KBr).



aqueous methanol as colorless needles (iSZ K).

Physicochemical Properties of iSZ, iSZ K and iSZ Na

Physicochemical properties of iSZ, iSZ K and iSZ Na are shown in Table 4. The properties of iSZ and iSZ Na are almost identical with those of free SZ and sodium salt of SZ[3], respectively, except for the values of optical rotation. The difference in the optical rotation was due to the configuration of alanine contained in SZ (D-isomer) and iSZ (L-isomer) as described below. The molecular formula of free iSZ,

Fig. 4. Infrared absorption spectrum of iSZ (sodium salt) (KBr).

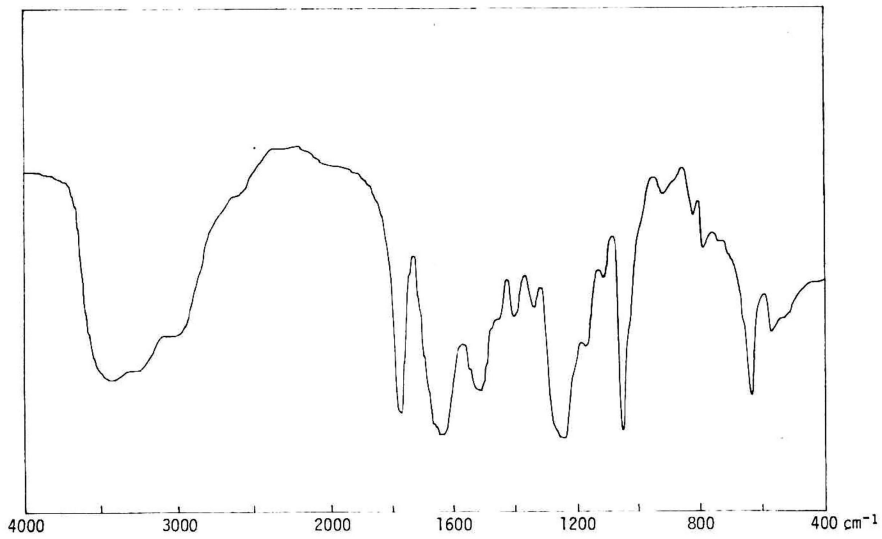
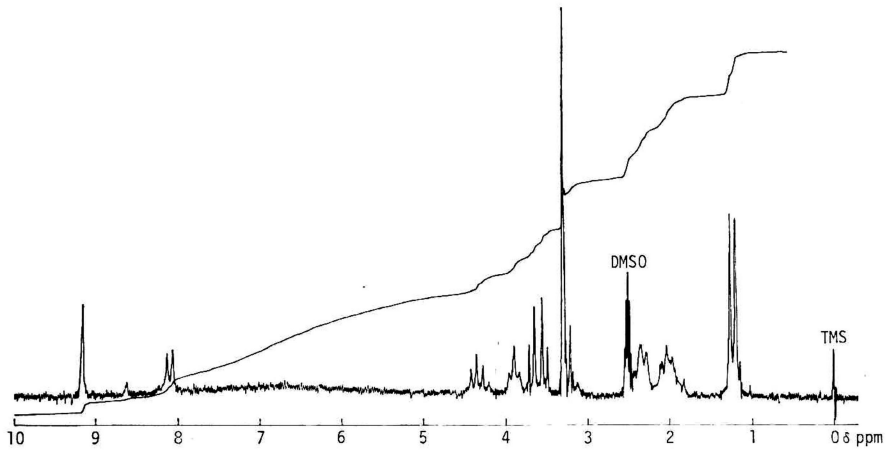


Fig. 5. ^{1}H NMR spectrum of iSZ (free acid) (DMSO-d_6).



$C_{12}H_{20}N_4O_9S$ and iSZ Na, $C_{12}H_{19}N_4O_9S \cdot Na$, were determined from elemental analysis. iSZ exhibited only end absorption in the ultraviolet absorption spectrum. Their infrared absorption spectra showed characteristic bands at 1790~1770 cm^{-1} (β-lactam C=O), 1660 cm^{-1} (amide C=O) and 1240, 1040~1050 and 630 cm^{-1} (SO_3^-) (Figs. 3 and 4). The ^{1}H NMR spectrum (in DMSO-d_6, 100 MHz) of iSZ is shown in Fig. 5, and the chemical shifts and coupling constants in Table 5. The presence of a doublet methyl (δ 1.24 ppm, 3H, d), two methylenes (δ 2.00 and 2.32 ppm, each 2H, m), a methoxyl (δ 3.31 ppm, 3H, s), a methylene of AB quartet (δ 3.53 and 3.69 ppm, 2H, q), two methines (δ 3.89 and 4.35 ppm, each 1H, m) and two amide protons (δ 8.11 and 9.16 ppm) is obvious. Upon addition of D_2O, two amide NH signals disappeared and the multiplet at δ 4.35 ppm collapsed to quartet (J=7 Hz).

iSZ was readily soluble in water, soluble in dimethylsulfoxide, dimethylformamide, slightly soluble in methanol, tetrahydrofuran, and practically insoluble in ethanol, acetone, ethylacetate, chloroform and

Table 5. Chemical shifts and coupling constants in ^1H NMR spectrum (DMSO-d_6, 100 MHz).

	3′	3″	4″	3	4		2″	2′	2′	3
	CH_3	CH_2	CH_2	OCH_3	CH_2		CH	CH	NH	NH
δ ppm	1.24	2.00	2.32	3.31	3.53	3.69	3.89	4.35	8.11	9.16
	3H	3H	2H	3H	1H	1H	1H	1H	1H	1H
	d	m	m	s	d	d	m	m	d	s
JHz	7				6	6			7	

Table 6. Thin-layer chromatographic properties of iSZ.

Solvent system	Rf
Cellulose*	
n-Propanol - H_2O (4: 1)	0.17
n-Propanol - acetonitrile - H_2O (1: 1: 1)	0.77
n-Propanol - ethanol - H_2O (5: 2: 3)	0.48
n-Butanol - acetic acid - H_2O (2: 1: 1)	0.22
DEAE cellulose**	
0.05 M Phosphate buffer, pH 6.8	0.72

* Cellulose f spot film, Tokyo Kasei Co., Japan.
** DEAE cellulose spot film, Tokyo Kasei Co., Japan.

other organic solvents. It showed a positive color reaction to ninhydrin but gave negative reactions with SAKAGUCHI's and GREIG-LEABACK's reagents. iSZ was relatively stable in neutral and weakly acidic solutions, but unstable in alkaline and strongly acidic solutions. Chromatographic properties of iSZ is shown in Table 6. The Rf values of iSZ were identical with those of SZ. Optical rotations of iSZ and iSZ Na were $[\alpha]_D^{25}+4.5°$ (*c* 1.01, H_2O) and $[\alpha]_D^{23}+8.5°$ (*c* 0.91, H_2O), respectively. These values were different from those of SZ {powder, $[\alpha]_D^{20}+94°$ (*c* 0.35, H_2O)} and its sodium salt {$[\alpha]_D^{20}+85°$ (*c* 0.37, H_2O)}.

Table 7. Comparison of antimicrobial activity of iSZ with SZ.

Organism	MIC (μg/ml)	
	iSZ	SZ
Pseudomonas fluorescens IFO 3081	800	100
Pseudomonas aeruginosa (Ps) IFO 3080	1600	800
Pseudomonas aeruginosa (PsC^{68})	0.78	0.78
Escherichia coli NIHJ JC-2	100	12.5
Escherichia coli (LD-2)	50	6.25
Escherichia coli (PG-8)	0.78	0.39
Serratia marcescens IFO 12648	100	25
Proteus mirabilis ATCC 21100	200	3.13
Proteus vulgaris IFO 3988	100	6.25
Salmonella typhimurium IFO 12529	100	6.25
Klebsiella pneumoniae IFO 3317	800	12.5
Enterobacter cloacae IFO 12937	50	25
Alcaligenes faecalis IFO 13111	25	25
Comamonas terrigena IFO 12685	25	12.5
Acinetobacter calcoaceticus IFO 13006	25	12.5
Staphylococcus aureus FDA 209P	200	200
Sarcina lutea IFO 3232	400	200
Bacillus subtilis PCI 219	100	50
Bacillus cereus FDA 5	400	800

Configuration of Alanine and Glutamic Acid in iSZ

Alanine and glutamic acid were obtained by hydrolysis of iSZ as follows.

iSZ (250 mg) was heated at 105°C for 3 hours in 10 ml of 6 N HCl. After removing HCl by evaporation, the amino acids were adsorbed on a column of Amberlite IR 120 (H^+, 8 ml) and eluted with 1 N NH_4OH. The fractions positive in ninhydrin reaction were combined and NH_4OH was removed by evaporation. The resulting solution was loaded on a column of Dowex 1×2 (OH^-, 15 ml). The fractions corresponding to alanine and glutamic acid (monitored by thin-layer chromatography) which were eluted

Table 8. Comparison of sensitivity of isosulfazecin (iSZ) to β-lactamase with those of benzylpenicillin (Pc G), cephalosporin C (CPC), cephamycin C (CMC) and sulfazecin (SZ).

Figures are the diameter (mm) of inhibition zones on plates seeded with *E. coli* PG-8.

Enzyme	units/ml	iSZ	Pc G	CPC	CMC	SZ
None		25.5	28.5	30	30	25
Penicillinase	0.05	25.5	0*	30	30	25
	5	25	0	29	30	25
Cephalosporinase	0.0035	25	11	9.5	15.5	24.5
	0.35	24.5	0	0	0	23.5

* 0: No activity detected.

with 0.1 N HCl were obtained. Each fraction was decolorized by passing through a column of activated charcoal (2 ml). Crystals were obtained on concentrating the column effluent. The optical rotations of the isolated alanine and glutamic acid were $[\alpha]_D^{23}+14.7°$ (*c* 0.62, 6 N HCl) and $[\alpha]_D^{23}-29.1°$ (*c* 0.58, 6 N HCl), respectively. These facts suggested that, in contrast to SZ which contains D-alanine and D-glutamic acid, iSZ contains L-alanine and D-glutamic acid as the constituents.

Biological Properties of iSZ

The antimicrobial spectrum of iSZ was compared with that of SZ (Table 7). iSZ inhibited *Alcaligenes, Acinetobacter, Enterobacter* and β-lactam antibiotic-sensitive mutants as strongly as SZ. Against other Gram-negative bacteria, however, the activity of iSZ was weaker than that of SZ. Both antibiotics showed weak activities against Gram-positive bacteria. iSZ also induced protoplast formation of susceptible bacteria (data not shown). Although iSZ was partially inactivated by excess β-lactamases incorporated in assay plates (see Ref. 1, data not shown), it was far more resistant to penicillinase and cephalosporinase than benzylpenicillin, cephalosporin C and cephamycin C (Table 8).

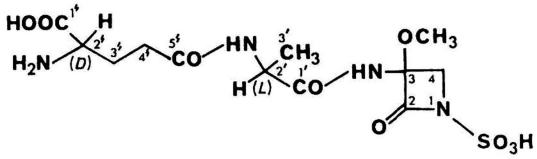
Discussion

The newly found bacterial β-lactam antibiotic, iSZ, is a stereochemical isomer of SZ which is the β-lactam antibiotic first discovered from bacterial orgin[1,2,3]. iSZ, as well as SZ, was elaborated by bacteria of the genus *Pseudomonas*. Though the taxonomical properties of iSZ- and SZ-producer's were different, they had a common property to grow at pH as low as 4.0. According to BERGEY's Manual of Determinative Bacteriology, 8th ed., species of *Pseudomonas* are described not to grow at such a low pH. Thus iSZ- and SZ-producer can be regarded as unique organisms among the genus *Pseudomonas* in their acidophilic or aciduric property. The iSZ-producer was named *Pseudomonas mesoacidophila*. Its detailed taxonomical studies will be published elsewhere. Although we do not know how the acidophilicity of organisms is related to the productivity of β-lactam antibiotics, our trial of isolating β-lactam antibiotic-producing bacteria on an acidified isolation medium has been successfully accomplished.

iSZ is a water-soluble and acidic antibiotic containing sulfur, alanine and glutamic acid as the constituents. None of the hitherto-known antibiotics except SZ[1,2,3] have that particular combination of characters. IR and ^1H NMR spectra and chromatographic properties of iSZ were almost identical with those of SZ. iSZ and SZ were, however, different in their optical rotation. The difference is due to the configuration of the alanine moiety; D-alanine is contained in SZ, while L-alanine in iSZ. Thus iSZ is a new antibiotic 3-γ-D-glutamyl-L-alanylamino-3-methoxyazetidin-2-one-1-sulfonic acid (Fig. 6) which is a stereoisomer of SZ. Although SZ and iSZ are different only in the configuration of one of the constituent amino acids, iSZ-producers produced only iSZ but did not produce SZ concomitantly and *vice versa*.

The activity of iSZ against bacteria, especially enteric bacteria, was far less than that of SZ. This difference is due to the replacement of D-alanine residue in SZ by L-alanine. The conformation of alanine residue adjacent to azetidinone ring may determine the antibacterial activity of SZ-group antibiotics through changes either in the permeability or in the binding capacity to the target enzyme of the antibiotic molecule.

Fig. 6. Structure of iSZ.



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