SULFAZECIN, A NOVEL β-LACTAM ANTIBIOTIC OF BACTERIAL ORIGIN

ISOLATION AND CHEMICAL CHARACTERIZATION

MITSUKO ASAI, KONOMI HAIBARA, MASAYUKI MUROI, KAZUHIKO KINTAKA
and TOYOKAZU KISHI

Microbiological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd.,
Jusohonmachi, Yodogawa-ku, Osaka 532, Japan

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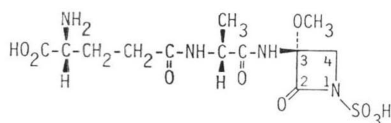
Sulfazecin, a new water-soluble acidic antibiotic, which is active against Gram-negative bacteria, was isolated as crystals from the culture broth of *Pseudomonas acidophila* G-6302 by use of anion exchange resin and activated charcoal. The molecular formula of sulfazecin was determined to be $C_{12}H_{20}N_4O_9S$ from physicochemical data. The IR and NMR spectra suggested that this antibiotic has a β-lactam ring, methoxyl and sulfonate groups. On acidic hydrolysis it gave D-alanine and D-glutamic acid. These chemical and physicochemical data indicated that sulfazecin is a new β-lactam antibiotic.

In the course of our screening for new cell wall inhibitors a new β-lactam antibiotic, sulfazecin (**1**), which is a water soluble acidic substance, was obtained from the culture broth of *Pseudomonas acidophila* sp. nov. strain G-6302[1]. It is active against Gram-negative bacteria but is only weakly active against most Gram-positive bacteria[1]. This paper describes its isolation and chemical characterization. A preliminary account of part of this work has appeared previously[2].

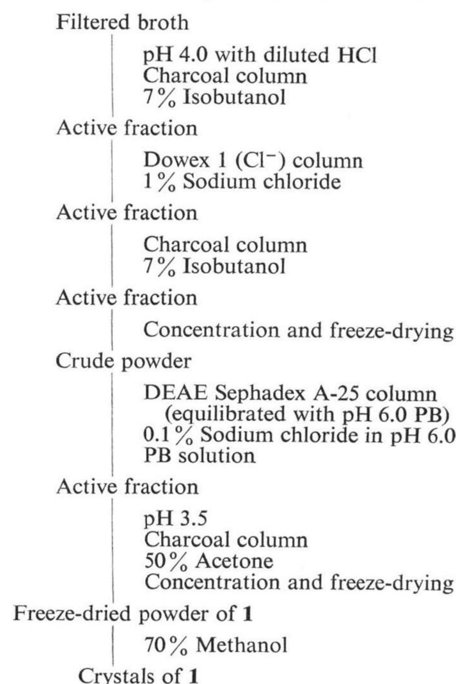


Isolation

Activity against *Escherichia coli* LD2[1] and *Proteus mirabilis* ATCC 21100 and a color reaction with ninhydrin reagent were employed to monitor sulfazecin (**1**) during its isolation from the culture broth of *Pseudomonas acidophila* G-6302. Since **1** is a water-soluble and acidic substance, **1** was isolated by the usual method for such products (Chart 1).

The culture broth was filtered with filter aid and the filtrate was acidified to pH 4 with diluted HCl and applied on a column of activated charcoal. The column was washed with water and eluted with 7% aqueous isobutanol. Then

Chart 1. Isolation procedure for sulfazecin.



the active fractions were passed through a column of Dowex-1 (Cl^-) and the adsorbed antibiotic was eluted with a 0.1% aqueous sodium chloride solution. The active fractions were desalted through a charcoal column, concentrated and freeze-dried to obtain a crude powder. Further purification was carried out by ion exchange chromatography using Sephadex DEAE A-25, which was equilibrated with 0.05 M phosphate buffer (PB) of pH 6.0. The crude powder was dissolved in 0.01 M PB (pH 6.0) and applied on a column of the buffered anion exchange Sephadex. The column was washed with 0.05 M PB, pH 6.0, and then **1** was eluted with the same buffer containing 0.1% sodium chloride. The fraction with antibacterial activity was desalted with a charcoal column, concentrated *in vacuo* and the active principle freeze-dried. The purified powder of **1** was dissolved in a small amount of water and methanol was added to give sulfazecin (**1**) as colorless needles. **1** thus obtained was dissolved in water and the solution was adjusted to pH 6.5 with diluted NaOH. Freeze-drying of the solution yielded monosodium salt of sulfazecin (**2**) as a colorless powder.

Physicochemical Properties of Sulfazecin (1) and its Sodium Salt (2)

Physicochemical properties of **1** and **2** are shown in Table 1. Sulfazecin (**1**) was obtained as a colorless powder by freeze-drying and was crystallized as colorless needles from 70% aqueous methanol. The crystals of **1**, mp 168～170°C, $[\alpha]_D^{25}$ +82° (*c* 1.0, H_2O), contained one mole of methanol per molecule, as evidenced by the presence of a signal of O–CH_3 (3.20 ppm, 3H, s) in its ^{1}H-NMR spectrum. Its molecular formula, $C_{12}H_{20}N_4O_9S$ ($CH_3OH \cdot \frac{1}{2}H_2O$) was determined from elemental analysis, ^{1}H-NMR and ^{13}C-NMR spectral data. **1** is readily soluble in water; soluble in dimethylsulfoxide (DMSO) and dimethylformamide; slightly soluble in methanol and tetrahydrofuran; practically insoluble in ethanol, acetone, ethyl acetate, chloroform and other organic solvents. It showed a positive color reaction to ninhydrin but gave negative reactions with SAKAGUCHI's and GREIG-LEABACK's reagents. **1** had pKa' values of 3.4 (COO^-) and 9.2 (NH_3^+). Besides, its 1% aqueous solution was highly acidic (pH 2.2), suggesting the presence of another strongly acidic function. **1** exhibited only end absorption in the ultraviolet (UV) spectrum. The infrared (IR) spectrum of **1** showed characteristic absorption bands at 1780 cm^{-1} (β-lactam C=O), 1660 cm^{-1} (amide C=O), and 1245, 1038 and 625 cm^{-1} (SO_3^-) (Figs. 1 and 2).

Table 1. Physicochemical properties of sulfazecin.

	Sulfazecin (1)	Sulfazecin Na (2)
Appearance	Colorless needles	Colorless powder
M.p. (dec.)	168～170°C	
$[\alpha]_D$ (H_2O)	+82° (*c* 1.0)	+85° (*c* 0.37)
Anal.	C H N S	C H N S Na
Found	35.51 5.61 12.93 7.45	33.08 5.07 12.73 7.73 5.19
Calcd. for	$C_{12}H_{20}N_4O_9S \cdot CH_3OH \cdot \frac{1}{2}H_2O$	$C_{12}H_{19}N_4O_9SNa \cdot H_2O$
	C H N S	C H N S Na
	35.69 5.76 12.81 7.33	33.03 4.85 12.84 7.35 5.27
UV	end absorption	end absorption
IR ν_{max}^{KBr} (cm^{-1})	1,780, 1,660, 1,538, 1,265, 1,245, 1,220, 1,038, 625	1,770, 1,640, 1,530, 1,245, 1,050, 632

Fig. 1. IR spectrum of sulfazecin (**1**) (freeze-dried powder).

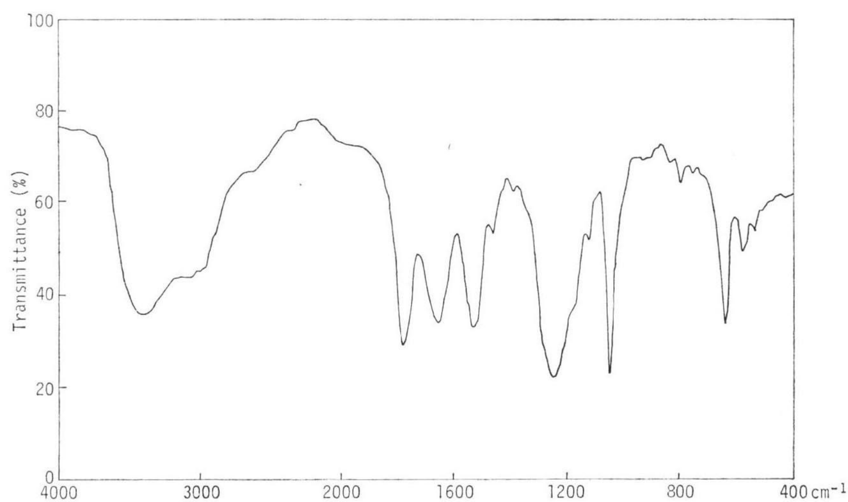


Fig. 2. IR spectrum of sulfazecin (**1**) (crystal).

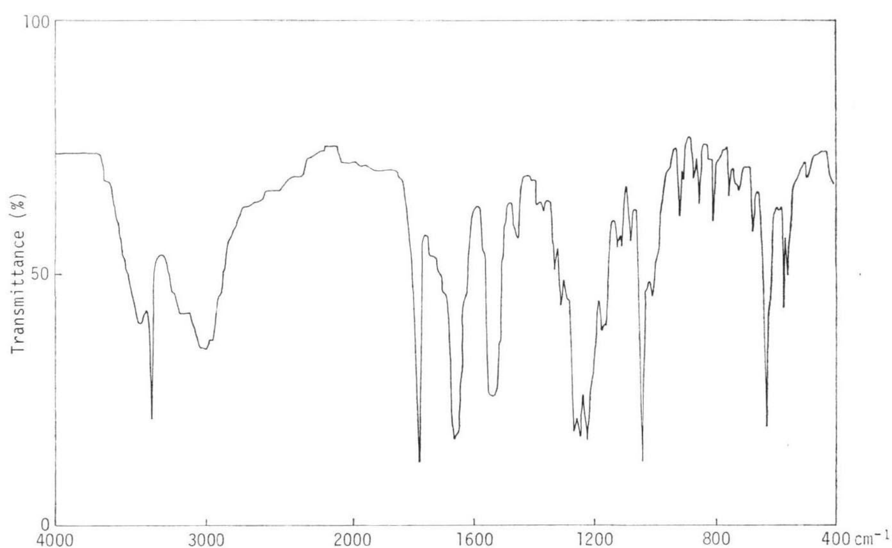


Table 2. Thin-layer chromatographic properties.

	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 PB pH 6.8		0.72

* Cellulose f spot film, Tokyo Kasei Co.

** DEAE cellulose spot film, Tokyo Kasei Co.

Fig. 3. IR spectrum of sulfazecin monosodium salt (**2**) (freeze-dried powder).

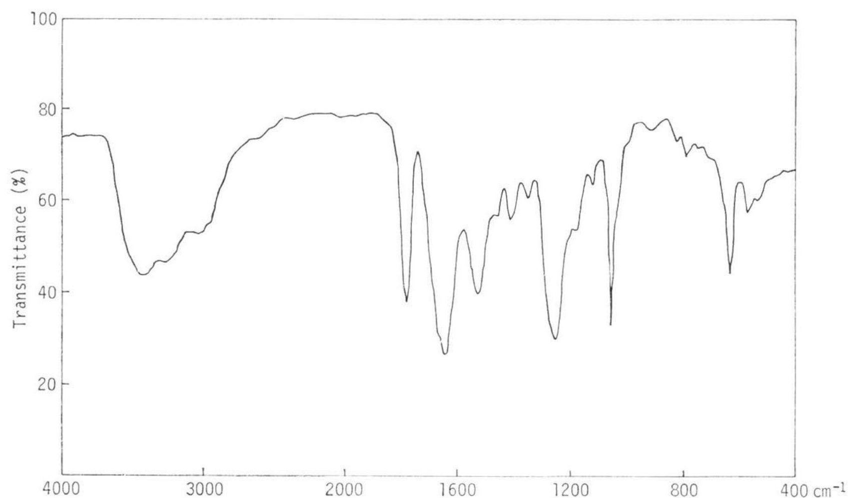
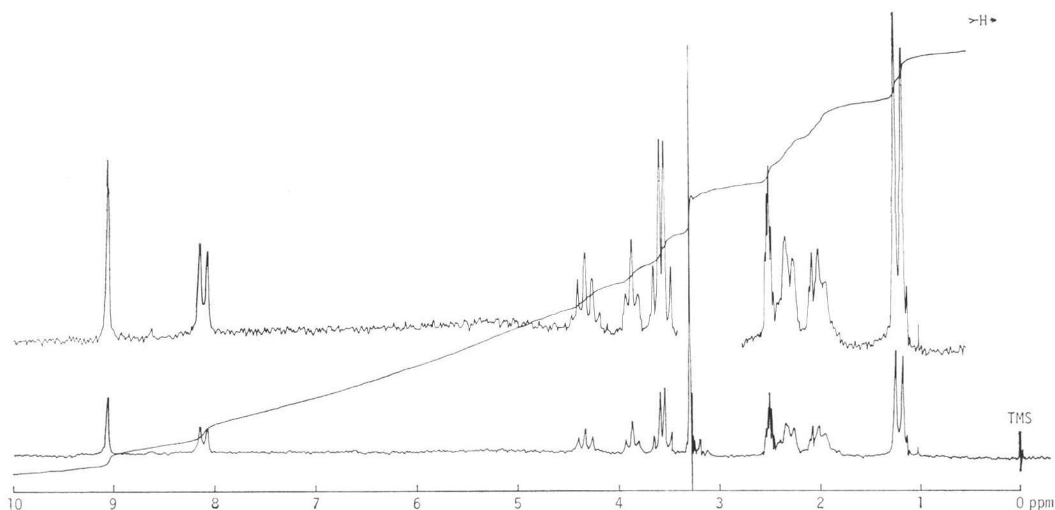


Fig. 4. ^{1}H-NMR spectrum of sulfazecin (**1**) (freeze-dried powder).



1 was relatively stable in neutral and weakly acidic solutions, but unstable in alkaline and strongly acidic solutions; 100%, 73% and 36% of the original activity were retained after heating at 37°C for 3 hours in aqueous solutions of pH 3.5 ~ pH 6.5, pH 2 and pH 8, respectively. Chromatographic data are presented in Table 2.

Sulfazecin monosodium salt (**2**), $C_{12}H_{19}N_4O_9SNa \cdot H_2O$, was obtained as a colorless powder and did not show a sharp melting point (browning at 170°C). **2** had $[\alpha]_D^{20}$ +85° (*c* 0.37, H_2O) and exhibited only end absorption in the UV spectrum. The IR spectrum of **2** (Fig. 3) was almost identical with that of **1**.

The ^{1}H-NMR spectrum (in d_6-DMSO, 100 MHz) (Fig. 4) of **1** obtained by freeze-drying exhibited the presence of a doublet methyl (δ 1.23 ppm, 3H, d), two methylenes (δ 2.02 and 2.31 ppm, each 2H, m), a methoxyl (δ 3.31 ppm, 3H, s), a methylene of AB quartet (3.52 and 3.63 ppm, 2H, each d), two

methines (δ 3.87 and 4.34 ppm, each 1H, m) and two amide protons (δ 8.12 and 9.07 ppm) as shown in Fig. 4. Upon addition of D_2O, two amide NH signals disappeared and the multiplet at 4.34 ppm collapsed to quartet (J=7 Hz). The ^{1}H-NMR spectrum of crystalline **1** was similar to that of freeze-dried powder except for the presence of a signal of methoxyl (δ 3.20 ppm, 3H, s) due to methanol. The ^{13}C-NMR spectrum of **1** (in D_2O) showed 12 carbon signals at δ 17.2 (q), 27.1 (t), 32.1 (t), 50.9 (d), 53.8 (q), 55.0 (d), 55.2 (t), 91.7 (s), 162.7 (s), 174.4 (s), 175.2 (s) and 176.8 ppm (s). On acid hydrolysis with 6 N HCl at 110°C, **1** gave D-alanine (**3**) and D-glutamic acid (**4**).

Comparison of Sulfazecin with the Known Antibiotics

Since sulfazecin is an acidic water-soluble substance and contains sulfur and a β-lactam ring as the constituents, it should be compared with a number of β-lactam antibiotics such as penicillin, cephalosporin[3] and other β-lactam antibiotics[4]. However, the absence of characteristic UV absorption maxima differentiates sulfazecin (**1**) from cephalosporin[5], thienamycin antibiotics[6,7], olivanic acid derivatives[8,9] and PS-5[10]. The facts that **1** gives a signal of OCH_3 in the ^{1}H-NMR spectrum and that it gives D-alanine and D-glutamic acid on acid hydrolysis also differentiate sulfazecin from any of the known naturally occurring penicillins, cephalosporins and other β-lactam antibiotics. Moreover sulfazecin is different from any of the known metabolites containing β-lactam structures such as tabtoxin[11,12] produced by *Pseudomonas* and X-372 A[13] in its physicochemical and biological properties. Sulfazecin is thus concluded to be a new antibiotic. Structural elucidation of sulfazecin will be reported elsewhere.

Experimental

Melting points were determined with a Mettler FP-5 instrument heating at 3°C/minute. IR spectra were recorded with a Hitachi 285 grating infrared spectrophotometer. NMR spectra were obtained using a Varian XL-100-12 instrument; chemical shifts (δ) are reported in ppm down field from an internal TMS reference.

Isolation of Sulfazecin (1)

The cultured broth (237 liters) of *Pseudomonas acidophila* G-6302 was mixed with 3 kg of Hyflo-Supercel (Johns-Manville Products, U.S.A.). The mixture was filtered in a filter press to yield 210 liters of filtrate. The filtrate was adjusted to pH 4.0 with 1 N HCl and passed through an activated charcoal column (12 liters). The column was washed with water (20 liters) and elution was carried out with 7% aqueous isobutanol (24 liters). Each fraction (3 liters) of effluent was tested for activity against *Escherichia coli* and *Proteus mirabilis*; **1** was eluted from the column in fractions 3~6. The active fractions were pooled and passed through a Dowex 1×2 (Cl^-) column (3 liters). The column was washed with water (10 liters). It was then eluted with a 1% aqueous sodium chloride solution (12 liters) while 1 liter fractions were collected. Fractions 4~8 were combined and again passed through an activated charcoal column (3 liters). The column was washed with water (8 liters) and then **1** was eluted with 7% aqueous isobutanol (6 liters). The active fractions (3~5, each 1 liter) monitored by color reaction with ninhydrin reagent were combined and concentrated *in vacuo*. The concentrate was lyophilized to give a crude powder of **1** (30.2 g). The crude powder of **1** (17 g) thus obtained was dissolved in 1 liter of 0.01 M phosphate buffer (pH 6.0) and applied on a column (400 ml) of Sephadex DEAE A-25, previously treated with 0.05 M phosphate buffer (pH 6.0). The column was washed with the same buffer (1200 ml) and then eluted with 0.1% sodium chloride in buffer solution, the eluate being collected in 250 ml fractions. The combined fractions 2~4 containing **1** were adjusted to pH 3.5 with 1 N HCl and run onto a column (400 ml) of activated charcoal, and it was washed with water and eluted with 50% aqueous acetone, successively. The active eluate was concentrated *in vacuo* and lyophilized to give a pure colorless powder of **1** (1.5 g). *Anal.* (dried *in vacuo* (60°C, 8 hours) over P_2O_5) Calcd. for $C_{12}H_{20}N_4O_9S\cdot H_2O$:

C, 34.78; H, 5.35; N, 13.52; S, 7.74. Found: C, 34.83; H, 5.41; N, 13.22; S, 7.65. The pure powder of **1** (1 g) dissolved in water (10 ml) was mixed with 25 ml of methanol and cooled to give crystalline **1** (920 mg), mp 168~170°C (decomp.), *Anal.* (dried *in vacuo* (60°C, 8 hours) over P_2O_5) Calcd. for $C_{12}H_{20}N_4O_9S\cdot\frac{1}{2}H_2O$: C, 35.69; H, 5.76; N, 12.81; S, 7.33. Found: C, 35.51, 35.56; H, 5.61, 5.61; N, 12.93, 12.98; S, 7.45, 7.59.

Sulfazecin Monosodium Salt (**2**)

To sulfazecin (**1**) (440 mg) dissolved in 5 ml of cold water was added 10 ml of 0.1 N NaOH dropwise under stirring. The mixture was lyophilized to give **2** (420 mg) as a colorless powder. *Anal.* (dried *in vacuo* (60°C, 8 hours) over P_2O_5) Calcd. for $C_{12}H_{19}N_4O_9SNa\cdot H_2O$; C, 33.03; H, 4.85; N, 12.84; S, 7.35; Na, 5.27. Found: C, 33.08, 33.32; H, 5.07, 4.97; N, 12.73, 13.27; S, 7.73, 7.43; Na, 5.19, 5.31.

Acid Hydrolysis of Sulfazecin

1) **1** (2 mg) was hydrolyzed with 6 N HCl at 110°C for 16 hours in a sealed tube. Amino acid determination on the acid hydrolyzates using a Hitachi KLA-3B instrument yielded 1.89 μmole/mg of glutamic acid and 0.94 μmole/mg of alanine.

2) **1** (540 mg) dissolved in 60 ml of 6 N HCl was refluxed for 1 hour. The reaction mixture was evaporated *in vacuo* almost to dryness to remove HCl. The residue was dissolved in water, passed through an Amberlite IR 120 (H^+) column (15 ml), and eluted with 1 N NH_4OH (40 ml). The eluate was evaporated *in vacuo* to remove NH_3. The concentrate (10 ml) was passed through a Dowex 1×2 (OH^-) column (15 ml) and eluted with 0.1 N HCl, the eluate being collected in 3 ml fractions. Fractions 4 and 5 were pooled and passed through an activated charcoal column (2 ml), and the effluent was concentrated *in vacuo* to give crystals of D-alanine (56 mg), $[\alpha]_D^{23}-12.1°$ (*c* 1.28, 6 N HCl). *Anal.* Calcd. for $C_3H_7NO_2$: C, 40.44; H, 7.92; N, 15.72. Found: C, 40.25; H, 7.88; N, 15.25. Fractions 7 and 8 were pooled and concentrated *in vacuo*. The concentrate was kept standing to afford colorless needles of D-glutamic acid (110 mg), $[\alpha]_D^{23}$ −29.8° (*c* 1.04, 6 N HCl). *Anal.* Calcd. for $C_5H_9NO_4$: C, 40.81; H, 6.16; N, 9.52. Found: C, 40.58; H, 6.25; N, 9.42.

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