DISCOVERY, ISOLATION AND STRUCTURE OF NOVEL CEPHAMYCINS OF *STREPTOMYCES CHARTREUSIS*

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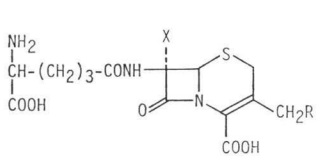
By the use of HPLC technique after treatment with β-lactamases, two novel cephamycins, SF-1623 and SF-1623B, were discovered and isolated from the fermentation broth of *Streptomyces chartreusis* SF-1623. The structures of SF-1623 and SF-1623B were determined to contain 3-sulfothiomethyl and 3-hydroxymethyl groups respectively, by chemical and enzymatic transformation reactions. Studies on the fermentation condition and process for the large scale preparation of antibiotic SF-1623 are also described.

During our screening program for new β-lactam antibiotics, we have developed a convenient technique to detect β-lactams by the use of HPLC after treatment with β-lactamases. By this method, it was found that a *Streptomyces* strain isolated from a soil sample collected at Oki Island, Shimane Prefecture, Japan, produced a variety of β-lactam antibiotics depending upon culture conditions. When cultured in a medium containing thiosulfate, a main product was a new cephamycin designated as antibiotic SF-1623[1]. Addition of L-cysteine instead of thiosulfate resulted in the production of another novel cephamycin, antibiotic SF-1623B[2]. The third β-lactam described as SF-1623C turned out to be deacetylcephalosporin C[3]. In addition, this strain produced cephamycins A and B[4]. This paper deals with the discovery, isolation and structure determination of the two novel cephamycins. The isolation of 7-methoxydeacetylcephalosporin C (SF-1623B) was recently reported by SOOD *et al.*[5].

Characterization of the Producing Organism, SF-1623

Strain SF-1623 was isolated from a half-diluted inorganic salts - starch agar containing 10 μg/ml of cephaloridine. From the taxonomic characteristics, strain SF-1623 was assigned to genus *Streptomyces*, among which *Streptomyces chartreusis*[4,6] was most closely related. Therefore, *S. chartreusis* ISP 5085 was directly compared with strain SF-1623 by simultaneous cultivation. Morphological, cultural and physiological properties of the two strains were in good agreement except for the formation of soluble pigments on sucrose - nitrate agar and tyrosine agar in strain SF-1623. The difference was not

Fig. 1. Chemical structures of cephem antibiotics produced by *Streptomyces chartreusis* SF-1623.



	X	R
SF-1623	OCH_3	SSO_3H
SF-1623B	OCH_3	OH
SF-1623C	H	OH
Cephamycin A	OCH_3	$OCOC(OCH_3)=CH-C_6H_4-OSO_3H$
Cephamycin B	OCH_3	$OCOC(OCH_3)=CH-C_6H_4-OH$

sufficient to designate strain SF-1623 as a new species, and it was named *Streptomyces chartreusis* SF-1623. It has been deposited in the American Type Culture Collection with accession number ATCC 21999.

Production on Antibiotics SF-1623 and SF-1623B

Because of the low productivity of the original strain, an attempt was made to obtain a high-yield mutant by UV irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoiminourea treatment. As a result, a highly productive mutant designated AP-26 was obtained and used in the following investigations.

Various carbon and nitrogen sources were examined for the effective production of antibiotic SF-1623. The basic medium used contained dextrin 4.0%, soybean meal 3.0%, sodium thiosulfate 0.3% and calcium carbonate 0.15% (pH 7.0). Among carbon sources listed in Table 1, a combination of sucrose 2.0% and soybean oil 2.0% was most effective for antibiotic production. With regards to nitrogen sources tested, a combination of soybean meal, fish meal and sodium nitrate brought about the highest titer. Effect of various sulfur sources on the production medium is shown in Table 2. It was found that sodium thiosulfate was indispensable not only for the production of antibiotic SF-1623, but also for the increase of titer of other cephamycins. A potential thiosulfate donor, L-sulfocysteine was also effective in the production of SF-1623.

Table 1. Effects of carbon sources* on the production of antibiotic SF-1623.

Carbon source (%)		pH	SF-1623 (μg/ml)
Glycerol	(4)	8.2	54
Glucose	(4)	7.2	38
Sucrose	(4)	7.5	105
Maltose syrup	(4)	7.6	100
Dextrin	(4)	7.2	155
Starch	(4)	7.2	75
Soybean oil	(4)	8.3	92
Sucrose+maltose syrup	(2+2)	7.4	60
Sucrose+dextrin	(2+2)	7.2	100
Sucrose+soybean oil	(2+2)	7.0	480
Dextrin+maltose syrup	(2+2)	7.2	170
Dextrin+soybean oil	(2+2)	7.2	130
Maltose syrup+soybean oil	(2+2)	7.0	380

* Basal medium: soybean meal 3.0%, $Na_2S_2O_3 \cdot 5H_2O$ 0.3% and $CaCO_3$ 0.15% (pH 7.0). All carbon sources were sterilized separately, and added to the basal medium before inoculation.

A well-grown agar slant of strain SF-1623 AP-26 was inoculated into a seed medium containing sucrose 1.0%, soybean meal 3.0% and a stainless steel-coil, adjusted to pH 7.0 before sterilization. The coil was used to prevent pellet formation. The seed culture was shaken at 28°C for 30 hours, and then added in the proportion of 1.0% to the production medium. It consisted of sucrose 2.0%, soybean oil 2.0%, soybean meal 2.0%, fish meal 2.0%, sodium nitrate 0.15%, sodium thiosulfate 0.3% and calcium

Table 2. Effects of sulfur sources*1 on the production of antibiotic SF-1623 and cephamycin B.

Sulfur source	pH	Growth*2 (%)	SF-1623 (μg/ml)	Cephamycin B (μg/ml)
None	6.5	50	0	24
Sodium thiosulfate	7.3	55	440	100
Sodium sulfide	6.7	50	0	20
Sodium sulfate	6.5	52	0	30
DL-Methionine	7.3	45	0	16
L-Cysteine	8.0	44	0	45
L-Sulfocysteine			180	

*1 Basal medium: sucrose 2.0%, soybean oil 2.0%, soybean meal 2.0%, fish meal 2.0%, sodium nitrate 0.15% and calcium carbonate 0.15% (pH 7.0). All sulfur sources (final concentration 0.3%) were sterilized separately, and added to the basal medium before inoculation.

*2 Packed mycelial volume.

Fig. 2. Fermentation time course of antibiotic SF-1623 (Fermentation I).

Production medium: sucrose 2.0%, soybean oil 2.0%, soybean meal 2.0%, fish meal 0.5%, sodium nitrate 0.15%, calcium carbonate 0.15%, sodium thiosulfate 0.3% (24 hours, 48 hours) (pH 7.0).

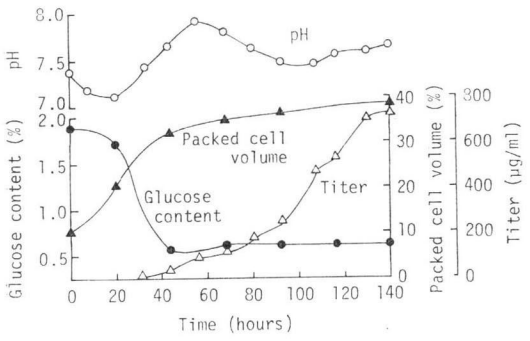
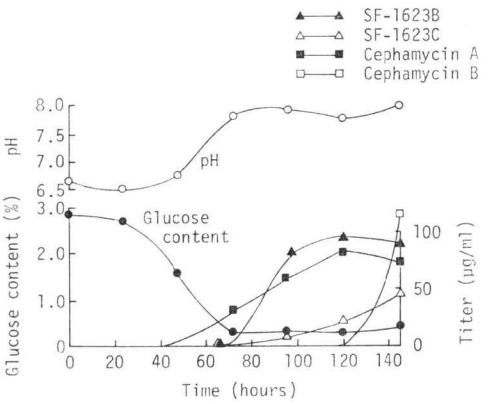


Fig. 3. Fermentation time course of antibiotic SF-1623B (Fermentation II).

Production medium: sucrose 2.0%, soybean meal 2.0%, soybean oil 2.0%, fish meal 2.0%, calcium carbonate 0.15%, L-cysteine 0.4% (pH 7.0).



carbonate 0.15% (pH 7.0) (Fermentation I). The fermentation time course of antibiotic SF-1623 is illustrated in Fig. 2. A 570-liter jar fermenter was operated at 28°C and 170 rpm at the aeration rate of 300 liters/minute. Sodium thiosulfate was added twice at 24 hours and 48 hours. The antibiotic production started 30 hours after inoculation, and gradually rose as the fermentation progressed, reaching 750 μg/ml after 140 hours.

Fig. 3 indicates a time course of SF-1623B in the production medium containing L-cysteine in stead of sodium thiosulfate as a sulfur source (Fermentation II). HPLC analysis revealed that antibiotic SF-1623B in place of SF-1623 was produced, in addition to cephamycins A and B, and SF-1623 C (deacetylcephalosporin C).

Isolation and Chemical Characterization of Antibiotics SF-1623 and SF-1623 B

Antibiotic SF-1623 could be isolated directly (A), or more conveniently by way of *N*-trichloroethoxycarbonyl derivative in an intermediate step (B). The latter was applicable to a large scale preparation.

A) The culture filtrate of Fermentation I (750 μg/ml, 20 liters) was passed through a column of Amberlite XAD-2 (2 liters). The filtrate was applied to a column of Amberlite IRA-68 (Cl^-, 1 liter) and the column was eluted with 0.5 M NaCl. The active eluates were collected (5 liters), and charged on a column of activated carbon (300 ml), being eluted with 50% aqueous acetone. The fractions containing SF-1623 (1 liter) were evaporated to remove acetone and chromatographed over DEAE-Sephadex A-25 (Cl^-, 200 ml). After washing with 0.05 M NaCl, antibiotic SF-1623 was eluted with 0.15 M NaCl. The active eluate was desalted over activated carbon (100 ml), and subjected to microcrystalline cellulose chromatography (Avicel, 400 g), being developed with *n*-BuOH - AcOH - H_2O (3 : 1 : 1). The active fractions thus obtained were decolorized over activated carbon, and evaporated to dryness to give the sodium salt of SF-1623 (750 mg). A part (190 mg) of this preparation was dissolved in water, and passed through a column of Amberlite IR-120 (H^+, 1.4×3 cm). The active effluent was concentrated and lyophilized to give the free acid of SF-1623 (50 mg). Mp 75~80°C with decomposition.

Table 3. Physico-chemical properties of antibiotics SF-1623 and SF-1623B.

	SF-1623	SF-1623B
Mp	160~165°C (dec.)	170~173°C (dec.)
$[\alpha]_D^{20}$	+58° (*c* 1.29, H_2O)	+168° (*c* 1.0, H_2O)
UV (H_2O) ($E_{1cm}^{1\%}$)	240 (sh), 271 nm (230)	241 (147), 264 nm (183)
IR (KBr)	3370, 1750 (β-lactam), 1610 (amide I), 1530 (amide II), 1400, 1210 (as SO_2), 1020 (as SO_2) cm^{-1}	3400, 1760 (β-lactam), 1610 (amide I), 1530 (amide II), 1410, 1360, 1080 cm^{-1}
NMR (D_2O)	5.13 s (H-6), 4.02 s (H-3′), 3.81 t (CH), 3.60 q (H-2), 3.50 s (OCH_3), 2.45 t, 1.85 m (CH_2) ppm	5.09 s (H-6), 4.17 s (H-3′), 3.68 t (CH), 3.47 s (OCH_3), 3.44 q (H-2), 2.44 t, 1.80 (CH_2) ppm
Molecular weight	525 (titration)	412 (titration)
Molecular formula	$C_{15}H_{21}N_3O_{10}S_3$ (499) (free acid)	$C_{15}H_{20}N_3O_8SNa$ (425)
Elementary analysis		
Calcd.;	C 36.4, H 4.2, N 8.4, S 19.3.	C 42.35, H 4.71, N 9.88, S 7.53.
Found;	C 35.9, H 4.0, N 7.9, S 18.7.	C 43.08, H 4.98, N 9.62, S 7.29.
Rf (silica gel TLC)*	0.18	0.31

* *n*-BuOH - AcOH - H_2O, 2: 1: 1

Table 4. Chemical stability of SF-1623, SF-1623B and related cephem antibiotics.

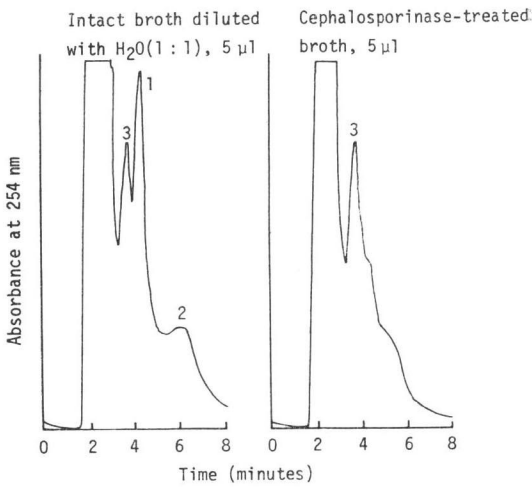
Antibiotic	Residual antibiotic content*			
	Heating at 60°C for 30 minutes		Heating at 100°C for 10 minutes	
	pH 2	pH 7	pH 2	pH 7
SF-1623	100%	100%	80%	90%
SF-1623B	59	95	<2	85
Cephamycin B	10	15	<2	<2
Cephamycin C	80	100	<2	10
7-Methoxycephalosporin C	85	80	8	<2
Cephalosporin C	65	95	<2	<2

* Initial concentration of antibiotics in water was 200 μg/ml. Residual activity was assayed against *Vibrio percolans* ATCC 8461.

Fig. 4. HPLC analysis of culture broth of *Streptomyces chartreusis* SF-1623 (Fermentation I).

1. Antibiotic SF-1623, 2. cephamycin B, 3. *p*-hydroxy-α-methoxycinnamic acid.

Column: Zipax SAX 0.2×100 cm; solvent: 0.1 M NaH_2PO_4 containing 0.002 M $NaClO_4$ (pH 4.5).



The physico-chemical properties of SF-1623 are summarized in Table 3. As shown in Table 4, antibiotic SF-1623 is extremely stable to heat and acid. This rendered the antibiotic more suitable for recovery from the fermentation broth than less stable cephem antibiotics such as cephamycins A and B. Table 5 summarizes the retention times of antibiotic SF-1623 and related cephalosporins on HPLC. Fig. 4 illustrates a HPLC pattern of the fermentation broth. A peak assigned to antibiotic SF-1623 was stable against penicillinase of *Bacillus cereus*, but disappeared by treatment with large excess of cephalosporinase of *Citrobacter freundii* GN 346.

B) The broth filtrate of Fermentation I (234 μg/ml, 760 liters) was passed through a column of

Table 5. Retention times of SF-1623, SF-1623B and related cephem antibiotics in HPLC.

Column: Zipax SAX 0.2×100 cm Developer: 0.1 M NaH_2PO_4 containing 0.002 M $NaClO_4$ (pH 4.5)		Column: Zipax SAX 0.2×200 cm Developer: 0.32 M Na borate (pH 9.6)	
Antibiotic	Retention time	Antibiotic	Retention time
SF-1623	4.0 minutes	SF-1623B	7.5 minutes
SF-1623B	2.3	SF-1623C	7.9
Cephalosporin C	2.8	Deacetylcephalosporin C lactone	5.6
3′-Deacetoxy-3′-sulfothio-cephalosporin C	4.3	Cephamycin C	8.2
Cephamycin B	5.3	7-Methoxycephalosporin C	8.6
Ethoxycarbonyl-SF-1623	4.1*	3′-Carbamoyloxy-3′-deacetoxy-cephalosporin C	8.8
Cephamycin B	3.3*	Cephalosporin C	9.3
Cephamycin A	6.1*	Cephamycin B	29.2

* Developer: 0.1 M NaH_2PO_4 containing 0.02 M $NaClO_4$.

Amberlite XAD-2 (90 liters), and adsorbed on a column of Amberlite 68 (Cl^-, 60 liters). The 0.1 M ammonium sulfate eluate (385 liters) was concentrated and methanol was added to precipitate inorganic salts. The filtrate (142 liters) was concentrated and desalted over activated carbon to give a concentrated solution of SF-1623 (6 liters). Excess trichloroethoxycarbonyl chloride in acetone was added under ice-cooling, while the solution was maintained at pH 7.5~8.0 by the addition of 20% sodium carbonate. After the reaction was completed (5 hours), the solution was concentrated and applied to a column of Diaion HP-20 (3 liters) and the column was developed with water. The active eluate was decolorized over activated carbon (500 ml) and lyophilized to give *N*-trichloroethoxycarbonyl SF-1623 (31.4 g, purity 89.7%). Rechromatography over Diaion HP-20 gave a pure sample, Rf value 0.48 in silica gel TLC. UV λ_{max}; 271 nm ($E^{1\%}_{1cm}$ 135) in water.

Anal. Calcd. for $C_{18}H_{19}N_3O_{12}S_3Cl_3Na_3 \cdot 2H_2O$: C 27.83, H 2.98, N 5.41.
Found: C 28.03, H 3.41, N 5.98.

Conversion of *N*-trichloroethoxycarbonyl SF-1623 into SF-1623 was achieved by treatment with zinc powder in 90% acetic acid at room temperature for 5 hours, followed by DEAE-Sephadex A-25 chromatography and carbon chromatography. But, it could be used as such in the derivatization studies described in the accompanying paper[7].

C) Antibiotic SF-1623B was isolated from the fermentation broth containing L-cysteine instead of thiosulfate (Fermentation II). The culture filtrate (150 μg/ml, 150 liters) was passed through a column of Amberlite XAD-2 (15 liters). Antibiotic SF-1623B was found in the effluent, and other β-lactam antibiotics co-produced were retained on the column. The effluent was adsorbed on a column of Dowex 1X2 (Cl^-, 7 liters) and the column was developed with 0.05 M NaCl. The active fraction was desalted over activated carbon (1 liter) and chromatographed on a column of DEAE-Sephadex A-25 (Cl^-, 50 ml) eluted with 0.03 M NaCl. The eluate (400 ml) was desalted, concentrated and subjected to microcrystalline cellulose chromatography (Avicel, 400 g), developed with a mixture of *n*-BuOH - AcOH - H_2O (2: 1: 1). Concentration and lyophilization of the active eluate gave the sodium salt of SF-1623B (430 mg).

The physico-chemical property of SF-1623B is listed in Table 3. It is less stable than SF-1623 in acidic solution, but equally stable to heat at pH 7 (Table 4). The retention times on HPLC are shown in Table 5. Under the conditions employed, SF-1623B was differentiated from closely related cephalosporins.

The antibiotics remaining in the above Amberlite XAD-2 column was eluted with 60% aqueous acetone. The antibiotics in the active eluate were fractionated into SF-1623C and cephamycins A and B by column chromatography over DEAE-Sephadex A-25 (Cl^-, 100ml) with 0.05 M NaCl as a developer. Further purification over Sephadex G-10 gave 280 mg of SF-1623C, which was identical with an authentic sample of deacetylcephalosporin C derived from cephalosporin C.

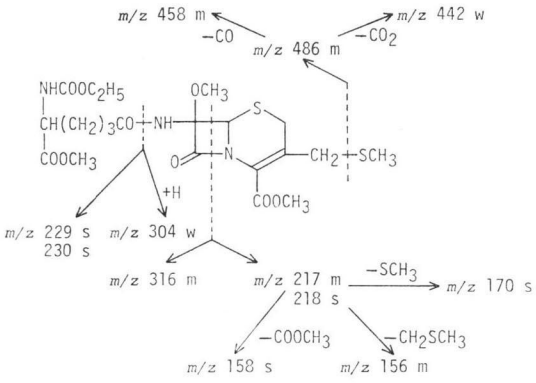
Structural Studies

Two antibiotics SF-1623 and SF-1623B showed a carbonyl IR band around 1750~1760 cm^{-1}, and positive azide-iodine color reaction, characteristic of β-lactams. Amino acid analyses of the acid hydrolysates of SF-1623 and SF-1623B revealed the presence of one mole of α-aminoadipic acid in both molecules. The 100 MHz PMR spectral data of both antibiotics (Table 3) resembled those of cephamycins A and B, showing signals due to α-aminoadipoyl, 7α-methoxy, β-lactam ring and methylene protons at C-3, but lacked in signals due to the C-3 acyloxy substituent. The presence of a sulfothio group in SF-1623 was suggested by 1) movement to the anode of 3,500 volts paper electrophoresis at pH 1.9 (cephalosporin C migrated to the cathode), 2) IR bands at 1210 and 1020 cm^{-1}, assignable to be asymmetric and symmetric SO_2 stretching modes and 3) elemental analysis of sulfur (Table 3).

The structure of SF-1623 was confirmed by the chemical transformation reaction which follow. Treatment of SF-1623 with ethoxycarbonyl chloride gave a *N*-ethoxycarbonyl derivative (**1**), which on reaction with dithiothreitol was converted into thiol (**2**) and disulfide (**3**). Reaction of compound **2** with diazomethane gave a *O*,*S*-trimethyl derivative (**4**). As shown in Fig. 5, mass spectrometry of compound **4** gave a fragmentation pattern, in agreement with the structure proposed for **4**, indicating the sulfur substitution at the C-3 methylene group. The disulfide (**3**), prepared in higher yield from compound **1** by treatment with sodium sulfide or more desirable sodium *p*-chlorothiophenolate, was reversed to compound **1** on reaction with sodium bisulfite, which is a typical cleavage reaction of S-S linkage. Finally, treatment of cephamycin B with sodium thiosulfate in water gave SF-1623, identical in all respects with the antibiotic isolated from the culture broth.

Fig. 5. Mass fragmentation pattern of compound **4** derived from SF-1623.

Each fragment was consistent with high-resolution mass data.



Isolation of the cephem antibiotics containing sulfur substituents at C-3 have been reported so far in cases of methylthio,[8] 2-amino-2-carboxy-1,1-dimethylethylthio[9] and various heterocyclic-thio groups[10]. In all cases, replacement of acyloxy groups with the respective thiol compounds was most probable. The fact that antibiotic SF-1623 was produced only in the thiosulfate-containing medium suggested that inorganic thiosulfate was a direct source of the Bunte salt in SF-1623, possibly by non-enzymatic conversion of the alkoxy side chain of cephamycins A and B or their biogenetic precursors with thiosulfate anion. However, the role of thiosulfate was not limited to a thiosulfate donor, but contributed to the β-lactam production, because the production of other cephem antibiotics such as cephamycin B was also increased by the addition of thiosulfate (Table 2). In this respect, the production of cephamycins by *Streptomyces chartreusis* was different from that by *Cephalosporium* sp., where

sodium thiosulfate did not affect cephalosporin biosynthesis[11].

The hydroxymethylene structure at C-3 of SF-1623B was supported by the chemical shift of methylene signals at 4.17, and microbiological preparation from cephamycin B. Enzymatic hydrolysis of cephamycin B with a crude esterase of *Aspergillus fumigatus* 2469 gave SF-1623B at high conversion rate (75~100%). The identification was made by comparison of HPLC, UV, IR and PMR spectra. In addition to *Aspergillus* sp., *Mucor lipolyticus* Aac-0102 and *Penicillium chrysogenium* IAM 7106 produced esterases capable of hydrolyzing cephamycins A and B and their *N*,*O*-ethoxycarbonyl derivatives.

The origin of SF-1623B was not determined, but it was of interest to know that deacetyl-7-methoxycephalosporin C (SF-1623B) and deacetylcephalosporin C (SF-1623C) are found in the culture broth of *Streptomyces chartreusis*, without any detection of biogenetically related acetoxy derivatives[12].

Antibacterial Activities

Table 6 summarizes the minimum inhibitory concentration (MIC) of SF-1623 and SF-1623B. Though MIC values were not low in general, antibiotic SF-1623 showed high activity against strains of Gram-negative bacteria such as *Vibrio percolans*, *Proteus mirabilis* and *Proteus vulgaris*. The activity of SF-1623B was much weaker than that of SF-1623.

Table 6. Antibacterial activity of antibiotic SF-1623 and SF-1623B.

Test organism	MIC*1 (μg/ml)	
	SF-1623	SF-1623B
Staphylococcus aureus 209P JC-1	>200	>100
Bacillus subtilis ATCC 6633	>200	>100
Escherichia coli No. 29	50	>100
Klebsiella pneumoniae PCI-602	25	>100
Salmonella typhi O-901-w	12.5	>100
Proteus mirabilis GN 79	3.2	50
Proteus vulgaris OX-19	0.78	25
Proteus rettgeri J-0026	25	100
Vibrio percolans ATCC 8461	0.39	12.5
Alcaligenes faecalis ATCC 8750	6.25	—*2

*1 Heart-infusion agar and bacterial inoculum of 10^6 CFU were used.
*2 Not determined.

Experimental

NMR spectra were recorded on a Varian XL-100 spectrometer; chemical shifts (δ) are given in ppm downfield from external D_2O or internal TMS. Mass spectra were determined with a JMS-0lSG mass spectrometer. IR, UV spectra and optical rotations were determined on Hitachi Model 215, Model 200–20 and Perkin-Elmer Model 141 instruments, respectively. Amino acids were analyzed by using a JLC-6AH amino acid analyzer. Melting points were measured in glass capillaries with a Yamato apparatus and are uncorrected. Thin-layer chromatography was carried out on Merck silica gel 60 F-254 plates. A mixture of *n*-BuOH - AcOH - H_2O (2: 1: 1) was used as a developing solvent, unless otherwise specified. Spots were detected by spraying a mixed solution of 0.1% NaN_3 and 0.5% I_2 in 95% EtOH.

Screening and Assay Methods of New β-Lactam Antibiotics

HPLC Analysis: A DuPont 830 Liquid Chromatograph was used, equipped with a UV detector at 254 nm, and operated at room temperature. Analysis was carried out by using Zipax SAX (0.2×100 or 0.2×200 cm), and 0.1 N NaH_2PO_4 containing 0.002 M $NaClO_4$ (pH 4.5) or 0.3 N Na-borate at pH 9.6 as a mobile phase at a flow rate of 0.6 ml/minute. A culture filtrate was divided into two parts. One was treated with an equal volume of cephalosporinase solution prepared from *Citrobacter freundii* GN 346 or penicillinase from *Bacillus cereus* (Sigma Chemical Company) at 37°C for 30 minutes before injection to HPLC. The other was treated similarly without β-lactamase. Any peak that showed a decrease in intensity by penicillinase was tentatively assigned to a penam antibiotic, and that decreased by cephalosporinase to a cephem antibiotic. A typical HPLC pattern of culture broth is shown in Fig. 4, and retention times of SF-1623, SF-1623B and related cephem antibiotics are listed in Table 5.

Microbial Assay: Antibiotics SF-1623 and SF-1623B were assayed by the conventional paper disc-agar diffusion method by using *Vibrio percolans* ATCC 8461 as a test organism. For the selective determination of SF-1623 in the presence of other cephem antibiotics, a culture filtrate was heated at 80°C for 10 minutes, and then assayed by the paper disc method as described above. Most activities of other cephem antibiotics were lost under these conditions, while more than 90% of the activity of SF-1623 remained. Antibiotic SF-1623C and cephamycins A and B were determined by the paper disc method by using *Bacillus stearothermophilus* as a test organism, against which SF-1623 and SF-1623B were essentially inactive.

Preparation of Methyl 7β-(5-D-Ethoxycarbonylamino-5-methoxycarbonyl-valeramido)-7α-methoxy-3-methylthiomethyl-3-cephem-4-carboxylate (**4**)

To an aqueous solution of SF-1623 (2.5 g) in H_2O (110 ml) were gradually added $NaHCO_3$ (17.5 g) and a solution of ethoxycarbonyl chloride (8 ml) in acetone (60 ml). The mixture was stirred for 1 hour, and washed with *n*-BuOH (300 ml). The aqueous layer was acidified with 5 N HCl (15 ml), and extracted twice with *n*-BuOH (each 200 ml). The organic layer was reextracted with H_2O at pH 6, and the aqueous solution was concentrated, and applied to a column of DEAE-Sephadex A-25 (Cl^-, 4×21 cm). The 0.4 M NaCl eluates were collected, concentrated and desalted by the addition of MeOH. Yield of crude *N*-ethoxycarbonyl SF-1623 (**1**) was 2.15 g. A part of this powder (500 mg) was dissolved in H_2O (20 ml), and subjected to column chromatography over Diaion HP-20 (1.7×18 cm), developed with H_2O. The main fractions were combined and evaporated to dryness to give 147 mg of **1**. Mp 180°C with decomposition; $[\alpha]_D^{20}$ +44.6° (*c* 1.0, H_2O), UV λ_{max} 242 nm ($E_{1cm}^{1\%}$ 108), 269 (138) in MeOH. Rf on silica gel TLC, 0.43.

Anal. Calcd. for $C_{18}H_{22}N_5O_{12}S_3Na_3 \cdot 2H_2O$: C 32.10, H 3.89, N 6.24.
Found: C 32.51, H 4.01, N 6.42.

To a solution of compound **1** (500 mg) in H_2O (20 ml) was added Na_2CO_3 (60 mg) and dithiothreitol (100 mg) under ice-cooling. The mixture was acidified with 1 N HCl, and extracted twice with ethyl acetate. The organic layer was reextracted with H_2O at pH 5. The aqueous layer was concentrated and applied to a column of Sephadex G-10 (2×43 cm) developed with H_2O. The fractions exhibiting positive thiol reaction by dithio-bis-2-nitrobenzoic acid at Rf 0.65 on TLC were collected, and evaporated to dryness to yield 171 mg of 3-mercaptomethyl derivative (**2**). Fractions showing Rf 0.44 on TLC were combined and evaporated to afford 3,3′-bismethylene disulfide (**3**) (10 mg).

A part of compound **2** (86 mg) was dissolved in ethyl acetate and excess diazomethane in ethyl ether - ethanol (1 : 1) was added. The mixture was immediately evaporated, and the residue was chromatographed on a column of silicic acid (1.5×10 cm), developed with benzene containing 20% acetone. The main eluate was evaporated to give compound **4** (35 mg). $[\alpha]_D^{20}$ +109° (*c* 1.15, MeOH), UV λ_{max} 245 nm (shoulder), 271 ($E_{1cm}^{1\%}$ 125) in MeOH, PMR ($CDCl_3$) δ 2.59 (SCH_3), 3.83, 3.86 (OCH_3).

Anal. Calcd. for $C_{21}H_{31}N_5O_9S_2$: C 47.27, H 5.86, N 7.87, S 12.02.
Found: C 47.58, H 5.83, N 7.72, S 11.86.

Preparation of 1, 1′-Bis[7β-(5-D-ethoxycarbonylamino-5-carboxy-valeramido)-7α-methoxy-4-carboxy-3-cephem-3-yl]dimethylenedisulfide Tetrasodium Salt (**3**)

To a solution of **1** (500 mg) in H_2O (20 ml) was added sodium *p*-chlorothiophenolate (700 mg) under ice-cooling. After standing for 30 minutes at room temperature, 1 N H_3PO_4 was added to adjust pH to 3.5, and washed twice with ethyl acetate. The aqueous layer was acidified, and extracted twice with ethyl acetate (each 30 ml). The organic layer was reextracted with H_2O at pH 7, and the aqueous extract was subjected to a column chromatography over Sephadex G-10 (2.0×43 cm). The main eluate was evaporated to give 162 mg of compound **3**. $[\alpha]_D^{23}$ +4.4° (*c* 1.0, MeOH), UV λ_{max} 245 nm (shoulder), 276 ($E_{1cm}^{1\%}$ 109) in MeOH.

Anal. Calcd. for $C_{36}H_{44}N_6O_{18}S_4Na_4 \cdot 2H_2O$: C 39.13, H 4.38, N 7.61.
Found: C 39.25, H 4.43, N 7.59.

Tetramethyl ester of compound **3** was prepared by treating **3** with diazomethane in ethyl ether - ethanol (1 : 1), followed by chromatographic purification over silicic acid, developed with 25% acetone - benzene. Mp 75~80°C (sintered) and decomposed at 156°C. $[\alpha]_D^{20}$ −2.5° (*c* 1.21, MeOH). UV λ_{max}

281 nm ($E^{1\%}_{1cm}$ 150) in MeOH. PMR ($CDCl_3$) δ 3.83, 3.86 ($COOCH_3$).

Anal. Calcd. for $C_{40}H_{56}N_6O_{18}S_4$: C 46.32, H 5.44, N 8.10, S 12.36.
Found: C 46.36, H 5.77, N 7.90, S 12.11.

A mixture of compound **3** (100 mg) and $NaHSO_3$ (30 mg) in H_2O (10 ml) was heated at 60°C for 6 hours, and passed through a column of Diaion HP-20 (50 ml). The active aqueous eluate was evaporated to give a white powder (35 mg), which was identical with compound **1**, in TLC, HPLC and IR analyses. When Na_2SO_3 was used in stead of $NaHSO_3$, no conversion of compound **3** to **1** was observed.

Enzymatic Hydrolysis of Cephamycin B

Spores of *Aspergillus fumigatus* 2469 were inoculated into the liquid medium (500 ml) containing soluble starch 4.0%, soybean meal 3.0%, KH_2PO_4 0.3% and $(NH_4)_2SO_4$ 0.3% (pH 6.5), and cultured at 25°C for 48 hours. The fermentation was transferred to the production medium (20 liters) having the same composition as the seed culture, and fermented at 25°C for 96 hours under aeration. To the culture filtrate (12 liters) was added $(NH_4)_2SO_4$ until 70% saturation, and the mixture was allowed to stand at 5°C overnight. The precipitate was collected, and dissolved in 500 ml of H_2O. Insoluble materials were removed by filtration, and the filtrate (600 ml) was dialyzed at 5°C for 24 hours. In the crude esterase preparation (1 liter) thus obtained, was dissolved cephamycin B (5 g, purity 50%), and the mixture was adjusted to pH 7.3, and allowed to react at 45°C for 6 hours. After passage through Amberlite XAD-2 (100 ml), the effluent was applied on a column of Dowex 1X2 (Cl^-, 50 ml), and eluted with 0.05 M NaCl. The eluate (250 ml) was adsorbed on activated carbon (20 ml), and eluted with H_2O. The desalted active fractions were lyophilized to give 770 mg (42%) of a white powder, mp 165~172°C, which was identical with antibiotic SF-1623B by UV, IR, PMR and HPLC analyses.

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