SF-1771, A NEW ANTIBIOTIC RELATED TO BLEOMYCIN

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A new antibiotic, substance SF-1771, has been isolated from the fermentation broth of *Streptomyces toyocaensis* SF-1771. The antibiotic is highly active against Gram-positive and -negative bacteria and effective against sarcoma 180 cells of ascites type in mice. It belonged to the phleomycin-bleomycin group antibiotics, but has been differentiated from the known antibiotics by the physicochemical properties, chromatographic behaviours and amino acid analysis.

In the course of our screening for new antibiotics, a novel basic antibiotic was isolated from the culture filtrate of *Streptomyces toyocaensis* SF-1771. The strain was isolated from a soil sample collected at Takehara city, Hiroshima prefecture, Japan. This paper deals with the production, isolation, purification and physico-chemical and biological properties of substance SF-1771.

Characterization of the Producing Microorganism

For the taxonomic characterization of the strain SF-1771, the methods of the International *Streptomyces* Project (ISP) by SHIRLING and GOTTLIEB[1] were used, with additional media recommended by WAKSMAN[2]. The strain SF-1771 showed the following morphological, cultural and physiological characteristics: Aerial mycelium indicated monopodial branching with spiral spore chains on most of the media tested. The spores were in chains of more than ten, ellipsoidal in shape, $0.8 \sim 1.1 \times 1.2 \sim 1.4\ \mu$ in size and had spiny surfaces. On most media, the aerial mass color was gray to brownish gray and the reverse color was pale yellow to grayish yellow. The strain SF-1771 showed good growth with abundant aerial mycelia in most of media except for sucrose-nitrate agar. No soluble pigment was formed. The strain utilized D-glucose, D-fructose, D-mannose and inositol but did not utilize D-xylose, L-arabinose, rhamnose, sucrose and raffinose. LL-Diaminopimeric acid was detected in the whole cell hydrolyzates. Based on the characteristics described above, the strain SF-1771 evidently belonged to genus *Streptomyces*, among which, *Streptomyces toyocaensis*[3] was most closely related. Therefore, *Streptomyces toyocaensis* ISP 5030 was directly compared with the strain SF-1771 by simultaneous cultivation, and the results are shown in Table 1. Except for utilization of L-arabinose and cultural characteristics on oatmeal agar and nutrient agar, morphological and physiological properties were in good agreement between the two strains. The differences were not sufficient to designate the strain SF-1771 as a new species, and it was named *Streptomyces toyocaensis* SF-1771.

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Production of SF-1771 Substance

Streptomyces toyocaensis SF-1771 was grown at 28°C for 7 days on yeast extract-starch agar slants. The inoculum was prepared by suspending the spores from each slant in 10 ml of sterile water, and planting in medium containing 1.0% soluble starch and 3.0% soybean meal (pH 7.0) in shaking flasks. The seed culture which was prepared by incubation at 28°C for 40 hours in a reciprocal shaker was transferred into a 50-liter jar fermentor, containing 35 liters of the medium containing 4.0% sucrose, 1.0% soybean oil, 3.0% soybean meal, 2.0% wheat embryo, 0.6% sodium chloride and 0.003% $CuSO_4 \cdot 5H_2O$ (pH 7.0). Fermentation was carried out at 28°C for 114 hours under aeration. The antibiotic titer was assayed against *Bacillus subtilis* ATCC 6633 by the usual paper-disc method.

Table 1. Difference between strain SF-1771 and *Streptomyces toyocaensis* ISP 5030.

	Strain SF-1771	*S. toyocaensis*
Reverse color in oatmeal agar	grayish yellow	yellow to brownish yellow
Soluble pigment in oatmeal agar	none	pale yellow
Aerial mycelium in nutrient agar	cottony, pale gray	scant, white
Utilization of L-arabinose	negative	positive

Isolation and Purification of SF-1771 Substance

The culture broth fermented for 114 hours (pH 6.8) was filtered with diatomaceous earth (5% w/v). The filtrate (20 liters) was adsorbed on a column of Amberlite XAD-2 (2 liters). The column was washed with 20 liters of water and the antibiotic adsorbed on the resin was eluted with 14 liters of 50% aqueous acetone (pH 3.0). The active fractions (9 liters) were adjusted to pH 6.0 with 1 N sodium hydroxide and concentrated to remove acetone. This was passed through a column of CM-Sephadex C-25 (250 ml), the column was washed with 2.5 liters of water, then with 0.1 M sodium chloride, and the antibiotic was eluted with 0.2 M aqueous sodium chloride. Effluents were collected in 20 ml portions, No. 125~150 were combined and adsorbed on a column of Diaion HP-20 (0.3 liter). The column was washed with 200 ml of water and eluted with 500 ml of 50% aqueous methanol. The active fraction was concentrated to a small volume and lyophilized to give a yellowish green powder of crude SF-1771 substance (320 mg). The crude powder was further purified by a column chromatography of CM-Sephadex C-25, developed with 0.15 M aqueous sodium chloride. Active eluate was passed through a column of HP-20 for desalting. The 50% methanol eluate was concentrated to dryness for further purification. This was dissolved in 70% aqueous methanol, passed through a 400-ml column of Sephadex LH-20 and developed with 90% aqueous methanol. Evaporation of solvent from the active effluent gave a blue powder of substance SF-1771 as a copper-chelated hydrochloride (42 mg).

A pale yellow powder of copper-free SF-1771 substance was obtained by treating the methanolic solution of copper-complex with hydrogen sulfide followed by chromatography over Sephadex LH-20. So far, no minor component has been detected during column chromatography.

Physico-chemical Properties

SF-1771, obtained as a blue amorphous hydrochloride, was readily soluble in water, soluble in methanol, slightly soluble in ethanol and insoluble in other organic solvents. It was stable in acidic solution but unstable in alkaline solution. It did not give a definite melting point, but gradually changed to brown around 185°C and decomposed at 208°C.

Fig. 1. Ultraviolet absorption spectrum of SF-1771 in water.

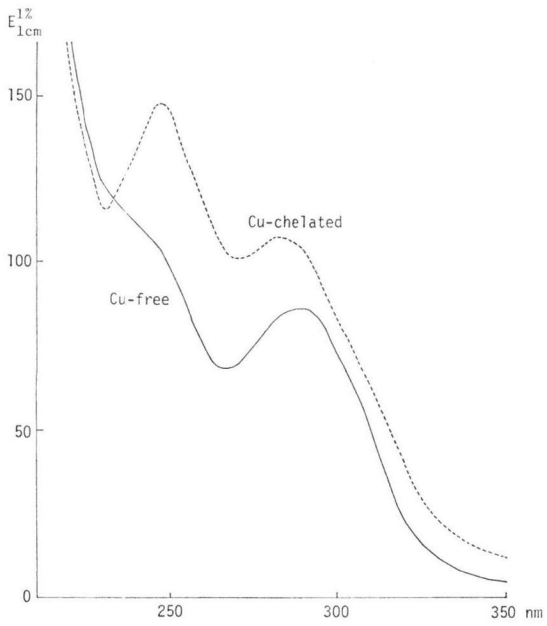


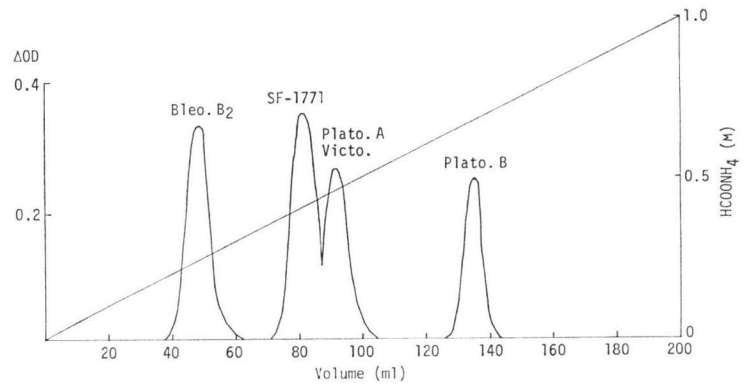
Table 2. Rf values of SF-1771 substance and related compounds in silica gel TLC.

	Solvent system and Rf		
	A	B	C
SF-1771 substance	0.68	0.57	0.83
Bleomycin A_2	0.37	0.36	—
Bleomycin B_2	0.64	0.65	—
Bleomycin A_5	0.54	0.52	—
Bleomycin B_4	0.54	0.51	—
Victomycin	0.64	0.51	—
Platomycin A	0.64	0.53	—
Platomycin B	0.52	0.45	—
Zorbonomycin B	0.65	—	0.88
Tallysomycin A	0.18	0.04	0.80
Tallysomycin B	0.21	0.06	0.79

Solvent A: 10% Ammonium acetate - methanol (1 : 1).
B: Methanol - 10% ammonium acetate - 10% ammonium water (10 : 9 : 1).
C: *n*-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12).

Fig. 2. CM-Sephadex chromatography of SF-1771, bleomycin (Bleo), victomycin (Victo), platomycins (Plato.).

Column: CM-Sephadex C-25 (i.d. 8 mm × 15 cm)
Elution: 0~1.0 M ammonium formate
Flow rate: 15 ml/hour



The elemental analysis was: C, 38.70; H, 5.42; N, 13.03; O, 29.62; S, 3.62; Cl, 6.07 and Cu, 3.53%. From the elemental analysis, approximate molecular formula $C_{58}H_{94}N_{17}O_{33}S_2Cu \cdot 3HCl$ was suggested for SF-1771.

As shown in Fig. 1, copper-chelated SF-1771 hydrochloride showed UV maxima at 248 nm ($E^{1\%}_{1cm}$ 146) and 282~284 nm ($E^{1\%}_{1cm}$ 106). The intensity ratio of the two absorption maxima at 248 nm and 282~284 nm was 1.38. SF-1771 hydrochloride (copper-free) showed $[\alpha]^{20}_D$ −18.4° (*c* 1, H_2O). Color reactions to GREIG-LEABACK, LEMIEUX, EHRLICH were positive but negative to ninhydrin and SAKAGUCHI

Fig. 3. IR spectrum of SF-1771 hydrochloride (KBr pellet).

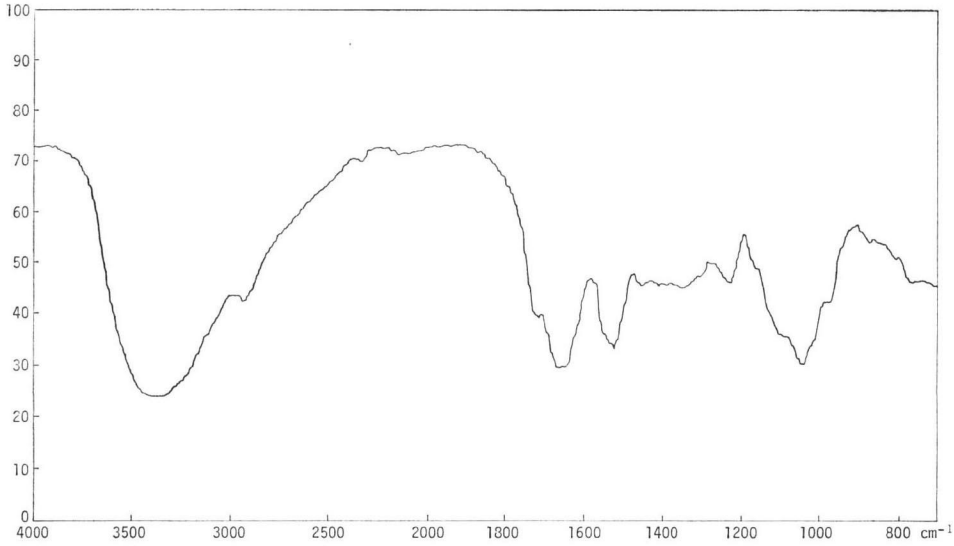
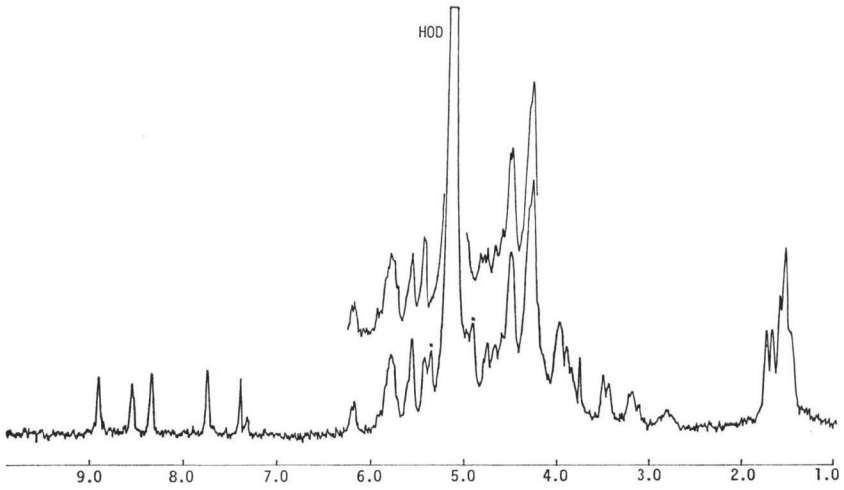


Fig. 4. ^{1}H-NMR spectrum of SF-1771 (Cu-free) (100 MHz in D_2O).



ppm from external TMS

reactions. Rf values on silica gel TLC and relative elution volume on a CM-Sephadex column of SF-1771 and some other related compounds are shown in Table 2 and Fig. 2. The IR spectrum of copper-free form in KBr pellet is presented in Fig. 3. Strong absorptions are observed at 3400~3200 cm^{-1} (OH/NH), 1720 cm^{-1} ($OCONH_2$), 1650 and 1550 cm^{-1} (CONH) and 1120~1020 cm^{-1} (C–O–).

The ^{1}H-NMR spectrum of copper-free hydrochloride is shown in Fig. 4.

Biological Properties of SF-1771

The minimum inhibitory concentration (MIC) of SF-1771 was determined by the agar dilution method, and the results are given in Table 3. It showed strong antibacterial activity against Gram-

Table 3. Antimicrobial spectrum of copper-free SF-1771.

Test organisms	MIC (mcg/ml)
Staphylococcus aureus JC-1	0.78
Staphylococcus aureus S-424	50
Staphylococcus epidermidis ATCC 14990	12.5
Staphylococcus epidermidis 109	12.5
Streptococcus faecalis ATCC 8043	>100
Bacillus anthracis No. 119	6.25
Escherichia coli JC-2	0.39
Escherichia coli No. 29	1.56
Escherichia coli RGN 823	0.39
Escherichia coli JR 66/W 677	0.39
Citrobacter freundii GN 346 (CSase; H)	0.78
Salmonella typhi 0-901-W	0.39
Salmonella typhimurium LT-2	0.39
Salmonella enteritidis No. 11	0.39
Sarcina lutea	12.5
Shigella sonnei EW 33 Type 1	0.78
Klebsiella pneumoniae PCI 602	0.78
Klebsiella pneumoniae 22#3038	100
Proteus vulgaris OX 19	1.56
Proteus rettgeri J-0026	50
Proteus morganii Kono	3.13
Serratia marcescens MB-3848	50
Pseudomonas aeruginosa MB-3829	>100
Pseudomonas cepacia M-0527	25

positive and -negative bacteria. Table 4 showed the antitumor activity against sarcoma 180 of mouse ascites type. SF-1771 (copper-free) was most effective by intraperitoneal administration of 4 mg/kg/day. It was inactive against leukemia L-1210 in mice.

The LD_{50} of copper-chelated and copper-free forms were about 10 mg/kg and 20 mg/kg respectively, by intravenous injection to mice. It showed negative mutagenecity test (*rec* assay) when examined by the streak and paper disc method using a mutant of *Bacillus subtilis* as a test organism. The relative rate of inactivation against rat liver enzyme of SF-1771 (copper-free) and bleomycin A_2 was compared.

This antibiotic was evidently more stable than bleomycin A_2 against 100S fraction of rat liver homogenate, as shown in Table 5.

Table 4. Antitumor activity of SF-1771 (Cu-free) against sarcoma 180 ascites type.

Dose (mg/kg/day)*	Mean survival days	ILS (%)
8	31.4	78.4
4	36.8	109.1
2	37.2	111.4
1	29.0	64.8
Control	17.6	—

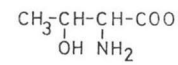
* The daily intraperitoneal injection of SF-1771 (Cu-free) for 3 days was started 24 hours after the intraperitoneal transplantation of 1.14×10^6 tumor cells.

Table 5. Inactivation of SF-1771 substance (Cu-free) by 100S fractions of rat liver homogenates.*

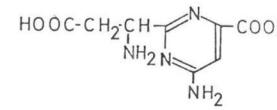
Substance	Survival of activity (%)				
	1 hr.	3 hrs.	6 hrs.	10 hrs.	24 hrs.
SF-1771 (Cu-free)	100	100	80	52	28
Bleomycin A_2 (Cu-free)	76	64	40	34	<6

* The inactivating reaction was carried out at 34°C, pH 7.5 (1/30 M phosphate buffer) and the remaining activity of the antibiotic was assayed by the paper disc method.

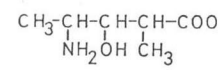
I



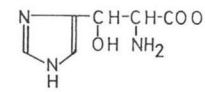
Demethyl-II



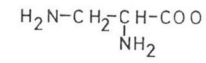
III



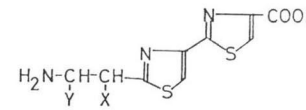
IV



V



VI′



Discussion

From the physico-chemical and biological properties described above, SF-1771 substance seems to belong to the bleomycin-phleomycin antibiotic. From the ratio of two UV maxima at 248 and 282~284 nm (1.38), SF-1771 substance is more similar to bleomycins[4], cleomycin[5], phleomycin (C,D_2 and F)[6], zorbonomycin[7], victomycin[8], platomycin (A and B)[9] and tallysomycins[10] rather than phleomycins[6], zorbamycin[7], zorbonomycin C[7], YA-56X[11] and SS-70[12] (ratio of 2.7~3.0).

The negative SAKAGUCHI reaction of SF-1771 further differentiated the B group of bleomycins, victomycin and platomycins, all of which gave positive SAKAGUCHI reaction[13,14]. SF-1771 seemed to be different from any other bleomycin group antibiotics including zorbonomycin B and tallysomycins as judged from various chromatographic systems.

The novelty of SF-1771 was strongly supported by degradation studies of it. Gulose and 3-O-carbamoyl-mannose, common sugar moieties of all bleomycins, were found to be sugar components of SF-1771 by GC-MS analysis of its methanolyzate, however, SF-1771 was found to possess unique amino acid components. It is still not clear whether an amino sugar is present, as found in tallysomycins, or not, however, CMR spectrum of SF-1771 suggested the presence of an extra sugar at 96.0 ppm. TLC (Avicel) coupled with high voltage electrophoresis (pH 1.8) of acid hydrolyzate of SF-1771 revealed the presence of six ninhydrin positive spots, in which four spots were identified as threonine (**I**)*, 4-amino-3-hydroxy-2-methyl-*n*-valeric acid (**III**)*, β-hydroxy-histidine (**IV**)* and β-aminoalanine (**V**)*. The spot corresponding to β-amino-β-(4-amino-6-carboxy-5-methyl-pyrimidin-2-yl)-propionic acid (**II**)* was not detected, however, the compound with lowest mobility on TLC seemed to be demethyl-**II**[15a,b] judging from its ninhydrin-coloration and its Rf value.

Finally, this amino acid was identified as β-amino-β-(4-amino-6-carboxy-pyrimidin-2-yl)-propionic acid (demethyl-**II**) by the direct comparison with an authentic sample[16]. In the ^{1}H-NMR spectrum of SF-1771, no aromatic methyl signal was observed around 3 ppm region and instead, an extra olefin proton assignable to H-5 in demethyl-II was observed in the lowest field region of the spectrum. All bleomycin group antibiotics so far reported possessed **II** in common and demethyl-**II** was only found, in the biosynthetic intermediates of bleomycin isolated from the culture broth of *Streptomyces verticillus*.[15a,b] Therefore, SF-1771 is the first bleomycin-phleomycin group antibiotic possessing demethyl-**II** instead of **II**. ^{1}H-NMR spectrum of SF-1771 also suggested the presence of a bithiazole component, however, 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid (**VI**)* could not be detected in acid hydrolyzate. Since 2′-polyfunctionally substituted bithiazole carboxylic acid (**VI′**) was reported to decompose during acid hydrolysis[18], SF-1771 was considered to contain **VI′** type of compound. It is also interesting that no terminal amine component found in bleomycins was found in SF-1771.

We have also isolated another bleomycin group antibiotic possessing demethyl-**II**, SF-1961, whose detail is given in the following paper[19].

Acknowledgement

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* These designations adopted here for the component in SF-1771 correspond to those in bleomycin group antibiotics[17a,b].

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