PRODUCTION OF ANTIBIOTIC SU-2 COMPLEX BY A 2-DEOXYSTREPTAMINE IDIOTROPH OF *MICROMONOSPORA SAGAMIENSIS*

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A 2-deoxystreptamine idiotrophic mutant of *Micromonospora sagamiensis*, KY 11509, was found to produce unknown antibacterial substances, which were named SU-2 complex. Each component, SU-1, SU-2 and SU-3 were isolated from a culture broth of KY 11509. Chromatographic data suggested that these components were new antibiotics. The antibiotics exhibited potent and broad spectrum of antibacterial activity.

The amount of SU-1, SU-2 and SU-3 production reached their maximum level (197, 82 and 58 μg/liter, respectively) in 3 to 4 days. Addition of cobalt chloride markedly stimulated SU-1 production but suppressed SU-2 and SU-3 production. Isolation of a mutant possessing a higher productivity of SU-2 complex is also described.

Mutants blocked in antibiotic biosynthesis have been widely used not only to study the biosynthetic pathway but also to produce useful new antibiotics or to improve fermentation processes[1]. In 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics, DOS idiotrophic mutants, that are prototrophic but produce the antibiotic of the parents only in the presence of DOS, were derived from various microorganisms, and effectively used for biosynthetic studies[1] and preparation of novel antibiotics by the technique of mutational biosynthesis[2,3]. DOS idiotrophs of *Micromonospora sagamiensis* has been isolated to study sagamicin biosynthesis[4,5] and to prepare a new antibiotic, 2-hydroxysagamicin[6]. In the course of these studies, we found that a DOS idiotroph, KY11509, produced a small amount of new antibiotic complex, each component of which was designated SU-1, SU-2 and SU-3 (previously named SUM-3)[7,8]. In this report, we describe the isolation, biological properties and fermentation of SU-2 complex. The structure elucidation studies will be described in the succeeding paper[9].

Materials and Methods

Microorganisms

A DOS idiotrophic mutant of *M. sagamiensis*, KY11509[10], was used in this experiment.

Media

Seed and fermentation media of the following compositions were used. Seed medium; 2% Stabilose K (soluble starch), 0.5% glucose, 0.5% peptone, 0.5% yeast extract, 0.3% meat extract, and 0.2% calcium carbonate (pH 8.0). Fermentation medium; 4% Stabilose K, 1% soy bean meal, 2% Pharmamedia (cotton seed flour), 0.1% corn oil, 0.5% casein, 0.2% phytate (1 Ca, 2 Mg), 0.015% $FeSO_4 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.025% KH_2PO_4 (pH 8.0).

Culture Condition

Shake flask fermentation: First seed culture were developed in a large test tube (2.5 × 19 cm) containing 10 ml of the seed medium by shaking at 30°C for 3 days. One milliliter of the seed culture was transferred into a large test tube containing the seed medium and incubated at 30°C for 3 days. Five milliliters of the second seed culture was transferred to 50 ml of the fermentation medium in a 300-ml

flask. The flask was incubated with shaking at 220 rpm at 30°C.

Tank fermentation: Seed culture developed in 2-liter flasks for 2 days were transferred to 13.5 liters of the seed medium in 30-liter jar fermentor. After stirring at 34°C at 300 rpm with a continuous air flow of 15 liters/minute for 16 hours, the inoculum was transferred to 135 liters of the fermentation medium in a 300-liter tank and the fermentation was conducted at 34°C by stirring at 230 rpm with aeration of 150 liters/minute.

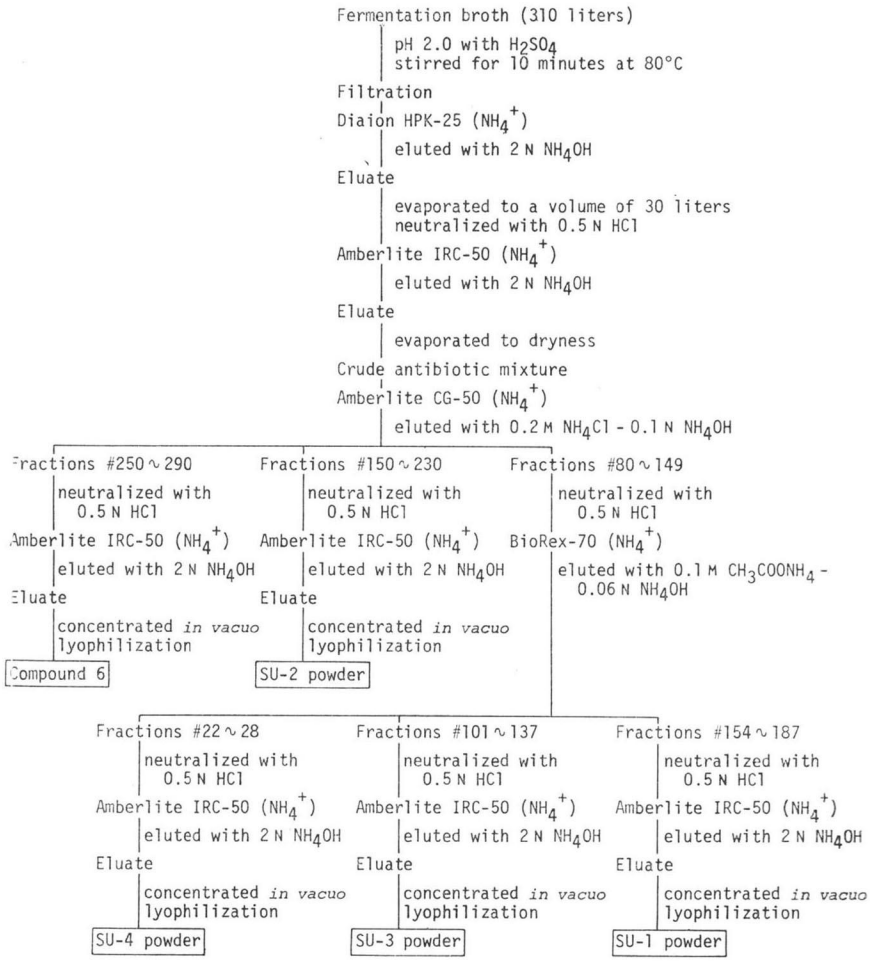
Mutation

Mutation and selection of the mutants with increased biotransformation activities from DOS into sagamicin were described in a previous paper[6].

Detection of Antibiotics

The fermentation broth (120 ml) was acidified to pH 2.0 with oxalic acid and stirred at 30°C for 1 hour. After centrifugation, the supernatant was neutralized with aqueous ammonia, and applied to 15 ml of Amberlite IRC-50 ion-exchange resin (NH_4^+ form) column. The resin was washed with 45 ml of distilled water, eluted with 105 ml of 0.2 M ammonium chloride - 0.1 N ammonium hydroxide solution, and washed again with 45 ml of distilled water. SU-2 complex was then eluted with 45 ml of 2 N ammonium hydroxide, and the eluate was evaporated to dryness to obtain the crude antibiotic mixture. The crude material was dissolved in 0.1 ml distilled water. The antibiotic was detected by bioauto-

Fig. 1. Isolation of SU-2 components.



graphy against *Bacillus subtilis* ATCC 6633 after silica gel thin-layer chromatography (TLC) or paper chromatography. The amount of the antibiotics was determined by *in-situ* fluorometric measurements of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole derivatives of the antibiotics formed after silica gel TLC[11].

Isolation of the SU-2 Complex

The procedure for the isolation of the individual antibiotics is shown schematically in Fig. 1. Tank fermentation broths (310 liters) were acidified to pH 2.0 with sulfuric acid and stirred for 10 minutes at 80°C. After filtration, the filtrate was applied to Diaion HPK-25 resin (NH_4^+) column (10 liters) and eluted with 60 liters of 2 N ammonium hydroxide. The eluate was evaporated under reduced pressure to a volume of 30 liters, neutralized with 0.5 N hydrochloric acid and then applied to Amberlite IRC-50 resin (NH_4^+) column (4 liters). The resin was washed with distilled water and eluted with 12 liters of 2 N ammonium hydroxide. The eluate was evaporated to dryness to obtain the crude antibiotic mixture. The crude material was further chromatographed on Amberlite CG-50 resin (NH_4^+) column (200 ml). Elution was performed with 0.2 M ammonium chloride - 0.1 N ammonium hydroxide solution. Two hundred and ninety fractions (20 ml each) were collected. Fractions containing SU-2 (Fractions 150~230) and compound **6** (Fractions 250~290) were each combined, neutralized with 0.5 N hydrochloric acid, and applied to Amberlite IRC-50 resin (NH_4^+) column (50 ml). Each resin was washed with distilled water and eluted with 150 ml of 2 N ammonium hydroxide. The eluate was each evaporated under reduced pressure to a small volume and lyophilized to yield pure SU-2 base (44 mg) and compound **6** (1 mg). Fractions containing SU-1 and SU-3 (Fractions 80~149) were combined, neutralized with 0.5 N hydrochloric acid, and chromatographed on a 10-ml BioRex-70 (NH_4^+) column. The column was eluted with 0.1 M ammonium acetate - 0.06 N ammonium hydroxide solution. Two hundred fractions (5 ml each) were collected. Fractions containing SU-1 (Fractions 154~187) and SU-3 (Fractions 101~137) were each combined, desalted with Amberlite IRC-50 (NH_4^+) column and lyophilized to yield pure white powders of SU-1 (10 mg) and SU-3 (15 mg). Fractions 22~28, which contained an antibiotic designated SU-4, were combined and desalted to yield pure white powder of SU-4 (1 mg).

Results

Isolation of SU-2 Complex

As shown in Fig. 2, 6 compounds with antibacterial activity were detected in a culture broth of KY 11509. Five were isolated from 310 liters of tank fermentation broth as described in Materials and Methods. Comparative Rf values of SU-2 components and other aminoglycoside antibiotics on paper and silica gel thin-layer chromatography are shown in Table 1. SU-1, SU-2 and SU-3 was differentiated from gentamicin C, sagamicin, fortimicin, sisomicin group antibiotics, or other aminoglycoside antibiotics, suggesting that each SU-2 component was a new antibiotic. A minor component SU-4 was also assumed to be a new antibiotic from the chromatographic behavior. Compound **6** was identical to sagamicin in the chromatographic behavior, and mass and proton NMR spectra[12]. Compound **5** has not been isolated yet.

Antibacterial Activities of SU-2 Components

The antibacterial spectrum of each component is shown in Table 2. Each component showed a potent antibacterial activity against a broad range of Gram-positive and Gram-negative bacteria, although the potency was slightly lower than that of sagamicin. SU-3 was found to be active against some sagamicin- and gentamicin-resistant strains, *i.e. Pseudomonas aeruginosa* KY8565 and *Escherichia coli* KY8563 which possess aminoglycoside 3-*N*-acetyltransferase III and II [AAC (3)-III and -II], respectively.

Table 1. Comparative Rf value of SU-2 components and other aminoglycoside antibiotics on paper- and thin-layer chromatography

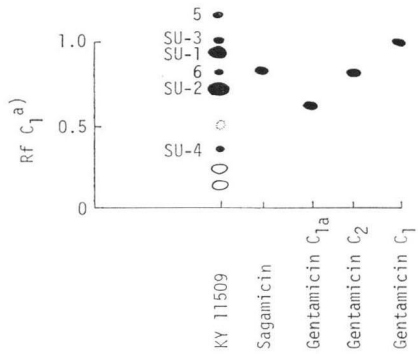
Antibiotics	(1) Rf	(2) Rf C_1[a)]
SU-1	0.45	0.92
SU-2	0.18	0.75
SU-3	0.51	1.04
SU-4	0.05	0.37
Sagamicin	0.49	0.86
Antibiotic G-52	0.49	0.79
Gentamicin C_{1a}	0.18	0.63
Sisomicin	0.18	0.67
Gentamicin C_2	0.38	0.79
Verdamicin	0.38	0.86
Gentamicin C_1	0.59	1.00
Fortimicin A	0.37	
Fortimicin B	0.65	
Other aminoglycosides[b)]	0.00~0.10	

(1) Paper chromatography; chloroform - methanol - 17% NH_4OH (2: 1: 1), lower phase.
(2) Thin-layer chromatography; chloroform - methanol - conc. NH_4OH (1: 1: 1), lower phase.
a) $\frac{\text{distance of component from origin}}{\text{distance of gentamicin } C_1 \text{ from origin}}$
b) Other aminoglycosides tested are streptomycins A and B, bluensomycin, ribostamycin, lividomycins A, B and D, hygromycin B, kasugamycin, butirocins A and B, gentamicins A, B and B_1, neomycins A, B and C, antibiotic No. 460, kanamycins A, B and C, paromomycin, nebramycin complex, tobramycin, apramycin, myomycin, seldomycin factors 1, 2, 3 and 5.

Fig. 2. Silica gel thin-layer chromatogram of the products in a culture broth of *Micromonospora sagamiensis* KY 11509.

M. sagamiensis KY 11509 was cultivated in 300-ml flasks containing 50 ml of the fermentation medium at 30°C for 3 days.

TLC solvent; chloroform - methanol - conc. NH_4OH (1: 1: 1), lower phase.

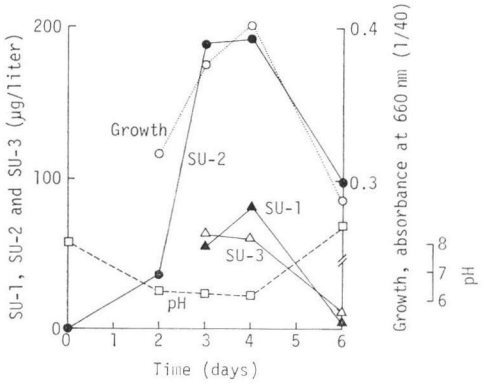


a) $\frac{\text{distance of component from origin}}{\text{distance of gentamicin } C_1 \text{ from origin}}$

●: compounds with antibacterial activities
○: compounds positive to RYDON-SMITH without antibacterial activities

Fig. 3. Time course of SU-2 fermentation.

M. sagamiensis KY 11509 was cultivated in 300-ml flasks containing 50 ml of the fermentation medium at 30°C.



Production of SU-2 Complex in the Flask Fermentation

The production of SU-2 complex was examined in 300-ml flask. The time course of the fermentation is shown in Fig. 3. The amounts of each component reached their maximum level (SU-2, 197 μg/liter; SU-1, 82 μg/liter; SU-3, 58 μg/liter) in 3 to 4 days, and then rapidly decreased, accompanying lysis of cells and rise of pH.

Addition of cobalt chloride significantly affected the production of each component (Table 3). In the absence of cobalt ion, 190 μg/liter of SU-2 was produced and the amounts of SU-1 and SU-3 production were each approximately one-forth that of SU-2. Addition of cobalt chloride (500 μg/liter) stimulated SU-1 production by approximately 3.5 times (160.5 μg/liter), whereas SU-2 and SU-3 production were suppressed to a level of 88.0 and 20.7 μg/liter, respectively. The effects of cobalt ion on SU-2 components production resembled to those on sagamicin and gentamicin C production[13]. The results

Table 2. Antibacterial activity of SU-2 components.

Test organisms	Minimum inhibitory concentration			
	SU-1	SU-2	SU-3	SU-4
Staphylococcus aureus 209P	2.5	0.62	0.62	1.25
S. epidermidis	0.62	0.31	0.31	1.25
Streptococcus faecalis ATCC 10541	>10	12.5	12.5	10
Bacillus subtilis ATCC 6633	0.31	0.31	0.31	0.62
Escherichia coli NIHJ JC-2	0.62	0.62	0.62	2.5
Klebsiella pneumoniae 8045	0.62	0.31	0.31	1.25
Pseudomonas aeruginosa #1	5.0	2.5	2.5	2.5
Serratia marcescens T-55	1.25	5.0	1.25	5.0
Proteus mirabilis 1287	1.25	0.62	1.25	2.5
E. coli KY 8348 [AAC(3)-I]	>10	5.0	3.12	2.5
E. coli KY 8563 [AAC(3)-II]	>10	10	5.0	>10
P. aeruginosa KY 8565 [AAC(3)-III]	>10	10	12.5	>10
E. coli KY 8356 [ANT(2'')]	>10	>10	3.12	>10
S. aureus KY 8970 [ANT(4')]	2.5	0.31	0.31	2.5

Table 3. Effect of cobalt chloride on the production of SU-2 components.

$CoCl_2$ μg/liter	pH	Growth[a)]	SU-1 (μg/liter)	SU-2 (μg/liter)	SU-3 (μg/liter)
—	6.1	0.38	45.9	190.0	50.0
500	6.2	0.36	160.5	88.0	20.7

KY 11509 was cultivated in 50 ml of the fermentation medium in the presence or absence of cobalt chloride in 300-ml flask at 30°C for 3 days.

a) growth: absorbance at 660 nm of the fermentation broth diluted to 1/40.

Table 4. SU-2 and SU-3 productivity of mutant G-660 and KY 11509.

	SU-2 (μg/liter)	SU-3 (μg/liter)
KY 11509	232	44
G-660	258	211

Fermentation was carried out in 300-ml flask containing 50 ml of the fermentation medium at 30°C for 3 days.

suggest that the SU-2 complex comprises aminoglycosides closely related to sagamicin and gentamicin.

A Mutant with Higher Productivity of SU-2 Complex

Mutant G-660 which possesses a high biotransformation activity from DOS into sagamicin was derived from KY11509 after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. The mutant was found to produce higher yields of SU-2 complex than KY11509 (Table 4). The total amount of SU-2 complex in G-660 was 1.6 fold that in KY 11509. In particular, SU-3 production in G-660 increased approximately 5 fold that in KY 11509.

Discussion

KY 11509, which was isolated as a DOS idiotrophic mutant, produced antibiotic SU-2 complex in the medium without supplemental DOS. The amount of antibiotic production was too small to detect under standard assay conditions. Many DOS idiotrophs have been reported to be isolated from various aminoglycoside antibiotic producing strains[2,3]. The authors also isolated several DOS idiotrophic mutants other than KY 11509 from *M. sagamiensis*. It may be worth examining whether these DOS produce idiotrophs new types of antibiotics. In fact, FUJIWARA *et al.*[14] recently reported that a new antibiotic S-11-A was produced by a DOS idiotroph of a xylostasin producing strain of *Bacillus circulans*.

S-11-A was identified as 1-deamino-1-hydroxyxylostasin.

Addition of cobalt chloride stimulated SU-1 production but suppressed SU-2 and SU-3 production. Cobalt ion significantly affected the production of sagamicin, gentamicin C_{1a}, C_2 and C_1 in *M. sagamiensis*: in the presence of cobalt ion, production of sagamicin and gentamicin C_{1a} was suppressed and that of gentamicin C_1 and C_2 was stimulated[13]. The *C*-methylation at the 6′-position and the 4″-position in sagamicin biosynthesis was dependent on cobalt ion[15]. It appeared that the stimulation of the *C*-methylation steps by cobalt ion caused the increase of gentamicin C_1 and C_2 production, resulting in the decrease of sagamicin and gentamicin C_{1a} production. Thus, it is suggested that *C*-methylation steps are involved in SU-2 complex synthesis in KY 11509. The biosynthetic mechanism of SU-2 complex production will be described in a paper of this series[16].

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