MICROBIAL CONVERSION OF SAFRAMYCIN A TO 25-DIHYDROSAFRAMYCIN A AND 21-DECYANO-25-DIHYDROSAFRAMYCIN A (25-DIHYDROSAFRAMYCIN B) AND THEIR BIOLOGICAL ACTIVITIES

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25-Dihydrosaframycin A(AR_1) and 21-decyano-25-dihydrosaframycin A(AR_3) were produced by the microbial conversion of saframycin A(SA). Efficient conversion of SA to AR_1 and AR_3 was observed with *Rhodococcus amidophilus* IFM 144. Though the antimicrobial activity of AR_1 was one tenth that of SA, the *in vitro* antitumor activity of AR_1 was found to be equivalent to that of SA. In contrast, AR_3 was biologically less active.

Microbial conversion has been studied extensively[1~3] for many classes of organic compounds, namely alkaloids, antibiotics and steroids.

For antitumor antibiotics, considerable attention has been given to the microbial reduction of carbonyl groups in relation to antitumor activities.[4~8]

The antitumor activity of a novel antibiotic, saframycin A, with a unique structure consisting of a dimer of 6-methyl-7-methoxy-1,2,3,4-tetrahydroisoquinoline-5,8-dione, as the basic skeleton, has been reported.[9~12]

In our studies on the microbial conversion of SA into antibiotics possessing antitumor spectra different from those of the parent compound (SA), reduction of the side chain carbonyl group of SA was observed.

In this paper, we report our studies on the microbial conversion of SA to AR_1 and AR_3 and their biological properties.

Materials and Methods

Microorganisms and Cultivation

Rhodococcus amidophilus IFM 144 was maintained on slants of 1% glycerol nutrient medium until used. The strain was grown in 500-ml Erlenmeyer flasks, each containing 100 ml of a medium consisting of: meat extract 1%, peptone 1%, yeast extract 1%, NaCl 0.3% (pH 7.2). After 48 hours incubation at 27°C on a rotary shaker at 250 rpm, the culture was centrifuged, washed and suspended in a small volume (23 ml) of 0.1 M phosphate buffer (pH 7.5).

Microbial Conversion of Saframyicn A(SA)

Two ml of the suspension was mixed with 2 ml of the 10% aqueous methanol solution of SA (0.5 mg/ml) in a test tube and the mixture incubated at 37°C for 18 hours. The reaction mixture was centrifuged at 500 rpm, and the supernatant extracted with ethyl acetate. The ethyl acetate layer was concentrated *in vacuo*, and the residue extracted with 1 N HCl solution. After adjustment to pH 7.5 with conc.

NH_4OH, the extract was re-extracted with ethyl acetate and the solvent layer concentrated to dryness. The residue was further purified by silica gel TLC (GFC_{254}, Merck) using chloroform - acetone (1:1, v/v) as solvent. The bioconversion products on TLC plates were monitored using a dual wave-length chromatogram scanner (Shimadzu CS-910) at 270 nm.

Preparative TLC plates developed with the solvent system described above were used to obtain a sample for physicochemical analysis.

Chemical Reduction of Saframycin A (SA) and Saframycin B (SB)

One hundred mg of SA or SB was dissolved in 10 ml of 10% aqueous methanol solution, and to the solution was slowly added 10 mg of $NaBH_4$. After stirring at room temperature for 10 minutes, the reaction mixture was extracted with ethyl acetate. The solvent layer was washed with distilled water and concentrated *in vacuo*. The residue was further purified by preparative TLC using ethyl acetate - benzene (2:1, v/v) as a solvent system.

Antimicrobial Activity of AR_1 and AR_3

Twenty two bacteria and eight fungi maintained in our laboratory were used in this experiment. Minimum inhibitory concentration (MIC) was determined by the agar streak method using 0.5% nutrient agar for bacteria and Sabouraud dextrose agar for fungi. The results were observed after incubation at 37°C for 48 to 72 hours.

In vitro Cytotoxicity Determination

In vitro antitumor activity was determined using a cultured cell line of L1210 mouse leukemia in suspension as described by ARAI *et al.*[12] ED_{50} was determined by the method of ISHIWATA.[13]

Spectroscopic Measurements

Melting points were determined on a Yamato melting point apparatus (Model MP-21, Yamato Kagaku, Japan) and are uncorrected. Optical rotations were measured on a DPI-4 polarimeter (Jasco, Japan). Ultraviolet absorption spectra (UV) were determined on a Hitachi 323 spectrophotometer (Hitachi, Japan) and infrared absorption spectra (IR) were taken on an IRA-2 spectrometer (Jasco, Japan). NMR spectra were determined on a JNM-PFT-100 (JEOL, Japan) with tetramethylsilane (TMS) as a internal standard. Mass spectra were determined on a JSM-01SG-2 double focusing mass spectrometer (JEOL, Japan) using field desorption method.

Results and Discussion

As shown in Fig. 1, two major and one minor peaks, AR_1, AR_2 and AR_3 were detected on TLC chromatography after conversion by *R. amidophilus* IFM 144. The Rf values of AR_1, AR_2 and AR_3 were 0.48, 0.35 and 0.28 respectively (Table 1). The microbial conversion of SA to AR_1, AR_2 and AR_3 was observed with species of *Nocardia, Rhodococcus* and *Mycobacterium*, but not with the 200 ISP (International Streptomyces Project)[14] *Streptomyces* strains tested (unpublished data by YAZAWA,

Table 1. Rf values of saframycins on TLC.

	Saframycin component					
	A	B	AR_1 (AH_2)	AR_3 (BH_1)	AH_1	BH_2
1*	0.61	0.40	0.48	0.28	0.54	0.29
2	0.50	0.14	0.21	0.06	0.29	0.06
3	—	—	0.29	0.07	0.35	0.10
4	—	—	—	0.17	—	0.20

* Solvent system and carrier: 1) acetone - $CHCl_3$, 1:1 (v/v), silica gel (Merck), 2) benzene - ethyl acetate, 1:2 (v/v), silica gel (Merck), 3) ethyl acetate, silica gel (Merck), 4) acetone - $CHCl_3$, 1:1 (v/v), alumina (Merck).

Fig. 1. Microbial conversion pattern of saframycin A by *R. amidophilus* (A: saframycin A, AR_1: saframycin AR_1, AR_2: saframycin AR_2, AR_3: saframycin AR_3).

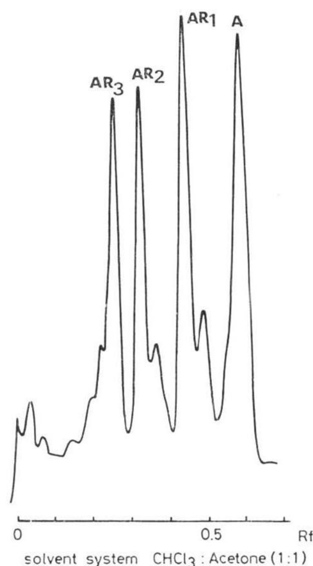
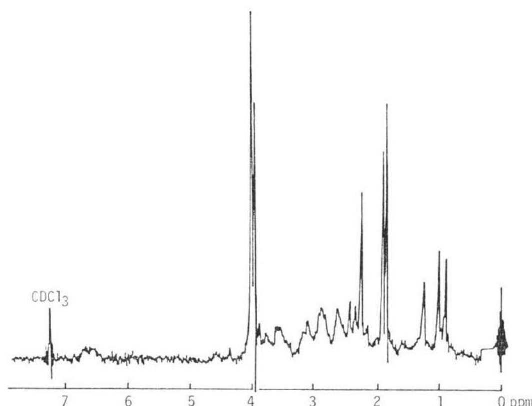


Fig. 2. ^1H NMR spectrum of saframycin AR_1 (100 Mz, $CDCl_3$, 25°C).



K.; K. TAKAHASHI, T. ASAOKA, Y. MIKAMI and T. ARAI).

Conversion products AR_2 and AR_3 were obtained from the basic fraction of the reaction mixture, whereas conversion product AR_1 was obtained from the neutral fraction. When 100 mg of SA was used, the yields of AR_1, AR_2 and AR_3 were 48, 8, and 21 mg, respectively. AR_2 has not been characterized yet because of insufficient material. The physical constants of AR_1 and AR_3 are as follows.

AR_1: yellow powder; mp 142~145°C; $[\alpha]_D^{25}$ +21.4° (MeOH); molecular formula $C_{29}H_{32}N_4O_8 \cdot \frac{1}{3}H_2O$: Calcd.; C 61.04, H 5.77, N 9.82, Found; C 61.34, H 5.77, N 9.37; mass spectrum: *m/z* 564 (M^+); UV λ_{max}^{MeOH} nm (log ε) 268 (4.01); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3430, 1660, 1620; ^1H NMR spectrum (100 MHz, $CDCl_3$, 25°C) δ 0.92 (3H, d, *J*=6Hz, $-CO-CH-CH_3$), 1.86 (3H, s, Ar-CH_3), 1.90 (3H, s, Ar-CH_3), 2.28 (3H, s, N-CH_3), 3.94 (3H, s, Ar-OCH_3), 4.00 (3H, s, Ar-OCH_3) due to six methyl groups (Fig. 2). The ^{13}C NMR spectrum of AR_1 is shown in Table 2.

AR_3: yellow powder; mp 123~126°C; $[\alpha]_D^{25}$ −76.7° (MeOH); molecular formula $C_{28}H_{33}N_3O_8 \cdot \frac{1}{2}H_2O$: Calcd.; C 61.30, H 6.25, N 7.64, Found; C 61.27, H 6.32, N 7.47; mass spectrum: *m/z* 539 (M^+); UV λ_{max}^{MeOH} nm (log ε) 269 (4.12); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3400, 1660, 1620; ^1H NMR spectrum (100 MHz, $CDCl_3$, 25°C) δ 0.95 (3H, d, *J*=6 Hz, $CO-CH-CH_3$), 1.88 (3H, s, Ar-CH_3), 1.92 (3H, s, Ar-CH_3), 2.29 (3H, s, N-CH_3), 4.00 (3H, s, Ar-OCH_3), 4.02 (3H, s, Ar-OCH_3) due to six methyl groups (Fig. 3). The ^{13}C NMR spectrum of AR_3 is shown in Table 2.

The fact that the UV and IR spectra of AR_1 and AR_3 were very similar to those of SA suggested that AR_1 and AR_3 have the basic skeleton of SA. In the ^1H NMR spectra, a new doublet was observed at δ 0.92 (*J*=6 Hz) in AR_1, and at δ 0.95 (*J*=6 Hz) in AR_3. The doublet could be attributed to a methyl proton of $-CH(OH)-CH_3$[15]. This partial structure was further supported by the ^{13}C NMR spectra of AR_1 and AR_3. The signal at δ 196.7 (s, CO) in SA disappeared in AR_1 and AR_3, while a new signal due to the carbon attached to hydroxyl group[16] appeared at δ 68.1 (d) in AR_1 and δ 68.2 (d) in AR_3. Consequently, the presence of a $-CH(OH)-CH_3$ group in AR_1 and AR_3 clearly indicated that the C-25 ketone of the pyruvoylamine group of SA was reduced to a carbinol[17]. Furthermore, in the spectra of

Table 2. ^{13}C NMR spectra of saframycins.

Carbon number*	Saframycin component				
	A	B	AR_1 (AH_2)	AH_1	AR_3 (BH_1)
-*CO*-CH_3	196.7 (s)	196.5 (s)	—	—	—
5 or 15	186.5 (s)	187.0 (s)	186.6 (s)	186.6 (s)	187.1 (s)
	185.2 (s)	185.7 (s)	185.6 (s)	185.7 (s)	186.0 (s)
8 or 18	183.4 (s)	182.8 (s)	182.5 (s)	182.5 (s)	182.7 (s)
	180.8 (s)	181.3 (s)	180.9 (s)	180.9 (s)	181.4 (s)
NH-CO-	160.2 (s)	160.1 (s)	174.5 (s)	174.4 (s)	174.6 (s)
7 or 17	155.9 (s)	156.1 (s)	156.4 (s)	156.2 (s)	156.8 (s)
	155.6 (s)	155.5 (s)	155.6 (s)	155.3 (s)	155.7 (s)
10 or 20	141.6 (s)	142.8 (s)	141.7 (s)	141.8 (s)	142.8 (s)
	141.2 (s)	141.6 (s)	140.3 (s)	140.7 (s)	140.6 (s)
9 or 19	135.6 (s)	136.6 (s)	136.4 (s)	136.3 (s)	137.4 (s)
	135.6 (s)	136.3 (s)	135.9 (s)	135.7 (s)	136.8 (s)
6 or 16	129.2 (s)	129.7 (s)	128.8 (s)	129.0 (s)	128.6 (s)
	128.3 (s)	127.7 (s)	127.6 (s)	127.7 (s)	126.9 (s)
-CN	116.7 (s)	—	117.0 (s)	116.9 (s)	—
-*CH*(OH)-CH_3	—	—	68.1 (d)	68.4 (d)	68.2 (d)
7 or 17-OCH_3	61.1 (q)	60.9 (q)	61.1 (q)	61.2 (q)	60.9 (q)
	61.0 (q)	60.9 (q)	61.1 (q)	61.2 (q)	60.9 (q)
21	58.3 (d)	58.7 (t)	58.2 (d)	58.5 (d)	58.6 (t)
1, 3, 11 or 13	56.3 (d)	57.4 (d)	56.4 (d)	56.9 (d)	58.2 (d)
	54.6 (d)	56.9 (d)	54.6 (d)	54.6 (d)	56.8 (d)
	54.3 (d)	54.8 (d)	54.3 (d)	54.5 (d)	55.0 (d)
	54.4 (d)	52.2 (d)	53.8 (d)	54.3 (d)	52.3 (d)
N-CH_3	41.6 (q)	41.2 (q)	41.7 (q)	41.7 (q)	41.3 (q)
22	40.7 (t)	40.4 (t)	40.1 (t)	40.1 (t)	40.7 (t)
4	25.6 (t)	25.6 (t)	25.5 (t)	25.3 (t)	26.1 (t)
-CO-*CH_3*	24.3 (q)	24.2 (q)	—	—	—
-CH(OH)-*CH_3*	—	—	21.6 (q)	21.7 (q)	22.8 (q)
14	21.6 (t)	22.7 (t)	21.0 (t)	21.3 (t)	21.0 (t)
6 or 16-CH_3	8.7 (q)	8.7 (q)	8.8 (q)	8.9 (q)	8.5 (q)
	8.7 (q)	8.7 (q)	8.8 (q)	8.9 (q)	8.5 (q)

(s): singlet, (d): doublet, (t): triplet, (q): quartet. Value in ppm from TMS.
* See Reference 11.

AR_3, the signal at δ 116.7 (s, CN) of SA due to the cyano group disappeared, and a new signal at δ 58.6 (t, CH_2) was observed. These spectral data indicated that AR_3 should have the same carbon skeleton as saframycin B (SB). Moreover, the molecular formulas and mass spectra of AR_1 and AR_3 supported the structure containing one more hydroxyl group than SA and SB, respectively.[10,11] Therefore, the structures of AR_1 and AR_3 were determined as 25-dihydrosaframycin A and 21-decyano-25-dihydro-saframycin A (25-dihydrosaframycin B), respectively (Fig. 4).

The chemical reduction of SA with $NaBH_4$ gave two products, saframycin AH_1(AH_1) and saframycin AH_2(AH_2) in a 1: 1 ratio. The physical constants of AH_1 and AH_2 are as follows:

AH_1: yellow powder; mp 164~167°C; $[\alpha]_D^{25}$ −1.8° (MeOH); UV λ_{max}^{MeOH} nm(log ε) 268 (4.11); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3440, 1660, 1620; ^{1}H NMR spectrum (100 MHz, $CDCl_3$, 25°C) δ 1.18 (3H, d, J=6 Hz),

Fig. 3. ^{1}H NMR spectrum of saframycin AR_3 (100 Mz, $CDCl_3$, 25°C).

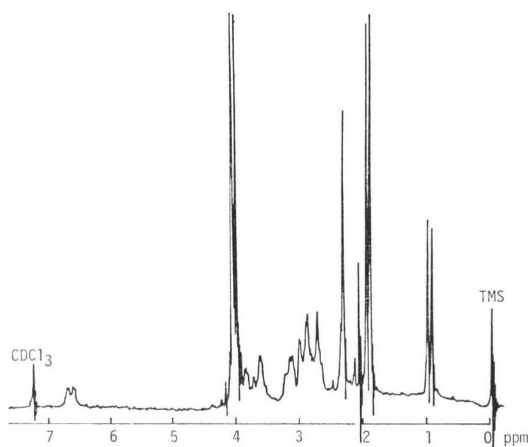


Fig. 4. Structures of saframycin A, B, AR_1 and AR_3.

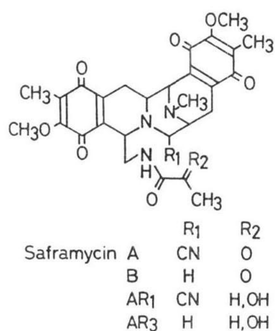


Table 3. Antimicrobial spectra of saframycin AR_1 and AR_3.

Test organism	AR_1	AR_3
Staphylococcus aureus FDA 209P	1*	50
Staphylococcus aureus Smith	1	50
Staphylococcus albus	1	50
Streptococcus faecalis	10	50
Streptococcus salivarius	10	50
Micrococcus flavus	1	50
Bacillus subtilis PCI 219	1	10
Bacillus cereus	N.D.	10
Corynebacterium diphtheriae	0.01	1
Corynebacterium xerosis	10	50
Mycobacterium sp. 607	>50	>100
Mycobacterium avium	>50	>100
Nocardia asteroides	>50	50
Escherichia coli F_1	>50	>100
Salmonella typhimurium	1	50
Shigella dysenteriae	25	>100
Klebsiella pneumoniae	5	10
Brucella abortus	5	10
Serratia marcescens	>50	>100
Pseudomonas aeruginosa	>50	>100
Mucor mucedo	>50	>100
Saccharomyces cerevisiae	>50	>100
Rhodotorulla glutinis	>50	>100
Aspergillus niger	>50	>100
Aspergillus oryzae	>50	>100
Penicillium glaucum	>50	>100
Trichophyton mentagrophytes	>50	>100
Candida albicans 7N	>50	>100

* MIC, μg/ml, N.D., Not determined.
Nutrient agar (0.5%) for bacteria, and Sabouraud glucose (2%) agar for fungi were used.

1.86 (3H, s), 1.90 (3H, s), 2.30 (3H, s), 3.94 (3H, s), 4.00 (3H, s); mass spectrum: m/z 564 (M^+).

AH_2: yellow powder; mp 142~144°C; $[\alpha]_D^{25}$ +20.2° (MeOH); UV λ_{max}^{MeOH} nm (log ε) 268 (4.10); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3440, 1660, 1620; ^{1}H NMR spectrum (100 MHz, $CDCl_3$, 25°C); δ 0.96 (3H, d, J=6 Hz), 1.85 (3H, s), 1.90 (3H, s), 2.26 (3H, s), 3.95 (3H, s), 4.00 (3H, s); mass spectrum m/z 564 (M^+). The ^{13}C NMR spectra of AH_1 and AH_2 are shown in Table 2. These physicochemical data of AH_1 and AH_2 indicated that these two compounds are stereoisomers at C-25; furthermore AH_2 was identified as AR_1 by TLC, mass, ^{1}H NMR, ^{13}C NMR and IR spectra.

On the other hand, the chemical reduction of SB with $NaBH_4$ under the conditions described above gave two products, saframycin $BH_1(BH_1)$ and saframycin $BH_2(BH_2)$ in a 10: 1 ratio. The physical constants of BH_1 are as follows: yellow powder; mp 124~127°C; $[\alpha]_D^{25}$ −74.2° (MeOH); UV λ_{max}^{MeOH} nm (log ε) 269 (4.12); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3400, 1660, 1620; ^{1}H NMR spectrum (100 MHz, $CDCl_3$, 25°C): δ 0.95 (3H, d, J=6 Hz), 1.88 (3H, s), 1.92 (3H, s), 2.29 (3H, s), 4.00 (3H, s), 4.02 (3H, s); mass spectrum 539 (M^+). The physical data of BH_1 and the ^{13}C NMR data (Table 2) showed that the reduction occurred at the same site as that observed in the conversion of SA into AH_1 and AH_2. BH_2 could not be

purified further due to the lack of sufficient material.

Moreover, BH_1 was found identical with AR_3 by comparison of TLC, ^1H NMR, ^{13}C NMR and IR spectra. These results further confirmed the structure of AR_3 as 25-dihydrosaframycin B(21-decyano-25-dihydrosaframycin A). BH_2 is assumed to be a stereoisomer of BH_1. The absolute configuration of the newly formed asymmetric carbon at the C-25 site remains to be determined.

As shown in Table 3, AR_1 showed higher activity against Gram-positive bacteria than against Gram-negative bacteria, and no activity against fungi. *Corynebacterium diphtheriae* was most sensitive to AR_1, and completely inhibited by 0.01 μg/ml. Compared to the parent compound (SA), AR_1 was 5 to 10 times less active against microorganisms. AR_3 proved to be 10 to 50 times less active than AR_1.

The ED_{50} of SA, AR_1 and AR_3 against L1210 mouse leukemia cell line was 0.003 μg/ml, 0.004 μg/ml, and 0.35 μg/ml, respectively. In our preliminary study of AR_1 and AR_3 against L1210 mouse leukemia *in vivo*, ILS (increase in life span) values of 156.8% and 143.2% were obtained for daily doses of 1.5 mg/kg and 1.0 mg/kg of AR_1. These values are comparable to those for SA. On the other hand, for a daily dose of 5.0 mg/kg, AR_3 did not show any antitumor activity.

Therefore, the reduction of the carbonyl group in the side chain of SA resulted in a marked loss of antimicrobial activity, and a negligible loss of antitumor activity. Reduction of the C-21 carbon on the basic skeleton of SA, at the attachment site of the cyano group, which leads to the release of cyanide, resulted in almost complete loss of biological activity. This also confirms the importance of the cyano group for the manifestation of the biological activity of SA, as described previously.[18]

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