PRODUCTION OF DEOXYFRENOLICIN AND A NEW ANTIBIOTIC, FRENOLICIN B BY *STREPTOMYCES ROSEOFULVUS* STRAIN AM-3867

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Two antibiotics of frenolicin group, antibiotic AM-3867 I and II were isolated from the fermentation broth of *Streptomyces roseofulvus* strain No. AM-3867, a soil isolate. The former was a new antibiotic designated as frenolicin B and its structure containing γ-lactone was determined, while the latter was identified as deoxyfrenolicin having been chemically prepared from frenolicin.

In the previous screening for new antimycoplasmic antibiotics, we discovered quinone antibiotics, nanaomycins A, B, C, D obtained from the culture broth of *Streptomyces rosa* var. *notoensis* and clarified these structures and antimicrobial activities *in vitro*[1~5]. In the subsequent screening program, *Streptomyces roseofulvus* strain AM-3867 isolated from a soil sample collected at Chino City, Nagano Prefecture, Japan was found to produce two quinone antibiotics, antibiotic AM-3867 I and II. It was later shown that antibiotic AM-3867 I was a new antibiotic, while antibiotic AM-3867 II was identified as deoxyfrenolicin[6], an antibiotic previously prepared chemically from frenolicin produced by *Streptomyces fradiae*[7].

The present paper deals with the taxonomy of the producing strain, as well as the production, isolation, structures and antimicrobial activities *in vitro* of two antibiotics.

Taxonomic studies

Morphological characteristics: Morphological characteristics of strain No. AM-3867 were observed on cultures incubated at 27°C for 14 days on various media, such as oatmeal agar and glycerol-asparagine agar. Microscopic observations of morphological characteristics were made with both optical and electron microscopes. Well-branched substrate mycelia were formed on most agar media. The aerial mycelia were irregularly abundantly branched, and the chains of spores were Rectus-Flexibilis and had ten or more spores. Conidia were oval or cylindrical ($0.5 \sim 0.6\,\mu \times 0.7 \sim 1.0\,\mu$) with smooth surface. Sclerotia, sporangia and flagellated spores were not observed.

Cultural characteristics, physiological properties and utilization of carbon sources: The color terms recorded for each culture were described according to Color Harmony Manual (Container Corporation of America). The cultural characteristics are shown in Table 1. The physiological properties and utilization of carbon sources of strain No. AM-3867 are summarized in Tables 2 and 3, respectively.

Cell wall analysis: LL-Diaminopimelic acid was found as a component of the cell wall of strain

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No. AM-3867.

The strain No. AM-3867 exhibits the following properties: Cell wall analysis, LL-diaminopimelic acid; spore chains, Rectus-Flexibilis with smooth surface; color of substrate mycelium, colorless to yellowish brown; color of aerial mycelium, brownish white to pale orange; melanoid pigment not observed.

From these data, it was concluded that strain No. AM-3867 belongs to the genus *Streptomyces* and is similar to *Streptomyces roseofulvus*[8]. Culture characteristics of *S. roseofulvus* ISP 5172 were then compared with those of strain No. AM-3867. Both strains exhibited somewhat different behavior on peptonization of milk, utilization of carbon sources (L-arabinose, sucrose, raffinose) and cultural characteristics of glycerol-asparagine agar. However, morphological and cultural characteristics and physiological properties of both strains closely resembled each other. On the basis of these results, it should be reasonable to conclude that strain No. AM-3867 is identified as a strain of *Streptomyces*

Table 1. Cultural characteristics of *Streptomyces* sp. AM-3867

Sucrose-nitrate agar	G: thin, colorless R: pearl pink (3ca) AM: moderate, velvety, pearl pink (3ca) SP: —	Tyrosine agar (ISP)	G: thin, colorless R: pearl pink (3ca) AM: poor, velvety, pearl pink (3ca) SP: ±
Glucose-nitrate agar	G: moderate, colorless R: pearl pink (3ca) AM: poor, velvety, pearl pink (3ca) SP: —	Nutrient agar	G: moderate, light maize (2ea) R: light maize (2ea) AM: moderate, velvety, white (a) SP: light maize (2ea)
Glycerol-calcium malate agar	G: moderate, pearl pink (3ca) R: pearl pink (3ca) AM: moderate, velvety, flesh pink (4ca) SP: flesh pink (5ca)	Glucose-peptone agar	G: moderate, wrinkled, light rose beige (4ec) R: rose beige (4gc) AM: moderate, velvety, light ivory (2ca) SP: light amber (3ic)
Glucose-asparagine agar (ISP)	G: thin, maple sugar (3ie) R: yellow maple (3ng) AM: moderate, velvety, flesh pink (4ca) SP: —	Yeast extract-malt extract agar (ISP)	G: moderate, maple sugar (3ie) R: topaz (3ne) AM: poor, velvety, pearl pink (3ca) SP: topaz (3pe)
Glycerol-asparagine agar (ISP)	G: moderate, colorless R: pearl pink (3ca) AM: moderate, velvety, pearl pink (3ca) SP: —	Oatmeal agar (ISP)	G: thin, light tan (3gc) R: pearl pink (3ca) AM: abundant, velvety, flesh pink (4ca) SP: ±
Inorganic salts-starch agar (ISP)	G: moderate, light tan (3gc) R: pearl pink (3ca) AM: abundant, velvety, flesh pink (4ca) SP: flesh pink (4ca)	Peptone-yeast extract iron agar (ISP)	G: moderate, bamboo (2gc) R: bamboo (2gc) AM: very poor SP: ±

Abbreviations; G (Growth), R (Reverse), AM (Aerial mycelium), and SP (Soluble pigment)

Table 2. Physiological properties of *Streptomyces* sp. AM-3867

	Growth
Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	−
Hydrolysis of starch	+
Liquefaction of gelatin	±
Peptonization of milk	+
Coagulation of milk	−
Cellulolytic activity	−
Temp. range of growth	20~35°C

Table 3. Utilization of carbon sources by *Streptomyces* sp. AM-3867

	Growth
Non-carbon	−
D-Glucose	+
L-Arabinose	±
Sucrose	±
D-Fructose	+
D-Xylose	+
Rhamnose	+
i-Inositol	−
Raffinose	±
D-Mannitol	−

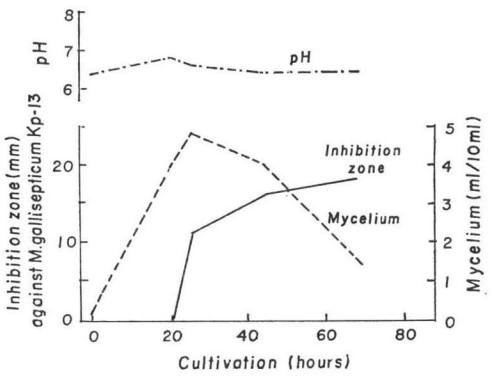
roseofulvus. The strain has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan and assigned as *Streptomyces* sp. AM-3867 with an accession number of FERM-P 4359.

Production and Isolation of Antibiotic AM-3867

Strain No. AM-3867 was maintained on KRAINSKY's agar medium or as freeze-dried stock. The stock culture was inoculated into 100 ml of seed medium (1% glucose, 2% starch, 0.5% yeast extract, 0.5% peptone, 0.4% $CaCO_3$) in a SAKAGUCHI flask and incubated at 27°C. A 48-hour culture (200 ml) was transferred into 20 liters of production medium in a 30-liter jar fermentor and the fermentation was carried out for 68 hours under the following conditions: Temperature, 27°C; aeration, 10 liters/min.; agitation, 250 r.p.m.; pressure, 0.5 kg/cm^2; and antifoam agent, Adekanol LG-109 (Asahi Electro-Chemical Co., Ltd.). The composition of production medium was 3.0% liver oil (Ritak R-10, Riken-vitamin Co., Ltd.), 0.5% peptone, 0.3% yeast extract, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ (pH 7 before sterilization).

A typical time course of antibiotic AM-3867 production in a 30-liter jar fermentor is shown in Fig. 1. The antibiotic production started about 24 hours after inoculation, then gradually increased and reached a maximum at 68 hours. The total amount of antibiotic AM-3867 accumulated during the 68 hours was about 200 μg/ml. The concentration of antibiotic AM-3867 was assayed by the paper disc method using *Mycoplasma gallisepticum* KP-13[2].

Fig. 1. Time course of antibiotic AM-3867 production by *Streptomyces roseofulvus* strain AM-3867.



The components of antibiotic AM-3867 were detected on silica gel TLC eluting with benzene - acetone (10 : 1, v/v); these were designated as AM-3867 I (Rf 0.55) and II (Rf 0.42). Culture broth (20 liters) of *S. roseofulvus* strain No. AM-3867 was centrifuged to obtain culture supernatant, which was used as a starting material for the isolation of antibiotic AM-3867. After the supernatant solution (17 liters) was adjusted to pH

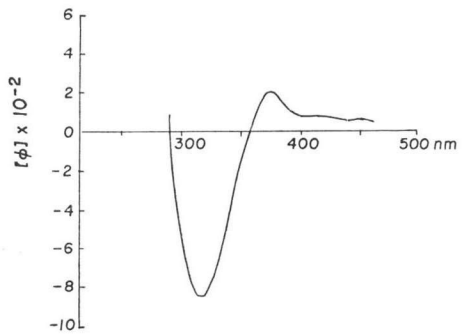
2.0 with 3 N hydrochloric acid, the antibiotics were extracted with 5 liters *n*-butyl acetate and then transferred into 2 liters of 1% sodium bicarbonate solution. The water layer was adjusted to pH 2.0 with 3 N hydrochloric acid and antibiotic AM-3867 in aqueous solution was extracted with 3 liters ethyl acetate. The ethyl acetate solution was dried over anhydrous sodium sulfate and the solvent completely removed under reduced pressure to give a reddish brown paste (13 g).

The paste was dissolved in a small amount of chloroform, and then chromatographed on silica gel (400 g) eluting with a mixture of chloroform and ethyl acetate. Antibiotic AM-3867 I and II were successively eluted from the column. The first active fraction was eluted with chloroform - ethyl acetate (50 : 1), concentrated to a small volume and then rechromatographed on silica gel (50 g) with benzene. The active fraction was concentrated *in vacuo* to dryness to give a yellowish brown powder. The dried powder was recrystallized from a solvent mixture of cyclohexane and ethyl acetate to afford orange columns (200 mg) of antibiotic AM-3867 I. The second active fraction eluted with chloroform - ethyl acetate (5 : 1) was concentrated *in vacuo* to give a reddish brown powder. The dried powder was recrystallized from benzene to afford yellow needles (80 mg) of antibiotic AM-3867 II.

Physicochemical Properties, Identification and Structures

Antibiotic AM-3867 II showed the following physicochemical properties: M.P. 173~175°C, $[\alpha]_D^{25}$ +118.4° (*c* 1.0, methanol), UV spectrum: λ_{max}^{MeOH} nm (ε); 245 (10,263), 272 (11,946), 418 (4,092). IR spectrum: ν_{max}^{KBr} cm^{-1}; 3100, 1715, 1665, 1650, 1630, 1605, 1565. The molecular formula $C_{18}H_{18}O_6$ was determined on the basis of elemental analysis (C 64.24, H 5.46, N 0%) and its mass spectrum (M^+, *m/e* 330). The physicochemical properties of AM-3867 described above indicated that it is most likely to be deoxyfrenolicin, derived by a chemical treatment of antibiotic frenolicin[7]. Its identity was confirmed with an authentic sample (IR spectrum and TLC). On the other hand, the absolute configuration of two quinone antibiotics, nanaomycin D produced by *Streptomyces rosa* var. *notoensis*[2] and kalafungin produced by *S. tanashiensis*[9~11] has been studied by ORD[5]. Nanaomycin D shows a negative COTTON effect and its enantiomer, kalafungin shows a positive COTTON effect. The ORD curve of antibiotic AM-3867 II showed a positive COTTON effect with a trough, $[\phi]$ −878 at 316 nm and a peak $[\phi]$ +176 at 374 nm (*c* 0.21, MeOH), as shown in Fig. 2. Consequently, it was concluded that antibiotic AM-3867 II is identical with deoxyfrenolicin.

Fig. 2. ORD curve of antibiotic AM-3867 II (deoxyfrenolicin)



The physicochemical properties of antibiotic AM-3867 I are summarized in Table 4. The molecular formula $C_{18}H_{16}O_6$ was determined on the basis of elemental analysis (C 65.96, H 4.87, N 0%) and its mass spectrum (M^+, *m/e* 328.094). The UV and IR spectra are shown in Figs. 3 and 4, respectively. These data suggested that antibiotic AM-3867 I is a quinone-related compound with a juglone moiety[12] and its structure closely resembles that of antibiotic AM-3867 II, deoxyfrenolicin. Since antibiotic AM-3867 I is less polar than deoxyfrenolicin and its IR spectrum shows no carboxyl absorption but a new

carbonyl band at 1770cm^{-1} presumably due to a lactone carbonyl group, the structure of antibiotic AM-3867 I was suspected to be structure **I** (Fig. 5) having a γ-lactone ring instead of a carboxymethyl group. This structure was further supported by the following chemical transformation as well as by consideration concerning the structural relationships between nanaomycins A and D[5], or griseusins A and B[13]. It was reported that griseusin B was quantitatively converted into griseusin A in pyridine and its mechanism was elucidated[13]. In a similar manner, treatment of deoxyfrenolicin with pyridine at room temperature overnight gave antibiotic AM-3867 I. It was reasonably concluded from the above data that the structure of AM-3867 I is represented by **I** (Fig. 5). Since this antibiotic is a new

Table 4. Physical and chemical properties of antibiotic AM-3867I (frenolicin B)

Appearance	Orange column
Melting point	157~159°C
Elemental analysis	Found: C, 65.96%; H, 4.87% Calcd. for $C_{18}H_{16}O_6$: C, 65.85%; H, 4.91%
Molecular weight (Mass, M^+)	Found: *m/e*, 328.094 Calcd. for $C_{18}H_{16}O_6$: *m/e*, 328.094
Molecular formula	$C_{18}H_{16}O_6$
UV λ_{max}^{MeOH}nm(ε)	260 (8,954), 270 (9,020), 425 (4,920)

Fig. 3. UV spectra of frenolicin B

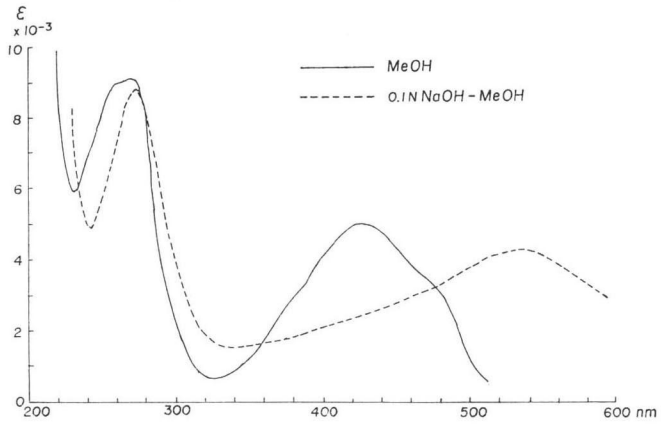


Fig. 4. IR spectrum of frenolicin B (KBr)

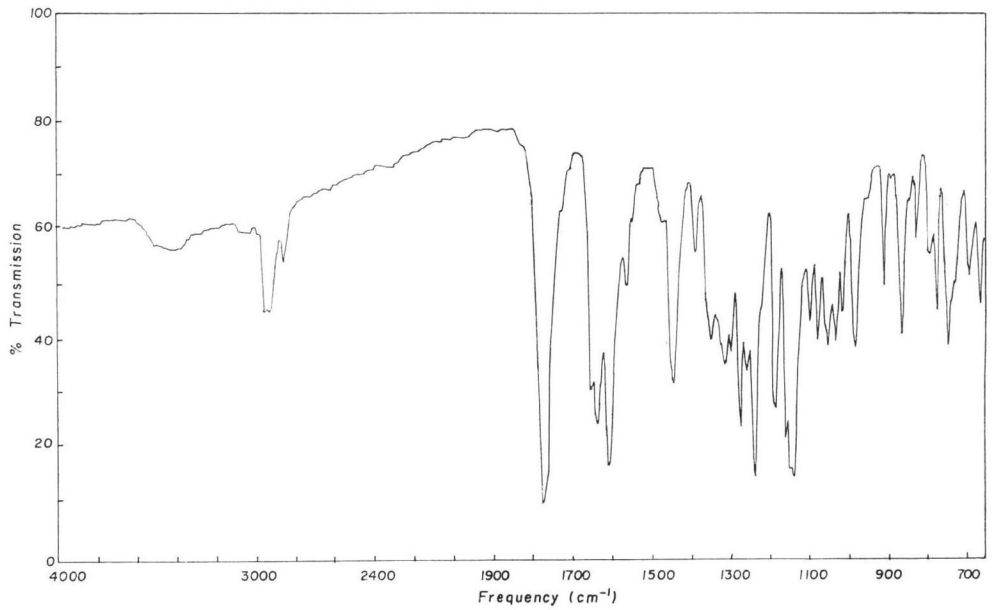
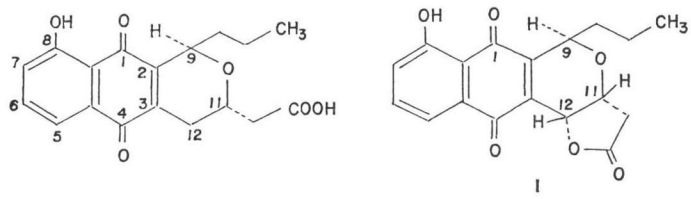


Table 5. Antimicrobial activity of frenolicin B and deoxyfrenolicin

Test organism	Medium*	Minimal inhibitory concentration (μg/ml)	
		Frenolicin B	Deoxyfrenolicin
Mycoplasma gallisepticum Kp-13	E	3.16	0.78
Mycoplasma gallisepticum S-6	E	3.16	0.78
Mycoplasma gallisepticum 333p	E	3.16	0.78
Mycoplasma pneumoniae	E	3.16	0.4
Acholeplasma laidlawii (A) PG8	E	6.25	1.56
Acholeplasma laidlawii (B) Bml	E	50	50
Candida albicans	P	0.78	25
Saccharomyces cerevisiae	P	0.78	25
Microsporum gypseum	P	0.1	12.5
Penicillium chrysogenum	P	6.25	50
Trichophyton interdigitale	P	0.1	12.5
Trichophyton mentagrophytes	P	12.5	25
Piricularia oryzae	P	0.1	0.78
Aspergillus fumigatus	P	6.25	25
Aspergillus niger	P	100	100

* Abbreviations used. E; Eiken PPLO agar (pH 7.8, 8 days, 37°C),
P; glucose-potato agar (pH 6.4, 3 days, 27°C)

Fig. 5. Structures of deoxyfrenolicin and frenolicin B



Deoxyfrenolicin (9R,11R)[6] I Frenolicin B (9R,11R,12R)

frenolicin group antibiotic, it is designated as frenolicin B.

Antimicrobial Activity of Frenolicin B and Deoxyfrenolicin

The antimicrobial spectra of frenolicin B and deoxyfrenolicin were determined by the conventional agar dilution method using glucose-potato agar for fungi (27°C, 72 hours) and Eiken PPLO agar for mycoplasmas (37°C, 8 days). Both antibiotics were active against mycoplasma and fungi as shown in Table 5. Frenolicin B was active against fungi at lower concentration than deoxyfrenolicin, while the former was less active against mycoplasma than the latter.

Discussion

In the screening for new antimycoplasmic antibiotics, we discovered two quinone antibiotics, frenolicin B and deoxyfrenolicin. Deoxyfrenolicin has been obtained from frenolicin by hydrogenation with 10% palladium charcoal catalyst in methanol[14]. Deoxyfrenolicin is more active against fungi than frenolicin. However, there has been no previous report describing the production of deoxyfrenolicin by a microorganism. Frenolicin B was found to be active against fungi at lower concentrations than deoxyfrenolicin.

Previous reports show that the quinone antibiotics are produced by different species belonging to

the genus *Streptomyces*. These quinone antibiotics produced by the genus *Streptomyces* can be classified into two groups on the basis of the absolute configurations on C-9 and C-11. Thus kalafungin type antibiotics (kalafungin, frenolicin, deoxyfrenolicin, frenolicin B and medermycin[15]) have configurations 9R and 11R and nanaomycin type antibiotics (nanaomycins, griseusins A and B) have configurations 9S and 11S. The production of these two types of antibiotics by the genus *Streptomyces* is of interest in regard to the biosynthesis of a series of the antibiotics belonging to these groups.

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