DEMETHOXYRAPAMYCIN (AY-24,668), A NEW ANTIFUNGAL ANTIBIOTIC

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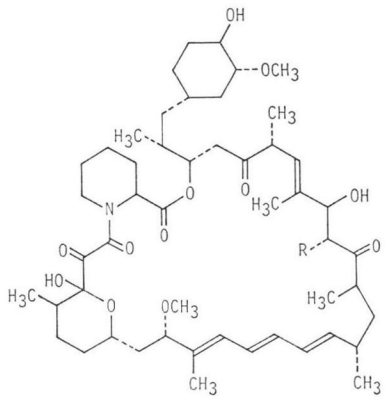
Demethoxyrapamycin is a new antifungal antibiotic which is co-produced with rapamycin by *Streptomyces hygroscopicus*. It was isolated as a minor component during recovery of rapamycin. Its antifungal and antitumor activity is compared with that of rapamycin.

The production, isolation and structure of rapamycin have been reported previously[1,2,3]. The rapamycin producing organism *Streptomyces hygroscopicus* NRRL 5491 also produces a structurally related antifungal compound AY-24,668. Initially, it was isolated as a minor component from the fermentation broths of rapamycin. FINDLAY *et al.*[4] recently reported the structure of AY-24,668 as 29-demethoxyrapamycin (Fig. 1). In this paper, we are describing the change in fermentation conditions of rapamycin to produce larger amounts of AY-24,668 and facilitate its isolation. The biological activities of AY-24,668 and its comparison with that of rapamycin is also reported.

Fig. 1.



Rapamycin R=OCH_3
AY-24,668 R=H

Production of AY-24,668

The producing strain, *Streptomyces hygroscopicus* NRRL 5491, was grown and maintained on tomato paste - oatmeal agar, as previously described.[1] Good growth and sporulation were obtained in 7 ~ 15 days of incubation at 25°C. Spores from one slant were suspended in 10 ml of sterile distilled water to constitute the first spore inoculum.

To a 500-ml Erlenmeyer flask, 50 g of "pot" barley and 25 ml of tap water were added. The contents were mixed and autoclaved at 121°C for 45 minutes using the fast exhaust cycle of the autoclave. The flasks were cooled and shaken to break the clumps. Each flask was inoculated with 2 ml of a spore suspension obtained from a slant. The inoculated flasks were incubated at 25°C and 70% relative humidity for 5 days and were shaken once a day during incubation. Then, 50 ml of sterile distilled water was added to each flask and the spores were suspended by agitation on a gyrotory shaker for 5 minutes. This suspension was used as the spore inoculum without any further adjustment.

Unbaffled, 2-liter Erlenmeyer flasks were filled with 500 ml of an inoculum medium consisting of (g/liter): soy peptone 10, "Cerelose" 20, baker's yeast 5, sodium chloride 2, $ZnSO_4 \cdot 7H_2O$ 0.05, $MgSO_4$

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0.125, $MnSO_4$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.02, DF-143PX (antifoam) 1 ml, and tap water to 1 liter (pH 7.0). The flasks were sterilized at 121°C for 45 minutes, cooled to 25°C and inoculated with 5% spore inoculum. The flasks were incubated at 25°C for 24 hours on a gyrotory shaker at 250 rev/minute, 5 cm-stroke.

Fermenters (Model MF-128S, New Brunswick Scientific Co)., 28-liter capacity, were filled with 20 liters of the same medium and autoclaved at 121°C for 30 minutes with agitation, cooled to 25°C and inoculated with 400 ml (2%) of the first stage inoculum. The fermenters were incubated for 18~20 hours at 25°C under agitation and aeration of 400 rev/minute and 1 v/v/minute respectively.

Fermenters (Model F-250, New Brunswick Scientific Co.), 250-liter capacity, equipped with automatic antifoam addition system and pH recorders-controllers were filled with 160 liters of the production medium consisting of (g/liter): soy peptone 10, baker's yeast 6, L-lysine, 6, "Cerelose" 20, K_2HPO_4 2.5, KH_2PO_4 2.5, NaCl 5.0, $MgSO_4$ 0.125, $ZnSO_4 \cdot 7H_2O$ 0.05, $MnSO_4$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.02, glycerol 30 ml, soybean oil 20 ml, DF-143PX antifoam 2 ml, and tap water to 1 liter (pH 6.4 after sterilization). The fermenters were sterilized at 121°C for 30 minutes with agitation, cooled to 32°C and inoculated with 4% of the second stage inoculum. The fermentation was run at 32°C under an agitation of 250 rev/minute and aeration of 1 v/v/minute. The optimum incubation temperature for the production of rapamycin is 25°C.[2] At elevated incubation temperature of 32°C, the production of rapamycin is inhibited by 70%, whereas the production of AY-24,668 is not affected. After 30~35 hours incubation, the pH started to drop but was controlled at 6.0 by on-demand addition of ammonia gas injected into the medium through air filter. The maximum titers were obtained in 96 hours. In a typical run AY-24,668 was produced at levels of 40 μg/ml, whereas under the same conditions of fermentation concurrently 170 μg/ml of rapamycin were produced.

Reverse phase high performance liquid chromatography was used to determine the antibiotic titer. A 5-ml sample of fermentation broth was diluted to 50 ml with methanol and shaken vigorously for 10 minutes. The methanolic extract was filtered through a Millipore filter and injected into a HPLC "Bondapak" C18 column through a 20 μl loop. 70% Methanol in water was used as solvent. The titers of antibiotic AY-24,668 were estimated from the peak heights of standards and unknown broth samples by interpolation.

Isolation of AY-24,668

The fermentation broth was adjusted to pH 4.0 with a 30% solution of sulfuric acid and filtered on a vacuum rotary filter coated with Celite. The mycelium containing the antibiotic was extracted twice by stirring for 2 hours with 2 volumes of trichloroethane. The trichloroethane extracts were pooled and evaporated to a small volume under reduced pressure, dehydrated with anhydrous sodium sulfate and further concentrated to an oily residue. A typical 400-liter fermentation run yielded about 1.4 kg of oily residue. A heavy glass column (2 m×10.5 cm I.D.) was packed with 7 kg (5 times the weight of oily residue) of dry silica gel G (Merck). A solvent mixture of 20% acetone in hexane was introduced under 0.84 kg/cm^2 nitrogen pressure. After the column was equilibrated with the solvent the oily residue, dissolved in 1 v/v of 20% (v/v) acetone in hexane, was introduced into the column at a rate of 3 liters per hour. Pressure chromatography was continued with a solvent mixture of 20% acetone in hexane until the antibiotic AY-24,668 appeared in the eluate. At this point the solvent was changed to 25% acetone in hexane. The fractions containing AY-24,668 were combined and evaporated to dryness. AY-24,668 was crystallized from the dry residue with ether.

Physico-Chemical Properties of AY-24,668

AY-24,668 (demethoxyrapamycin) is a white crystalline solid melting at 122~124°C. It is freely soluble in methanol, ethanol, acetone, chloroform, methylene chloride, trichloroethane, dimethylformamide, dimethyl sulfoxide; sparingly soluble in ether, and practically insoluble in water. FINDLAY *et al.*[4] have recently elucidated its structure and found it to be 29-demethoxyrapamycin. Optical rotation is $[\alpha]_D^{25}$ −124.4° in methanol. Demethoxyrapamycin forms a transcient pink solution when heated with 0.1 N methanolic NaOH, which changes to yellow.

Biological Activity of Demethoxyrapamycin

29-Demethoxyrapamycin was compared to rapamycin and amphotericin B against clinical isolates of *Candida albicans*; the results are shown in Table 1. It is about 1/4 as active against *Candida albicans* as rapamycin, but nearly as active as amphotericin B. Demethoxyrapamycin (AY-24,668) was found to be active against experimental systemic candidal infections in mice. When formulated as a suspension in 5% acacia, it gave a PD_{50} of 38.17±16.0 mg/kg (i.p.). LD_{50} i.p. in mice was >900 mg/kg.

Table 1. *In vitro* activity (MIC, μg/ml) of rapamycin, 10-demethoxyrapamycin (AY-24,668) and amphotericin B (AM-B) against clinical isolates of *Candida albicans*[a].

Strain No.	Rapamycin	AY-24,668	AM-B
618	0.08	0.32	0.32
620	0.04	0.32	0.16
621	>10	>10	2.50
623	<0.02	0.32	0.16
624	<0.02	2.50	0.32
668	<0.02	0.16	1.25

[a] MIC determined after 48 hours incubation at 37°C in Sabouraud liquid medium (BBL).

Rapamycin shows marked activity against transplanted tumors[5]. Demethoxyrapamycin (AY-24,668) shows only marginal activity against P388 lymphocytic leukemia (T/C (%)=135) and has no activity against B16 melanocarcinoma and Colon 38 solid tumor.

The mechanism of action of demethoxyrapamycin was compared to that of rapamycin[6]. The results are shown in Table 2. No significant difference was observed between rapamycin and demethoxyrapamycin.

Table 2. Inhibitory effect (% inhibition) of rapamycin AY-22,989 on macromolecular biosynthesis in *Candida albicans*.

Antibiotics	μg/ml	Na [1-^{14}C]-acetate into the total lipid fraction	[U-^{14}C]-leucine into protein	[2-^{14}C]-uridine into RNA	[U-^{14}C]glucose into		
					Whole cell	Mannan	Glucan
Rapamycin	1.0	25.4	64.2	81.0	17.2	46.2	11.2
	0.1	25.0	32.0	30.9	9.3	—*	—
	0.02	20.9	2.8	6.6	0	—	—
AY-24,668	1.0	38.6	52.9	72.6	14.9	51.3	20.5
	0.1	28.5	28.4	11.1	7.9	—	—
	0.02	—	11.2	2.5	0.9	—	—

* —: not done.

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