RAPAMYCIN (AY-22,989), A NEW ANTIFUNGAL ANTIBIOTIC

II. FERMENTATION, ISOLATION AND CHARACTERIZATION

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Rapamycin is a new antifungal antibiotic produced by *Streptomyces hygroscopicus* NRRL 5491. It was isolated from the mycelium by solvent extraction, purified by silica gel column chromatography and crystallized as a colorless solid which melts at 183~185°C and has the empirical formula $C_{56}H_{89}NO_{14}$. From its characteristic ultraviolet absorption spectrum rapamycin can be classified as a triene. It is highly active against various *Candida* species, especially *Candida albicans*. Its activity is compared with that of amphotericin B, candicidin and nystatin.

In a previous publication,[1] a strain of *Streptomyces hygroscopicus*, newly isolated from an Easter Island soil sample, was reported to inhibit *Candida albicans*, *Microsporum gypseum* and *Trichophyton granulosum*. The active principle was isolated and found to be a new antibiotic of unknown structure; it was named rapamycin. In the present paper, we are describing the fermentation of rapamycin in agitated-aerated vessels, an improved process for its isolation and purification as well as its physical-chemical characteristics. Comparison of its activity with that of other antifungal antibiotics is also reported.

Production of Rapamycin

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 ROUX bottle were suspended in 50 ml of sterile distilled water to constitute the spore inoculum.

Unbaffled, 500-ml Erlenmeyer flasks were filled with 100 ml of an inoculum medium consisting of (g/liter): soybean meal ("Special X", Archer Daniels Midland Co., Minneapolis, Minn.), 40; "Cerelose" (a pharmaceutical grade of glucose), 20; $(NH_4)_2SO_4$, 3; $CaCO_3$, 1.5; and tap water to 1 liter (pH 7.0). The flasks were sterilized at 121°C for 30 minutes, cooled to 25°C and inoculated with 4 ml of the spore inoculum. The inoculated flasks were incubated for 24 hours at 25°C on a gyrotory shaker at 240 rev/min, 2″-throw, to constitute the first-stage inoculum.

Unbaffled, 24-liter round bottom flasks were filled with 3.4 liters of the same medium and autoclaved at 121°C for 30 minutes. The flasks were agitated to resuspend the solids and autoclaved for an additional period of 1 hour at 121°C, cooled to 25°C and inoculated with 78 ml (2 %) of the first-stage inoculum. The inoculated flasks were incubated for 18 hours at 25°C on a reciprocating shaker at 65 strokes per minute and 4″-throw. These flasks were used to inoculate the production stage.

Fermenters (model F-250, New Brunswick Scientific Co.), 250-liter capacity, equipped with automatic antifoam addition system and pH recorder-controller, were filled with 160 liters of the production medium consisting of (g/liter): soybean meal ("Special X"), 30; "Cerelose," 20; $(NH_4)_2SO_4$, 5; KH_2PO_4, 5; Mazer DF-143PX (antifoam), 0.5 ml; and tap water to 1 liter. The fermenters were sterilized at 121°C for 30 minutes under an agitation of 150 rev/min, cooled to 25°C and pH of medium adjusted to 6.1 by addition of 10 N NH_4OH solution. The fermenters were inoculated with 3.2 liters (2 %) of the second-stage inoculum. The fermentation was run at 25°C under an agitation of 200 rev/min and an aeration of 0.25 v/v/min. Sterile Mazer DF-143PX antifoam was added on demand. After 30~35 hours of incubation the pH started to drop but was controlled at 6.0 by addition of 10 N NH_4OH solution on demand. After 48 hours of incubation, a 40 % sterile solution of "Cerelose" was added continuously at the rate of 3.85 % per day. The antibiotic titres were determined every 24 hours starting at 48 hours. The maximum titers were usually obtained in 96 hours. The results of a typical fermentation are shown in Table 1.

Table 1. Production of rapamycin in aerated-agitated fermenters

Fermentation time (hours)	pH	Packed cell volume (%)	Potency (μg/ml)
48	6.0	23	20
72	6.0	60	63
96	6.3	50	87

Conventional paper disc-agar diffusion assays were used to determine the antibiotic titre. A 10-ml sample of fermentation broth was centrifuged at 2,500 rev/min for 15 minutes. The supernate was discarded and the mycelial pellet suspended in 250 ml of methanol and shaken vigorously. The extract was filtered. Filter paper discs, 13 mm in diameter, were dipped in the extract and placed on filter paper to dry. Similar discs were dipped in standard solutions containing 10, 5, 2.5 and 1.25 μg rapamycin/ml. All the discs were deposited on agar plates seeded with the test strain of *Candida albicans* AY F-598. The inhibition zone diameters obtained for the standard solutions after overnight incubation were plotted against log concentration on semi-logarithmic paper and titre of fermentation broths read from the standard curve and corrected for dilution.

Isolation of Rapamycin

The fermentation broth was adjusted to pH 4.0 with a 30 % sulfuric acid solution and filtered on a vacuum rotary filter coated with Celite. The mycelium, containing the antibiotic, was extracted twice by stirring for 1 hour with 1½ volume 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 160-liter fermentation run yielded about 500 g of oily residue. The residue was extracted twice with one volume of methanol. The methanolic extracts were pooled and evaporated to dryness to yield about 50 g of oily residue containing rapamycin. The residue was dissolved in 10 v/w of a solvent mixture consisting of 15 % acetone in hexane. To this solution, 2 weights of silica gel G (Merck) per weight of oil were added and the mixture stirred gently for 50 minutes. The mixture was filtered and silica gel with adsorbed rapamycin washed onto a column with several volumes of 15 % acetone in hexane. The antibiotic was eluted with 25 % acetone in

hexane and the eluant evaporated to dryness. The residue was dissolved in ether from which pure rapamycin crystallized out. The recoveries were about 40% based on broth assay.

Physical and Chemical Properties of Rapamycin

Rapamycin is a white crystalline solid melting at 183~185°C. It is freely soluble in methanol, ethanol, acetone, chloroform, methylene dichloride, trichloroethane, dimethyl formamide, dimethyl sulphoxide; sparingly soluble in ether, and practically insoluble in water.

Rapamycin analysed for $C_{56}H_{89}NO_{14}$ (E.W. 999). Calcd: C, 67.2; H, 8.9; N, 1.4; Found: C, 67.24; H, 8.93; N, 1.39.

Fig. 1. Ultraviolet absorption spectrum of rapamycin (AY-22,989).

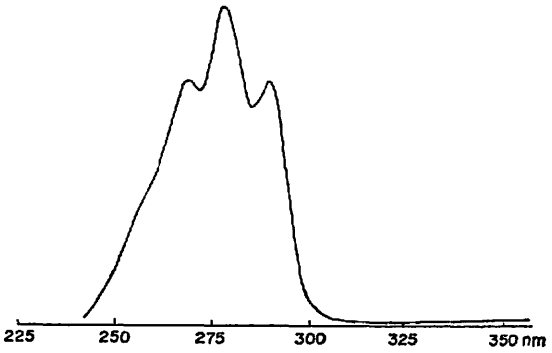


Fig. 2. Infrared spectrum of rapamycin (AY-22,989).

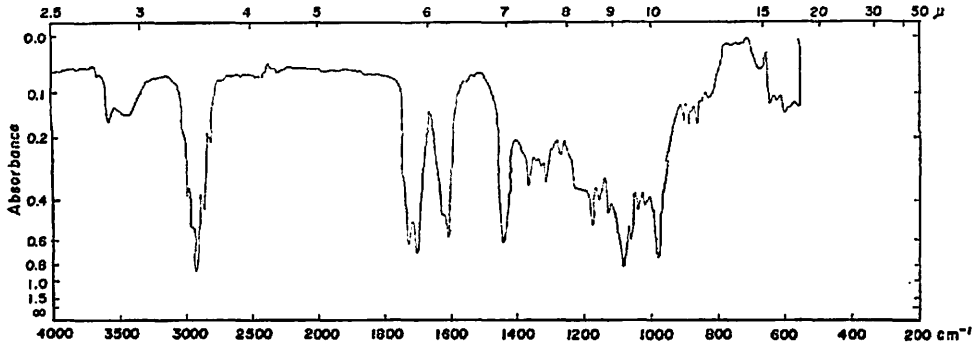
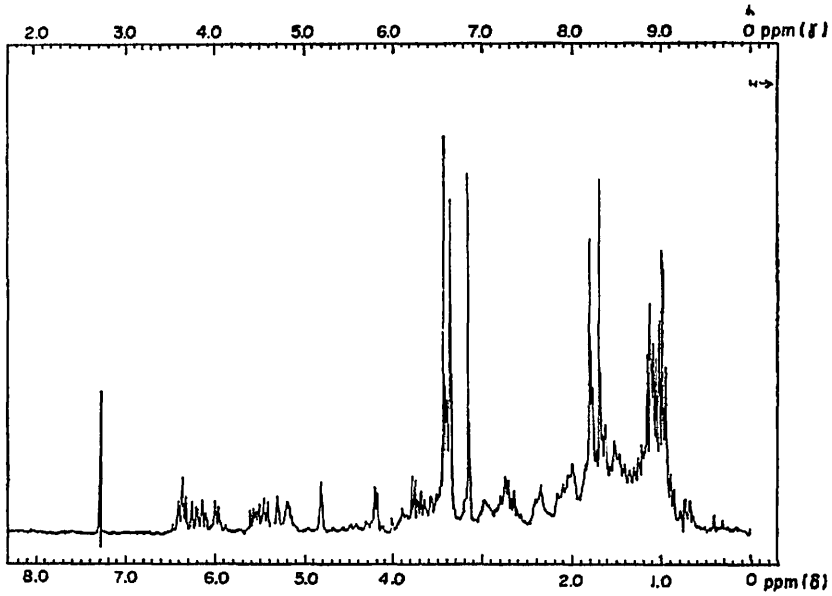


Fig. 3. NMR (200 MHZ) spectrum of rapamycin (AY-22,989).



The ultraviolet spectrum (Fig. 1) shows λ_{max} at 288, 277 and 267 nm with $E^{1\%}_{1cm}$ 416, 514 and 417 respectively.

The infrared spectrum (Fig. 2) shows OH at 3500, a band at 1730 (possibly lactone carbonyl) and at 1700 (carbonyl), and a band between 1610 and 1630 cm^{-1} (C=C).

NMR spectrum (200 MHZ) of rapamycin is shown in Fig. 3. It shows vinylic protons between δ 5~6.5, methoxyl between δ 3.1~3.6 and vinylic at δ 1.8.

Optical rotation is $[\alpha]_D^{25}$ −58.2 in methanol. Rapamycin forms a yellow chromophore when dissolved in 0.1 N methanolic NaOH and heated at 60°C; this property is the basis of a colorimetric assay.

Table 2. Comparison of rapamycin with other trienes

Antibiotics	Appearance	m.p.	Molecular or equivalent weight	Molecular or equivalent formula	U.V. λ_{max} (nm)	Anti-microbial spectrum	Toxicity i.p. mice (LD_{50} mg/kg)	Ref.
Rapamycin (AY-22,989)	Colorless, crystalline	183~185°C	999	$C_{56}H_{89}NO_{14}$	267,277,288	Antifungal: strongly candicidal	600	
Mycotrienin	Yellow powder	149~150°C	683	$C_{36}H_{50}N_2O_8$	262,272,282	Antifungal	15	2
Trienine	Off-white powder	163~165°C	1,400	—	257,267,278	Antitumor	—	3
Proticin	—	—	582	$C_{31}H_{44}O_7PNa$	264,272,284	Antibacterial	>150 (i.v.)	4
MM8	Yellow powder	—	—	—	262,270,282	Antifungal against filamentous fungi	—	5
Resistaphylin	Colorless, crystalline	91~ 92°C	462.5	$C_{24}H_{34}N_2O_7$	230,267,275, 285	Antibacterial	—	6

On the basis of its ultraviolet spectrum, rapamycin can be classified as a triene, but comparison with other known trienes (Table 2) shows it to be novel compound.

Antimicrobial Activity of Rapamycin

Rapamycin inhibits yeasts and filamentous fungi. Results reported in Table 3 using the agar-diffusion assay indicate that rapamycin would be as active against the dermatophytes as it is against *Candida albicans*. However, activity expressed as minimum inhibitory concentration in broth has already been shown[11] to be much higher against yeast than against the dermatophytes;

Table 3. Antifungal activity of rapamycin

Concentration (μg/ml)	Zones of inhibition (mm)			
	Candida albicans	*Microsporum gypseum*	*Trichophyton mentagrophytes*	*Aspergillus fumigatus*
0 (control)	0	0	0	0
0.5	21	18	20	>40
1.0	23	22	25	—
2.5	27	26	30	—
5.0	28	32	32	—

—: not determined.

Table 4. Activity of rapamycin and amphotericin B against clinical isolates of various *Candida* species

Candida species (AY Nos.)	Minimum inhibitory concentration (μg/ml)	
	Rapamycin	Amphotericin B
C. albicans F-598	<0.02 to 0.2	2.5
C. albicans F-634 (ATCC 11651)	0.08 to 0.32	2.5
C. catenulata F-662	<0.1	2.0
C. internodia F-670	<0.1	<0.1
C. lipolytica F-669	2.5	4.0
C. monosa F-664	<0.1	0.2
C. parapsilosis F-665	<0.1	>1.0
C. pseudotropicalis F-666	>5.0	3.0
C. stellatoidea F-667	<0.1	0.7
C. tropicalis F-668	<0.1	2.5

Table 5. Activity of rapamycin compared to that of nystatin and candicidin against various strains *Candida albicans*

Candida albicans Strain Nos.	Incubation time (hour or day)	Minimum inhibitory concentration (MIC) (μg/ml)		
		Rapamycin	Nystatin	Candicidin
F-612	48 hours	0.0025	5.0	0.08
	8 days	0.02	>10.0	1.2
F-615	48 hours	0.0025	5.0	0.16
	8 days	0.32	>10.0	0.62
F-619	48 hours	<0.00063	5.0	0.08
	8 days	0.04	>10.0	1.25
F-620	48 hours	<0.00063	5.0	0.08
	8 days	0.02	>10.0	0.62
F-621	48 hours	>10.0	>10.0	2.5
	8 days	>10.0	>10.0	>10.0
F-623	48 hours	0.00125	10.0	0.04
	8 days	0.02	>10.0	0.16
F-624	48 hours	<0.00063	5.0	0.08
	8 days	0.00125	>10.0	1.25
F-626	48 hours	<0.00063	5.0	0.02
	8 days	0.01	>10.0	0.04
F-004	48 hours	0.04	2.5	0.02
	8 days	>10.0	10.0	0.04

these contradictory results were explained by the instability of the antibiotic in broth over long period of incubation required for the growth of dermatophytes: uninoculated SABOURAUD dextrose broth containing 5 μg of rapamycin per ml lost 80 % of its activity after 7 days of incubation.

Rapamycin was compared to amphotericin B against clinical isolates of various *Candida* species, and the results are shown in Table 4; the minimum inhibitory concentration of rapamycin is much lower than that of amphotericin B, except for *Candida pseudotropicalis*. When compared with nystatin and candicidin using the same method, rapamycin again appeared somewhat more active against clinical isolates of *Candida albicans* (Table 5). Rapamycin was also found active against candidal infections in mice; the results will be reported in a subsequent publication.[7]

Acknowledgements

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