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[54] **RAPAMYCIN AND PROCESS OF PREPARATION**

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Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 293,699, Sept. 29, 1972, abandoned.

[52] **U.S. Cl.** ..... **424/122;** 195/80 R

[51] **Int. Cl.²** **A61K 35/00**

[58] **Field of Search** 424/122; 195/80

[56] **References Cited**

OTHER PUBLICATIONS

Miller, The Pfizer Handbook of Microbial Metabolites McGraw-Hill Book Co. Inc., N.Y., N.Y., 1961, p. 580.

Primary Examiner—Jerome D. Goldberg

[57] **ABSTRACT**

Antibiotic rapamycin is producible by culturing *Streptomyces hygroscopicus* NRRL 5491 in an aqueous nutrient medium. Rapamycin has antifungal properties. Methods for its preparation and use are disclosed.

4 Claims, 2 Drawing Figures

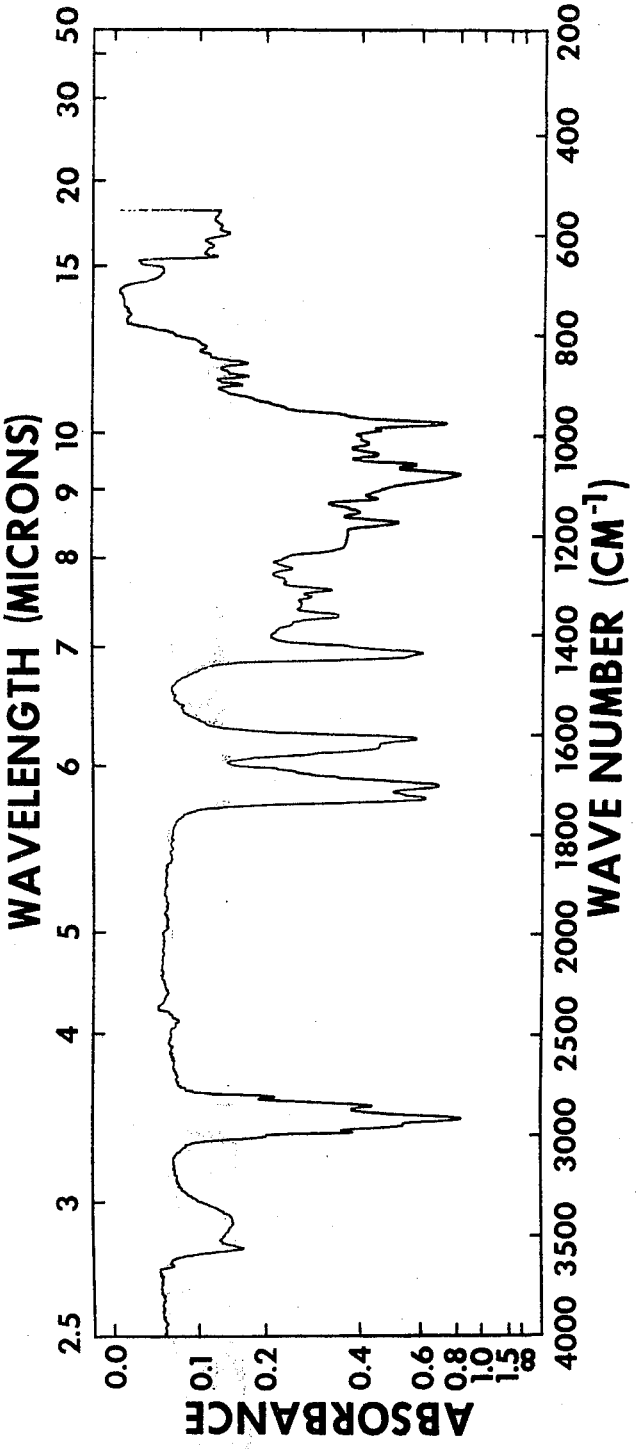

FIG. 1

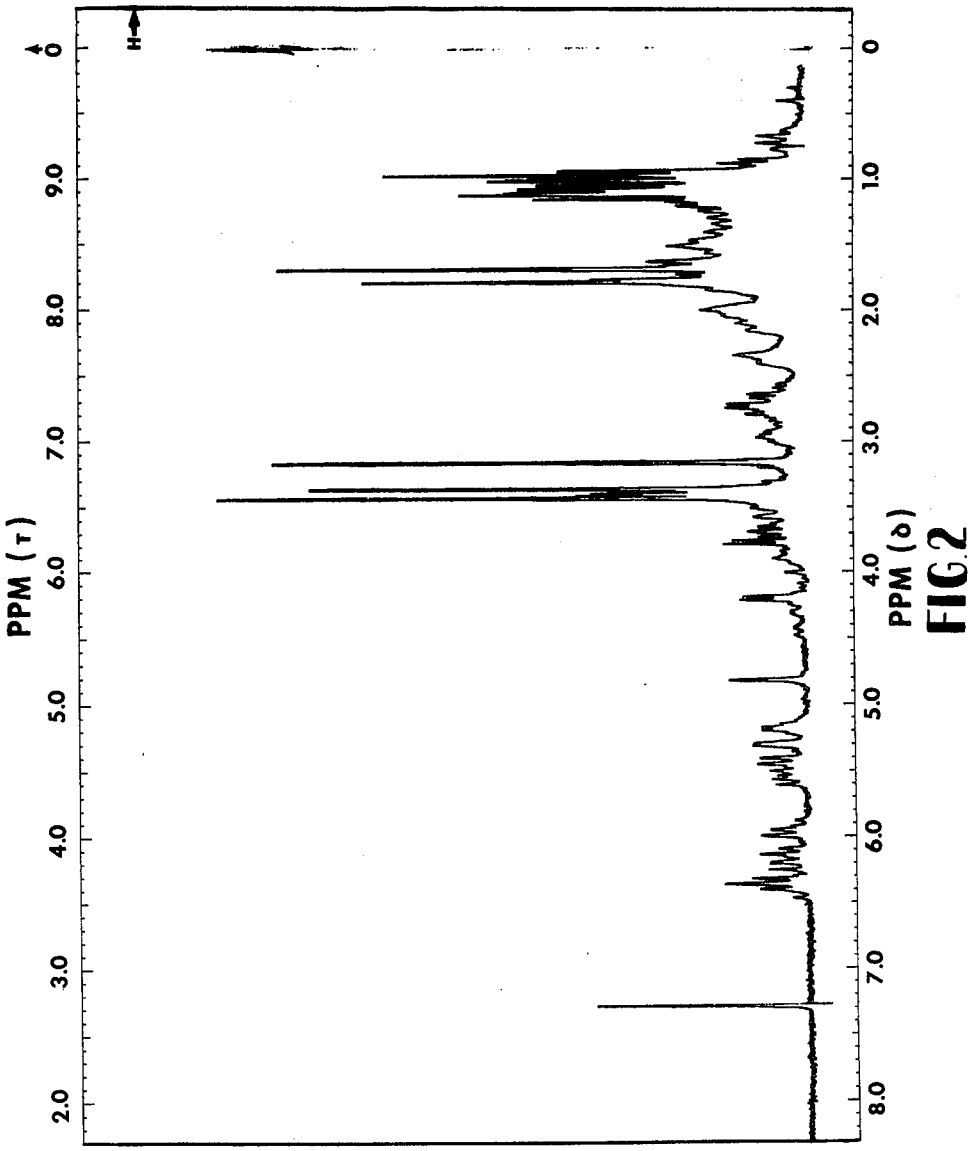

FIG.2

RAPAMYCIN AND PROCESS OF PREPARATION

This application is a continuation-in-part of our earlier application Ser. No. 293,699, filed Sept. 29, 1972 now abandoned.

BACKGROUND OF THE INVENTION

a. Field of Invention

This invention relates to an antibiotic, a new composition of matter calling rapamycin, and to a process for its preparation.

b. Description of Prior Art

The antibiotic of this invention is readily distinguished from prior art compounds of its class by its profound antifungal activity and its relatively low order of toxicity.

More explicitly, the ultra violet spectrum of rapamycin, noted herein, indicates that this compound belongs to the class of antibiotics known as triene antibiotics. In this particular class there are only five compounds reported previously. Trienine, A. Aszalos et al., J. Antibiotics, 21, 611 (1968) is a triene antibiotic with antitumor activity which also shows marked activity against gram positive organisms and only marginal activity against Candida strains. The antifungal triene reported by J. J. Armstrong, et al., Nature, 206, 399 (1965) and Mycotrienin reported by C. Coronelli et al., J. Antibiotics, 20, 329 (1967) are probably identical. Both have low antifungal activity (MIC against *C. albicans:* 5 μg/ml) and high toxicity (LD_{50} in mice: 15 mg/kg). The remaining two antibiotics - Resistaphylin, S. Aezaiva et al., J. Antibiotics, 24, 393 (1971) and Proticin, G. Nesemann et al., Naturwissenschaften, 59, 81 (1972)- are readily distinguished from the compound of the present invention in that they exhibit antibacterial without any antifungal activity.

BRIEF SUMMARY OF THE INVENTION

Rapamycin is a chemical compound producible by culturing a rapamycin-producing organism in an aqueous nutrient medium. The compound has the property of adversely affecting the growth of fungi, for example, *Candida albicans* and *Microsporum gypseum*. Accordingly, rapamycin may be used to prevent the growth of or reduce the number of certain fungi in various environments.

The rapamycin - producing organism used for this invention, *Streptomyces hygroscopicus* NRRL 5491, was obtained from Easter Island soils and samples thereof have been deposited without restrictions with the Northern Utilization and Research Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill., U.S.A.

It is to be understood that the invention is not limited to the use of the particular organism herein described, but includes variations and mutants obtained by natural selection or by treatment of the microorganism with, for instance, ultraviolet rays, X-rays, N-methyl-N'-nitro-N-nitroso-guanidine, manganese chloride, camphor, nitrogen mustards, and the like, as well as polyploids of the various mutants.

Streptomyces hygroscopicus NRRL 5491 develops abundantly in culture media usually employed for cultivation of other organisms of the same genus. It is capable of growing at temperatures ranging from 20° to 35°C., preferably at about 28°C, on Czapek's agar, glucose asparagine agar, glycerol asparagine agar

starch agar and peptone beef agar. Also, the organism grows very well on yeast extract agar, malt extract agar, starch-inorganic salts agar, oatmeal agar, oatmeal-tomato agar and Bennet's agar. On potato slices there is no aerial mycelium, but substrate growth is well developed and buff in color. On all media, the aerial growth is at first white then grayish with black spots. Sporophores are often compact, forming a spiral of more than ten spores. Substrate growth is light yellow to almost colorless and in some media pale brown. Occasionally a yellowish pigment is produced. The organism is H_2S- and melanine-negative.

Carbohydrate utilization by *Streptomyces hygroscopicus* NRRL 5491 was studied in carbon utilization agar (ISP Medium 9) according to the procedure standardized by the International Streptomyces Project (ISP).

The best utilized carbohydrates were D-glucose, inositol, D-fructose and D-mannitol; less well utilized carbohydrates were rhamnose, raffinose, xylose, starch and arabinose. Carbohydrates not utilized were sucrose and cellulose.

The environment and nutritional requirements for the fermentation of *Streptomyces hygroscopicus* NRRL 5491 are similar to those necessary for the production of antibiotics by other aerobic microorganisms. Thus, aerobiosis can be sustained in a liquid nutrient medium inoculated with a sterile culture incubated in flasks placed on shaking machines. For industrial production, metal tanks with internal aeration and agitation by means of paddles can be substituted. Rapamycin is also produced by surface cultivation. The microorganism requires as nutrient elements assimilable carbon and organic nitrogenous substances. The presence of mineral salts is desirable. Cultivation is best effected when the initial pH of the culture medium is between 6.5 and 7.5, the optimum pH being around 6.8–7.3.

The utilizable sources of assimilable carbon for the production of the antibiotic are very diverse, there being included sugars (for example, glucose, D-fructose, D-mannitol, maltose, arabinose, rhamnose, raffinose, xylose, and the like), dextrin, starches of different types of origin, glycerol (and other polyalcohols), inositol and animal and vegetable fats, as well as esters thereof. The sources of organic assimilable nitrogen which actively stimulate growth and favor production of rapamycin are substances such as soybean meal, cotton meal and other vegetable meals (whole or partially or totally defatted), meat flours or animal viscera, various peptones, casein hydrolysates, soybean hydrolysates, yeast hydrolysates, lactalbumin, wheat glutins, distillers solubles, corn steeps, molasses, urea and amino acids.

Mineral salts, such as the chlorides, nitrates, sulfates, carbonates and phosphates of sodium, potassium, ammonium and calcium, should be included in appropriate concentrations. The nutritive medium should contain a number of trace elements such as magnesium, iron, manganese, and zinc.

The inoculum of the above medium for the fermentation is provided with a fresh slant of *Streptomyces hygroscopicus* NRRL 5491.

Under the described conditions and with the temperature of cultivation at about 20°–35°C, preferably at about 25°C, maximum production of rapamycin in tanks is obtained in from about 2 to about 8 days. Alternatively, the pH may be controlled during fermentation in tanks and maintained at about pH 6.0 and glu-

cose may be added continuously from about 2 days after beginning to the end of fermentation, thus obtaining maximum yields in about 4 to 5 days.

Thereafter, a variety of procedures may be employed in the isolation and purification of rapamycin, for example, solvent extraction, partition chromatography, silica gel chromatography, liquid-liquid distribution in a Craig apparatus, and crystallization from solvents. Solvent extraction procedures are preferred for commercial recovery inasmuch as they are less time consuming and less expensive.

Generally speaking, rapamycin may be harvested by one of the following methods.

a. The fermentation mixture is extracted with a substantially water-immiscible solvent, preferably a lower alkanol, for example n-butanol, n-pentanol or the commercial mixture of pentanols known as "Pentasol" or n-hexanol, or a substantially water-immiscible lower alkyl lower alkanoate, for example, ethyl acetate, butyl acetate, amyl acetate or the commercially available mixture of amyl acetates, or a substantially water-immiscible halogenated aliphatic hydrocarbon, for example, chloroform, methylene dichloride or dichloroethane. The extracts are dried and concentrated under reduced pressure to yield an oily residue which is in turn extracted with a water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. Said last-named extracts are filtered through diatomaceous earth ("Celite"), and the filtrate concentrated under reduced pressure to yield an oily residue containing crude rapamycin.

b. The fermentation mixture is filtered through a pad of diatomaceous earth (Celite) and the filter cake containing the mycelium is extracted as described below under (c). The filtrate, i.e. the mycelium-free fermentation mixture, is extracted several times with a substantially water-immiscible solvent, for example, a lower alkanol, lower alkyl lower alkanoate or halogenated aliphatic hydrocarbon as exemplified above in section (a). The extracts are dried and concentrated under reduced pressure to yield an oily residue which is extracted with a water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. Said last-named extracts are treated in the same manner as described above under (a) to yield an oily residue containing crude rapamycin.

c. The mycelium is separated from the fermentation mixture and extracted with a suitable water-miscible solvent, preferably a lower alkanol, for example methanol or ethanol. The extract is concentrated by evaporation to the aqueous phase, which in turn is extracted with a substantially water-immiscible solvent, such as a lower alkyl lower alkanoate, halogenated aliphatic hydrocarbon or a substantially water-immiscible lower alkanol as described above or an aromatic hydrocarbon, for example benzene or toluene. The latter extract is evaporated under reduced pressure to yield an oily residue containing crude rapamycin.

The crude rapamycin obtained by any of the processes described in sections (a), (b) or (c) is then purified by a variety of methods, for example, see above. Preferred methods include absorption of the crude rapamycin on an absorbent, for instance charcoal or silica gel from a solution in a substantially non-polar, first solvent, followed by elution therefrom with a second solvent, more polar than said first solvent.

DETAILS OF THE INVENTION

Rapamycin is useful as an antifungal agent against a number of pathogenic fungi; for example, *Candida albicans*, and other Candida species, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Aspergillus sp.*, and *Sporotrichum sp.*.

The inhibitory activity of rapamycin is especially pronounced against *Candida albicans* and said last organism may be used advantageously for assay purposes.

The antifungal activity of this compound is demonstrable in standard tests used for this purpose, for example, in the tests described in "Antiseptics, Disinfectants, Fungicides and Sterilization", G. F. Reddish, Ed., 2nd ed., Lea and Febiger, Philadelphia, 1957 or by D. C. Grove and W. A. Randall in "Assay Methods of Antibiotics", Med. Encycl. Inc., New York 1955.

When the antibiotic of this invention is employed as an antifungal agent in warm-blooded animals, e.g. rats, it may be used alone or in combination with pharmaceutically acceptable carriers, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard biological practice. For example, an antifungally effective amount of the antibiotic may be administered orally in solid form containing such excipients as starch, sugar, certain types of clay and so forth. Similarly, such an amount may also be administered orally in the form of solutions or suspensions, or the antibiotic may be injected parenterally. For parenteral administration the antibiotic may be used in the form of a sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

The dosage of the present antibiotic will vary with the form of administration and the particular compound chosen. Furthermore, it will vary with the particular host under treatment. Generally, treatment is initiated with small dosages substantially less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. In general, the compound of this invention is most desirably administered at a concentration level that will generally afford antifungally effective results without causing any harmful or deleterious side effects and preferably at a level that is in a range of from about 1.0 mg to about 250 mg per kilo per day, although as aforementioned variations will occur. However, a dosage level that is in the range of from about 10 mg to about 100 mg per kilo per day is most desirably employed in order to achieve effective results.

In addition, the agent may be employed topically. For topical application it may be formulated in the form of solutions, creams, or lotions in pharmaceutically acceptable vehicles containing 0.1–5 per cent, preferably 2 per cent of the agent, and may be administered topically to the infected area of the skin.

Rapamycin may also be used for cleaning and disinfecting laboratory equipment, surgical instruments, locker rooms, or shower rooms of sensitive fungus organisms. For such purposes it is preferred to use 0.1–10% solutions of rapamycin in a lower alkanol, preferably methanol, diluted with 10–100 volumes of water containing 0.001–0.1% of a non-ionic surface-ac-

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