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Production of Rifamycins by a *Nocardia* Sp.

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Abstract

Rifamycins, which was recently discovered by Sensi et al. in the culture broth of a *Streptomyces* sp., has been obtained from the culture broth of a *Nocardia* sp., C-521 strain. The taxonomy and fermentation of the new *Nocardia* sp. were described. To isolate the antibiotic complex, the broth was extracted with petroleum ether. The petroleum ether extract was further purified by silica gel chromatography to give C-521 substance A, which was proved to be identical with rifamycin O. By distribution of the petroleum ether-insoluble residue between ethyl acetate and phosphate buffer solution (pH 7.0—7.2), a crude product of C-521 substance B was obtained from the latter. This was presumed to be rifamycin B from the chemical behavior. Evaporation of the ethyl acetate layer followed by further purification through countercurrent distribution, afforded another new antibiotic (C-521 substance C). Antibactrial spectrum of the C-521 substance A (rifamycin O) was presented.

I. Introduction

During our antibiotic screening program, a soil isolate identified as a *Nocardia* sp. was found to produce rifamycin O and several related antibiotics. Rifamycin complex was isolated by Sensi et al.[1] from the fermentation broth of *Streptomyces meditarranei*, and it was reported that rifamycin B [2] can be converted into rifamycin SV [3] through rifamycin O [4] and rifamycin S. Among these rifamycins, rifamycin

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1) P. Sensi, P. Margalith and M. T. Timbal: Farmaco (Ed. Sc.), **14** (2), 146 (1956); P. Margalith and H. Pagani: Microbiol., **9** (4), 320 (1961); Structures: W. Oppolzer, V. Prelog and P. Sensi: Experientia, **20**, 336 (1964). J. Leitich, W. Oppolzer and V. Prelog: ibid., **20**, 343 (1964).
2) P. Sensi, A. M. Greco and R. Ballotta: Antibiotics Annual, 1959—1960, 262 (1960); P. Margalith and H. Pagani: Appl. Microbiol., **9** (4), 325 (1961).
3) P. Sensi, R. Ballota, A. M. Greco and G. G. Gallo: Farmaco (Ed. Sc.), **16** (3), 165 (1961).
4) P. Sensi, R. Ballotta and A. M. Greco: Farmaco (Ed. Sc.), **15** (4), 228 (1960).

SV was shown to be of therapeutic value and it is now employed for the treatment of staphylococcal infections and of tuberculous and leprotic infections. S. Sugawara et al.[5] described the production of rifamycin O by a strain of *Streptomyces*. However, to our knowledge, it has never been reported that rifamycin complex is produced by a *Nocardia*. This paper describes the taxonomic characteristics of the strain, production of rifamycin complex, isolation of rifamycin O and another antibiotic and their chemical and biological properties.

II. Taxonomy

The strain, C-521, was isolated from a soil at Ueno Park, Tokyo, in 1962, and was determined to be a strain of Genus *Nocardia* based on the following characterization. Identity was checked with species described in "Classification of *Nocardia* according to the System of Waksman and Henrici" in the Vol. II of "The *Actinomyces*" by S. Waksman 1961. It has been revealed that the strain belongs to BII group, however, that isolate appeared to differ morphologically and physiologically from any previously described species.

This microorganism is characterized by yellowish vegetative mycelium and colorless to pale orange aerial growth. The appearance is suggestive of a *Nacardia*. Microscopic observation revealed that no sporophore is produced on mycelium, and the division velocity is faster than that of *Streptomyces*. The width of mycelia is about 0.8—1.0 μ. Ziel-Neelsen method for staining acid-fast bacilli is negative.

The cultural characteristics of the strain C-521 are recorded in Table 1.

The strain produces nitrite from nitrate, but solubilizatin of calcium malate, tyrosinase reaction and melanin formation are not observed. The sugar utilization pattern of the strain in the synthetic medium of Pridham Gottlieb[6] as follows: Positive: glucose, fructose, galactose, rhamnose, xylose, arabinose, lactose, mannose, maltose, mannnitol, saccharose, raffinose, inulin, glycerol and inositol. Negative: sorbitol, salicin and dulcitol.

Infrared spectral studies of the mycelia of C-521 according to the method of Arai et al.[7] gave the absorptions characteristic of *Nocardia* type A for the first region, C or D for second and third region and D or E for the fourth conformation region, and gas chromatography of the amino acid components of the mycelia by the method of Becker et al.[8] indicated the presence of arabinose, mannose, galactose and glucose. This result suggests that C-521 is more closely related with *Mycobacteria* than with *Actinomyces* and *Streptomyces*. Furthermore, when the mycelia was hydrolyzed

5) S. Sugawara, K. Karasawa, M. Watanabe and T. Hidaka: J. Antibiotics, A **17** (1), 29 (1964).

6) T. G. Pridham and D. Gottlieb: J. Bact., **56**, 104 (1948).

7) T. Arai, S. Kuroda and Y. Koyama: J. Gen. Appl. Microbiol., **9**, 119 (1963).

8) B. Becker, M. P. Lechevalier, R. E. Gordon and H. A. Lechevalier: Appl. Microbiol., **12**, 421 (1964).

Table 1. Cultural Characteristics of C-521 Strain.

Medium	Characteristics
1. Glycerol nitrate agar 27°C	Pale yellowish orange to yellowish orange vegetative; white to pale orange aerial mycelium; pale-orange soluble pigment.
2. Glucose-asparagin agar 27°C	Yellowish orange to dark orange vegetative, pale orange to pale brown aerial mycelium; yellow to pale brown soluble pigment.
3. Starch agar 27°C	Pale yellowish brown to dull yellowish orange vegetative, white to brownish white aerial mycelium; no soluble pigment; weak hydrolysis.
4. Calcium malate agar 27°C	Colorless growth; no aerial mycelium; no soluble pigment.
5. Peptone solution (sodium nitrate 0.2%), 27°C	Colorless growth; no aerial mycelium; no soluble pigment.
6. Nutrient agar 37°C	Dark yellowish orange vegetative mycelium; no aerial mycelium; no soluble pigment.
7. Loeffler's coagulated serum 37°C	Orange to deep yellowish orange wrinkles vegetative; no aerial mycelium; no soluble pigment; no liquefaction.
8. Blood agar 37°C	Cream to yellowish brown vegetative mycelium; no aerial mycelium; positive hemolysis.
9. Gelatin 27°C	Colorless growth; no aerial mycelium; no soluble pigment; no liquefaction.
10. Egg medium 37°C	Orange to deep yellowish orange growth wrinkled; no aerial mycelium; no soluble pigment.
11. Skimmed milk 37°C	Cream to dull yellowish orange ring; no aerial mycelium; no soluble pigment; positive coagulation and liquefaction.
12. Potato plug 27°C	Dull yellowish orange to yellowish orange wrinkled growth; scanty white aerial mycelium; no soluble pigment.
13. Carrot plug 27°C	Yellowish orange to dark reddish orange growth; white aerial mycelium; dark reddish orange soluble pigment.
14. Cellulose medium 27°C	Dull orange to yellowish red growth; white to pale orange aerial mycelium; no soluble pigment; no cellulose decomposition.

with 6 N hydrochloric acid and the hydrolyzate was subjected to paper chromatography, the presence of D,L-diaminopimelic acid was detected, indicating that the strain C-521 belongs to *Nocardia*. *Streptomyces* and *Actinomyces* sp. generally afford L,L-diaminopimelic acid.

III. Fermentation

Our medium for the fermentation consists of: glucose 1.0, soybean meal 1.5, glycerol 0.5 and sodium chloride 0.3 %. In the laboratory, the culture was grown in 500-ml. flasks containing 100-ml. of medium, inoculated generally with spore suspension and incubated at 28°C on a rotatory shaker. In the pilot plant, a 30-L fermentor was used, equipped for aseptic operation with the usual aeration, agitation, temperature and antiform controls. In both cases of shaking and tank fermentation, antibiotic activity reached a maximum in about three days.

IV. Isolation and Properties

The culture was harvested after reaching maximum activity. Mycelium was removed by filtration and the filtrate (about 12 L) was extracted thrice with 1/6 volumes of chloroform. The combined chloroform extracts were evaporated in vacuum to give a dark-brown oily residue (4 g., 44 units/mg., 60 % on the basis of activity; rifamycin O 1mg. = 1000 units). When the residue was extracted with petroleum ether (200 ml.), C-521 substance A (rifamycin O) could be separated from other antibiotics. Evaporation of the petroleum ether extract was followed by silica gel chromatography (Mallinckrodt Co., 100 mesh). After washing the column with petroleum ether, elution was effected with benzene. The active fractions were combined, concentrated in vacuum and allowed to stand at room temperature to give a crude yellow solid of rifamycin O; yield 59.0 mg (33.5 %). Washing with ether followed by recrystallization from a small quantity of methanol gave fine yellow needles, which darkened at about 180—185°C; UV absorption maxima in ethanol: 275 mμ ($E^{1\%}_{1cm}$ 520), 375 mμ ($E^{1\%}_{1cm}$ 87.6), $[\alpha]^{24}_{D}$ + 71.0 (c 1.0 chloroform).[9]

Anal. Found: C, 61.72; H, 7.00; N, 1.67 %. Calcd. for $C_{39}H_{49}NO_{14}$: C, 61.98; H, 6.53; N, 1.85 %.

The ultraviolet and infrared spectra of the product were identical to those of rifamycin O.

A portion (1.5 g.) of the residual paste (2.1 g), which was insoluble in petroleum ether and separated from rifamycin O, was dissolved in ethyl acetate (200 ml.) and shaken thrice with 50 ml. of phosphate buffer solution (pH 7.0—7.2). The buffer

9) Reported $[\alpha]^{20}_{589}$ +71.5 (c 1, dioxane), UV maxima (in pH 7.3 buffer): 226 mμ ($E^{1\%}_{1cm}$ 365), 273 mμ ($E^{1\%}_{1cm}$ 440), and 370 mμ ($E^{1\%}_{1cm}$ 60): see Ref. 4.

extract was acidified at pH 2.0, extracted with ethyl acetate and the organic layer was dried over anhydrous sodium sulfate. Concentration of the solvent gave a crude powder (365 mg.) of weakly antibacterial activity, which (C-521 substance B) was presumed from the chemical behavior to be crude rifamycin B.

The ethyl acetate layer separated from the phosphate solution was concentrated to a small volume and to this was added ten volumes of petroleum ether to give a dark-brown paste. The precipitate was extracted with benzene and the benzene extract was evaporated in vacuum to leave a dark brown residue (750 mg.) having strongly antibacterial activity. The residue was further purified by countercurrent distribution, using petroleum ether-benzene-0.01 N hydrochloric acid-methanol (15 : 5 : 5 : 10) and fourty transfers. The distribution is shown in Table 2.

Table 2. Countercurrent distribution of the benzene-soluble fraction.

Tube No.	Yield, mg.	Activity, u/mg.*	Yield, %**
1 — 5	17.6	320	3.2
6 — 20	42.1	1820	43
21 — 34	31.4	40	0.7
35 — 37	64.2	36	1.3

*The reference standard is the crystals of rifamycin O 1000 units per mg.

**Yields based upon the petroleum ether-insoluble paste (1.5 g., 118 units per mg.) separated from rifamycin O.

The combined fractions of tube No. 6—20 afforded a dark glassy solid. Further treatment with ethyl acetate-petroleum ether (1 : 14) gave a yellowish brown powder, which inhibited the growth of *Micrococcus pyogenes* var. *aureus* 209 P at a dilution of 0.1 mcg./ml.. The product (C-521 substance C) gave positive Tollens, Fehling and ferric chloride tests and negative ninhydrin test. Bioautographic behavior, using water containing 3.0 % ammonium chloride and 1.0 % ascorbic acid and *Micrococcus pyogenes* var. *aureus* 209 P, was similar to that of rifamycin S and SV, however, the ultra and visible spectra of the product showed such a characteristic which is not shown by rifamycin S or SV ; ultraviolet and visible absorption maxima in methanol : 270 mμ ($E^{1\%}_{1cm}$ 244), 314 mμ ($E^{1\%}_{1cm}$ 174) and 410—450 mμ broad shoulder ($E^{1\%}_{1cm}$ 61). Thin layer chromatography of the product by use of silica gel plates and by development with acetone indicated a slight difference from rifamycin S or SV.

Though all attempts to obtain the abovementioned product in a pure crystalline form have so far been unsuccessful, the abovementioned chemical and physical properties of the product suggested it to be a new antibiotic.

V. Antibacterial Activity

A general dilution technique was used to determine the minimum inhibitory concentration (MIC).

Table 3. Antibiotic Spectrum of C-521 Substance A (Rifamycin O).

Organism	MIC, mcg./ml.	Medium
Micrococcus pyogenes var. *aureus* 209P	0.4	Bouillon
Micrococcus pyogenes var. *aureus*		
resistant to five antibiotics*	3.1	〃
resistant to chloramphenicol	0.4	〃
resistant to streptomycin	0.4	〃
resistant to erythromycin	0.2	〃
resistant to novobiocin	0.4	〃
Streptococcus hemolyticus	25	Bouillon-horse serum (10%)
Streptococcus faecalis	6.3	〃
Diplococcus pneumoniae I	50	〃
Bacillus subtilis	50	Bouillon
Escherichia coli	> 50	〃
Candida albicans	> 50	Sabouraud liquid
Mycobacterium tuberculosis $H_{37}Rv$	3.1	Dubos-Davis modification
Mycobacterium tuberculosis ATCC 607	25 - 50	Glycerol-bouillon

*Penicillin, streptothricin, tetracycline, erythromycin and carbomycin.

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