RESISTAPHYLIN, A NEW ANTIBIOTIC. I

PRODUCTION, ISOLATION AND PROPERTIES

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Resistaphylin is a new antibacterial antibiotic produced by an identified species of *Streptomyces antibioticus* No. K-869. The active substance in the fermented broth can be isolated by solvent extraction followed by chromatography on an alumina column. It exhibits marked inhibitory activity against Gram-positive bacteria. Resistaphylin is a new antibiotic, because it differs from other antibiotics in certain physico-chemical properties.

This communication describes the production, isolation and characterization of resistaphylin.

Production and Isolation of Resistaphylin

1. Fermentation

The inoculum of *Streptomyces antibioticus* No. K-869 was prepared in K-1 flasks[1] on a rotary shaker at 27°C for 48 hours. The growth (300 ml) was used to inoculate a jar fermentor containing 15 liters of medium after growing at 27°C for 48 hours with adequate aeration and agitation. The culture broth in the jar fermentor was transferred to a tank fermentor containing 400 liters of medium. The culture was grown in the large fermentor at 27°C for approximately 68~72 hours using usual conditions of aeration with agitation.

The medium used throughout had the following composition (g per liter): glucose 45, soy bean flour 40, yeast extract 3, $(NH_4)_2 \cdot SO_4$ 2, NaCl 2, KCl 4 and $CaCO_3$ 2.

Antibiotic activity was determined using a paper-disc bioassay with *Staphylococcus aureus* as the indicator organism.

2. Extraction and Purification

The harvested broth (400 liters) was filtered using diatomaceous earth. The clarified broth was isolated with ethyl acetate, and the mycelial cake was extracted with acetone, concentrated *in vacuo*, and further extracted with ethyl acetate. The ethyl acetate extracts were combined and washed with distilled water, and then concentrated under reduced pressure. Addition of hexane to the concentrated extract precipitated about 30 g of crude powder.

The crude powder was dissolved in a small amount of ethyl acetate, and then passed through an acidic alumina column. The column was then washed several time with benzene. Development and elution were carried out with ethyl acetate. The eluted active fractions were combined and concentrated to a small volume. Addition of hexane to the concentrated extract precipitated about 3.55 g of pure resistaphylin. The isolation process is outlined in Fig. 1.

Physico-chemical Properties of Resistaphylin

Resistaphylin is a colorless crystalline powder showing the following physical and chemical properties:

(1) Solubility: Soluble in chloroform, ace-

Fig. 1. Extraction and purification of resistaphylin

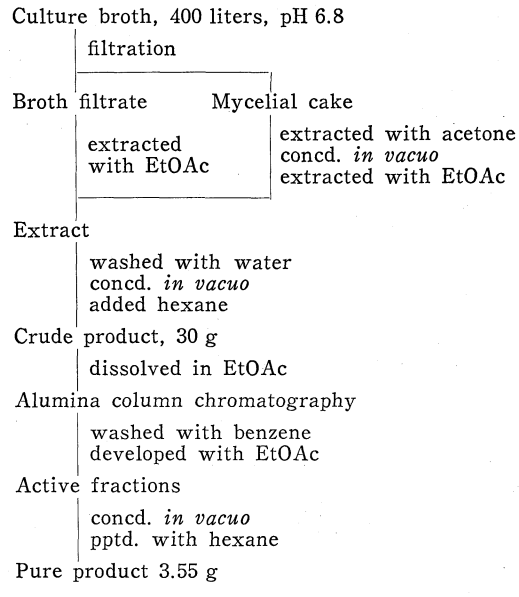


Fig. 2. Ultraviolet absorption spectrum of resistaphylin in methanol.

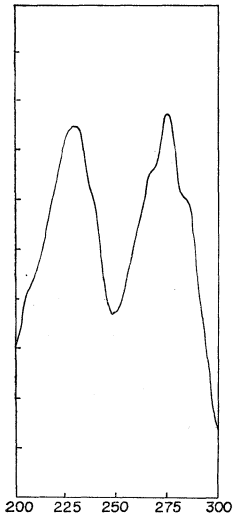
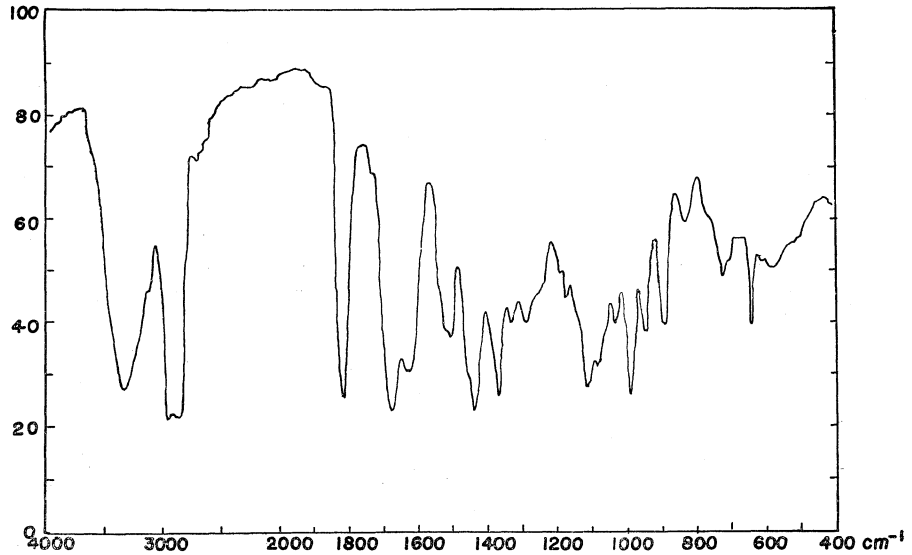


Fig. 3. Infrared absorption spectrum of resistaphylin (in nujol)



tone, ethyl acetate, ethanol, and pyridine. Slightly soluble in benzene, ether and water. Insoluble in hexane and petroleum ether.

(2) Melting point: 91~92°C.

(3) Optical rotation: $[\alpha]_D^{22}$ +65° (*c* 1, methanol).

(4) Color reaction: Positive FEHLING, TOLLENS and LEMIEUX reactions. Negative ninhydrin, $FeCl_3$, biuret and SAKAGUCHI reactions.

(5) Thermostability: When resistaphylin was dissolved in methanol-water and kept at 100°C for 5 minutes, it was stable at neutral, but unstable at acidic and basic pH.

(6) Elemental analysis:

Found: C 62.10, N 7.41, N 6.05, O 24.40 %

Calcd. for $C_{24}H_{34}N_2O_7$: (M. W. 462.53).

C 62.32, H 7.41, N 6.06, O 24.21 %

(7) The ultraviolet spectrum of resistaphylin in methanol (Fig. 2) shows maxima at

Fig. 4. NMR spectrum of resistaphylin (in $CDCl_3$)

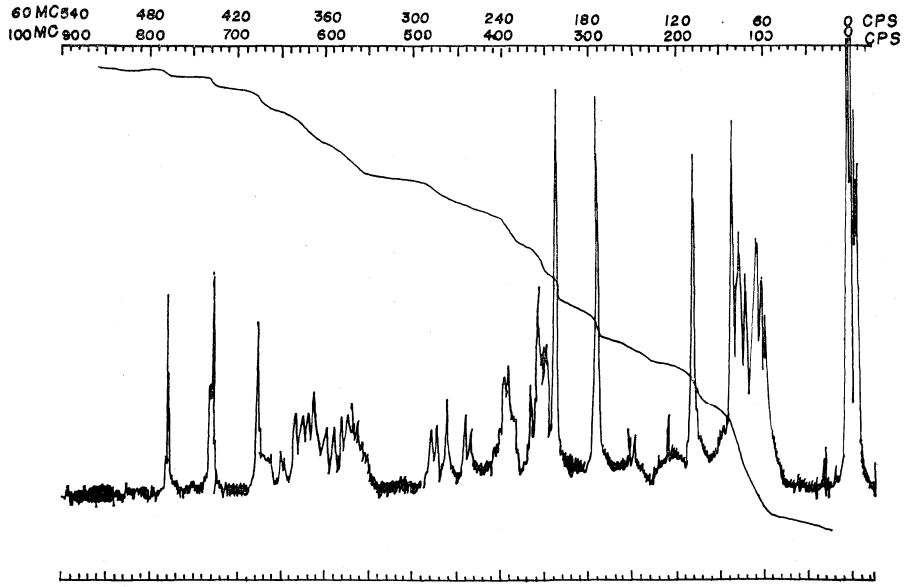


Table 1. Thin-layer chromatography of resistaphylin

Solvent system	Rf value	Solvent system	Rf value
Ethyl acetate	0.45	Acetone	1.00
Benzene	0.00	Ethyl acetate, benzene (1 : 1)	0.00
Chloroform	0.00	Methanol, ethyl acetate (1 : 10)	0.90

The movement of resistaphylin was indicated by bioautography against *Staphylococcus aureus*.

230 mμ ($E^{1\%}_{1cm}$ 748), 267 mμ (shoulder), 275 mμ ($E^{1\%}_{1cm}$ 750) and 285 mμ (shoulder), indicating the presence of a triene group[2] in the molecule.

(8) The infrared spectrum (Fig. 3): It shows the presence of OH and NH functions at 3330 cm^{-1}, β-lactone function at 1820 cm^{-1} and amide group at 1690 and 1630 cm^{-1}. The strong band at 995 cm^{-1} is attributable to the triene grouping (CH out-of-plane deformation vibration).

(9) NMR spectrum (Fig. 4): It shows signals at 3.37 ppm due to methoxyl group, and at 1.3~0.9 ppm due to CH_3-C group (in $CDCl_3$, 100 MHZ).

(10) Bioautography of resistaphylin. The Rf values of resistaphylin on silica gel TLC in various solvents are shown in Table 1.

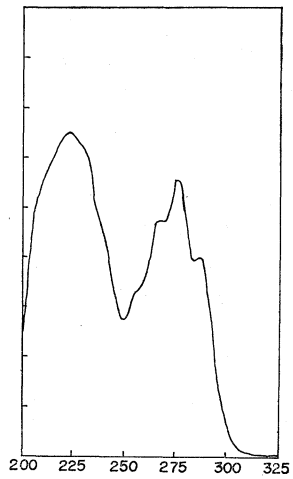
(11) Acetylation: Resistaphylin (200 mg) was acetylated with 2 ml of acetic anhydride in 2 ml of pyridine. After keeping at room temperature over-night the reaction mixture was concentrated *in vacuo*, and the residue was treated with hexane to give 164 mg crude acetate. The crude acetate was purified by chromatography using a neutral alumina column with benzene - ethyl acetate (1 : 1) as an eluting solvent the acetylated was crystallized resistaphylin from chloroform - ether to give colorless crystals m.p. 79~80°C, and was biologically inactive.

Found : C 61.57, H 7.28, N 5.52, O 24.89 %
Calcd. for $C_{26}H_{36}N_2O_8$: (M.W. 504.56)
C 61.89, H 7.19, N 5.55, O 25.37 %

Molecular weight determination by the RAST method using 3-bromocamphor gave a value of 486.

The ultraviolet spectrum of acetylated resistaphylin in methanol (Fig. 5) shows maxima at 223 mμ ($E^{1\%}_{1cm}$ 650), 267 mμ (shoulder), 275 mμ ($E^{1\%}_{1cm}$ 550) and 285 mμ (shoulder).

Fig. 5. Ultraviolet absorption spectrum of acetylated resistaphylin in methanol.



Antimicrobial Spectrum of Resistaphylin

The minimum inhibitory concentration of resistaphylin against various microorganisms was determined using a serial agar dilution method with brain heart infusion agar plate, except on potato sucrose agar for *Xanthomonas oryzae*, on glycerine nutrient agar for

Table 2. Antimicrobial spectrum of resistaphylin

Test organisms	Minimum inhibition concentration (mcg/ml)
Bacillus subtilis	0.05
Bacillus cereus ATCC-10702	0.1
Staphylococcus aureus	0.006
Staphylococcus aureus (resistant)*	0.025
Staphylococcus epidermidis	0.006
Sarcina lutea	0.005
Micrococcus flavus	0.025
Escherichia coli	100
Klebsiella pneumoniae	100
Pseudomonas aeruginosa	100
Shigella dysenteriae	100
Xanthomonas oryzae	0.05
Mycobacterium smegmatis	100
Trichophyton asteroides	100
Trichophyton rubrum	100
Cryptococcus neoformans	50
Candida albicans	100
Aspergillus fumigatis	100

* Resistant against penicillin, chloramphenicol, tetracycline, erythromycin, kanamycin, streptomycin and sulfonamide.

Mycobactrium smegmatis and on SABOURAUD's agar plate for yeasts. The results are given in Table 2.

Resistaphylin was very active against Gram-positive bacteria, and showed only slight activity against some mycobacteria, fungi and yeast.

Discussion

Resistaphylin contains a conjugated triene system and is very active against Gram-positive bacteria. Mycotrienin[3] has been reported to have a conjugated triene system in the molecule and extracted with organic solvent.

However, mycotrienin is active against fungi and yeast whereas resistaphylin is active primarily against Gram-positive bacteria.

Acknowledgement

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