DACTYLARIN, A NEW ANTIPROTOZOAL ANTIBIOTIC FROM *DACTYLARIA LUTEA*

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A new crystalline antibiotic, dactylarin, $C_{16}H_{16}O_6$, was isolated from the culture broth of the hyphomycete *Dactylaria lutea* ROUTIEN. Isolation, purification, properties and structure of the new antibiotic are described. Dactylarin exhibits antiprotozoal activity against *Leishmania braziliensis* and *Entamoeba invadens* and is slightly active against Gram-positive bacteria.

Our screening for new antiprotozoal antibiotics as described by NEMEC *et al.*[1] has revealed a very high incidence of cultures with antiprotozoal activity in the *Fungi imperfecti*. Nearly all species of the *Fungi imperfecti* tested are "predacious fungi" characterized by their ability to trap and kill nematodes and/or protozoa[2], *Dactylaria lutea* ROUTIEN, obtained from the culture collection of the Centraalbureau voor Schimmelcultures in Baarn. The Netherlands, and previously reported to exhibit antiprotozoal activity[1] was studied in more detail. Isolation and purification by chromatography using silica gel gave an active red crystalline substance which we call dactylarin. On the basis of physical and chemical data, the structure of dactylarin has been established. With regard to its physico-chemical characteristics and its biological properties, we conclude that dactylarin is a new antibiotic. In this paper, the fermentation, isolation procedure and characterization of dactylarin are reported.

Production and Isolation

Dactylaria lutea was maintained on corn meal agar slants after cultivation for 7 days at 24°C. The slants were rinsed with water and 10 ml of the spore suspension were used for inoculating 100 ml of nutrient medium in 500 ml shake flasks. The malt extract medium diluted with tap water to 3.5°Bx was found to be suitable for development of inoculum and production of dactylarin. Prior to sterilization, the pH of the medium was adjusted to 6.5. The seed culture was incubated on a reciprocal shaker at 29°C for 6 days. From the inoculum thus obtained, 10 ml portions were used to inoculate flasks containing per 100 ml of the medium for submerged production of the antibiotic. The highest antibiotic activity was found after 11~14 days of cultivation on a reciprocal shaker at 29°C.

The antiprotozoal activity was found mainly in the broth filtrate and was assayed during the fermentation and isolation process by the estimation of its inhibitory effect against *Leishmania braziliensis*.

Thin-layer chromatographic methods were used for identification of the antibiotic during fer-

mentation. Due to the UV absorbing chromophores in the molecule of dactylarin, visualization of the spots could be achieved by the luminescent indicator method using silica gel foils with an inert luminescent indicator (Silufol UV_{254}^{R}, product of Lachema, Brno, Czechoslovakia); spots were then visualized under UV light.

The culture filtrate was acidified to pH 3 with 2 N HCl and then extracted twice, each time using one-third volume of ethyl acetate per volume of filtrate. After separation, the extracts containing the active substance were pooled and filtered and the yellow solution was shaken with one-third volume of water adjusted with sodium hydroxide to pH 9. The antibiotic was thus reextracted into the aqueous phase which turned intensly red. Following separation, the aqueous layer was acidified to pH 3 and the active substance reextracted with an equal volume of ethyl acetate. The solvent phase was separated and concentrated to dryness under reduced pressure. The residue was dissolved in a small volume of ethyl acetate and chromatographed on a column of silica gel which was developed with a chloroform-ethyl acetate mixture (1 : 1). The active fractions were collected, concentrated to a small volume *in vacuo* and crystallized. Crude crystals of dactylarin were obtained by keeping the solution in a refrigerator for one or two days. Red crystalline plates were obtained by recrystallization from hot chloroform-ethyl acetate or chloroform-*n*-butanol followed by two washings with ethanol and ether.

Physical and Chemical Properties

Dactylarin is a weakly acidic compound which forms red plates, m.p. 201~205°C (with decomposition). It is unstable in strongly acidic solutions. Dactylarin is quite soluble in acetone, ethyl acetate and dimethylsulphoxide, less soluble in *n*-butanol and chloroform, slightly soluble in methanol, benzene and water, and insoluble in petroleum ether and ethylether. The antibiotic is an optically inactive compound. The R_f values in TLC using silica gel (Silufol UV_{254}^{R}) were:

		R_f
n-butanol-chloroform	1:9	0.59
methanol-chloroform	1:9	0.73
acetone -chloroform	7:3	0.76

Elemental analysis of the antibiotic was as follows:

Found:	C 62.76,	H 5.31,	O 31.93
Calcd. for $C_{16}H_{16}O_6$:	C 63.15,	H 5.30,	O 31.55

The antibiotic contains no nitrogen, sulphur or halogens. The empirical formula $C_{16}H_{16}O_6$ was established by the parent peak in the mass spectrum at *m/e* 304 and by elemental analysis. Specific rotation and ORD measurements were made in ethyl acetate on a Jasco UV/ORD-5 spectropolarimeter. The IR spectrum was recorded in a KBr-disc and in nujol on a Carl Zeiss UR-20 instrument. The NMR spectrum of the antibiotic was recorded on a Tesla BS 487 A instrument (80 MHz) in d_6-DMSO solution containing HMDS. The UV spectrum was measured in *n*-butanol on a Perkin-Elmer model 450 spectrophotometer; the mass spectrum was measured in an MCH 1306(USSR) spectrometer. The ionization energy was maintained at 70 eV.

The IR spectrum, presented in Fig. 1, shows an absorption band at 1645 cm^{-1} assigned to the stretching vibration of a conjugated carbonyl group and two absorption bands at 3460 cm^{-1} and 3515 cm^{-1} assigned to the stretching vibration of O-H bonds. The band at higher wavenumbers can

Fig. 1. Infrared spectrum of dactylarin

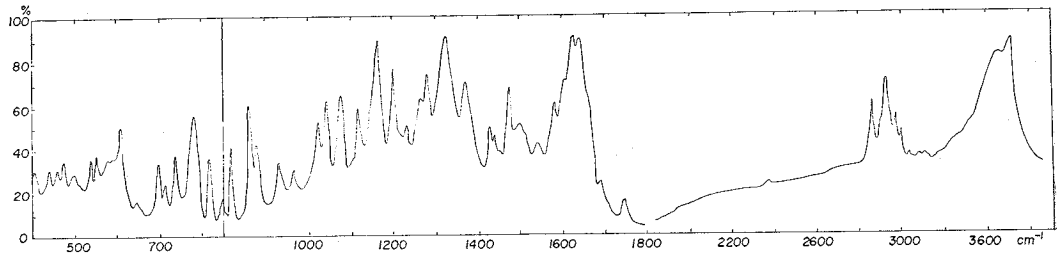


Fig. 2. NMR spectrum of dactylarin (in d_6-DMSO)

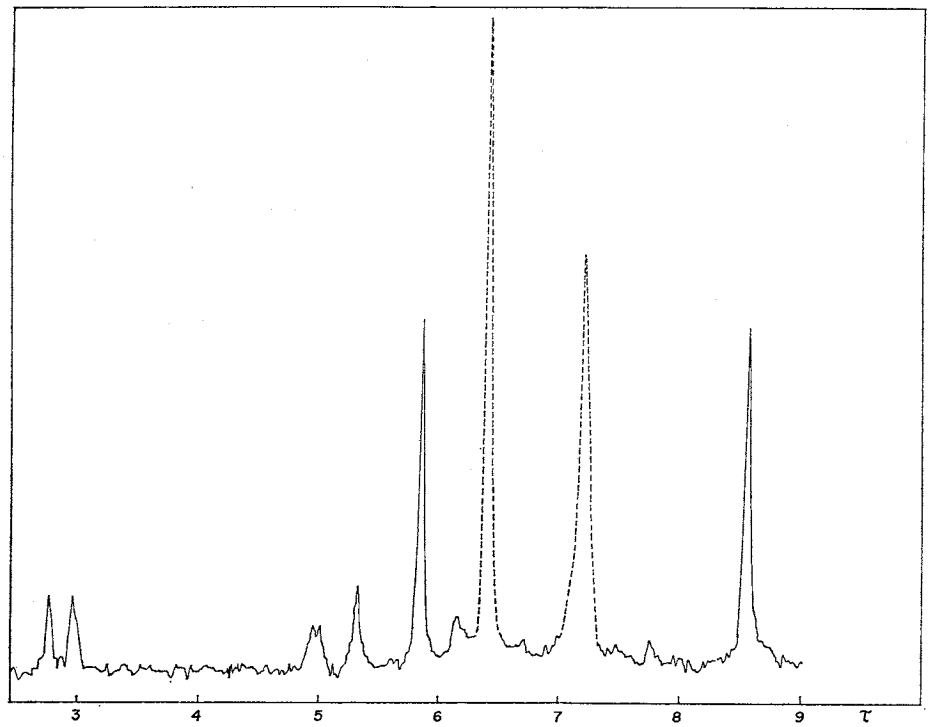
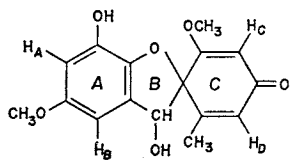


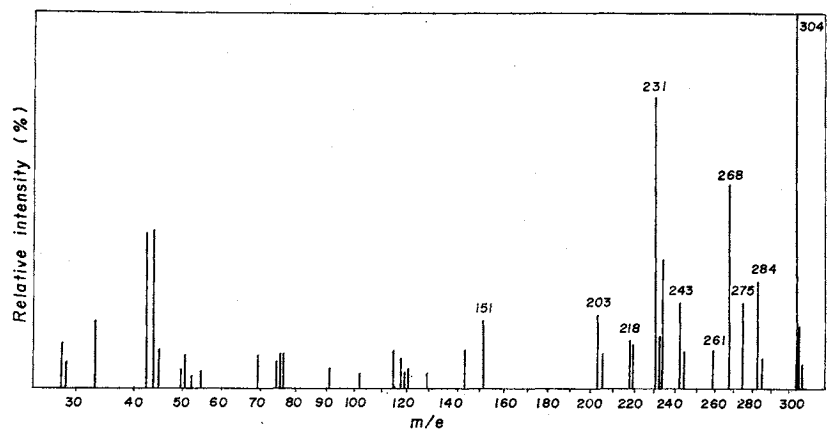
Fig. 3. The structure of dactylarin



be assigned to the stretching vibration of the O-H group intramolecularly bounded to an oxygen atom. Well-resolved bands in the region of 2860~3100 cm^{-1} clearly indicate the presence of C-H bonds. The bands at 2862 cm^{-1} and 2935 cm^{-1} are assigned to the stretching vibration of C-H bonds in methyl groups. Other bands in this region can be assigned as follows: the band at 2925 cm^{-1} (-C-H) and the bands at higher wavenumbers are assigned to the stretching vibration of =C-H bonds.

The UV and visible spectra suggest that the antibiotic is an aromatic compound containing a conjugated carbonyl group. The following λ_{max} (in phosphate buffer at pH 7.38) are observed: 218 nm (ε=29.800); 266 nm (ε=13.600); 290 nm (ε=8.650) and 435 nm (ε=3.720). In 0.1 N aqueous NaOH a shift to longer wavelengths is observed pointing out the presence of phenolic group.

Fig. 4. Mass spectrum of dactylarin



In the NMR spectrum of dactylarin, shown in Fig. 2, only singlets are observed indicating isolated protons. The singlet at -2.45τ can be assigned to the OH proton of the phenolic group situated in ortho position in respect to the ethereal oxygen (Fig. 3). Two singlets at 2.76 and 2.97τ could reasonably be assigned to the H_A and H_B aromatic protons, respectively. The singlets at 4.95 and 5.01τ are assigned to the H_C and H_D protons of the ring C and the signal at 5.32τ is assigned to the secondary hydroxyl proton and a sharp singlet at 5.87τ is due to the OCH_3 protons. The signal of the >CH–group is observed at 6.15τ. The singlets at 7.22 and 8.57τ are assigned to the signals of protons of the $-OCH_3$ and $-CH_3$ groups, respectively. Unfortunately, the signal at 7.22τ is overlapped by absorption by the solvent. The NMR spectrum of dactylarin recorded for the trifluoracetic solution points out that the antibiotic is being decomposed.

Table 1. Antimicrobial spectrum of dactylarin

Test organism	Minimum inhibitory concentration (mcg/ml)
Leishmania braziliensis	0.5
Entamoeba invadens	2.5
Trypanosoma cruzi	100
Tetrahymena pyriformis	>200
Staphylococcus aureus Sta-2	25
Bacillus subtilis SDPC	50
Bacillus cereus	50
Escherichia coli	>100
Proteus vulgaris B-16	>100
Saccharomyces cerevisiae	>100
Kloeckera corticis	>100
Oospora lactis	>100
Torulopsis utilis	>100
Candida albicans	>100
Candida parapsilosis	>100
Candida pseudotropicalis	>100
Aspergillus niger	>100
Aspergillus fumigatus	>100
Penicillium chrysogenum	>100

The mass spectrum of dactylarin, reported in Fig. 4, exhibits, in addition to the molecular ion at *m/e* 304, a number of prominent peaks, M-20 (*m/e* 284), M-29 (*m/e* 275), M-36 (*m/e* 268), M-61 (*m/e* 243), M-73 (*m/e* 231), M-87 (*m/e* 218), M-101 (*m/e* 203) and M-153 (*m/e* 151).

On the basis of the data presented, the structure of dactylarin could be represented as shown in Fig. 3.

Biological Properties

The antimicrobial spectrum of dactylarin is shown in Table 1. Antibacterial and antifungal activities were determined by the agar dilution streak method using meat-extract peptone medium for bacteria and malt extract medium for fungi. Incubation was at 29°C for 24~72 hours. Antiprotozoal activity was determined using the method described by NEMEC *et al.*[3]

Dactylarin has strong inhibitory activity *in vitro* against *Leishmania braziliensis* and *Entamoeba invadens* and is slightly active against Gram-positive bacteria. No activity was observed against *Trypanosoma cruzi*, *Tetrahymena pyriformis*, Gram-negative bacteria, yeasts or molds.

Discussion

Up to the present investigation, no antibiotic had been isolated from *Dactylaria lutea* ROUTIEN, which belongs to the group of "predacious fungi".

Dactylarin is not identical with any known antibiotic exhibiting antiprotozoal activity or with any described fungal metabolite. On the basis of its physico-chemical and biological properties, dactylarin is considered to be a new antibiotic[4,5].

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