FUSIDANE ANTIBIOTICS PRODUCED BY DERMATOPHYTES

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Isolates of Microsporum canis, Microsporum gypseum and Epidermophyton floccosum were observed to produce antibacterial activities under cross-resistance to fusidic acid. The activity from E. floccosum was shown to be due to fusidic acid, diketofusidic acid and 3-ketofusidic acid. Possible contributions of these antibiotics to microbial interaction during dermatophytosis is discussed.

Fusidic acid is a therapeutically useful antibiotic, being particularly active against penicillin resistant staphylococcal infections, and has been isolated from Fusidium coccineum3, Mucor ramannianus2, Cephalosporium lamellaecula3 and Paecilomyces fusidioides4. The structure of fusidic acid (Fig. 1) is characterised by the presence of the cyclopentanoperhydrophenanthrene ring system. Other antibiotics possessing this ring system, and therefore belonging to the fusidane (protostane) class of natural products, include helvolic acid, cephalosporin P, and viridominic acids A, B and C5.

As a result of a program initiated in our laboratory to screen dermatophytes for the synthesis of non-β-lactam antibiotics, isolates of Microsporum canis, Microsporum gypseum and Epidermophyton floccosum were observed to produce antibacterial activity which was under cross-resistance to fusidic acid. A detailed study of the activity produced by one of the isolates of E. floccosum (strain 8051) revealed that it was due to the presence of three antibiotics which we refer to as EF1, EF2 and EF3. In this paper we describe the isolation and characterisation of these substances and present evidence for their identity with fusidic acid, diketofusidic acid and 3-ketofusidic acid respectively. The possible contribution of the three antibiotics to microbial interaction during dermatophytosis is discussed.

Materials and Methods

Unless otherwise stated chemicals and solvents were of analytical grade and supplied by BDH Chemicals Ltd., Poole, United Kingdom.

Dermatophyte Cultures
The dermatophytes studied were cultured from tinea lesions at St. John's Hospital for Diseases of the
Skin. Isolates were allocated consecutive domestic code numbers. All cultures were maintained on Sabouraud agar (Oxoid) slopes.

**Antibiotic Production Medium**

Optimum antibiotic production was obtained using the fermentation unit medium (FUM) of HOLT and MACDONALD without phenylacetylethanolamine. Fermentations were performed in 2-liter conical flasks containing 400 ml of FUM. Each flask was inoculated with mycelium grown for 5 days at 30°C in a 100-ml conical flask containing 20 ml of Sabouraud broth and incubated for 7 days on a rotary shaker (150 rpm and a 5-cm eccentric throw) at 30°C.

**Spectroscopic Analysis of EF1 and EF2**

Infra-red spectra were determined in KBr discs using a Perkin-Elmer 457 spectrophotometer. Proton magnetic resonance spectra were measured in deuterated chloroform on a Perkin-Elmer R12B 60 MHz spectrophotometer using tetramethylsilane as the internal standard.

**High Performance Liquid Chromatography (HPLC)**

A 5 µl sample of the antibiotic to be assayed (1 mg/ml in methanol) was injected onto a 5 µm Spherisorb ODS column (125 × 4.6 mm) and eluted at a flow rate of 2 ml/minute with methanol - 0.1 M aqueous sodium chloride (70: 20). Detection was at 230 nm.

**Thin-layer Chromatography (TLC)**

Plastic backed TLC sheets (Merck, Silica gel 60) were used without prior activation. The solvent systems employed were those of GODTFREDSEN et al. Detection was by bioautography against Bacillus subtilis NCTC 8236.

**Sodium Borohydride Reduction of EF2 and EF3**

Sodium borohydride (1 mg) in water (20 µl) was added to a solution of the antibiotic (1 mg) in 2-propanol (200 µl). The mixture was allowed to stand at room temperature for 30 minutes and then acidified (pH 3.0), diluted with water (1 ml) and extracted with diethyl ether (1 ml). After removal of the ether in vacuo, the residue was dissolved in 100 µl of methanol and analysed by HPLC and TLC.

**Oxidation of EF1 and EF3**

Potassium dichromate solution (10 µl) (3 g of potassium dichromate dissolved in 2 ml of sulfuric acid and then added to 6 ml of water) was added to a solution of the antibiotic (2 mg) in acetone (200 µl). After 5 minutes at room temperature, the mixture was diluted with water (1 ml) and extracted with diethyl ether (1 ml). After removal of the ether in vacuo, the residue was dissolved in 100 µl of methanol and analysed by HPLC and TLC.

**Results**

Isolation of EF1, EF2 and EF3

The fermentation liquor was separated from the mycelium by centrifugation, adjusted to pH 4 with 6 M HCl, and the three antibiotics extracted into diethyl ether. The solvent extract was washed successively with 0.1 M NaHCO₃ and 0.1 M HCl and then concentrated in vacuo to yield a yellow oil. This residue was dissolved in chloroform and applied to a column of Silica gel 60 (Merck) developed with the same solvent. EF3 emerged from the column first followed by EF2. Elution of EF1 was accomplished by changing the solvent system to 50% chloroform in ethyl acetate (v/v). Each antibiotic was then purified to homogeneity by repeated chromatography over Silica gel 60 using the following solvent systems for EF1, EF2 and EF3 respectively; ethyl acetate - pyridine (99: 1), chloroform - ethyl acetate - acetic acid (90: 9: 1) and chloroform - acetic acid (99: 1).

In a typical extraction 18 mg of EF1, 3 mg of EF2 and 0.5 mg of EF3 were obtained from 10 liters of fermentation liquor.
Spectroscopy

The IR spectra of EFI is shown in Fig. 2. The spectra of EFI and fusidic acid were identical while that of EF2 differed only in the absence of the -OH stretching absorption at 3500 cm⁻¹.

Table 1 shows the chemical shifts for the indicated protons in the 1H NMR spectra of EFI, EF2 and fusidic acid. Assignments were made on the basis of GODTFREDSEN et al. The spectra of EFI and fusidic acid were identical. The signals due to the H-3 and H-11 protons were not present in the 1H NMR spectrum of EF2 while the Me-18 and Me-19 protons resonated at lower fields and the Me-32 protons exhibited a diamagnetic shift. These changes are indicative of the replacement of the -OH group on C-11 with a carbonyl function. In addition, the paramagnetic shift exhibited by the Me-30 protons of EF2 suggests a carbonyl rather than a hydroxyl group at C-3.

Thin-layer Chromatography

The Rf values of the antibiotics tested are given in Table 2. EFI, EF2 and EF3 exhibited the same mobilities as fusidic acid, 3,11-diketofusidic acid and 3-ketofusidic acid respectively.

High Performance Liquid Chromatography

EFI, EF2, EF3, 11-ketofusidic acid and 3-epifusidic acid exhibited the following retention times; 11.10, 5.89, 7.13, 7.90 and 5.72 minutes. Fusidic acid, 3,11-diketofusidic acid and 3-ketofusidic acid co-eluted with EFI, EF2 and EF3 respectively.

Oxidation of EFI and EF3

Oxidation of EFI and EF3 yielded a single antibiotic in each case which was identical to 3,11-diketofusidic acid by HPLC and TLC.
Reduction of EF2 and EF3

Analysis by HPLC and TLC indicated that both EF2 and EF3 were reduced by sodium borohydride to a mixture of fusidic acid and 3-epifusidic acid. The ratio of fusidic acid to 3-epifusidic acid was approximately 10:90 as estimated by comparison of the areas of the peaks eluted during HPLC.

Discussion

The antibiotics EF1 and EF2 produced by *E. floccosum* 8051 have been shown to be identical to fusidic acid and 3,11-diketofusidic acid respectively. Oxidation of EF3 gave 3,11-diketofusidic acid indicative that this antibiotic is a monoketo derivative of fusidic acid. EF3 was indistinguishable from 3-ketofusidic by HPLC and TLC but was clearly different from 11-ketofusidic acid. In addition, sodium borohydride reduction of both EF3 and 3-ketofusidic acid yields predominantly 3-epifusidic acid and a small amount of fusidic acid, whereas reduction of 11-ketofusidic acid gives mainly fusidic acid with a minor amount of 11-epifusidic acid\(^5\)). Therefore, although it was not possible to isolate sufficient EF3 for detailed spectroscopic analysis we believe that this antibiotic is most probably 3-ketofusidic acid.

Although certain strains of *M. gypseum*, *Trichophyton longifusum* and *E. floccosum* have previously been reported to produce an antibiotic with TLC mobility similar to fusidic acid\(^7,8,9\)) the results presented in this paper represent the first conclusive evidence for the production of fusidane antibiotics by a dermatophyte species. A total of ten isolates of *E. floccosum* have been examined by HPLC and TLC for their ability to produce antibiotics EF1, EF2, and EF3. Three isolates produced both EF1 and EF2 while 5 isolates produced only EF1. Production of EF3 was not observed, although this probably reflected the inability of the chromatographic techniques to detect the very low levels in which this antibiotic was produced. All ten isolates produced an antibiotic which was sensitive to \(\beta\)-lactamase (Type 1 from *Bacillus cereus*). In addition, two independent isolates of *M. canis* and an isolate of *M. gypseum* produced an antibiotic which was chromatographically identical to EF1.

Our results suggest that the ability of *E. floccosum* to produce both fusidane antibiotics and penicillins *in vitro* is widespread. This poses the interesting question as to whether a similar production occurs *in vivo*, i.e. in the natural lesion of tinea. Uri et al.\(^{10,11}\) demonstrated penicillin production in fragments of skin from patients infected with *E. floccosum* and it is of interest to note that these authors also referred to the production of antibiotics which were not destroyed by penicillinase. Tinea lesions are frequently subject to bacterial colonisation, particularly by *Staphylococcus aureus*\(^12\)). In tinea pedis two extremes of the disease exist, dermatophytosis simplex in which fungi are dominant and dermatophytosis complex in which *Brevibacterium* prevail\(^13\)). Antibiotic production by *E. floccosum in vivo* therefore has the potential to exert a powerful selective effect on the type of bacterial flora associated with the tinea lesion, especially as the synergistic action of penicillin with fusidic acid has been reported\(^14\)). Indeed both Wallerström\(^15\) and Youseff et al.\(^16\) observed an increase in the resistance of staphylococci to both penicillin and fusidic acid as a result of dermatophyte infection.

Acknowledgments

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