FUSIDANE ANTIBIOTICS PRODUCED BY DERMATOPHYTES

M. J. PERRY**, A. HENDRICKS-GITTINS, L. M. STACEY,
M. W. ADLARD and W. C. NOBLE*

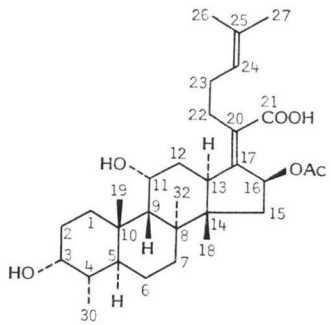
The Polytechnic of Central London,
115, New Cavendish St., London WlM 8JS, United Kingdom
*The Institute of Dermatology, St. JOHN's Hospital for Diseases of the Skin,
Homerton Grove, London E9 6BX, United Kingdom
**National Institute for Biological Standards,
Holly Hill, London NW3, United Kingdom

(Received for publication June 6, 1983)

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 coccineum*[1], *Mucor ramannianus*[2], *Cephalosporium lamellaecula*[3] and *Paecilomyces fusidioides*[4]. 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_1 and viridominic acids A, B and C[5].

Fig. 1. Fusidic acid.



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[8] 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 eccenctric 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.*[5]. 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 $NaHCO_3$ 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.

Fig. 2. The IR spectrum of EF1.

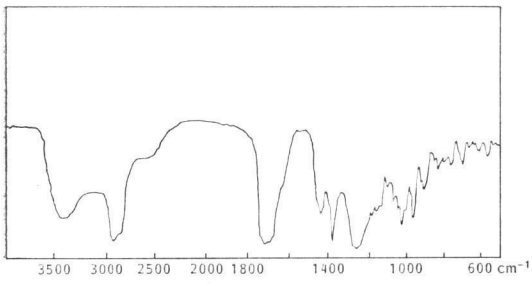


Table 1. Chemical shifts (δ) for the indicated protons in the NMR spectra.

Location of protons	EF1	EF2	Fusidic acid
Me-18	0.89	1.04	0.89
Me-19	0.96	1.14	0.96
Me-26	1.58	1.58	1.58
Me-27	1.66	1.65	1.66
Me-30[a]	0.89 d	1.04 d	0.89 d
Me-32	1.36	1.19	1.36
H-3	3.75 m	—	3.75 m
H-11	4.32 m	—	4.32 m
H-13	3.05 m	2.97 m	3.05 m
H-16[b]	5.86 d	5.91 d	5.86 d
H-24	5.10 m	5.13 m	5.10 m
AcO-16	1.95	1.99	1.95

Multiplicity of signals: Unless indicated all signals are singlets, d; doublet, m; multiplet.

[a] Doublet signals (J=6.5~7.0 Hz).

[b] Doublet signals (J=7.5~8.0 Hz).

Table 2. Thin-layer chromatography of EF1, EF2, EF3 and fusidic acid derivatives.

	Rf value*			
	A	B	C	D
EF1	0.51	0.33	0.40	0.76
EF2	0.78	0.66	0.60	0.80
EF3	0.82	0.71	0.70	0.85
11-Ketofusidic acid	0.72	0.63	0.65	0.78
3-Epifusidic acid	0.59	0.45	0.42	0.75

* Solvent A: Ether - acetic acid (100: 0.5).

B: Ether - dichloromethane - acetic acid (50: 50: 0.5).

C: Dichloromethane - methanol - acetic acid (95: 5: 0.5).

D: Cyclohexane - chloroform - methanol - acetic acid (20: 80: 2.5: 10).

Spectroscopy

The IR spectra of EF1 is shown in Fig. 2. The spectra of EF1 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 EF1, EF2 and fusidic acid. Assignments were made on the basis of GODTFREDSEN *et al.*[5]. The spectra of EF1 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. EF1, EF2 and EF3 exhibited the same mobilities as fusidic acid, 3,11-diketofusidic acid and 3-ketofusidic acid respectively.

High Performance Liquid Chromatography

EF1, 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 EF1, EF2 and EF3 respectively.

Oxidation of EF1 and EF3

Oxidation of EF1 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 β-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 YOUSSEF *et al.*[16] observed an increase in the resistance of staphylococci to both penicillin and fusidic acid as a result of dermatophyte infection.

Acknowledgments

We thank the NRDC for financial support and Leo Pharmaceutical Products for the gift of fusidic acid, 3-ketofusidic acid, 11-ketofusidic acid, 3,11-diketofusidic acid and 3-epifusidic acid.

References

1) GODTFREDSEN, W. O.; S. JAHNSEN, H. LORCK, K. ROHOLT & L. TYBRING: Fusidic acid: A new metabolite. Nature 193: 987, 1962
2) VANDERHAEGHE, H.; P. VAN DIJCK & P. DE SOMER: Identity of ramycin with fusidic acid. Nature 205: 710, 1965
3) ABILDGAARD, K.: Nouveau procede pour preparer l'acide fusidique. French Patent 1,326,076, 1963
4) NICOT, J.: The identity of the organism which produced fusidic acid, an antistaphylococcic antibiotic. C. R. Acad. Sci. Paris 267: 290~292, 1968
5) GODTFREDSEN, W. O.; N. RASTRUP-ANDERSEN, S. VANGEDAL & W. D. OLLIS: Metabolites of *Fusidium coc-*

cineum. Tetrahedron 35: 2419~2431, 1979

6) HOLT, G. & K. D. MACDONALD: Penicillin production and its mode of inheritance in *Aspergillus nidulans*. Antonie van Leeuwenhoek 34: 409~416, 1968
7) ELANDER, R. P.; R. S. GORDEE, R. M. WILGUS & R. M. GALE: Synthesis of an antibiotic closely resembling fusidic acid by imperfect and perfect dermatophytes. J. Antibiotics 22: 176~178, 1969
8) HALLER, B. & W. LOEFFLER: Metabolic products of microorganisms. 71. Fusidic acid from dermatophytes and other fungi. Arch. Mikrobiol. 65: 181~194, 1969
9) WALLERSTRÖM, A.: Production of antibiotics by *Epidermophyton floccosum*. 5. Relation between the 'epidermophyton factor' (EPF) and some steroid antibiotics, particularly fucidin. Acta Patholog. Microbiol. Scand. 78: 395~400, 1970
10) URI, J.; S. SZATHMARY & Zs. HERPAY: Production of an antibiotic by dermatophytes living in horn products. Nature 179: 1029~1030, 1957
11) URI, J.; S. SZATHMARY & Zs. HERPAY: Antibiotic production by fungi with respect to the location of their natural existence; demonstration of antibiotic production by the horny tissue of live dermatophytes. Die Pharmazie 12: 485~488, 1957
12) MARPLES, M. J. & M. J. BAILEY: A search for the presence of pathogenic bacteria and fungi in the interdigital spaces of the foot. Brit. J. Dermatol. 69: 379~388, 1957
13) LEYDEN, J. J. & A. M. KLIGMAN: Interdigital athletes foot. The interaction of dermatophytes and resident bacteria. Arch. Dermatol. 114: 1466~1472, 1978
14) WATERWORTH, P. M.: Apparent synergy between penicillin and erythromycin or fusidic acid. Clin. Med. 70: 941~953, 1963
15) WALLERSTRÖM, A.: Production of antibiotics by *Epidermophyton floccosum*. 2. Microflora in epidermophyton-infected skin and its resistance to antibiotics produced by the fungus. Acta Patholog. Microbiol. Scandinavica 74: 531~542, 1968
16) YOUSSEF, N.; C. H. E. WYBORN, G. HOLT, W. C. NOBLE & Y. M. CLAYTON: Ecological effects of antibiotic production by dermatophyte fungi. J. Hyg. Camb. 82: 301~307, 1979