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FAERIEFUNGIN: A NEW BROAD-SPECTRUM ANTIBIOTIC FROM *STREPTOMYCES GRISEUS* VAR. *AUTOTROPHICUS*[1,2]

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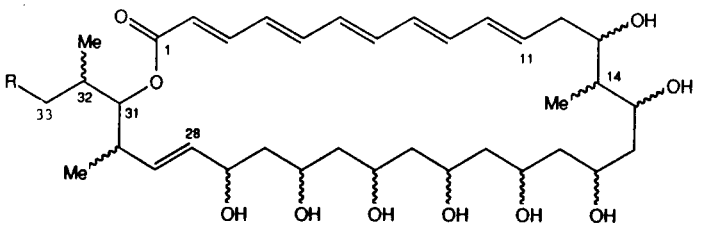
ABSTRACT.—Faeriefungin, a polyol polyene macrolide lactone antibiotic, was isolated from the mycelium of *Streptomyces griseus* var. *autotrophicus*, MSU-32058/ATCC 53668, collected from the soil sample of a fairy ring in an old lawn in Lansing, Michigan. Faeriefungin has some properties similar to the previously reported polyene macrolides, mycoticin and flavofungin, but possesses different physiochemical and biological properties. *Aspergillus, Fusarium, Microsporum, Trichophyton,* and *Alternaria* spp. were completely inhibited by faeriefungin at 3.2 μg/ml, *Candida* spp. at 5.5 μg/ml, and *Pythium, Phialophora, Leptosphaeria* spp., and some selected Gram-negative penicillin-resistant strains of *Neisseria gonorrhoeae* at 16.0 μg/ml. At a concentration of 100 ppm, it caused 100% mortality of mosquito larvae (*Aedes aegypti*, Rockefeller strain) and free-living nematodes (*Panagrellus redivivus*). Unlike the related polyene macrolides, faeriefungin is crystalline and stable with broad-spectrum antimicrobial and insecticidal activity. Preliminary cytotoxicity studies with human erythrocytes and rat liver epithelial cells indicated that faeriefungin and amphotericin B have comparable toxicity. Solution nmr study has indicated that faeriefungin is a mixture of two compounds, faerifungins A [**1**] and B [**2**], and that they differ in the attachment of a H or an Me at C-33.

Nystatin, produced by *Streptomyces noursei*, was the first of the polyene macrolide series showing antifungal and antibiotic activities (1). Most of the polyene macrolides discovered since are produced by a variety of soil-bound *Streptomyces* spp. (2,3). One of these, mycoticin, actually a 1:1 mixture of two compounds, mycoticins A and B, is produced by *Streptomyces ruber* (ATCC 3348) and was first described by Burke *et al.* (4) and later characterized by Wasserman *et al.* (5). Flavofungin, obtained from *Streptomyces flavofungini*, was found to be a 9:1 mixture of mycoticins A and B, and this mixture showed different biological properties from mycoticin (6–8). Mycoticin, for example, showed only antifungal activity, whereas flavofungin exhibited antifungal and antiviral activity (7); neither compound showed antibacterial activity. This paper describes a new polyene macrolide lactone, faeriefungin, somewhat similar to mycoticin and flavofungin, but with different physiochemical and biological properties.



1 R=H
2 R=Me

[1]Journal Article No. 12988 of the Michigan Agricultural Experiment Station.

[2]Some of the results contained herein were presented at The 29th Annual Meeting of the American Society of Pharmacognosy, July 17–21, 1988, Park City, Utah.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on either a Varian XL-400, a GE Omega 500, or a Bruker AM-500 spectrometer in DMSO-d_6 solution at 25° and referenced to the solvent at 39 ppm (^{13}C) and 2.49 ppm (^{1}H). The spectrum of faeriefungin acetate was recorded in $CDCl_3$ solution with 7.27 and 77 ppm (^{1}H and ^{13}C, respectively) as reference. Spectra at 10 ppm and 200 ppm were used for ^{1}H and ^{13}C, respectively. Downfield regions (up to 14 ppm of ^{1}H and 220 ppm of ^{13}C spectra) were examined and no resonances were observed. One-dimensional ^{1}H decoupling and ^{13}C DEPT (9) spectra, 2D ^{1}H-^{1}H COSY, ^{1}H-^{13}C correlation (10–12) and ^{1}H-^{1}H TOCSY (13) maps (Figure 1) were all recorded using standard pulse sequences.

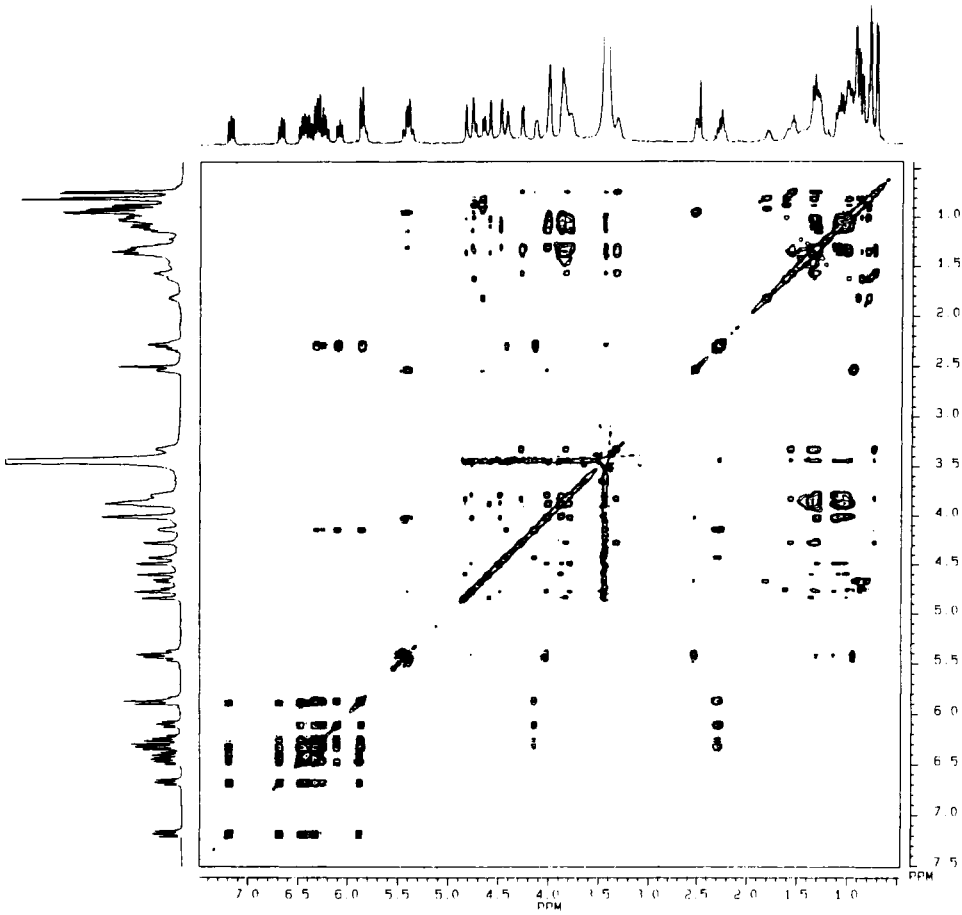


FIGURE 1. TOCSY map of faeriefungin with standard pulse sequence on Bruker AM-500 MHz spectrometer in DMSO solution at 25°.

Ir spectra were obtained with a Perkin-Elmer model 1170 FT-IR spectrophotometer, and uv spectra were recorded on a Gilford Resonse 11 UV spectrophotometer. Optical rotations were recorded at room temperature (22–24°) using a Perkin-Elmer model 141, KL 9467 polarimeter. Cd was measured on a CARY model 60 spectrophotometer. Melting points were recorded on a Thomas model 40 micro hot stage apparatus and are uncorrected. All mass spectra were recorded on either a VG 7070 (EBQQ) or a VG 70-250-HF (EB) mass spectrometer. X-ray powder diffraction was performed on a Philip APD 3720 model spectrometer.

ISOLATION AND CULTURE.—Producer strain MSU-32058/ATCC 53668 was isolated from a soil sample collected from the center of a fairy ring in an old lawn. Physiological and biochemical tests were done as described by Mishra *et al.* (14). These tests identified the producing organism as a variant of *Streptomyces griseus*.

FERMENTATION.—Cultures of *S. griseus* var. *autotrophicus* were grown in 2-liter baffle-bottomed Erlenmeyer flasks, each containing 400 ml of A-9 medium (peptone 4 g, glucose 10 g, "Brer Rabbit green label" molasses 20 g, distilled H_2O 1 liter). The inoculated flasks were placed on a rotary shaker at 150 rpm at 26° for 7 days. Larger batches were grown in a 130-liter fermenter containing 100 liters of A-9 medium, aerated at 100 liters/min and stirred at 100 rpm at 26° for 5 days. The culture broth was centrifuged, and faeriefungin was extracted from the mycelial biomass.

PRODUCTION OF COMPOUNDS FROM *S. RUBER* (ATCC 3348).—*S. ruber*, the mycoticin-producing ATCC strain, was cultured as in the case of our strain, *S. griseus* (ATCC 53668), in A-9 medium and in the medium originally used by Burke *et al.* (4). The compounds produced by *S. ruber*, in our laboratory, were mycoticin N (from A-9 medium) and mycoticin B (from Burke's medium; bactopeptone 2%, glucose 2%, Na_2HOP_4 0.2%, KCl 0.02%, $MgSO_4$ 0.02%, and NaCl 0.02%, pH adjusted to 6.5–7.0). These compounds were isolated from the respective cell mass by the method identical to that used for faeriefungin.

ANTIFUNGAL BIOASSAY.—Cultures of *Candida albicans* (MSU-SM 543), grown 48 h and mostly in yeast phase, and a 5–7-day-old well sporulating culture of *Aspergillus fumigatus* (MSU-SM 920) were initially used. The culture broth of *S. griseus* (30 μl) was placed in the center of a Petri dish containing 20 ml Emmons medium (neopeptone 10 g, glucose 20 g, bacto agar 18 g, distilled H_2O 1 liter, pH 6.8). The medium was pre-mixed with the cell suspension of the test organisms to get a final concentration of 10^3 colony forming units/ml (CFU/ml). An equal volume of the uninoculated culture medium was used as a control. The inoculated plates were incubated at 26° for 48 h. A clear zone of inhibition characterized by absence of the fungal growth around the broth droplet was recorded. Bioassay of the purified compound was done in Emmons liquid medium (same as described above, but devoid of agar). A known amount of the pure faeriefungin was dissolved in DMSO and serial dilutions prepared in the same solvent. A 20-μl aliquot of each solution was mixed with 2 ml of Emmons liquid medium seeded with ca. 2×10^3 CFU/ml of the test organism. The inoculated tubes were vortexed and incubated at 26°. Similarly, inoculated tubes without faeriefungin served as controls. Depending on the growth characteristics of the test species, results were recorded after 2–4 days. The lowest concentration of the faeriefungin that totally inhibited growth of the test organism was recorded as the MIC for that species.

ANTIBACTERIAL ASSAY.—The antibacterial activity of faeriefungin was tested by the agar dilution method (15). A known amount of pure faerifungin was dissolved in 100% DMSO, diluted into 20% DMSO, and serial two-fold dilutions at concentrations from 2560 to 40 μg/ml prepared in 20% DMSO. Mueller-Hinton agar plates containing 4, 8, 16, 32, 64, 128, and 256 μg/ml faeriefungin as well as control plates with 20% DMSO were prepared and used within 48 h.

Test organisms were grown to log phase in Mueller-Hinton broth [plus supplements (16) if required] at 37°. The organisms (10^4 CFU each) were spot inoculated onto each antibiotic-containing agar plate and control plates. Plates were incubated overnight at 37° and scored for growth of each test organism. The MIC for each species represents the lowest concentration of faeriefungin at which complete inhibition of growth occurred.

INSECTICIDAL AND NEMATICIDAL ASSAYS.—The bioassay for insecticidal properties was conducted on instar mosquito larvae, *Aedes aegypti*, reared from the mosquito eggs (University of Davis California Straw, courtesy of Dr. Fumio Matsumara). Fifteen larvae (2–3 days old) were placed in 90 ml of distilled H_2O in 180-ml clear plastic urinalysis cups. Ten ml of the culture broth was added to each cup with gentle stirring. Controls received 10 ml of the growth medium. The cups were covered and left at room temperature. The number of dead larvae was recorded at 2-, 4-, 24-, and 48-h intervals. The experiment was later repeated with pure faeriefungin.

Nematicidal activity was performed on the free-living nematode (*Panagrellus redivius*, MSU culture, courtesy of Dr. George Bird). The nematodes were reared in Erlenmeyer flasks (250 ml) containing 5 ml of A-9 medium. The cultures (2–3 weeks old, 0.25 ml) were diluted with 4.75 ml of sterile A-9 medium. Aliquots of this nematode suspension (45 μl) containing 30–50 nematodes at various developmental stages were transferred into each well (0.7 cm diameter × 1.0 cm deep) of a 96-well Corning flat-bottomed tissue culture plate. Culture broth (5 μl) was added to each well and mixed gently. The inoculated plates were held in a glass container at ca. 100% relative humidity. The experiment was repeated with pure faeriefungin. The nematodes were examined with an inverted microscope at 40X. Mortality and mobility were recorded after 4, 24, 38, and 96 h.

CYTOTOXICITY ASSAYS.—*Human erythrocytes.*—A suspension of 1.0 ml blood in 20 ml physiological saline was prepared. A solution of faeriefungin in DMSO with varying concentrations (100 μl each) was added to 2 ml of the erythrocyte suspension, and turbidity was read at 10, 30, and 60 min. Amphotericin B was used as the positive control in addition to the solvent control. The concentrations which gave a clear solution, due to lysing of RBC, are an indication of the degree of toxicity of the test compounds to the erythrocytes.

WB-S cells.—The cells (MSU Clinical Center, WB-5344) (17) were plated out in 35 × 10 mm Corning tissue culture dishes at 2×10^5 cells per plate and incubated at 37° with 5% CO_2 (24 h). Each dish contained 2 ml of Dulbecco's Modified Eagles Medium (D-MEM) with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Solutions of faeriefungin and the control drug, amphotericin B, were prepared in DMSO, at different concentrations, and 100 μl of each concentration and pure solvent was mixed with 1.9 ml D-MEM, separately, containing 5% fetal calf serum alone. The dishes containing the WB-S cells, after 24 h incubation, were rinsed 3 × 2 ml with CaMgPSB (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.2 g, $MgCl_2 \cdot 6H_2O$ 0.1 g, H_2O 1 liter); the test media were added, and the dishes were incubated at 37° and 5% CO_2 for 1 h. The media from the plates were then decanted into 12 × 75 mm glass tubes kept on ice and centrifuged (4°). The supernatant was poured into another 12 × 75 mm glass tube and kept on ice until all the samples were ready. These supernatant solutions were then kept at room temperature for LDH (lactate dehydrogenase) assay (using Sigma LDH assay kit) since liver LDH decomposes at low temperatures. The plates were rinsed with 3 × 2 ml CaMgPBS. Trypan blue (1 ml) was added to each plate. Plates were incubated 5 min at room temperature, and approximately 500 cells per plate were counted.

ISOLATION OF FAERIEFUNGIN.—Microbial cells were centrifuged (15 min, 10,000 rpm) from 12 liters of the *S. griseus* broth after 7 days of culture and filtered (sintered glass funnel, fine) under vacuum to obtain the cells as dry as possible. The cell mass (210 g wet wt) was homogenized in MeOH (4 liters) for 10 min and vacuum filtered (sintered glass, fine). The residue in the funnel was washed further with MeOH (200 ml), combined with the original filtrate, and evaporated under reduced pressure at 40°. The resulting yellow solid was redissolved in boiling MeOH (250 ml), cooled to room temperature in the dark (2 h), and filtered. The fine pale yellow needles thus obtained, dried under vacuum in a desiccator over Drierite (8 h), were pure faeriefungin (18 g). The cell-free broth (12 liters) was extracted with $CHCl_3$-MeOH (4:1) (3 × 150 ml), and the organic extracts were combined and evaporated in vacuo. The resulting yellow solid was crystallized from boiling MeOH as above to afford another batch of yellow needles of faeriefungin (300 mg).

FAERIEFUNGIN.—An inseparable 1:1 mixture of faeriefungin A [**1**] $C_{36}H_{58}O_{10}$ and faeriefungin B [**2**] $C_{37}H_{60}O_{10}$; mp 210–213°, charring at 310°; uv λ max (MeOH) 363 and 259 nm (ε = 63413 and 4649); ir (KBr) 3375, 3000, 2950, 2925, 2900, 2850, 1678, 1605, 1560, 1420, 1350, 1300 cm^{-1}; fabms *m/z* $[M+H]^+$ 651.4107 (25) (calcd 651.4105 $C_{36}H_{59}O_{10}$), 665.4237 (25) (calcd 665.4233 $C_{37}H_{61}O_{10}$), 536 (20), 464 (5), 393 (100), 299 (20), 205 (100); ^1H and ^{13}C nmr see Table 1. Faeriefungin was converted to a new compound in all organic solvents studied under laboratory light or sunlight with no conversion in the dark. The uv and ms of this converted product are the same as the parent compound.

OPTICAL ACTIVITY.—Solutions of faeriefungin, mycoticin N, and mycoticin B were made in pyridine, dioxane (0.2%), and MeOH (0.15%) separately. The rotations of these solutions were recorded at times t = 0 and 10 min, and $[\alpha]^{25}D$ values were calculated. The $[\alpha]^{25}D$ values at 0 and 10 min are: faeriefungin −70.5 and −70.5 (pyridine), −40.4 and −52.5 (dioxane), −34.0 and −35.3 (MeOH); mycoticin N −74.5 and −74.5 (pyridine), −58.0 and −58.0 (dioxane), −44.7 and −42.0 (MeOH); mycoticin B −72.0 and −73.5 (pyridine), −50.0 and −51.5 (dioxane), −38.7 and −38.7 (MeOH).

CIRCULAR DICHROISM OF FAERIEFUNGIN AND MYCOTICIN N.—Cd values were obtained for MeOH solutions of faeriefungin (1.4 mg/100 ml) and mycoticin N (2.0 mg/100ml). Faeriefungin: λ max (ΔE) 380 (−14.6), 374 (−16.3), 368 (−11.8), 362 (−13.5), 360 (−13.5), 354 (−10.1), 349 (−10.1), 344 (−9.6). Mycoticin N: 377 (−11.4), 372 (−11.4), 370 (−16.6), 365 (−15.8), 360 (−15.0), 355 (−13.4), 348 (−7.1), 339 (−3.9). For comparison of cd values of faeriefungin and the octa-acetates of faeriefungin, mycoticin, and flavofungin in dioxane see Table 2.

X-RAY (POWDER) DIFFRACTION STUDIES OF FAERIEFUNGIN AND MYCOTICIN N.—Pure, recrystallized (from MeOH) samples of faeriefungin and mycoticin N were made into a paste with distilled H_2O on separate glass slides. Diffraction of the air-dried samples was measured under the following conditions: generator settings, 35 kV, 20 mA; step size, count time, 0.020°, 1.00 s; angle range (2θ), 2.010–59.990°; A1, A2 wavelengths, 1.54056–1.54435°; and range in d-spacing, 1.5408–43.9165; A monochromator was used for both samples.

ACETYLATION OF FAERIEFUNGIN.—Faeriefungin (200 mg) was dissolved in a mixture of pyridine (10 ml) and Ac_2O (3 ml) and kept in the dark (48 h). The reaction mixture was then poured into crushed ice and H_2O (100 ml), stirred, and kept at room temperature (1 h). The resulting precipitate was filtered off, washed with H_2O, dried under vacuum over Drierite, and recrystallized from MeOH: pale yellow plates (210 mg); mp 132–137°; fabms *m/z* $[M+H]^+$ 989 (5), 1000 (50); ^1H-nmr ($CDCl_3$) signals at 4.45, 4.25, 4.85, 4.60, 5.02, 4.02, 4.50, and 4.77 assigned for the -OH groups in the natural product are missing, 8 methyl singlets at 2.08, 2.01, 2.00, 1.99, 1.96, 1.95, and 1.94 for the octa-acetates.

TABLE 1. ^{1}H- and ^{13}C-nmr Data for Faeriefungins A and B.

Atom	H	$^{3}J_{HH}$	C	^{13}C-1	^{13}C-2	^{13}C-1,2	$^{1}J^{13}C$-^{13}C	^{13}C-1,p
1	—	—	166.65	x[a]	—	x	77	
2	5.89	15.2	120.03	—	x	x	77	
3	7.19	11.2	145.17	x	—	x	56	
4	6.14	14.8	129.39	—	x	x	56	
5	6.68	11.2	141.40	x	—	x	58	
6	6.32	14.5	131.23	—	x	x	58	
7	6.47	11.0	137.80	x	—	x	58	
8	6.25	14.8	130.40	—	x	x	58	
9	6.28	11.0	135.7	x	—	x	57	
10	6.11	14.0	130.7	—	x	x	57	
11	5.86	—	135.2	x	—	x	43	
12	2.28		38.0	—	x	x	43	
13	4.15		67.9	—	—	—		x
14	1.59		46.2	—	—	x		
15	3.31		68.7	x	—	x	38	
16	1.35		43.9	—	x	x	38	
17	3.82		66.4	x	—	x	38	
18	1.1, 1.3		47.2	—	x	x	38	
19	3.84		67.5	x	—	x	40	
20	1.1, 1.3		46.7	—	x	x	40	
21	3.92		61.9	x	—	x	38	
22	1.1, 1.3		46.3	—	x	x	38	
23	3.84		62.3	x	—	x	38	
24	1.1, 1.3		42.6	—	x	x	38	
25	3.78		69.2	x	—	x	38	
26	1.05, 1.3		45.8	—	x	x	38	
27	4.02		69.5	x	—	x	47	
28	5.46		133.0	—	x	x	47	
29	5.42		130.0	—	—	—		x
30	2.54		35.7	—	x	—		
31(A)	4.63	10	80.1	—	—	—		
31(B)	4.76	10	78.2	—	—	x		
32(A)	1.82		29.5	—	—	—		
32(B)	1.62		34.4	—	—	—		
32-Me(A)	0.91	7	19.5	—	—	—		
32-Me(B)	0.88	7	14.5	—	—	—		
30-Me(A)	0.95	7	11.5	—	x	—		
30-Me(B)	0.95	7	11.5	—	—	—		
14-Me	0.74	7	10.2	—	—	—		
33(A)	0.82	7	18.5	—	—	—		
33(B)	1.00		25.0	—	—	—		
33-Me(B)	0.88	7	10.5	—	—	—		
13-OH	4.45	3						
15-OH	4.25	6.8						
17-OH	4.85	2						
19-OH	4.60	2						
21-OH	3.85							
23-OH	4.02							
25-OH	4.50	4.0						
27-OH	4.77	2.8						

[a]x indicates signal enhancement.

RESULTS AND DISCUSSION

BIOLOGICAL INVESTIGATION.—Strain MSU-32058/ATCC 53668 grows well on most of the laboratory and fermentation media. On YMG agar (yeast 4 g, maltose 10 g,

TABLE 2. Cd of Octa-acetylmycoticin, Octa-acetylflavofungin, Octa-acetylfaeriefungin, and Faeriefungin in Dioxane.

λ max (ΔE)							
Octa-acetylmycoticin[a]		Octa-acetylflavofungin[b] 5.8 mg/ml		Octa-acetylfaeriefungin 1.8 mg/ml		Faeriefungin 1.7 mg/ml	
210	+0.85	208	+2.34	—	—	—	—
224	−2.04	224	−2.34	—	—	—	—
260	−3.73	260	−5.19	—	—	—	—
345	−1.86	343	−2.51	347	−11.3	387	−10.2
362	−2.04	371	−2.18	363	−19.2	366	− 9.7
380	−1.86	389	−2.51	385	−11.9	347	−10.2

[a]Values are from Bognar *et al.* (8).
[b]Values are from Bognar *et al.* (8).

glucose 4 g, and agar 18 g per liter), it grows rapidly, colonies reaching a diameter of ca. 1 cm in a week. The gross appearance of the colonies is closely comparable to those of *Nocardia autotrophica* (Gordon) (18). This strain decomposed adenine, casein, hypoxanthine, urea, and xanthine but not tyrosine. It hydrolyzed starch, produced nitrate from nitrite, and did not grow in the presence of lysozyme. Production of acid was observed when adonitol, cellobiose, glucose, glycerol, inositol, galactose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, trehalose, and xylose were added to the medium. No acid was produced with arabinose, erythritol, rhamnose, and sorbitol. Analysis of whole-cell hydrolysate revealed presence of DAP (DL-2,6,diaminopimelic acid). A comparison of these morphological and physiological characteristics with those of published species suggested that strain MSU 32058/ATCC 53668 is closely related to *S. griseus*. However, the gross resemblance of its colonies with those of *N. autotrophica* and its inability to decompose tyrosine and the production of acid with melezitose are unusual characteristics for this species. Therefore, this strain is classified as *S. griseus* var. *autotrophicus* var. nov.

The wild type of *S. griseus* produced 1.5–1.8 g/liter of pure faeriefungin in A-9, YMG, and potato dextrose (PD) media after 5–6 days of fermentation. The culture broth, as well as the pure compound, inhibited the growth of several bacteria and a wide range of filamentous and yeast-like fungi (Table 3). To date, all fungal species studied were inhibited by this compound. A comparison of faeriefungin with other polyene antibiotics and synthetic antifungal drugs against four species of human fungal pathogens proved that faeriefungin is a superior antifungal compound against all tested organisms except *C. albicans* (Table 4). It also showed nematicidal and mosquitocidal activity at 100 ppm (Table 3) whereas mycoticin and flavofungin did not exhibit such biological activity.

In contrast to the other polyene macrolides, such as nystatin, which have no antibacterial activity, faeriefungin inhibited the growth of a variety of bacterial species (Table 3). All species of Gram-positive bacteria tested, including methicillin-resistant *Staphylococcus aureus* strains, were inhibited by 16–32 μg/ml faeriefungin. Among the Gram-negative species tested, all *Enterobacteriaceae* and *Pseudomonadaceae* were completely resistant to faeriefungin at the concentrations tested. Isolates of *Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae,* and *Branhamella catarrhalis*, including penicillin-resistant strains, were susceptible to faeriefungin, although generally at higher concentrations than those which inhibited Gram-positive bacteria. The basis for

TABLE 3. Minimum Inhibitory Concentration of Faeriefungin Against Selected Strains of Bacteria, Fungi, Insects, and Nematodes.

Organism	MIC (μg/ml)
Fungi:	
Aspergillus fumigatus MSU-SM 920	3.2
Aspergillus flavus MSU-SM 921	3.2
Aspergillus niger MSU-SM 922	3.2
Candida albicans MSU-SM 543	5.5
Candida tropicalis MSU-SM 544	5.5
Cryptococcus neoformans MSU-SM 714	5.5
Microsporum canis MSU-SM 816	3.2
Trichophyton rubrum MSU-SM 916	3.2
Alternaria solani MSU-SM 1212	3.2
Fusarium oxysporum MSU-SM 1322	3.2
Fusarium moniliforme MSU-SM 1323	3.2
Pythium ultimum MSU-SM 2146	12.0
Phialophora graminicola MSU-SM 2378	12.0
Leptosphaeria korrae MSU-SM 3146	12.0
Gram-positive bacteria:	
Staphylococcus aureus ATCC 25923, 29213	16.0
Staphylococcus epidermidis MHM[a]-9-25, MHM-6505	16.0
Streptococcus pyogenes MHM-1645, MHM-81-4	16.0
Streptococcus agalactiae MHM-3-43, MHM-81-7	16.0
Streptococcus faecalis ATCC 29212, MHM-3129	16.0–32.0
Streptococcus pneumoniae ATCC 6303, MHM-DP11	16.0
Listeria monocytogenes MHM-LM1	32.0
Gram-negative bacteria:	
Escherichia coli ATCC 25922, MHM-DS13	not active
Enterobacter aerogenes MHM-24-20, MHM-10-58	not active
Klebsiella pneumoniae MHM-H3-46, MHM-81-9	not active
Proteus mirabilis ATCC 7002	not active
Pseudomonas aeruginosa ATCC 27853, MHM-81-5	not active
Neisseria gonorrhoeae ATCC 19424, 31426	16.0–64.0
Neisseria meningitidis MHM-6682, MHM-6857	32.0–64.0
Branhamella catarrhalis ATCC 25238	64.0
Haemophilus influenzae MHM-HI-1, HI-2	64.0–128.0
Insects:	
Aedes aegypti (LC_{50}) see text	100
Panagrellus redivivus (LC_{50}) see text	100

[a]MHM = Personal Collection of Dr. Martha H. Mulks, Department of Microbiology and Public Health, MSU.

the different susceptibilities of Gram-positive bacteria, fastidious Gram-negative bacteria, and enteric Gram-negative species is not understood.

Preliminary cytotoxicity experiments with RBC and WB-S cells (11) indicated that faeriefungin is less toxic than amphotericin B (Table 5). Both compounds induced vacuolization of WB-S cells at 5 ppm and the cells appeared completely nonviable by vital dyes.

CHEMICAL ANALYSES.—The initial ^1H- and ^{13}C-nmr experiment suggested that faeriefungin was an inseparable 1:1 mixture of two polyene macrocyclic lactones faeriefungins A [**1**] and B [**2**] and a member of the same family of molecules as flavofungin and mycoticin (4–8). Mass spectral analysis further supported this contention, providing molecular compositions for the two compounds as $C_{36}H_{58}O_{10}$ and $C_{37}H_{60}O_{10}$.

The assignment of the ^1H-nmr spectrum of faeriefungin was based on the ^1H-^1H COSY map and 1D ^1H decoupling data. The resonances of polyene protons, the protons

TABLE 4. Comparison of Faeriefungin and Mycoticin N Toxicity with Known Polyene and Non-polyene Antifungal Compounds.

Substance	Organism			
	Candida albicans	*Aspergillus fumigatus*	*Microsporum canis*	*Trichophyton rubrum*
	Minimum inhibitory concentration (μg/ml)			
Faeriefungin	5.5	3.2	3.2	3.2
Mycoticin N	64	16	16	16
Amphotericin B	3.2	80	16	16
Nystatin	3.2	100	100	100
Pimaricin	5.5	16	16	16
Ketoconazole	0.5–500	1–500	5.5–80	5.5–80
Miconazole	1–50	8–500	16	80
Clotrimazole	1–50	1–500	16	80

directly coupled to the olefinic protons (at C-12 and C-30), as well as the protons at C-13, C-14, 14-Me, C-15, C-27, C-31, 13-OH, 15-OH, and 27-OH, were assigned in this way without ambiguity. Two distinct signals at 4.63 and 4.76 ppm for H-31 were of equal intensity but integrated roughly half the value of the other methine protons. This finding suggested that faeriefungin was actually a 1:1 mixture of two compounds, assigned A and B. The enlarged portion of the TOCSY map (Figure 2) gave two resonances at 1.82 and 1.62 ppm coupled to H-31 (A) and H-31 (B), respectively, for H-32 (A) and H-32 (B). Two methyl groups at δ 0.91 and δ 0.81, both coupled to H-32 (A), were assigned as 32-Me (A) and C-33 (A). The signal for H_2-33 (B) and 33-Me (B) appeared as a multiplet at 1.00 ppm. These were further coupled to H-32 (B), and $C_{33}H_2$ (B) was further coupled to a methyl group at δ 0.82 assigned as C_{33}-CH_3 (B). The above assignments established the difference between faeriefungins A [**1**] and B [**2**].

TABLE 5. Cytotoxicity of Faeriefungin Against WB-S Cells and Human Erythrocytes.

Compound	Concentration (ppm)	% viable WB-S	Erythrocyte toxicity at 1 h[a]
DMSO	—	100	NT
Faeriefungin	50	0	T
	20	0	T
	5	95[b]	NT
	0.5	100	NT
	0.05	100	NT
Mycoticin N	50	0	T
	20	0	T
	5	95[b]	NT
	0.5	100	NT
	0.05	100	NT
Amphotericin B	50	0	T
	20	0	T
	0	0	T
	0.5	100	NT
	0.05	100	NT

[a]T = toxic; NT = nontoxic.
[b]Cell vacuolization.

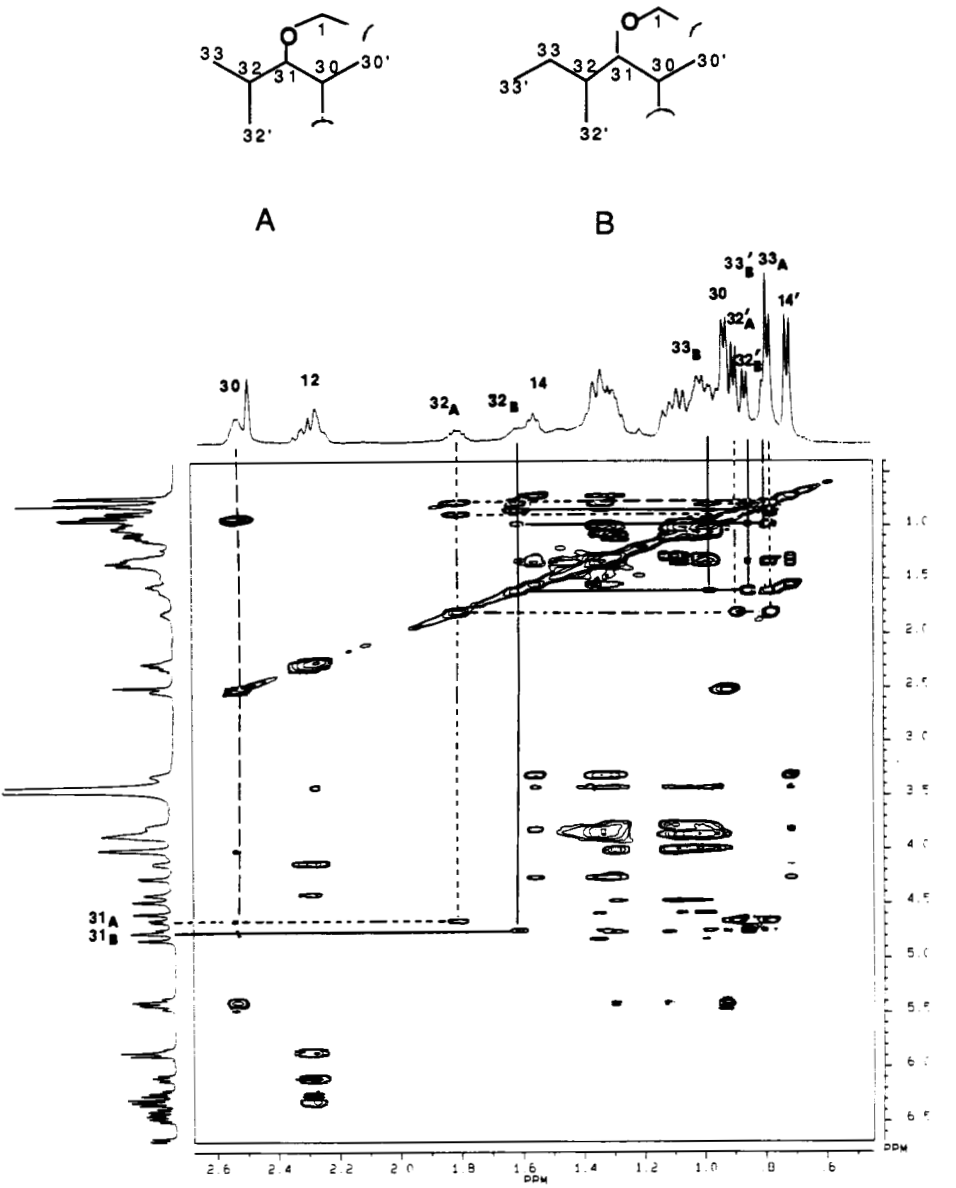


FIGURE 2. Enlargement of upfield portion of Figure 1. The dotted lines indicate the connection of the side chain of the A form [**1**], and the solid lines indicate the connection of the B form [**2**]. The dashed line indicates the direction of coupling between C-30 and C-32 (A) and C-31 (B).

The assignments of the methine and the hydroxyl protons at C-17, C-19, C-21, C-23, and C-25 were not clear from the COSY spectrum. Additional information was obtained from the TOCSY experiment (Figure 3) (13,20,21). The long-range coupling between H-15 and H-17, 15-OH and H-17 established the 17-OH and H-17 assignments. The relay between 17-OH and 19-OH assigned 19-OH and H-19. 25-OH and H-25 were detected from the relayed cross peaks between 27-OH and H-25, H-27 and H-25. 23-OH and H-23 were assigned from the relayed cross peak between H-23 and H-25 and 23-OH and 25-OH. The resonances of 21-OH and H-21 were obscured by other protons, but the carbon spectrum indicated the position of C-21 and a variable

temperature experiment identified OH-21 at 3.85 ppm. This signal was found to move up field with increasing temperature.

The TOCSY map provided further information for the assignment of protons at C-13 and C-30. The cross peak between protons at C-30 and C-31 was weak in the COSY spectrum but stronger in the TOCSY experiment (Figures 2 and 3). The cross peak between H-13 and H-14 was not observed in either the COSY or TOCSY experiments, but the relayed cross peaks among H-13 and 14-Me, 13-OH and H-14, H-13 and H-11 and H-13 and H-10 were observed in TOCSY in addition to the cross peak between H-12 and H-13 (Figure 3). The cross section plotted parallel to the F1 axis at the F2 frequencies of H-14, 14-Me, H-12, H-11, and H-10 (Figure 4), confirmed the assignment for H-13. However, it is not yet clear why the intensities of the relays of H-13 to either 14-Me, H-11, or H-10 were stronger than that of the cross peak of the three bonds coupled to H-13 and H-14. The coupling constant of H-13 and H-14 coupling was found to be very small (<2 Hz), which could cause the low intensity of these cross peaks.

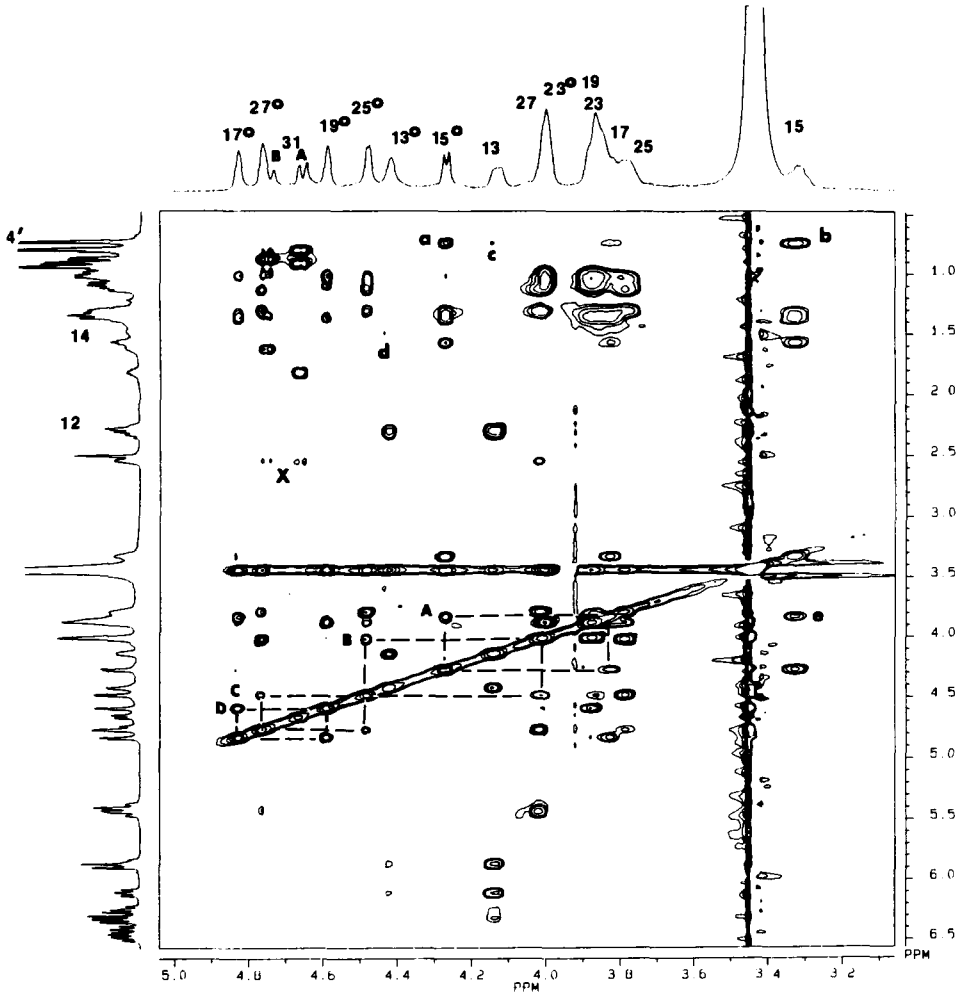


FIGURE 3. Enlargement of hydroxyl portion of Figure 1. The labeled cross peaks are the relays between (a) 15-OH and 14-Me (b) H-15 and 14-Me (c) H-13 and 14-Me (d) 13-OH and H-14. The cross peak labeled by X indicates the direct coupling between C-30 and C-31 (A) and C-31 (B). The cross peaks connected by dashed lines are the relays between (A) 15-OH and 17-OH. The superscript o indicates the hydroxyl proton at that carbon.

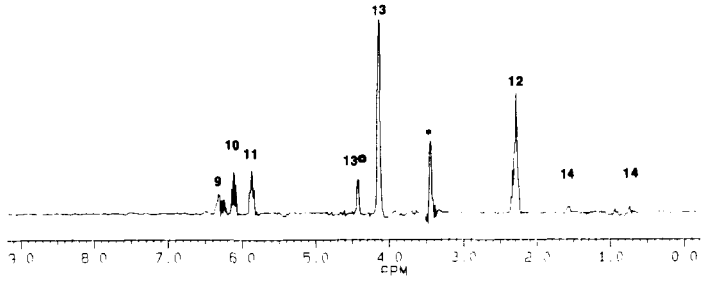


FIGURE 4. The cross section parallel to F1 axis of Figure 1 at the F2 frequency of H-13. The peaks are the cross peaks between the labeled protons and H-13. The superscript o indicates the hydroxyl proton at that carbon.

The ^{13}C spectrum of faeriefungin was assigned through the 1D-DEPT (9) experiment and 2D ^{1}H-^{13}C correlation map (10–12). The DEPT spectrum distinguished the multiplicities of the individual carbons. There were 12 olefinic signals corresponding to the C-2–C-11, C-28, and C-29 carbons. Two methine signals that appeared at 80 ppm were at half the intensity of the other 8 methine carbons (odd-numbered carbons in the ring between C-13 and C-27), which were within 60–70 ppm. The methine carbons at δ 46.2, 35.7, 29.5, and 34.4 were assigned to C-14, C-30, C-32 (A), and C-32 (B), respectively. The 12 methylene carbons for C-12 and the even-numbered carbons between C-16 and C-26 appeared between 38 and 50 ppm. The C-33 (B) methylene carbon appeared at δ 25.0. Seven methyl carbons at high field were assigned as 14-Me, 30-Me (A), 30-Me (B), 32-Me (A), 32-Me (B), C-33 (A), and Me-33 (B). The 2D ^{1}H-^{13}C correlation map made it possible to extend the information obtained from both the ^{1}H and ^{13}C spectra. The resonances of C-31 (A) and C-32 (A), for example, were distinguished from C-31 (B) and C-32 (B) through proton correlation. The methine carbons (within 60–70 ppm) were also identified individually. However, some of the methylene assignments (between 40–50 ppm) were tentative due to the overlap of the proton resonances.

Based on an apparent polyketide biosynthetic pathway, [1-^{13}C], [2-^{13}C], [1,2-^{13}C] acetate and [1-^{13}C] propionate were fed to the fungal culture. The faeriefungin isolated from these feeding experiments amplified ring carbons consistent with a polyketide biosynthesis and supported the previous spectral assignment. There were only two signals enhanced in the nmr spectra due to [1-^{13}C] propionate feeding. The enhancement confirmed the assignment of C-13 and C-29 (Figure 5A). The signals of the odd-numbered carbons in the ring, except C-13, C-29, and C-31, were enhanced in 1-^{13}C acetate feeding (Figure 5B), while the even-numbered carbons of the ring, except C-14, were enhanced with the 2-^{13}C acetate feeding (Figure 5C). The enriched carbons appeared as triplets in the 1,2-^{13}C-acetate-fed material due to ^{13}C-^{13}C coupling between the adjacent carbon pairs. All the ring carbons except C-13, C-14, C-29, and C-31 showed this coupling (Figure 5D). All the observed enhancements matched the expectations based on a polyketide biosynthesis. The summarized nmr data of faeriefungin are listed in Table 1.

The glaring exceptions in the feeding experiments were the lack of incorporation in the carbons of the side chain. Small enhancements were seen in C-31 (B) and C-30 with [1,2-^{13}C] acetate feeding (Figure 5C). Enhancements in C-30 and some of the terminal methyls are shown in the [1,2-^{13}C] acetate feeding experiments, but lack of detectable ^{13}C-^{13}C coupling (Figure 5D) suggested extensive metabolism before incorporation. Further experiments detailing the biosynthetic pathway of faeriefungin will be reported elsewhere.

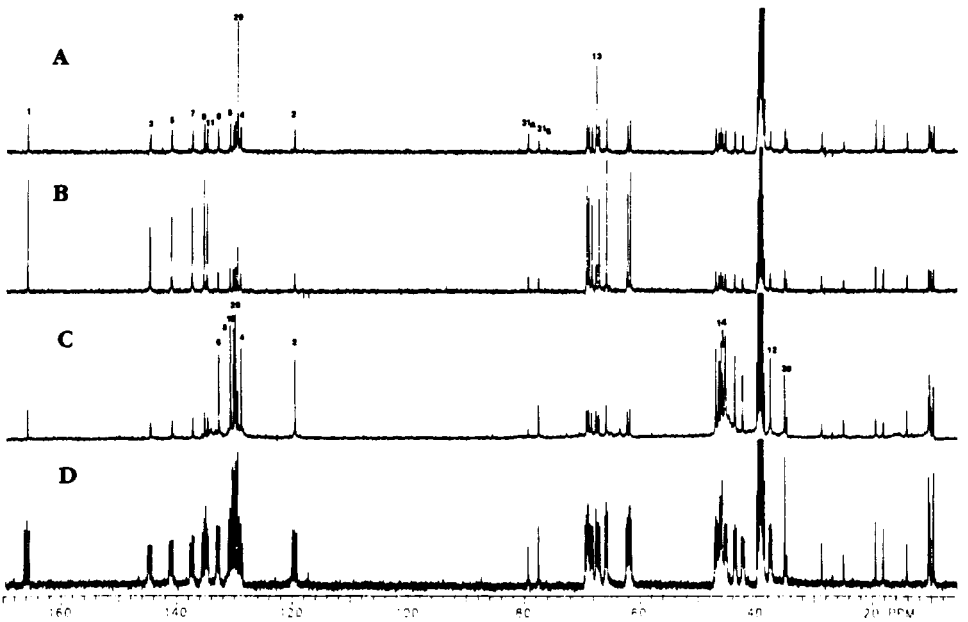


FIGURE 5. Nmr spectra of: (**A**) 1-^{13}C-propionate-, (**B**) 1-^{13}C-acetate-, (**C**) 2-^{13}C-acetate-, and (**D**) 1,2-^{13}C-acetate-fed faeriefungin; all are taken in DMSO solution. The unlabeled resonances in the region of 60 to 70 ppm are the odd-numbered methine carbons (C-15 to C-27), the resonances in the 40 to 50 ppm region are the even-numbered methylene carbons (C-14 to C-26).

Initial experiments exploring conformational dynamics and conformation of the macrolide were not successful. Temperature-dependent experiments in a range of 19 to 40° shifted the hydroxyl protons upfield with increasing temperature, but the other protons were unaffected. There was no detectable evidence of intramolecular hydrogen bonding or conformational changes within the temperature range explored. The coupling constants $^{3}J_{HH}$ of the polyene protons indicated an all *trans* conformation.

The unique properties of faeriefungin are further substantiated by its optical rotation, cd, X-ray (powder) diffraction, and chemical properties. Faeriefungin gave an $[\alpha]^{25}D$ of -40.5 at time $t=0$ in dioxane compared to the $+63.5$ by mycoticin. No $[\alpha]^{25}D$ value was reported for flavofungin in dioxane. Faeriefungin and flavofungin showed $[\alpha]^{25}D$ values of -70.5 and -88, respectively, in pyridine. No rotation data for mycoticin in pyridine have been reported. It is important to note that the ATCC strain of *S. ruber*, the original producer of mycoticins A & B with $[\alpha]^{25}D$ value of $+63.5$, did not produce the expected compound, mycoticin. However, this strain now produces only mycoticin N, a compound with $-[\alpha]^{25}D$ instead of the reported positive value. A comparison of the cd values of the octa-acetates of faeriefungin, flavofungin (8), and mycoticin (8) octa-acetates revealed remarkable differences (Table 2). There were no cd transitions in the 260–210 nm region of faeriefungin octa-acetate or faeriefungin itself. The X-ray powder diffraction spectra of faeriefungin and mycoticin N also showed significant differences.

The structure and absolute stereochemistry of mycoticin have been recently established based on degradation and systematic synthesis of diastereomeric combinations of the polyol segment (22,23). Acetylation of faeriefungin in pyridine/Ac_2O afforded an octa-acetate like mycoticin and flavofungin. Unlike mycoticin, faeriefungin did not yield methylenedioxy or acetonide derivatives (22,23). Under these conditions, faeriefungin rearranges; this is possibly due to random *trans*-acylation resulting in a ring contraction. The major difficulty encountered with faeriefungin is an isomeriza-

tion to a new molecule and rearrangement in solution under acid and light. It is, however, stable when stored as a crystalline solid under laboratory conditions or at 4° for long periods of time.

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LITERATURE CITED

1. E.L. Hazen and R. Brown, *Science,* **112**, 423 (1950).
2. K.S. Gopalakrishnan, N. Narasimhachari, V.B. Joshi, and M.J. Thirumalachar, *Nature,* **218**, 597 (1968).
3. G.H. Wagman, R.T. Testa, M. Patel, J.A. Marquez, E.M. Oden, J.A. Waitz, and M.J. Weinstein, *Antimicrob. Agents Chemother.*, **7**, 457 (1975).
4. R.C. Burke, J. Swartz, S.S. Chapman, and W. Huang, *J. Invest. Dermatol.*, **23**, 163 (1954).
5. H.H. Wasserman, J.E. VanVerth, D.J. McCaustland, I.J. Borowitz, and B. Kamber, *J. Am. Chem. Soc.*, **89**, 1535 (1967).
6. J. Uri and I. Bekesi, *Nature,* **181**, 908 (1958).
7. M.A. Schneider, E.B. Stilbans, L.A. Rachkovskaya, and V.A. Poltorak, *Antibiotiki (Moscow),* **28**(5), 352 (1983).
8. R. Bognar, B.O. Brown, W.J.S. Lockley, S. Makleit, T.P. Toube, B.C.L. Weedon, and K. Zsupan, *Tetrahedron Lett.*, 471 (1970).
9. D.M. Doddrell, D.T. Pegg, and M.R. Bendall, *J. Magn. Reson.*, **48**, 323 (1982).
10. A.D. Bax and G.A. Morris, *J. Magn. Reson.*, **42**, 51 (1981).
11. A.D. Bax, *J. Magn. Reson.*, **53**, 517 (1983).
12. J.A. Wilde and P.H. Bolton, *J. Magn. Reson.*, **59**, 343 (1984).
13. A.D. Bax and D.G. Davis, *J. Magn. Reson.*, **65**, 355 (1985).
14. S.K. Mishra, R.E. Gordon, and D.A. Barnett, *J. Clin. Microbiol.*, **11**, 728 (1980).
15. J.A. Washington, "Manual of Clinical Microbiology," 4th ed., American Society for Microbiology, Washington, DC., 1985, p. 967.
16. J.S. Knapp and K.K. Holmes, *J. Infect. Dis.*, **132**, 204 (1975).
17. M. Tsao, J.D. Smith, K.G. Nelson, and J.W. Grisham, *Exp. Cell Res.*, **154**, 38 (1984).
18. R.E. Gordon, S.K. Mishra, and D.A. Barnett, *J. Gen. Microbiol.*, **109**, 69 (1978).
19. R. Cleeland and E. Grunberg, "Antibiotics in Laboratory Medicine," Williams and Wilkins, Baltimore, 1980, p. 506.
20. A.D. Bax and D.G. Davis, *J. Am. Chem. Soc.*, **107**, 2820 (1985).
21. L. Brauschweiler and R.R. Ernst, *J. Magn. Reson.*, **53**, 521 (1983).
22. S. Schreiber and M. Goulet, *Tetrahedron Lett.*, **28**, 6001, 6005 (1987).
23. S. Schreiber and M. Goulet, *J. Am. Chem. Soc.*, **109**, 8120 (1987).

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