Fulvoferruginin, a Carotane Antibiotic from *Marasmius fulvoferrugineus* Gilliam [1]

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Fulvoferruginin, Carotane Derivative, Sesquiterpenoid, Antibiotic, *Marasmius fulvoferrugineus,* Basidiomycete

The structure of fulvoferruginin (**1**), a carotane derivative from cultures of *Marasmius fulvoferrugineus,* has been established by spectroscopic investigations and an X-ray structure analysis. **1** exhibits antibacterial, antifungal, and cytotoxic activities.

Introduction

Marasmius fulvoferrugineus is a rather small agaric with a conspicuous reddish brown pileus which can be found quite frequently in the Great Smoky Mountains and the southern United States. Its natural habitats are plant debris or wood in mixed forests. It has been described as a new species by Gilliam [2]. The species most closely related is *Marasmius siccus* (Schwein.) Fr. which differs in the orange colour of the pileus and the size of the basidiospores. Only few antibiotic metabolites have been reported from *Marasmius* species. Among them are sesquiterpenoids like marasmic acid from *M. conigenus* [3] and the alliacols A and B from *M. alliaceus* [4, 5], 6-methyl-1,4-naphthoquinone from *M. graminum* [6], and acetylenes like scorodonin from *M. scorodonius* [7]. In the following we wish to describe the fermentation, isolation, structural elucidation and biological characterization of fulvoferruginin, the first carotane sesquiterpenoid from a basidiomycete [8].

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Materials and Methods

Marasmius fulvoferrugineus strain 8661

Mycelial cultures of *M. fulvoferrugineus* were obtained from spore prints of fruiting bodies collected in the Great Smoky Mountains, U.S.A. Herbarium specimen and strain 8661 are deposited in the collection of the LB Biotechnologie der Universität Kaiserslautern.

Fermentation and isolation

For maintenance on agar slants and submerged cultivation *M. fulvoferrugineus* was grown in a yeast extract-malt extract-glucose (YMG) medium composed of (g/l): Yeast extract 4, malt extract 10, and glucose 4. The medium (M2A) used for the production of fulvoferruginin contained (g/l): maltose 30, glucose 10, yeast extract 1, peptone 2, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 1, $FeCl_3$ 0.01, $ZnSO_4$ 0.002, and $CaCl_2$ 0.055. A well grown seed culture of *M. fulvoferrugineus* (200 ml) in YMG-medium was used to inoculate 20 l of M2A medium in a Biolafitte C6 fermentation apparatus. After 10–12 days of fermentation (22 °C, 120 rpm, 2 l air/min) the mycelia were separated from the culture fluid and discarded. The culture fluid (19 l) was extracted with ethyl acetate (2 × 5 l). The resulting crude extract (1.3 g) was applied to a col-

umn (Merck silica gel 60; 6 × 19 cm) and eluted with toluene–acetone–ethanol (70:30:2). The fractions exhibiting antifungal activity were pooled and the solvent evaporated. Fulvoferruginin was purified from the enriched product (480 mg) by preparative HPLC [Merck LiChrosorb Si 60, 7 μm, 25 × 250 mm. Mobile phase: gradient (% 2-propanol in cyclohexane): 0–60 min, 10–55%. Flow: 3 ml/min. Detection at 254 nm. Retention time fulvoferruginin: 33 min]. Yield: 250 mg of colourless crystals.

*Physico-chemical properties of fulvoferruginin (**1**)*

Colourless needles, m.p. 127 °C, R_f 0.56 [silica gel, toluene–Me_2CO–AcOH (70:30:1), detection: lilac-blue spot upon spraying with 1% vanillin in conc. H_2SO_4], $[\alpha]_D^{20}$ −65.4 (*c* 0.13, MeOH); UV (MeOH) λ_{max} nm (log ε) 247 (3.63); CD (MeOH): $[\theta]_{235.5}$ 0, $[\theta]_{243}$ -5.10×10^3, $[\theta]_{250}$, 0 $[\theta]_{264}$ 17.32×10^3, $[\theta]_{289}$ 0; IR (KBr) cm^{-1} 3490(sst), 3420(sh), 3020(sh), 2960(m), 2930(sh), 2870(sh), 1715(sst), 1640(w), 1580(st), 1445(m), 1405(st), 1370(sh), 1350(w), 1320(w), 1270(st), 1210(sh), 1180(sst), 1135(m), 1110(sh), 1060(m), 1025(m), 980(m), 950(m), 930(m), 840(w, br), 810(w), 745(m), 700(m), 660(w, br); ^1H and ^{13}C NMR spectra see Table I; HR-MS (70 eV, DI 180 °C): *m/z* (relative intensity, %) 248 (4, M + 2), 247 (3, M + 1), 246.1255 (16, M^+, calcd for $C_{15}H_{18}O_3$ 246.1255), 228 (5), 215 (5), 213 (12), 186 (6), 185 (8), 159 (8), 157 (9), 153 (5), 148 (11), 145 (8), 143 (10), 142 (6), 141 (5), 139 (15), 138 (80, $C_8H_{10}O_2$), 133 (10), 132 (12), 131 (12), 129 (8), 128 (6), 123 (10), 121 (9), 120 (8), 119 (11), 117 (15), 110 (25), 109 (100, C_7H_9O), 108 (33), 107 (5), 105 (19), 95 (8), 93 (10), 92 (5), 91 (25), 81 (5), 79 (10), 77 (9), 67 (6), 65 (6), 44 (5), 43 (9), 41 (7).

*X-ray structural analysis of **1***

Crystal data: $C_{15}H_{18}O_3$; M = 246.3; monoclinic $P2_1$ with a = 10.237(4), b = 7.328(4), c = 9.859(3), β = 116.17(2)°, V = 663.7(9) pm^3; F(000) = 264; D_{calcd} = 1.23 mg/m^{-3}; Z = 2; λ(CuKα) = 1.54184; μ(CuKα) = 6.5 cm^{-1}; T = 293 K.

Data collection and structure refinement: A prismatic crystal with dimensions 0.32 × 0.18 × 0.144 mm was chosen for data collection. Cell parameters were obtained from a least-squares fit to the settings of 25 reflections in the range $15° \leq \theta \leq 25°$ centered on an Enraf-Nonius CAD4 diffractometer using CuKα radiation. The intensity data were collected in the ω-scan mode for reflection width 0.90 + 0.14 tanθ at variable speeds between 1.08 and 5.03 deg·min^{-1}. A total of 1041 independent reflections were collected for $2\theta \leq 120°$. Three monitor reflections were measured at regular intervals; crystal decay was not observed. Lorentz, polarization and empirical absorption corrections based on ω-scan data were applied to the reflection intensities. On the basis of the criterion $I \geq 1.0\sigma(I)$, 799 reflections were retained for use in the structure refinement. The structure was solved by direct methods (SHELXS) and refined by full matrix least-squares with the SHELX-76 system. All hydrogen atoms could be located in difference syntheses and were included in the final cycles with group isotropic temperature factors. Whereas the position of H 10, H 141 and H 142 were fixed, the remaining protons were allowed to ride on their respective carbon atoms with d(C–H) = 1.08 Å and idealized H–C–C angles. Anisotropic temperature factors were introduced for all hydrogen atoms. The terminal reliability indices were R = 0.0500 and $R_w = [\Sigma w(|F_o| - |F_c|)^2/\Sigma w|F_o|^2]^{1/2}$ = 0.0489 with weights given by the expression $w = (\sigma^2(F_o) + 0.0002\,F_o^2)^{-1}$. Inversion of the configuration led to a deterioration in the generalized R factor which was significant at the 95% level. The more probable absolute configuration is displayed in Fig. 1; positional parameters with equivalent isotropic temperature factors are listed in Table II. Table III contains bond length (Å) and angles (°) in the molecule. Further details of the crystal structure determination may be obtained from Fachinformationszentrum Energie, Physik, Mathematik, D-7514 Eggenstein-Leopoldshafen by providing the deposition number CSD 54752, the authors and the journal citation.

Biological assays

Antibiotic content in fermentations and fractions after chromatography was determined by paper disc/agar diffusion assay using *Paecilomyces varioti* as test organism. The antimicrobial spectra, cell culture, cytotoxicity, and macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma were measured as described previously [9, 10], HeLa cells were grown in Ham's F 12 medium

containing 10% fetal calf serum and 100 µg/ml of streptomycin sulfate and 65 µg/ml of penicillin G in a humidified atmosphere containing 5% of CO_2 at 37 °C.

Results and Discussion

Structure determination

Fulvoferruginin (**1**), $C_{15}H_{18}O_3$, exhibits IR bands at 3490 and 1715 cm^{-1} which suggest the presence of a hydroxy and an α,β-unsaturated ester or lactone group. According to the ^1H and ^{13}C NMR data (Table I) the antibiotic contains three double bonds which can be defined as an *exo* methylene group, a (*Z*)-CH=CH− and a −CH=C(CH_3)− unit. In the aliphatic region signals for an isolated −CH_2CH_2− moiety, a tertiary methyl group at δ 0.95 and two vicinal methine protons at δ 2.44 (d, J = 12.5 Hz) and 4.65 (m) are visible. By means of COLOC experiments [11] 1,3-correlations between the tertiary methyl group and the (*Z*)-CH=CH−, −CH_2CH_2− and −CH−CH− units were established. In the same manner the presence of an α-methylene lactone moiety with a tertiary alcohol group in β-position and the conjugation of the two remaining double bonds was deduced. From this evidence Formula 1 can be proposed for fulvoferruginin.

The 12.5 Hz coupling between protons 5-H and 6-H is in accord with their *trans* relationship. The vinylic proton 4-H forms a dihedral angle of nearly 90° with 5-H which explains the small coupling of

Table II. Positional parameters with equivalent isotropic temperature factors (Å^2 × 10^3).

	x/a	y/b	z/c	U_{eq}
O5	0.5954(4)	1.0000	0.2403(4)	77(2)
O10	0.7236(4)	0.7296(8)	0.5445(4)	77(2)
O15	0.4155(5)	1.0366(9)	0.2992(5)	115(4)
C1	0.9627(7)	0.6192(10)	0.2802(6)	74(4)
C2	0.9927(6)	0.7740(12)	0.2333(6)	74(4)
C3	0.8992(7)	0.9307(10)	0.1574(6)	68(3)
C4	0.7644(6)	0.9682(10)	0.1387(6)	67(4)
C5	0.6713(6)	0.8630(9)	0.1920(6)	58(3)
C6	0.7520(5)	0.7334(10)	0.3197(5)	51(2)
C7	0.8163(6)	0.5701(10)	0.2707(6)	63(3)
C8	0.8246(7)	0.4251(11)	0.3865(7)	84(4)
C9	0.6782(7)	0.4507(10)	0.3939(7)	82(4)
C10	0.6601(6)	0.6571(10)	0.3938(6)	65(3)
C11	0.5083(6)	0.7320(13)	0.3162(6)	74(4)
C12	0.9729(9)	1.0626(12)	0.0923(8)	102(5)
C13	0.7187(8)	0.5034(10)	0.1078(7)	82(4)
C14	0.3878(8)	0.6392(13)	0.2819(9)	116(6)
C15	0.4994(7)	0.9272(12)	0.2862(7)	84(4)

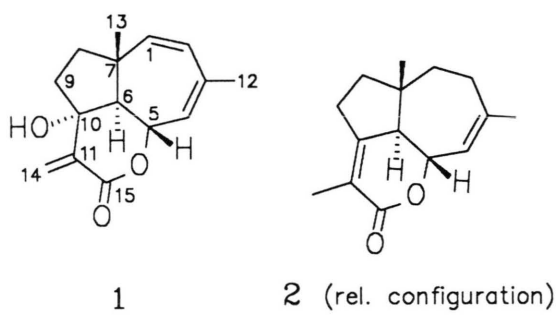
Table I. ^1H and ^{13}C NMR data of fulvoferruginin (**1**) (400 and 100.6 MHz, respectively; MeOH-d_4 as solvent and internal standard; recorded on a Bruker WM 400 instrument)*.

H	δ [ppm]	J [Hz]	C	δ [ppm]	J [Hz]
1-H	6.15 dt	11.2/1	C-1	143.65 Dd	159/8
2-H	5.65 dd	11.2/1.25	C-2	126.55 Dm	154
			C-3	133.94 br, m	
4-H	5.72 m		C-4	125.27 D"t"	158/6
5-H	4.65 dm	12.5	C-5	78.58 Dd	147/6
6-H	2.44 dd	12.5/0.75	C-6	57.53 D(br)	134
			C-7	45.24 br, s	
8-H^a	2.15 ddd	13.5/12/6.5/0.5	C-8	38.42 Tm	134
8-H^b	1.78 dd	12/6.5			
9-H^a	2.45 m		C-9	36.17 Td	131/4
9-H^b	1.98 ddt	13.5/6.5/0.75			
			C-10	80.65 br, s	
			C-11	144.82 br, s	
12-CH_3	1.95 "t"	≈2	C-12	27.89 Qddd	126/8/5/1
13-CH_3	0.95 s		C-13	19.76 Q(br)	128
14-H^a	6.00 s		C-14	121.78 T	162
14-H^b	5.78 s				
			C-15	171.81 dd	12/8

* The assignments were confirmed by selective decouplings in the ^1H-coupled ^{13}C NMR spectrum and a 2D $^{13}C-^1H$ correlation.

Table III. Bond lengths (Å) and angles (°) in **1**.

C(15)–O(5)	1.359(8)	C(5)–O(5)	1.472(6)
C(15)–O(15)	1.223(7)	C(10)–O(10)	1.435(6)
C(11)–C(15)	1.456(10)	C(10)–C(11)	1.502(8)
C(14)–C(11)	1.316(9)	C(6)–C(10)	1.529(7)
C(9)–C(10)	1.524(8)	C(5)–C(6)	1.501(6)
C(7)–C(6)	1.542(7)	C(4)–C(5)	1.490(8)
C(3)–C(4)	1.338(7)	C(2)–C(3)	1.471(8)
C(12)–C(3)	1.531(8)	C(1)–C(2)	1.312(8)
C(7)–C(1)	1.504(8)	C(8)–C(7)	1.534(8)
C(13)–C(7)	1.550(7)	C(9)–C(8)	1.545(9)
C(5)–O(5)–C(15)	113.7(5)	O(15)–C(15)–O(5)	115.0(7)
C(11)–C(15)–O(5)	117.7(5)	C(11)–C(15)–O(15)	127.3(7)
C(10)–C(11)–C(15)	114.9(6)	C(15)–C(11)–C(14)	119.2(7)
C(14)–C(11)–C(10)	125.8(7)	C(11)–C(10)–O(10)	106.7(5)
C(6)–C(10)–O(10)	105.4(4)	C(6)–C(10)–C(11)	109.8(4)
C(9)–C(10)–O(10)	111.5(5)	C(9)–C(10)–C(11)	117.5(6)
C(9)–C(10)–C(6)	105.3(5)	C(5)–C(6)–C(10)	113.9(4)
C(7)–C(6)–C(10)	107.6(5)	C(7)–C(6)–C(5)	112.4(4)
C(6)–C(5)–O(5)	109.5(4)	C(4)–C(5)–O(5)	105.8(4)
C(4)–C(5)–C(6)	115.0(5)	C(3)–C(4)–C(5)	128.8(6)
C(2)–C(3)–C(4)	129.1(6)	C(12)–C(3)–C(4)	118.8(6)
C(12)–C(3)–C(2)	112.1(6)	C(1)–C(2)–C(3)	130.8(6)
C(7)–C(1)–C(2)	124.5(6)	C(1)–C(7)–C(6)	110.3(5)
C(8)–C(7)–C(6)	101.4(5)	C(8)–C(7)–C(1)	113.1(5)
C(13)–C(7)–C(6)	113.6(4)	C(13)–C(7)–C(1)	108.0(5)
C(13)–C(7)–C(8)	110.6(5)	C(9)–C(8)–C(7)	102.3(5)
C(8)–C(9)–C(10)	103.9(5)		



1 2 (rel. configuration)

Formula.

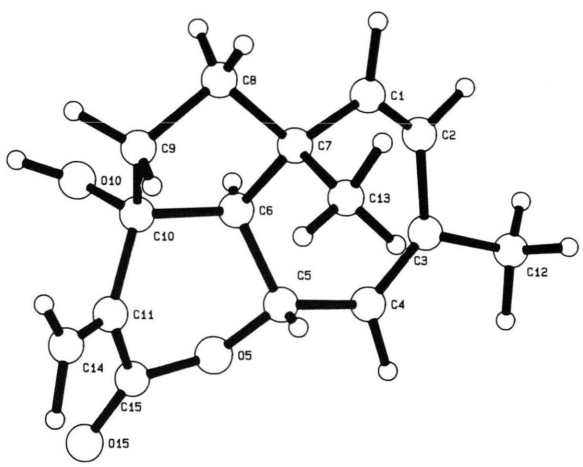


Fig. 1. Molecular structure of fulvoferruginin.

1 Hz between these protons. The NOE enhancement of the H-5 signal on irradiation at the frequency of the angular methyl group establishes the *cis* relationship between these substituents.

The structure and relative stereochemistry of **1** was confirmed by a single crystal X-ray analysis (Fig. 1). The fused five-membered ring system in **1** displays an envelope conformation with C8 displaced 0.657 Å from the best least-squares plane through the remaining ring atoms (distances: C7 −0.010, C6 0.016, C10 −0.016, C9 0.010 Å). A distorted boat conformation is observed for the six-membered ring with the following distances from a least-squares plane: C5 −0.035, C6 0.150, C10 0.156, C11 −0.355, C15 0.186, O5 0.198 Å. Bond distances and angles are typical. The crystal structure contains no hydrogen bonds between neighbouring molecules.

The more probable absolute configuration of fulvoferruginin as deduced from the anomal X-ray dispersion is given in the Formula. Fulvoferruginin displays in its CD spectrum a strong positive Cotton effect at 264 nm (Fig. 2).

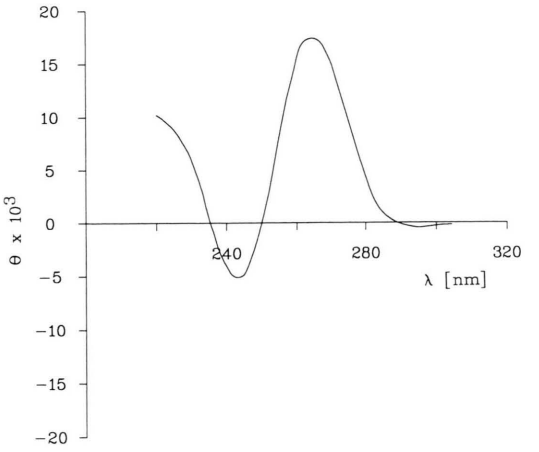


Fig. 2. CD spectrum of fulvoferruginin in MeOH.

Fulvoferruginin (**1**) is closely related to hercynolactone (**2**) which has been isolated from liverworths by Huneck *et al.* [12].

The antimicrobial activities of fulvoferruginin in the serial dilution and plate diffusion assays are shown in Tables IV and V. The antibiotic exhibits modest activity against Gram-positive bacteria with minimal inhibitory concentrations (MICs) of 20–100 µg/ml. The antifungal activity is most pronounced against *Paecilomyces varioti* with a MIC of 1–5 µg/ml. The cytotoxic activity towards Ehrlich ascitic tumor (ECA) cells (murine) and HeLa cells (human) is shown in Table VI. 50%

Table IV. Minimal inhibitory concentrations of fulvoferruginin in the serial dilution assay.

	MIC [µg/ml]
Bacteria	
Acinetobacter calcoaceticus	>100
Bacillus brevis	50–100
Bacillus licheniformis	50–100
Bacillus subtilis	50–100
Corynebacterium insidiosum	20–50
Micrococcus luteus	50–100
Mycobacterium phlei	20–50
Staphylococcus aureus	50–100
Fungi	
Candida albicans	20–50
Mucor miehei	50–100
Nematospora coryli	20–50
Paecilomyces varioti	1–5
Penicillium notatum	20–50
Rhodotorula glutinis	>100
Saccharomyces cerevisiae is 1*	20–50

* Obtained from Prof. F. Lacroute, Straßburg.

Table V. Antifungal activity of fulvoferruginin in the plate diffusion assay. Paper discs of 6 mm diameter were used.

Test organism	Diameter inhibition zone [mm] µg/disc			
	10	20	50	100
Alternaria porri	–	–	–	10
Ascochyta pisi	10	31	52	
Aspergillus ochraceus	–	–	15	20
Botrytis cinerea	–	10	38	
Cladosporium cladosporioides	–	10	28	34
Curvularia lunata	–	–	8	10
Epicoccum purpurascens	35	40	60	
Eurotium cristatum	–	–	10	15
Fusarium fujikuroi	–	–	–	–
Fusarium oxysporum	–	–	–	7
Neurospora crassa	–	–	30	60
Paecilomyces varioti	33	40	43	50
Penicillium islandicum	–	7	20	
Phoma clematidina	–	20	55	
Verticillium sp.	–	–	10	30

Table VI. Cytotoxic activity of fulvoferruginin.

Fulvoferruginin [μg/ml]	Lysis of cells [%] Cell line HeLa	ECA
0.5	0	0
1	<10	0
5	50	0
10	>80	50
25		>80

lysis of cells is observed at 5 μg/ml (HeLa) and 10 μg/ml (ECA). When tested according to [9] the incorporation of [^{14}C]leucine, [^{14}C]uridine, and [^{14}C]thymidine into trichloroacetic acid-precipitable material (protein, RNA, DNA) in ECA cells was inhibited 50% at concentrations of 10–20 μg/ml. Like other α,β-unsaturated lactones fulvoferruginin readily reacts with cysteine or other thiols yielding adducts which are devoid of antimicrobial and cytotoxic activity.

Acknowledgements

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