Octadeca-8,11-dienoic Acid Methylester, a New Fatty Acid Metabolite from *Fistulina hepatica*

Veneta Ivanova[1,*], Mariana Kolarova[1], Krasja Aleksieva[1], Rolf Schlegel[2], Peter Schumann[2] and Udo Graefe[2]

[1]*Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G.Bonchev-str., Bl. 26, 1113 Sofia, Bulgaria*

[2]*Leibniz Institute for Natural Product Research and Infection Biology-Hans-Knoell-Institute, Beutenbergstrasse 11a, D-07745 Jena, Germany*

Abstract: A new fatty acid metabolite, octadeca-8,11-dienoic acid methylester (**1**) was isolated together with the known ergosterol (**2**) from *Fistulina hepatica.* The edible mushroom *Fistulina hepatica* is a relatively rarely occurring representative of the wood-rotting basidiomycetes for which no characteristic metabolites have been reported so far. The compounds **1** and **2** were purified by solvent extraction, silica gel column chromatography and preparative HPLC consecutively. The structures of the both compounds were unequivocally determined on the basis of NMR spectroscopic methods including 1D (^{1}H , ^{13}C and DEPT) and 2D (COSY, HSQC and HMBC) and by mass spectrometry data (ESI, EI and HREI-MS). The new fatty acid methylester **1** displays no antimicrobial properties against *Staphylococcus aureus*, *Bacillus cereus, Bacillus subtilis, Pseudomonas aeriginosa* and *Candida albicans*, but very low activity against *Escherichia coli* and *Proteus vulgaris*.

Keywords: *Fistulina hepatica*, octadeca-8,11-dienoic acid methylester, ergosterol.

INTRODUCTION

Large quantity of natural products with different structure types have not only been focused on the study of functional molecules for the organism in the fields of chemical defense, chemical regulation and signal transduction, but also played a vital role in the discovery of new pharmacy. Germany, with its climatic conditions and flora diversity, is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. Studies conducted on mushrooms proved their antioxidant [1], antitumor [2], and antimicrobial properties, as well as their interesting contents in nutraceuticals [3]. Furthermore, mushrooms are becoming important in our diet for their nutritional and organoleptic characteristics [4]. Many authors have been interested in the bioactive properties and chemical profile of wild and commercial mushrooms; regarding chemical characterization, special attention have been dedicated to the determination of proteins, carbohydrates, individual sugars, fatty acids, phenolic compounds, carotenoids, ascorbic acid and tocopherols [5-11]. Other authors also analyzed ergosterol, vitamin D2, nucleosides and nucleobases in mushrooms [12-15]. A comparative study was developed on the total fatty acids composition of twelve wild edible mushroom species (*Suillus bellini*, *Suillus luteus*, *Suillus granulatus*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, *Tricholoma equestre*, *Fistulina hepatica*, *Cantharellus cibarius*, *Amanita caesarea* and *Hydnum rufescens*). By GC-MS analysis was established that polyunsaturated and monounsaturated fatty acids, valuable healthy compounds for humans, predominated over saturated fatty acids for all the studied mushroom species. *R. cyanoxantha* presented the highest fatty acids amounts, while *B. edulis* was the poorest species [16].

In this paper, we describe the isolation, structure elucidation and the antimicrobial properties of the related new octadeca-8, 11-dienoic acid methylester (**1**) and the known ergosterol (**2**), using high resolution EI-MS, ESI-MS and extensive NMR spectroscopic analysis.

EXPERIMENTAL

General Experimental Procedures

Electrospray mass spectra (ESI-MS) were recorded on a LCQ Finnigan Mass Spectrometer. ESI-HR mass spectra were recorded on a APEX IV, 7T, FT-ICR MS Bruker Daltonik. Electron Impact Mass-spectra (EI-HRMS) were recorded on a double focusing sector field mass spectrometer AMD-402 (AMD Inectra, Harpstedt, Germany). ^{1}H (300 and 600 MHz) and ^{13}C (75.5 and 125.7 MHz) NMR spectra were measured on a Bruker AMX 300 and on a Varian Inova 600 (599.740 MHz) spectrometer in $CDCl_3$ and CD_3OD. The chemical shifts

*Address correspondence to this author at the Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G.Bonchev-str., Bl. 26, 1113 Sofia, Bulgaria; Tel: +359 2 979 3144; E-mail: vntivanova@yahoo.com

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are expressed in δ values with TMS as an internal standard. ^{1}H-^{1}H COSY, HSQC and HMBC were obtained by conventional methods. UV-Vis spectroscopy was recorded on a Specord 2000 instrument (Analytik Jena, Germany). IR spectra were recorded on a Beckman DU 601 and Shimadzu IR scanning spectrophotometers. Gas-chromatography was performed with a Shimadzu instrument, coupled to a FID detector.

Producing Organism

The edible mushroom *Fistulina hepatica* grows widespread in the Northern hemisphere on oak trees and attracts attention due to its excellent taste and high nutrient value. A single fruit-body of *Fistulina hepatica* was collected in the forestal region of Seitenbrueck near Jena, Germany.

Isolation of a New Fatty Acid, Octadeca-8,11-dienoic Acid Methylester (1) and Ergosterol (2) from the Basidiomycete Fistulina hepatica

The biomass of *Fistulina hepatica* (550 g) was extracted threetimes with 1000 ml methanol. The combined methanol extracts were concentrated *in vacuo*, and the remaining watery phase was extracted with 3 x 500 ml ethyl acetate. After evaporation of the solvent the crude product (1.2 g) was dissolved in chloroform and chromatographed on a silica gel 60 (70-230 mesh) column (5 cm x 300 cm). The metabolites were eluted from the column by stepwise use of n-hexane (800 ml), chloroform (1000 ml), chloroform-methanol (9:1/750 ml; 7:3/1000 ml; 5:5/500 ml; 2:8/500 ml; 1:10/300 ml) and methanol (800 ml). The compound **2** was eluted with chloroform-methanol (7:3/1000 ml) and the fraction was evaporated to dryness under reduced pressure, dissolved in methanol and cristalised. Compound **1** was eluted with chloroform-methanol (1:10/300 ml) and detected by its diagnostical reddish staining by 3% vanillin/methanol/H_2SO_4 as reagent. The final separation and purification of **1** by preparative HPLC on Spherisorb ODS2 (column 12.5 mm × 250 mm), using an acetonitrile-water (83:17, v/v) and monitoring at 210 nm (flow rate, 10.0 ml min^{-1}) delivered the pure octadeca-8,11-dienoic acid methylester **1** (8.0 mg) as waxy colorless solid.

Analytical HPLC

Analytical HPLC analysis of pure octadeca-8,11-dienoic acid methylester **1** was carried out on a Spherisorb ODS2 (column 12.5 mm × 250 mm). Mobile phase: acetonitrile-water (83:17, v/v). Flow rate: 1.0 ml/min. Detection: 210 nm (diode array detector). Compound **1** was eluted with a retention time (t_R) of 2.10 minutes.

Thin-Layer Chromatography (TLC)

TLC was carried out on silica gel plates (Merck 60, F_{254}) with n-butanol-acetic acid-water [5:1:4 v/v, (upper phase)] of compound **1** and chloroform-methanol (9:1 v/v) for compound **2.** The chromatographic spots were visualized by spraying with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and ammonium molybdate, and heating at 120 ^{o}C for 3-5 minutes. In this systems the compounds **1** and **2** possessed an R_f values of 0.45 for **1** and 0.67 for **2**, and gave a blue-violet, rsp. a blue color.

Antimicrobial Activity of Compound 1

Antimicrobial activity was determined by agar diffusion test according to European Pharmacopoia, 1997 [17]. Test organisms were suspended in the melted nutrient agar (Serva) and poured into petri dishes. Holes of 8 mm diameter were cut in the agar and filled with 100 µl of a 100 mg l^{-1} solution of the compound. Inhibition zones were read after incubation for 18 h at 37 ^{o}C for bacterial strains and 30 ^{o}C for yeasts.

RESULTS AND DISCUSSION

Compound **1** was obtained as a waxy colorless solid, which was soluble in methanol, ethanol, dimethylsulfoxide, N,N-dimethylformamide and ethyl acetate, but insoluble in water, ether and *n*-hexane. It gave a blue-violet and blue color positive reactions with 3% vanillin-sulfuric acid and ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}$]. The physico-chemical properties of octadeca-8,11-dienoic acid methylester (**1**) are summarized in Table **1**. The presence of carboxyl acid ester group was attested by ν_{max} 1734 cm^{-1} in the IR spectrum. The chemical constitution and stereochemistry of **1** were settled on the basis of electrospray (ESI-MS), high-resolution electron impact mass spectrometry (EI-HRMS) and one- and two dimensional NMR measurements (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC).

The (+)-ESI mass spectrum of **1** displayed *pseudo*-molecular ion at *m/z* 317.3 $(M+Na)^+$. The molecular weight (294 g/mol) and the chemical formula $C_{19}H_{34}O_2$ were readily determined by high-resolution electron impact mass spectrometry (EI-HRMS, *m/z* 294.25460).

Table 1: Physico–Chemical Properties of Octadeca-8,11-dienoic Acid Methylester (1)

Appearance	waxy colorless solid
Molecular formula	$C_{19}H_{34}O_2$
Molecular weight	294 g/mol
EI-HRMS (m/z):	calcd.: 294.25321
	found: 294.25460
UV-Vis λ_{max} CH_3OH (nm)	< 230
IRν_{max} (KBr, cm^{-1})	524, 624, 651, 717, 979, 1090, 1182, 1249,
	1380, 1404, 1457, 1579, 1734, 2913, 2925,
	3425
R_f on TLC	0.45
[silica gel sheets Merck, Mobile phase:n-butanol-acetic acid-water, 5:1:4 v/v, (upper phase)]	
T_R on analytical HPLC (min)	2.10
[Spherisorb ODS2 (column 12.5 mm × 250 mm) Mobile phase: acetonitrile-water (83:17, v/v)]	

This formula indicated 3 double bond equivalents. EI-MS of **1** exhibited a strong ion peak at *m/z* 294.2, together with fragment peaks at *m/z* 270.2 ($C_{17}H_{34}O_2$), 239.2 ($C_{16}H_{31}O$), 227.1 ($C_{14}H_{27}O_2$) and 143.1 ($C_8H_{15}O_2$).

In the ^{1}H NMR spectrum of compound **1**, four olefinic protons appeared at δ_H 5.34 (H-8), 5.33 (H-9), 5.34 (H-11) and 5.31 (H-12). The remaining signals of the proton spectrum included a large signals at δ_H 1.27÷2.35 due to a number of overlapping methylene signals as well, as a methyl protons at δ_H 0.95 and the protons of the methoxy group at δ_H 3.64. The all-*cis* configuration at the two double bonds (C-8, C-9, C-11, C-12) was proved by $^3J_{H\text{-}8,\ H\text{-}9}$ = 6.0 Hz and $^3J_{H\text{-}11,\ H\text{-}12}$ = 6.0 Hz, typical for a *cis*-olefin. Catalytical hydrogenation of **1** by Pt/H_2 yielded stearic acid methylester as was shown by GC measurement reffering to authentic material.

The ^{13}C and DEPT NMR spectral data of **1** presented in Table **2** suggested the presence of one carboxylic acid ester group (δ_C 180.34), four sp^2 carbons (δ_C 129.06, 129.86, 130.86 and 130.93), one methyl group (δ_C 14.20), one methoxyl (δ_C 51.90) and 12 methylene groups). The chemical shifts of all proton and carbon atoms of **1** are summarized in Table **2**.

For the structural assignment of compound **1**, the ^{1}H-^{1}H COSY and ^{13}C-^{1}H long- range heteronuclear coupled NMR spectra (HMBC) were of pivotal importance.

According to the ^{1}H-^{1}H COSY spectra, the methyl group (H_3-18) at δ_H 0.95 was linked to the methylene group (H_2-17) at δ_H 1.30 adjacent to the methylene group at δ_H 1.60 (H_2-16). The methylene group (H_2-15) at δ_H 2.35 was linked to the methylene group (H_2-14) at δ_H 1.36 adjacent to the methylene group (H_2-13) group at δ_H 2.05. The COSY experiment demonstrated also couplings from the olefinic proton of H-11 at δ_H 5.34 to the protons of the methylene group (H_2-10) at δ_H 2.75. Similarly a coupling was established from the olefinic proton of H-8 at δ_H 5.34 to the protons of the methylene group (H_2-7) at δ_H 2.05.

A heteronuclear multiple bond correlation (HMBC) experiment showed long-range couplings from the protons of H_3-19 at δ_H 3.64 and H_2-2 at δ_H 1.88 to the carbonyl carbon C-1 at δ_C 180.3. Further correlations are demonstrated in Table **2** and Figure **2**.

Thus octadeca-8,11-dienoic acid (**1**) appears as a new olefinic fatty acid occurring as methylester (Figure **1**) in *Fistulina hepatica*. **1** enlarges the spectrum of the know unsaturated fatty acids from basidiomycetes. The NMR data enabled doubtlessly to distinguish between **1** and related fatty acids such as octadeca-9,12-dienoic acid (linolenic acid) as a frequently occurring plant product. Obviously in *Fistulina hepatica* there are desaturating enzymes introducing two isolated (Z)-double bonds into a C18-aliphatic acid. The new fatty acid methylester **1** displays no antimicrobial properties against *Staphylococcus aureus*, *Bacillus cereus, Bacillus subtilis, Pseudomonas aeriginosa* and *Candida albicans*, but very low activity (≥1000 µg/ml) against *Escherichia coli* and *Proteus vulgaris*. But due to the activated C-10 methylene group **1** should readily be oxidized in presence of singlet oxygen and thus could

Table 2: ^{1}H and ^{13}C NMR Chemical Shifts of Octadeca-8,11-dienoic Acid Methylester (1) in CD_3OD (600 MHz and 125.7 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_H, *J* (Hz)	HMBC (H→C)	δ_C	Groups
1	-		180.3	C=O
2	1.88 (2H, t,br)	C-1	23.9	CH_2
3	1.27 (2H, m)		30.9	CH_2
4	1.27 (2H, m)		30.9	CH_2
5	1.27 (2H, m)		30.9	CH_2
6	1.36 (2H, m)	C-5	30.2	CH_2
7	2.05 (2H, m)	C-8, C-9	28.0	CH_2
8	5.34 (1H, dd, 6.0, 6.0 Hz)		130.9	HC=
9	5.33 (1H, dd, 6.0, 4.0 Hz)		129.1	HC=
10	2.75 (2H, d,d,d, 6.0, 4.0, 1.0)	C-9	27.0	CH_2
11	5.34 (1H, dd, 6.0, 6.0 Hz)		130.9	HC=
12	5.31 (1H, dd, 6.0, 4.0 Hz)		129.0	HC=
13	2.05 (2H, d, 4.0 Hz)	C-11, C-12, C-14	28.0	CH_2
14	1.36 (2H, m)		30.2.	CH_2
15	2.35 (2H, m)	C-14, C-16, C-17	35.0	CH_2
16	1.60 (2H, m)	C-17	26.0	CH_2
17	1.30 (2H, m)		23.4	CH_2
18	0.95 (3H, dd, 7.0 Hz)	C-17	14.2	CH_3
19	3.64 (3H, s)	C-1	51.9	H_3C-O

*Coupling constants in Hz; s: singlet, d: doublet, t: triplet, m: multiplet, br: broad.

be considered as a powerful radical scavenger and valuable antioxidant food additive.

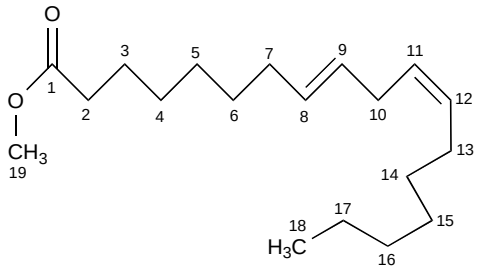

Figure 1: Structure of octadeca-8,11-dienoic acid methylester (**1**).

By Gas-chromatography of the fraction, eluted with methanol from the silica gel 60 (70-230 mesh) column from *Fistulina hepatica* was established also presence of the following fatty acids: dodecanoate (C12:0), 34.1%; hexadecanoate (C16:0), 21.9%; octadecanoate (C18:0), 9.2%; octadecenoate acid (C18:1, *cis* 9), 10.6% and octadecadienolic acid (C18:2, *cis* 9,12), 1.5%.

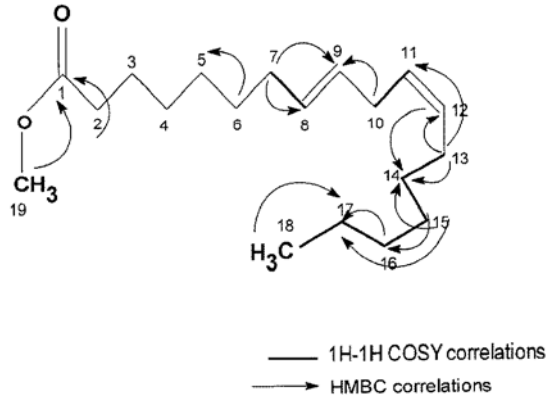

Figure 2: Instructive ^{1}H-^{1}H COSY and HMBC correlations of octadeca-8,11-dienoic acid methylester (**1**).

Compound **2** was isolated as a colorless crystals, which were soluble in chloroform, ethyl acetate but insoluble in lower alcohols and water. The chemical constitution of **2** was settled on the basis of high-resolution electron impact mass spectrometry (EI-HRMS) and one- and two dimensional NMR measurements (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, HSQC

Table 3: ^{13}C NMR and DEPT Chemical Shifts of Ergosterol (2) in $CDCL_3$ (125.7 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_C	Groups	Assignment	δ_C	Groups
1	40.8	CH_2	13-CH_3	12.0	CH_3
2	70.5	CH-O	14	54.5	CH
3	31.9	CH_2	15	23.0	CH_2
4	38.4	CH_2	16	40.0	CH_2
5	37.0	Cq	17	55.7	CH
5-CH_3	16.3	CH_3	18	40.4	CH
6	141.3	Cq	18-CH_3	21.1	CH_3
7	119.6	HC=	19	132.0	HC=
8	116.3	HC=	20	135.5	HC=
9	139.8	Cq	21	42.8	CH
10	46.3	CH	21-CH_3	17.6	CH_3
11	21.1	CH_2	22	33.1	CH
12	28.3	CH_2	22-CH_3	19.9	CH_3
13	42.8	Cq	22-CH_3	19.6	CH_3

and HMBC). The molecular weight (396 g/mol) and the chemical formula $C_{28}H_{44}O$ were readily determined by high-resolution electron impact mass spectrometry (EI-HRMS, *m/z* 396.24360). This formula indicated 7 double bond equivalents. The chemical shifts of all carbon atoms of **2** are summarized in Table **3**. On the basis of the above evidence the structure of **2** was established as known ergosterol [ergosta-5,7,22-trien-3β-ol] (Figure **3**). It is a sterol found in *Fistulina hepatica*. Ergosterol does not occur in plant or animal cells. It is a component of yeast and fungal cell membranes, serving the same function cholesterol serves in animal cells. However, ergosterol is not produced by all fungi and the ergosterol concentrations are known to vary between the same species depending on the physiological state of the fungus. Ergosterol is an unsaturated hydrocarbon of the vitamin D group isolated from yeast, mushrooms, ergot, and other fungi. When treated with ultraviolet irradiation it is converted into vitamin D_2 [18, 19].

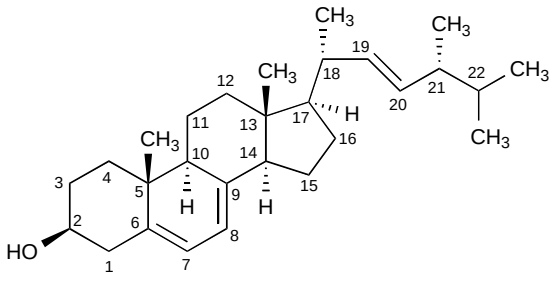

Figure 3: Structure of ergosterol (**2**).

CONCLUSIONS

The edible mushrooms are known for their high content of steroidal compounds and unsaturated fatty acids. From a single fruit-body of the basidiomycete *Fistulina hepatica*, collected in the forestal region of Seitenbrueck, Germany were isolated for the first time a secondary metabolites which were separated and purified by preparative chromatographic methods. The structures of the both compounds were determined on the basis of NMR spectroscopic methods including 1D (^1H, ^{13}C and DEPT) and 2D (COSY, HSQC and HMBC) and by mass spectrometry data (ESI, EI and HREI-MS). The new fatty acid metabolite, octadeca-8,11-dienoic acid methylester (**1**) was isolated together with the known ergosterol (**2**). The new unsaturated fatty acid is suggested to contribute much to the taste and flavour of basidiomycetes as food components and to pharmacological effects as well. The new fatty acid methylester (**1)** displays very low activity (≥1000 µg/ml) against *Escherichia coli* and *Proteus vulgaris*. Ergosterol (**2**) does not occur in plant or animal cells. It is a component of yeast, mushrooms and other fungal cell membranes.

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