Unusual Bioactive 4-Oxo-2-alkenoic Fatty Acids from *Hygrophorus eburneus*

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Z. Naturforsch. **60b,** 25 – 32 (2005); received July 7, 2004

Dedicated to Dr. Helmut Besl on the occasion of his 60th birthday

From fruit bodies of the basidiomycete *Hygrophorus eburneus* (Bull.: Fr.) Fr. (Tricholomataceae) eight fatty acids (C_{16}, C_{18}) with γ-oxocrotonate partial structure could be isolated. Initial tests demonstrate their bactericidal and fungicidal activity. The structures of (2*E*,9*E*)-4-oxooctadeca-2,9,17-trienoic acid (**1**), (2*E*,11*Z*)-4-oxooctadeca-2,11,17-trienoic acid (**2**), (*E*)-4-oxohexadeca-2,15-dienoic acid (**3**), (*E*)-4-oxooctadeca-2,17-dienoic acid (**4**), (2*E*,9*E*)-4-oxooctadeca-2,9-dienoic acid (**5**), (2*E*,11*Z*)-4-oxooctadeca-2,11-dienoic acid (**6**), (*E*)-4-oxohexadec-2-enoic acid (**7**), and (*E*)-4-oxooctadec-2-enoic acid (**8**) were elucidated on the basis of their spectroscopic data.

Key words: Hygrophorus eburneus, Basidiomycetes, 4-Oxo-2-alkenoic Fatty Acids

Introduction

Hygrophorus eburneus (Bull.: Fr.) Fr. (synonym: *H. cossus* sensu auct. eur. plur.) is an obligate mycorrhizal fungal species – mostly associated with *Fagus* species and belongs to the family Tricholomataceae in Agaricales [1]. The white fruit bodies are covered with a viscid or glutinous velum universale and are characterized by a rather weak to strong aromatic-acidulous smell which sometimes reminds of the characteristic smell of the caterpillars of *Cossus cossus* L.

In continuation of our research on (bioactive) secondary metabolites in *Hygrophorus* species [2] we discovered only very few reports on chemical analysis of secondary metabolites from fruit bodies of the genus *Hygrophorus*. Most articles deal with the common fungal sterol ergosterol and its derivatives [3 – 5]. Muscaflavine and hygrophoric acid were isolated from some *Hygrophorus* species [6]. Indole and 3-chloroindole from *Hygrophorus paupertinus* A. H. Smith & Hesler are the reason for its disagreeable odour [7]. Fungicidal cyclopentenone derivatives could be isolated from various *Hygrophorus* species [2]. Volatile compounds from some *Hygrophorus* species, among other fungi, were investigated by GC-MS using steam distillation [8 – 11].

Only two reports are dealing with *H. eburneus* and *H. cossus*, respectively. As flavour compound in the odour of *H. cossus*, tridecanal could be detected after steam distillation [10]. A second report describes the isolation and structure elucidation of hygrophamide as (2*S*, 3*S*, 4*R*, 2'*R*)-2-(2'-hydroxy-9'-*Z*-ene-tetracosanylamino)-octadecane-1,3,4-triol (**15**) [12]. Strangely, the authors named the investigated species *Hygrophorus eburnesus* Fr. (collected at Lijiang of Yunnan Province in July 2002, People's Republic of China). Surprisingly, this species is not described in relevant Chinese mycological literature or data bases [13, 14].

In this report on *Hygrophorus* species we describe the isolation and structure elucidation of unusual fatty acids (C_{16} or C_{18}) with γ-oxocrotonate partial structure **1** – **8**. Additionally, some compounds possess an internal *E* or *Z* configured optional double bond or a terminal one. Initial tests demonstrate their fungicidal activity.

Results and Discussion

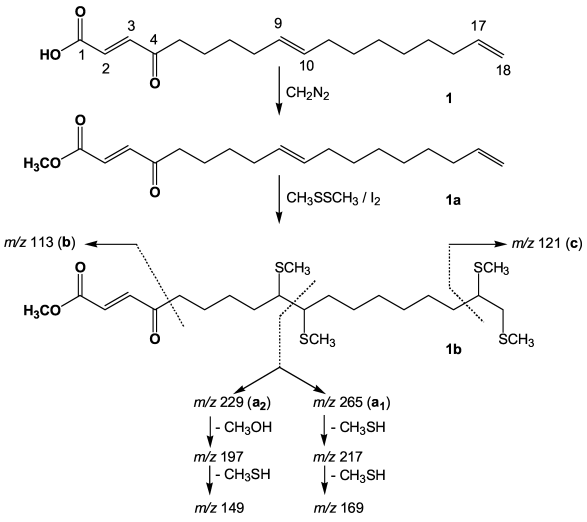
The compounds **1** – **8** exhibit nearly the same R_f-values (0.55 – 0.60) on silica TLC-plates and could be detected by UV light (fluorescence quenching at

Table 1. ^{1}H NMR and ^{13}C NMR data in $CDCl_3$ of **1**, 400 MHz or 500 MHz.

C	δ C	δ H	^{1}H -^{1}H COSY selected	ROESY selected	HMBC selected
1 (COOH)	169.0				
2 (CH)	140.8	7.116 *d* (16.0)	H-3		
3 (CH)	129.7	6.673 *d* (16.0)	H-2		
4 (CO)	199.5				H-5; H-6
5 (CH_2)	41.6	2.656 *t* (7.3)	H-6	H-6, H-7	H-6; H-7
6 (CH_2)	23.2	1.62 – 1.71 *m*	H-5; H-7	H-5, H-7, H-8	H-5; H-7; H-8
7 (CH_2)	29.1	1.24 – 1.42 *m*	H-8	H-5, H-6, H-8	H-5; H-6; H-8; H-9
8 (CH_2)	27.2	1.98 – 2.09 *m*	H-7; H-9	H-6, H-7, H-9	H-6; H-7; H-9
9 (CH)	130.5	5.29 – 5.42 *m* (18.2)	H-11	H-8	H-8
10 (CH)	128.9	5.29 – 5.42 *m* (18.2)	H-11	H-11	H-11
11 (CH_2)	26.9	1.98 – 2.09 *m*	H-10; H-12	H-10, H-12	H-12
12 – 15 (CH_2)	29.6 – 28.9	1.24 – 1.42 *m*			
16 (CH_2)	33.8	1.98 – 2.09 *m*		H-15	H-17; H-18
17 (CH)	139.2	5.813 *ddt* (17.1/10.2/6.7)	H-16; H-18	H-18	H-16
18 (CH_2)	114.1	H-18*Z*: 4.994 *dtd* (17.1/1.9/1.4) H-18*E*: 4.951 *dtd* (10.2/1.7/1.4)	H-17	H-17	H-16

254 nm) or after spraying with potassium permanganate. After separation by HPLC, compound **1** was obtained as a slightly yellow solid. Its UV spectrum exhibits one absorption band at $\lambda_{max} = 219$ nm. The IR spectrum presents a strong hydroxyl absorption band at 2924 cm^{-1}; bands at 1684 cm^{-1} and 1640 cm^{-1} belong to carbonyl groups. The negative ion electrospray (ESI) mass spectrum shows two characteristic ions at *m/z* 291 $[M\text{-}H]^-$ and *m/z* 247 $[M\text{-}H\text{-}CO_2]^-$. The molecular formula $C_{18}H_{28}O_3$ was deduced from negative ion ESI-FT-ICR-MS (*m/z* 291.19631 $[M\text{-}H]^-$, calcd. for $C_{18}H_{27}O_3$ 291.19657).

The signals at $\delta = 168.9$ and 199.5 ppm belong to a carboxyl group and a carbonyl group, respectively. The signals at $\delta = 129.7$ ppm and $\delta = 140.8$ ppm which show HSQC correlations with the doublets at $\delta = 7.11$ ppm and $\delta = 6.67$ ppm (1H, *d*, $J_{A,B} = 16.0$ Hz) indicate a *trans* configured double bond-positioned between the carboxyl and carbonyl group. Methine ^{13}C NMR signals at $\delta = 130.5$ ppm and $\delta = 128.9$ ppm demonstrate the presence of a non-terminal double bond, which is correlated to the NMR signal at $\delta = 5.29 - 5.42$ Hz in the HSQC spectrum. The vicinal coupling constant $J = 18.2$ Hz of these two protons was derived from the corresponding ^{13}C satellites in the ^{1}H NMR spectrum and proved the *trans* configuration of the double bond. The signals from the three spin system at $\delta = 5.81$ ppm (1H, *ddt*, $J = 17.1/10.2/6.7$ Hz), $\delta = 4.99$ ppm (1H, *dtd*, $J = 17.1/1.9/1.4$ Hz) and $\delta = 4.95$ ppm (1H, *dtd*, $J = 10.2/1.7/1.4$ Hz) are due to a terminal double bond. In Table 1 the ^{1}H NMR and ^{13}C NMR signals of **1** are summarized.



Scheme 1. Structure of **1** and its derivatives.

To determine the position of the double bond at $\delta = 130.5$ ppm, compound **1** was treated with diazomethane (**1a**) followed by addition of dimethyl disulfide (**1b**). Derivative **1b** was subjected to GC-EIMS analysis immediately (R_t **1b**: 34.8 min). The EI mass spectrum exhibits the $[M]^+$ ion at *m/z* 494, and significant key ions at *m/z* 265 (type $\mathbf{a_1}$) and *m/z* 229 (type $\mathbf{a_2}$) resulting from the α-cleavage between the

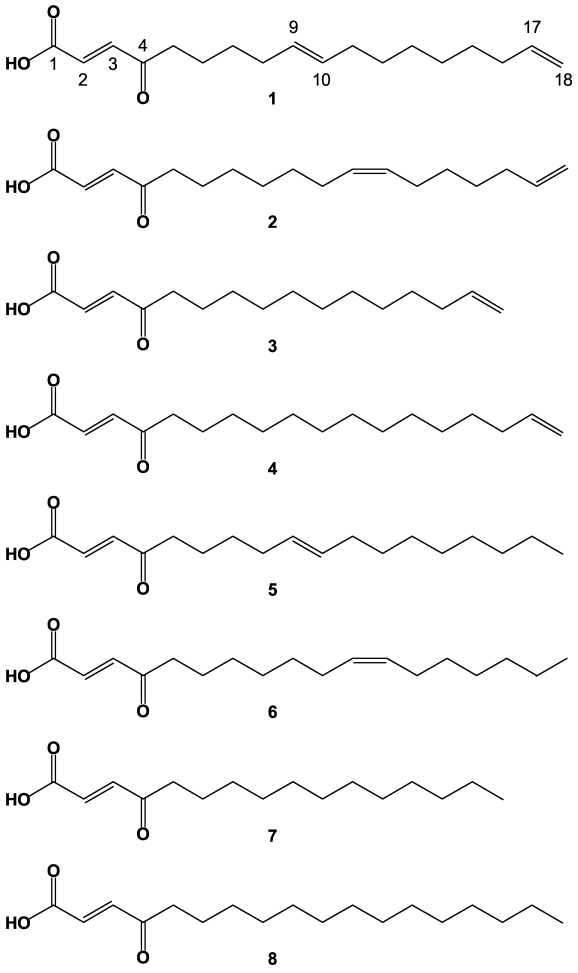
Table 2. ^{13}C NMR data of compounds **2**–**8** in $CDCl_3$, 400 MHz.

C	**2**	**3**	**4**	**5**	**6**	**7**
1	168.6	168.5	168.3	169.8	169.7	169.1
2	140.9	140.9	140.6	140.9	141	140.8
3	129.4	129.3	129.8	129.7	129.8	129.7
4	199.6	199.7	199.9	199.5	199.7	199.8
5	41.7	41.7	41.7	41.6	41.7	41.7
6	23.2	23.6	23.6	23.2	23.6	23.6
7	29.5	29.5	29.7	29.5	29.7	31.9
8	29.3	29.4	29.6	27.2	29.5	29.6
9	29.2	29.4	29.5	130.6	29.3	29.6
10	29	29.3	29.4	128.9	29	29.6
11	129.9	29.1	29.3	26.9	130.1	29.4
12	128.7	29.1	29.1	31.9	129.6	29.3
13	27	28.9	29.1	29.7	27.1	29.3
14	28.5	33.8	29	29.3	31.8	29.1
15	27.1	139.2	28.9	29.3	29.3	22.7
16	33.7	114.1	33.8	29.1	27.2	14.1
17	139.1		139.3	22.7	22.6	
18	114.2		114.1	14.1	14.1	

two carbons bearing a methylthio group. While the ion at *m/z* 113 (**b**) characterizing the 4-oxo-2-enoic acid moiety is a key fragment for all detected fatty acids, the ion at *m/z* 121 (**c**) represents a specific ion for a terminal double bond as in **1b**. These data indicate that the additional double bond in **1** is located between C-9 and C-10 (Scheme 1). From all data, the structure of compound **1** was established as (2*E*,9*E*)-4-oxooctadeca-2,9,17-trienoic acid.

The spectral data of compounds **2**–**8** are closely related to **1**. The UV spectra of **2**–**8** exhibit one absorption band at $\lambda_{max} = 219$ nm. Their IR spectra present strong absorption bands for a hydroxyl and for carbonyl groups. As in compound **1**, the negative ion ESI mass spectra show two characteristic ions representing the $[M\text{-}H]^-$ ion and $[M\text{-}H\text{-}CO_2]^-$ ion. The molecular formulas were deduced from negative ion ESI-FT-ICR-MS data.

The double bond of compounds **2**–**8** at C-2 in all cases is *trans* configured ($^3J_{H-2,H-3} \sim 16$ Hz). The methine signals of C-11 and C-12 in compound **2** and **6**, respectively, indicate the presence of a non-terminal double bond which is correlated to NMR signals at H-11 and H-12 in the HSQC spectra. The vicinal coupling constants $J = 10.2$ Hz of these two protons in **2** and **6** were derived from the corresponding ^{13}C satellites and proved the *cis* configuration of the double bond, in contrast to compound **1** and **5** with *trans* configured double bond (Table 3). The signals from the three spin system in compounds **2** (H-17, H-18*E*, H-18*Z*), **3** (H-15, H-16*E*, H-16*Z*), and **11**



(H-17, H-18*E*, H-18*Z*) are resulting from a terminal double bond. Other than compounds **2**–**4**, the acids **5**–**8** do not possess a terminal double bond. The ^{13}C-NMR spectra of compounds **2**–**7** are given in Table 2; for compound **8** due to the very low yield, no ^{13}C data were available. From the mass spectral data, the length of the fatty acid chain in compounds **3** and **7** could be determined as C_{16}, for all others as C_{18}. For determination of the position of the non terminal double bond in **2**, **5**, and **6** the compounds were derivatized with diazomethane, followed by addition of dimethyl disulfide. Immediate GC-EI-MS analysis gave characteristic fragments in the MS spectrum. The spectra showed the major peak ion for **2b** at *m/z* 494 $[M]^+$ (R_t **2b**: 34.2 min) and for **5b** and **6b** at *m/z* 402 $[M]^+$ (R_t **5b**: 26.4 min; R_t **6b**: 26.5 min). From the fragmentation pattern, the position of the double bond in **2** and **6** could be located between C-11 and

Table 3. ^{1}H NMR data of compounds **2** – **8** in $CDCl_3$, 400 MHz.

	2 δ H	**3** δ H	**4** δ H	**5** δ H	**6** δ H	**7** δ H	**8** δ H
2	7.139 (*d*, 16.0)	7.123 (*d*, 16.0)	7.103 (*d*, 16.0)	7.128 (*d*, 16.0)	7.132 (*d*, 15.9)	7.114 (*d*, 16.0)	7.120 (*d*, 16.0)
3	6.692 (*d*, 16.0)	6.675 (*d*, 16.0)	6.672 (*d*, 16.0)	6.674 (*d*, 16.0)	6.674 (*d*, 16.0)	6.670 (*d*, 16.0)	6.678 (*d*, 16.0)
5	2.649 (*t*, 7.3)	2.646 (*t*, 7.4)	2.642 (*t*, 7.4)	2.656 (*t*, 7.3)	2.652 (*t*, 7.2)	2.645 (*t*, 7.3)	2.644 (*t*, 7.4)
6	1.60 – 1.68 (*m*)	1.6 – 1.67 (*m*)	1.60 – 1.68 (*m*)	1.62 – 1.70 (*m*)	1.61 – 1.69 (*m*)	1.60 – 1.69 (*m*)	1.59 – 1.73 (*m*)
7	1.30 – 1.43 (*m*)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	1.24 – 1.43 (*m*)	1.25 – 1.36 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
8	1.30 – 1.43 (*m*)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*) (*m*)	1.98 – 2.09 (*m*)	1.25 – 1.36 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32
9	1.30 – 1.43 (*m*)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	5.29 – 5.42 (*m*, 18.3)	1.25 – 1.36 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
10	2.00 – 2.08 (*m*)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	5.29 – 5.42 (*m*, 18.3)	1.98 – 2.04 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
11	5.30 – 5.41 (*m*, 10.2)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	1.98 – 2.09 (*m*)	5.30 – 5.40 (*m*, 10.2)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
12	5.30 – 5.41 (*m*, 10.2)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	1.24 – 1.43 (*m*)	5.30 – 5.40 (*m*, 10.2)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
13	2.00 – 2.08 (*m*)	1.24 – 1.40 (*m*)	1.23 – 1.40 (*m*)	1.24 – 1.43 (*m*)	1.98 – 2.04 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
14	1.30 – 1.43 (*m*)	2.01 – 2.07 (*m*)	1.23 – 1.40 (*m*)	1.24 – 1.43 (*m*)	1.25 – 1.36 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
15	2.00 – 2.08 (*m*)	5.814 (*ddt*, 17.1/10.3/7.6)	1.23 – 1.40 (*m*)	1.24 – 1.43 (*m*)	1.25 – 1.36 (*m*)	1.24 – 1.42 (*m*)	1.24 – 1.32 (*m*)
16	2.00 – 2.08 (*m*)	H-16Z: 4.993 (*ddt*, 17.1/ 1.8/1.7) H-16E: 4.930 (*ddt*,10.3/ 1.8/1.4)	1.99 – 2.07 (*m*)	1.24 – 1.43 (*m*)	1.25 – 1.36 (*m*)	0.880 (*t*, 6.7)	1.24 – 1.32 (*m*)
17	5.827 (*ddt*, 17.2/ 10.1/6.5)		5.816 (*ddt*, 17.1/ 10.2/6.7)	1.24 – 1.43 (*m*)	1.25 – 1.36 (*m*)		1.24 – 1.32 (*m*)
18	H-18Z: 5.014 (*dtd*,17.2/ 1.8/1.7) H-18E: 4.951 (*dtd*, 10.1/ 1.9/1.7)		H-18Z: 4.993 (*ddt*, 17.1/ 2.1/1.6) H-18E: 4.928 (*ddt*, 10.2/ 2.1/1.2)	0.879 (*t*, 6.7)	0.882 (*t*, 6.8)		0.880 (*t*, 6.7)

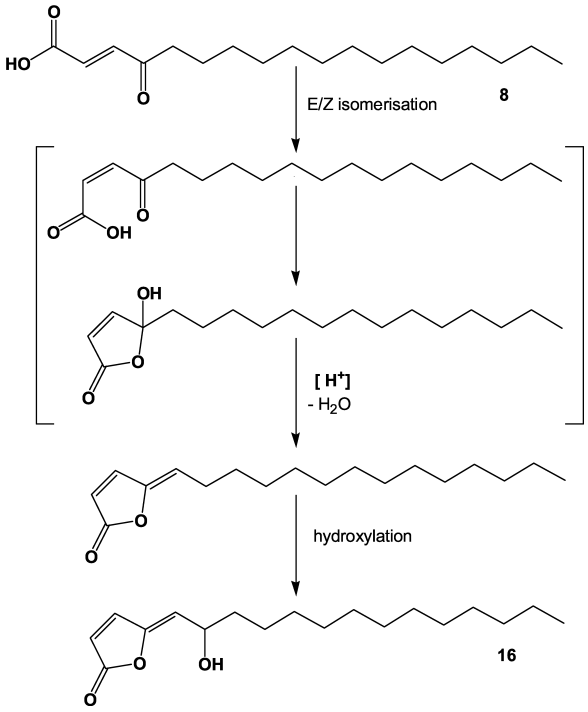
C-12 in similar manner as described for compound **1** above (Scheme 1), and in **5** between C-9 and C-10. In conclusion, from the data the structures of compounds **2** – **8** can be established as (2*E*,11*Z*)-4-oxooctadeca-2,11,17-trienoic acid (**2**), (*E*)-4-oxohexadeca-2,15-dienoic acid (**3**), (*E*)-4-oxooctadeca-2,17-dienoic acid (**4**), (2*E*,9*E*)-4-oxooctadeca-2,9-dienoic acid (**5**), (2*E*,11*Z*)-4-oxooctadeca-2,11-dienoic acid (**6**), (*E*)-4-oxohexadec-2-enoic acid (**7**), and (*E*)-4-oxo-octadec-2-enoic acid (**8**).

Initial tests of fungicidal activity were carried out by the method of Gottstein a semiquantitative test that allows a relative estimation of the activity of compounds with similar diffusion characteristics [15].

Table 4. Inhibition area in mm^2 of **2**, **3**, **5**, **6**, and **7** after application of 0.5 μg to 100 μg. A larger area correlates with higher activity.

	5 μg	10 μg	20 μg	40 μg	80 μg	100 μg
(**2**)	201	283	415	530	706	907
(**3**)	201	283	314	314	452	615
(**5**)	254	314	346	490	706	706
(**6**)	201	314	380	530	706	706
(**7**)	153	153	176	201	254	254

The phytopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth. was used as test organism. All compounds tested exhibit a similar antifungal activity against *C. cucumerinum*. The size of the inhibition area of **2**, **3**, **5**, **6**, and **7** is given in Table 4. However, those

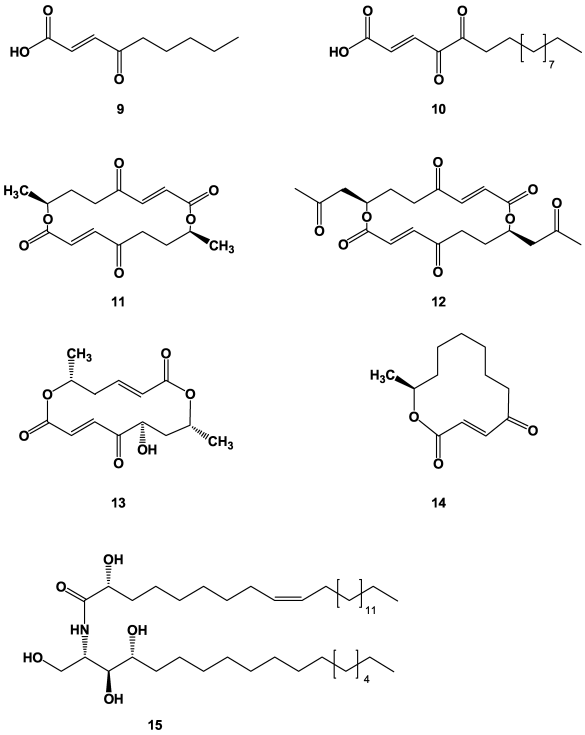


Scheme 2. Hypothetical relationship of **8** to hygrophorone G^{12} (**16**).

with an internal double bond (**2**, **5**, **6**) appear to be a little more active. In addition to the fungizidal properties, bacterizidal effects were observed as well.

A biosynthetic relationship of the 4-oxo-2-alkanoic fatty acids to the hygrophorones might be possible (Scheme 2) [2].

There have been some reports of fatty acids from natural resources with γ-oxocrotonate partial structure and derivatives therefrom. (*E*)-4-Oxonon-2-enoic acid (**9**) isolated from *Streptomyces olivaceus* exhibits high bactericidal activity against Gram-positive and Gram-negative bacteria [16]. Podoscyphic acid (**10**) obtained from fermentations of the basidiomycete *Podoscypha* spec. inhibits the avian myeloblastosis virus and moloney murine leukemia virus reverse transcriptase [17]. From the phytopathogenic fungus *Pyrenophora avenae* the cyclic lactone pyrenophorin (**11**) with fungicidal and cytostatic properties could be isolated [18]. Very similar to **11** is vermiculin (**12**) from *Penicillium vermiculatum* with antibiotic and antiprotozoic features [19]. From the culture filtrate of the phytopathogenic fungi *Colletotrichum capsici* the dimeric cyclic bislactone colletoketol (**13**) could be isolated [20]. The antibiotic macrolide patulolid



A (**14**) is described from cultures of *Penicillium urticae* [21]. As aforementioned, the ceramide named hygrophamide (**15**) from the doubtful Chinese species *Hygrophorus eburnesus* shows some structural similarity with the herein reported fatty acids from *Hygrophorus eburneus* [12].

Experimental Section

General

1D NMR spectra (^{1}H, ^{13}C) were recorded from a Varian Unity 400 at 400 MHz for ^{1}H, and at 100 MHz for ^{13}C NMR. 2D NMR spectra (HSQC, HMBC, COSY, ROESY) were recorded from a Varian Inova 500 at 500 MHz for ^{1}H. Chemical shifts in ppm were referenced to the internal TMS ($\delta = 0$) for ^{1}H and $CDCl_3$ ($\delta = 77.0$ ppm) for ^{13}C, respectively.

Preparative HPLC were performed on a Varian ProStar 218 system with a PrepStar 330 photodiode array detector using a Nucleosil 100-7 C-18 column (250 × 21 mm, Macherey-Nagel).

The high resolution negative ion ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Infinity^{TM} cell, a 7.0 Tesla superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 μl/h.

The GC-MS measurements were performed with a GC-MS system (Voyager, ThermoQuest): 70 eV EI, source temp. 200 °C, column DB-5MS (30 m×0.25 mm, 0.25 μm film thickness), injection temperature 250 °C, interface temperature 300 °C, carrier gas He, flow rate 1.0 ml/min, constant flow mode, splitless injection, column temperature programm: 60 °C for 1 min, then raised to 300 °C at a rate of 10 °C/min, then isothermal at 300 °C for 20 min.

IR spectra were measured with an Bruker IFS 28 infrared spectrophotometer as film on NaCl. UV spectra were obtained in methanol from a Jasco J-710 UV spectrophotometer.

Melting points were obtained on a VMTG apparatus (Leica, Germany) and are uncorrected.

Column chromatography was carried out on silica gel 60 (0.063 – 0.200 mm, Merck, Germany). TLC was carried out on plates precoated with silicagel 60 F_{254} (Merck). Potassium permanganate was used as spray reagent (5% $KMnO_4$ in 5% aqueous Na_2CO_3 solution).

Fungal material

Fresh fruit bodies *of Hygrophorus eburneus* (Bull.: Fr.) Fr. were collected near Freyburg, Saxony-Anhalt, Germany under *Fagus* spec. (14.10.2003, leg. A. Teichert, T. Lübken, N. Arnold; det. N. Arnold). Voucher specimen are deposited at the Leibniz-Institute of Plant Biochemistry Halle (Saale), Germany (IPB).

Extraction and isolation

Frozen fruitbodies of *H. eburneus* (246 g) were extracted at room temperature with ethyl acetate (1 l). The light yellow solution was concentrated to dryness *in vacuo* to produce an oily residue (1.28 g). The crude extract was subjected to column chromatography on silica gel eluting with solvent mixture of ethyl acetate/methanol/water (6:1:1, v/v). Fractions containing **1** – **8** (TLC: $R_f = 0.55 - 0.60$; silica gel 60 F_{254}, solvent system ethyl acetate/methanol/water (6:1:1, v/v)) were further purified by preparative HPLC using a Nucleosil 100 C18 column (7 μm, 250 × 21 mm ID, Macherey & Nagel, Germany) using H_2O + 0.2% TFA (A) and CH_3CN + 0.2% TFA (B) as solvents (linear gradient: 0 – 40 min, 65% B – 68% B; isocratic flow: 40 – 50 min, 32% A and 68% B, flow rate 27 ml/min). Fraction I at 17.7 min contains 3.3 mg **3**, fraction II at 20.4 min contains 2.6 mg **2**, fraction III at 21.0 min contains 5.9 mg **1**, fraction IV at 27.8 min contains 6.3 mg **7**, fraction V at 30.7 min contains 11.6 mg **6**, fraction VI at 32.1 min contains 12.6 mg **5**, fraction VII at 35.3 min contains 2.6 mg **4**, fraction VIII from 37 – 45 min contains **8**. Fraction VIII was further purified in a similar manner by HPLC using H_2O + 0.2% TFA (A) and CH_3CN + 0.2% TFA (B) as solvents (linear gradient: 0 – 40 min, 65% B – 85% B, flow rate 27 ml/min) and yield 1 mg compound **8** at 31.8 min.

Methylation of compounds **1**, **2**, **5**, *and* **6**

In a diazomethane-generator (Aldrich) ∼ 50 ng of compound **1**, **2**, **5**, and **6**, respectively, dissolved in 3 ml diethyl ether, were placed in the outside tube. 1 mmol (133 mg) of MNNG reagent (1-methyl-3-nitro-1-nitrosoguanidine) is placed in the inside tube with 0.50 ml of water. The two parts are assembled and held together by tightening the screw cap. The lower part is immersed in an ice bath and 1 ml of 40% sodium hydroxide is injected. After 2 h reaction time 2 ml acetic acid was added and the diethyl ether solution was removed and evaporated to dryness to yield the methylation products **1a**, **2a**, **5a**, and **6a**, respectively, which immediately were derivatized further.

Methylsulfanylation of compounds **1a**, **2a**, **5a**, *and* **6a**

According to the method of Francis and Veland the samples **1a**, **2a**, **5a**, and **6a** were dissolved in 300 μl dimethyl disulfide [22]. 50 μl of an iodine solution (60 mg I_2 in 1 ml diethyl ether) was added and stirred in a gas tight vial for 48 h at room temperature. After the reaction was finished, the samples were treated with sodium thiosulfate solution (0.5 g in 10 ml distilled water) and extracted with 500 μl *n*-hexane. The organic layers were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The resulting compounds **1b**, **2b**, **5b**, and **6b**, respectively, were analyzed by GC-MS.

*(2E,9E)-4-Oxooctadeca-2,9,17-trienoic acid (***1***):* Slightly yellow solid. – TLC: $R_f = 0.55$. – HPLC: $R_t = 21.0$ min. – M.p. 95 – 105 °C. UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 219$ nm (3.58). – IR (film): $\nu = 3065$, 3000, 2924, 2852, 1684, 1669, 1640, 1623, 1458, 1436, 1404, 1277, 1170, 991, 910 cm^{-1} Negative ion ESI-MS: *m/z* 291 $[M\text{-}H]^-$, 247 $[M\text{-}H\text{-}CO_2]^-$. Negative ion ESI-FT-ICR-MS: *m/z* 291.19631 $[(M\text{-}H)^-$, calcd. for $C_{18}H_{27}O_3{}^-$ 291.19657]. ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 1.

*(2E,11Z)-4-Oxooctadeca-2,11,17-trienoic acid (***2***):* Slightly yellow solid. – TLC: $R_f = 0.60$. – HPLC: $R_t = 20.4$ min. – M.p. 90 – 110 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 220$ nm (3.36). – IR (film): $\nu = 3070$, 2926, 2854, 1693, 1681, 1650, 1402, 1204, 1140, 989, 910, 800, 723 cm^{-1}. Negative ion ESI-MS: *m/z* 291 $[M\text{-}H]^-$, 247. $[M\text{-}H\text{-}CO_2]^-$. Negative ion ESI-FT-ICR-MS: *m/z* 291.19629 ($[M\text{-}H]^-$, calcd. for $C_{18}H_{27}O_3{}^-$ 291.19657). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(E)-4-Oxohexadeca-2,15-dienoic acid (***3***):* Slightly yellow solid. – TLC: $R_f = 0.55$. – HPLC: $R_t = 17.7$ min. – M.p. 95 – 110 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 219$ nm (3.30). – IR (film): $\nu = 3068$, 2913, 2848, 1684, 1663, 1623, 1470, 1439, 1412, 1279, 1219, 1000, 911, 756, 715 cm^{-1}. Negative ion ESI-MS: *m/z* 265 $[M\text{-}H]^-$, 221 $[M\text{-}H\text{-}CO_2]^-$. Negative ion ESI-FT-ICR-MS: *m/z* 265.18062 ($[M\text{-}H]^-$,

calcd. for $C_{16}H_{25}O_3^-$ 265.18092). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(E)-4-Oxooctadeca-2,17-dienoic acid (**4**):* Slightly yellow solid. – TLC: $R_f = 0.60$. – HPLC: $R_t = 35.3$ min. – M.p. 106 – 108 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 220$ nm (3.18). – IR (film): $\nu = 3060$, 2912, 2848, 1682, 1664, 1470, 1412, 1279, 1219, 1000, 911, 715 cm^{-1}. – Negative ion ESI-MS: $m/z = 293$ [M-H]$^-$, 249 [M-H-CO_2]$^-$. Negative ion ESI-FT-ICR-MS: *m/z* 293.21180 ([M-H]$^-$, calcd. for $C_{18}H_{29}O_3^-$ 293.21222). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(2E,9E)-4-Oxooctadeca-2,9-dienoic acid (**5**):* Slightly yellow solid. – TLC: $R_f = 0.60$. – HPLC: $R_t = 32.1$ min. – M.p. 90 – 110 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 219$ nm (3.72). – IR (film): $\nu = 3064$, 3000, 2919, 2850, 1669, 1680, 1620, 1463, 1434, 1401, 1287, 1170, 1008, 977, 793, 720 cm^{-1}. Negative ion ESI-MS: *m/z* 293 [M-H]$^-$, 249 [M-H-CO_2]$^-$. Negative ion ESI-FT-ICR-MS: *m/z* 293.21188 ([M-H]$^-$, calcd. for $C_{18}H_{29}O_3^-$ 293.21222). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(2E,11Z)-4-Oxooctadeca-2,11-dienoic acid (**6**):* Slightly yellow solid. – TLC: $R_f = 0.60$. – HPLC: $R_t = 30.7$ min. – M.p. 90 – 105 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 220$ nm (3.84). – IR (film): $\nu = 3065$, 3001, 2952, 2851, 1681, 1667, 1624, 1466, 1438, 1403, 1376, 1278, 1246, 1216, 1170, 1006, 937, 758 cm^{-1}. – Negative ion ESI-MS: *m/z* 293 [M-H]$^-$, 249 [M-H-CO_2]$^-$. Negative ion ESI-FT-ICR-MS: *m/z* 293. 21184 ([M-H]$^-$, calcd. for $C_{18}H_{29}O_3^-$ 293.21222). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(E)-4-Oxohexadec-2-enoic acid (**7**):* Slightly yellow solid. – TLC: $R_f = 0.55$. – HPLC: $R_t = 27.8$ min. – M.p. 90 – 110 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 219$ nm (3.80). – IR (film): $\nu = 3065$, 2913, 2848, 1682, 1664, 1559, 1471, 1413, 1203, 1138, 1000, 910, 715 cm^{-1}. – Negative ion ESI-MS: *m/z* 267 [M-H]$^-$, 223 [M-H-CO_2]$^-$. Negative ion ESI-FT-ICR-MS: *m/z* 267.19626 ([M-H]$^-$, calcd. for $C_{16}H_{27}O_3^-$ 267.1965). – ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 2 and Table 3.

*(E)-4-Oxooctadec-2-enoic acid (**8**):* Slightly yellow solid. – TLC: $R_f = 0.60$. – HPLC: $R_t = 31.8$ min. – M.p. 95 – 110 °C. – UV/vis (CH_3OH): $\lambda_{max}(\lg\varepsilon) = 219$ nm (3.44). – IR (film): $\nu = 3050$, 2914, 2848, 1682, 1661, 1470, 1413, 1380, 1250, 1210, 1180, 990, 715 cm^{-1}. – Negative ion ESI-MS: *m/z* 295 [M-H]$^-$, 251 [M-H-CO_2]$^-$. Negative ion ESI-FT-ICR-MS: *m/z* 295.22750 ([M-H]$^-$, calcd. for $C_{18}H_{31}O_3-$ 295.22787). ^{1}H NMR ($CDCl_3$) see Table 2.

*9,10,17,18-Tetrakis-methylsulfanyl-4-oxo-octadec-2-enoic acid methyl ester (**1b**):* GC: $R_t = 34.8$ min. – 70 eV EI-MS (*m/z* (rel. int., %)): 494 (M$^+$, 2), 320 (3), 265 (**a$_1$**, 4), 237 (3), 229 (**a$_2$**, 2), 221 (2), 219 (4), 218 (6), 217 [(**a$_1$**-MeSH), 36], 216 (3), 211 (5), 207 (6), 205 (4), 197 [(**a$_2$**-MeOH), 10], 189 (3), 181 (9), 171 (8), 170 (8), 169 [(**a$_1$**-2MeSH), 62], 155 (5), 153 (8), 149 [(**a$_2$**-MeOH-MeSH), 22], 147 (4), 141 (7), 131 (5), 121 (**c**, 32), 119 (4), 113 (**b**, 43), 109 (19), 107 (9), 105 (6), 101 (6), 95 (19), 93 (29), 91 (10), 87 (44), 85 (14), 81 (28), 80 (5), 79 (28), 78 (2), 77 (8), 75 (10), 74 (8), 73 (10), 69 (6), 67 (34), 66 (2), 65 (3), 63 (5), 61 (CH_2=S$^+$-Me, 100), 55 (27).

*11,12,17,18-Tetrakis-methylsulfanyl-4-oxo-octadec-2-enoic acid methyl ester (**2b**):* GC: $R_t = 34.2$ min. – 70 eV EI-MS (*m/z* (rel. int., %)): 494 (M$^+$, 1), 399 (5), 281 (4), 257 (**a$_2$**, 4), 239 (6), 237 (**a$_1$**, 35), 225 [(**a$_2$**-MeOH), 3], 221 (6), 209 (7), 207 (9), 191 (10), 190 (4), 189 [(**a$_1$**-MeSH), 27], 177 [(**a$_2$**-MeOH-MeSH), 6], 169 (5), 149 (7), 147 (7), 143 (6), 141 [(**a$_1$**-2MeSH), 58], 131 (12), 129 (6), 127 (5), 121 (**c**, 8), 119 (4), 113 (**b**, 32), 109 (8), 107 (8), 105 (6), 99 (4), 97 (7), 96 (5), 95 (35), 94 (7), 93 (45), 91 (12), 87 (28), 86 (2), 85 (17), 81 (37), 80 (5), 79 (21), 77 (6), 75 (8), 74 (6), 73 (14), 71 (7), 69 (7), 67 (28), 61 (CH_2=S$^+$-Me, 100), 59 (10), 55 (24).

*9,10-Bis-methylsulfanyl-4-oxo-octadec-2-enoic acid methyl ester (**5b**):* GC: R_t = 26.4 min. – 70 eV EI-MS (*m/z* (rel. int., %)): 402 (M$^+$, 7), 229 (**a$_2$**, 5), 227 (4), 197 [(**a$_2$**-MeOH), 23], 181 (24), 175 (4), 173 (**a$_1$**, 100), 169 (23), 153 (18), 149 [(**a$_2$**-MeOH-MeSH), 33], 145 (4), 131 (4), 121 (13), 113 (**b**, 45), 87 (9), 85 (5), 83 (15), 81 (6), 69 (21), 61 (CH_2=S$^+$-Me, 30), 55 (13).

*11,12-Bis-methylsulfanyl-4-oxo-octadec-2-enoic acid methyl ester (**6b**):* GC: $R_t = 26.5$ min. – 70 eV EI-MS (*m/z* (rel. int., %)): 402 (M$^+$, 7), 257 (**a$_2$**, 14), 225 [(**a$_2$**-MeOH), 8], 209 [(**a$_2$**-MeSH), 26], 191 (34), 177 [(**a$_2$**-MeOH-MeSH), 17], 159 (6), 149 (14), 146 (11), 145 (**a$_1$**, 100), 131 (22), 129 (6), 113 (**b**, 41), 109 (10), 97 (39), 95 (11), 93 (8), 87 (18), 85 (10), 81 (27), 69 (13), 67 (16), 61 (CH_2=S$^+$-Me, 62), 55 (57).

Bioactivity evaluation

Fungicidal activity

A dilution series of **1**, **2**, **5**, **6**, and **7** in methanol containing substance in a range from 0.5 μg to 100 μg was spotted on 0.5 mm thin layer silica plates and sprayed with an aqueous, nutritive suspension of the phytopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth. After two days in a wet chamber (> 95% humidity) the plates were overgrown with a dark gray coloured mycelium. Areas with sufficient fungicidal compound were recognizable as white spots (inhibition area). A relative quantitative estimation can be deduced from the size and intensity of the spots.

Acknowledgements

The authors are indebted to Mrs. Monika Kummer for implementation of antifungal tests, Mrs. Christine Kuhnt for measuring GC-MS, as well as Mrs. Maritta Süsse for IR and UV measurements.

The work was financially supported by the Kultusministerium of Saxony-Anhalt (HWP) and the Deutsche Forschungsgemeinschaft (DFG, AR 358/3-1).

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