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Secondary metabolites from *Ganoderma lucidum* and *Spongiporus leucomallellus*

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Abstract

The hydrodistillates and solvent extracts of the fruit bodies of *Ganoderma lucidum* (Fr.) P. Karst. and *Spongiporus leucomallellus* (Murril) A. David were investigated. The constituents in both oils comprised hydrocarbons, monoterpenes, sesquiterpenes, and fatty acids. Major volatiles of *G. lucidum* were *trans*-anethol, *R*-(−)-linalool, *S*-(+)-carvone and α-bisabolol, while the essential oil of *S. leucomallellus* contained relatively large amounts of *R*-(−)-1-octene-3-ol, *R*-(−)-linalool, 1-hepten-3-one and (*Z*)-nerolidol. From the *n*-hexane extract of *G. lucidum*, the steroid ester ergosta-7,22-diene-3β-yl pentadecanoate could be identified. From *S. leucomallellus* two constituents showing structures of 3,4-*seco*-lanostane type triterpene acids were identified as (+)-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene-3,26-dicarboxylic acid and (+)-20-hydroxy-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene3,26-dicarboxylic acid, respectively. Cytotoxicity and antimicrobial activity of selected compounds were investigated using standard tests.

Keywords: *Ganoderma lucidum*; *Spongiporus leucomallellus*; Volatiles; Ergosta-7,22-diene-3β-yl pentadecanoate; Spongiporic acid A and B; Biological testing

1. Introduction

Ganoderma lucidum (Fr.) P. Karst. belongs to the Ganodermataceae (Basidiomycetes) (Laux, 2001). The fungus grows mostly saprophytic on deciduous trees like oak and beech (Jahn, 1979) causing white rot. *G. lucidum*, known from traditional Chinese medicine, is an important species with high economic importance (Bankhofer et al., 2000). During previous investigations, several compounds with biomedicinal properties like triterpenoids (Kim and Kim, 1999) and polysaccharides (Bao et al., 2002) have been isolated from this species. There is no report, however, on the composition of its essential oil. Our investigation on the fruit bodies of *G. lucidum* resulted in the identification of 65 constituents in the essential oil and the isolation of ergosta-7,22-diene-3β-yl palmitate (**1**), ergosta-7,22-diene-3β-yl linoleate (**2**) and a new steroid ester, ergosta-7,22-diene-3β-yl pentadecanoate (**3**).

Spongiporus leucomallellus (Murril) A. David belongs to the Polyporaceae s. lat. (Basidiomycetes) (Laux, 2001). This fungus lives saprophytic mostly on wet, old pines (Jahn, 1979) causing brown rot. Eburicoic acid, tumulosic acid, and trametenolic acid have been identified as methyl esters in *Spongiporus* species (Yokohama and Natori, 1974), but there are no reports on *S. leucomallellus*.

Here we describe the identification of 42 constituents in the essential oil and the isolation of ergosterol (**5**), ergosta-7,22-diene-3β-ol (**6**), lanosta-8(9),24(28)-diene-3β-ol (**7**), ergosterol peroxide (**8**), and 5,8-*epi*-dioxy-24-methylcholesta-6,9(11),22-triene-3β-ol (**9**), eburicoic acid (**10**) as well as two new triterpenes, (+)-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene-3,26-dicarboxylic acid (**11**) and (+)-20-hydroxy-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene-3,26-dicarboxylic-acid (**12**).

Compound **9** was tested for its anticarcinogenic activity and compound **11** for its antimicrobial activity.

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2. Results and discussion

2.1. Ganoderma lucidum

2.1.1. Volatile constituents

The essential oil of *G. lucidum* was analyzed by GC (Fig. 1) and GC–MS. Using a non-polar stationary phase (CP-Sil-5), mass spectra and retention indices were compared with a library of mass spectra of authentic compounds established under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2004). Overall, 65 compounds could be identified. Employing enantioselective gas chromatography, enantiomeric compositions of linalool and carvone were determined according to the methods of Krüger (1995) and König (1992), respectively (Fig. 2).

The volatile components identified in *G. lucidum* and their relative proportions are listed in Table 1. A corresponding gas chromatogram is depicted in Fig. 1. Major constituents in the essential oil were *trans*-anethol (9.1%), *R*-(−)-linalool (4.4%), *S*-(+)-carvone (4.4%) and α-bisabolol (2%).

2.1.2. Triterpenoid esters

Ergosta-7,22-diene-3β-yl palmitate (**1**, Lin et al., 1990), ergosta-7,22-diene-3β-yl linoleate (**2**, Lin et al., 1991), a new steroid ester, **3** (Fig. 3) and ergosta-7-ene-3β-yl linoleate (**4**, Rösecke and König, 2000) were isolated from the *n*-hexane extract (Fig. 3) of dried fruit bodies.

Ergosta-7,22-diene-3β-yl pentadecanoate **3** was isolated as a solid by semi-preparative HPLC-UV from the *n*-hexane extract of dried fruit bodies of *G. lucidum*. It exhibited a negative optical rotation, a melting point of 91 °C, and its mass spectrum showed a molecular ion peak at *m/z* 622. High resolution EI-MS of **3** showed a signal at *m/z* 622.5688, suggesting a molecular formula of $C_{43}H_{74}O_2$, a steroidal compound with seven degrees of unsaturation.

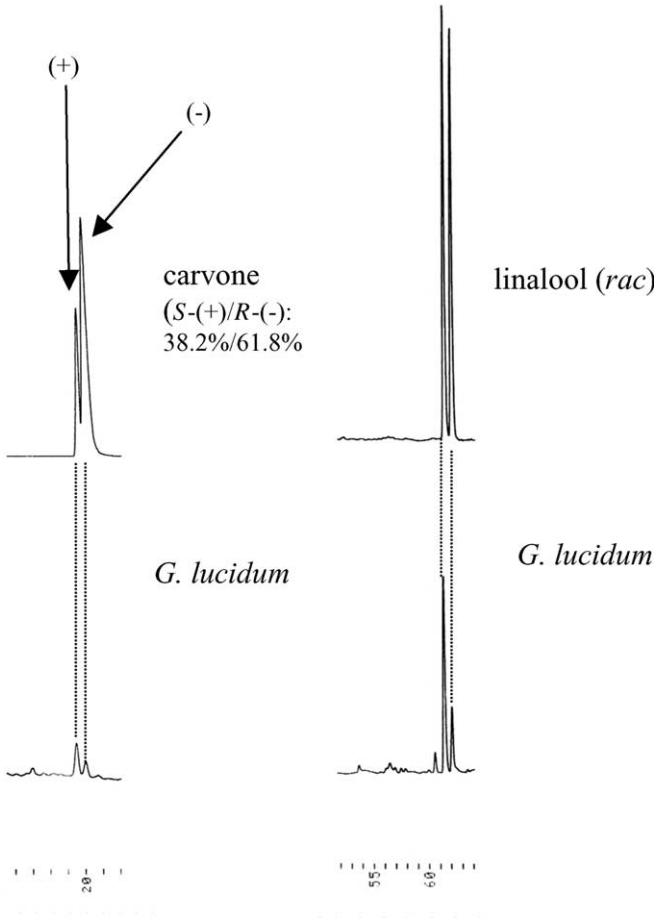


Fig. 2. Enantioselective GC analysis of carvone and linalool in *G. lucidum*.

Its ^{1}H NMR (Table 2) and HMQC data indicated the presence of a total of 74 protons. The identified signals represent two tertiary methyls at δ 0.60 (3H, *m*) and 0.75 (3H, *m*), four secondary methyls of which two were at δ

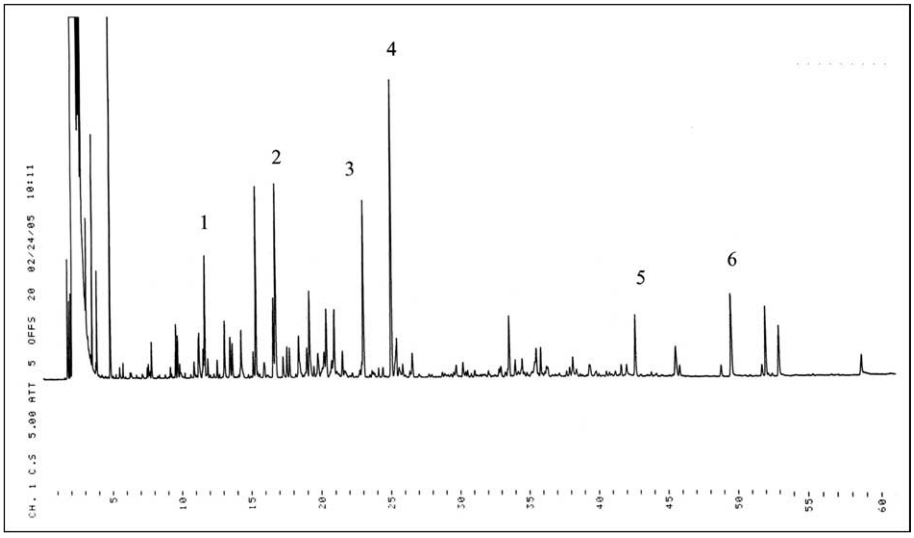
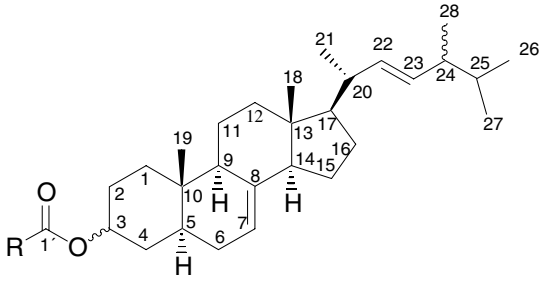


Fig. 1. Gas chromatogram of the essential oil from *G. lucidum* 1 = 2-pentylfuran; 2 = linalool; 3 = carvone; 4 = *trans*-anethol; 5 = α-bisabolol; 6 = *n*-pentanoic acid.

Table 1
Composition of the hydrodistillates from *G. lucidum* and *S. leucomallellus*

Compound	KI[b]	Species	
		G. lucidum (%)[a]	*S. leucomallellus* (%)[a]
2-Heptanone	871	+	–
n-Heptanal	882	0.7	–
n-Nonane	906	+	–
α-Pinene	936	1.1	+
(*E*)-2-heptenal	942	–	+
Camphene	950	–	+
1-Heptene-3-one	956	+	5.1
R-(−)-1-Octene-3-ol	962	–	37.5
S-(+)-1-Octene-3-ol		–	2.9
3-Octanone	969	–	2.3
6-Methyl-5-heptene-2-one	972	1.2	–
2-Pentylfuran	981	2.8	2.4
p-Methylanisol	1004	0.4	–
Phenylacetaldehyde	1012	1.6	+
1,8-Cineol	1024	1.2	–
Limonene	1025	–	+
(*E*)-β-Ocimene	1029	–	+
(*E*)-2-Octenal	1034	1.0	1.6
1-Nonen-3-ol	1058	–	0.9
Fenchone	1069	0.7	–
cis-Linalool oxide (furanoid)	1072	–	+
o-Cymene	1076	+	–
n-Nonanal	1076	2.3	0.8
R-(−)-Linalool	1086	4.4	15.8
S-(+)-Linalool		1.5	1.5
α-Thujone	1089	+	–
α-Pinene epoxide (isomer)	1116	+	–
Camphor	1123	+	–
trans-Pinocarveol	1126	+	–
Menthone	1136	0.3	–
Pinocarvone	1137	+	–
(*E*)-2-Nonenal	1139	–	0.9
Isomenthone	1146	+	–
n-Nonanol	1149	1.1	–
Borneol	1150	+	–
Isopinocamphone	1151	+	–
n-Octanoic acid	1156	+	+
Terpinene-4-ol	1164	2.1	–
Estragol	1175	+	–
Dillether	1176	+	–
α-Terpineol	1176	2.7	+
2-Decanone	1176	–	+
n-Decanal	1180	0.8	0.2
(*E*,*E*)-2,4-Nonadienal	1188	–	0.3
R-(−)-Carvone	1214	0.9	–
S-(+)-Carvone		4.4	–
Piperitone	1226	+	–
(*E*)-2-Decenal	1240	+	0.7
Geranial	1244	+	–
n-Nonanoic acid	1263	0.3	–
trans-Anethol	1263	9.1	–
2,4-Decadienal	1270	–	0.7
2-Undecanone	1273	1.8	0.2
Undecanal	1290	0.4	–
(*E*,*E*)-2,4-Decadienal	1291	0.8	1.7
α-Ylangene	1376	+	–
α-Copaene	1379	–	+
Daucene	1380	+	–
Sandvicene	1399	–	+
Geranylacetone	1430	+	+
trans-α-Bergamotene	1430	1.8	–
Cyclofarnesa-5(14), 8,10-triene	1441	–	+
Isobazzanene	1442	+	+
β-Barbatene	1445	–	+
(*E*)-β-Farnesene	1446	+	–
ar-Curcumene	1473	0.8	–
α-Muurolene	1496	–	+
Cuparene	1498	+	–
β-Bisabolene	1503	0.9	–
β-Bazzanene	1519	+	–
δ-Cadinene	1520	+	–
(*Z*)-Nerolidol	1522	–	2.9
(*E*)-Nerolidol	1547	+	–
n-Dodecanoic acid	1554	+	–
β-Irone	1566	+	–
Cedrol	1603	+	–
T-Cadinol	1633	–	+
2-Himalachene-7β-ol	1642	0.5	–
Bisabolol oxide B	1654	0.9	–
α-Bisabolol	1673	2.0	–
n-Tetradecanoic acid	1748	1.4	0.60
Drimenol	1750	0.5	–
n-Pentadecanoic acid	1823	0.5	1.6
n-Hexanoic acid	1951	2.1	+
Linoleic acid	2095	1.1	0.9
n-Tetracosane	2400	–	+
Dotriacontane	3200	–	+

[a] Relative percentage of the identified volatiles based on GC-FID, components marked as + are present in less than 0.1%.
[b] Kovats index.



(**1**) R= -$(CH_2)_{14}$-CH_3

(**2**) R= -$(CH_2)_7$-CH=CH-CH_2-CH=CH-$(CH_2)_4$-CH_3

(**3**) R= -$(CH_2)_{13}$-CH_3

Fig. 3. Isolated steroid esters from *G. lucidum*.

0.89–0.95 (3H, *d*, each), one at δ 1.01 (3H, *d*) and one at δ 1.11 (3H, *d*), along with one primary methyl at δ 0.92 (3H, *m*), nine methylenes at δ 1.17/1.97 (2H, *m*), 1.40/1.67 (2H, *m*), 1.41 (2H, *m*), 1.54/1.93 (2H, *m*) 1.57 (2H, *m*), 2.27 (2H, *m*), 1.66/1.38 (2H, *m*), 1.65/0.93 (2H, *m*), and 1.69 (2H, *m*), three olefinic methine multiplets at δ 5.19 (1H, *m*) and two at δ 5.27 (3H, *m*), and eight methine multiplets δ at 1.25

Table 2
^{1}H and ^{13}C NMR data of compounds **3**, **11** and **12** (F.a.: Fatty acid)

Atom no.	**3** (benzene-d_6)			**11** (pyridine-d_5)			**12** (methanol-d_4)		
	$\delta^{1}H$	M (*J*)	$\delta^{13}C$	$\delta^{1}H$	M (*J*)	$\delta^{13}C$	$\delta^{1}H$	M (*J*)	$\delta^{13}C$
1	1.65, 0.93	*m*	37.4	2.58, 2.50	*m*	31.0	2.24, 1.72	*m*	30.7
2	1.40, 1.67	*m*	30.2	2.14, 1.90	*m*	37.2	1.84, 1.58	*m*	36.2
3	4.93	*m*	73.3	–	–	177.5	–	–	177.9
4	1.54, 1.93	*m*	28.05	–	–	150.1	–	–	149.20
5	1.53	*m*	49.70	2.34	*m*	51.5	2.29	*m*	51.2
6	1.66,1.38	*m*	30.0	2.06, 2.55	*m*	29.4	2.04, 2.60	*m*	28.8
7	5.19	*br s*	117.9	5.24	*br s*	118.8	5.31	*br s*	117.7
8	–	–	139.5	–	–	142.7	–	–	142.1
9	1.27	*m*	40.4	–	–	138.4	–	–	137.6
10	–	–	34.4	–	–	39.5	–	–	38.4
11	1.41	*m*	22.0	5.34	*br s*	121.1	5.39	*br s*	120.0
12	1.97,1.17	*m*	40.1	2.16	*m*	39.6	2.37, 2.30	*m*	38.9
13	–	–	33.7	–	–	45.4	–	–	44.8
14	1.81	–	55.6	–	–	51.3	–		50.6
15	1.69	*m*	25.8	1.62	*m*	32.0	1.75	*m*	31.2
16	1.57	*m*	23.5	2.5	*m*	29.0	1.97	*m*	22.3
17	1.25	*m*	56.4	1.66	*m*	52.3	2.12	*m*	53.5
18	0.60	*s*	12.6	0.69	*s*	17.6	0.84	*s*	17.9
19	0.75	*s*	13.4	1.09	*s*	23.1	0.99	*s*	21.4
20	2.05	*m*	41.2	2.29	*m*	34.7	–	–	75.3
21	1.11	*d* (6.7)	21.5	1.07	*d* (6.4)	20.5	1.35	*s*	26.0
22	5.27	*m*	136.3	2.74, 2.85	*m*	45.7	2.90	*m*	49.4
23	5.27	*m*	132.3	–	–	202.3	–	–	202.3
24	1.92	*m*	43.5	–	–	151.0	–	–	150.3
25	1.50	*m*	33.6	4.13	*q* (7.3)	42.0	3.59	*q* (6.0)	41.5
26	0.89–0.95	*d* (2.2)	19.2–21.9	–	–	177.4	–	–	177.7
27	0.89–0.95	*d* (2.2)	19.2–21.9	1.56	*d* (7.1)	17.6	1.30	*d* (6.1)	15.5
28	1.01	*d* (6.9)	18.0	0.97	*s*	25.1	0.90	*s*	33.9
29	–	–	–	4.82, 4.77	*s* *s*	113.0	4.68, 4.62	*s* *s*	111.4
30	–	–	–	1.72	*s*	23.0	1.67	*s*	21.1
31	–	–	–	6.37, 6.02	*s* *s*	125.2	6.25, 5.96	*s* *s*	125.5
1 (F.a.)	–	–	172.7	–	–	–	–	–	–
2	2.27	*m*	35.3	–	–	–	–	–	–
3–14 (F.a.)	1.14–1.44	*m*	28.7–34.5	–	–	–	–	–	–
15 (F.a.)	0.92	*m*	20.55	–	–	–	–	–	–

(1H, *m*), 1.27 (1H, *m*), 1.50 (1H, *m*), 1.53 (1H, *m*), 1.81 (1H, *m*), 1.92 (1H, *m*), 2.05 (1H, *m*), and 4.93 (1H, *m*). The ^{13}C NMR (Table 2) contained the signals of a total of 43 carbons. Identified signals were seven methyl carbons at δ 12.6, 13.4, 18.0, two at 19.2–21.9, 20.55, and 21.5, four methine carbons of which three were olefinic methines at δ 117.9, 132.3, and 136.3 and one olefinic quaternary at δ 139.5, eight methine carbons at δ 33.6, 40.4, 41.2, 43.5, 49.7, 55.6, 56.4, 73.3, one carbonyl carbon at δ 172.7 and two quaternary carbons at δ 33.7 and 34.4. The ^{1}H–^{1}H-COSY of **3** (Fig. 4) confirmed the presence of three partial structures: (i) CH_2–CH_2–CH–(O–C=O–$(CH_2)_{13}$–CH_3)–CH_2–CH–CH_2–CH, (ii) CH–CH_2–CH_2, and (iii) CH–CH_2–CH_2–CH–CH–CH_3–CH–CH–CH–CH_3–CH–$(CH_3)_2$. The connectivity of each partial structure was clarified by the HMBC spectrum as shown in Fig. 4.

Analysis of connectivities in the ^{1}H–^{1}H-COSY and HMBC spectra confirmed that three of the seven unsaturations were due to double bonds, and the remaining four were due to four rings. The presence of four rings,

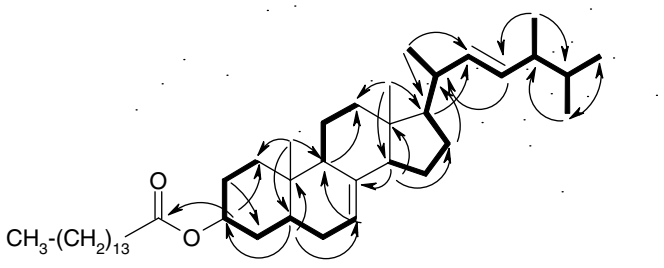


Fig. 4. Long-range ^{1}H–^{13}C (arrows) and 1H–1H (bold lines) correlations of **3**.

the carboxy carbon as well as the fragment ion at *m*/*z* 255 in the mass spectrum, characteristic for a monounsaturated steroid after loss of the side chain (Orcutt and Richardson, 1970), suggested a steroid ester. The fragment-ion *m*/*z* 380 was regarded as the result of a McLafferty-rearrangement involving a methylene group with loss of the fatty acid part ($[M - C_{15}H_{30}O_2]^{+}$).

Loss of the side chain at position C-17 was also observed at *m*/*z* 495 ($[M - C_9H_{19}]^{+}$) due to fragmentation described

by Wyllie and Djerassi (1968) in steroids having a double bond in position C22. The compound proved to be ergosta-7,22-diene-3β-yl pentadecanoate. Derivatization reactions were performed to confirm the postulated structure. Transesterification with trimethylsulphonium hydroxide (TMSH) followed by GC–MS analysis of the reaction products confirmed the presence of pentadecanoic acid. Subsequent silylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) produced a compound with a mass spectrum that strongly resembled the trimethylsilyl (TMS) ether of ergosta-7,22-diene-3β-ol (Masslib Database, 2004).

2.2. S. leucomallellus

2.2.1. Volatile constituents

The essential oil of *S. leucomallellus* was analyzed by GC (Fig. 5) and GC–MS, and the data of individual compounds were compared with library data (Joulain and König, 1998; Hochmuth et al., 2004). For the analysis of the enantiomers of 1-octene-3-ol the method described by König et al. (1990) was modified (Fig. 6).

The volatile components identified in *S. leucomallellus* and their relative proportions are listed in Table 1. Overall, 42 compounds could be identified. Major constituents in the essential oil were *R*-(−)-1-octene-3-ol (37.5%), *R*-(−)-linalool (15.8%), 1-heptene-3-one (5.1%) and (*Z*)-nerolidol (2.9%).

2.2.2. Triterpenoids

The dichloromethane extract yielded ergosterol (**5**), ergosta-7,22-diene-3β-ol (**6**, Ramirez et al., 2003), lanosta-8(9),24(28)-diene-3β-ol (**7**, Harref and Lavergne, 1985), ergosterol peroxide (**8**, de Queiroz et al., 2001) and 5,8-*epi*-dioxy-24-methylcholesta-6,9(11),22-triene-3β-ol (**9**), eburicoic acid (**10**, Kamalov et al., 2000), and two new lanostane type triterpenes, **11** and **12** (Fig. 7).

2.2.3. Spongiporic acid A *11*

Upon repeated column chromatography on silica gel and RP-18 HPLC columns (see Section 3.3.2), compound **11** was obtained as a white solid. It exhibited a positive optical rotation. An IR spectrum of the compound showed broad absorption bands in the carbonyl and double bond regions.

Its mass spectrum showed a molecular ion peak at *m*/*z* 496. High resolution value of the molpeak in direct inlet ESI-MS (positive ion mode) gave an ion peak at *m*/*z* 519.3110 ($[M + Na]^+$) suggesting the molecular formula of $C_{31}H_{44}O_5$, a compound with 10 degrees of unsaturation (double bonds and/or rings).

Its ^{1}H NMR (Table 2) and HMQC data indicated the presence of a total of 42 protons. The identified signals were four tertiary methyls at δ 0.69 (3H, *s*), 0.97 (3H, *s*), 1.09 (3H, *s*,), and 1.72 (3H, *s*), two secondary methyls at δ 1.07 (3H, *d*) and 1.56 (3H, *d*), two olefinic methylenes at δ 4.82/4.77 (2H, *s*, *s*), and 6.37/6.02 (2H, *s*, *s*) seven methylenes at δ 1.62 (2H, *m*), 1.90/2.14 (2H, *m*), 2.06/2.55 (2H, *m*), 2.16 (2H, *m*), 2.50 (2H, *m*), 2.50/2.58 (2H, *m*), and 2.74/2.85 (2H, *m*), two olefinic methines at δ 5.24 (1H, *br s*) and 5.34 (1H, *br s*), four methines at δ 1.66 (1H, *m*), 2.29 (1H, *m*), 2.34 (1H, *m*) and 4.13 (1H, *q*). The ^{13}C NMR (Table 2) contained the signals of a total of 31 carbons. Identified signals were six methyl carbons at δ 17.6, 17.6, 23.0, 23.1, 20.5, 25.1, two olefinic methylenes at δ 113.0, 125.2, seven methylenes at δ 29.0, 29.4, 31.0, 32.0, 37.2, 39.6, 45.7, two olefinic methine carbons at δ 118.8, 121.1, four methines at δ 34.7, 42.0, 51.5, 52.3, four olefinic quaternary carbons at δ 138.4, 142.7, 150.1, 151.0, three quaternary carbons at δ 39.5, 45.4, 51.3, two carboxy carbons at δ 177.4, 177.5 and one keto carbon at 202.3. The ^{1}H–^{1}H-COSY of **11** (Fig. 8) confirmed the presence of five partial structures: (i) CH_2–CH_2, (ii) CH–CH_2–CH, (iii) CH–CH_2, (iv) CH_2–CH_2–CH–CH–CH_3–CH_2, and (v) CH_3–CH. The connectivity

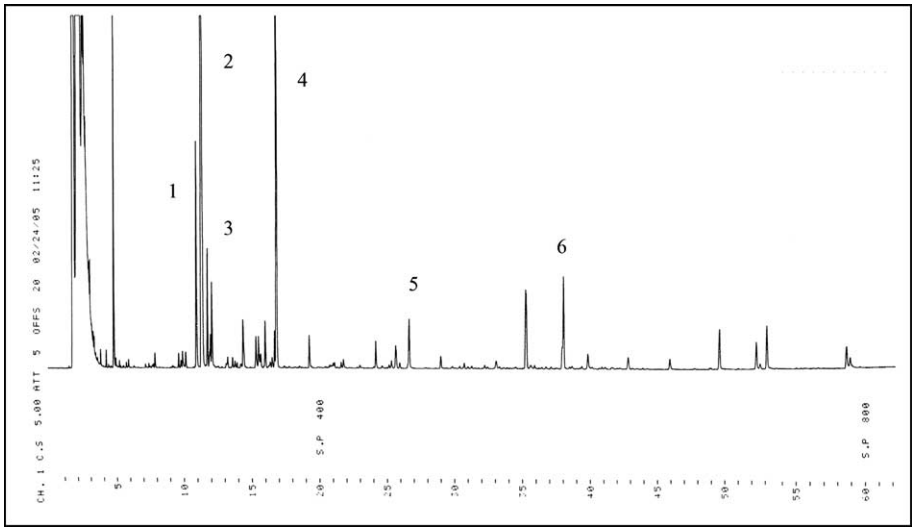


Fig. 5. Gas chromatogram of the essential oil from *S. leucomallellus* 1 = 1-heptene-3-one; 2 = 1-octene-3-ol; 3 = 3-octanone; 4 = linalool; 5 = (*E*,*E*)-2,4-decadienal; 6 = (*Z*)-nerolidol.

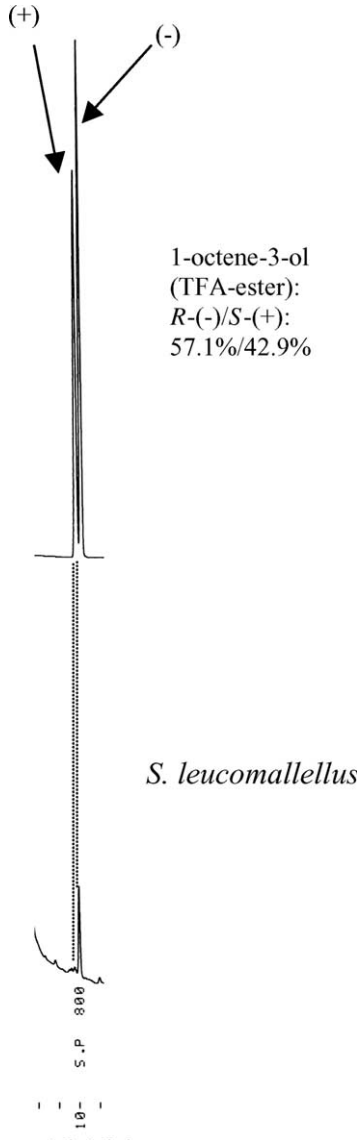


Fig. 6. Enantioselective GC analysis of 1-octene-3-ol (TFA-derivative) in *S. leucomallellus*.

of each partial structure was clarified by the HMBC spectrum as shown in Fig. 8. The NOESY spectrum showed the typical configuration of the lanostane skeleton. In this structure it is possible for the methine (H_1-5) to interact with the methyl (H_3-19; Fig. 9).

In the EI-spectrum, the fragment ion at *m/z* 478 shows the loss of water ($[M - H_2O]^+$) described before in systems with a double bond in β-γ-position to a carboxy group suggesting this fragmentation to take place at position C-28 (Lauwers et al., 1973; Herrmann and Schwarz, 1976; Holmes et al., 1979). The fragment ions *m/z* 423, 327 and 169 represent the loss of the chain C-1 to C-3 ($[M - C_3H_5O_2]^+$), the loss of the complete side chain C-20 to C-26 ($[M - C_9H_{13}O_3]^+$), and the loss of the triterpene skeleton at position 20 ($[M - C_{22}H_{31}O_2]^+$), respectively, due to σ-fragmentation. The origin of the fragmentation *m/z* 423 could also be explained with an allylic fragmentation with the loss of the chain C-1 to C-3 ($[M - C_3H_5O_2]^+$). Analysis of connectivities in the ^1H–^1H-COSY and HMBC spectra confirmed that seven of the 10 desaturations were due to double bonds and the remaining three were due to three rings. The compound revealed to be a 3,4-*seco*-lanostane type triterpene. 3,4-*seco*-Lanostane triterpenes have been reported as natural products from other fungi (Takaaki et al., 1993; Rösecke and König, 1999). Thus, compound **11** proved to be (+)-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene-3,26-dicarboxylic acid for which we propose the name (+)-spongiporic acid A.

2.2.4. Spongiporic acid B 12

Upon repeated column chromatography on silica gel and RP-18 HPLC columns (see section 3.3.2), compound **12** was obtained as a white solid. It exhibited a positive optical rotation.

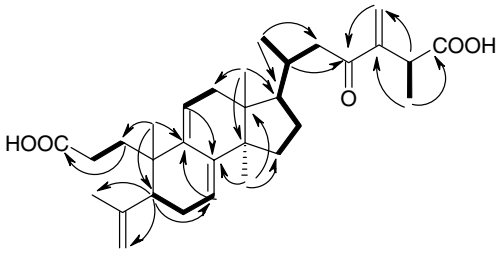


Fig. 8. Long-range ^1H-^{13}C (arrows) and ^1H–^1H (bold lines) correlations of **11**.

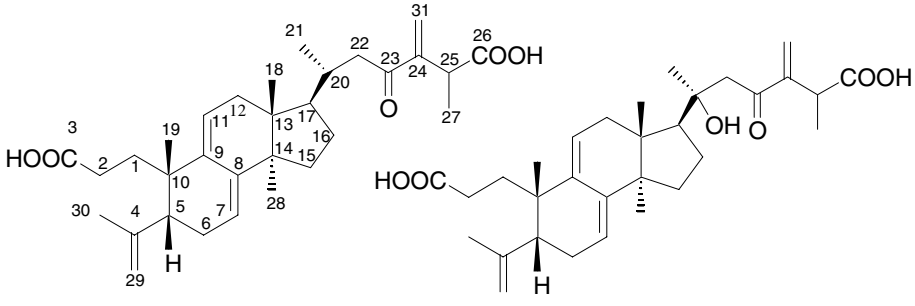


Fig. 7. Isolated triterpenes from *S. leucomallellus*.

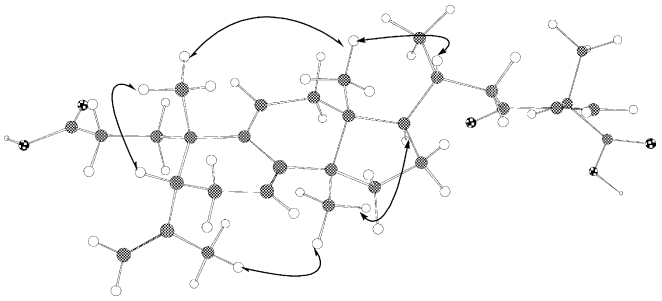


Fig. 9. Molecular model (AM1, MOPAC) of **11** with relevant NOEs.

Its ^{1}H NMR (Table 2) and HMQC data indicated the presence of a total of 41 protons. The identified signals were five tertiary methyls at δ 0.84 (3H, *s*), 0.90 (3H, *s*), 0.99 (3H, *s*), 1.35 (3H, *s*), 1.67 (3H, *s*), one secondary methyl at δ 1.30 (3H, *d*), two olefinic methylenes at δ 4.62/4.68 (2H, *s*, *s*), 5.96/6.25 (2H, *s*, *s*), seven methylenes at δ 1.75 (2H, *m*), 1.72/2.24 (2H, *m*), 2.04/2.60 (2H, *m*), 2.30/2.37 (2H, *m*), 1.97 (2H, *m*), 1,58/1.84 (2H, *m*), and 2.90 (2H, *m*), two olefinic methines at δ 5.32 (1H, *br s*) and 5.39 (1H, *br s*), three methines at δ 2.12 (1H, *m*), 2.29 (1H, *m*) and 3.59 (1H, *q*).

The ^{13}C NMR (Table 2) contained the signals of a total of 31 carbons. Identified signals were six methyl carbons at δ 15.5, 17.9, 21.1, 21.4, 26.0, 33.9, two olefinic methylenes at δ 111.4, 125.5, seven methylenes at δ 22.3, 28.8, 30.7, 31.2, 36.2, 38.9, 49.4, two olefinic methine carbons at δ 117.7, 120.0, three methines at δ 41.5, 51.2, 53.5, four olefinic quaternary carbons at δ 137.6, 142.1, 149.2, 150.3, one oxygenated quaternary carbon at δ 75.3, two quaternary carbons at δ 38.4, 44.8, 50.6, two carboxy carbons at δ 177.7, 177.9 and one keto carbonyl carbon at 202.3. The ^{1}H–^{1}H-COSY of **12** confirmed the presence of five partial structures, of which 4 showed a strong resemblance with partial structures of **11**. The partial structure (iv) CH_2–CH_2–CH (Fig. 10) differs from the partial structure (iv) described for the compound **11**. The HMBC-spectrum shows strong coupling of the methylene protons of carbon C-16 with an oxygenated quaternary carbon that could be identified as C-20.

High resolution value of the molpeak in direct inlet ESI-MS (negative ion mode) gave an ion peak at *m*/*z* 511.2999 ($[M - H]^-$) suggesting the molecular formula of $C_{31}H_{44}O_6$, a compound with 10 degrees of unsaturation (double bonds and/or rings). Analysis of connectivities in the ^{1}H–^{1}H-COSY and HMBC spectra confirmed that seven of the 10 unsaturations were due to double bonds and the three remaining were due to three rings. The compound proved to be a 3,4-*seco*-lanostane type triterpene. The compound is homologous to compound **11**. The NOESY-spectrum reveals the same relative configuration as compound **11**. Thus, compound **12** was shown to be (+)-20-hydroxy-23-oxo-3,4-*seco*-lanosta-4(28),7(8),9(11),24(31)-tetraene-3,26-dicarbo- xylic acid for which we propose the name (+)-spongiporic acid B.

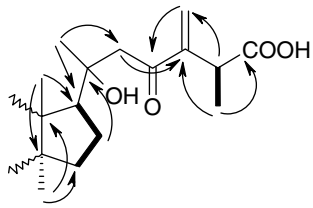


Fig. 10. Long-range ^{1}H–^{13}C (arrows) and ^{1}H–^{1}H (bold lines) correlations of **12**.

2.2.5. Biological activities

Using the agar diffusion assay according to Anke et al. (1989), spongiporic acid A (**11**) was examined for bactericidal and fungicidal activity. At a dose of 10 μg/disc the compound showed weak activity against *Proteus vulgaris* and beginning of inhibition of *Bacillus subtilis* and *B. brevis*. At a dose oPf 50 μg/disc activity against *P. vulgaris* rose, and clear inhibition zones against *B. brevis*, *B. subtilis*, and the fungus *Paecilomyces variotii* were observed. Beginning inhibition was observed with *Acinetobacter calcoaceticus*.

Cytotoxical tests according to Zapf et al. (1995) of 5,8-*epi*-dioxy-24-methylcholesta-6,9(11),22-triene-3β-ol (**9**) revealed a weak anticancerogenic activity against HeLa-S3 cervix carcinoma and Jurkat T-cell leukemia cells. Proliferation of Jurkat and HeLa-S3 cells was reduced to 90% with concentrations of 10 and 25 μg/ml, respectively.

3. Experimental

3.1. Fungal material

Samples of *G. lucidum* were purchased from Fungi Perfecti (Olympia WA, USA). The fungi grew on a matrix of Red Alder (*Alnus rubra*), containing wood chips and saw dust.

A sample of *S. leucomallellus* was collected near Trittau, Germany, growing on a dead pine. Voucher specimens of *G. lucidum* and *S. leucomallellus* are deposited at the Institute of Organic Chemistry, University of Hamburg (GD2 and SP2, respectively).

3.2. Methods

Melting points were measured using a Electrothermal Melting Point apparatus. NMR spectra were recorded with a Bruker WM 400 or 500 MHz spectrometer (Rheinstetten, Germany) in either deuteriobenzene, deuteriochloroform, deuteriomethanol or deuteriopyridine. Chemical shift values are reported with reference to TMS, and the coupling constants are given in Hertz. HR-EI-MS was performed with a VG 70-SE Micromass double focussing mass spectrometer (Manchester, UK). ESI-MS measurements were performed with a MAT 95XL double focussing Thermo-Quest Finnigan mass spectrometer (Bremen, Germany).

Optical rotations were measured as solutions in methanol or chloroform using a Perkin–Elmer 341 polarimeter at 589 nm and 20 °C. The IR-spectrum of (+)-spongiporic acid A was measured as a solid with a Bruker IFS 28 spectrometer with an attenuated total reflection (ATR) unit: Golden Gate (Diamond), single reflection.

3.3. Hydrodistillation, extraction, and isolation procedure

3.3.1. G. lucidum

Fruit bodies of *G. lucidum* (304.7 g) were cleaned, pulverized, and homogenized. One part, 28.9 g, was hydrodistilled in a Clevenger type apparatus for 2.5 h, and a slightly yellowish oil was collected in *n*-hexane (HPLC grade). The oil was analyzed by using a Carlo Erba HRGC with FID-Detector and a HP- 5890 GC coupled to a VG Analytical 70-250S mass spectrometer (EI, 70 eV). The chromatographs were fitted with a 25 m × 0.25 mm CP-Sil-5 CB fused silica capillary column. For conventional GC, the injector and detector temperatures of the GC-FID were 200 and 250 °C, respectively. The carrier gas was hydrogen with a flow rate of 2 ml/min. The column temperature was programmed from 50 to 250 °C at a rate of 3 °C/min. For GC–MS, the injector temperature was 200 °C. The carrier gas was helium with a flow rate of 2 ml/min. The column temperature was programmed from 80 to 270 °C at a rate of 10 °C/min. Retention indices and mass spectra of the components were compared with library spectra generated under identical experimental conditions (Joulain and König, 1998; Hochmuth et al., 2004).

For the enantioselective analysis, carvone and linalool as major chiral substances in the essential oil were isolated. The oil was subjected to TLC on silica gel with *n*-hexane/ethyl acetate (80/20, v/v) as solvent. Standard substances carvone (*rac*) and linalool (*rac*) were applied at one margin of the TLC-plate. The spots of the standard substances were detected separately from the sample spots. Detection was performed with anisaldehyde/sulphuric acid-reagent. R_f values of carvone (*rac*) and linalool (*rac*) were 0.38 and 0.28, respectively. For isolation of carvone and linalool, bands with the diameter from the standard spots were cut from the plates and extracted with dichloromethane. The volume of the solutions was reduced to 0.5 ml under a stream of dry nitrogen. The carvone and linalool solutions were analyzed with a Carlo Erba Fractovap Series 2150 equipped with a FID-detector. The chromatograph was fitted with a 25 m × 0.25 mm oktakis(6-methyl-2,3-pentyl)-γ-cyclodextrin (CD) or heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-β-CD capillary column for the analysis of the enantiomers of carvone and linalool, respectively. The injector and detector temperatures were 200 °C. The carrier gas was hydrogen with a flow rate of 2 ml/min. For the analysis of carvone, the oven temperature was 100 °C isothermal. For the analysis of linalool the column temperature was programmed from 40 °C (20 min isotherm) to 160 °C at a rate of 1 °C/min. Under these conditions the α-value for carvone (rt at about 19 min) was $\alpha = 1.016$ [rt *R*-(−)/rt *S*-(+)] while the α-value for linalool (rt > 60 min) proved to be $\alpha = 1.060$ [rt *S*(+)/rt *R*(−)].

The second part of the pulverized fruit bodies (275.8 g) was extracted with *n*-hexane for 15 min while ultrasonicating. The extract was filtered. This procedure was repeated twice. The solvent was removed under reduced pressure to give a yellowish oily residue. Part of the extract was subjected to column chromatography on silica gel (Merck silica gel 60 [0.04–0.063 mm]) with *n*-hexane as eluent containing increasing amounts of ethyl acetate. Seven fractions were collected. Fractions 1 and 2 containing steroid esters eluted with pure 100% hexane and 5% ethylacetate in hexane, respectively. These fractions were combined, and a part was subjected to semi-preparative HPLC with UV-detection ($\lambda = 210$ nm) with an analytical RP-18 (Macherey und Nagel (Düren, Germany) EC 250/4, Nucleosil 100-5, C18, 250 × 4 mm [5 μm]) column using acetonitrile/*tert*-butylmethyl ether (60/40, v/v) as the eluent with a flow rate of 1 ml/min. Ergosta-7,22-diene-3β-yl palmitate (**1**), ergosta-7,22-diene-3β-yl linoleate (**2**), ergosta-7,22-diene-3β-yl pentadecanoate (**3**) and ergosta-7-ene-3β-yl linoleate (**4**) were isolated. The amounts of the isolated compounds were 1 mg for **1** and **2**, 2.7 mg for **3**, and 0.8 mg for **4**.

The transesterification method reported by Schulte and Weber (1989) was modified: 100 μg of the fraction were dissolved in 100 μl *tert*-butyl methyl ether in a 2-ml screw cap vial. 50 μl TMSH-Reagent (Fluka) were added. After closing of the vial and shaking, 1 μl of the solution was analyzed by GC. The remaining solution was dried and 100 μl MSTFA-Reagent (Macherey-Nagel) were added. The solution was analyzed after 30 min using the GC–MS-instrument mentioned above. 1 μl of the solution was injected. The oven temperature was programmed from 130 to 300 °C at a rate of 20 °C/min. The injector temperature was kept at 250 °C.

3.3.2. S. leucomallellus

Fruit bodies of *S. leucomallellus* (471.1 g) were cleaned, pulverized and homogenized. One part, 71.4 g, was hydrodistilled in a Clevenger type apparatus for 2.5 h and a slightly yellowish oil was collected in HPLC grade *n*-hexane. The oil was analyzed as described above.

For the enantioselective analysis, 1-octene-3-ol and linalool (see section 3.3.1) as major chiral substances in the essential oil were isolated. The oil was subjected to TLC on silica gel. The solvent and detection are the same as described above. The R_f-value of 1-octene-3-ol was 0.35. The R_f-value of linalool was 0.28. The isolated 1-octene-3-ol was transformed to the TFA-ester (Knapp, 1979). The derivatization was performed in a 7-ml vial. 0.1 ml of a 0.05-M triethylamine solution in dichloromethane followed by 10 μl of trifluoroacetic acid anhydride were added. The closed vial was shaken for 1 min. After 30 min, the organic phase was separated, dried with sodium sulfate (anhydrous), filtered and analyzed after 30 min using enantioselective gas chromatography with an oktakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-CD column as described above.

The oven temperature was kept at 60 °C. Under these conditions, the α-value of the TFA-esters of 1-octene-3-ol (rt at around 10 min) was $\alpha = 1.030$ [rt *R*-(−)/rt *S*-(+)]. The α-value of linalool was $\alpha = 1.060$ (see Section 3.3.1).

The second part of the pulverized fruit bodies (275.82 g) was extracted with dichloromethane for 15 min while ultrasonicating. The extract was filtered. This procedure was repeated twice. The solvent was removed under reduced pressure to give a yellowish oily residue. Part of the extract was subjected to column chromatography on silica gel (Merck silica gel 60 [0.04–0.063 mm]) with *n*-hexane as the eluent containing increasing amounts of ethyl acetate. Nine fractions were collected. Ergosterol (**5**), ergosta-7,22-diene-3β-ol (**6**), lanosta-8(9),24(28)-diene-3β-ol (**7**), ergosterol peroxide (**8**) and 5,8-*epi*-dioxy-24-methylcholesta-6,9(11), 22-triene-3β-ol (**9**) were obtained from fraction 4, which eluted with hexane and ethylacetate (70/30, v/v). Isolation of the compounds was achieved by using semi preparative HPLC with UV detection on a RP-18 analytical column (Macherey und Nagel EC 250/4, Nucleosil 100-5, C18, 250 × 4 mm [5 μm]), acetonitrile/*tert*-butylmethyl ether (80/20, v/v) as an eluent with a flow of 1 ml/min, detection at 210 nm. Eburicoic acid (**10**) was isolated from fraction 5, which eluted with hexane and ethylacetate (60/40, v/v). Isolation of the compound was achieved by using semi preparative HPLC with UV detection: Silica gel analytical column (Knauer Si 60, 250 × 4 mm [7 μm]), dichloromethane/methanol (90/10, v/v) as the eluent with a flow of 1 ml/min and a wavelength of 254 nm. Compound **11** was isolated from fraction 6, which was obtained with hexane:ethylacetate 1:1. Isolation of the compound was achieved by using semi-preparative HPLC with UV detection and a RP-18 analytical HPLC column, methanol/water (80/20, v/v) as the eluent with a flow of 1 ml/min and detection at 254 nm. Compound **12** was isolated from fraction 9, that eluted with 100% methanol by using semi-preparative HPLC with UV detection on a RP-18 analytical column (mentioned above), methanol/water (80/20) as an eluent with a flow of 1 ml/min, and detection at 254 nm. The amounts of the isolated compounds were 1 mg for **5**, **6**, **7**, **8**, **10** and **12**, 3.2 mg for **9**, and 9 mg for **11**.

3.3.3. Tests for biological activities

Tests for antimicrobial activities were carried out at the IBWF Institute of Biotechnology and Drug Research according to the method of Anke et al. (1989). Results are listed in Table 3. Tests for cytotoxic activities were measured at the IBWF according to the method of Zapf et al. (1995). Jurkat cells (ATCC TIB 152, T-lymphocytes, human) and HeLa-S3 cells (ATCC CCL 2.2, cervix carcinom human) were used. Results are listed in Table 4.

3.4. Ergosta-7,22-diene-3β-yl pentadecanoate *3*

White crystals; m.p. 91 °C; $[\alpha]_{20}^{589} = -9.5$ ($c = 0.06$, benzene); ^{1}H and ^{13}C NMR (C_6D_6) (see Table 2); MS (EI, 70 eV), *m/z* (rel. inten.): 622 [M^+] (11), 607(0.04), 524(3), 495(7), 381(15), 255(36), 84(96), 49 (100), 40(82); HRMS $m/z = 622.5688$ [M^+] (calc. for $C_{43}H_{74}O_2$: 622.2568).

Table 3
Antimicrobial activity of (+)-spongiporic acid A in the agar diffusion tests

Test organism	Inhibition zone (mm) after treatment with (+)-spongiporic A (**11**) (μg/disc)	
	50	10
Staphylococcus aureus	–	–
Proteus vulgaris	13	9
Acinetobacter calcoaceticus	+	–
Candida albicans	–	–
Aspergillus fumigatus	–	–
Bacillus subtilis	10	+
Bacillus brevis	11	+
Micrococcus luteus	–	–
Enterobacter dissolvens	–	–
Penicillium notatum	–	–
Paecilomyces variotii	12i	–
Mucor miehei	–	–
Nematospora coryli	–	–

Table 4
Cytotoxic activities (inhibitor concentration) of 5,8-*epi*-dioxy-24-methylcholesta-6,9(11),22-triene-3β-ol (**9**)

Cell line	IC90 (μg/ml)
Jurkat	10
HeLa-S3	25

3.5. Spongiporic acid A *11*

White crystals; m.p. 182 °C; $[\alpha]_{20}^{589} = +59$ ($c = 0.18$, methanol); IR_{max}^{solid} cm^{-1} : 1706(CO), 1667 (C=C); ^{1}H and ^{13}C NMR (pyridin-d_5) (see Table 2); MS (EI, 70 eV), *m/z* (rel. inten.): 496 [M^+] (9), 478(18), 423(40), 405(19), 339(4), 327(7), 281(15), 151 (100), 145(39), 81(35), 69(43), 55 (41); MS (ESI, positive ion mode), *m/z* 519 [M + Na]$^+$; HRMS $m/z = 519.3110$ [M + Na]$^+$ (calc. for $C_{31}H_{44}O_5Na$: 519.3086).

3.6. Spongiporic acid B *12*

White crystals; m.p. 218 °C; $[\alpha]_{20}^{589} = +145$ ($c = 0.02$, methanol); ^{1}H and ^{13}C NMR (methanol-d_4) (see Table 2), MS (ESI, negative ion mode), *m/z* 511 [M − H]$^-$, HRMS $m/z = 511.2999$ (calc. for $C_{31}H_{43}O_6$: 511.3059).

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running GC–MS. We also thank the Institute for Biotechnology and Drug Research (IBWF), Kaiserslautern (Germany) for the biological testing of spongiporic acid A and 5,8-*epi*-dioxy-24-methylcholesta-6,9(11),22-triene-3β-ol.

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