9β-Hydroxymarasmic Acid and Other Sesquiterpenoids from Submerged Cultures of a Basidiomycete [1]

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9β-Hydroxymarasmic acid, a new sesquiterpenoid, marasmic acid and isovelleral were isolated from cultures of a basidiomycete. Comparison of the antimicrobial activity of the three natural compounds together with two *n*-butyl ether derivatives of marasmic acid revealed MICs against bacteria in the range of 0.2–20 μg/ml. The antifungal, cytotoxic and phytotoxic activities of isovelleral were similar to those exhibited by marasmic acid, whereas the marasmic acid derivatives were considerably less active than the parent compound. Isovelleral was the only compound to show hemolytic activity.

Introduction

From submerged cultures of a basidiomycete three metabolites exhibiting antimicrobial activity were isolated. Elucidation of the structures revealed, that two of the compounds were identical to sesquiterpenoids described previously, namely isovelleral (**1**) [2, 3] and marasmic acid (**2**) [4, 5]. The third metabolite proved to be a new marasmane derivative (**3**).

Since marasmic acid, which had been isolated by our group from cultures of several mushrooms [6], exhibited pronounced antitumor activity in the screen of the NCI (J. Duros, personal communication) comparison of the biological activities of the three compounds was carried out. In the present paper the production, isolation and structure elucidation of the new marasmane derivative together with the biological activities of isovelleral and marasmic acid will be described.

Experimental

Culture media

Bacteria were grown on nutrient broth. All fungi were grown on YMG-medium [7]. For the evaluation of the antimicrobial activity the same media were used. Ehrlich ascites tumor cells (ECA cells) were grown as described earlier [8]. The composition of the BAF-medium is given in [9].

Assays

Tests for cytotoxic activity were carried out as previously described [8]. Tests for hemolytic activity were carried out as in ref. [10]. For phytotoxic activity six seeds of the plants were placed on filter discs (13 mm ∅; 1 mm thick) bearing the compounds. The discs were soaked with water (150 μl) and incubated in a humid chamber at 20 °C for three days in the dark, thereafter in the light. After 48 h, 72 h and 96 h the growth of the seedlings was compared with the control without antibiotics. Tests were carried out in triplicates.

Results

*Producing organism, fermentation and isolation of **1**, **2** and **3***

The producing organism, strain HA 2-83, was isolated as a culture contaminant growing on a culture of a *Hygrophorus* species. The mycelia bear clamp connections and form brown-black sclerotia. Abundant production of sclerotia was observed upon storage of the culture at 4 °C. Formation of conidia or fruit bodies was never observed, therefore the sexual state of the fungus is unknown. The formation of clamp connections puts the strain among the basidiomycetes.

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The production of antimicrobial compounds was best on BAF-medium at 22 °C. A typical fermentation diagram in a 20 l jar is shown in Fig. 1. Due to the two carbon sources present in the medium the mycelial growth exhibited diauxic characteristics. Production of antibiotics was followed by using the agar diffusion assay with *Paecilomyces variotii* and *Bacillus brevis* as test organisms. The appearance of the compounds was monitored by extracting aliquots of the broth with EtOAc, concentrating the solvent, spotting on a silica gel TLC plate and developing in toluene–Me_2CO–AcOH (70:30:1). The compounds were visualized with UV light at 254 nm or by spraying with vanillin–H_2SO_4. During the 20 l fermentations no antimicrobial activity could be detected in mycelial extracts and on the chromatograms no spots corresponding to **2** or **3** could be seen. Whereas all fermentations contained **2** and **3** in the culture filtrate, **1** was obtained only from 100 l fermentations. A fraction (20%) of **1** was located in the mycelium and extracted with Me_2CO, the remainder was obtained from the extracts of the broth. The isolation and separation procedure is depicted in Fig. 2. The production of **2** started after two to three days and continued for five to six days. When **1** was present its production started together with **2**. Traces of **3** were detected two days after the onset of the production of **2**. Its ratio in the mixture increased from 15% on day 7 to 25% on day 9, whereas the total amount of the two compounds decreased on the 9th day. Fermentors were harvested when the carbon source in the medium was exhausted.

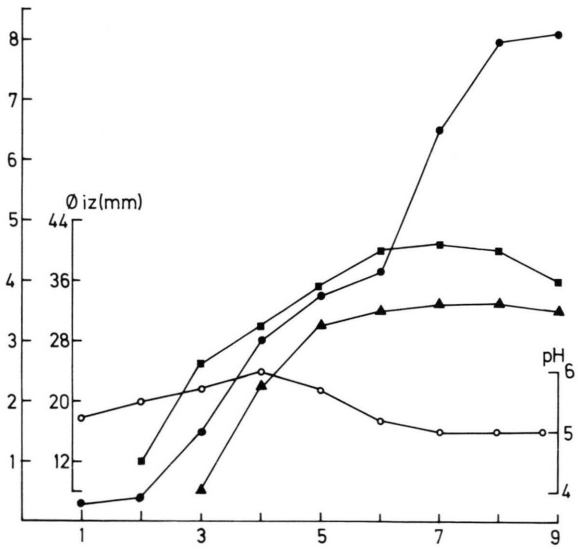


Fig. 1. Fermentation diagram of strain HA2-83 in BAF-medium. ●——● mycelial weight (MW; g/l); ■——■ antifungal activity (assayed with *P. variotii*); ▲——▲ antibacterial activity (assayed with *B. brevis*). The antimicrobial activities are given as ∅ inhibition zone (iz). ○——○ pH.

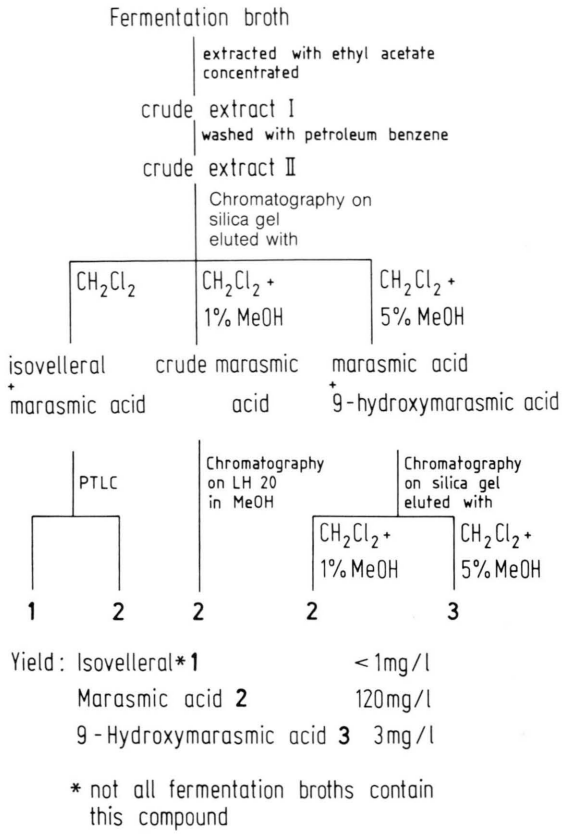


Fig. 2. Isolation and separation of **1**, **2**, and **3**.

On one occasion the culture broth was extracted with *n*-butanol and two "new" active compounds were detected on TLC. These compounds proved to be the epimeric 5-*O*-butyl derivatives of marasmic acid (**4a**, **4b**) and were considered to be artifacts of the isolation procedure. Since they exhibited antimicrobial activity, they were included in the biological tests.

Physico-chemical properties of the compounds

Isovelleral (**1**), oil, was identical in its spectral data and its specific rotation, $[\alpha]_D^{20}$ +293° (*c* 0.1, $CHCl_3$), with the compound from *Lactarius vellereus* [3, 11]. Marasmic acid (**2**), colourless crystals, m.p. 174 °C,

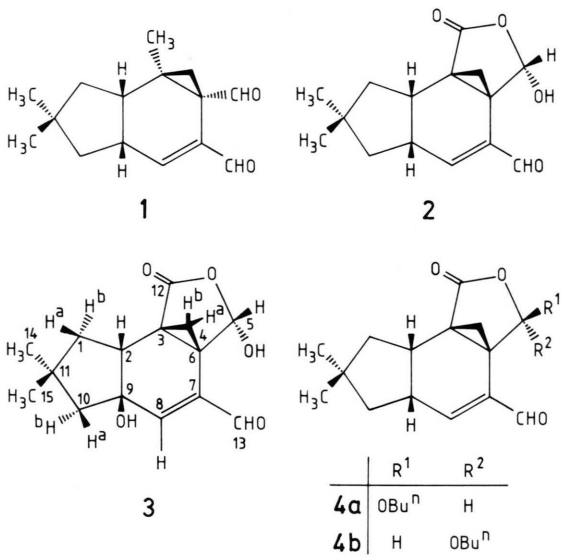
was in every respect identical with an authentic sample [5, 6].

*9β-Hydroxymarasmic acid (**3**)*

Light-yellow oil, R_f 0.47 [silica gel; toluene–Me_2CO–AcOH (70:30:1)], $[\alpha]_D^{20}$ +12.0° (*c* 0.2, Me_2CO); UV λ_{max} nm (log ε) 215 (3.63), 237 (3.69); CD (EtOH): $[\theta]_{216}$ $+38.81\times10^3$, $[\theta]_{231}$ 0, $[\theta]_{244}$ -27.31×10^3, $[\theta]_{280}$ 0, $[\theta]_{325}$ $+3.01\times10^3$, $[\theta]_{367}$ 0, $[\theta]_{382}$ -0.56×10^3; IR (KBr) cm^{-1} 3440 (sst), 2980 (st), 2950 (m), 2890 (w), 2870 (w), 1732 (sst), 1680 (sst), 1642 (w), 1467 (m), 1440 (m), 1379 (m), 1320 (w), 1271 (m), 1220 (st), 1152 (m), 1100 (st), 1075 (w), 1051 (m), 1040 (m), 1011 (st), 961 (st), 914 (m), 899 (m), 841 (m), 816 (m), 779 (m), 740 (m); HR-MS (70 eV, DI 180 °C): *m/z* (relative intensity, %) 278.1154 (70, M^+, calcd for $C_{15}H_{18}O_5$ 278.1154), 260 (42, $C_{15}H_{16}O_4$), 245 (15, $C_{14}H_{13}O_4$), 232 (100, $C_{14}H_{16}O_3$), 214 (27, $C_{14}H_{14}O_2$), 208 (21, $C_{10}H_8O_5$), 204 (50, $C_{13}H_{16}O_2$), 187 (45, $C_{13}H_{15}O$), 161 (35, $C_{11}H_{13}O$), 135 (69, $C_8H_7O_2$), 115 (37, C_9H_7), 107 (52, C_7H_7O), 77 (83, C_6H_5), 55 (51, C_4H_7), 51 (25, C_4H_3), 43 (53, C_2H_3O).

*5-O-Butylmarasmic acid (**4a**, **4b**)*

By extraction of the culture broth with *n*-butanol a 3:1 mixture of epimers **4a** and **4b** was obtained which could be separated by HPLC (LiChrosorb Si 60 Merck, 7 µm; *n*-hexane, 10% EtOAc, 0.02% iPrOH). **4a**: $[\alpha]_D^{20}$ +44° (*c* 0.1, $CHCl_3$), R_f 0.85 [silica gel, toluene–Me_2CO–AcOH (70:30:1)]; **4b**: $[\alpha]_D^{20}$ +180° (*c* 0.1, $CHCl_3$), R_f 0.84; UV (mixture of **4a** and **4b**) λ_{max} (log ε) 238 nm (3.97); IR (mixture, KBr) cm^{-1} 3520–3340 (m, br.), 2950 (sst), 2930 (sst), 2870 (st), 1765 (sst), 1670 (sst), 1630 (m), 1455 (m), 1325 (m), 1085 (st), 915 (st), 902 (st). Selected ^1H NMR data (400 MHz, $CDCl_3$) **4a**: δ 0.93 (t, 3H), 1.00, 1.06 (each s, 6H), 1.17 (d, *J* = 5.2 Hz, 4-H_a), 1.41 (m, 2H), 1.67 (m, 2H), 1.94 (d, *J* = 5.2 Hz, 4-H_b), 3.81 (t, 2H), 5.77 (s, 5-H), 6.35 (d, *J* = 2.2 Hz, 8-H), 9.48 (s, 13-H); **4b**: δ 0.87 (t, 3H), 1.03, 1.07 (each s, 6H), 1.04 (d, *J* = 5 Hz, 4-H_a), 1.26 (m, 2H), 1.35 (d, *J* = 5 Hz, 4-H_b), 1.47 (m, 4H), 3.53 (dt, *J* = 9.5, 6.5 Hz, OCH_aH), 3.76 (dt, *J* = 9.5, 6.5 Hz, OCH_bH), 5.68 (s, 5-H), 6.39 (d, *J* = 2.5 Hz, 8-H), 9.43 (s, 13-H); HR-MS (mixture, direct inlet 180°, 70 eV): *m/z* (relative intensity, %) 318.1837 (43, M^+, calcd for $C_{19}H_{26}O_4$ 318.1831), 274 (42, $C_{18}H_{26}O_2$), 262 (22, $C_{15}H_{18}O_4$), 246 (15, $C_{15}H_{18}O_3$), 245 (49, $C_{15}H_{17}O_3$), 244 (100, $C_{15}H_{16}O_3$), 217 (20, $C_{14}H_{17}O_2$), 218 (49, $C_{14}H_{18}O_2$), 217 (20), 216 (16), 215 (13), 203 (12), 190 (18), 175 (17), 173 (16), 162 (19), 161 (13), 134 (17), 105 (28).



Elucidation of the structures

Metabolite **3**, $C_{15}H_{18}O_5$, resembles in its UV and IR spectra marasmic acid (**2**). In the NMR spectra of **3** (Table I) differences are visible which can be explained by the presence of an additional hydroxy group at C-9. It causes a downfield shift of one of the cyclopropyl protons in the ^1H NMR spectrum from δ 1.23 (**2**) to 1.52 ppm (**3**) and should therefore be β-oriented. This assignment is supported by the accordance of the CD spectra of **2** and **3** (Fig. 3). **3** is

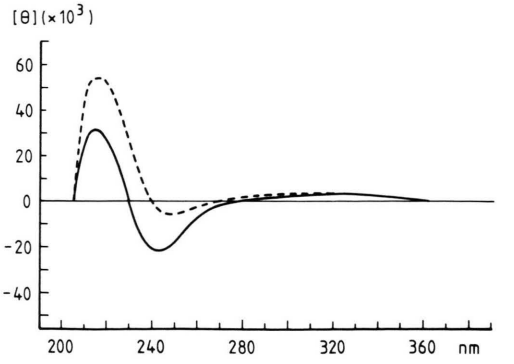


Fig. 3. CD spectra of marasmic acid (**2**) [——] and 9β-hydroxymarasmic acid (**3**) [– – – –] in MeOH; recorded on a Circular Dichrographe Jouan-Roussel III.

Table I. ^1H and ^{13}C NMR data of 9β-hydroxymarasmic acid (**3**) (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^a	1.27 m		C-1	45.92	D 130
1-H^b	2.10 ddd	12.9/7.5/1.2			
2-H	2.99 dd	12.9/7.5	C-2	40.54	D 134
			C-3	30.48*	
4-H^a	1.42 d	4.5	C-4	26.69	T 166
4-H^b	1.52 d	4.5			
5-H	6.08 s (br.)		C-5	97.66	D 180
			C-6	30.69*	
			C-7	139.20	d 26
8-H	6.62 d	0.5	C-8	149.80	D 160
			C-9	78.92	m
10-H^a	1.95 d	13.8	C-10	57.11	D 128
10-H^b	2.01 dd	13.8/1.2			
			C-11	36.15	
			C-12	175.19	m
13-H	9.60 s		C-13	193.28	Dd 178/8
14-CH_3	1.03 s		C-14	31.14	Q
15-CH_3	1.25 s		C-15	30.89	Q

* Signals may be interchanged.

closely related to pilatin, a 9β,10α-dihydroxymarasmic acid derivative from cultures of *Flagelloscypha pilatii* [1]. The two epimers of 5-*O*-butylmarasmic acid were separated by HPLC. In their MS spectra loss of butanol from the molecular ion *m/z* 318 leads to the base peak *m/z* 244. The ^1H NMR spectra ($CDCl_3$) of the epimers exhibit characteristic differences which may be used to assign the stereochemistry at C-5. In epimer **4b** the signals of the *n*-butoxy chain are considerably shielded which is in accord with the α-configuration. Interestingly, the OCH_2-protons give rise to two doublets of triplets at δ 3.53 and 3.76 whereas in **4a** the corresponding signal appears as a sharp triplet at δ 3.81. In epimer **4a** the β-oriented ether moiety causes a remarkable downfield shift of the neighbouring cyclopropane proton to δ 1.94 as compared to δ 1.35 in **4b**.

Biological properties

The antimicrobial spectra of all compounds are depicted in Tables II and III. Isovelleral (**1**) and marasmic acid (**2**) exhibited similar spectra of activity including the cytotoxic activity, which is given in Table IV. The main difference was the higher lytic activity of **1**. This was confirmed using bovine erythrocytes (Table V). Neither **2** nor any of its derivatives showed hemolytic activity at concentrations up to 100 μg/ml, whereas 50 μg/ml of **1** caused almost com-

Table III. Antifungal activity of **1**, **2**, **3**, **4a**, and **4b** in the plate diffusion assay. Filter discs (6 mm ∅) bearing 20 μg of the compounds were placed onto YMG-agar seeded with the test organism.

Organism	Inhibition zone [mm]				
	1	**2**	**3**	**4a**	**4b**
Absidia glauca	12	8	–	–	–
Alternaria porri	19	11	–	–	–
Curvularia lunata	29	20	8	–	–
Epicoccum purpurascens	8	8	–	–	–
Fusarium fujikuroi	10	–	–	–	–
Mucor miehei	–	15	–	10	16
Neurospora crassa	11	18	8	–	–
Paecilomyces variotii	–	11	–	8	12
Penicillium notatum	–	10	–	–	11
Phoma clematidina	9	8	–	–	–
Venturia inaequalis	8	–	–	–	–
Verticillium spec.	8	–	–	–	–

Table II. Minimal inhibitory concentrations (MIC) of **1**, **2**, **3**, **4a**, and **4b** against bacteria and yeasts in the serial dilution assay; 10^6 cells/ml.

Organism	MIC [μg/ml]				
	1	**2**	**3**	**4a**	**4b**
Bacteria					
Acinetobacter calcoaceticus	1	1	15	20	10
Bacillus brevis	0.2	0.2	5	10	2
B. subtilis	0.5	0.5	10	10	5
Proteus vulgaris	10	5	20	20	10
Micrococcus luteus	10	10	20	50	10
Staphylococcus aureus	5	5	20	20	10
Yeasts					
Nematospora coryli	0.5	0.5	5	10	10
Saccharomyces cerevisiae iS1	0.1	2	20	5	5
Rhodotorula glutinis	20	>100	>100	>100	>100

plete lysis. The introduction of a hydroxyl function at C-9 reduced the inhibitory activity of marasmic acid in all of our test systems, whereas the mutagenic activity in the Ames test was increased [13]. The epimeric *n*-butyl ethers (**4a**, **4b**) are both less active than the parent compound, this is also true for the phytotoxic activity which is given in Table VI.

Table IV. Cytotoxic and lytic activity of **1**, **2**, **3**, **4a**, and **4b** against Ehrlich ascitic tumor cells (5×10^5 cells/ml).

Compound	IC_{50} [μg/ml]	Lysis [μg/ml]
Isovelleral (**1**)	2	10
Marasmic acid (**2**)	2	25
9-Hydroxymarasmic acid (**3**)	30	≥50
n-Butyl ether **4a**	50	≥50
n-Butyl ether **4b**	10	50

Table V. Hemolytic activity of **1**, **2**, **3**, **4a**, and **4b** (bovine erythrocytes).

Compound	Concentration [μg/ml]	Hemolysis [%]
Isovelleral (**1**)	10	10
Isovelleral (**1**)	50	88
Isovelleral (**1**)	100	100
Marasmic acid (**2**)	10	3
Marasmic acid (**2**)	50	5
Marasmic acid (**2**)	100	6
9-Hydroxymarasmic acid (**3**)	10	3
9-Hydroxymarasmic acid (**3**)	50	3
9-Hydroxymarasmic acid (**3**)	100	5
n-Butyl ether **4a**	10	4
n-Butyl ether **4a**	50	5
n-Butyl ether **4a**	100	5
n-Butyl ether **4b**	10	6
n-Butyl ether **4b**	50	5
n-Butyl ether **4b**	100	5

Discussion

Marasmic acid has been found in cultures of several basidiomycetes *e.g. Marasmius conigenus*, *Lachnella villosa* and *Peniophora laeta* and its biological activities are well established [6]. Comparison of the culture morphology of known producers of marasmic acid with our strain HA 2-83 revealed no similarities. Strain HA 2-83 is not identical with any of them. Isovelleral so far had only been found in fruit bodies of mushrooms belonging to the Russulaceae, where it possibly takes part in the defense system [14]. Since these basidiomycetes can not been grown in artificial cultures, the present paper is the first report on its production in submerged cultures and on its co-occurrence with marasmic acid. A biogenetic relationship of the two compounds has been discussed before [15, 16]. 9β-Hydroxymarasmic acid is a new naturally occurring sesquiterpenoid, which is produced towards the end of the fermentation. Isovelleral adsorbed on alumina can be easily transformed into its 9-hydroxy derivative by autoxidation [17]. Under the same conditions or after incubation for several days in culture broth without mycelia no conversion of marasmic acid into **3** was observed [11]. The semisynthetic *n*-butyl ethers of marasmic acid showed significantly lower inhibitory activities, especially epimer **4b**. In this compound the reactive α,β-unsaturated aldehyde moiety is sterically more shielded due to the α-orientation of the *n*-butyl chain.

Acknowledgements

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Table VI. Phytotoxic activity of **1**, **2**, **3**, **4a**, and **4b** against seeds of *Setaria italica* and *Lepidium sativum*. (Concentrations needed for complete inhibition of germination or for 50% reduction of growth; μg/assay.)

Compound	Inhibition of			
	Germination	Growth	Germination	Growth
	Setaria italica		*Lepidium sativum*	
Isovelleral (**1**)	10	5	20	5
Marasmic acid (**2**)	50	20	30	10
9-Hydroxymarasmic acid (**3**)	150	50	100	30
n-Butyl ether **4a**	>250	150	250	50
n-Butyl ether **4b**	>250	150	100	30

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