6,9-Dihydroxy-3(15)-caryophyllen-4,8-dione – a New Antibiotic from a *Marasmius* Species

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6,9-Dihydroxy-3(15)-caryophyllen-4,8-dione (**1**), a new cytotoxic caryophyllane sesquiterpene, was isolated from fermentations of a tropical *Marasmius* species. The structure was established by spectroscopic methods. **1** exhibits strong cytotoxic effects on different cell lines, but only weak antimicrobial activities. **1** weakly inhibits the incorporation of leucine and thymidine into proteins and DNA of mammalian cells and interferes with the aggregation of human and bovine platelets.

Introduction

In our search for new bioactive metabolites from basidiomycetes, a new caryophyllane sesquiterpene, 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**), was isolated from fermentations of a *Marasmius* species from New Caledonia. Caryophyllane sesquiterpenoids have been described as widespread constituents of several plants, e.g. *Jasminum*, *Lavendula* and *Juniperus.* Previously, two derivatives similar to **1**, naematolin (**2**) and naematolon (**3**), have been isolated from several *Hypholoma* (= *Naematoloma*) and a *Panus* species (Ito *et al.*, 1967; Doi *et al.*, 1986; Backens *et al.*, 1984; Lorenzen *et al.*, 1994a). For naematolin (**2**), cytotoxic, antiviral and vasodilatoric activities have been described (Ito *et al.*, 1967; Backens *et al.*, 1984), whereas naematolon (**3**) has been reported to exhibit cytotoxic and antimicrobial activities (Backens *et al.*, 1984) and as inhibitor of platelet aggregation (Lorenzen *et al.*, 1994a). In the following we describe the production, isolation, structure elucidation and biological properties of 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione.

Materials and Methods

General

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, UV and IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer, and the optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C. For analytical HPLC a Hewlett Packard 1090 series II instrument was used.

Producing organism

The basidiomycete *Marasmius* sp. strain 96115 was isolated from the spore print of a fruiting body collected in New Caledonia. The specimen showed all essential characteristics of the genus (Singer, 1996). The species, however, could not be identified. The strain and voucher specimen are deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern.

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Fermentation and isolation

For maintenance on agar slants and submerged cultivation, *Marasmius* sp. was grown in YMG medium composed of : yeast extract 0.4%, malt extract 1%, glucose 0.4%, pH 5.5 and agar 1.5% for solid media. Fermentations were carried out in a Biolafitte C6 fermenter containing 20 l of YMG medium with aeration (3 l air/min) and agitation (120 rpm) at 27 °C. 250 ml of a well grown culture in YMG medium were used as inoculum.

After 25 days of fermentation the culture fluid was separated from the mycelia by filtration. Compound **1** was removed from the culture fluid (15 l) by adsorption to HP 21 resin (Mitsubishi, column: 30×300 mm) and eluted with acetone. The crude extract (2372 mg) was applied onto a column (60×265 mm) containing silica gel (0.063–0.2 mesh, Merck 60) and eluted with 700 ml cyclohexane:ethylacetate 30:70. 1268 mg of a crude product were obtained and applied onto another silica gel column (30×240 mm, 0.063–0.2 mesh, Merck 60). Elution with cyclohexane:ethylacetate 30:70 yielded 351 mg of pure **1**.

6,9-Dihydroxy-3(15)-caryophyllen-4,8-dione (**1**) was obtained as a colourless oil. $[\alpha]_D$ +33° (*c* 0.25 in $CHCl_3$–CH_3OH 1:1). UV (MeOH), λ_{max} (ε): 236 nm (2,750). IR (KBr): 3430, 2925, 1700, 1430, 1260, 1125, 1055 and 1010 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm), mult., *J* (Hz): 6.16, d, J_{2-15a} = 1.0, 15-Ha; 5.75, d, J_{2-15b} = 1.8, 15-Hb; 4.25, d, J_{9-10} = 11.1, 9-H; 4.00, ddd, J_{5a-6} = 5.4; J_{5b-6} = 7.0, J_{6-7} = 1.9, 6-H; 3.56, ddd, J_{1a-2} = 8.2, J_{1b-2} = 9.1, J_{2-10} = 10.5, 2-H; 3.10, dd, J_{5a-5b} = 12.0, J_{5a-6} = 5.4, 5-Ha; 2.98, dq, J_{6-7} = 1.9, J_{7-14} = 7.0, 7-H; 2.68, dd, J_{5a-5b} = 12.0, J_{5b-6} = 7.0, 5-Hb; 2.42, ddd, J_{1b-10} = 1.3, J_{2-10} = 10.5, J_{9-10} = 11.1, 10-H; 1.99, dd, J_{1a-1b} = 12.2, J_{1a-2} = 8.2, 1-Ha; 1.96, ddd, J_{1a-1b} = 12.2, J_{1b-2} = 9.1, J_{2b-10} = 1.3, 1-Hb; 1.30, d, J_{7-14} = 7.0, 14-H_3; 1.28, s, 13-H_3; 1.21, s, 12-H_3. ^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 220.7, C-8; 201.2, C-4; 150.6, C-3; 124.0, C-15; 75.8, C-9; 71.9, C-6; 51.9, C-10; 48.2, C-7; 45.6, C-5; 35.2, C-1; 34.5, C-11; 31.6, C-13; 30.4, C-2; 25.4, C-12; 16.0, C-14. CIMS (CH_4), *m/z* (rel. int.): 267 (32%, $M + H^+$), 249 (100%, $M - H_2O + H^+$), 231 (45%), 209 (60%), 194 (69%), 191 (62%), 175 (44%), 163 (29%), 149 (28%).

Biological assays

The tests for cytotoxic effects (Zapf *et al.*, 1995) and the antimicrobial activity in the serial dilution assay (Anke *et al.*, 1989) have been described elsewhere. The test for inhibitory effects on the synthesis of macromolecules was carried out as reported previously (Lorenzen *et al.*, 1994a).

The inhibitory effect on the aggregation of human and bovine platelets was determined as follows: Concentrated human platelets (2×10^9 cells/ml) were provided by the Universitätsklinikum Homburg/Saar. Citrate – dextrose – buffer (ACD solution) was used as anticoagulans. Bovine platelet rich plasma (PRP) and bovine platelet poor plasma (PPP) were prepared as described before (Lorenzen *et al.*, 1994b). The PRP contained about $4–5\times10^5$ platelets/ml. The aggregation assay was carried out in a spectrophotometer (Hitachi, model 100–60) with temperated (37 °C) and stirred cuvettes. 1.5 ml bovine PRP or concentrated human platelets were preincubated for 10 minutes with the tested compound. The aggregation of bovine platelets was stimulated by collagen and ADP, whereas the aggregation of human platelets was induced by different agonists using, besides collagen and ADP, thrombin and the thromboxane A2 analogue U46619. After adding the agonists the change of transmittance was monitored at 600 nm with PPP as blank.

For assays of extracts and fractions for aggregation inhibiting activities 150 µl of bovine PRP or concentrated human platelets were placed in 96-well microtiter plates, preincubated with the samples, and the change of transmittance during aggregation was measured with an eight-channel photometer (Labsystems iEMS Reader MF), then.

Results and Discussion

Fermentation and isolation

A typical fermentation of *Marasmius* sp. 96115 in 20 liters of YMG medium is shown in Fig. 1. As detected by analytical HPLC, the production of 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**) started after 8 days, reached a maximum of about 32 mg/l after 25 days of fermentation and decreased afterwards. The extraction of the culture

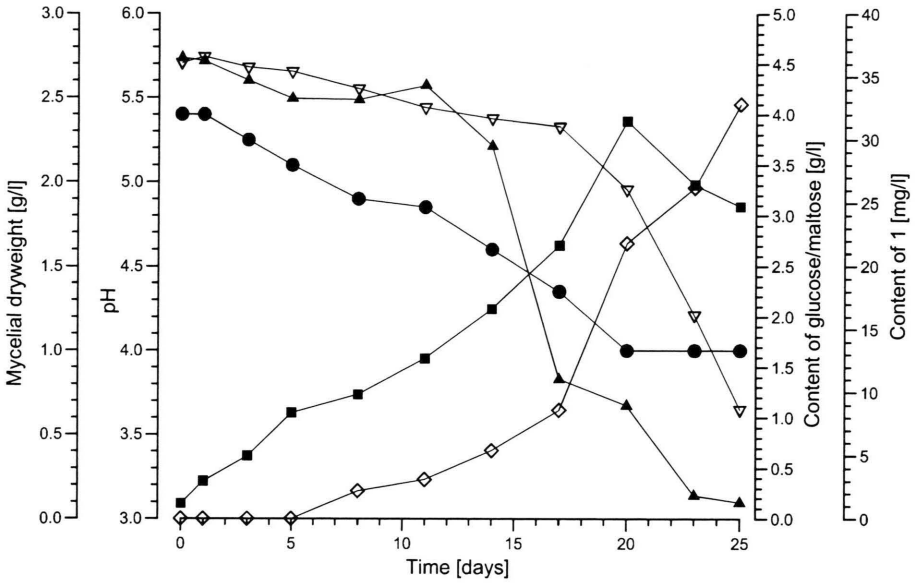
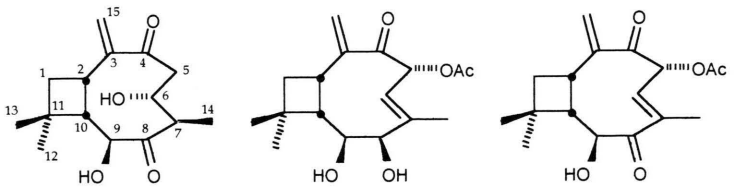


Fig. 1. Fermentation of *Marasmius* sp. 96115 in 20 l of YMG-medium. ●, pH; ▲, content of glucose; ▽, content of maltose; ■, mycelial dry weight; ◇, content of **1**.

broth and isolation of **1** is described in the experimental section.

Structural elucidation

The elucidation of the structure of 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**) is based on NMR spectroscopic data, of which 1D ^1H and ^{13}C data are given in Materials and Methods and pertinent 2D HMBC and NOESY data given in Fig. 3. The scalar ^1H-^1H couplings from 1-H_2 *via* 2-H and 10-H to 9-H, as well as from 5-H *via* 6-H and 7-H to 14-H_3 demonstrated the presence of two spin systems which could be joined together in a 9-membered ring by the HMBC couplings shown in Fig. 3 and the long-range $^1H-^1H$ couplings observed between 2-H and 15-H_2. The presence of a cyclobutane ring was established by the HMBC correlations from both 12-H_3 and 13-H_3 to C-1, C-10 and C-11, as well as to each others carbons. The stereostructure of **1** (shown in Fig. 2) was suggested by the NOESY correlations observed (see Fig. 3) and by the magnitude of certain $^1H-^1H$ couplings. NOESY correlations between 13-H_3 and 1-Hβ, 2-H and 10-H shows that the two rings are *cis*, and a NOESY correlation between 12-H_3 and 9-H together with the large J_{9-10} (12.2 Hz) suggest that 9-H is in the α position and axial. 9-H also gives a strong NOESY correlation to 7-H which in turn correlates to 5-Hα. The two protons in the exomethylene group correlate to 1-Hα and 5-Hα, respectively. 6-H gives NOESY correlations to both 5-Hα and 5-Hβ, and to 7-H as well as 14-H_3, and the inspection of a Dreiding model of **1** having a conformation that satisfies the above mentioned NOESY data shows that the configura-



1 **2** **3**

Fig. 2.

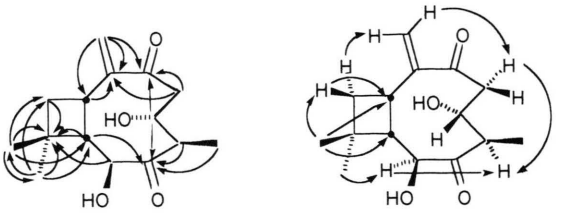


Fig. 3. Pertinent HMBC (left) and NOESY (right) correlations observed with 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**).

tion of C-6 must be as suggested. This will also be in accord with the small J_{6-7} (1.9 Hz) observed, and allows the α,β-unsaturated ketone to adopt a planar configuration. The absolute stereochemistry of **1** was not determined, although it is assumed to be the same as that of naematolin (**2**). The structural similarities between 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**), naematolin (**2**) and naematolon (**3**) indicate that the conjugated exomethylene ketone functionality is at least partly responsible for the biological activities.

Table I. Cytotoxic activities of **1**.

Cells	IC_{50} [μM]	IC_{100}[a] [μM]
HeLa S3[1]	19	>37.6
HL 60[2]	3.8	37.6
L 1210[3]	1.9	9.4

[a] Complete lysis of cells after 48 h.
[1] Epitheloid carcinoma, cervix human; ATCC CCL 2.2.
[2] Promyelocytic leukemia, human; ATCC CCL 240.
[3] Lymphocytic leukemia, mouse; ATCC CCL 219.

Table II. Effect of **1** on the incorporation of ^{14}C-labelled precursors in DNA, RNA and proteins of L1210 cells.

Concentration of **1** [μM]	Incorporation of ^{14}C-labelled Thymidine [%]	Incorporation of ^{14}C-labelled Uridine [%]	Incorporation of ^{14}C-labelled Leucine [%]
0	100	100	100
0.2	89.8	97.8	82.4
0.4	94.6	92.4	82.0
1.9[a]	90.3	97.4	82.5
3.8	90.6	89.0	67.4
9.4[b]	87.0	92.7	60.0
18.8	34.5	79.7	34.6

[a] This concentration is corresponding to the IC_{50} in the cytotoxicity test.
[b] This concentration is corresponding to the IC_{100} in the cytotoxicity test.
Controls (100% incorporation): thymidine 8765 cpm; uridine 8807 cpm; leucine 12071 cpm

Biological properties

6,9-Dihydroxy-3(15)-caryophyllen-4,8-dione (**1**) showed strong cytotoxic activities on L 1210 and HL 60 cells with IC_{50}'s of 1.9 and 3.8 μM, respectively, whereas lower cytotoxic effects were observed with HeLa S3 cells (Table I). The influence of **1** on the incorporation of ^{14}C-labelled thymidine, uridine and leucine into DNA, RNA and proteins was tested with L1210 cells, and the results are shown in Table II. At lower concentrations of **1** no significant inhibition of the biosyntheses of macromolecules could be observed, although at concentrations higher than 9.4 μM (corresponding to the IC_{100} in the cytotoxicity test with L1210 cells) **1,** as expected, inhibited the incorporation of leucine and thymidine into proteins and DNA significantly. The effect of **1** on the aggregation of human and bovine platelets stimulated with different agonists is shown in Table III. **1** preferentially inhibited the ADP and collagen induced aggregation while no inhibition on thrombin induced aggregation was observed. The effects were more pronounced on human as compared to bovine platelets.

The antimicrobial activity of **1** was tested in the serial dilution assay. **1** exhibited neither antibacterial nor antifungal activities at concentrations up to 376 μM (100 μg/ml) against the following microorganisms: *Escherichia coli* K12, *Arthrobacter citreus*, *Bacillus brevis*, *Bacillus subtilis*, *Corynebacterium insiduosum*, *Micrococcus luteus*, *Mycobacterium phlei*, *Nematospora coryli*, *Saccharomyces cerevisiae* is 1, *Paecilomyces variotii*, *Penicillium notatum*, *Mucor miehei*, *Rhodotorula glutinis*.

Compared to the structurally similar caryophyllanes naematolin (**2**) and naematolon (**3**), stronger cytotoxic effects could be observed for 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**). In contrast to **1**, **2** and **3** inhibited preferentially the DNA and RNA syntheses (Backens *et al.*, 1984). The antimicrobial activity of naematolon is very weak (Backens *et al.*, 1984), whereas for naematolin (**2**) and 6,9-dihydroxy-3(15)-caryophyllen-4,8-dione (**1**) no antibacterial or antifungal activity could be

Table III. Effect of **1** on platelet aggregation.

Inducer	Human platelets IC_{50}* [μM]	[μg/ml]	Inducer	Bovine platelets IC_{50}* [μM]	[μg/ml]
Collagen [0.4 mg/ml]	47.0	(12.5)	Collagen [0.4 mg/ml]	56.4	(15)
ADP [33 μM]	47.0	(12.5)	ADP [16 μM]	75.2	(20)
Thrombin[1] [0.13 U/ml]	>376	(>100)	Thrombin[1] [0.1 U/ml]	n.t.	n.t.
U 46 619[2] [1.3 μM]	150.4	(40)	–		

IC_{50} values of **1** for the inhibition of aggregation of human and bovine platelets stimulated by different inducers.
n.t., Not tested.
* 50% inhibition of aggregation.
– Inducer not applicable.
[1] Human thrombin was obtained from Fluka. 1 U hydrolyzes 1 μmol/min. Tos–Gly–Pro–Arg–pNA.AcOH (Chromozym TH) at pH 8.4 and 37 °C.
[2] Thromboxane A_2 analogue (UpJohn).

detected. The influence on bovine and human platelet aggregation of **1** and **3** (Lorenzen *et al.*, 1994a) differs for the used agonists. Like **2** and **3**, **1** reacts with sulfhydryl groups of e.g. dithiothreitol or cysteine by forming inactive adducts, a typical reaction for α,β-unsaturated ketones (Backens *et al.*, 1984).

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