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Bioactive Triterpenes from the Fungus *Piptoporus betulinus*

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Abstract: Phytochemical investigation of the ethyl acetate extract of the fruiting bodies from the basidiomycete *Piptoporus betulinus* led to the isolation of a new bioactive lanostane triterpene identified as 3β-acetoxy-16-hydroxy-24-oxo-5α-lanosta-8- ene-21-oic acid (**1**). In addition, ten known triterpenes, polyporenic acid A (**5**), polyporenic acid C (**4**), three derivatives of polyporenic acid A (**8, 10, 11**), betulinic acid (**3**), betulin (**2**), ergosterol peroxide (**6**), 9,11-dehydroergosterol peroxide (**7**), and fomefficinic acid (**9**), were also isolated from the fungus. All isolated compounds were tested for antimicrobial activity against some Gram-positive and Gram-negative bacteria as well as against a fungal strain. The new triterpene and some of the other compounds showed antimicrobial activity against Gram-positive bacteria.

Keywords: *Piptoporus betulinus*; triterpenoids; antibacterial activity; iceman "Ötzi".

1. Introduction

Mushrooms, similar to plants, have a great potential for the production of bioactive metabolites. The responsible bioactive compounds belong to several chemical groups, very often they are polysaccharides or triterpenes [1]. Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds are reported from mushrooms and that these compounds offer potential benefits for humans [1]. The mushroom *Piptoporus betulinus* (Bull.: Fr.) P. Karst. (Polyporaceae), birch polypore, grows as trunk parasite and saprophyte on *Betula pendula* Roth. and *B. pubescens* Ehrh. (Betulaceae). Fruiting bodies of this fungus are used in the European ethnomedicine for the treatment of cancer and stomach diseases. The mushroom is also known as fungus of the iceman "Ötzi" from the copper age found frozen in a glacier in 1991, who carried *P. betulinus* fruiting bodies attached to his clothing on his journey in the Alps [1]. Some triterpenes from *P. betulinus* are already known, e.g. the polyporenic

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acids A and C [2-3]. Here we describe the isolation of a new triterpene together with ten known compounds from the fruiting bodies of this mushroom.

2. Materials and Methods

2.1 .General

The high resolution positive ion ESI mass spectra were obtained from a Bruker Apex III 70 e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. All 1H and 2D spectra were recorded on a Varian/Agilent VNMRS 600 NMR spectrometer operating at 599.832 MHz using a 5 mm inverse detection cryoprobe. Internal reference: 1H: TMS = 0 ppm; 13C: CD3OD = 49.0 ppm.

2.2 . Plant Material

Fruiting bodies of *Piptoporus betulinus* were collected in April 2009 from *Betula pendula* Roth. (Betulaceae) nearby Hanshagen, 10 km south from Greifswald in the northeast of Germany. Samples were identified by Professor Hanns Kreisel, Institute for Microbiology, University Greifswald. The fruiting bodies were cut, dried in an oven at 40°C and stored at room temperature. Voucher specimens (Nr. 48957) are deposited at the Department for Pharmaceutical Biology, Institute for Pharmacy, University of Greifswald, Germany.

2.3 . Extraction and Isolation

The dried powder of fruiting bodies (500 g) was extracted successively in a Soxhlet apparatus with n-hexane, methanol and water. The solutions obtained were concentrated under reduced pressure. The methanol extract (38 g) was dried and then partitioned between water (20 g) and ethyl acetate (16 g). The major anti-bacterial activity was found in the ethyl acetate part. The ethyl acetate extract was fractionated by column chromatography (CC) using silica gel 60 (0.040-0.063 mm mesh size, Merck, Germany). Elution was performed first by ethyl acetate/toluene (7:3) and then by dichloromethane containing increasing amounts of methanol; dichloromethane/methanol 10:1, dichloromethane/methanol/water 40:12:1 and at last dichloromethane/methanol/water 65:35:8. Analytical thin layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Germany) was used to identify similar fractions. The fractions obtained from ethyl acetate/toluene (7:3), dichloromethane/methanol 10:1 and dichloromethane/methanol/water 40:12:1 appeared to be the most active and were subjected to column chromatography with long and thin columns (60 x 1.2 cm and 50 x 2.0 cm) (silica gel 0.015-0.040) and after that subjected to solid phase extraction (C18-E cartridges and MeOH/H_2O gradient). The obtained fractions were then separated by preparative HPLC to yield eleven compounds. The structural identification of the compounds was performed by spectroscopic methods as described in the next section.

2.4 . Antimicrobial assays

The antimicrobial tests were carried out against the Gram-positive bacteria *Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 6538, against the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 22853 and *Escherichia coli* ATCC 11229 as well as against the fungal strain *Candida maltosa* SBUG 700.

The antimicrobial activity of the samples was tested by the agar diffusion assay. The bacterial strains were cultivated on NA agar II medium (Merck, Germany). Ampicillin was used as positive control for Gram-positive bacteria, gentamicin for Gram-negative bacteria and nystatin for fungi. The diameter of zone of inhibition was measured in mm including the disc. Minimal inhibitory concentration (MIC) was determined using broth dilution method with 96-wells microtiter plates.

3. Results and Discussion

3.1. Structure elucidation

The fractionation and purification of the ethyl acetate extract of the fruiting bodies of *Piptoporus betulinus* resulted in the isolation of eleven triterpenoic compounds. The compounds are 3β-acetoxy-16α hydroxyl-24-oxo-5α-lanosta-8-ene-21-oic acid, a not previously described compound (**1**; 2,8 mg), betulin (**2**; 3,9 mg), betulinic acid (**3**; 5,2 mg), polyporenic acid C (**4**; 3,1 mg), polyporenic acid A (**5**; 2,8 mg), ergosterol peroxide (**6**; 3,3 mg), 9,11-dehydroergosterol peroxide (**7**; 4,1 mg), (25S)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**8**; 5,1 mg), fomefficinic acid (**9**; 3,6 mg), (25S)-(+)-12α-hydroxy-3α-malonyloxy-24-methyllanosta-8,24(31)-dien-26-oic acid (**10**; 4,4 mg) and (25S,3´S)-(+)-12α-hydroxy-3α-(3`-hydroxy-3`-methyl glutaryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**11**; 2,6 mg).

The HR-EI-MS of compound **1** showed the *quasi*-molecular ion peak at *m/z* 553.3495 $[M-Na]^+$ ion corresponding to a molecular formula of 1 as $C_{32}H_{50}O_6$ (calc. 553.34996, Δ0.8 ppm) Due to the small available amount of compound **1**, no one-dimensional ^{13}C NMR spectrum with reasonable S/N could be obtained. However, with exception of C-20 and C-21, all ^{13}C chemical shifts could be extracted from the hetero nuclear 2D NMR correlation spectra. According to the molecular formula derived from HR-MS, the two missing positions comprised two carbon, two hydrogen and two oxygen atoms. H-20 (2.346 ppm, ddd, J=11.2,11.2,3.5 Hz) could be identified according to its COSY correlations with H-17 (2.07 ppm), H-22A (2.20 ppm) and H-22B (1.72 ppm). Thus, C-21 has to be a quaternary carbon and part of a carboxylic acid function. The high-frequency shift of H-23A/B (2.54 ppm) and H-25 (2.65 ppm) is caused by the adjacent ketone group at C-24. Due to the HMBC correlation between H-3 and the acetyl carbonyl signal at 172.9 ppm, the hydroxyl group at C-3 is acetylated. The relative configuration of compound **1** was determined via the observed NOE correlations (Tab. 1). Particularly, the β axial oriented methyl group at C-4 (Me-28) could be assigned by its strong NOE with Me-19, whereas the second geminal methyl group at C-4 (Me-29) shows an NOE with H-3. The latter has also an NOE correlation with H-5α, supporting it's α axial position. The β position of H-16 was derived from its NOE with Me-18. The structures of compound **1** and daedaleanic acid, which comes from the fungus *Daedalea dickisii* [4] are quite similar, the only difference is the β-OAc attached to C-3 in case compound **1**, whereas daedaleanic acid shows a 3α-OH group.

The structures of compounds **2** – **11** were identified by comparison with literature. Only compounds **4**, **5**, **10** and **11** have been isolated before from the fungus *Piptoporus betulinus* [3].
Betulin (**2**), lup-20(29)-ene-3β,28-diol, which is found in birch bark can be easily converted to betulinic acid (**3**), a more active compound that is well known for its anti-inflammatory [5,6], antiviral [7] and antineoplastic activities [8,9]. Derivatives of betulin possess hepatoprotective and anti-HIV activity [10], anti-inflammatory and immunomodulatory effects [11-13]. Fomefficinic acid (**9**) was isolated from the fungus *Fomes officinalis.* Its occurrence in *P. betulinus* is described here for the first time [14]. Polyporenic acid C (**4**) exhibits cytotoxic and anti-inflammatory activities [15,16]. Ergosterol peroxide (**6**) has been isolated from a variety of fungi and has been reported to exhibit immunosuppressive, antiviral, and antitumor activities [1]. 9,11-dehydroergosterol peroxide (**7**) was isolated from *Ganoderma lucidum* and showed cytotoxic activity [17]. Compound (**8**) which was isolated by Wangun et al. 2004 exhibited a stronger anti-inflammatory activity than indomethacin [18].

Compounds **1 – 11** were tested for their antibacterial activity. The new compound **1** shows antimicrobial activity against both Gram-positive bacterial strains and weaker activity against the Gram-negative strains (Tab. 2). Compounds **4**, **6**, **7**, **8** and **11** exhibit only weak or very weak antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* and no activity against the Gram-negative strains. The MIC values of compound **1** are 98 µg/ml against *Staphylococcus aureus* and about 200 µg/ml against *Bacillus subtilis.*

Table 1. NMR data of compound **1** (600 MHz, CD_3OD).

Pos.	^{13}C[a] δ[ppm]	^{1}H[b] δ[ppm] m J [Hz]	selected NOE correlations	selected HMBC correlations (H to C)
1	36.4	1.77/1.27		
2	25.1	1.66/1.66		
3	82.3	4.45	H-5α, Me-29	OAc (C=O)
4	38.9	---		
5	52.0	1.157 dd (12.8/2.0)	H-3α, Me-29	
6	19.2	1.72/1.57		
7	27.6	2.10/2.07		
8	136.0	---		
9	135.5	---		
10	38.2	---		
11	21.5	2.05/1.97		
12	30.2	1.80/1.451 dd (13.2/9.0)		
13[c]	47.0	---		
14[c]	49.3	---		
15	43.6	2.18/1.28		
16	77.5	4.111 brdd (8.1/6.4)		
17	57.2	2.07		
18	17.8	0.760 s	H-15β, H-16β, H-20	12, 13, 14, 17
19	19.6	1.019 s	H-1β, H-2β, H-6β, H-11β, Me-28	1, 5, 9, 10
20	n.d.	2.346 ddd (11.2/11.2/3.5)		
21	n.d.	---		
22	27.0	2.20/1.72		
23	39.1	2.54		
24	216.9	---		
25	42.0	2.648 sept (6.7)		
26[d]	18.6	1.069 d (6.9)		24, 25, 27
27[d]	18.6	1.063 d (6.9)		24, 25, 26
28	17.0	0.908 s	H-6β, Me-19	3, 4, 5, 29
29	28.5	0.891 s	H-3α, H-5α, H-6α	3, 4, 5, 28
30	25.5	1.126 s	H-7α, H-12α,, H-15α	8, 13, 14, 15
Oac	21.1/172.9	2.035 s/---		

[a] All ^{13}C chemical shifts are derived from HSQC and HMBC correlation peaks. [b] All ^{1}H chemical shifts with only two decimal places are chemical shifts of HSQC correlation peaks. [c,d] May be interchanged.

Table 2. Antibacterial activity of the isolated substances using agar diffusion test.

Bacterial pathogen	control	Compounds										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Bacillus subtilis	++++	+++	-	-	++	-	+	+	+	-	-	+
Staphylococcus aureus	++++	++	-	+	++	-	-	-	-	-	-	+
Pseudomonas aeruginosa	++++	+	-	-	-	-	-	-	-	-	-	-
Escherichia coli	++++	+		-	-	-	-	-	-	-	-	-
Candida maltosa	++++	-	-	-	-	-	-	-	-	-	-	-

++++ Strong activity (Diameter of inhibition zone: more than 15 mm)
+++ Medium activity (Diameter of inhibition zone: between 8 and 15 mm)
++ Weak activity (less than 8 mm)
+ Very weak (trace of activity)

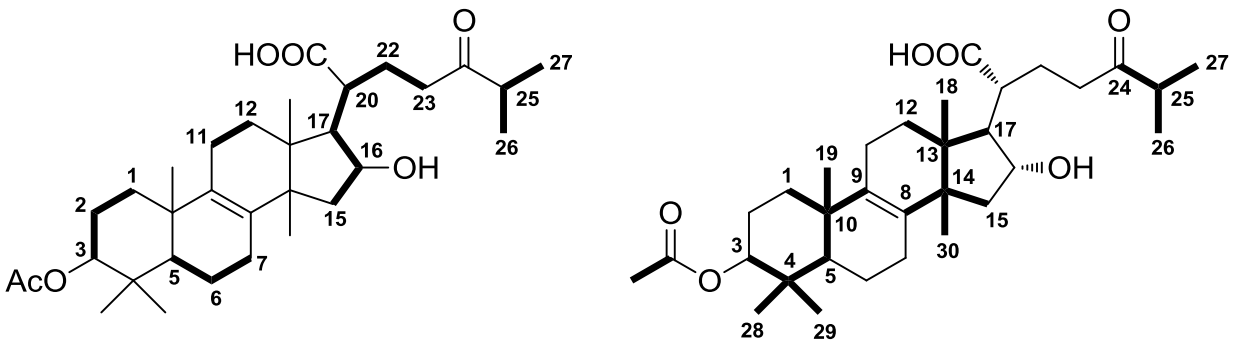

Figure 1. Partial structure (bold lines) established based on H^1, H^1 COSY (left) and H^1, C^{13} HMBC correlation (right).

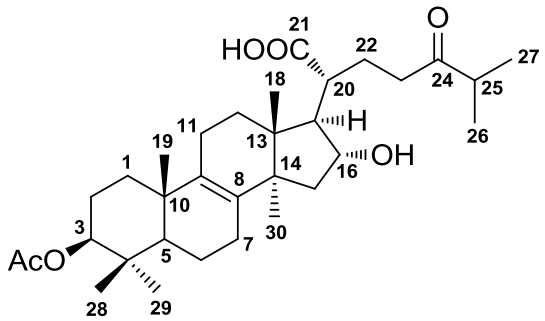

Figure 2. Structure of compound **1:** 3β-acetoxy-16 α hydroxyl-24-oxo-5α-lanosta-8-ene-21-oic acid.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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