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Antibacterial triterpenes from the threatened wood-decay fungus *Fomitopsis rosea*

Milena Popova [a], Boryana Trusheva [a], Melania Gyosheva [b], Iva Tsvetkova [c], Vassya Bankova [a,*]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str. Bl. 9, 1113 Sofia, Bulgaria
[b] Institute of Botany, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 23, 1113 Sofia, Bulgaria
[c] Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

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ABSTRACT

From a biologically active extract from the fungus *Fomitopsis rosea*, two new lanostane triterpenes were isolated, 3α-(3′-butylcarboxyacetoxy)oxepanoquercinic acid C **1** and 3α-hydroxy-24-methylene-23-oxolanost-8-en-26-carboxylic acid **2**, along with three known triterpenes, **3**–**5**, and the epidioxy sterol derivative **6**. The structures of the compounds were elucidated based on their spectral properties. All triterpenes demonstrated antibacterial activity against *S. aureus* but possessed no antiradical activity against DPPH radicals.

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1. Introduction

The wood-decay fungus *Fomitopsis rosea* (Alb. et Schw. Fr.) Karst (Polyporales, Basidiomycota) occurs in the boreal zone of Europe, Asia and North America and in the mountainous areas in continental Europe. It is a characteristic and indicator species in old-growth spruce forests but it has been negatively affected by forestry and is now Red-Listed in many European countries, including Bulgaria [1,2]. To the best of our knowledge, its secondary metabolites have never been studied. It is related to the red-belted polypore *F. pinicola*, a bracket fungus used in folk medicine in different cultures as haemostatics, anti-inflammatory and as a tonic [3]. *F. pinicola* is known to be a rich source of triterpenic compounds [4–6], many of them possessing antimicrobial and antiinflammatory activities [7–9]. In the present paper, we report on the isolation of two novel triterpenes (**1**) and (**2**) along with 4 known compounds from *F. rosea* fruiting bodies.

2. Experimental

2.1. General

Optical rotation: Perkin-Elmer 241. IR: Bruker IFS 113v. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz): Bruker AV 600. EIMS: Hewlett Packard MS 5973 HRESIMS: Micromass LCT TOF.

2.2. Mushroom material

Fruiting bodies of *F. rosea* were collected in October 2006 at Parangalitsa Biosphere Reserve locality, South-West Rila Mts., Bulgaria. The fungi were identified by Dr. Melania Gjosheva.

2.3. Extraction and isolation

The fresh fruiting bodies (338 g) were cut into small pieces, extracted three times with chloroform, and the extract evaporated *in vacuo* to yield 111.5 g dry extract. 39 g of the chloroform extract was subjected to column chromatography on silica gel with mobile phase light petroleum–acetone (10:1–1:1) to give five major fractions A–F. Fraction B (2.7 g)

* Corresponding author. Tel.: +359 2 9606 149; fax: +359 2 8700 225.
E-mail address: bankova@orgchm.bas.bg (V. Bankova).

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eluted with light petroleum–acetone 2:1, was rechromatographed on a silica gel column with mobile phase light petroleum–ethyl acetate (20:1–1:1). Fraction B3 (eluted with mobile phase light petroleum– ethyl acetate 2:1) after final purification by PTLC (silica gel, chloroform–ethyl acetate 1:1) yielded 11.4 mg **1** and 30.3 mg **3**. Fraction B2 eluted with light petroleum–ethyl acetate 5:1 after final purification by PTLC (silica gel, chloroform–ethyl acetate 1:0.6) yielded 12.3 mg **4**.

Fraction C, 1.6 g (eluted with light petroleum–acetone 5:3) was subjected to Lobar column chromatography (Silica gel), eluted with chloroform–methanol (95:5–1:1). Fraction C1 eluted with chloroform–methanol 95:5 was rechromatographed on silica gel (chloroform–methanol–water 60:15:4) and after final purification by PTLC (silica gel, chloroform–methanol–water 60:19:1, three-fold development), 6.2 mg **2** were isolated. Fraction C3 eluted with chloroform–methanol 80:20 was purified by PTLC (silica gel plates Merck, eluent chloroform–methanol–water 60:22:4) to yield 59 mg of **5**.

From fraction B, eluted with light petroleum–acetone 5:2, by preparative TLC (silica gel, light petroleum–ethyl acetate 2:1) 9.7 mg **6** was isolated.

3α-(3′-Butylcarboxyacetoxy)oxepanoquercinic acid C (**1**). Amorphous powder $[\alpha]^{21} - 5.9$ ($c = 0.34$ in:MeOH). IR ($CHCl_3$) cm^{-1}: 3460, 1780, 1747, 1733, 1148. EI MS m/z (%) 644 (M^+., 2), 626 ([M-H_2O]$^+$, 19), 611 ([M-H_2O-CH_3]$^+$, 484 ([M-

Table 1
NMR data of **1** in $CDCl_3$, and **2** in CD_3OD, δ in ppm.

	1			2		
C	δ_H[a]	δ_C[b]	HMBC(H→C)	δ_H[a]	δ_C[b]	HMBC(H→C)
1	1.35 m	30.9		1.37 m	31.8	
	1.44 m			1.51 m		
2	1.63 m	23.1		1.65 m	24.0	
	1.79 m			1.79 m		
3	4.65 br s	79.5	C-1, C-5, C-1′	4.66 br. s	79.7	C-1, C-5
4		36.8			37.8	
5	1.35 m	45.3	C-4, C-6, C-10	1.38 m	46.5	
6	1.37 m	17.8		1.65 m	19.1	
	1.51 m			1.73 m		
7	1.95 m	25.6		2.07 m	27.0	
8		133.8			135.3	
9		135.0			136.0	
10		36.8			38.0	
11	1.69 m	29.4		2.10 m	22.0	
	2.31 m					
12	4.28 t (8.3)	75.8	C-11	1.68 m	32.1	
13		52.7			45.7	
14		49.2			51.1	
15	1.16 m	31.8		1.22 m	31.8	
	1.69 m			1.71 m		
16	1.69 m	23.6		1.68 m	29.3	
				1.94 m		
17	2.58 m	43.0	C-16, C-20	1.69 m	51.8	
18	0.70 s	11.8	C-13, C-14	0.79 s	16.3	C-12, C-13, C-14, C-17
19	0.95 s	18.9	C-1, C-9, C-10, C-5	1.04 s	19.4	C-1, C-5, C-9, C-10
20	2.17 m	28.1		2.08 m	35.0	
21	0.92 d (6.0)	19.7	C-20, C-22	0.89 d (6.6 Hz)	20.2	C-17, C-20, C-22
22	1.69 m	40.7	C-17, C-20, C-23	2.58 dd (10.2, 16.2)	46.0	
	1.87 dd (6.6; 15)			2.72 dd (2.4, 16.2)		
23		110.1			204.5	
24	2.07 dq (6.6; 10.8)	50.6	C-25		152.7	
25	2.17 dq (7.2, 10.8)	42.3	C-24, C-26, C-27	3,53 q (7.2)	44.5	C-24, C-26, C-27
26		177.6			181.2	
27	1.19 d (7.2)	13.7	C-24, C-25, C-26	1.26 d (7.2)	17.4	C-24, C-25, C-26
28	0.86 s	21.8	C-3, C-4, C-29	0.95 s	22.3	C-3, C-4, C-5, C-29
29	0.82 s	27.6	C-5, C-4, C-3 C-28	0.92 s	28.2	C-3, C-4, C-5, C-28
30	0.91 s	24.7	C-8, C-13, C-14, C-15	0.95 s	24.6	C-14, C-15
31	1.04 d (6.6)	13.1	C-23, C-24, C-25	5.83 d (1.2 Hz)	123.4	C-23, C-24, C-25
				6.10 d (1.2 Hz)		
1′		166.3				
2′	3.32 br s	42.1	C-1′, C-3′			
3′		166.9				
1″	4.05 t (6.6)	65.4	C-3′			
2″	1.54 m	30.5	C-1″, C-3″, C-4″			
3″	1.30 m	19.1	C-2″, C-4″			
4″	0.86 t (7.2)	13.7				

[a] ^{1}H NMR, 600 MHz.
[b] ^{13}C NMR, 125 MHz.

160], 8), 469 (32), 451 (100). HRESIMS *m/z*: 667.4118 $[M+Na]^+$, calc. 667.4128 for $C_{38}H_{60}O_8Na$. For ^1H and ^{13}C NMR and HMBC spectral data see Table 1.

3α-Hhydroxy-24-methylene-23-oxolanost-8-en-26-carboxylic acid (**2**) Colorless crystals MP 193–197 °C. $[\alpha]^{21}0$ ($c = 0.25$ in:MeOH). IR (KBr) cm^{-1}:3420, 1723, 1706, 1604, 1162. EI MS *m/z* (%) 484 ($M^{+\cdot}$, 7), 469 ($[M\text{-}CH_3]^+$, 25), 466 ($[M\text{-}H_2O\text{-}CH_3]^+$, 18), 69 (100). HRESIMS *m/z*: 507.3442 $[M+Na]^+$, calc. 507.3446 for $C_{31}H_{48}O_4Na$. For ^1H and ^{13}C NMR and HMBC spectral data see Table 1.

2.4. Antimicrobial tests

For the investigation of the antibacterial activity, the agar cup method [10] was used with test strains *Staphylococcus aureus* 209, *Escherichia coli* WF+ and *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, institute for State Control of Drugs, Sofia). An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the cup). 0.1 ml of test solution containing 0.4 mg of each substance in ethanol was applied to every cup (concentration of the test solution 4 mg/ml). Control experiments with solvent showed that it does not have any activity. Streptomycin and nystatin were used as positive controls.

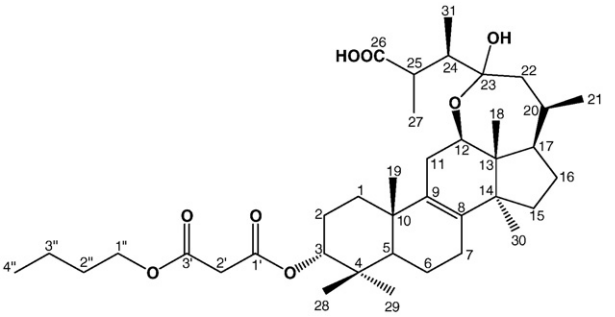
2.5 DPPH free radical scavenging activity

DPPH free radical scavenging activity was measured according to the procedure described in [11]. The decrease of the absorption at 516 nm of the DPPH· solution after addition of the tested solution was measured. An aliquot (2960 µl) of 0.1 mM ethanolic DPPH· solution was mixed with 40 µl of a 0.65 mg/ml solution of the extract and of a 3.5 mM solution of the individual substances. The radical scavenging activity was expressed as percentage decrease with respect to control values. Caffeic acid was used as positive control.

3. Results and discussion

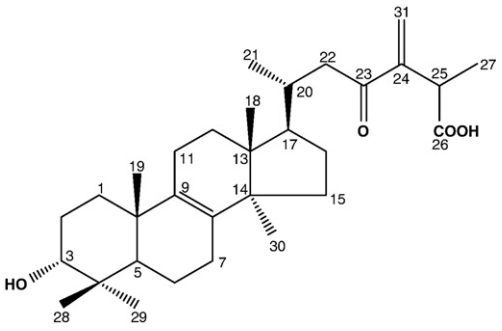
The chloroform extract of the fresh fruiting bodies of *F. rosea* was fractionated by silica gel column chromatography and some of the resulting fractions were purified by further column chromatography and preparative TLC to afford the triterpenic compounds **1**–**5** and the sterol derivative **6**.

Compound **1** possessed a molecular formula $C_{38}H_{60}O_8$ as deduced from the HRESIMS which was in agreement with the ^{13}C NMR spectra (Table 1). The latter disclosed 38 carbon signals: 9 CH_3 groups, 12 CH_2, 7 CH and 10 quaternary C atoms. The ^1H and ^{13}C NMR spectra of **1** were similar to those of 3α-oxepanoquercinic acid C **3** (12β,23-epoxy-3α,23-dihydroxy-24-methylen-23-lanost-8-en-26-carboxylic acid), isolated from another wood-rotting fungus, *Dedalea quercina* [4]. There were seven more signals in the ^{13}C NMR spectrum of **1**, compared with that of **3**. Three of them could be attributed to a malonate moiety, where both carboxyl groups were esterified (δ_C 166.3, 166.9). Of the remaining four additional signals, three were CH_2 groups, one of them adjacent to an oxygen atom (δ_C 65.4), and one CH_3 group, leading to the suggestion that the compound is a monobutyl ester of the malonic acid. Long range ^1H–^{13}C couplings (HMBC) confirmed the position of the butyl group to be at one of the carboxyl groups of the malonate moiety. The position of the monobutylmalonate moiety was determined to be 3, based on long range coupling between H-3 and one of the carboxyl carbons of the malonate in the HMBC spectrum (Table 1). The relative stereochemistry is analogous to oxepanoquercinic acid C as given by Röesecke and König [4] and Quang et al. [12], according to the NMR data. Thus, **1** was identified as a 3′-butyl ester of 3α-carboxyacetoxyoxepanoquercinic acid **C**, a new natural compound. To the best of our knowledge, it is the first butyl ester of a lanostane derivative, isolated from fungi.



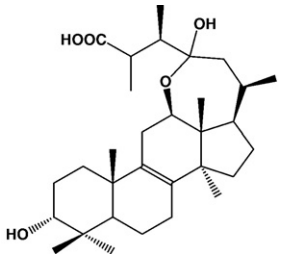
1)

For compound **2**, a molecular formula $C_{31}H_{48}O_4$ was deduced from the HRESIMS. Its ^{13}C NMR spectrum showed four olefinic carbons (δ_C 123.4, 135.3, 136.0, 152.7) one acid (181.2) and one ketone carbon signal. The chemical shift of the ketone at δ_C 204.5 and the olefinic methylene group (δ_C 123.4), together with the observed long-range correlations indicated a lanost-8-ene structure with a 23-oxo-24-methylene functionalized side chain and an acid function in position 26 which is common for previously identified triterpenic acids from wood-rotting fungi [4]. Analysis of the NMR spectra (DEPT, HMBC, HMQC) and their comparison with the data of other isolated fungal lanostane derivatives [14] indicated the structure 3α-hydroxy-24-methylene-23-oxolanost-8-en-26-carboxylic acid [24(31)-dehydroquercinic acid]. Stereochemistry in position 3 was proved to be α, according to the typical shifts for carbons of the A ring [8]. The acid **2** is a new natural product, its malonate half-ester at position 3 has previously been isolated from wood-rotting fungi *Ganoderma* (unidentified species) [15] and *Daedaleopsis confragosa* var. *tricolor* [3].

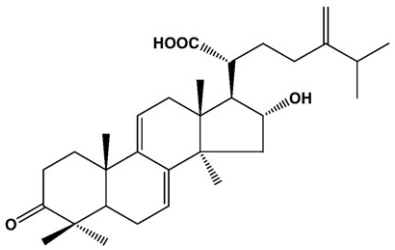


2)

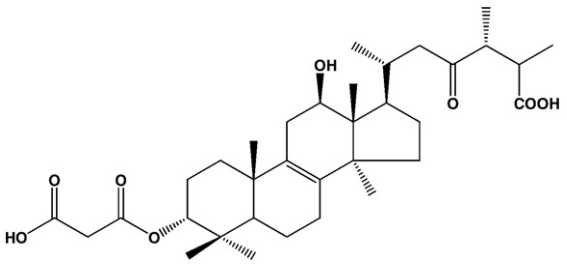
Compounds **3** 3α-oxepanoquercinic acid C [4], **4** polyporenic acid C [14,16], **5** 3α-carboxyacetoxyquercinic acid C [3] and **6** 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol [13] were identified by comparison of their spectral data (MS, ^{1}H and ^{13}C NMR) with literature data.



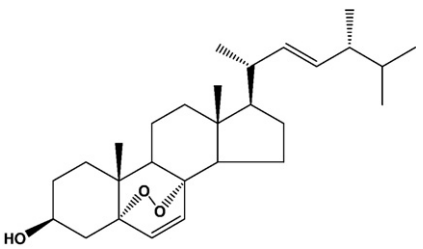
3)



4)



5)



6)

The chloroform extract and the isolated triterpenes were tested for their action against *S. aureus* and *C. albicans* (Table 2.). Neither the extract nor the isolated compounds suppressed the growth of *E. coli.* Only the extract showed mild activity against *Candida*. The chloroform extract and all the tested triterpenes suppressed *S. aureus* and the extract demonstrated the highest inhibitory zone. Compound **2** was the most active individual component, followed by **5**. Interestingly, both compounds lack the oxepane ring and possess a carboxyl group in position 26, which might be of importance for their antibacterial action.

Table 2
Antibacterial activity of chloroform extract and isolated compounds of *F. rosea* (at 400 μg in the cup).

Substance	Zone of inhibition (mm)	
	S. aureus	*C. albicans*
Chloroform extract	25 ± 1	15 ± 1
1	17 ± 1	0
2	24 ± 0	0
3	19 ± 1	0
4	14 ± 0	0
5	20 ± 0	0
Streptomycin [a]	28 ± 1	–
Nystatin [b]	–	31 ± 1

Tests were done in triplicate, values are mean ± S.D.
[a] 100 μg in the cup.
[b] 50 I.U.

There are communications claiming antioxidant activity of extracts of mushrooms known to contain significant amounts of lanostane triterpenes [17] and for this reason the chloroform extract of *F. rosea* and compounds **1**–**5** were tested also for their ability to scavenge DPPH radicals. No significant antiradical activity was registered. These results are consistent with the data recently published by Zhou et al. [18], reporting the lack of antiradical activity against DPPH in lanostanes isolated from *Poria cocos*.

It is interesting to note that the triterpenes found in *F. rosea* are similar to the structures isolated from *Daedalea quercina*, rather than to those of *F. pinicola.* All the identified lanostanes of *F. rosea* have an additional C atom at position 24, which is lacking in most of the metabolites found in *F. pinicola.* [3,7]. The only common constituent of *F. rosea* and *F. pinicola* so far is polyporenic acid **C 4**, which was isolated also from *D. quercina*. These facts could be regarded as a confirmation of the close relationship between the two genera *Fomitopsis* and *Daedalea* [19].

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