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Anti-inflammatory and Anti-hyaluronate Lyase Activities of Lanostanoids from *Piptoporus betulinus*

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The isolation and full characterisation of 3α-acetylpolyporenic acid A (**1**) and (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**2**) together with the known lanostanoid **3** and polyporenic acid C (**4**) are reported. While **1**～**4** show only weak cyclooxygenase I inhibition activity, they prove to be promising anti-inflammatory agents by inhibiting 3α-hydroxysteroid dehydrogenase. **1**～**4** also possess a remarkable selective activity towards bacterial hyaluronate lyase while not affecting bovine hyaluronidase.

Anti-inflammatory compounds can interfere with many steps of pathophysiological processes. For example, a compound might block the biosynthesis of pro-inflammatory mediators by direct interaction with a key enzyme (*e.g.* inhibition of 3α-hydroxysteroid dehydrogenase and cyclooxygenase I) or by decreasing enzyme expression (*e.g.* steroidal anti-inflammatory compounds) or reducing substrate levels (*e.g.* decreased release of arachidonic acid). An anti-inflammatory compounds may also act by immunostimulation (*e.g* maturation of myeloid cells, stimulated phagocytosis) promoting an increased removal of signal molecules, which results in a less aggressive inflammatory response to allergens[1].

It is well known that certain anti-inflammatory drugs, such as salicylates[2] and indomethacin[3] possess a hyaluronidase inhibitory activity. These drugs may exert a portion of their inflammatory activity by preventing the generation of small hyaluronic acid fragments. Various antiallergenic drugs function as hyaluronidase inhibitors, such as disodium cromoglycate and tranilast[4,5]. In this communication we report on the isolation, structural elucidation, anti-inflammatory and anti-hyaluronate lyase activities of lanostanoids from the fungus *Piptoporus betulinus*.

Fruiting bodies of *Piptoporus betulinus* were collected in a forest district near Jena (Thuringia, Germany) and deposited to the strain collection of the Hans-Knöll-Institute for Natural Products Research, Jena, Germany. One kilogram of fresh fruiting bodies was cut in small species, dried and crushed. The resulting powder was extracted three times with ethyl acetate, chloroform and methanol (3×2 lites, 3 days each). The ethyl acetate extract (3 g of a brown solid), which exhibited strong anti-inflammatory activities, was subjected to silica gel chromatography (silica gel 60, Merck, 0.063～0.1 mm, column 4×60 cm), using a gradient of $CHCl_3$ - MeOH (v/v=9 : 1, 8 : 2, 1 : 1) as eluent. Bioassay-guided fractionation using a 3α-hydroxysteroidehydroxygenase (3α-HSD)-assay[6] and monitoring by mass spectrometry led to the detection of four active substances with *m*/*z*=529, 587, 645 and 483 in the positive mode.

Final purification of the compounds (yield: 8 mg of **1**, 15 mg of **2**, 40 mg of **3** and 30 mg of **4**) was achieved by preparative HPLC (Spherisorb ODS-2 RP_{18}, 5 μ (Promochem), 250×25 mm, acetonitrile/H_2O, v/v=83 : 17; flow rate: 10 ml/minute, UV-detection at 210 nm).

Compound **1** was obtained as a colourless oil ($[\alpha]_D^{22}$ +30°; MeOH, *c*=0.18). Its molecular formula ($C_{33}H_{52}O_5$), determined by HR-EIMS *m*/*z* 551.3701 $(M+Na)^+$, is consistent with ^1H and ^{13}C-NMR data (Table 1). The ^1H-NMR spectrum of **1** shows 50 non-exchangeable protons, including two olefinic protons and seven methyl groups. Analyses of the ^{13}C-NMR, DEPT 135 and HMQC spectra of **1** indicate the presence of eight methyl carbons, ten methylene carbons (one of which is sp^2 hybridized), six methine carbons (two of which are oxygenated), seven quaternary carbons (three of which are sp^2 hybridized), a carbonyl carbon and a carboxyl carbon δ 171.0 and δ 178.7 respectively. The occurrence of a carbonyl was confirmed by the IR spectrum, which shows strong absorptions at

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Table 1. ^{1}H and ^{13}C NMR data of **1** and **2** (500 MHz, in $CDCl_3$, chemical shifts in ppm, coupling constants in Hz, TMS as internal standard).

	1		**2**	
Position	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H
1	30.5 (t)	1.49 (m)	30.4 (t)	1.50 (m)
2	23.2 (t)	1.61 (m); 1.69 (m)	23.1 (t)	1.62 (m); 1.90 (m)
3	78.0 (d)	4.65 (brs)	79.7 (d)	4.70 (brs)
4	36.7 (s)	-	36.8 (s)	-
5	45.3 (d)	1.49 (m); 1.61 (m)	45.4 (d)	1.44 (m)
6	17.9 (t)	1.49 (m)	17.9 (t)	1.44 (m); 1.60 (m)
7	25.9 (t)	2.02 (m)	25.9 (t)	2.01 (m)
8	134.7 (s)	-	134.8 (s)	-
9	132.9 (s)	-	132.8 (s)	-
10	36.7 (s)	-	36.6 (s)	-
11	32.5 (t)	2.09 (m); 2.63 (m)	32.5 (t)	2.10 (m); 2.60 (m)
12	73.4 (d)	4.00 (d, J = 8.12 Hz)	73.4 (d)	4.00 (d , J= 8.0 Hz)
13	49.5 (s)	-	49.5 (s)	-
14	49.6 (s)	-	49.6 (s)	-
15	31.9 (t)	1.19 (m); 1.67 (m)	31.9 (t)	1.19 (m); 1.65 (m)
16	27.7 (t)	1.35 (m); 2.10 (m)	27.7 (t)	1.37 (m); 1.65 (m)
17	43.1 (d)	2.02 (m)	43.1 (d)	2.01 (m)
18	16.1 (q)	0.60 (s)	16.1 (q)	0.60 (s)
19	18.7 (q)	0.96 (s)	18.7 (q)	0.97 (s)
20	35.9 (d)	1.40 (m)	35.9 (d)	1.40 (m)
21	17.8 (q)	1.00 (d, J = 6.5 Hz)	17.8 (q)	1.00 (d, J = 6.5 Hz)
22	34.2 (t)	1.24 (m); 1.65 (m)	34.2 (t)	1.21 (m); 1.65 (m)
23	31.8 (t)	2.02 (m); 2.21 (m)	31.8 (t)	2.01 (m); 2.20 (m)
24	148.1 (s)	-	148.1 (s)	-
25	45.1 (d)	3.16 (q, J = 7.0 Hz)	45.1 (d)	3.15 (q , J = 7.0 Hz)
26	178.7 (s)	-	179.0 (s)	-
27	16.2 (q)	1.30 (d, J = 7.0 Hz)	16.2 (q)	1.32 (d, J = 7.0 Hz)
28	21.7 (q)	0.85 (s)	21.6 (q)	0.92 (s)
29	27.5 (q)	0.90 (s)	27.5 (q)	0.86 (s)
30	24.5 (q)	1.00 (s)	24.4 (q)	1.07 (s)
31	111.0 (t)	4.93 (brs);4.97(brs)	111.4 (t)	4.93 (brs);4.97(brs)
1'	171.0 (s)	-	166.0 (s)	-
2'	21.3 (q)	2.06 (s)	41.7 (t)	3.38 (s)
3'			167.2 (s)	-
4'			52.3 (q)	3.70 (s)

Abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, brs: broad, m: multiplet.

1708 cm^{-1}. ^{1}H-^{1}H COSY spectra helped identifying the coupling systems H15/H16/H17/H20; H21/H20/H22/H23/H31, H6/H7, and H1/H2/H3. By means of C,H long range coupled NMR spectra (HMBC) all connectivities of protons and carbons were fully assigned. The correlation of H31 (δ 4.93, 4.97) with C24 (δ 148.1) and C23 (δ 31.8), the correlation of H25 (δ 3.16) with C26 (δ 178.7), C27 (δ 16.2), C23 (δ 31.8), C24 and C31 (δ 111.4) and the correlation of H27 (δ 1.30) with C24 (δ 148.1), C25 (δ 45.1) and C26 unequivocally revealed the connection of the carboxyl carbon in C25.

The observed HMBC correlations of H12 δ 4.00 with C9

(δ 132.9), C11 (δ 32.5), C13 (δ 49.6) and C14 (δ 49.6) helped identifying the position of the hydroxymethine C12HOH. The C,H long range HMBC showed also the correlation of H3 at δ 4.65 and H2′ at δ 2.06 with the carbonyl at δ 171.0. This unambiguously indicated that the carbon C3 is connected to an acetate group. Consequently, compound **1** was identified as 3α-acetylpolyporenic acid A. Alkaline hydrolysis of **1** provided the known polyporenic acid A, which indicates that **1** possesses the same absolute configuration[7].

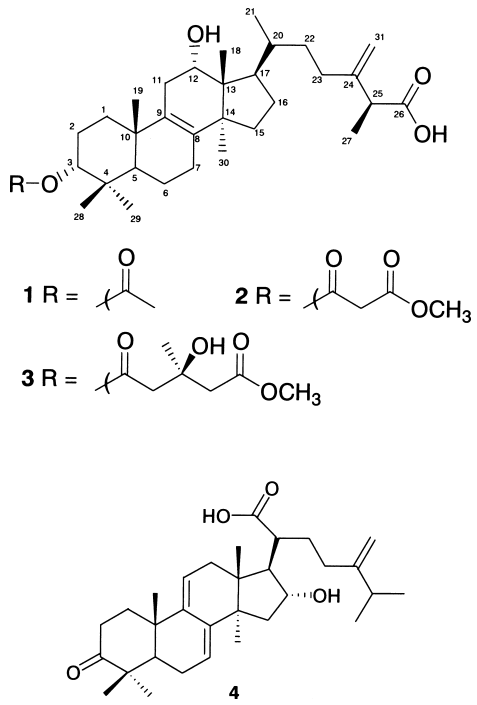
^{1}H and ^{13}C NMR spectra of **2**, which was obtained as a colourless oil ($[\alpha]_D^{22}$ +19.2°; MeOH, c=0.50), are almost identical with those obtained from **1**. However, it appeared that the C3 of **2** was substituted by a functional other than acetate group. The ^{1}H NMR spectrum of **2** indicates the presence of two additional singlets (2H) at δ 3.39 and (3H) at δ 3.70. In the ^{13}C NMR spectrum additional signals are observed at δ 41.7 (CH_2), 52.3 (CH_3O), 166.0 (C=O) and 167.2 (C=O). C,H long range HMBC data helped elucidating the correlation of the protons of the additional methylene with the two additional carbonyls, and the correlation of the protons of the additional methoxy moiety with the carbonyl at δ 167.2. Thus, the substituent at C3 could be identified as methyl malonate ($-CO_2CH_2CO_2CH_3$), which is supported by IR (strong absorption at 1722 cm^{-1}) and MS data (pseudo molecular ion peak at m/z 609.3756 $[M+Na]^+$). Consequently, compound **2** was identified as (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid.

To our knowledge this is the first isolation and full characterization of **1** and **2**. Yet, it is important to note that methyl ester derivatives of **1** and **2** have been previously isolated from the methylated extract of *Piptoporus betulinus*[8].

All physicochemical and spectrometric data obtained for compounds **3** and **4** proved to be identical with those reported for (25*S*,3′*S*)-(+)-12α-hydroxy-3α-(3′-hydroxy-4′methoxycarbonyl-3′-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid and polyporenic acid C, which have been reported to suppress 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced edema of mouse ears[9].

While compounds **1**~**4** exhibited only weak inhibition activity of cyclooxygenase I, they proved to be promising

Fig. 1. Structures of **1**~**4**.



anti-inflammatory agents in our 3α-hydroxysteroiddehydrogenase (3α-HSD)-assay according to the method of PENNING[6]. Most remarkably, **1**, **3**, and **4** inhibit 3α-HSD at a level comparable with that of the indomethacin reference, and **2** even exhibits a stronger activity than that of indomethacin (Table 2).

Anti-hyaluronate lyase activities were measured spectrophotometrically based on the precipitation of undigested hyaluronan by *N*-cetyl-*N*-trimethylammonium-bromide and the measurement as turbidity (E_{600nm}) using the method described by DI FERRANTE *et al.*[10] Although **1**~**4** showed no activity against the bovine Hyaluronidase, they are potent inhibitors of *a* hyaluronate lyase from *Streptococcus agalactiae* (Table 3). This enzyme acts as surface antigen, which is responsible for the degradation of hyaluronan (hyaluronic acid), the most abundant matrix of connective tissues, predominantly into disaccharide units. The degradation of hyaluronan facilitates the invasion of

Table 2. Inhibitory activity of **1**~**4** against 3α-hydroxysteroid dehydrogenase.

	1	**2**	**3**	**4**	Indomethacin
IC_{50} (μg/ml)	8.5	4.0	5.5	17.5	6.5

Table 3. Inhibitory activity of **1**～**4** against *Streptococcus agalactiae* hyaluronate lyase.

	1	**2**	**3**	**4**
IC_{50} (μM)	40.0	3.5	51.0	12.5

bacterial pathogens, mostly Gram-positive *Streptococci* into the host tissues.[11~13] To date only saccharic acid is known to selectively inhibit bacterial hyaluronate lyase, while not affecting the enzymatic activity of testicular hyaluronidase[14]. Thus, **1**～**4** appear to be a new class of compounds possessing a similar selectivity. Strikingly, the methylated derivatives of **3** and **4** exhibited no inhibition towards the bacterial hyaluronate lyase, bovine hyaluronidase, and 3α-HSD indicating that the carboxyl moieties of **1**～**4** are essential for activity.

In conclusion, we have isolated and fully characterized 3α-acetylpolyporenic acid A (**1**) and (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**2**) together with the known lanostanoid **3** and polyporenic acid C (**4**) from *Piptoporus betulinus*. While **1**～**4** show only weak cyclooxygenase I inhibition activities, they prove to be promising anti-inflammatory agents by strongly inhibiting 3α-hydroxysteroid dehydrogenase. Strikingly, lanostanoids **1**～**4** selectively inhibit bacterial hyaluronate lyase while not affecting bovine hyaluronidase. Further structure-activity studies are in progress.

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