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BRASSINOSTEROIDS, STEROLS AND LUP-20(29)-EN-2α,3β,28-TRIOL FROM *RHEUM RHABARBARUM*

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Key Word Index—*Rheum rhabarbarum*; Polygonaceae; panicles; brassinosteroids; phytosterols; triterpenoids; lup-20(29)-en-2α,3β,28-triol.

Abstract—The new pentacyclic triterpene lup-20(29)-en-2α,3β,28-triol and its 3,28-dipalmitoyl ester were identified from panicles of *Rheum rhabarbarum* on the basis of their spectral data (^{1}H NMR, ^{13}C NMR, MS, IR). Besides the brassinosteroids brassinolide, castasterone and 24-epicastasterone, the phytosterols campesterol, stigmasterol, sitosterol and isofucosterol were identified from the same plant material.

INTRODUCTION

Brassinosteroids represent a class of naturally occurring phytohormones with high plant growth regulating and anti-stress activity [1–3]. In a continuation of our work on the distribution of brassinosteroids, we have investigated the panicles of *Rheum rhabarbarum* L. (rhubarb), widely used as a cultivated plant. In the course of these studies, the known members brassinolide, castasterone and 24-epicasterone were identified. As potential biogenetic precursors the phytosterol pattern was determined [4]. Furthermore, the new pentacyclic triterpene lup-20(29)-en-2α,3β,28-triol and its 3,28-dipalmitoyl ester were isolated and their structures elucidated by spectral data.

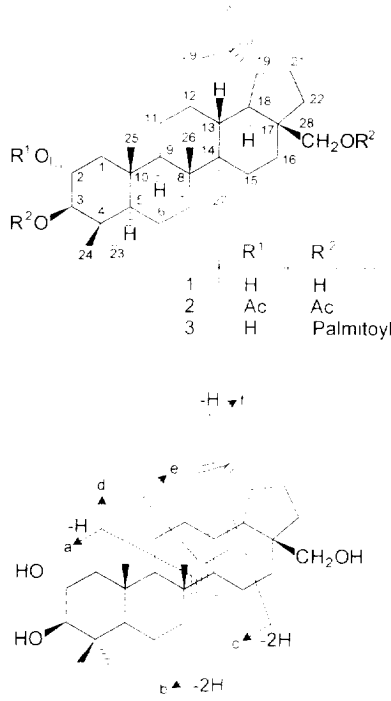
RESULTS AND DISCUSSION

Panicles of *R. rhabarbarum* were extracted with methanol and the extracts concentrated *in vacuo*. The aqueous residue was extracted with chloroform and the chloroform extract partitioned between *n*-hexane and 80% methanol. The aqueous methanol fraction was subjected to a silica gel column eluted stepwise with increasing concentrations of methanol in chloroform. The fractions eluted with 4 and 5% methanol displayed bioactivity in the rice lamina inclination test. Further purification was carried out by LH-20 Sephadex chromatography, DEA chromatography and preparative reversed-phase HPLC. The bioactive HPLC-fractions were combined and analysed by GC-MS after methaneboronation.

Brassinolide (fractions 21–22, M^+ *m/z* 528), castasterone (fractions 25–28, M^+ *m/z* 512) and 24-epicastasterone (fractions 27–28, M^+ *m/z* 512) were identified by their GC-MS data in comparison with authentic samples. The estimated ratio of castasterone/24-epicastasterone was *ca* 10:1. The co-occurrence of castasterone and its 24-epimer was recently described by us for seeds of *Ornithopus sativus* [5] as well as *Beta vulgaris* [6]. These findings suggest that 24-epicastasterone, first detected as a native brassinosteroid only in the green alga *Hydrodictyon reticulatum* [7], is distributed fairly widely in higher plants.

A portion of the residue of the *n*-hexane phase was saponified and the unsaponified lipids were extracted with chloroform. The residue of the chloroform layer was chromatographed on a silica gel column using a *n*-hexane–ethylacetate gradient system. Campesterol (5.4%), stigmasterol (3.9%), sitosterol (87.7%) and isofucosterol (3.0%) were identified as their acetates by capillary GC and GC-MS.

Repeated silica gel column chromatography of the saponified *n*-hexane layer led to the isolation of the new pentacyclic triterpene **1**. The molecular formula of compound **1** was assigned to be $C_{30}H_{50}O_3$ by HR-EIMS (M^+, *m/z* 458.3769). The IR spectrum shows absorptions assignable to hydroxyl and methylene functions. The mass spectral fragmentation of **1** is mainly characterized by bond cleavages in ring C leading to the key ions of type **a** (*m/z* 223), **b** (263), **c** (304), **d** (234), **e** (220) and **f** (207) (Scheme 1) [8]. Therefore, two hydroxy groups are located in rings AB and one in rings DE, respectively. The prominent ion at *m/z* 427 $[M - CH_2OH]^+$ is evidence for the presence of a primary hydroxy function. The ^{1}H NMR spectrum of the triterpenoid **1** shows 10 non-overlapped signals in the high field region between δ2.4 and 4.7 and many overlapped signals below δ2 with seven methyl group singlets (Table 1). Three of the non-overlapped signals disappear in the presence of CD_3OD and represent hydroxy protons (δ3.34, 3.43, 3.52). The ^{13}C NMR spectrum of **1** shows two signals in the down field region (δ108.8, 150.0) (Table 2). Heteronuclear shift correlation via ^1J (C, H) (HETCOR) indicates the con-



Scheme 1. Mass spectral fragmentation of the triterpene **1**.

nectivity of the proton signals at δ4.57 and 4.68 with the carbon signal at δ108.8. A complete assignment of all proton signals to the carbon signals was carried out by a HETCOR experiment and by the homonuclear 2D H,H-COSY spectrum (especially couplings to the hydroxy protons); the remaining above mentioned non-overlapped protons are readily assigned to one hydroxymethylene group (δ3.29, *t* and δ3.75, *t*, J = 11 Hz) and two hydroxymethine groups (δ2.92, *dd*, J = 10 Hz; δ3.63, *m*, J = 4 Hz). The proton–proton coupling network was analysed by the H,H-COSY spectrum. The connectivities of the rings of the triterpenoid skeleton and the position of the hydroxy groups as well as of the side chains were detected by the H,H-delayed COSY 45 technique [9] and by heteronuclear multiple bond connectivity (HMBC) experiments [10]. On considering the two- and three-bond correlations of the methyl proton signals with the corresponding carbon signals for example, it is possible to assign both the methyl proton and the carbon signals of the triterpenoid skeleton without additional information. A complete assignment of all carbon and proton signals (the partially overlapped included) is thus possible (Tables 1 and 2). Finally, the configuration of the hydroxy and methyl groups had to be determined. This was carried out by analysing the

Table 1. ^{1}H NMR data of compounds **1**–**3** ($CDCl_3$, TMS as int. standard)

H	**1**	**2**	**3**	Palmitic acid moieties of **3**
1α	0.83 *m*	1.00 *m*	0.85 *m*	C-3:H-2′:2.32 (2H, *d*)
1β	2.01 *dd*	2.07 *dd*	2.11 *dd*	H-3′:1.61 (2H, *m*)
2β	3.63 *m*	5.08 *m*	3.77 *m*	(CH_2):1.2–1.3 *m*
3α	2.92 *dd*	4.71 *d*	4.48 *d*	CH_3:0.87 (3H, *s*)
5α	0.82 *m*	0.92 *m*	0.78 *m*	C-28:H-2″:2.39 (2H, *d*)
6α, β	1.42/1.54 (2H, *m*)	1.42/1.52 (2H, *m*)	1.44/1.54 (2H, *m*)	H-3″:1.63 (2H, *m*)
7α, β	1.38/1.43 (2H, *m*)	1.38/1.43 (2H, *m*)	1.3–1.4 *m*	(CH_2):1.2–1.3 *m*
9α	1.36 *m*	1.34 *m*	1.3–1.4 *m*	CH_3:0.88 (3H, *s*)
11α, β	1.28/1.43 (2H, *m*)	1.25/1.41 (2H, *m*)	1.2–1.4 *m*	
12α, β	1.09/1.65 (2H, *m*)	1.12/1.66 (2H, *m*)	1.10/1.65 (2H, *m*)	
13β	1.66 *m*	1.67 *m*	1.67 *m*	
15α	1.06 *m*	1.04 *m*	1.07 *m*	
15β	1.75 *ddd*	1.68 *m*	1.71 *m*	
16α	1.19 *ddd*	1.24 *m*	1.22 *m*	
16β	1.97 *m*	1.83 *dm*	1.83 *dm*	
18α	1.58 *m*	1.59 *m*	1.60 *m*	
19β	2.41 *m*	2.43 *m*	2.45 *m*	
21α	1.39 *m*	1.42 *m*	1.42 *m*	
21β	1.98 *m*	1.97 *m*	2.00 *m*	
22α	1.03 *m*	1.09 *m*	1.08 *m*	
22β	1.90 *m*	1.76 *dd*	1.77 *dd*	
CH_3-23	1.01 (3H, *s*)	0.87 (3H, *s*)	0.88 (3H, *s*)	
CH_3-24	0.79 (3H, *s*)	0.88 (3H, *s*)	0.86 (3H, *s*)	
CH_3-25	0.91 (3H, *s*)	0.96 (3H, *s*)	0.90 (3H, *s*)	
CH_3-26	1.05 (3H, *s*)	1.02 (3H, *s*)	1.03 (3H, *s*)	
CH_3-27	1.00 (3H, *s*)	0.95 (3H, *s*)	0.98 (3H, *s*)	
CH_2-28	3.29 *d*/3.75 *d*	3.83 *d*/4.24 *d*	3.83 *d*/4.27 *d*	
29 (E)	4.57 *d*	4.57 *d*	4.59 *d*	
29 (Z)	4.68 *m*	4.68 *m*	4.69 *m*	
CH_3-30	1.68 (3H, *s*)	1.66 (3H, *s*)	1.68 (3H, *s*)	
		1.95 (3H, *s*)/2.04 (3H, *s*)/2.06 (3H, *s*)		

NOE's using ROESY measurements [11]. Thus, we found strong NOE's between H-2β and CH_3-25, H-3α and CH_3-23, CH_3-25 and CH_3-26, H-13β and H-19β as well as between CH_2-28 and H-13β/H-15β. All the above data can be accomodated only in a lup-20(29)-en-2α,3β,28-triol structure for compound **1**.

Acetylation of triterpene **1** yielded a triacetate as indicated by a molecular ion at *m/z* 584 in the MS of compound **2**. The ^{1}H NMR spectrum of **2** confirmed the suspected positions of the hydroxy groups. A comparison of the ^{1}H NMR data of compound **1** with those of **2** clearly established that the hydroxy groups are located in position 2α, 3β and 28 (Table 1). This was in agreement with the observed deshielding effects on the H-2β, H-3α and H-28 protons.

Repeated silica column chromatography of the residue of the *n*-hexane layer led to the isolation of compound **3** (MS, M^+ at *m/z* 934). The positions of the two palmitic acid moieties were deduced from the ^{1}H and ^{13}C NMR data (Tables 1 and 2). On considering the three-bond correlations of the proton signals H-3β and H-28 with the corresponding carbonyl carbon signals of the palmitic acid moieties, the determination of the positions of the palmitic acid units was achieved. This was in agreement with the deshielding effects on the H-3β and H-28 protons compared with those of compound **1**. Alkaline hydrolysis of **3** led to the triterpene lup-20(29)-en-2α,3β,28-triol (**1**). Therefore, the triterpenoid ester **3** was assigned to be 3,28-dipalmitoyl lup-20(29)-en-2α,3β,28-triol. The hitherto only known lupane type triterpene with a 2α,3β-dihydroxy structural feature is lup-20(29)-en-2α,3β,diol isolated from the bark of *Pterocarpus santalinus* [12] and from buds of *Rhododendron macrocepalum* [13].

Table 2. ^{13}C chemical shifts of compounds ($CDCl_3$, solvent as int. standard, δ77.0 ppm)

C	1	2	3
1	46.2	44.3	48.1
2	68.2	70.3	67.8
3	82.8	80.7	84.7
4	37.8	39.4	39.3
5	54.9	55.1	55.3
6	17.8	18.2	18.3
7	33.6	34.1	34.0
8	38.8	41.0	41.0
9	49.9	50.4	50.3
10	37.8	38.4	38.4
11	20.4	21.0	20.9
12	24.7	25.1	25.1
13	36.8	37.6	37.5
14	40.4	42.8	42.8
15	26.4	27.1	27.0
16	29.2	29.8	29.3
17	42.2	46.4	46.4
18	48.3	48.8	48.8
19	47.5	47.7	47.7
20	150.0	150.1	150.0
21	28.7	29.6	29.6
22	33.4	34.6	34.6
23	27.7	28.4	28.4
24	16.5	17.5	17.2
25	15.8	17.2	17.5
26	15.2	16.1	16.1
27	14.0	14.8	14.7
28	58.9	62.8	62.5
29	108.8	110.0	109.9
30	18.2	19.2	19.1
CO	—	170.5/170.9	174.3 175.2
CH_3-Ac	—	21.1/21.2	
C-2′/C-2″	—	—	34.5 34.7
$-CH_2-$			29–30
CH_3	—		14.1

EXPERIMENTAL

General. Mps: uncorr.; NMR (Varian Unity 500): 499.85 MHz (^{1}H) and 125.7 MHz (^{13}C), TMS as int. standard; MS (AMD 402, AMD Intectra GmbH): 70 eV EIMS (probe, DIS), HR-EIMS (resolution 7500); IR (Bruker IFS 28): KBr discs; CC: silica gel: Merck 60, 0.063–0.2 mm; GC-MS (MD-800, Fisons Instruments): EI (70 eV), source temp. 200°, column DB-5MS (J&W, 15 m × 0.32 mm, 0.25 μm film thickness), inj. temp. 260°, interface temp. 300°, carrier gas He, flow rate 1 ml min^{-1}, splitless injection; column temp. program (brassinosteroids): 170° for 1 min, then raised to 290° at a rate of 30 min^{-1} and held on this temperature for 20 min.

Plant material. The panicles of *Rheum rhabarbarum* L. collected in May 1993 were identified by Dr Peter Hanelt (Institute of Plant Genetics and Cultivated Plants, Gatersleben, Germany).

Bioassay for brassinosteroids. The rice lamina inclination test was carried out using the cultivar 'Koshihikari' as described previously [14].

Extraction and isolation. Panicles of *R. rhabarbarum* (805 g fr. wt) were dried and powdered. The dried material (141 g) was extracted (×3) with MeOH. The combined MeOH extracts were evapd to dryness *in vacuo*. The residue was partitioned (×3) between H_2O and $CHCl_3$. The $CHCl_3$ phase was dried with Na_2SO_4. After removal of the Na_2SO_4 by filtration, the $CHCl_3$ was evapd. The residue (9.32 g) was partitioned between *n*-hexane (200 ml) and 80% MeOH (200 ml). The *n*-hexane phase was partitioned a second time with 80% MeOH and the combined 80% MeOH frs were concd (4.03 g).

Purification of the phytosterols and triterpenes. A 2.5 g sample of the dried *n*-hexane phase (5.31 g) were saponified (5% KOH in 80% EtOH for 3 hr). The unsaponifiable lipid was extracted with $CHCl_3$. The $CHCl_3$ phase was dried with Na_2SO_4. The residue (2.38 g) was chromatographed on a silica gel column (119 g) successively eluted with *n*-hexane, *n*-hexane–EtOAc (9:1, 7:3, 5:5, 3:7, 1:9) and EtOAc in 50 ml frs. The frs were monitored by TLC using $CHCl_3$–MeOH (49:1) as developing system. The sterol frs 29–33 eluting with *n*-hexane–EtOAc (7:3) were combined and concd. The residue (144 mg) was dissolved in 6 ml MeOH with warming and filtered off. The soln was concd and dried *in vacuo* yielding a crystalline residue (30 mg). A portion of

this sterol mixture was acetylated and then examined by capillary GC as described previously [6] and GC-MS.

The frs 39–50 eluting with *n*-hexane–EtOAc (5:5–3:7) were combined and evapd. The residue (205 mg) was further chromatographed on a silica gel column using $CHCl_3$ in 3 ml frs. Frs 83–102 were combined and, after evaporation of the solvent, the residue (71 mg) was recrystallized in MeOH. The crystalline residue (48 mg) was identified as lup-20(29)-en-2α,3β,28-triol (**1**).

Lup-20(29)-*en*-2α,3β,28-*triol* (**1**). White needles, mp 258–260° (MeOH), $[\alpha]_D$ +11° ($CHCl_3$–MeOH 9:1; *c* 0.595), IR ν_{max} cm^{-1}: 3404 (*br* OH), 2941, 2868, 1641 (exo $=CH_2$), 1457, 1374, 1046, 965, 879 (exo $=CH_2$). EI-MS, *m/z* (rel. int.): 458.3769 $[M]^+$ (39) (calc. 458.3778 for $C_{30}H_{50}O_3$), 443 (11), 440 $[M - H_2O]^+$ (19), 427.3576 $[M - CH_2OH]^+$ (100) (calc. 427.3576 for $C_{29}H_{47}O_2$), 415.3201 $[M - C_3H_7]^+$ (25) (calc. 415.3189 for $C_{27}H_{43}O_3$), 409 $[M - CH_2OH - H_2O]^+$ (32), 401 (15), 397 (14), 383 (16), 304.2396 **c** (10) (calc. 304.2390 for $C_{20}H_{32}O_2$), 263.2017 **b** (9) (calc. 263.2022 for $C_{17}H_{27}O_2$), 234.1992 **d** (14) (calc. 234.2001 for $C_{16}H_{26}O$), 233 (11), 223.1737 **a** (19) (calc. 223.1776 for $C_{14}H_{23}O_2$), 220.1865 **e** (8) (calc. 220.1903 for $C_{15}H_{24}O$), 207.1743 **f** (8) (calc. 207.1748 for $C_{14}H_{23}O$), 205.1586 [**a** – H_2O] (31) (calc. 205.1593 for $C_{14}H_{21}O$), 203.1786 [**b** – CH_2OH] (34) (calc. 203.1772 for $C_{15}H_{23}$), 189 [**f** – H_2O] (34), 177 (19), 161 (11), 147 (11), 133 (16), 121 (20), 107 (20).

Acetylation of compound **1** (Ac_2O/pyridine, 12 hr at room temp.) yielded compound **2**.

Lup-20(29)-*en*-2α,3β,28-*triol triacetate* (**2**). Resin, $[\alpha]_D$ −8 ($CHCl_3$; *c* 0.63), IR ν_{max} cm^{-1}: 2946, 1742 (–O–$COCH_3$), 1637 (exo $=CH_2$), 1458, 1367, 1248, 1037, 883 (exo $=CH_2$). EI-MS, *m/z* (rel. int.): 584 $[M]^+$ (10), 524 $[M - HOAc]^+$ (63), 511 $[M - CH_2OAc]^+$ (18), 482 (27), 464 $[M - 2HOAc]^+$ (100), 449 (21), 421 (22), 404 ($[M - 3HOAc]^+$, 15), 391 (19), 389 (13), 361 (8), 323 (7), 276 **b** (7), 247 [**a** – HOAc] (13), 227 (13), 216 [**b** – HOAc] (24), 215 (25), 203 [**b** – CH_2OAc] (65), 189 [**f** – HOAc] (74), 187 [**a** – 2HOAc] (66), 173 (27), 159 (29), 147 (23), 133 (34), 121 (24), 109 (17).

Isolation and purification of 3,28-*dipalmitoyl-lup*-20(29)-*en*-2α,3β,28-*triol* (**3**). A 2.5 g sample of the *n*-hexane layer were chromatographed on a silica gel column (125 g) which was eluted with *n*-hexane, *n*-hexane–EtOAc (9:1, 7:3, 5:5, 3:7, 1:9), EtOAc and EtOAc–MeOH (1:1) in 50 ml frs. The frs were monitored by TLC using $CHCl_3$ as developing system. Frs 25–27 eluting with *n*-hexane–EtOAc (9:1–7:3) were combined and evapd. The residue (211 mg) was further chromatographed on a silica gel column (12.7 g) using $CHCl_3$ as eluent in 2 ml frs. Frs 19–24 were combined and evapd. The residue (78 mg) was investigated by spectroscopic methods.

3,28-*Dipalmitoyl-lup*-20(29)-*en*-2α,3β,28-*triol* (**3**). Waxy, $[\alpha]_D$ +5° ($CHCl_3$–MeOH 9:1, *c* 0.75). IR ν_{max} cm^{-1}: 3447 (*br* OH), 2923, 2853, 1734 (–O–CO–CH_2–), 1635 (exo $=CH_2$), 1465, 1375, 1176, 1114, 1046, 884 (exo $=CH_2$), 721; EI-MS, *m/z* (rel. int.): 934 $[M]^+$ (1.3), 678 $[M - C_{15}H_{31}COOH]^+$ (33), 660 (8), 440 (5), 422 $[M - 2C_{15}H_{31}COOH]^+$ (100), 409 (26), 407 (16), 394 (7), 379 (14), 256 $[C_{15}H_{31}COOH]^+$ (10), 217 (23), 205 [**a** – $C_{15}H_{31}COOH]^+$ (56), 203 (44), 189 (34), 177 (17), 161 (11), 147 (13), 135 (19), 121 (19), 109 (17).

Alkaline hydrolysis of compound **3** (5% KOH in 80% EtOH for 3 hr) yielded triterpene **1**.

Purification of the brassinosteroids. The residue resulting from the 80% MeOH fr. was charged onto a column prepared by swelling of 20 g silica gel in $CHCl_3$. Elution was carried out step-wise with10 frs (150 ml) of MeOH in $CHCl_3$ (0, 2, 3, 4, 5, 7, 10, 15, 20, 50%). The bioactive fractions eluted with 4 and 5% MeOH were combined and evapd. The residue (151 mg) was further purified by a LH-20 Sephadex chromatography (bed vol. 320 ml) using MeOH–$CHCl_3$ (4:1) as eluent. The eluates were collected in 6 ml fractions. The biologically active fr. 39 (elution vol./total column vol. 0.73) was evapd and the residue (9.7 mg) dissolved in MeOH and run on a DEA ion exchange chromatography (200 mg Bondesil 40 μm). The residue resulting from the DEA chromatography (4.5 mg) was chromatographed by preparative HPLC under conditions reported previously [6]. The bioactive fractions were pooled and concd, and examined by GC-MS. The methaneboronation of the brassinosteroids was carried out with pyridine containing methaneboronic acid at 70° for 30 min [15].

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