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Phyteumosides A and B: New Saponins with Unique Triterpenoid Aglycons from *Phyteuma orbiculare* L.

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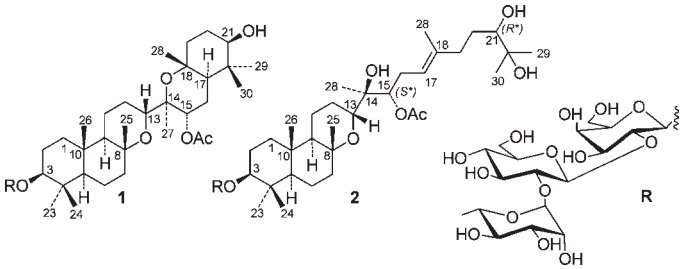
ABSTRACT



Phyteumosides A (1) and B (2), two saponins with unprecedented triterpenoid aglycons, were isolated from the aerial parts of *Phyteuma orbiculare* (Campanulaceae). Their structures were elucidated by spectroscopic and chemical methods and corroborated by X-ray diffraction analyses of the aglycons obtained through enzymatic hydrolysis. The aglycon of 1 can be considered as an incompletely cyclized onoceroid or gammaceroid triterpene with two additional tetrahydropyran rings arising from oxygen bridges. Compound 2 possesses a new 17-polypodene aglycon.

The round-headed rampion (*Phyteuma orbiculare* L., Campanulaceae) is a perennial herb which grows in subalpine and alpine regions of Central Europe. The leaves and the flowers were eaten in the past by the population of the Valais region (Switzerland) as a salad. In a study of forgotten traditional food plants, we investigated the aerial parts of *P. orbiculare*. No data have been reported on the secondary metabolites of this species nor the entire genus *Phyteuma*, but plants of the family Campanulaceae are known to contain triterpene saponins derived from oleanolic acid.[1] Here we report the isolation and structure elucidation of two new triterpene glycosides, phyteumosides A (**1**) and B (**2**), which possess unique triterpenic aglycons.

The aerial parts (226 g) of *P. orbiculare* were collected in June 2009 near Orsières in Valais, Switzerland. The dried plant material was defatted with CH_2Cl_2 and subsequently extracted with MeOH. Fractionation of the MeOH extract (43.7 g) by a combination of gel filtration on Sephadex LH-20 (MeOH) and flash chromatography on RP-18 (MeOH/H_2O gradient) afforded compounds **1** (51 mg) and **2** (40 mg). Both compounds gave purple spots on TLC after staining with vanillin/sulfuric acid.

The molecular formula of compound **1** ($[\alpha]^{20}_D$ −4.0 (*c* 0.27, MeOH)) was established as $C_{50}H_{84}O_{20}$ from the pseudomolecular $[M + H]^+$ ion at *m*/*z* 1005.5677 (calcd 1005.5629) in the HR-ESI-MS spectrum. ESI-MS^2 and MS^3 experiments in positive and negative modes gave fragment ions at *m*/*z* 857 ($[M - H - 146]^-$), 695

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Table 1. ^{1}H NMR and ^{13}C NMR Data of the Aglycon Portions of Phyteumosides A (**1**) and B (**2**)[a]

	phyteumoside A (**1**)				phyteumoside B (**2**)			
position	δ_H mult[b]	δ_C mult		HMBC	$\delta_H$mult[b]	δ_C mult		HMBC
1α	0.78	37.8	(t)		0.82	38.0	(t)	
1β	1.39				1.42			
2α	2.16, br m	27.0	(t)		2.16, br m	26.9	(t)	
2β	1.78				1.67			
3	3.28, dd (4.2, 11.5)	90.0	(d)	C-1′ C-2 C-4 C-23 C-24	3.35, dd (4.2, 11.8)	90.0	(d)	C-1′ C-2 C-4 C-23 C-24
4		40.0	(s)			40.0	(s)	
5	0.82	56.1	(d)		0.86	56.2	(d)	
6α	1.25	20.0	(t)		1.27	20.0	(t)	
6β	1.57				1.60			
7α	1.43	42.5	(t)		1.44	42.6	(t)	
7β	1.84				1.75			
8		75.5	(s)			75.3	(s)	
9	1.08	58.3	(d)	C-7 C-8 C-12	1.10, br d (12.2)	58.2	(d)	C-7 C-8 C-12
10		36.6	(s)			36.6	(s)	
11α	1.53	19.1	(t)		1.53	19.0	(t)	
11β	1.28				1.28			
12α	1.47	27.6	(t)		1.80	26.9	(t)	
12β	2.04				2.18			
13	3.83, dd (2.0, 11.3)	73.7	(d)		3.94, dd (1.9, 11.5)	73.1	(d)	
14		77.8	(s)			75.0	(s)	
15	5.75, dd (3.5, 4.6)	71.5	(d)	C-17 Ac(CO)	4.00	76.7	(d)	C-17 Ac(CO)
16α	1.87	23.1	(t)		1.82	27.5	(t)	
16β	2.05				2.06			
17	1.91	45.9	(d)	C-18 C-15	5.59	121.9	(d)	C-18 C-15
18		75.4	(s)			138.3	(s)	
19α	1.67	41.3	(t)		2.33, ddd (7.0, 9.6, 14.6)	38.0	(t)	
19β	1.83				2.62, ddd (4.6, 9.6, 14.2)			
20α	1.92	30.2	(t)		2.81	28.7	(t)	
20β	1.75				2.81			
21	3.54, dd (1.7, 10.4)	78.0	(d)	C-29 C-30	3.74, dd (1.4, 11.5)	78.8	(d)	C-29 C-30
22		38.9	(s)			72.9	(s)	
23	1.25, s	27.0	(q)	C-3 C-4 C-5	1.31, s	27.0	(q)	C-3 C-4 C-5
24	0.99, s	16.8	(q)	C-3 C-4 C-5	1.00, s	16.9	(q)	C-3 C-4 C-5
25	1.22, s	21.1	(q)	C-7 C-8 C-9	1.24, s	21.5	(q)	C-7 C-8 C-9
26	0.64, s	16.1	(q)	C-5 C-9 C-10	0.63, s	16.1	(q)	C-5 C-9 C-10
27	1.34, s	19.9	(q)	C-13 C-14	1.46, s	19.8	(q)	C-13 C-14
28	1.38, s	24.2	(q)	C-17 C-18 C-19	1.45, s	16.8	(q)	C-17 C-18 C-19
29	1.15, s	28.2	(q)	C-17 C-21 C-22 C-29	1.48, s	25.9	(q)	C-21 C-22 C-29
30	0.95, s	15.8	(q)	C-17 C-21 C-22 C-30	1.51, s	26.6	(q)	C-21 C-22 C-30
Ac(CO)		170.4	(s)			170.9	(s)	
Ac(Me)	2.09, s	21.6	(q)	Ac(CO)	2.03, s	21.4	(q)	Ac(CO)

[a] ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), in pyridine-d_5 (δ in ppm, J in Hz). [b] Multiplicities of overlapped signals are omitted.

([M − H − 146 − 162]$^-$), and 653 ([M − H − 146 − 162 − 42]$^-$) and m/z 535 ([M + H − 146 − 162 − 162]$^+$), suggesting the presence of one acetyl group, one terminal rhamnose, and two hexose moieties. Acid hydrolysis of **1** (1 mg) with 2 N TFA (100 °C, 2 h) afforded L-rhamnose, D-galactose, and D-glucose, which were identified by GC−MS analysis after derivatization with L-cysteine methyl ester and silylation.[2]

The ^{13}C NMR spectrum of **1** exhibited 50 resonances including 10 methyl, 11 methylene, 22 methine, and 7 quaternary carbons. Among the signals assigned to the aglycon, four methine and three quaternary carbons were oxygenated (Table 1). The multiplicities together with the nine degrees of unsaturation suggested the presence of five rings in the aglycon portion, from which two were oxygen heterocycles. The ^{1}H NMR spectrum displayed eight tertiary methyl groups at δ_H 1.38, 1.34, 1.25, 1.22, 1.15, 0.99, 0.95, 0.64 ppm and confirmed the presence of an acetyl group (δ_H 2.09 ppm) (Table 1).

The carbon backbone and the substitution pattern of the aglycon were deduced from HSQC, HMBC (Table 1), and HSQC-TOCSY NMR data (Supporting Information). However, since no HMBC correlation was detected from H-13 to C-8, the exact connectivity of the epoxy bridges (8,13:14,18 or 8,14:13,18) could not be assigned. The NMR data were compatible with two pentacyclic scaffolds containing two tetrahydropyran or oxepan rings, respectively.

This ambiguity was finally resolved by an X-ray diffraction analysis of the aglycon (Figure 1). Treatment of

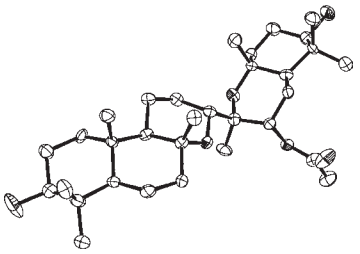

Figure 1. ORTEP drawing of aglycon **1a** with 50% probability displacement ellipsoids.

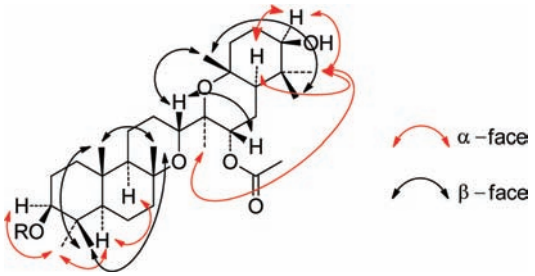

Figure 2. ROESY Correlations of phyteumoside A (**1**).

1 (20.0 mg) with a mixture of β-D-glucuronidase, hesperidinase, and β-galactosidase in acetate buffer (pH = 4.4) at 38 °C for 72 h yielded 7.0 mg of aglycon **1a** (ESI-MS: *m*/*z* 533.3 $[M + H]^+$; colorless needles from acetone/water).

The analysis also definitively confirmed the relative configuration which had been inferred from the 2D-ROESY correlations (Figure 2). Thus, the structure of **1a** is (3*S**,8*R**,13*R**,14*R**,15*S**,17*R**,18*R**,21*R**) 15-*O*-acetyl-8,13;14,18-diepoxy-17,18-diepi-13,18-seco-onocerane-3,15,21-triol.

With respect to the glycosidic portion, the ^{1}H NMR spectrum revealed the presence of three anomeric protons at δ_H 4.80, 5.57, and 6.29 ppm assigned to galactose, glucose, and rhamnose, respectively (Table 2). Vicinal coupling constants, *J*(1,2), of the anomeric H-atoms (δ_H 5.57 and 4.80 ppm (*J* = 7.6 Hz)) indicated a diaxial coupling and β-configuration for the galactose and glucose residues. The α-configuration of rhamnose was established on the basis of the ^{13}C NMR chemical shifts of C-3 and C-5.[3] The identity of the oligosaccharide chain was established by ^{1}H−^{1}H-COSY, HSQC, HMBC, HSQC-TOCSY, 1D TOCSY, and ROESY experiments. Starting from the anomeric protons of each sugar and from the methyl group of the rhamnose, all protons and carbons could be assigned within each spin system. In particular, correlations of H-1/H-2 and H-2/H-3 in the COSY spectrum along with the small coupling constant for H-3/H-4 (*J* = 2.9 Hz) supported the assignment of β-galactopyranose protons, while the signals δ_H 5.57, 4.20, 4.14, 3.98, and 3.62 ppm showed the typical spin system of a β-glucopyranosyl unit.

Table 2. ^{1}H and ^{13}C NMR Data of the Glycosidic Portion of **1**[a,b]

	position	δ_H[c] mult	δ_C
Gal	1	4.80, d (7.6)	106.0
	2	4.65, dd (7.7, 9.3)	77.8
	3	4.38, dd (2.9, 9.4)	76.7
	4	4.31, br s	70.8
	5	3.96	76.8
	6	4.34, dd (4.0, 8.8)	62.8
		4.34, dd (4.0, 8.8)	
Glc	1	5.57, d (7.6)	102.4
	2	4.20, dd (7.6, 9.0)	79.8
	3	4.14, dd (8.8, 9.0)	78.5
	4	3.98, dd (8.6, 9.2)	73.1
	5	3.62, ddd (5.5, 9.6, 9.2)	77.4
	6	4.09	63.6
		4.26, dd (9.6, 12.0)	
Rha	1	6.29, br s	102.3
	2	4.67	73.1
	3	4.69	73.0
	4	4.26	74.7
	5	4.93, dq (6.2, 9.2)	69.9
	6	1.73, d (6.2)	19.2

[a] ^{1}H NMR(500 MHz) and ^{13}C NMR (125 MHz), in pyridine-d_5 (δ in ppm, *J* in Hz). [b] Data of **2** showed deviations of < 0.01 (^{1}H) or < 0.04 (^{13}C) ppm and are provided in the Supporting Information. [c] Multiplicities of overlapped signals are omitted.

HMBC correlations (^3J) observed between H-1 of glucose (δ_H 5.57 ppm) and C-2 of galactose (δ_C 77.8 ppm) and between H-1 of rhamnose (δ_H 6.29 ppm) and C-2 of glucose (δ_C 79.8 ppm) enabled the sugar chain to be assigned as [α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl]. ROESY correlations further supported the interglycosidic linkages. The sugar chain was linked to C-3 of the aglycon based on the correlation between H-1 of galactose (δ_H 4.80 ppm) and C-3 (δ_C 90.0 ppm). This trisaccharidic moiety has not been yet reported.

Phyteumoside B (**2**) ($[\alpha]^{20}_D$ −28.9 (*c* 0.11, MeOH)) was assigned the molecular formula $C_{50}H_{86}O_{21}$ from the pseudomolecular $[M + H]^+$ ion at *m*/*z* 1023.5790 (calcd 1023.5734) in the HR-ESI-MS spectrum. The presence of D-galactose, D-glucose and L-rhamnose was established by acid hydrolysis and GC analysis. The ^{1}H and ^{13}C NMR signals assigned to the glycosidic portion were identical to those observed in **1** revealing the same oligosaccharide chain. The position of the glycosidic chain was inferred from the HMBC correlation between H-1 of the galactose and C-3 of the aglycon.

With regard to the aglycon portion, the multiplicities together with the eight degrees of unsaturation suggested the presence of three rings and a double bond. While the NMR signals including 2D correlations (Table 1, Figure 3) of rings A and B and the tetrahydropyran ring fused to ring B remained practically unchanged compared to **1**, significant differences were observed for the rest of the molecule. The methyls Me-29 and Me-30 were attached to an oxygenated carbon at δ_C 72.9 ppm and presented no HMBC correlation with C-17 in contrast to compound **1** which confirmed

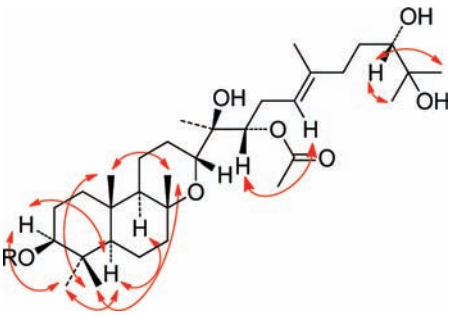

Figure 3. ROESY Correlations of phyteumoside B (**2**).

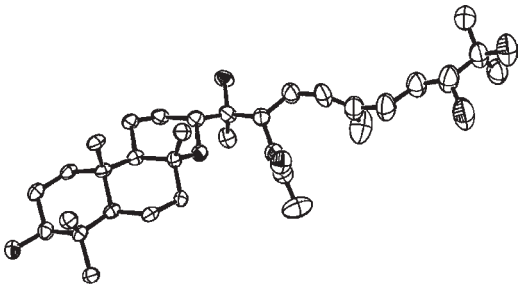

Figure 4. ORTEP drawing of aglycon **2a** with 50% probability displacement ellipsoids.

the absence of cyclization between C-17 and C-22. The double bond could be assigned to C-17(18) from the C-15 to C-17 spin system detected in the HSQC-TOCSY spectrum and the HMBC correlations from Me-28 to C-17, C-18 and C-19. This implied the absence of an oxygen bridge between C-14 and C-18. The position of the acetyl group was confirmed from the HMBC correlation between H-15 and the acetyl CO.

The complete structure of the aglycon of **2** including its relative configuration was also established by X-ray diffraction analysis (Figure 4).

Incubation of **2** (15 mg) with a mixture of glycosidases under the same condition as for **1** and subsequent purification by semipreparative HPLC afforded 1.5 mg of aglycon **2a** (ESI-MS: *m*/*z* 551.2 $[M + H]^+$, colorless needles from $MeCN/H_2O$).

The structure of **2a** was established as (3*S**,8*R**,13*R**, 14*R**,15*S**,21*R**) 15-*O*-acetyl-8,13-epoxy-17-polypoden-3,15,21,22-pentol. It is noteworthy that the relative configuration of **1a** and **2a** is identical.

The structure of **1** can be regarded as derived from a new secoonoceroid (or bissecogammaceroid) skeleton, but the reversed configuration at C-17 and C-18 is, to our knowledge, unique in these groups of triterpenes. The existence of two tetrahydropyran rings in the center of the cyclized carbon chain is also unprecedented. Interestingly, gammacerane triterpenes have been mainly reported from sediments,[4] bacteria,[5] and ferns.[6] In higher plants, few gammacerane triterpenoids have been reported in taxonomically scattered species including *Abies* species (Pinaceae),[7] *Ailanthus grandis* (Simaroubaceae),[8] and *Coriandrum sativum* (Apiaceae).[9] Onoceroids are rare in nature and mostly found in club mosses and ferns.[10] One representative, α-onocerin, has been, however, reported in various higher plants, in particular *Ononis* species (Fabaceae).[11] There are only few natural products with structurally analogous features as in **1** and **2**. Labdane diterpenes such as microtropiosides A-F[12] and tarapacol[13] possess the same tricylic system as **1** and **2**. Compound **1** exhibits also some similarities with colysanoxide, an onoceroid triterpene from fern species of the genus *Colys*, possessing a tetrahydropyrane ring fused to the ring E as in **1**.[14] However, the relative configuration at C-17 and C-18 in colysanoxide is opposite to **1**.

Biosynthetically, both aglycons seem to derive from an unrearranged squalene molecule, which underwent incomplete cyclization. The presence of OH groups at both C-3 and C-21 in **1** would agree with cyclization of a squalene bisepoxide from both ends as described for the onocerane skeleton.[15] Interestingly, triterpenes with the same tricyclic system as in **2** have been obtained by incubation of squalene diols with a squalene cyclase from *Alicyclobacillus acidcaldarius*.[16]

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Supporting Information Available. Experimental procedures, MS and NMR spectra of compounds **1** and **2**, X-ray crystallographic data (CIF), and NMR data of aglycons **1a** and **2a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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