Rivalosides A and B, Two 19-Oxo Triterpenoid Saponins from *Galium rivale*

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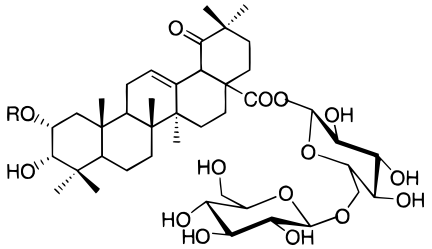
Two new oleanene-type triterpene glycosides, rivaloside A (2α-acetoxy-3α-hydroxy-19-oxo-olean-12-en-28-oic acid 28-*O*-*β*-glucopyranosyl-(1→6)-*β*-D-glucopyranoside) (**1**) and rivaloside B (2α,3α-dihydroxy-19-oxo-olean-12-en-28-oic acid 28-*O*-*β*-glucopyranosyl-(1→6)-*β*-D-glucopyranoside) (**2**), were isolated from aerial parts of *Galium rivale*. The structures were determined on the basis of spectroscopic data and chemical transformations. Under alkaline or acid hydrolysis, rivalosides A and B undergo allylic rearrangement with migration of the double bond. These compounds are the first representatives of 19-oxo substituted oleanenes, and they seem to be promising as taxonomic markers for *G. rivale*.

Galium rivale (Sibth. and Sm.) Griseb. (Rubiaceae) is an extremely variable species and represents an interesting taxonomic problem. To our knowledge, only tannins[1] and phenols[2] have been found in *G. rivale*. In a continuation of the chemical investigation of the genus *Galium*,[3−6] we have studied *G. rivale* collected from Slavyanka Mountain, Bulgaria, and herein we report the isolation of two new oleanene-type triterpene saponins both having a 19-oxo function.

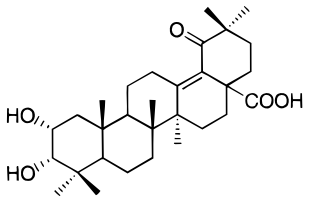
A MeOH extract of dried aerial parts of *G. rivale*, upon repeated charcoal chromatography and ascending droplet counter current chromatography (DCCC), afforded two new triterpenoid saponins, rivalosides A (**1**, 0.40% dry wt) and B (**2**, 0.05%).

Rivaloside A (**1**), an amorphous solid, had $[\alpha]_D$ +3.66° and a molecular formula of $C_{44}H_{68}O_{16}$, as determined from its positive ion HRFABMS (*m*/*z* 853, $[M + H]^+$). Its spectral features suggested **1** to be a triterpenoid saponin. The presence of a fragment at *m*/*z* 529 $[M + H - 324]^+$, in the FABMS indicated the presence of two linked hexose units in the molecule. The IR bands at 3600−3400, 1740, 1730, 1705, 1240, 1090, and 1045 cm^{-1} were characteristic of hydroxyl, esters, and ketone groups. Of the 44 carbons displayed in the ^{13}C NMR spectrum, 30 were assigned to the aglycon part, two to an acetyl group (*δ* 21.3 and 170.6), and the remaining 12 to the sugar moiety. The presence of a methyl singlet at *δ* 1.96 in the ^1H NMR spectrum of **1** confirmed the acetyl group. Acid hydrolysis of **1** afforded glucose, which was confirmed by TLC, high-performance anion exchange (HPAE-PAD), and GC (alditol acetate), and an artifactual aglycon (**3**). Compound **3** was also obtained after alkaline hydrolysis. The signals of the anomeric protons at *δ* 6.28 (d, *J* = 8.1 Hz) and 5.07 (d, *J* = 7.7 Hz) in the ^1H NMR spectrum of **1**, showed that both glucose units had the *β*-configuration. A combination of 2D NMR experiments (COSY, TOCSY, HMQC, and HMBC) allowed us to assign all signals in the ^1H and ^{13}C NMR spectra and to define the 1−6 connection between the two glucose units. The chemical shift of one anomeric carbon (*δ* 96.2) suggested the presence of an ester-linked sugar unit. HMBC correlation observed between the anomeric proton (*δ* 6.28) and the carboxyl group (*δ* 175.0) defined the connection between the aglycon and the sugar units. Its position at C-28 was further confirmed by alkaline hydrolysis. The ^1H and ^{13}C NMR spectra of **1** (Table 1), which displayed seven tertiary methyl groups, a trisubstituted double bond, two oxymethines, and an oxo group, were indicative of a Δ^{12} oleanene skeleton. The COSY-45 spectrum indicated that the oxymethine proton at *δ* 3.80 was coupled with the second oxymethine at *δ* 5.44, which in turn was coupled to nonequivalent methylene protons (*δ* 1.80, 1.65). These data placed the two hydroxyl groups in ring A. HMBC correlation observed between the olefinic proton H-12 (*δ* 5.44) and the carbon at *δ* 56.2 (d), which was correlated (HMQC) with the proton at *δ* 4.09, allowed assignment of the ^{13}C and ^1H chemical shifts at the 18-position. The oxo group was placed at C-19 based on the presence of HMBC correlation between the methine at *δ* 4.09 (H-18) and the carbon at *δ* 214.1 (s). Other HMBC correlations (Table 1) led to structure **1**. The relative stereochemistry of the hydroxyl groups was determined from the examination of the artifactual aglycon **3**.



1 R = Ac

2 R = H



3

Compound **3** contained seven methyl, nine methylene, four methine, and 10 quaternary carbon atoms instead of the expected DEPT pattern. Lack of the vinylic H-12 signal

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Table 1. NMR Spectral Data of **1** in Pyridine-d_5 Solution[a]

carbon no.	^{13}C	^{1}H	HMBC (J_{C-H} = 10 Hz)
1	38.7 t	1.80 m, 1.65m	3.80 (H-3), 1.03 (H-25)
2	71.7 d	5.44[b] br s	3.80 (H-3), 1.80−1.65 (H-1)
3	76.1 d	3.80 br s	1.80−1.65 (H-1), 1.18 (H-23), 0.92 (H-24)
4	39.4 s		1.65 (H-5), 1.18 (H-23), 0.92 (H-24)
5	48.7 d	1.65 m	3.80 (H-3), 1.18 (H-23)
6	18.3 t	1.10 m	1.65 (H-5)
7	33.6 t	1.32 m	1.65 (H-5)
8	38.9 s		1.98 (H-9), 1.02 (H-26)
9	47.8 d	1.98 m	5.44 (H-12), 1.80−1.65 (H-1)
10	39.9 s		1.96 (H-11), 1.10 (H-6), 1.03 (H-25)
11	24.0 t	1.96 m, 1.30 m	5.44 (H-12)
12	128.2 d	5.44[b] br s	4.09 (H-18)
13	134.5 s		4.09 (H-18), 1.12 (H-30)
14	42.8 s		5.44 (H-12), 4.09 (H-18), 2.08 (H-16), 1.98 (H-9)
15	28.1 t	2.24 m	
16	27.5 t	2.08 br d (13.4), 1.65 m	4.09 (H-18)
17	51.5 s		4.09 (H-18), 1.55 (H-21)
18	56.2 d	4.09 br s	5.44 (H-12)
19	214.1 s		4.09 (H-18)
20	44.2 s		1.12 (H-30), 1.09 (H-29)
21	34.7 t	1.55 m	2.27−1.80 (H-22)
22	31.5 t	2.27 m, 1.80 m	
23	29.4 q	1.18 s	3.80 (H-3), 1.65 (H-5), 0.92 (H-24)
24	22.2 q	0.92 s	3.80 (H-3), 1.65 (H-5), 1.18 (H-23)
25	16.8 q	1.03 s	1.98 (H-9), 1.80−1.65 (H-1)
26	17.7 q	1.02 s	
27	26.3 q	1.06 s	
28	175.0 s		6.28 (H-1?), 4.09 (H-18), 2.08−1.65 (H-16)
29	25.8 q	1.09 s	
30	23.8 q	1.12 s	1.55 (H-21)
Glc			
1′	96.2 d	6.28 d (8.1)	4.12 (H-2′)
2′	73.8 d	4.12[b]	4.20 (H-4′)
3′	78.5 d	4.20[b]	
4′	71.0 d	4.20[b]	4.75−4.35 (H-6′)
5′	78.7 d	4.15[b]	
6′	69.4 t	4.75 d (11.4), 4.35[b]	5.07 (H-1′)
Glc′			
1″	105.3 d	5.07 d (7.7)	4.75−4.35 (H-6′), 4.00 (H-2″)
2″	75.2 d	4.00 dd (7.7, 6.7)	4.20 (H-4″)
3″	78.5 d	4.20[b]	
4″	71.6 d	4.20[b]	4.49−4.35 (H-6″)
5″	78.2 d	3.89 m	
6″	62.7 t	4.49 d (11.7), 4.35[b]	4.20 (H-4″)
*C*OCH$_3$	170.6 s		1.96
CO*C*H$_3$	21.3 q	1.96 s	

[a] Chemical shifts (δ) are referred to TMS. Multiplicities are indicated by usual symbols. Coupling constants (Hz) are in parentheses. [b] Overlapped signals.

in the ^{1}H NMR, the DEPT data for a four-substituted double bond, the appearance of a methylene signal at δ 36.8 (C-12), and the lack of the methine signal at δ 56.2 (C-18) suggested migration of the 12,13-double bond. Consequently, under both alkaline and acid hydrolysis conditions, an allylic rearrangement occurred with migration of the double bond to the 13,18-position. The process influenced by the 19-oxo substituent is favored by formation of a conjugated oxo system (UV maximum at 256 nm). The EIMS of **3** suggested the presence of two hydroxyl groups and a COOH group (*m/z* 468 $[M - H_2O]^+$, 450 $[M - 2H_2O]^+$, 405 $[M - 2H_2O - COOH]^+$). The fragments at *m/z* 223 $[C_{14}H_{23}O_2]^+$, 205 $[223 - H_2O]^+$, 187 $[223-2H_2O]^+$ (A/B rings) and *m/z* 262 $[C_{16}H_{22}O_3]^+$, 217 $[262 - COOH]^+$, 189 $[217 - CO]^+$ (D/E rings) indicated the presence of two hydroxyl groups in ring A or B and of one oxo group in ring D or E. The ^{1}H NMR spectrum of **3** showed signals due to carbinylic protons at δ 3.74 (d, J = 2.7 Hz, H-3) and 4.29 (ddd, J = 11.5, 3.1, 2.7 Hz, H-2). The chemical shift and the coupling constant of the H-3 signal showed its equatorial position. The magnitude of the coupling constants associated with H-2 indicated that the proton had a trans-diaxial relationship with one H-1 proton, and therefore the hydroxyl group at C-2 was equatorial. NOEs between the axial Me-4 (δ 0.84) and H-2 and between the equatorial Me-4 (δ 1.24) and H-3 gave further support to the proposed relative streereochemistry. The carbon chemical shifts, regarding rings A/B of **3**, were almost identical with those published for thomandertriol[7] (2α,3α,19α-trihydroxyolean-12-ene). The deshielded C-2 signal (δ 71.7) and shielded C-3 signal (δ 76.1) and the chemical shift of H-3 (δ 5.44) observed in the NMR spectra of **1** determined the positions of the acetoxy and hydroxyl groups at the 2- and 3-position, respectively. Thus, the structure of compound **1** was elucidated as 2α-acetoxy-3α-hydroxy-19-oxo-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Rivaloside B (**2**), an amorphous solid, had $[\alpha]_D$ −15.41° and a molecular formula of $C_{42}H_{66}O_{15}$, as determined from FABMS and NMR data. Analysis of its ^{1}H and ^{13}C NMR spectra showed that **2** was closely related to **1**, except for the absence of signals due to the acetoxy group. Alkaline and acid hydrolysis of **2** yielded the same compound (**3**) that was obtained from **1**. Hence, the structure of **2** was established as 2α,3α-dihydroxy-19-oxo-olean-12-en-28-oic acid-*O*-β-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Rivalosides A (**1**) and B (**2**) are the first 19-oxo-oleanene triterpenoids to be isolated from a natural source. Our studies of more than 20 *Galium* species showed a general lack of triterpene saponins. The presence of triterpene saponins in *G. rivale*, moreover in considerable concentrations, appeared to be a promising chemotaxonomic marker for this species.

Experimental Section

General Experimental Procedures. Melting points were measured on a Kofler apparatus and were uncorrected. UV spectra were obtained on a Varian DMS 90 spectrophotometer. IR spectra were recorded on a Bio-Rad FTS-7 FT-IR spectrometer. Optical rotations were measured on a JASCO DIP 370 polarimeter, using a 10-cm microcell. LRMS and HRMS were recorded on a JEOL JMS D-300 and an AEI MS-50, respectively. FABMS were obtained on a VG-ZAB instrument equipped with a FAB source at 25 keV (2 μA) using glycerol as matrix. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, with TMS as internal standard on a Bruker AM 500 instrument, under Aspect X32 control. The 2D NMR spectra were obtained using Bruker's microprograms. DCCC was performed on a Buchi 670 apparatus by ascending mode. Aluminum sheets coated with Si gel 60 F_{254} were used for TLC.

Plant Material. *G. rivale* was collected at florescence from Slavyanka Mountain and identified by Dr. M. Anchev. The voucher specimen A94101 was deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM).

Extraction and Isolation. Dry aerial parts of *G. rivale* (60 g) were extracted twice with MeOH, and the concentrated extract (6.0 g) was partitioned between $Cl(CH_2)_2Cl$ and H_2O. The aqueous phase (4.8 g) was treated with charcoal (31 g) and eluted with H_2O (600 mL), MeOH−H_2O (1:19) (300 mL), MeOH−H_2O (3:7) (300 mL), MeOH−H_2O (1:1) (300 mL), MeOH (300 mL), MeOH−Me_2CO (1:1,) (300 mL), MeOH−$Cl(CH_2)_2Cl$ (1:1) (300 mL). The combined 50% MeOH (0.3 g), MeOH (0.3 g), MeOH−Me_2CO (0.3 g) and MeOH−$Cl(CH_2)_2Cl$ (0.5 g) were separated by ascending DCCC with $CHCl_3$−MeOH−H_2O−*i*PrOH (5:6:4:1). Fractions 46−53 (31 mg) contained **2**. The DCCC stationary phase was collected in fractions of 100 mL; fractions 3−4 (243 mg) contained pure **1**.

Sugar Identification. Compound **1** or **2** (5 mg) was heated with 2 M CF_3COOH (1 mL containing 0.9 mg of myo-inositol as an internal standard) for 8 h at 100 °C. The neutral monosaccharides released were converted into acetylated alditols by reduction with $NaBH_4$ followed by acetylation with acetic anhydride−pyridine. The alditol acetates obtained were analyzed by GC on a Hewlett-Packard 5890A gas chromatograph equipped with a flame-ionization detector, HP Ultra-2 capillary column, and HP 3393A recording integrator. Temperature was programmed to hold for 1 min at 175 °C, increased at 10°/min to 290 °C, and held for 3 min. Individual alditol acetates were identified by comparison of their retention times with those of authentic samples.

The water-soluble residue of acid hydrolysis was analyzed on HPAE-PAD (Dionex) equipped with a Carbopac PA1 column eluted with 15 mM NaOH (1 mL min^{-1}) and with a pulsed amperometric detector, giving D-glucose, identified by comparison of its retention time with that of authentic sample.

Rivaloside A (1): amorphous solid; $[\alpha]_D$ +3.66° (*c* 0.2; MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (2.08) nm; IR (Nujol) ν_{max} 3600−3400, 1740, 1730, 1705, 1640, 1240, 1090, 1045 cm^{-1}; NMR data, see Table 1; FABMS *m*/*z* 853.4586 $[M + H]^+$ (calcd for $C_{44}H_{69}O_{16}$, 853.4581), 529 $[M + H - 324]^+$.

Rivaloside B (2): amorphous solid, $[\alpha]_D$ −15.41° (*c* 0.2; MeOH); IR (Nujol) ν_{max} 3600−3400, 1730, 1705, 1640, 1090, 1045 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 6.27 (1H, d, J = 8.0 Hz, H-1′), 5.41 (1H, br s, H-12), 5.02 (1H, d, J = 7.6 Hz, H-1″), 4.30 (1H, ddd, J = 11.5, 3.1, 2.7 Hz, H-2), 3.72 (1H, d, J = 2.7 Hz, H-3), 1.21 (3H, s, H-23), 1.12 (3H, s, H-30), 1.10 (3H, s, H-29), 1.05 (3H, s, H-27), 1.03 (3H, s, H-26), 0.96 (3H, s, H-25), 0.86 (3H, s, H-24); ^{13}C NMR (pyridine-d_5) δ 214.2 (s, C-19), 175.1 (s, C-28), 134.5 (s, C-13), 128.5 (d, C-12), 105.4 (d, C-1″), 96.3 (d, C-1′), 79.4 (d, C-3), 78.8 (d, C-5′), 78.6 (d, C-3′ and C-3″), 78.3 (d, C-5″), 75.3 (d, C-2″), 73.9 (d, C-2′), 71.6 (d, C-4″), 71.1 (d, C-4′), 69.5 (t, C-6′), 66.2 (d, C-2), 62.8 (t, C-6″), 56.3 (d, C-18), 51.6 (s, C-17), 48.9 (d, C-5), 48.0 (d, C-9), 44.3 (s, C-20), 43.0 (t, C-1), 42.9 (s, C-14), 40.0 (s, C-10), 38.9 (s, C-4), 38.8 (s, C-8), 34.8 (t, C-21), 33.9 (t, C-7), 31.6 (t, C-22), 29.6 (q, C-23), 28.2 (t, C-15), 27.7 (t, C-16), 26.4 (q, C-27), 25.9 (q, C-29), 24.1 (t, C-11), 23.9 (q, C-30), 22.4 (q, C-24), 18.6 (t, C-6), 17.8 (q, C-26), 17.1 (q, C-25); FABMS *m*/*z* 833 $[M + Na]^+$, 811 $[M + H]^+$, 487 $[M + H - 324]^+$.

Alkaline Hydrolysis of Rivalosides A (1) and B (2). Rivaloside A (**1**, 30 mg) or rivaloside B (**2**, 5 mg) was dissolved in 0.1 N LiOH (1 mL), and the solutions were kept at 40 °C for 10 h. After neutralization the reaction mixtures were extracted with EtOAc. The EtOAc extracts were purified on a Si gel column using CH_2Cl_2−MeOH (4:1) as eluent to obtain **3** (13 and 2 mg, respectively, from **1** and **2**).

Acid Hydrolysis of Rivalosides A (1) and B (2). Rivaloside A (**1**, 60 mg) or rivaloside B (**2**, 5 mg), dissolved in HCl−H_2O−EtOH (2:1:2) was refluxed for 30 min. The reaction mixtures were extracted with EtOAc and the solvent evaporated. The water-soluble residue was analyzed by HPAE-PAD giving D-glucose. The EtOAc extracts were purified as described for the alkaline hydrolysis, to obtain **3** (28 and 2 mg, respectively, from **1** and **2**).

Compound 3: colorless needles (MeOH); mp 134−136 °C, $[\alpha]_D$ −84.72° (*c* 0.1; MeOH); UV (MeOH) λ_{max} (log ϵ) 256 (4.20) nm; IR (Nujol) ν_{max} 3600−3200, 1740, 1680 1615, cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 4.29 (1H, ddd, J = 11.5, 3.1, 2.7 Hz, H-2), 3.74 (1H, d, J = 2.7 Hz, H-3), 3.0 (1H, dt, J = 13.7, 3.5 Hz, H-12eq), 2.51 (1H, dt, J = 13.0, 3.1 Hz, H-16eq), 2.27 (1H, dt, J = 13.6, 3.3 Hz, H-22eq), 1.25 (3H, s, H-29), 1.24 (3H, s, H-23), 1.11 (3H, s, H-27), 1.07 (3H, s, H-30), 1.02 (3H, s, H-26), 0.86 (3H, s, H-25), 0.83 (3H, s, H-24); ^{13}C NMR (pyridine-d_5) δ 208.0 (s, C-19), 178.4 (s, C-28), 151.1 (s, C-13), 134.2 (s, C-18), 79.4 (d, C-3), 66.4 (d, C-2), 53.1 (s, C-17), 51.3 (d, C-9), 48.9 (d, C-5), 45.8 (s, C-14), 45.5 (s, C-20), 43.4 (t + s, C-1 and C-8), 39.1 (s, C-10), 38.9 (s, C-4), 36.4 (t, C-21), 35.2 (t, C-22), 34.7 (t, C-7), 32.2 (t, C-16), 29.6 (q, C-23), 28.6 (t, C-15), 27.4 (t, C-12), 25.9 (q, C-29), 24.7 (q, C-30), 23.3 (t, C-11), 22.3 (q, C-24), 21.0 (q, C-27), 18.4 (t, C-6), 17.8 (q, C-26), 17.7 (q, C-25); EIMS *m*/*z* 486 $[M]^+$ (40), 468 $[M - H_2O]^+$ (67), 450 $[M - 2\ H_2O]^+$ (100), 405 (54), 262 (47), 223 (11), 217 (82), 205 (65), 189 (22), 187 (49); HREIMS *m*/*z* 486.3340 (calcd for $C_{30}H_{46}O_5$, 486.3345).

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