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Steroidal glycosides from the underground parts of *Helleborus caucasicus*

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Abstract

Four polyhydroxylated and polyunsaturated furostanol glycosides (**1**–**4**), named caucasicosides A (**1**), B (**2**), C (**3**) and D (**4**), were isolated from the MeOH extract of the underground parts of *Helleborus caucasicus*, along with four spirostanol derivatives, a furostanol glycoside, a furospirostanol glycoside, 20-hydroxyecdysone and the bufadienolides hellebrigenin and deglucohellebrin. The structures of **1**–**4** were elucidated as furosta-5,20(22),25(27)-triene-1β,3β,11α,26-tetrol 26-*O*-β-D-glucopyranoside (**1**), 26-*O*-β-D-glucopyranosylfurosta-5,20(22),25(27)-triene-1β,3β,11α,26-tetrol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**2**), 26-*O*-β-D-glucopyranosyl-22α-methoxyfurosta-5,25(27)-diene-1β,3β,11α,26-tetrol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**3**), 26-*O*-β-D-glucopyranosylfurosta-5,20(22),25(27)-triene-1β,3β,26-triol 3-*O*-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1→2)-4-*O*-sulfo-α-L-arabinopyranoside (**4**). Structure elucidation was accomplished through the extensive use of 1D- and 2D NMR experiments including ^{1}H–^{1}H (COSY, 1D-TOCSY) and ^{1}H–^{13}C (HSQC, HMBC) spectroscopy along with ESI-MS and HR-ESI-MS. The aglycones of **1**–**4** have never been reported before.

Keywords: *Helleborus caucasicus*; Ranunculaceae; Furostanol glycosides; Spirostanol glycosides; Bufadienolides; Ecdysteroid

1. Introduction

Helleborus species are evergreen, rhizomatous plants belonging to the family Ranunculaceae. For some spp. interesting biological activities are reported. *H. foetidus* shows anti-insect and poisonous activity (Pascual-Villalobos and Robledo, 1998). *H. niger* is used in homeopathy and as an adjuvant therapy in the treatment of tumour patients in anthroposophical medicine in Germany (Bussing and Schweitzer, 1988). An original patent medicine utilizing purified *Helleborus* extracts having antiantalgic and antirheumatic activity (Kerek, 1981) has been prepared in Romania with the registered name Boicil (US Patent).

H. caucasicus is an endemic plant of Caucasian flora, widely distributed in the territory of West Georgia, characterized by a strong underground system. The MeOH extract of the roots and rhizomes of this plant showed very high cytotoxic activity against human lung cancer (A-549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) (Muzashvili et al., 2006). In an effort to characterize the chemical composition of this extract, we have undertaken the investigation of its steroidal constituents obtaining four new furostane glycosides along with 9 known compounds among which two bufadienolides and an ecdysteroid are included.

2. Results and discussion

The dried roots and rhizomes of *H. caucasicus* were submitted to sequential extraction with petroleum ether,

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chloroform and 80% methanol and the methanol extract was partitioned between 1-butanol and water. Chromatography of the 1-butanol extract over a silica gel column followed by RP-HPLC yielded compounds **1**–**4**.

Compound **1** showed a major ion peak at *m/z* 607 $[M+H]^+$ in the positive ESI-MS and a significant fragment at *m/z* 445 $[M+Na\text{-}162]^+$ attributable to the loss of a hexose unit. The molecular formula of **1** was unequivocally established to be $C_{33}H_{50}O_{10}$ by HR-MALDI-MS (*m/z* 607.3476 $[M+H]^+$). The ^1H NMR spectrum of **1** showed signals for three steroidal methyl groups at δ 1.66 attributable to a methyl on a sp^2 carbon and 0.74 and 1.17, three methine protons at δ 3.42, 3.46 and 4.00 indicative of secondary alcoholic functions, two methylene protons at δ 4.14 and 4.35 ascribable to a primary alcoholic function. Further features were a multiplet at δ 4.79 typical of H-16 of a pentacyclic steroidal saponin (Hamed et al., 2004), two signals at δ 4.96 and 5.12 indicative of an exomethylene group (Watanabe et al., 2003), a signal at δ 5.70 attributable to a further olefinic proton and the signal of an anomeric proton at δ 4.30. The ^{13}C NMR spectrum for the aglycone moiety displayed signals ascribable to six sp^2 carbons at δ 104.9, 112.0, 126.4, 139.9, 146.1 and 152.6, four oxygenated functions at δ 67.1, 68.6, 76.3, 85.4, and one primary alcoholic function at δ 72.4, suggesting the occurrence of a glycosidic furostanol skeleton (Skhirtladze et al., 2006). Full assignments of the proton and carbon resonances of the aglycone (Table 1) were secured by ^1H–^1H DQF-COSY and HSQC spectra. The occurrence of a $\Delta^{20,22}$ double bond was deduced from the HMBC spectrum which showed significative cross-peaks, due to $^2J_{C-H}$ and $^3J_{C-H}$ correlations between the proton signal of Me-21 (δ 1.66) and C-20 (δ 104.9)/C-22 (δ 152.6). HMBC correlations between M-19 (δ 1.17) and the olefinic resonance at δ 139.9 (C-5) suggested a C-5(6) double bond (Watanabe et al., 2003) while the exomethylene group was located at C-25 on the basis of the HMBC correlations between the primary alcoholic function signals (δ 4.14 and 4.35) and C-25 (δ 146.1) and C-27 (δ 112.0). The multiplet at δ 3.46 was attributed to H-3 while the

Table 1
^1H and ^{13}C NMR data of the aglycon portions of compounds **1**, **2**, **3**, and **4**

Pos.	**1**		**2**		**3**		**4**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	76.3	3.42 (1H, *dd*, 11.4, 4.4)	76.1	3.44 (1H, *dd*, 12.0, 3.9)	76.0	3.43 (1H, *dd*, 12.0, 3.9)	84.3	3.40 (1H, *dd*, 11.4, 3.5)
2	41.3	2.08[a] 1.59(1H, *q*-like, 11.7)	39.3	2.22[a] 1.70 (1H, *q*-like, 11.9)	39.3	2.17[a] 1.70 (1H, *q*-like, 11.9)	36.9	2.15[a] 1.70 (1H, *q*-like, 11.8)
3	68.6	3.46 (1H, *m*)	75.1	3.68 (1H, *m*)	75.0	3.68 (1H, *m*)	68.8	3.39 (1H, *m*)
4	43.7	2.27[a], 2.21[a]	39.7	2.47[a], 2.30[a]	39.6	2.47[a], 2.28[a]	43.2	2.28[a], 2.23[a]
5	139.9	–	139.4	–	139.4	–	139.6	–
6	126.4	5.70 (1H, *br d*, 5.7)	127.0	5.70 (1H, *br d*, 5.0)	127.0	5.71 (1H, *br d*, 5.6)	126.0	5.59 (1H, *br d*, 5.3)
7	33.7	2.07 (1H, *m*), 1.68 (1H, *m*)	33.1	2.05 (1H, *m*), 1.60 (1H, *m*)	32.7	2.04[a], 1.65 (1H, *m*)	33.3	1.56 (2H, *m*)
8	33.1	1.61 (1H, *m*)	33.2	1.60 (1H, *m*)	33.2	1.60 (1H, *m*)	33.6	1.54 (1H, *m*)
9	58.2	1.22 (1H, *t*, 10.2)	58.2	1.21[a]	58.3	1.21[a]	51.2	1.27 (1H, *m*)
10	46.1	–	45.9	–	45.9	–	50.9	–
11	67.1	4.00 (1H, *td*, 10.2, 5.3)	66.7	3.98 (1H, *td*, 10.1, 5.4)	66.5	3.97 (1H, *td*, 10.1, 5.4)	25.1	1.50 (2H, *m*)
12	49.7	2.21[a], 1.36 (1H, *t*, 10.2)	49.6	2.19[a], 1.35 (1H, *t*, 10.1)	49.9	2.15[a], 1.26 (1H, *t*, 10.1)	40.7	1.76 (1H, *m*), 1.35 (1H, *m*)
13	43.6	–	43.3	–	43.4	–	43.6	–
14	54.4	1.20[a]	54.4	1.19[a]	55.9	1.30[a]	56.3	1.06 (1H, *m*)
15	34.7	2.22[a], 1.43 (1H, *td*, 12.7, 5.7)	34.7	2.21[a], 1.42 (1H, *td*, 12.7, 5.7)	32.3	2.02[a], 1.30[a]	34.8	2.16[a], 1.43 (1H, *td*, 12.4, 5.3)
16	85.4	4.79 (1H, *m*)	85.8	4.78 (1H, *m*)	82.2	4.44 (1H, *m*)	85.2	4.74 (1H, *m*)
17	65.1	2.59 (1H, *d*, 10.1)	65.1	2.58 (1H, *d*, 10.3)	64.6	1.83[a]	65.3	2.55 (1H, *d*, 10.1)
18	14.1	0.74 (3H, *s*)	14.8	0.74 (3H, *s*)	16.9	0.88 (3H, *s*)	14.4	0.74 (3H, *s*)
19	13.4	1.17 (3H, *s*)	12.9	1.18 (3H, *s*)	13.1	1.19 (3H, *s*)	14.6	1.13 (3H, *s*)
20	104.9	–	104.9	–	40.7	2.25[a]	105.6	–
21	11.6	1.66 (3H, *s*)	11.0	1.65 (3H, *s*)	15.5	1.06 (3H, *d*, 6.9)	11.7	1.64 (3H, *s*)
22	152.6	–	152.7	–	112.8	–	152.1	–
23	24.7	2.32 (2H, *m*)	24.5	2.30 (2H, *m*)	28.1	2.24[a], 2.18[a]	24.5	2.27 (2H, *m*)
24	31.1	2.29 (2H, *m*)	31.1	2.29 (2H, *m*)	31.7	1.92 (2H, *m*)	31.6	2.29 (2H, *m*)
25	146.1	–	146.4	–	146.2	–	146.2	–
26	72.4	4.35 (1H, *d*, 12.5), 4.14 (1H, *d*, 12.5)	71.9	4.35 (1H, *d*, 12.5), 4.13 (1H, *d*, 12.5)	72.2	4.36 (1H, *d*, 12.5), 4.14 (1H, *d*, 12.5)	72.1	4.35 (1H, *d*, 11.8), 4.14 (1H, *d*, 11.8)
27	112.0	5.12 (1H, *br s*), 4.96 (1H, *br s*)	112.5	5.12 (1H, *br s*), 4.96 (1H, *br s*)	112.0	5.12 (1H, *br s*), 4.96 (1H, *br s*)	112.4	5.12 (1H, *br s*), 4.96 (1H, *br s*)
22-OMe					47.3	3.18 (3H, *s*)		

[a] Overlapped with other signals.

location of a hydroxyl group at C-1 was determined by the correlation between Me-19 (δ 1.17) and C-1 (δ 76.3). The multiplicity of H-1 (*dd*, $J = 4.4$ and 11.4 Hz) suggested the β-orientation of the C-1 hydroxyl group (Watanabe et al., 2003). The third secondary alcoholic function was located at C-11 on the basis of the carbon resonances of ring B and of the HMBC correlation between the signal at δ 4.00 (H-11) and the carbon resonances at δ 58.2 (C-9) and 49.7 (C-12) which gave in turn correlations with Me-19 (δ 1.17) and Me-18 (δ 0.74), respectively. The coupling constants of H-11 (δ 4.00, *td*, $J = 5.3$, 10.2 Hz) suggested the α-orientation of the C-11 hydroxyl group (Watanabe et al., 2005). The sugar unit occurring in **1** was identified as a glucose unit which was located at C-26 on the basis of the HMBC correlation between the signal at δ 4.30 (H-1_{glc}) and the carbon resonance at δ 72.4 (C-26). The configuration of the glucose unit was established as D after hydrolysis of **1** with 1 N HCl, trimethylsilation and determination of retention time by GC (Experimental (Section 3)).

Thus compound **1** was identified as furosta-5,20(22),25(27)-triene-1β,3β,11α,26-tetrol 26-*O*-β-D-glucopyranoside named caucasicoside A.

The ESI-MS (positive-ion mode) of compound **2** exhibited a pseudomolecular ion peak $[M+H]^-$ at *m/z* 915, ascribable to a molecular formula $C_{45}H_{70}O_{19}$. Further fragment ion peaks in the ESI-MS spectrum were observed at *m/z* 753 $[M+H-162]^-$, *m/z* 607 $[M+H-162-146]^-$ and *m/z* 445 $[M+H-162-162-146]^-$ corresponding to the successive loss of one hexosyl, one deoxyhexosyl and one hexosyl moieties. The molecular formula of **2** was confirmed as $C_{45}H_{70}O_{19}$ by HR-MALDI-MS (*m/z* 915.4588 $[M+H]^+$). This result suggested that saponin **2** contained three sugar units. ^1H and ^{13}C NMR data of **2** in comparison with those of **1** indicated that the two compounds differed only for the occurrence of a disaccharide moiety at C-3 which showed a downfield shift (δ 75.1) due to the glycosylation (Braca et al., 2004). The ^1H NMR spectrum for the sugar portion of compound **2** showed three anomeric proton signals at δ 4.30 (1H, *d*, $J = 8.1$ Hz), 4.52 (1H, *d*, $J = 7.9$ Hz) and 5.23 (1H, *d*, $J = 1.4$ Hz). The chemical shifts of all the individual protons of the two sugar unit were attributed on the basis of 2D-TOCSY and DQF-COSY spectral analysis, and the ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (Table 1). These data showed the presence of a terminal β-glucose (δ 4.30), a terminal rhamnose (δ 5.23) and a 2-substituted β-glucose (δ 4.52) as indicated by the downfield shift of C-$2_{glc'}$ (δ 78.7) signal. The α configuration of the rhamnose unit was deduced from the absence of intraresidual ROESY correlations between H-1_{rha} and H-3_{rha}/H-5_{rha}. An unambiguous determination of the linkage site was obtained from the HMBC spectrum, which showed key correlation peaks between the proton signal at δ 4.30 (H-1_{glc}) and the carbon resonance at δ 71.9 (C-26), the proton signal at δ 4.52 (H-$1_{glc'}$) and the carbon resonance at δ 75.1 (C-3),and between the proton signal at δ 5.23 (H-1_{rha}) and the carbon resonance at δ 78.7 (C-$2_{glc'}$) (Table 2). Also in this case, the D configuration of the glucose units and the L configuration of the rhamnose unit were determined by acid hydrolysis followed by GC analysis. Thus, compound **2** was defined as 26-*O*-β-D-glucopyranosylfurosta-5,20(22),25(27)-triene-1β, 3β,11α,26-tetrol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside named caucasicoside B.

The HR-MALDI mass spectrum of **3** (*m/z* 947.4846 $[M+H]^+$) supported a molecular formula of $C_{46}H_{74}O_{20}$. It was apparent from the NMR data (^1H, ^{13}C, 1D-TOCSY, DQF-COSY, HSQC, HMBC) of compounds **3** that this compound only differed from **2** by the absence of the $\Delta^{20,22}$ double bond and the presence of a methoxyl group at C-22 (Skhirtladze et al., 2006). The α orientations of Me-21 and the methoxyl group at C-22 were deduced from the ROESY correlations among H-17 (δ 1.83), Me-21 (δ 1.06) and the methoxyl group (δ 3.18). Therefore, **3** was deduced to be 26-*O*-β-D-glucopyranosyl-22α-methoxyfurosta-5,25(27)-diene-1β,3β,11α,26-tetrol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside named caucasicoside C.

Compound **3** can be considered as an artifact caused by the addition of methanol to the 20(22) double bond of **2** during the extraction of the plant material.

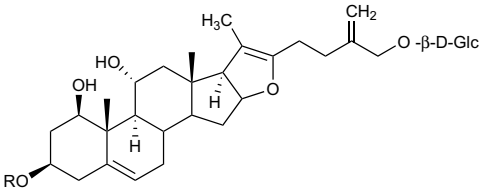
Compound **4** showed in the negative ESI-MS a major ion peak at *m/z* 1081 $[M+H]^-$ and a significant fragment in MS/MS analysis at *m/z* 949 $[M+H-132]^-$, *m/z* 919 $[M+H-162]^-$, *m/z* 859 [M+H-132-90], ascribable to the loss of a pentose, a deoxyhexose and a sulfate-pentose unit. Its molecular formula was established unequivocally as $C_{49}H_{76}O_{24}S$ by HR-MALDI-MS (*m/z* 1081.4528 $[M-H]^-$, calcd. for $C_{49}H_{77}O_{24}S$, 1081.4531). Acid hydrolysis of **4**, followed by treatment with $BaCl_2$, gave a white precipitate, thus demonstrating the presence of a sulfate residue. On the basis of the NMR data (^1H, ^{13}C, 2D-TOCSY, DQF-COSY, HSQC, HMBC) of **4** in comparison with those of **3** the aglycon of **4** was identified as furosta-5,20(22),25(27)-triene-1β,3β,26-triol. The downfield shift exhibited by C-1 (δ 84.3) suggested that this carbon was glycosylated. The ^1H NMR spectrum for the sugar portion of compound **4** showed four anomeric proton signals at δ 4.30 (1H, *d*, $J = 7.9$ Hz), 4.35 (1H, *d*, $J = 7.4$ Hz), 4.51 (1H, *d*, $J = 7.4$ Hz) and 5.32 (1H, *d*, $J = 1.4$ Hz). The chemical shifts of all the individual protons of the four sugar units were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analysis, and the ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (Table 1). These data showed the presence of a β-glucopyranosyl unit (δ 4.30), a β-xylopyranosyl unit (δ 4.51), an α-rhamnopyranosyl unit (δ 5.32), an unusual 4-sulfo-α-arabinopyranosyl unit (δ 4.35), as indicated by the downfield shift of H-4_{ara} (δ 4.55) if compared to H-4_{ara} (δ 3.90) of an unsubstituted model compound (Perrone et al., 2007). Glycosidation shifts were observed for C-2_{ara} (δ 75.3) and C-3_{rha}(δ 82.1). An unambiguous determination of the sequence

Table 2
^{1}H and ^{13}C NMR data of the sugar portions of **1**, **2**, **3**, and **4**

1				**2**				**3**				**4**			
Pos.	δ_C		δ_H, *J* in Hz	Pos.	δ_C		δ_H, *J* in Hz	Pos.	δ_C		δ_H, *J* in Hz	Pos.	δ_C		δ_H, *J* in Hz
				Glc ‘(C-3)	1	100.0	4.52 (*d*, 7.9)	Glc′ (C-3)	1	100.0	4.52 (*d*, 7.8)	Ara I (C-1)	1	100.4	4.35 (*d*, 7.4)
					2	78.7	3.39 (*t*, 8.0)		2	78.7	3.39 (*t*, 8.5)		2	75.3	3.71 (*dd*, 8.8, 7.4)
					3	79.0	3.50 (*t*, 9.1)		3	79.0	3.50 (*t*, 8.5)		3	74.8	3.79 (*dd*, 8.8, 3.9)
					4	71.4	3.29[a]		4	71.4	3.29[a]		4	77.3	4.55 *m*
					5	77.5	3.27 *m*		5	77.5	3.27 *m*		5	64.8	4.21 (*br d*, 12.3, 2.2)
					6	62.3	3.88 (*dd*, 12.1, 2.1) 3.68 (*dd*, 12.1,4.6)		6	62.3	3.88 (*dd*, 12.1, 2.1) 3.68 (*dd*, 12.1,4.6)				3.56 (*br d*, 12.3)
				Rha II (C-2 Glc I)	1	101.6	5.23 (*d*, 1.4)	Rha II (C-2 Glc I)	1	101.6	5.23 (*d*, 1.5)	Rha II (C-2 Ara I)	1	100.9	5.32 (d, 1.4)
					2	71.8	3.93 (*t*, 3.6)		2	71.8	3.93 (*t*, 3.4)		2	71.4	4.10 (t, 3.6)
					3	72.0	3.68 (*dd*, 9.3, 3.6)		3	72.0	3.68 (*dd*, 9.3, 3.4)		3	82.1	3.81 (dd, 9.0, 3.6)
					4	73.7	3.42 (*t*, 9.3)		4	73.7	3.42 (*t*, 9.3)		4	72.5	3.59 (t, 9.0)
					5	69.3	4.16 *m*		5	69.3	4.16 *m*		5	69.1	4.16 m
					6	17.4	1.28 (*d*, 6.1)		6	17.4	1.28 (*d*, 6.3)		6	18.2	1.28 (d, 6.1)
												Xyl III (C-3 RhaII)	1	106.4	4.51 (*d*, 7.4)
													2	75.2	3.32 (*dd*, 8.8, 7.4)
													3	78.0	3.37 (*t*, 8.8)
													4	70.7	3.52 *m*
													5	66.7	3.90 (*dd*, 10.5, 4.6) 3.27 (*t*, 10.5)
Glc (C-26)	1	103.0	4.30 (*d*, 7.4)	Glc (C-26)	1	102.7	4.30 (*d*, 8.1)	Glc (C-26)	1	102.7	4.30 (*d*, 7.7)	Glc (C-26)	1	102.7	4.30 (*d*, 7.9)
	2	74.8	3.24 (*dd*, 8.5, 7.4)		2	74.6	3.23 (*t*, 8.5)		2	74.6	3.23 (*t*, 8.5)		2	74.8	3.25 (*dd*, 8.5, 7.9)
	3	77.8	3.37 (*t*, 8.9)		3	77.9	3.36 (*t*, 8.9)		3	77.9	3.36 (*t*, 8.9)		3	77.8	3.38 (*dd*, 9.2, 8.5)
	4	71.3	3.31 (*t*, 9.1)		4	71.3	3.30 (*t*, 9.1)		4	71.3	3.30 (*t*, 9.1)		4	71.2	3.31 (*t*, 9.2)
	5	77.7	3.26 *m*		5	77.4	3.27 *m*		5	77.4	3.27 *m*		5	77.6	3.28 *m*
	6	62.8	3.90 (*dd*, 11.8, 2.6) 3.70 (*dd*, 11.8, 5.7)		6	62.3	3.88 (*dd*, 11.9, 2.0) 3.68 (*dd*, 11.9, 4.6)		6	62.3	3.88 (*dd*, 11.9, 2.0) 3.68 (*dd*, 11.9, 4.6)		6	62.8	3.90 (*dd*, 11.5, 2.0) 3.69 (*dd*, 11.5, 4.1)

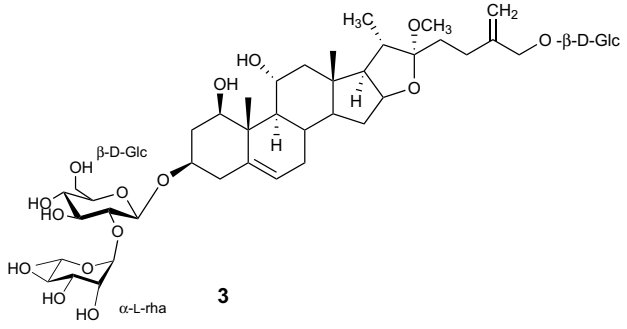
[a] Overlapped with other signals.

and linkage sites was obtained from the HMBC spectrum which showed key correlation peaks between the proton signal at δ 4.35 (H-1_{ara}) and the carbon resonance at δ 84.3 (C-1), 5.32 (H-1_{rha}) and 75.3 (C-2_{ara}), 4.51 (H-1_{xyl}) and 82.1 (C-3_{rha}), and between the proton signal at δ 4.30 (H-1_{glc}) and the carbon resonance at δ 72.1 (C-26). The sugar units of **4** were determined to be L-arabinose, D-glucose, L-rhamnose by acid hydrolysis followed by GC analysis. On the basis of all this evidence, the structure of **4** was deduced to be 26-*O*-β-D-glucopyranosylfurosta-5,20(22),25(27)-triene-1β, 3β,26-triol 3-*O*-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-4-*O*-sulfo-α-L-arabinopyranoside named caucasicoside D.

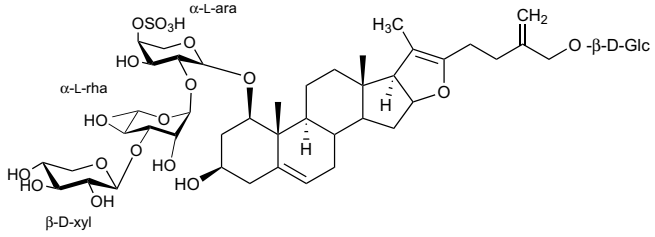


1 R = H

2 R = α-L-Rha-(1->2)- β-D-Glc-



3



4

Four spirostane derivatives namely spirosta-5,25(27)-diene-1β,3β,11α-triol (**5**) (Wissner and Kating, 1974), (23*S*,24*S*)-21-acetoxy-24-*O*-β-D-quinovopyranosylspirosta-5,25(27)-diene-1β,3β, 23,24,tetrol 1-*O*-β-D-apiofuranosyl-(1 → 3)-(4-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside (**6**) (Watanabe et al., 2003), (23*S*,24*S*)-21-acetoxy-24-*O*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosylspirosta-5, 25(27)-diene-1β, 3β,23,24-tetrol 1-*O*-β-D-apiofuranosyl-(1 → 3)-4-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside (hellebosaponin A) (**7**) (Mimaki et al., 2003), and (23*S*,24*S*)-21-acetoxy-24-*O*-β-D-fucopyranosylspirosta-5,25(27)-diene-1β,3β,23, 24-tetrol 1-*O*-β-D-apiofuranosyl-(1 → 3)-(4-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-α-L-arabinopyranoside (hellebosaponin B) (**8**) (Mimaki et al., 2003) were isolated. Furthermore the furostanol derivatives (25*S*)-22α,25-epoxyfurost-5-ene-3β,11α,26-triol 26-*O*-β-D-glucopyranoside (**9**) (Watanabe et al., 2005), 26-*O*-β-D-glucopyranosyl-22α-furosta-5,25(27)-diene-1β, 3β,11α,22α,26-pentaol (**10**) (Akin and Anil, 2007), along with 20-hydroxyecdysone (**11**) (Werawattanametin et al., 1986), and the bufadienolides 3β,5β,14β-trihydroxy-19-oxo-bufa-20,22-dienolide 3-*O*-β-D-glucopyranoside (hellebrigenin) (**12**) (Shimada et al., 1979) and 3β,5β,14β-trihydroxy-19-oxo-bufa-20,22-dienolide 3-*O*-α-L-rhamnopyranoside (deglucohellebrin) (**13**) (Kissmer and Wichtl, 1986) were isolated.

The co-occurrence of furostanol glycosides, spirostanol glycosides, bufadienolides and ecdysteroids is a typical finding of *Helleborus* genus (Meng et al., 2001; Watanabe et al., 2003, 2005). On the other hand compounds **6**–**8** which are polyoxygenated bidesmosidic spirostanol saponins characterized by the unusual hydroxylation of ring E at C-23 and C-24 have been previously reported only in *H. orientalis*, a deeply investigated *Helleborus* sp. indigenous to Greece and Turkey (Mimaki et al., 2003; Watanabe et al., 2003; Akin and Anil, 2007). Thus the steroidal profile of *H. caucasicus* appears similar to that of *H orientalis* but shows as particular feature the occurrence of compounds **1**–**4** characterized by polyhydroxylated and polyunsaturated aglycones never reported before.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO DIP 1000 polarimeter. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) at 300 K dissolving all the samples in CD_3OD (Carlo Erba, 99.8%). All of the 2D NMR spectra were performed on a 512 × 1024 data matrix and acquired in the phase-sensitive mode with the transmitter set at the solvent resonance; time proportional phase increment (TPPI) was used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, HSQC and HMBC spectra. The NMR data were processed using UXNMR software. The ROESY spectra were acquired with $t_{mix} = 400$ ms.

ESI-MS in the positive ion mode was performed using a Finnigan LCQ Deca ion trap instrument from Thermo Finnigan (San Jose, CA) equipped with Xcalibur software. Samples were dissolved in MeOH (Baker) and infused in the ESI source by using a syringe pump; the flow rate was 3 μl/min. The capillary voltage was 43 V, the spray voltage was 5 kV, and the tube lens offset was 30 V. The

capillary temperature was 280 °C. Data were acquired in MS1 and MS/MS scanning mode.

Exact masses were measured by a Voyager DE mass spectrometer (Applied Biosystems, Foster City, CA). Samples were analysed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and Angiotensin III at 931.5154 Da as internal standard. GC analysis was performed on a Termo Finnigan Trace GC apparatus using a 1-Chirasil-Val column (0.32 mm × 25 m). HPLC separations were carried out on a Waters 590 system equipped with a Waters R401 refractive index detector, a Waters XTerra Prep MSC_{18} column (300 × 7.8 mm i.d.) and a Rheodyne injector.

Column chromatography was performed over Silica gel (100/160 μm, Merck). TLC was performed on silica gel plates (Merck precoated silica gel 60 F_{254}) and developed in the solvent system, $CHCl_3$:MeOH:H_2O (26:14:3). All solvents for chromatographic separation were of analytical grade from Carlo Erba (Rodano, Italy). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

3.2. Plant material

The roots and rhizomes of *Helleborus caucasicus* A.Br were collected in Georgia in September 2005. Samples of *H. caucasicus* were identified by Dr. Jemal Aneli, Department of Botany, Institute of Pharmacochemistry, Tblisi, Georgia. A voucher specimen (No. 357), has been deposited at this Department.

3.3. Extraction and isolation

The air-dried roots and rhizomes (500 g) of *H. caucasicus* were defatted with petroleum ether (1.5 l) and then with chloroform (1.5 l) during 18 h. After drying the plant material was extracted with 80% methanol (2.4 l) three times at 60 °C. The collected extracts have been dried under vacuum and the concentrate was partitioned between water (20 g) and 1-butanol (49 g). Part of 1-butanolic extract (3 g) containing steroidal compounds was subjected to silica gel column chromatography (100 × 2.5 cm, 100/160 μm, Merck) eluting with isocratic system chloroform–methanol–water (26:14:3) yielding 150 fractions (8 ml each) which were combined in fractions A (900 mg), B (320 mg) and C (450 mg). Fractions A–C were separated on a C_{18} column (7.8 × 300 mm, LiChroprep RP18, 10 μm, XTerra), using different percentage of MeOH in isocratic conditions. From fraction A (53% MeOH as eluent, flow rate 2 ml/min) compounds **1** (8.2 mg, $t_R = 23.8$ min), **5** (12.3 mg, $t_R = 4.5$ min), **9** (7.6 mg, $t_R = 17.7$ min), **10** (9.3 mg, $t_R = 3.1$ min), **11** (11.5 mg, $t_R = 29.6$ min), **12** (2.0 mg, $t_R = 6.7$ min), **13** (2.3 mg, $t_R = 7.2$ min) were obtained; from fraction B (55% MeOH as eluent,) **2** (3.9 mg, $t_R = 18.0$ min), **3** (4.1 mg, $t_R = 20.8$ min), **6** (3.7 mg, $t_R = 11.1$ min), **7** (4.3 mg, $t_R = 8.8$ min), **8** (3.4 mg, $t_R = 6.7$ min) were isolated, while fraction C (50% MeOH) yielded **4** (8.5 mg, $t_R = 11.3$ min).

3.4. Furosta-5,20(22),25(27)-triene-1β,3β,11α, 26-tetrol 26-O-β-D-glucopyranoside (caucasicoside A, 1)

White powder; $C_{33}H_{50}O_{10}$; $[\alpha]_D^{24}$ − 35.5 (MeOH; *c* 0.05); for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 607 $[M+H]^+$; MS/MS *m/z* 445 $[M-162]^+$; HR-MALDI-MS $[M+H]^+$ *m/z* 607.3476 (calcd. for $C_{33}H_{51}O_{10}$, 607.3482).

3.5. 26-O-β-D-Glucopyranosylfurosta-5,20(22),25(27)-triene-1β,3β,11α,26-tetrol 3-O-α-L-rhamnopyranosyl-(1 → 2)-O-β-D-glucopyranoside (caucasicoside B, 2)

White powder; $C_{45}H_{70}O_{19}$; $[\alpha]_D^{24}$ − 63.0 (MeOH; *c* 0.05); for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z* 915 $[M+H]^+$; MS/MS *m/z* 753 $[M-162]^+$; *m/z* 607 $[M-162-146]^+$; *m/z* 445 $[M-162\times2-146]^+$; HR-MALDI-MS $[M+H]^+$ *m/z* 915.4588 (calcd. for $C_{45}H_{71}O_{19}$, 915.4590).

3.6. 26-O-β-D-Glucopyranosyl-22α-methoxyfurosta-5,25(27)-diene-1β,3β,11α,26-tetrol 3-O-α-L-rhamnopyranosyl-(1 → 2)-O-β-D-glucopyranoside (caucasicoside C, 3)

White powder; $C_{46}H_{74}O_{20}$; $[\alpha]_D^{24}$ − 73.7 (MeOH; *c* 0.07); for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; ESI/MS *m/z* 947 $[M+H]^+$; MS/MS *m/z* 785 $[M-162]^+$; *m/z* 639 $[M-162-146]^+$; *m/z* 477 $[M-162\times2-146]^+$; HR-MALDI-MS $[M+H]^+$ *m/z* 947.4846 (calcd. for $C_{46}H_{75}O_{20}$, 947.4852).

3.7. 26-O-β-D-Glucopyranosylfurosta-5,20(22),25(27)-triene-1β,3β,26-triol 3-O-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-[4-O-sulfo]-α-L-arabinopyranoside (caucasicoside C, 4)

White powder; $C_{49}H_{76}O_{24}$; $[\alpha]_D^{24}$ − 25.5 (MeOH; *c* 0.12); for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; ESI/MS *m/z* 1081 $[M+H]^+$; MS/MS *m/z* 919 $[M-162]^+$; *m/z* 949 $[M-132]^+$; *m/z* 869 $[M-132-80]^+$; *m/z* [M-132-80-146]$^+$; HR-MALDITOF-MS $[M+H]^+$ *m/z* 1081.4528 (calcd. for $C_{49}H_{77}O_{24}$, 1081.4531).

3.8. Acid hydrolysis

A solution (1 mg each) of compounds **1**, **2** and **4** in 1 N HCl (0.5 ml) was stirred at 80 °C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)-imidazole and pyridine (0.1 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue

was partitioned between H_2O and CH_2Cl_2 (1 ml, 1:1 v/v). The CH_2Cl_2 layer was analysed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. The peak of the hydrolysate of **1** was detected at 14.71 min (D-glucose). The peaks of the hydrolysate of **2** were detected at 14.72 min (D-glucose) and 9.68 and 10.71 (L-rhamnose). The peaks of the hydrolysate of **4** were detected at 14.74 min (D-glucose), 10.97 and 12.00 (D-xylose), 9.67 and 10.70 (L-rhamnose), 8.90 and 9.79 min (L-arabinose). Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine were detected at 8.80 and 9.72 min (D-arabinose), 14.71 min (D-glucose), 9.78 and 10.76 (D-rhamnose), 10.98 and 12.00 min (D-xylose), 8.92 and 9.80 min (L-arabinose), 14.64 min (L-glucose), 9.68 and 10.71 (L-rhamnose), and 11.03 and 12.06 min (L-xylose).

Since to authors' knowledge D-rhamnose is not commercially available, this monosaccharide was obtained by acid hydrolysis of methyl-3,4-di-*O*-benzyl-α-D-rhamnopyranosyde (Bedini et al., 2005) kindly gifted by Dr. Emiliano Bedini, Department of Organic Chemistry and Biochemistry, University of Naples Federico II.

3.9. Detection of the sulfate group

One milligram of **4** was refluxed with 10% HCl (4 ml) for 4 h and then extracted with Et_2O. An aliquot of the aqueous layer was treated with 70% $BaCl_2$ to give a white precipitate ($BaSO_4$).

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