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Structure and Absolute Configuration of Helosides A and B, New Saponins from *Chamaelirium luteum*

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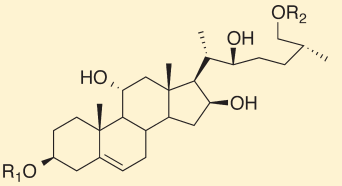
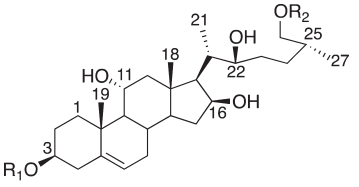
Supporting Information

ABSTRACT: Investigation of *Chamaelirium luteum* roots led to the isolation of two new steroidal saponins, helosides A (**1**) and B (**2**), that contain a previously unreported aglycone, helogenin (**3**). Their structures and absolute configuration were elucidated through MS-MS, NMR, chemical degradation, and X-ray crystallography.



1 R_1,R_2 = β-D-Glc
2 R_1 = β-D-Glc R_2 = H
3 R_1,R_2 = H

Chamaelirium luteum (L.) A. Gray, commonly known as "helonias" or "false unicorn", is a member of the family Melanthiaceae that is native to North America.[1] The roots and rhizomes of *C. luteum* have been used in traditional herbal medicine to treat female reproductive health problems[2] and are now found in a wide range of commercial botanical dietary supplements. The phytochemical profile of *C. luteum* has received very limited characterization, with most primary literature generated during the nineteenth century.[3,4] The major steroidal saponins of *C. luteum*, chamaelirosides A and B, were reported recently along with the total synthesis of their aglycone, (23*R*,24*S*)-chiograsterol B; these possess an unusual polyhydroxylated cholestane-type core.[5] The present investigation has led to the discovery of two new steroidal saponins, helosides A and B (**1** and **2**), for which the structure and stereochemistry were determined using MS-MS, 1D and 2D NMR, chemical degradation, and X-ray crystallographic methods.



1 R_1,R_2 = β-D-Glc
2 R_1 = β-D-Glc R_2 = H
3 R_1,R_2 = H

Compound **1** was isolated as an off-white solid, and positive-ion HRESIMS provided an ion at m/z 797.4304, corresponding to a molecular formula of $C_{39}H_{66}O_{15}$. Fragmentation by tandem ESIMS revealed the neutral loss of two 180 Da units, suggesting the presence of two hexose monosaccharides along with a C_{27} steroidal skeleton bearing five oxygens. The ^{1}H NMR spectrum (in pyridine-d_5) revealed two signals typical of the steroid angular methyl groups at δ_H 1.22 (s, H_3-18) and 1.28 (s, H_3-19), in addition to two secondary methyl groups at δ_H 0.99 (d, J = 6.7 Hz, H_3-27) and 1.18 (d, J = 7.0 Hz, H_3-21). A broad doublet at δ_H 5.42 (d, J = 5.7 Hz, H-6) indicated the presence of a double bond, which suggested a polyhydroxylated cholestene aglycone for **1**. The ^{13}C NMR spectrum displayed 39 signals, including four methyl groups, 11 methylenes, 21 methines, and only three quaternary carbons, at δ_C 38.9 (C-10), 43.4 (C-13), and 141.8 (C-5). Correlations observed in the HSQC and HMBC spectra allowed assignment of the steroid methyl groups as δ_C 14.8 (C-18), 15.0 (C-21), 17.7 (C-27), and 19.1 (C-19). The HMBC correlations of these characteristic signals were a helpful starting point for structure elucidation of the aglycone. For example, HMBC correlations from the methyl signal at δ_H 0.99 (H_3-27) revealed C-26 (δ_C 75.3), C-25 (δ_C 34.3), and C-24 (δ_C 31.6), indicating oxygenation of C-26. A combination of 1D TOCSY, COSY, HSQC, and HMBC experiments allowed complete assignment of the steroidal skeleton (Table 1). Four secondary hydroxy groups were found at C-3 (δ_C 78.3), C-11 (δ_C 68.2), C-16 (δ_C 71.7), and C-22 (δ_C 75.1), with one primary hydroxy group at C-26 (δ_C 75.3). The stereochemistry of the different ring junctions and substituents was assigned using a 2D ROESY experiment. ROE correlations observed between H_3-19/H-1β/H-8 and H-1α/H-3/H-9 indicated a *trans* B/C ring junction and the β-orientation of the oxygen-containing substituent at C-3, while correlations between H_3-18/H-15β and H-15α/H-14/H-16/H-17 showed a *trans* C/D ring junction and β-orientation of the substituents at C-16 and C-17. Intense ROE correlations between H-11/H_3-18/H_3-19 indicated the α-orientation of the C-11 hydroxy group. The unexpected downfield shift of H-1β to δ_H 3.21 (td, J = 3.4, 13.8 Hz), presumably due to a through-space interaction between H-1β and the 11α hydroxy group, was in agreement with literature data.[6] The relative configuration of C-20 was indicated by a ROE correlation between H_3-18 and H-20, while the configurations of C-22 and C-25 were unable to

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Table 1. NMR Spectroscopic Data for Saponins 1 and 2[a] and Their Aglycone 3[b] (δ in ppm, pyridine-d_5)

	1		2		3			1		2	
	^{1}H [δ, mult., J (Hz)]	^{13}C	^{1}H [δ, mult., J (Hz)]	^{13}C	^{1}H [δ, mult., J (Hz)]	^{13}C		^{1}H [δ, mult., J (Hz)]	^{13}C	^{1}H [δ, mult., J (Hz)]	^{13}C
1α	1.37 dt (3.6, 14.3)	39.7	1.37 m[c]	39.7	1.52 dt (3.6, 13.9)	40.1	3-*O*-β-D-Glc				
1β	3.21 td (3.4, 13.9)		3.22 dt (3.2, 13.9)		3.31 td (3.4, 13.9)		1′	5.07 d (7.8)	102.4	5.07 d (7.7)	102.4
2α	2.15 m	30.5	2.15 m	30.5	2.14 m	33.0	2′	4.06 t (8.0)	75.3[e]	4.05 t (8.3)	75.4
2β	1.91 m[c]		1.91 m[c]		1.97 m[c]		3′	4.31 t (8.9)	78.6[f]	4.31 t (8.7)	78.7
3	4.02 m[c]	78.3	4.02 m	78.3	3.92 qt (4.9, 10.2)	71.7[d]	4′	4.29 t (8.8)	71.7	4.29 t (8.7)	71.8
4α	2.78 ddd (2.1, 4.7, 13.1)	39.9	2.78 ddd (2.0, 4.6, 13.1)	39.9	2.71 m[c]	44.2	5′	3.97 m	78.5	3.97 m	78.5
4β	2.58 m[c]		2.58 m[c]		2.71 m[c]		6a′	4.53 br d (11.8)	62.8[g]	4.53 br d (11.9)	62.8
5		141.8		141.8		142.9	6b′	4.40 dd (4.4, 11.8)		4.40 dd (5.4, 11.9)	
6	5.42 d (5.7)	121.7	5.43 d (5.5)	121.7	5.51 d (5.6)	121.0					
7α	1.60 m	32.4	1.61 m	32.4	1.66 m[c]	32.5	26-*O*-β-D-Glc				
7β	1.93 m[c]		1.94 m[c]		2.00 m[c]		1″	4.81 d (7.8)	104.9		
8	1.53 m	31.8	1.54 m[c]	31.8	1.62 m[c]	32.0	2″	4.02 t (8.1)	75.4[e]		
9	1.28 m[c]	57.2	1.29 m[c]	57.2	1.37 t (10.2)	57.3	3″	4.24 t (8.9)	78.7[f]		
10		38.9		38.9		38.9	4″	4.22 t (8.6)	71.8		
11	4.31 m[c]	68.2	4.31 m[c]	68.2	4.37 ddt (5.3, 10.8, 10.8)	68.2	5″	3.93 m	78.5		
12α	1.58 m[c]	52.3	1.58 m[c]	52.4	1.61 m[c]	52.4	6a″	4.55 br d (11.9)	62.9[g]		
12β	2.61 dd (4.7, 12.0)		2.61 dd (4.8, 12.1)		2.65 dd (4.6, 12.0)		6b″	4.37 dd (5.7, 11.9)			
13		43.4		43.4		43.4					
14	1.08 ddd (7.4, 11.1, 13.2)	54.5	1.08 m[c]	54.5	1.12 m[c]	54.6					
15α	2.27 td (7.6, 12.7)	37.2	2.27 td (7.5, 12.7)	37.2	2.29 td (7.6, 13.0)	37.2					
15β	1.50 dt (4.5, 13.3)		1.50 dt (4.4, 13.1)		1.53 dt (4.5, 13.1)						
16	4.76 m	71.7	4.77 tt (3.8, 7.4)	71.7	4.78 tt (4.2, 7.3)	71.8[d]					
17	1.72 dd (6.9, 10.9)	58.1	1.73 dd (7.0, 11.1)	58.1	1.75 dd (6.9, 11.2)	58.1					
18	1.22 s	14.8	1.23 s	14.8	1.25 s	14.8					
19	1.28 s	19.1	1.28 s	19.1	1.41 s	19.3					
20	2.54 m[c]	36.1	2.60 m[c]	36.0	2.61 m	36.0					
21	1.18 d (7.0)	15.0	1.19 d (7.1)	15.1	1.20 d (7.1)	15.1					
22	4.15 m	75.1	4.21 m	75.4	4.21 m	75.4					
23a,b	1.74 m[c]	31.8	1.84 m[c]	32.0	1.84 m[c]	32.0					
24a	2.07 m	31.6	2.20 m	31.5	2.20 m	31.5					
24b	1.30 m[c]		1.39 m		1.39 m[c]						
25	1.96 octet (6.7)	34.3	1.91 m[c]	37.0	1.92 m[c]	37.0					
26a	3.92 dd (7.0, 9.5)	75.3	3.78 td (5.3, 10.5)	67.6	3.78 m	67.6					
26b	3.62 dd (5.9, 9.5)		3.69 td (5.5, 10.7)		3.69 m						
27	0.99 d (6.7)	17.7	1.09 d (6.7)	17.7	1.10 d (6.7)	17.6					

[a] Recorded at 750 MHz for ^{1}H NMR and 188 MHz for ^{13}C NMR. [b] Recorded at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. [c] Indicates overlapping signals. [d,e,f,g] Assignments are interchangeable within column.

be determined spectroscopically, due to the flexibility of the steroid side-chain. The ^{1}H NMR spectrum of **1** also contained two signals characteristic of the anomeric proton of a glycoside, at δ_H 4.81 (d, J = 7.8 Hz, H-1″) and δ_H 5.07 (d, J = 7.8 Hz, H-1′). These anomeric signals were correlated in the HSQC spectrum with signals at δ_C 104.9 and 102.4, respectively. Examination of the vicinal coupling constants obtained through 1D TOCSY experiments identified the monosaccharides as two β-glucopyranosyl residues. In the HMBC spectrum of **1**, correlations were observed between δ_H 5.07 (H-1′, 3-*O*-β-D-glucose) and δ_C 78.3 (C-3, aglycone) and between δ_H 4.81 (H-1″, 26-*O*-β-D-glucose) and δ_C 75.3 (C-26, aglycone), revealing the sugars to be

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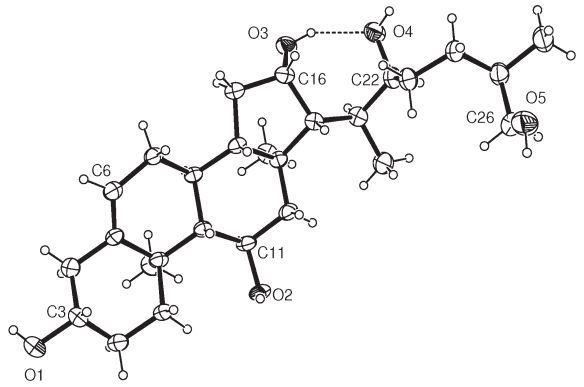

Figure 1. ORTEP view of the aglycone **3**. Ellipsoids are drawn at the 30% level of probability.

separately linked at positions C-3 and C-26 of the aglycone. The absolute configuration of the sugar units was determined by enantioselective GC analysis, following acid-catalyzed methanolysis of **1** and per-trifluoroacetylation of the resultant methyl-glycosides.[7] The structure of heloside A (**1**) was therefore elucidated as 3-*O*-β-D-glucopyranosyl-26-*O*-β-D-glucopyranosyl-(3β,11α,16β,22,26)-pentahydroxycholest-5-ene.

Compound **2** was isolated as an amorphous solid, and positive-ion HRESIMS provided an ion at *m*/*z* 635.3770, consistent with a molecular formula of $C_{33}H_{56}O_{10}$. The ^{1}H and ^{13}C NMR spectra for **2** (in pyridine-d_5) were very similar to those for **1**. However, the ^{1}H NMR spectrum displayed only one anomeric proton signal at δ_H 5.07 (d, J = 7.7 Hz, H-1′), which was correlated in the HSQC spectrum with a signal at δ_C 102.4. Enantioselective GC analysis and examination of vicinal coupling constants obtained through 1D TOCSY experiments were used to identify this sugar as a β-D-glucose residue. In the HMBC spectrum of **2**, a correlation was observed between δ_H 5.07 (H-1′, 3-*O*-β-D-glucose) and δ_C 78.3 (C-3, aglycone), revealing the linkage of the sugar residue to position C-3. In the ^{13}C NMR spectrum, the upfield shift of C-26 (δ_C 67.6), when compared with its value in **1** (δ_C 75.3), also indicated that a free hydroxy group was present at this position. Examination of COSY, HSQC, HMBC, and ROESY spectra allowed the complete assignment of **2** (Table 1), which differed from **1** only by lacking the C-26-linked glucose residue. The structure of heloside B (**2**) was therefore assigned as 3-*O*-β-D-glucopyranosyl-(3β,11α,16β,22,26)-pentahydroxycholest-5-ene.

To determine the configuration of C-22 and C-25 in saponins **1** and **2**, their aglycone, **3**, was obtained through acid hydrolysis of a crude *C. luteum* extract followed by purification via RP-HPLC. As expected, the ^{1}H and ^{13}C NMR data for **3** (Table 1) closely matched those of the steroid nucleus of saponins **1** and **2**. Crystallization of compound **3** via the slow evaporation of a methanol/water solution yielded white needles suitable for X-ray crystallography. The structure obtained agreed with the planar structure deduced from the NMR data above and revealed the 22*S* and 25*R* configuration of the steroidal side-chain (Figure 1).

Saponins **1** and **2** of *C. luteum* contain a polyhydroxylated cholestene aglycone, unusual in lacking the additional ring(s) characteristic of furo- and spirostanol saponins. The similar cholest-5-ene-3β,16β,22,26-tetrol aglycone (lacking C-11 hydroxylation) has been reported in saponins isolated from *Solanum lyratum* and *S. anguivi*,[8,9] *Allium tuberosum*,[10] and *Trillium erectum*.[11] The ^{1}H and ^{13}C NMR data for the side-chain of **1** and **2** are surprisingly in good agreement with those recorded for bethosides B and C from *Trillium erectum*, which were reported to contain the epimeric 22*R*, 25*R* configuration of the side-chain ($\Delta\delta_H \leq 0.04$ ppm, $\Delta\delta_C \leq 0.3$ ppm for the side-chain of the corresponding saponins).[11] These assignments were based upon work that had determined the C-22 configuration of the corresponding aglycone via Horeau's method.[8] Although this discrepancy remains to be resolved, absolute configurations that rely upon this previous work,[8] including our own,[11] should be treated with caution.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were performed on a melting-point apparatus (Dr. Tottoli) and are uncorrected. Optical rotations were measured using a 1 mL cell with a 10 cm path length on a JASCO P-2000 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV500 or AV750 spectrometer with the pyridine-d_5 signal (δ_H 8.71 ppm, δ_C 149.9 ppm) as internal standard. Low-resolution and tandem mass spectra were recorded on a Bruker ESQUIRE HCT instrument (positive- and negative-ion ESI). High-resolution mass spectra were acquired on a Bruker MicrOTOF-Q instrument (positive-ion ESI) with internal calibration using Agilent Tune-Mix. Semipreparative RP-HPLC was performed on a Shimadzu LC-20AT liquid chromatograph equipped with an ELSD-LT detector (52 °C, N_2 pressure: 200 KPa), column oven (40 °C), and a Phenomenex HPLC column (Luna C-18, 5 μm, 250 × 10 mm).

Plant Material. *Chamaelirium luteum* was sourced from Botanical Liaisons, Ltd., Boulder, CO (harvested in North Carolina, 2002), and Blessed Herbs, Ltd., Oakham, MA (purchased 2001). A specimen from Botanical Liaisons, Ltd. was deposited (accession PHARM-06275) at the Medicinal Plant Herbarium, Southern Cross University, Lismore, Australia, and verified by Dr. Hans Wohlmuth.

Extraction and Isolation. Powdered roots of *C. luteum* (80 g) were extracted (80% MeOH(aq), 800 mL) with sonication (3 × 10 min). Following filtration and removal of the solvent in vacuo, a portion of this crude extract (approximately 5%) was loaded onto a solid-phase extraction (SPE) cartridge (Phenomenex Strata C-18E, 55 μm, 70 Å, 1000 mg/6 mL). The SPE cartridge was washed with H_2O (10 × 6 mL), saponins **1** and **2** were eluted with 20% MeOH(aq) (10 × 6 mL), and the SPE cartridge was washed with 100% MeOH (10 × 6 mL). This procedure was repeated to purify the entire crude extract. The combined 20% MeOH fractions were concentrated in vacuo, dissolved (90% MeOH(aq), 15 mL), filtered, and purified by semipreparative RP-HPLC (gradient of 6% to 40% CH_3CN(aq) over 50 min, 2 mL/min). Two different pure fractions were collected, which corresponded to **1** (t_R 34.0 min, 21.9 mg) and **2** (t_R 38.9 min, 2.4 mg), respectively. To obtain the aglycone, **3**, crude extract (from 20 g of plant material) was dissolved in EtOH (60 mL) and HCl(aq) (32%, 8 mL) and heated under reflux for 2.5 h. After cooling, water (60 mL) was added and the reaction was extracted with diethyl ether (3 × 100 mL). The combined organic layers were washed with NaOH(aq) (5% w/v, 3 × 100 mL) and dried ($MgSO_4$) before removal of the solvent in vacuo. The crude residue was dissolved (90% MeOH(aq), 20 mL), filtered, and purified by semipreparative RP-HPLC (gradient of 30% to 70% CH_3CN(aq) over 40 min, 2 mL/min). Compound **3** was collected as a pure fraction (t_R 15.2 min, 27.2 mg).

*Heloside A (**1**):* off-white solid; mp 162−164 °C (dec); $[\alpha]^{25}_D$ −20.4 (*c* 0.11, CH_3OH); ^{1}H (pyridine-d_5, 750 MHz) and ^{13}C NMR (pyridine-d_5, 188 MHz), see Table 1; positive-ion ESIMS *m*/*z* 797, 617, 437; negative-ion ESIMS *m*/*z* 773, 611, 593, 449; HRESIMS *m*/*z* 797.4304 $[M + Na]^+$ (calcd for $C_{39}H_{66}NaO_{15}$, 797.4294).

*Heloside B (**2**):* amorphous solid; $[\alpha]^{28}_D$ −29.6 (*c* 0.24, CH_3OH); ^{1}H (pyridine-d_5, 750 MHz) and ^{13}C NMR (pyridine-d_5, 188 MHz), see Table 1; positive-ion ESIMS *m*/*z* 635; negative-ion ESIMS *m*/*z* 611; HRESIMS *m*/*z* 635.3770 $[M + Na]^+$ (calcd for $C_{33}H_{56}NaO_{10}$, 635.3766).

*Helogenin (**3**):* white solid; mp 216−217 °C; $[\alpha]^{24}_{D}$ −19.9 (*c* 0.12, CH_3OH); ^1H (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 1; positive-ion ESIMS *m/z* 451, 473, 489; negative-ion ESIMS *m/z* 449; HRESIMS *m/z* 473.3234 $[M + Na]^+$ (calcd for $C_{27}H_{46}NaO_5$, 473.3237); *anal.* C 68.89, H 10.52%, calcd for $C_{27}H_{46}O_5.H_2O$, C 69.19, H 10.32%.

Determination of Absolute Configuration of Sugar Units of 1 and 2. Each saponin (1 mg) was subjected to acid-catalyzed methanolysis, before per-trifluoroacetylation of the resultant methyl-glycosides and enantioselective GC analysis (Chirasil-L-Val capillary column) according to procedures used previously.[11] The retention times of the TFAA-derivatized standards were as follows: for the two anomers of D-glucose (26.09 and 29.87 min) and L-glucose (26.06 and 29.77 min). The hydrolysates from **1** and **2** had peaks with identical retention times to those from D-glucose, which co-eluted upon co-injection.

X-ray Crystallography. Crystals of **3** that were suitable for X-ray work were grown via the slow evaporation of a methanol/water solution. An entire sphere of data was collected at 293 K using Cu Kα radiation (1.5418 Å) with an Oxford Diffraction Gemini Ultra CCD diffractometer. Data reduction and empirical absorption corrections were performed with the CrysAlisPro package (Oxford Diffraction, ver. 171.33.42). The structure was solved by direct methods with SHELXS-86 and refined by full matrix least-squares analysis with SHELXL-97.[12] All non-H atoms were refined with anisotropic thermal parameters, while H atoms were constrained at calculated distances and refined with a riding model. The positions of hydroxyl H atoms were determined by refining the C−C−O−H dihedral angles to obtain the best position. The view of compound **3** was generated with ORTEP3,[13] and all calculations were carried out within the WinGX graphical user interface.[14] The absolute configuration of compound **3** was determined using the Bijvoet analysis of Hooft et al.[15] comprising 1638 Friedel pairs and using Student's *t* test statistics ($\nu = 15$). The Hooft *y* parameter was 0.00(6). The probability of the correct enantiomer was 1.000 for a two-component model. Crystallographic data in CIF format have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 800112).

*Crystal data of **3**:* $C_{27}H_{46}O_5 \cdot 1/2CH_3OH$, fw = 466.66, orthorhombic, $P2_12_12_1$ (no. 19), a = 6.1059(1) Å, b = 12.3606(2) Å, c = 34.9384(6) Å, V = 2636.89(8) Å^3, Z = 4, ρ_{calc} = 1.175 g cm^{-3}, N_{tot} = 35496, N_{unique} = 3965 (R_{int} 0.029), N_{obs} ($I > 2\sigma(I)$) = 3522, R_1 (obsd data) = 0.0539, wR_2 (all data) = 0.1866.

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR assignments including HMBC and ROESY correlations for **1** and **2** and ^1H and ^{13}C NMR spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org. Crystallographic data for **3** have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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