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Revision of the Absolute Configurations of Bethosides B and C and Their Aglycone

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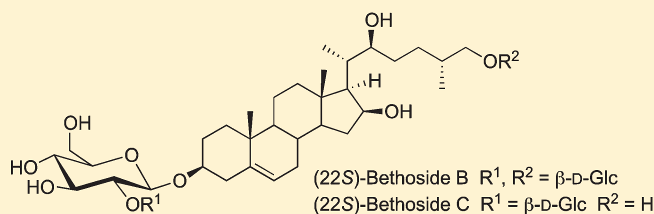
S Supporting Information

ABSTRACT: The absolute stereochemistry of the steroidal saponins bethosides B and C was previously assigned as (22*R*,25*R*) on the basis of work that employed Horeau's method. Our studies of helosides A and B created doubt about both the original assignment and consequently our conclusion that relied upon it. The absolute configurations of bethosides B and C are revised to (22*S*,25*R*) following X-ray crystallographic analysis of their aglycone. Synthesis and full spectral characterization of both the 22*R* and 22*S* aglycones is reported to facilitate future stereochemical assignments in this series of saponins.



Steroidal saponins are common phytochemical constituents of medicinal herbs. They consist of a steroidal nucleus bearing varying numbers of sugar moieties and can possess anti-inflammatory, hemolytic, cytotoxic, antifungal, and antibacterial properties.[1] Our recent investigation into the constituents of the medicinal plant *Chamaelirium luteum* (False Unicorn) resulted in the isolation of two steroidal saponins, helosides A and B (**1** and **2**, Figure 1).[2] These contain an unusual (22*S*,25*R*)-3β,11α,16β,22,26-pentahydroxy-cholest-5-ene skeleton (helogenin, **3**), which lacks the additional rings derived from the C-17 side chain that are characteristic of furo- and spirostanol saponins. The stereochemistry of the side chain in **3** was determined to be (22*S*,25*R*) by X-ray crystallographic analysis.[2]

The closely related 3β,16β,22,26-tetrahydroxy-cholest-5-ene skeleton (**6** is the 25*R* isomer), whose planar structure differs from **3** in lacking C-11 hydroxylation, has been reported as the aglycone in steroidal saponins isolated from *Solanum lyratum* and *Solanum anguivi* (Solanaceae),[3,4] *Allium tuberosum* (Alliaceae),[5] and *Trillium erectum* (Melanthiaceae).[6] Surprisingly, the ^{1}H and ^{13}C NMR spectroscopic data for the side chains of **1** and **2** were in good agreement with those for bethosides B and C isolated from *T. erectum* (**4** and **5**, Figure 1), despite the fact that we had deduced the latter to contain the epimeric 22*R* configuration in the side chain ($\Delta\delta_H \leq 0.04$ ppm, $\Delta\delta_C \leq 0.3$ ppm in C_5D_5N for positions 20 to 27 of the corresponding saponins, Table 1).[6] Our assignment of the 22*R* configuration in **4** and **5** was based upon assignment of the absolute configuration of their aglycone **6**.[3] This has been reported in work by Yahara et al., in which the stereochemistry of a saponin isolated from *S. lyratum* was determined by employing Horeau's method upon a derivative of its corresponding aglycone **6**.[3] A comparison of the limited characterization data (optical rotation and partial ^{1}H NMR data only) reported for synthetic 22*S*-**7** and 22*R*-**7**,[3] with the aglycone tetra-acetate **7** derived from **4** and **5**, had led to the assignment of the 22*R* configuration in these saponins.[6]

Realizing that close agreement of the ^{1}H and ^{13}C NMR spectroscopic data for **1** and **4**, and also **2** and **5**, may still be possible even in saponins differing in configuration at C-22, we investigated whether NMR spectral differences would be revealed by comparison of their acetylated aglycones. Conversion of helogenin **3** and the bethoside aglycone **6** to the corresponding penta-acetate **8** and tetra-acetate **7**, respectively, would also allow direct comparison with the partial characterization in the original literature report.[3] Owing to the small scale of our previous work on bethosides B and C,[6] we chose to isolate their aglycone **6** directly from the acid hydrolysate of a crude *T. erectum* extract via semipreparative reverse phase (RP) HPLC. The complete and previously unreported ^{1}H and ^{13}C NMR assignment of **6** was undertaken prior to derivatization (see Supporting Information). Once again, this revealed chemical shifts in close agreement with those for the side chain of the helogenin aglycone **3** ($\Delta\delta_H \leq 0.04$ ppm, $\Delta\delta_C \leq 0.2$ ppm in C_5D_5N for positions 20 to 27),[2] suggesting that in fact the 22*S* configuration is present in bethosides B and C (**4** and **5**) as well as helosides A and B (**1** and **2**). Conversion of **6** to the tetra-acetate **7** was followed by purification via semipreparative normal phase (NP) HPLC. Since only limited ^{1}H NMR shifts were available in the literature,[3,6] a complete ^{1}H and ^{13}C NMR assignment of **7** was obtained via a combination of 1D TOCSY, and 2D COSY, HSQC and HMBC experiments (see Supporting Information). In accord with our previous work,[6] the ^{1}H NMR spectroscopic

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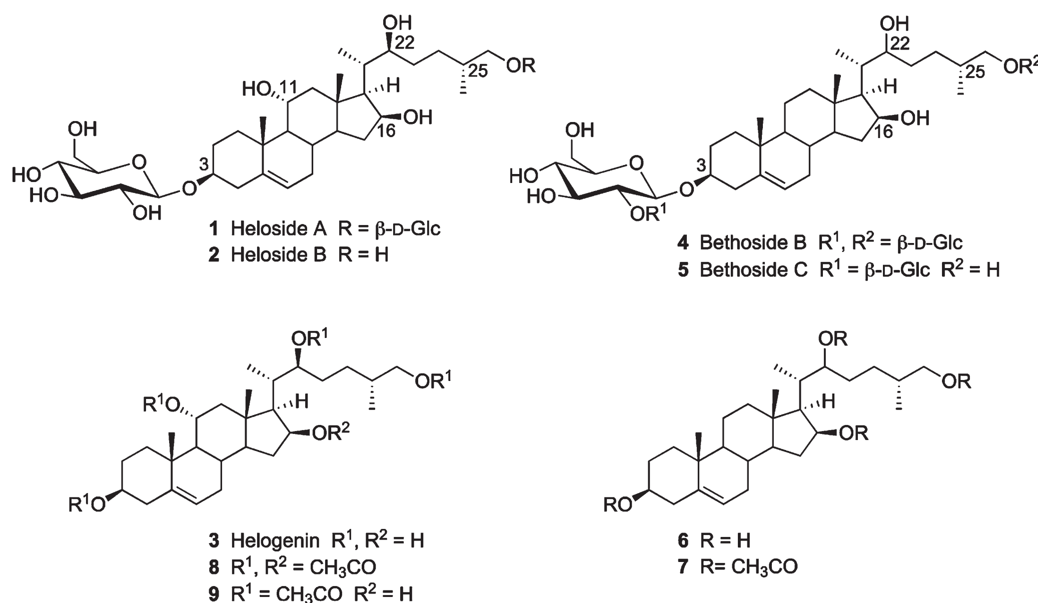


Figure 1. Steroidal saponins isolated from *C. luteum* and *T. erectum* and their derivatives.

Table 1. Comparison of Literature[2,6] NMR Spectroscopic Data for the Side Chain of Structurally Analogous Saponins 1/4 and 2/5 (δ in ppm, C_5D_5N)

	1[a]		4[b]		2[a]		5[b]	
	^{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
20	2.54 m	36.1	2.56 td (6.2, 7.4)	36.1	2.60 m	36.0	2.61 m	36.1
21	1.18 d (7.0)	15.0	1.19 d (7.1)	15.3	1.19 d (7.1)	15.1	1.21 d (7.1)	15.3
22	4.15 m	75.1	4.17 m	75.3	4.21 m	75.4	4.21 m	75.6
23a,b	1.74 m	31.8	1.78 m	31.9	1.84 m	32.0	1.87 m	32.1
24a	1.30 m	31.6	1.32 m	31.6	1.39 m	31.5	1.42 m	31.6
24b	2.07 m		2.10 m		2.20 m		2.22 m	
25	1.96 oct. (6.7)	34.3	1.97 m	34.3	1.91 m	37.0	1.93 m	37.0
26a	3.62 dd (5.9, 9.5)	75.3	3.63 dd (6.0, 9.4)	75.3	3.69 td (5.5, 10.7)	67.6	3.71 m	67.7
26b	3.92 dd (7.0, 9.5)		3.93 dd (7.3, 9.4)		3.78 td (5.3, 10.5)		3.80 m	
27	0.99 d (6.7)	17.7	1.00 d (6.8)	17.7	1.09 d (6.7)	17.7	1.12 d (6.7)	17.7

[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.

data for **7** were in better agreement with the selected chemical shifts reported by Yahara et al. for their synthetic 22*R*-**7** rather than 22*S*-**7**,[3] although our NMR analysis did reveal some discrepancies in the previous assignment. The chemical shifts of H-3 (δ_H 4.58 ppm, tt, *J* = 5.3, 10.8 Hz) and H-22 (δ_H 4.80 ppm, t, *J* = 6.9 Hz) had been mistakenly attributed to H-22 and H-16, respectively, with the result that the signal for H-16 (δ_H 5.18 ppm, dt, *J* = 4.4, 7.6 Hz) was absent from their data.[3] Additionally, the methyl singlet attributable to H_3-18 in **7** was observed at δ_H 0.85 ppm, as compared with δ_H 0.75 ppm reported for 22*R*-**7**.[3] The optical rotation of naturally derived **7** ($[\alpha]^{24}_D$ ~0, *c* = 0.10, $CHCl_3$) also differed from the literature report for 22*R*-**7** ($[\alpha]^{29}_D$ −61.1, *c* = 0.53, $CHCl_3$),[3] inconsistent with stereochemical coincidence.

Acetylation of helogenin **3** obtained from a crude *C. luteum* extract[2] was followed by purification via NP-HPLC to yield pure penta-acetate **8** along with the 16β-hydroxy tetra-acetate **9** (Figure 1). Examination of 1D TOCSY, and 2D COSY, HSQC and HMBC NMR spectra allowed the complete assignment of **8** and **9** (see Supporting Information). The ^{1}H and ^{13}C NMR spectroscopic data for **8** were in very close agreement with those of the naturally derived tetra-acetate **7** ($\Delta\delta_H \leq 0.02$ ppm, $\Delta\delta_C \leq 0.2$ ppm in $CDCl_3$ for positions 20 to 27), again strongly suggesting that 22*S* is the naturally occurring stereochemistry in bethosides B and C (**4** and **5**). Crystallization of aglycone **6** via the slow evaporation of a methanol/water solution pleasingly furnished fine, needle-like, single crystals of its trihydrate that were suitable for X-ray crystallography. The crystal structure analysis confirmed the constitution of **6** and, importantly, revealed the 22*S* absolute configuration of the steroidal side chain (Figure 2). The absolute configuration of the steroidal ring system was initially assumed but subsequently confirmed by the analysis of Hooft et al. using 736 Bijvoet pairs.[7] A cluster of water molecules associated with the hydroxyl groups on C-16, C-22, and C-26 form an extensive H-bonded network that bridges adjacent molecules of **6**. On the basis of this analysis, the structures of **4** and **5** should be revised to (22*S*)-bethoside B and (22*S*)-bethoside C, respectively.

Although it was now clear that 22*S* is the natural stereochemistry in bethosides B and C, it remained unclear whether NMR

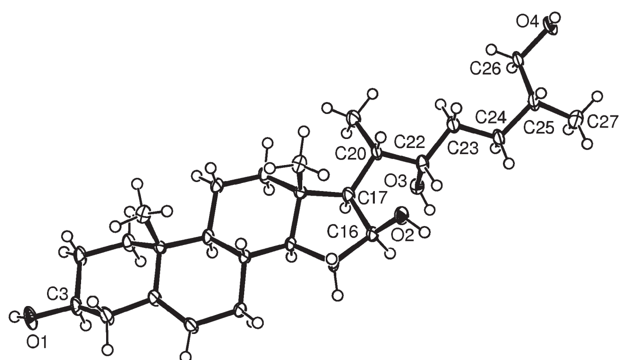
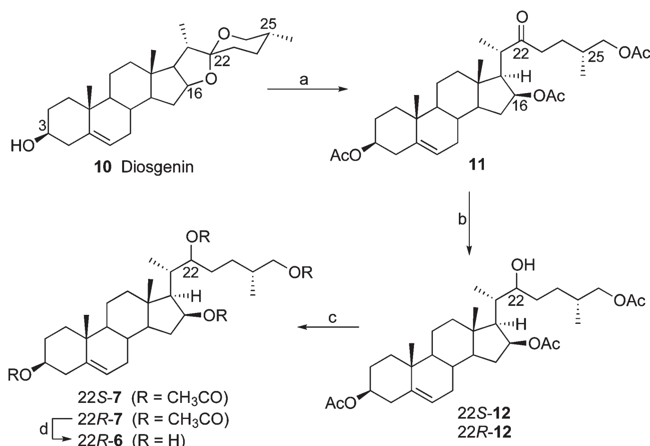


Figure 2. ORTEP view of natural **6** (trihydrate) (30% ellipsoids, water molecules omitted for clarity), the aglycone of bethosides B and C (**4** and **5**).

spectroscopy was useful in distinguishing the 22*R* and 22*S* epimers of their aglycone. We therefore synthesized both 22*S*-**7** and 22*R*-**7**, to enable a detailed comparison of their ^{1}H and ^{13}C NMR spectra. Spiroketal ring opening of diosgenin **10** as reported by Fernandez-Herrera et al.,[8,9] followed by reduction of ketone **11** to epimeric C-22 alcohols 22*S*-**12** and 22*R*-**12** and subsequent acetylation would provide the desired tetra-acetates 22*S*-**7** and 22*R*-**7** (Scheme 1). The required 25*R* configuration was already present in the spiroketal **10**, and the C-22 epimers were expected to be formed during nonstereoselective reduction of **11**. Yahara et al. had previously reported a somewhat more demanding route to 22*S*-**7** and 22*R*-**7** involving $AlCl_3$-catalyzed spiroketal ring opening of diosgenin acetate.[3] In their work, reduction of **11** was then followed by application of a modified Horeau's method to 22*S*-**12** and 22*R*-**12**.[3]

Attempted reduction of **11**, readily available from **10** by treatment with $BF_3 \cdot OEt_2/Ac_2O$,[8,9] with sodium borohydride alone was not successful. However, sodium borohydride in the presence of $CeCl_3 \cdot 7H_2O$ was tolerably efficient, and subsequent purification via NP-HPLC afforded triacetates 22*S*-**12** and 22*R*-**12** in an approximately 1:3 ratio in low yield along with a significant amount of recovered starting material **11** (55%). The ^{1}H NMR data of our 22*R*-**12** (*vide infra*) were in fair agreement with the partial characterization (limited ^{1}H NMR shifts only) reported by Yahara et al. for their purported 22*S* triacetate and not the compound they assigned as the 22*R* isomer.[3] Given the concordance of our 22*R*-**12** with Yahara's 22*S* isomer, we focused on comparison of our 22*S*-**12** (*vide infra*) with Yahara's 22*R* isomer. However, our NMR data for 22*S*-**12** do not match the data reported for their 22*R* triacetate (or indeed their 22*S* triacetate). In particular, the signals for H-16 (δ_H 5.22 ppm, dt, J = 4.1, 7.7 Hz) and H_3-18 (δ_H 0.87 ppm, s) in our 22*S*-**12** were reported as δ_H 4.93 ppm ($\Delta\delta_H = -0.29$ ppm)[3] and δ_H 0.74 ppm ($\Delta\delta_H = -0.13$ ppm),[3] respectively, for Yahara's 22*R* isomer. Additionally, the reported optical rotation of their putative 22*R* triacetate ($[\alpha]^{29}_D$ −69.2, c = 0.37, $CHCl_3$)[3] differed from that of our 22*S*-**12** ($[\alpha]^{24}_D$ −5.0, c = 0.09, $CHCl_3$). Acetylation of 22*S*-**12** and 22*R*-**12** afforded, respectively, the tetra-acetates 22*S*-**7** and 22*R*-**7**. The ^{1}H and ^{13}C NMR spectra of 22*S*-**7** were identical to those of **7** derived from the authentic aglycone of bethosides B and C, whose stereochemistry was above determined to be (22*S*,25*R*) by X-ray crystallography, and distinct from those of 22*R*-**7**. A region of the ^{1}H NMR spectra of natural **7**, 22*S*-**7**, 22*R*-**7**, and the helogenin penta-acetate **8** is shown in Figure 3 for comparison. For 22*R*-**7**, both the complete

Scheme 1[a]



[a] Reagents and conditions: (a) Fernandez-Herrera et al.[8,9] (b) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, CH_2Cl_2, 25°C. **11**: 55%, semi-preparative NP-HPLC. 22*S*-**12**: 3%. 22*R*-**12**: 10%. (c) Ac_2O, pyridine, 25°C. 22*S*-**7**: 53%. 22*R*-**7**: 66%. (d) LiOH, MeOH, THF, H_2O, 25°C, 39%.

^{1}H and ^{13}C NMR assignment (see Supporting Information) and optical rotation were in agreement with the partial characterization reported for the tetra-acetate assigned as 22*S* by Yahara et al.[3] As a final step, 22*R*-**7** was converted to the tetra-ol 22*R*-**6**, whose ^{1}H and ^{13}C NMR chemical shifts diverged from those of natural (22*S*) **6** most notably at positions 16 (δ_H 4.61 ppm, $\Delta\delta_H$ = −0.15 ppm; δ_C 71.4 ppm, $\Delta\delta_C$ = −0.1 ppm), 21 (δ_H 1.29 ppm, $\Delta\delta_H$ = +0.07 ppm; δ_C 13.7 ppm, $\Delta\delta_C$ = −1.6 ppm) and 22 (δ_H 4.48 ppm, $\Delta\delta_H$ = +0.25 ppm; δ_C 73.4 ppm, $\Delta\delta_C$ = −2.1 ppm).

Although the ^{1}H and ^{13}C NMR data for the *S. lyratum* saponin are surprisingly (given expected solubility) reported in $CDCl_3$[3] and are therefore not directly comparable, the ^{1}H NMR data provided for the tetra-acetate derived from its aglycone suggest that the stereochemistry of this saponin should also be revised to 22*S*. Due to the anomalous differences between the physical properties of our 22*S*-**7** and the 22*R* tetra-acetate reported by Yahara et al.[3] and without full characterization of the compounds in the literature, it is difficult to determine the basis for the misassignment. Certainly, problems with the application of Horeau's method to complex molecules have been reported previously.[10,11] The 3*β*,16*β*,22,26-tetrahydroxy-cholest-5-ene skeleton (**6** is the 25*R* isomer) is present in a number of steroidal saponins,[4,5,12] and the data presented herein allow the unambiguous determination of stereochemistry within either the aglycone tetra-ol or the tetra-acetate derivative of this recurring structural motif.

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. ^{1}H NMR spectra were recorded at 400, 500, or 750 MHz with the residual protonated signal in the $CDCl_3$ (δ_H 7.24 ppm) or C_5D_5N (δ_H 8.71 ppm) solvent as internal standard. ^{13}C NMR spectra were recorded at 100, 125, or 188 MHz with the central line of the $CDCl_3$ (δ_C 77.0 ppm) or C_5D_5N (δ_C 149.9 ppm) signal as internal standard. Low-resolution and tandem mass spectra were recorded on an ion trap spectrometer (positive- and negative-ion ESI). High-resolution mass spectra were acquired on a MicrOTOF-Q instrument (positive-ion ESI)

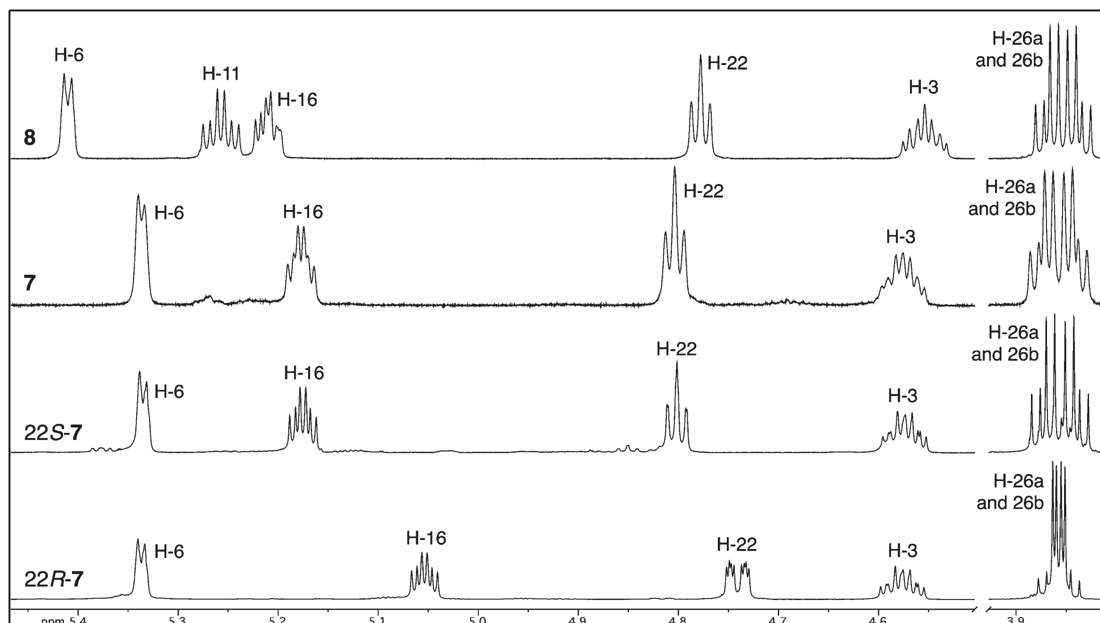


Figure 3. Segment of the ^{1}H NMR spectra of naturally derived **7** and **8** and synthetic 22*S*-**7** and 22*R*-**7** (750 MHz, $CDCl_3$).

with internal calibration. Semipreparative RP- or NP-HPLC was performed on an HPLC system equipped with an ELSD-LT detector (52 or 35 °C, N_2 pressure: 200 KPa), column oven (40 or 25 °C), and an HPLC column (C-18 or Silica, 5 μm, 250 × 10 mm).

Extraction and Isolation. Powdered roots of *T. erectum* (31 g) were extracted (85% aq MeOH, 400 mL) with sonication (3 × 10 min). Following filtration and removal of the solvent in vacuo, the crude extract was dissolved in aq MeOH (80%, 120 mL) and aq HCl (32%, 20 mL) and heated under reflux for 2.5 h. After cooling, water (150 mL) was added, and the reaction mixture extracted with diethyl ether (5 × 200 mL). The combined organic layers were washed with aq NaOH (5% w/v, 5 × 200 mL) and dried ($MgSO_4$) before removal of the solvent in vacuo. The crude residue was dissolved (90% aq MeOH, 30 mL), filtered, and purified by semipreparative RP-HPLC (gradient of 45% to 70% aq acetonitrile over 30 min, 2 mL/min). Compound **6** was collected as a pure fraction (t_R: 21.6 min, 27.4 mg).

(22*S*,25*R*)-3β,16β,22,26-Tetrahydroxy-cholest-5-ene (6). White solid; mp 209−210 °C; $[\alpha]^{25}_D$ −27.3 (*c* = 0.15, CH_3OH); ^{1}H NMR (C_5D_5N, 500 MHz) δ_H 6.43 (br d, *J* = 5.1 Hz, 1H), 6.17 (br d, *J* = 3.9 Hz, 1H), 5.98 (m, 1H), 5.93 (br d, *J* = 3.6 Hz, 1H), 5.41 (d, *J* = 4.7 Hz, 1H, H-6), 4.76 (tt, *J* = 3.8, 7.4 Hz, 1H, H-16), 4.23 (m, 1H, H-22), 3.84 (m, 1H, H-3), 3.80 (m, 1H, H-26b), 3.71 (m, 1H, H-26a), 2.55−2.67 (m, 3H), 2.30 (td, *J* = 7.6, 13.0 Hz, 1H, H-15b), 2.22 (m, 1H, H-24b), 2.03−2.12 (m, 2H), 1.75−2.02 (m, 6H), 1.67 (dd, *J* = 7.0, 11.0 Hz, 1H, H-17), 1.37−1.63 (m, 6H), 1.22 (d, *J* = 7.1 Hz, 3H, H_3-21), 0.90−1.21 (m, 4H), 1.19 (s, 3H, H_3-18), 1.11 (d, *J* = 6.7 Hz, 3H, H_3-27), 1.07 (s, 3H, H_3-19); ^{13}C NMR (C_5D_5N, 125 MHz) δ_C 142.1 (C-5), 121.3 (C-6), 75.5 (C-22), 71.5 (C-16), 71.3 (C-3), 67.6 (C-26), 58.2 (C-17), 55.1 (C-14), 50.7 (C-9), 43.6 (C-4), 42.7 (C-13), 40.5 (C-12), 37.9 (C-1), 37.2 (C-15), 37.1 (C-25), 37.0 (C-10), 36.1 (C-20), 32.7 (C-2), 32.3 (C-7), 32.1 (C-23), 32.0 (C-8), 31.6 (C-24), 21.2 (C-11), 19.7 (C-19), 17.7 (C-27), 15.3 (C-21), 13.5 (C-18); positive-ion ESIMS *m*/*z* 473, 457; negative-ion ESIMS *m*/*z* 433; HRESIMS *m*/*z* 457.3286 $[M + Na]^+$ (calcd for $C_{27}H_{46}NaO_4$, 457.3288).

X-ray Crystallography. Crystals of **6** that were suitable for X-ray work were grown via the slow evaporation of a methanol/water solution. Data were acquired with an Oxford Diffraction Gemini Ultra CCD diffractometer with Cu Kα radiation (1.5418 Å). Data reduction was performed with the CrysAlisPro software (Oxford Diffraction vers. 171.34.40). The structure was solved by direct methods with SHELXS-86 and refined with SHELXL-97.[13] The thermal ellipsoid plot was produced with ORTEP3,[14] and all calculations were carried out within the WinGX program.[15] Crystallographic data in CIF format have been deposited with the Cambridge Crystallographic Data Centre (CCDC No. 826278).

(22*S*,25*R*)-3β,16β,22,26-Tetra-acetoxy-cholest-5-ene (7). Compound **6** (4.1 mg, 9.4 μmol) was dissolved in pyridine (1 mL) and acetic anhydride (500 μL), and the mixture was stirred for 24 h at 25 °C. The solution was then concentrated to dryness under a stream of nitrogen, and the residue was dissolved (10% hexane in ethyl acetate, 2 mL), filtered, and purified by semipreparative NP-HPLC (isocratic conditions of 19% ethyl acetate in hexane, 2 mL/min) to yield **7** as a colorless oil (t_R: 46.3 min, 1.0 mg, 18%): $[\alpha]^{24}_D$ ~0 (*c* = 0.10, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 750 MHz) δ_H 5.34 (d, *J* = 4.5 Hz, 1H, H-6), 5.18 (dt, *J* = 4.4, 7.6 Hz, 1H, H-16), 4.80 (t, *J* = 6.9 Hz, 1H, H-22), 4.58 (tt, *J* = 5.3, 10.8 Hz, 1H, H-3), 3.84 and 3.87 (dABq, *J* = 6.4, 10.8 Hz, 2H, H-26a and H-26b), 2.42 (td, *J* = 7.6, 13.7 Hz, 1H, H-15b), 2.29 (m, 2H, H-4a and H-4b), 2.09 (qd, *J* = 6.1, 11.9 Hz, 1H, H-20), 2.025 (s, 3H), 2.019 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.98 (m, 1H, H-12b), 1.92 (m, 1H, H-7b), 1.80−1.87 (m, 2H), 1.73 (o, *J* = 6.6 Hz, 1H, H-25), 1.42−1.61 (m, 7H), 1.31 (m, 1H, H-24b), 1.27 (dd, *J* = 7.6, 11.2 Hz, 1H, H-17), 1.02−1.18 (m, 4H), 1.00 (s, 3H, H_3-19), 0.98 (d, *J* = 6.9 Hz, 3H, H_3-21), 0.90−0.96 (m, 2H), 0.90 (d, *J* = 6.7 Hz, 3H, H_3-27), 0.85 (s, 3H, H_3-18); ^{13}C NMR ($CDCl_3$, 188 MHz) δ_C 171.1, 170.8, 170.5, 169.9, 139.6 (C-5), 122.3 (C-6), 75.8 (C-16), 75.3 (C-22), 73.8 (C-3), 69.0 (C-26), 55.9 (C-17), 54.4 (C-14), 49.9 (C-9), 42.4 (C-13), 39.6 (C-12), 38.1 (C-4), 36.9 (C-1), 36.5 (C-10), 35.2 (C-15), 33.1 (C-20), 32.5 (C-25), 31.5 (C-7), 31.4 (C-8), 29.6 (2C, C-23 and C-24), 27.7 (C-2), 21.4, 21.3, 21.1, 20.9, 20.7 (C-11), 19.3 (C-19), 16.7 (C-27), 12.5 (C-18), 12.1 (C-21); positive-ion ESIMS *m*/*z* 625; negative-ion ESIMS *m*/*z* 601; HRESIMS *m*/*z* 625.3724 $[M + Na]^+$ (calcd for $C_{35}H_{54}NaO_8$, 625.3711).

(22*S*,25*R*)-3β,11α,16β,22,26-Penta-acetoxy-cholest-5-ene (8) and (22*S*,25*R*)-3β,11α,22,26-Tetra-acetoxy-16β-hydroxy-cholest-5-ene (9). Helogenin **3** (5.1 mg, 11.3 μmol) isolated from *C. luteum*[2] was dissolved in pyridine (1 mL) and acetic anhydride (500 μL), and the mixture was stirred for 24 h at 25 °C. The solution was then concentrated to dryness under a stream of nitrogen, and the residue was dissolved (10% hexane in ethyl acetate, 2 mL), filtered, and purified by semipreparative NP-HPLC

(isocratic conditions of 27% ethyl acetate in hexane, 2 mL/min). The first compound to elute was **8** (t_R: 40.7 min, 2.6 mg, 35%): colorless oil; $[\alpha]^{23}_D$ −14.5 (c = 0.26, $CHCl_3$); ^1H NMR ($CDCl_3$, 750 MHz) δ_H 5.41 (d, J = 5.5 Hz, 1H, H-6), 5.26 (dt, J = 5.3, 10.7 Hz, 1H, H-11), 5.21 (dt, J = 3.9, 7.6 Hz, 1H, H-16), 4.78 (t, J = 7.1 Hz, 1H, H-22), 4.55 (tt, J = 4.9, 11.4 Hz, 1H, H-3), 3.83 and 3.86 (dABq, J = 6.4, 10.8 Hz, 2H, H-26a and H-26b), 2.44 (m, 1H, H-15b), 2.25−2.33 (m, 3H), 2.07 (m, 1H, H-20), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.94 (m, 1H, H-7b), 1.78−1.85 (m, 2H), 1.73 (o, J = 6.6 Hz, 1H, H-25), 1.44−1.61 (m, 5H), 1.34 (t, J = 10.7 Hz, 1H, H-9), 1.21−1.32 (m, 3H), 1.15 (t, J = 11.4 Hz, 1H, H-12a), 1.08 (s, 3H, H_3-19), 1.04−1.05 (m, 3H), 0.96 (d, J = 7.0 Hz, 3H, H_3-21), 0.92 (s, 3H, H_3-18), 0.90 (d, J = 6.7 Hz, 3H, H_3-27); ^{13}C NMR ($CDCl_3$, 188 MHz) δ_C 171.1, 170.8, 170.5, 170.2, 169.9, 139.6 (C-5), 122.4 (C-6), 75.7 (C-16), 75.1 (C-22), 73.5 (C-3), 71.3 (C-11), 69.0 (C-26), 55.7 (C-17), 53.2 (C-14), 52.8 (C-9), 45.7 (C-12), 42.6 (C-13), 38.5 (C-4), 38.1 (C-1), 38.0 (C-10), 35.0 (C-15), 33.0 (C-20), 32.5 (C-25), 31.7 (C-7), 31.4 (C-8), 29.6 (2C, C-23 and C-24), 27.9 (C-2), 21.9, 21.4, 21.2, 21.1, 20.9, 19.2 (C-19), 16.7 (C-27), 13.2 (C-18), 12.0 (C-21); positive-ion ESIMS m/z 683, 699; negative-ion ESIMS m/z 659; HRESIMS m/z 683.3751 $[M + Na]^+$ (calcd for $C_{37}H_{56}NaO_{10}$, 683.3766). The second compound to elute was **9** (t_R: 43.5 min, 1.4 mg, 20%): colorless oil; $[\alpha]^{23}_D$ −39.5 (c = 0.14, $CHCl_3$); ^1H NMR ($CDCl_3$, 750 MHz) δ_H 5.42 (d, J = 5.7 Hz, 1H, H-6), 5.26 (dt, J = 5.3, 10.7 Hz, 1H, H-11), 5.19 (dd, J = 5.6, 8.2 Hz, 1H, H-22), 4.56 (tt, J = 5.4, 11.0 Hz, 1H, H-3), 4.44 (m, 1H, H-16), 3.98 (dd, J = 5.9, 10.8 Hz, 1H, H-26b), 3.74 (dd, J = 7.1, 10.8 Hz, 1H, H-26a), 2.28−2.34 (m, 2H), 2.26 (dd, J = 5.2, 12.0 Hz, 1H, H-12b), 2.23 (td, J = 7.6, 13.1 Hz, 1H, H-15b), 2.12 (m, 1H, H-20), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H), 1.97 (m, 1H, H-7b), 1.84 (td, J = 3.6, 13.8 Hz, 1H, H-1b), 1.75−1.82 (m, 2H), 1.50−1.63 (m, 5H), 1.22−1.35 (m, 3H), 1.07−1.18 (m, 4H), 1.09 (s, 3H, H_3-19), 1.00 (ddd, J = 7.2, 10.8, 13.2 Hz, 1H, H-14), 0.96 (d, J = 7.1 Hz, 3H, H_3-21), 0.95 (s, 3H, H_3-18), 0.90 (d, J = 6.7 Hz, 3H, H_3-27); ^{13}C NMR ($CDCl_3$, 188 MHz) δ_C 171.3, 171.2, 170.5, 170.2, 139.7 (C-5), 122.5 (C-6), 75.7 (C-22), 73.6 (C-3), 71.8 (C-16), 71.5 (C-11), 69.2 (C-26), 57.2 (C-17), 53.2 (C-14), 52.9 (C-9), 46.0 (C-12), 42.4 (C-13), 38.5 (C-4), 38.1 (2C, C-1 and C-10), 37.1 (C-15), 33.1 (C-20), 32.1 (C-25), 31.9 (C-7), 31.5 (C-8), 29.4 (2C, C-23 and C-24), 27.9 (C-2), 22.0, 21.4, 21.2, 21.0, 19.2 (C-19), 16.9 (C-27), 13.6 (C-18), 12.3 (C-21); positive-ion ESIMS m/z 641, 657; HRESIMS m/z 641.3663 $[M + Na]^+$ (calcd for $C_{35}H_{54}NaO_9$, 641.3660).

(22*S*,25*R*)-3β,16β,26-Triacetoxy-22-hydroxy-cholest-5-ene (22*S*-12) and (22*R*,25*R*)-3β,16β,26-Triacetoxy-22-hydroxy-cholest-5-ene (22*R*-12). Compound **11** (105 mg, 0.19 mmol) was dissolved in a mixture of MeOH and DCM (1:1, 10 mL), then $CeCl_3 \cdot 7H_2O$ (100 mg) and $NaBH_4$ (50 mg) were added, and the mixture stirred for 3 h at 25 °C. Following addition of DCM (100 mL), the mixture was washed with 1 N HCl (3 × 100 mL) and brine (3 × 100 mL) and then dried ($MgSO_4$), and the solvent was removed in vacuo. The residue was purified by flash chromatography (25% ethyl acetate in petroleum ether, silica gel 60) to yield a mixture of 22*S*-**12** and 22*R*-**12** (31.5 mg, 30%; recovered starting material, 55%), which was further purified by semipreparative NP-HPLC (gradient of 30−40% ethyl acetate in hexane over 10 min increasing to 50% over 25 min, 2 mL/min). The first compound to elute was 22*S*-**12** (t_R: 27.6 min, 3.2 mg, 3%): amorphous solid; $[\alpha]^{24}_D$ −5.0 (c = 0.09, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ_H 5.34 (d, J = 5.1 Hz, 1H, H-6), 5.22 (dt, J = 4.1, 7.7 Hz, 1H, H-16), 4.58 (m, 1H, H-3), 3.88 (dABq, J = 6.4, 10.9 Hz, 2H, H-26a and H-26b), 3.43 (t, J = 5.9 Hz, 1H, H-22), 2.39 (td, J = 7.5, 13.1 Hz, 1H), 2.24−2.34 (m, 2H), 2.03 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.79−2.02 (m, 5H), 1.75 (m, 1H), 1.64 (dd, J = 7.4, 12.3 Hz, 1H), 1.38−1.61 (m, 8H), 1.01 (s, 3H, H_3-19), 0.92 (d, J = 6.9 Hz, 3H, H_3-21), 0.91 (d, J = 6.7 Hz, 3H, H_3-27), 0.87 (s, 3H, H_3-18), 0.84−1.25 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 171.2, 170.5, 170.4, 139.7, 122.3, 75.3, 73.9, 72.8, 69.1, 55.6, 54.5, 49.8, 42.3, 39.5, 38.0, 36.9, 36.5, 35.0, 34.2, 32.8, 32.7, 31.6, 31.4, 30.1, 27.7, 21.4, 21.3, 20.9, 20.7 19.3, 16.8, 12.5, 10.8; positive-ion ESIMS m/z 583, 599; HRESIMS m/z 583.3610 $[M + Na]^+$ (calcd for $C_{33}H_{52}NaO_7$, 583.3605). The second compound to elute was 22*R*-**12** (t_R: 31.5 min, 11.0 mg, 10%): colorless oil; $[\alpha]^{24}_D$ 2.1 (c = 0.43, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ_H 5.34 (d, J = 4.5 Hz, 1H, H-6), 5.04 (dt, J = 4.0, 4.6 Hz, 1H, H-16), 4.58 (m, 1H, H-3), 3.86 and 3.92 (dABq, J = 6.1, 10.8 Hz, 2H, H-26a and H-26b), 3.44 (m, 1H, H-22), 2.38 (td, J = 7.5, 13.6 Hz, 1H), 2.04 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.71−2.33 (m, 9H), 1.00 (s, 3H, H_3-19), 0.95 (d, J = 6.9 Hz, 3H, H_3-21), 0.91 (d, J = 6.7 Hz, 3H, H_3-27), 0.88 (s, 3H, H_3-18), 0.82−1.65 (m, 14H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 171.3, 170.7, 170.5, 139.7, 122.2, 74.9, 73.8, 73.0. 69.3, 56.1, 54.5, 49.9, 42.8, 39.5, 38.0, 36.9, 36.5, 35.2, 32.6, 31.6, 31.4, 30.5, 29.7, 27.7, 27.6, 21.4, 21.3, 21.0, 20.7, 19.3, 16.8, 12.5, 12.1; positive-ion ESIMS m/z 583, 599; HRESIMS m/z 583.3588 $[M + Na]^+$ (calcd for $C_{33}H_{52}NaO_7$, 583.3605).

(22*S*,25*R*)-3β,16β,22,26-Tetra-acetoxy-cholest-5-ene (22*S*-7) and (22*R*,25*R*)-3β,16β,22,26-Tetra-acetoxy-cholest-5-ene (22*R*-7). Compound 22*S*-**12** (2.3 mg, 4.1 μmol) was dissolved in pyridine (1 mL) and acetic anhydride (500 μL), and the mixture was stirred for 48 h at 25 °C. The solution was then concentrated to dryness under a stream of nitrogen to yield 22*S*-**7** as a colorless oil (1.3 mg, 53%): $[\alpha]^{24}_D$ ~0 (c = 0.13, $CHCl_3$); ^1H ($CDCl_3$, 750 MHz) and ^{13}C NMR ($CDCl_3$, 188 MHz) were identical to those of **7** derived from the natural aglycone **6**; positive-ion ESIMS m/z 625; HRESIMS m/z 625.3715 $[M + Na]^+$ (calcd for $C_{35}H_{54}NaO_8$, 625.3711). Under identical conditions starting from 22*R*-**12** (8.1 mg, 14.5 μmol), 22*R*-**7** was obtained as a colorless oil (5.8 mg, 66%): $[\alpha]^{24}_D$ 7.8 (c = 0.58, $CHCl_3$); ^1H NMR ($CDCl_3$, 750 MHz) δ_H 5.33 (d, J = 4.9 Hz, 1H, H-6), 5.05 (dt, J = 3.9, 7.6 Hz, 1H, H-16), 4.74 (ddd, J = 2.1, 3.3, 11.1 Hz, 1H, H-22), 4.57 (m, 1H, H-3), 3.83 and 3.87 (dABq, J = 6.2, 10.8 Hz, 2H, H-26a and H-26b), 2.39 (td, J = 7.6, 13.6 Hz, 1H, H-15b), 2.19−2.32 (m, 3H), 2.08 (s, 3H), 2.04 (s, 3H), 1.79−2.02 (m, 4H), 2.01 (s, 6H), 1.72 (m, 1H, H-25), 1.04−1.63 (m, 13H), 1.00 (s, 3H, H_3-19), 0.90−0.98 (m, 2H), 0.95 (d, J = 6.9 Hz, 3H, H_3-21), 0.89 (d, J = 6.7 Hz, 3H, H_3-27), 0.87 (s, 3H, H_3-18); ^{13}C NMR ($CDCl_3$, 188 MHz) δ_C 171.4, 171.3, 170.6, 170.1, 139.7 (C-5), 122.2 (C-6), 75.0 (C-22), 73.9 (C-16), 73.8 (C-3), 69.2 (C-26), 56.3 (C-17), 54.5 (C-14), 49.9 (C-9), 43.0 (C-13), 39.6 (C-12), 38.0 (C-4), 36.9 (C-1), 36.5 (C-10), 35.4 (C-15), 33.8 (C-20), 32.3 (C-25), 31.5 (C-7), 31.4 (C-8), 29.8 (C-24), 27.7 (C-2), 24.6 (C-23), 21.4 (2C), 21.2, 20.9, 20.7 (C-11), 19.2 (C-19), 16.7 (C-27), 12.6 (C-21), 12.5 (C-18); positive-ion ESIMS m/z 625; HRESIMS m/z 625.3689 $[M + Na]^+$ (calcd for $C_{35}H_{54}NaO_8$, 625.3711).

(22*R*,25*R*)-3β,16β,22,26-Tetra-hydroxy-cholest-5-ene (22*R*-6). Compound 22*R*-**12** (4.3 mg, 7.7 μmol) was dissolved in MeOH (1 mL), THF (1 mL) and water (50 μL) before addition of LiOH (10 mg). The mixture was stirred for 24 h at 25 °C, then brine (1 mL) was added, and the reaction extracted into ethyl acetate (2 mL). The ethyl acetate layer was concentrated under a stream of nitrogen, then dissolved (90% aq MeOH, 2 mL), filtered, and purified by semipreparative RP-HPLC (conditions of 45% to 60% aq acetonitrile over 20 min, 2 mL/min) to yield 22*R*-**6** as an amorphous solid (t_R: 17.5 min, 1.3 mg, 39%): $[\alpha]^{23}_D$ −3.3 (c = 0.13, CH_3OH); ^1H (C_5D_5N, 750 MHz) δ_H 6.17 (br s, 1H), 5.95 (m, 2H), 5.82 (br s, 1H), 5.42 (d, J = 5.1 Hz, 1H, H-6), 4.61 (tt, J = 3.9, 7.6 Hz, 1H, H-16), 4.48 (m, 1H, H-22), 3.85 (m, 1H, H-3), 3.82 (td, J = 5.2, 10.3 Hz, 1H, H-26b), 3.73 (td, J = 5.3, 10.4 Hz, 1H, H-26a), 2.80 (m, 1H), 2.59−2.66 (m, 2H), 2.30 (td, J = 7.6, 12.7 Hz, 1H), 2.09 (m, 2H), 1.94−2.02 (m, 3H), 1.77−1.90 (m, 5H), 1.56−1.63 (m, 2H), 1.46−1.56 (m, 3H), 1.29 (d, J = 7.0 Hz, 3H, H_3-21), 1.26 (dd, J = 7.2, 11.3 Hz, 1H), 1.17 (s, 3H, H_3-18), 1.15 (d, J = 6.6 Hz, 3H, H_3-27), 1.12−1.20 (m, 2H), 1.06 (s, 3H, H_3-19), 1.01 (m, 1H), 0.90 (ddd, J = 7.6, 10.0, 13.5 Hz, 1H); ^{13}C NMR (C_5D_5N, 188 MHz) δ_C 142.1 (C-5), 121.2 (C-6), 73.4 (C-22), 71.4 (C-16), 71.3 (C-3), 68.1 (C-26), 59.8, 54.9, 50.7, 43.5, 43.0, 40.5, 37.9, 37.8, 37.5, 37.0 (2C), 32.7, 32.3, 32.0, 31.2, 29.6, 21.2, 19.7 (C-19), 17.5 (C-27), 13.7 (C-21), 13.5 (C-18); positive-ion ESIMS m/z 457; negative-ion ESIMS m/z 433; HRESIMS m/z 457.3306 $[M + Na]^+$ (calcd for $C_{27}H_{46}NaO_4$, 457.3288).

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR spectra of **6**−**9**, 22*S*-**12**, 22*R*-**12**, 22*S*-**7**, 22*R*-**7**, and 22*R*-**6**, and complete ^{1}H and ^{13}C NMR assignment of **6**−**9** and 22*R*-**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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