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Downeyoside A and B, Two New Sulphated Steroid Glucuronides from the Starfish *Henricia downeyae*

Elio Palagiano, Franco Zollo, Luigi Minale* and Luigi Gomez Paloma

Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II", Via D. Montesano 49, 80131, Napoli, Italy

Maria Iorizzi

Università degli Studi del Molise, Facoltà di Agraria, Via Tiberio 21/A, 86100 Campobasso, Italy

Patrick Bryan and James Mc Clintock

Department of Biology, University of Alabama at Birmingham, Alabama 35294-1170 U.S.A

Thomas Hopkins

Department of Biological Sciences, University of Alabama, Tuscaloosa, Alabama 35487-0344 U.S.A

Denis Riou and Christos Roussakis

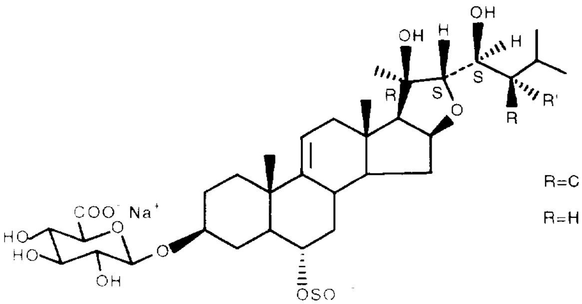
ISOMER, Faculté de Pharmacie, 1 rue Gaston Veil, University, 44035 Nantes, France

Abstract.- Two new sulphated polyhydroxylated steroid glucuronides, downeyoside A and B (**1**, **2**), C-24 methyl epimers possessing an ethereal ring linking C-16 to C-22 of the steroid, were isolated from the starfish *Henricia downeyae* collected from the Gulf of Mexico. Their structures were determined by spectral analysis and the stereochemistry of the highly oxygenated steroid side chain was derived from NMR data combined with molecular dynamics calculation. Compounds **1** and **2** were tested against non-small-cell lung human carcinoma cells and found to be cytotoxic with IC50 of 60 μg/ml and 36 μg/ml, respectively.

As a part of a project on the investigation of echinoderms from the Gulf of Mexico, we have examined a starfish, *Henricia downeyae* (family Echinasteridae, order Valvatida), and have isolated two new sulphated steroid glucuronides, named downeyoside A and B (**1**, **2**), together with eleven more novel steroid glycoside constituents and seven known ones. In this paper we describe the isolation and structure elucidation of **1** and **2**; the remaining *H. downeyae* constituents will be the subject of a further paper. *Henricia downeyae* was collected from the northern Gulf of Mexico in July 1993. Freeze-dried animals (150 g) were extracted with water and then with Me_2CO, the aqueous extracts were passed through a column of Amberlite XAD-2 and the organic material eluted with MeOH; the Me_2CO extract, after evaporation of the solvent, was partioned against organic solvents. Purification of Amberlite XAD-2 MeOH eluate combined with the *n*-BuOH fractions by gel filtration, droplet counter-current chromatography and reversed-phase hplc afforded compounds **1** (3.5 mg) and **2** (3.0 mg).

RESULTS AND DISCUSSION

Compound **1**, obtained as a glassy material, $[\alpha]_D$=-13.3° (in MeOH), gave FABMS (negative ion) pseudomolecular ion peaks at *m/z* 739 $[M_{Na}-H]^-$, corresponding to the anion of a monosodium salt, and 717 $[M_H-H]^-$, corresponding to the anion of the acid, suggestive of the presence of two anionic functions in the molecule, (ν_{max} 1240 cm^{-1} a sulphate, and 1700 cm^{-1} a carboxylic acid, δ_C 177.0 ppm). A fragment peak at *m/z* 541 corresponding to the loss of 176 mass units from 717 (and or 198 from 739), was interpreted as due to the loss of an uronic acid unit (and or a sodium uronate unit), which was identified as glucuronic acid after acid methanolysis of **1** and GLC of the silylated mixture.



R=CH_3, R'=H Downeyoside A **1**
R=H, R'=CH_3 Downeyoside B **2**

The nmr spectra of **1** supported the presence of a β-glucuronic acid unit and showed the presence of a trisubstituted double bond [1H, brd, δ 5.41, J=5.2 Hz, ^{13}C δ 118.8 (=CH), 146.4 (s)] as well as two oxygenated methine protons at δ 3.74 m, with the shape typical for a 3β-O- grouping and at δ 4.36 (dt, J=10.8, 4.4 Hz), already observed in the spectra of 6α-sulfoxysteroids[1]. Also present were singlets at δ 1.18 and 1.08 corresponding to Me-18 and Me-19 respectively. These nmr signals were indicative for $\Delta^{9(11)}$-3β,6α-dioxysteroidal tetracyclic nucleus, which is a common structural feature of the steroid aglycone of the asterosaponins[2]. The sulphate group in **1** was located at C-6 by considering the nmr signal of H-6 at δ 4.36 upfield shifted to δ 3.61 (dt, J=10.5, 4.0 Hz) in the desulphated analogue (solvolysis in dioxane-pyridine 50% of **1**), while the sugar moiety was located at C-3 because of the chemical shifts of C-3, downfield shifted to 78.8 ppm, and C-2 and C-4 upfield to 30.1 and 30.2 ppm, respectively, relative to 3β,6α-dihydroxysteroids [*e. g.* in 5α-cholestane-3β,6α-diol: C-2 : 31.3, C-3 : 71.1 and C-4 : 32.3[3]], (glycosidation shifts[4,5,6,7]). This was opposite of what found in the asterosaponins from starfishes, which have the sulphate at C-3 and the oligosaccharide at C-6 [2].

Continuing now with the analysis of ^1H nmr data for **1** (Table 1), two one-proton signals at 4.49 m, 3.99 (d, J=9.0 Hz) and 3.62 (dd, J=9.0, 4.6 Hz) denoted the presence of three oxygenated methine protons : in agreement with this the ^{13}C nmr spectrum showed three signals at δ 83.6, 83.2 and 75.0 ppm. The presence of a 20-hydroxy-24-methyl steroid side chain was indicated by a methyl singlet at δ 1.41 (Me-21), and by three methyl doublets at δ 0.89, 0.94 and 0.95; in agreement with the presence of a 20-hydroxy group the ^{13}C nmr spectrum contained a quaternary signal at δ 81.5 ppm

The sugar residue accounts for $C_6H_9O_6$ out of the $C_{34}H_{53}O_{11}$ SO_3^- molecular formula, as deduced from DEPT ^{13}C NMR and FABMS data, leaving $C_{28}H_{44}O_5$ SO_3^- for the aglycone. This implied a C_{28} monounsaturated steroid with three hydroxyl groups, one sulfoxy and one ethereal ring; in agreement with this the ^{13}C nmr showed six signals for oxygenated carbons (5 CH, one quaternary), and the chemical shift at δ 83.2 (CH) and 83.6 (CH), downfield relative to those expected for hydroxy-bearing carbons, were assigned to the ethereal carbons.

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
1	1.50,1.75 m	36.9	1.50,1.75 m	36.9
2	1.65, 2.07 m	30.1	1.65, 2.07 m	30.1
3	3.74 m	78.9	3.74 m	78.9
4	2.40, 1.32m	30.2	2.40, 1.32m	30.2
5	1.26 m	49.0	1.26 m	49.0
6	4.36 dt (4.4, 10.8)	78.4	4.36 dt (4.4, 10.8)	78.4
7	2.60 m, 1.07 m	40.7	2.60 m, 1.07 m	40.7
8	2.24 m	35.5	2.24 m	35.6
9	-	146.4	-	146.4
10	-	39.9	-	40.0
11	5.41 brd (5.2)	118.0	5.41 brd (5.2)	118.0
12	2.01, 2.28 dd (5.2, 10.0)	42.7	2.01, 2.28 dd (5.2, 10.0)	42.5
13	-	42.3	-	42.3
14	1.23	54.1	1.23	54.2
15		37.3		37.2
16	1.41, 2.24	83.6	1.41, 2.24	83.5
17	4.49 m	67.5	4.49 m	68.1
18	1.18 s	14.4	1.18 s	14.4
19	1.08 s	19.7	1.08 s	19.7
20	-	81.5	-	81.2
21	1.41 s	26.2	1.42 s	26.1
22	3.99 d (9.0)	83.2	3.93 d (9.3)	82.9
23	3.64 dd (9.0, 4.6)	75.0	3.80 dd (9.3, 1.3)	72.8
24	1.51 m	44.0	1.51 m	42.6
25	2.02 m	28.4	2.02 m	31.3
26	0.95 d (6.8)	22.7	0.96 d (6.5)	21.1
27	0.89 d (6.8)	18.9	0.98 d (6.8)	21.6
28	0.94 d (7.2)	11.2	0.90 d (6.5)	10.0

Spectral data of glucuronic acid unit: ^{1}H-nmr (CD_3OD) δH: 4.51 (1H, d, J=7.8 Hz, H-1'), 3.19 (1H, t, J=7.8, 8.0 Hz, H-2'), 3.45 (1H, H-3'), 3.45 (1H, H-4'), 3.60 (1H, H-5'), ^{13}C-nmr (CD_3OD) : C-1 102.2, C-2 75.1, C-3 78.7, C-4 73.7, C-5 75.8, C-6 177.0. The coupling constants are given in Hz and are enclosed in parentheses. The assigments were aided by 2D-COSY and HMQC.

Table 1 ^{1}H- and ^{13}C-NMR data for Downeyoside A **1** and B **2** (CD_3OD)

The ^{1}H-^{1}H COSY spectrum of **1** showed a correlation between the resonances at δ 4.49 m and the doublet at δ 1.90 (d, J=7.6, H-17), indicative for the location of an oxygenated function at C-16 in a β-configuration, because the signal of the Me-18 was observed shifted downfield to δ 1.18, and also allowed the assignment of all proton resonances from C-22 to C-28 indicating the presence of oxygenated functions at C-22 (δ 3.99, d, J=9.0 Hz) and C-23 (δ 3.64, dd, J=9.0, 4.6 Hz).

Acetylation of **1** with triethylamine-Ac_2O in the presence of cathalytic amounts of DMAP^8 gave a triacetate **1a** (see experimental part), FABMS (negative ion), *m/z* 825 $[M_{SO_3}]^-$ with the glucuronic acid converted into the corresponding 3-6 lactone 2,4-diacetate. The ^{1}H-nmr spectrum showed that the resonance frequency of the H-23 methine proton has moved downfield to δ 5.08 (dd, J=8.6, 4.0 Hz) while H-16 and H-22 methine proton remained essentially unshifted, δ 4.47 and δ 4.24 respectively, thus proving that the ethereal oxygen links C-16 to C-22. The stereochemistry of **1** was elucidated on the basis of NOEDS experiments (Fig. 1), and ^{1}H-^{1}H coupling analysis in combination with molecular dynamics and mechanics calculations.

On irradiation on 18-Me at δ 1.18 intense NOE's were observed with H-8 (δ 2.24) and H-22, thus revealing a *trans* C/D ring junction with H-22 β-oriented; the *cis* D/ethereal ring junction was deduced from the strong NOE observed between H-16 and H-17, and corroborated by the NOE between Me-21 and both H-16 and H-17; a strong NOE effect was also observed between Me-21 and H-23, which implied a rigid conformation of the side chain with Me-21 close to H-23. In order to obtain information on the stereochemistry at C-23, a combination of molecular dynamics and mechanics calculations in the force field CHARM^9 was performed on model compounds with 23(S)- and 23(R)-hydroxy substituents (see experimental part). The so obtained minimum energy conformation of the 23S-model compound (Fig.2) shows good agreement between the calculated H22/H23 J value (J=8.5 Hz) and the experimental one (J=8.7 Hz); in addition, such conformation (23S-model) fits well with the strong NOE observed between H-21 and H-23. The conformational search on the model with 23R-hydroxy substituent yielded several lowest force field conformations, whose calculated Boltzman-weighted H22/H23 coupling constant was in the range 4.0-5.0 Hz, away from the experimental value. We wish to point out that the proposed stereochemistry 20R,22S,23S for **1** is that expected from the anti attack of the 16β-hydroxy group on a (20R,22S,23S)-22,23-epoxysteroids, commonly found in starfish^2 and also co-occurring in *Henricia downeyae*. The configuration at C-24 does not change significantly the minimum conformations of both 23S- and 23R-models.

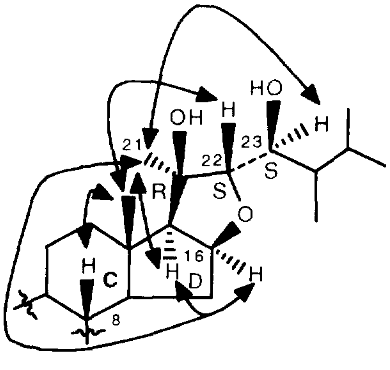


Fig. 1 ↷ Some NOE effects of Downeyoside A **1** and B **2**

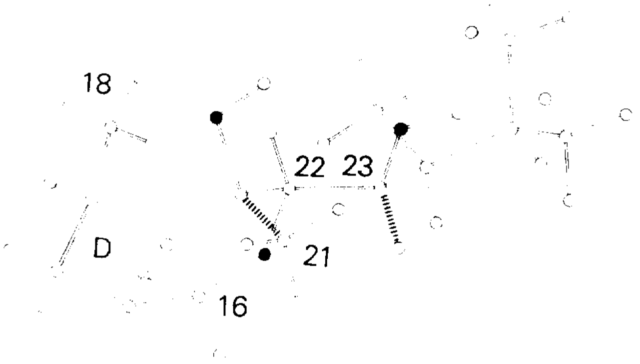


Fig. 2 Minimum energy conformation of Downeyoside A **1** obtained by molecular mechanics and dynamics calculation.

Coumpound **2**, obtained as a glassy material, $[\alpha]_D$=-18.5 (from MeOH), gave a FABMS (negative ion) pseudomolecular ion peak at *m/z* 717 $[M_H\text{-}H]^-$ and a fragment peak at *m/z* 541 (loss of 176 mass units) already observed in the spectrum of **1**. The ^{1}H- and ^{13}C-nmr spectra of **2** were almost superimposable on the corresponding spectra of **1** except small differences in the chemical shift around C-22/C28. Acetylation followed by ^{1}H nmr examination of **2**a (see experimental part) confirmed the presence of the hydroxyl group at C-23 ($\delta_{H\text{-}23}$: 5.20 vs 3.80 in **2**), and the ethereal oxygen between C-16 and C-22 ($\delta_{H\text{-}22}$: 4.13, $\delta_{H\text{-}16}$: 4.47 almost unshifted relative to **2**); NOEDS experiments gave the same results as in **1** confirming the stereochemistry at C-16, C-17, C-20, C-22 and C-23. Thus we have assigned the same structure as **1** but differing in the configuration at C-24 : *i.e.* **1** and **2** are C-24 methyl epimers. We do not have conclusive evidence for the assignment the configuration at C-24 in **1** and **2**. Sterol **1** and **2** were found cytotoxic against non-small-cell lung human carcinoma cells (NSCLC-N6) with IC50 of 60 µg/ml and 36 µg/ml , respectively. These new steroids are addition to the extensive list of hydroxylated sterols, some of which are cytotoxic and antiviral, isolated from marine sources[10,11].

EXPERIMENTAL

General Methods.

NMR measurements were performed on a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer, using the UXNMR software package. Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional sequence. The COSY spectra were obtained using a data set (t_1 x t_2) of 1024 X 512 points for a spectral data width of 2673.797 Hz (relaxation delay 1 sec.). The data matrix was processed using an unshifted sine bell window function, following transformation to give a magnitude spectrum with symmetrization (digital resolution in both F2 and F1 dimensions was 2.611 Hz/pt).

The {^1H, ^{13}C} shift correlation experiments, at 300 K, utilized a 5-mm probe with reverse geometry and the sample was not spun.

^1H-detected heteronuclear multiple quantum coherence (HMQC) experiments were performed according to Bax and Subramanian [12], using an initial BIRD pulse to suppress ^1H resonances not coupled to ^{13}C and a GARP sequence for ^{13}C decoupling during data acquisition. The spectral width in ^1H dimension was 2673.797 Hz; 200 experiments of 64 scans each (relaxation delay 3 s, delay after BIRD pulse 0.55 s, fixed delay t_1 3.7 ms) were acquired in 1K points. A sine square function was applied in t_2 dimension and a trapezoidal window in t_1 dimension (TM_1 0.03 Hz, TM_2 0.6 Hz) before Fourier transformation (digital resolution in F2 dimension 2.611 Hz/pt).

Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. Fast atom bombardment mass spectra (FABMS) were recorded in a glycerol-thioglycerol matrix in the negative ion mode on a VG AUTOSPEC instrument (CsI ions bombardment). Infrared spectra were done on FTIR Bruker ifs-48.

Animal Collection and Preliminary Experiments.

Henricia downeyae (family *Echinasteridae*, order *Valvatida*) was collected in the northern Gulf of Mexico in July 1993 and identified by Dr. T. Hopkins, University of Alabama. A voucher specimen is preserved at the Department of Biology, University of Alabama.

Extraction and Isolation.

The organism *Henricia downeyae* (150 g, lyophilized) was extracted with water and the aqueous extract was decanted and passed through a column of Amberlite XAD-2. This column was washed with distilled H_2O and eluted with MeOH to give, after removal of the solvent, a glassy material (2.0 g) The solid mass, left after water extraction, was extracted with Me_2CO and the extract, evaporated under vacuum was partioned between H_2O and Et_2O. The aqueous residue was then extracted with *n*-BuOH. Evaporation of the *n*-BuOH extract afforded 500 mg of glassy material. The combined MeOH, eluates from Amberlite, and *n*-BuOH extracts (2.5 g) were chromatographed on a column of Sephadex LH-60, followed by DCCC using *n*-BuOH-Me_2CO-H_2O (3:2:5) in the descending mode (the upper phase was used as stationary phase). Three main fractions were collected : 40-47 (57 mg), 48-53 (44.5 mg) and 54-59 (35.8 mg). Fractions 40-47 were purified by hplc on a Waters C_{18} μ-Bondapak column (30 cm x 3.9 mm i.d.; flow rate 1.5 ml m^{-1}) with MeOH-H_2O 30:70 as eluent to give pure **1** (3.5 mg) and **2** (3.0 mg).

Methanolysis of **1** *: Sugar analysis.*

A solution of **1** (1.0 mg) in anydrous 2.5 M HCl in MeOH (400 μl) was heated at 80° C in a stoppered reaction vial for 8 h. After having been cooled the reaction mixture was neutralized with Ag_2CO_3 and centrifuged, and the supernatant was evaporated to dryness under N_2. The residue was trimethylsilylated with TRISIL Z (Pierce Chemical Co.) for 15 min. at room temperature.

Glc analysis (SBP-1 capillary column, 30 m, i.d. 32 mm, 150° C, He carrier, flow 2 ml m^{-1}), gave peaks which co-eluted with those of methyl glucuronide.

Solvolysis of **1**.

A solution of **1** (0.5 mg) in pyridine (100 μl) and dioxane (100 μl) was heated at 160° for 2 h in a stoppered reaction vial. The residue was evaporated to dryness and purified by hplc (C_{18}-μ-Bondapak column 30 cm X 3.8 mm i.d.) with MeOH-H_2O (75:25) as eluent to give only one peak (desulfated of **1**). Spectral data :

FABMS (negative ion) *m/z* 657 $[M_H\text{-}H]^-$ ^{1}H-nmr (CD_3OD) δ_H : 1.00 (s, 19-Me), 3.54 (dt, J=4, 12 Hz, 6-H), 3.76 (m, 3-H), other signals are identical to those reported for natural **1** (see table 1).

Acetylation of **1** *and* **2** *to give* ***1a*** *and* ***2a***.

To a stirred solution of **1** (1.0 mg, 1.4 10 $^{-6}$ mol) in 150 µl Et_3N and 150 µl $(CH_3CO)_2O$ was added DMAP (0.1 mg) and the mixture was allowed to stand overnight at room temperature. The solution was partioned between H_2O and $CHCl_3$. The organic phase was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by hplc on a C_{18} µ-Bondapak (30 cm x 3.9 mm i.d.) with MeOH : H_2O (1 :1) flow rate 1 ml m^{-1}. Spectral data of **1a** : FABMS (negative ion) *m/z* 847 $[M_{Na}\text{-}H]^-$ and 825 $[M_H\text{-}H]^-$; ^{1}H-nmr (CD_3OD) δ_H : 0.87 (d, J=6.5 Hz, 26-Me), 0.92 (d, J=6.5 Hz, 27-Me), 0.98 (d, J=6.5 Hz, 28-Me), 1.06 (s, 19-Me), 1.18 (s, 18-Me), 1.34 (s, 21-Me), 1.88 (d, J=7.6 Hz, 17-H), 2.06 (s, 23-Ac), 3.74 (m, 3-H), 4.24 (d, J=8.6 Hz, 22-H), 4.36 (dt, J=4.4, 10.8 Hz, 6H), 4.49 (m, 16-H), 5.08 (dd, J=4.0, 8.6, 23-H), 5.40 (brd, J=5.2 Hz, 11-H), 5.27 (1H, d, J=4.9 Hz, H-1'), 5.03 (1H, t, J=4.5, Hz, H-2'), 2.07 (s, 2'-Ac), 3.45 (1H, H-3'), 5.32 (1H, t, J=4.0 Hz, H-4'), 2.11 (s, 4'-Ac), 5.85 (1H, d, J=3.7 Hz, H-5'). **2** was acetylated in the same manner. Spectral data of **2a** : FABMS (negative ion) *m/z* 847 $[M_{SO_3}\text{-}H]^-$ and 825 $[M_H\text{-}H]^-$; ^{1}H-nmr (CD_3OD) δ_H : 0.89 (d, J=6.5 Hz, 28-Me), 0.92 (d, J=6.5 Hz, 26-Me), 0.99 (d, J=6.5 Hz, 27-Me), 1.06 (s, 19-Me), 1.18 (s, 18-Me), 1.35 (s, 21-Me), 1.90 (d, J=7.6 Hz, 17-H), 2.05 (s, 23-Ac), 3.74 (m, 3-H), 4.13 (d, J=9.0 Hz, 22-H), 4.35 (dt, J=4.4, 10.8 Hz), 4.47 (m, 16-H), 5.20 (dd, J=1.3, 9.0, 23-H), 5.40 (brd, J=5.2 Hz), 5.27 (1H, d, J=4.9 Hz, H-1'), 5.03 (1H, t, J=4.5, Hz, H-2'), 2.07 (s, 2'-Ac), 3.45 (1H, H-3'), 5.32 (1H, t, J=4.0 Hz, H-4'), 2.11 (s, 4'-Ac), 5.85 (1H, d, J=3.7 Hz, H-5').

Molecular modelling.

Molecular mechanics and dynamics calculations were carried out on SGI Personal Iris 35G computer using the force field CHARM (QUANTA 3.3 software package). Global minimum energy conformations were obtained by performing a high-temperature molecular dynamics simulation (HTMDS) followed by energy minimization [9]. By means of a molecular dynamics simulation of 50 ps at 1000 K using the Verlet algorithm, 500 conformations of **1** were achieved. All the conformations of **1** were then subjected to an energy minimization (300 steps, conjugated gradient algorithm). Inspection of the minimized structures provided the lowest energy conformation of **1**.

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