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TRACE POLYHYDROXYLATED STEROIDS FROM STARFISH *HACELIA ATTENUATA*

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ABSTRACT.—Three very minor polyhydroxylated sterols, 24ξ-methyl-26,27-bisnor-5α-cholest-22-ene-3β,4β,6β,8,15α,16β,25-heptol (**5**), 24ξ-methyl-27-nor-5α-cholestane-3β,6β,8,15α,16β,26-hexol (**6**) and 24ξ-methyl-27-nor-5α-cholestane-3β,4β,6β,8,15α,16β,26-heptol (**7**) have been isolated from the Mediterranean starfish *Hacelia attenuata*, along with 24-O-α-arabinosyl-5α-cholestane-3β,6α,8,15β,24ξ-pentol (**8**). Their structures have been proposed on comparative spectral data.

Recently, we described the occurrence of a group of highly hydroxylated steroids, with moderate cytotoxicity, from the starfish *Hacelia attenuata* and *Protoreaster nodosus* (1). In particular, *H. attenuata* has given 5α-cholestane-3β,6β,15α,16β,26-pentol (**1**) (2), and *P. nodosus* has yielded 5α-cholestane-3β,6α,8,15α,16β,26-hexol (**2**) and the related heptol (**3**) and octol (**4**) (3). We now report the isolation of three further, very minor hydroxylated sterols from *H. attenuata*: 24ξ-methyl-26,27-bisnor-5α-cholest-22-ene-3β,4β,6β,8,15α,16β,25-heptol (**5**), 24ξ-methyl-27-nor-5α-cholestane-3β,6β,8,15α,16β,26-hexol (**6**), and 24ξ-methyl-27-nor-5α-cholestane-3β,4β,6β,8,15α,16β,26-heptol (**7**). The occurrence of polyhydroxylated sterols with side chains of the 24-Me-27-norcholestane and 24-Me-26,27-bisnor types may be of interest. C-26 and C-27 sterols, with the atom carbons missing in the ergostane side chain, are a widespread class of marine sterols of dietary origin, which have been found in nearly every marine invertebrate phylum (4).

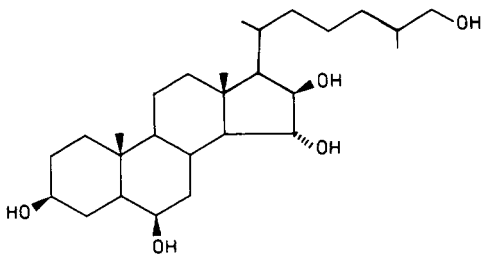
Furthermore, the investigation of the minor compounds of *H. attenuata* has resulted in the isolation of a novel steroidal glycoside, 24-O-α-arabinofuranosyl-5α-cholestane-3β,6α,8,15β,24ξ-pentol (**8**), named attenuatoside A-II. This represents a further member of a novel group of steroidal glycosides recently encountered in this starfish (1,5) and in *P. nodosus* (6). Its structure is closely related to that of attenuatoside A-I (**9**), the major glycoside of *H. attenuata* (5); both compounds have the same aglycone moiety; the difference resides in the sugar residue.

EXPERIMENTAL

The animals (*H. attenuata*) were collected in the Bay of Naples and lyophilized (1.2 kg). The lyophilized animals were first extracted in a Soxhlet apparatus with light petroleum (boiling point, 40-70°), then with methanol-chloroform (1:9), followed by methanol and water-methanol (1:1). The methanol-chloroform (1:9) extract (32 g) was chromatographed by lc on a prepak-500 SiO_2 column (Waters Associates LC/system) by using 10% methanol ion chloroform and increasing methanol content to 20%. After tlc monitoring (SiO_2 with $CHCl_3$-MeOH-H_2O, 80:18:2) the fractions (200 ml, each) were combined to give eight main fractions, A-H.

Fraction C (1.2 g) was rechromatographed on Sephadex LH-20 (80 x 4cm), and 20-ml fractions were eluted using methanol as solvent. Fractions 11-12 gave 121 mg of residue, which was submitted to droplet counter-current chromatography (7) (DCC-A apparatus, Tokyo Rikakikai, 250 tubes, solvent system: $CHCl_3$-MeOH-H_2O, (10:14:7), descending mode, flow rate 15-20 ml/h, the eluants were collected in 3-ml fractions and monitored by tlc SiO_2) to afford *attenuatoside A-II* (**8**, 15mg, 0.0012% yield).

Fraction D (1.5 g) was also rechromatographed on Sephadex LH-20 (80x4cm) as before. The 20-ml fractions 18-20 were chromatographed by hplc on a μ-Bondapack C-18 column (7.8 mm x 30 cm, Waters Model 6000 pump equipped with U6K injector and a Model 401 refractive index detector) using 35% water in methanol and yielded the sterol **6** (5 mg) along with major amounts of the glycoside **9**. Under the



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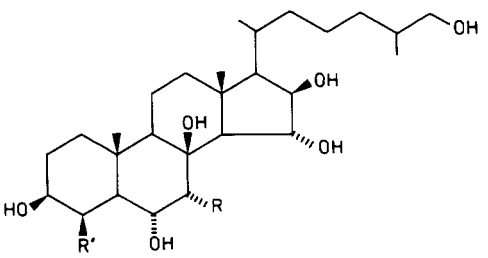
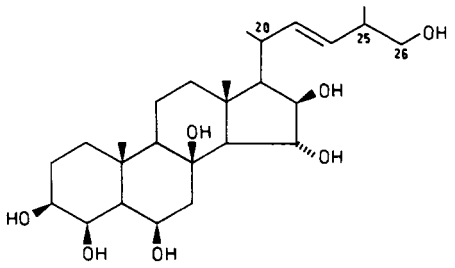


2 R = R' = H
3 R = OH ; R' = H
4 R = R' = OH



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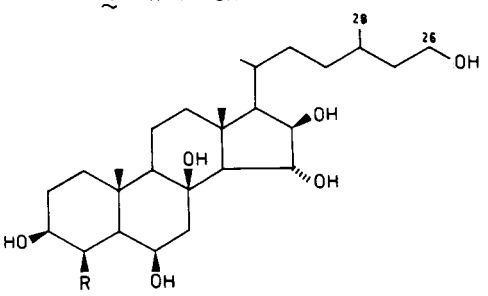
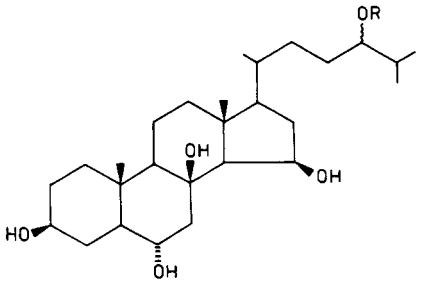


6 R = H
7 R = OH



8 R = α-arabinofuranosyl
9 R = 2-O-methyl-β-xylopyranosyl (1→2)α-arabinofuranosyl

same conditions as before, hplc of fractions 21-26 yielded the C-26 sterol (**5**) (3mg) and C-27 sterol (**7**), eluted in that order. The C-27 sterol (**7**) was further purified by preparative tlc on SiO_2 in $CHCl_3$-MeOH-H_2O, 80:18:2, to afford 4 mg of pure compound (single spot in tlc and single peak in hplc). Owing to the scarcity of material, the novel isolated compounds were not crystallized.

Spectral data were determined on the following instruments: ^{1}H-nmr and ^{13}C-nmr, Bruker WX-270; EI mass spectra, A.E.I. MS-30.

RESULTS AND DISCUSSION

24ξ-methyl-26,27-bisnor-5α-cholest-22-ene-3β,4β,6β,8,15α16β,25-heptol (**5**).—The spectral properties of compound **5** were indicative of a steroidal skeleton.

The ^{1}H-nmr spectrum (CD_3OD) contained two mutually coupled doublets of doublets at δ 4.18 (J = 10.0 and 2.5 Hz) and 3.92 (J = 7.5 and 2.5 Hz) already observed in the spectra of the previous polyhydroxysterols **1-4** and assigned to 15β- and 16α-protons. The spectrum also contained two one-proton double doublets at δ 2.48 (J = 14.0 and 2.5 Hz) and 1.62 (J = 14.0 and 3.0 Hz) coupled to each other by 14.0 Hz. Decoupling also proved that the small couplings were due to interactions with 6α-protons (δ 4.28, broad, W½ = 8 Hz), thus allowing the assignment of these peaks to the protons at C-7. This suggests the location of a hydroxyl group at C-8, which is a com-

mon feature of starfish-hydroxylated sterols (1,3). In agreement with the introduction of the hydroxyl group at C-8 was the downfield shift of 15β-H (δ 4.18) when compared with the resonance of the corresponding proton (δ 3.84) in the pentol **1**.

The remaining signals due to protons attached to carbon having hydroxyl groups were observed at δ 4.09 (1H) and 3.65-3.45 (complex signal, 3H). The quite narrow nature of the signal at δ 4.09 ($W½=8$ Hz) suggests the equatorial proton associated with 4β-hydroxyl group (8) and the complex signal spread out between δ 3.65-3.45 could be caused by protons associated with 3β- and 26-hydroxyl groups, which are common elements of starfish-hydroxylated sterols (1,3). The presence of only two three-protons methyl doublets at δ 0.97 and 1.05 was consistent with the previous assumption of a primary hydroxyl function (probably located at C-26). The ^{1}H-nmr spectrum displayed CH_3-18 and CH_3-19 singlets at δ 1.17 and 1.47 in agreement with the postulation of a 3β,4β,6β,8,15α,16β-hexahydroxycholestane structure [calcd based on Zürker's table (9) and the more recent data of Bridgeman *et al.* (8): CH_3-18: 1.21, CH_3-19: 1.48]. Perhaps the most significant feature of the ^{1}H-nmr spectrum was an eight-line pattern centered at δ 5.35 and 5.55 (23,22-H) with J of 7.5 (couplings 22-20-H and 23-25-H) and 15 (coupling 22-23-H *trans*) Hz, which indicated a >C(20)H-C(22)H=C(23)H-C(24)H< partial structure. This could be expanded to the $-CH(CH_3)-CH=CH-CH(CH_3)-CH_2OH$ side chain on the basis of decoupling experiments and the use of pyridine solvent.

In pyridine, a spectrum with isolated signals for all protons of the chain was obtained. Sequential irradiation of each band allowed all signals to be interrelated. A one-proton multiplet, appearing at δ 2.53 (24-H), when irradiated, collapsed one doublet Me at δ 1.10, transformed the eight-line pattern centered at δ 3.64 (J=13,7 Hz) and 3.74 (J=13,7 Hz) into an AB quartet (J=13 Hz, 26-H_2), and simplified the double doublet (J=15, 7.5 Hz) at δ 5.58 into a doublet with J=15 Hz (23-H); further decoupling experiments indicated that the CH_3-21 (d at δ 1.23 in this condition) was coupled (J=6.5 Hz) to the 20-H (m at δ 3.00), which was itself coupled (J=8 Hz) to the 22-H (dd, J=15, 8 Hz, at δ5.82). Hence, the presence in this novel steroid of a shortened side chain was firmly established. We would note that, in pyridine, both the angular methyl resonances shifted significantly downfield (CH_3-19, δ 1.90; CH_3-18, δ 1.72, cf. 1.47 and 1.17 in CD_3OD), in agreement with the proposed hydroxylation pattern with both angular methyl groups subjected to 1,3-diaxial interaction with hydroxyl groups.

Treatment with excess acetic anhydride at room temperature produced a triacetate showing three acetate methyl singlets in the ^{1}H-nmr (δ 2.03, 2.07 and 2.12). The protons α to the acetoxy groups were centered at δ 4.99 (1H, dd, J=11.0, 2.5 Hz, 15β-H), 4.75 (1H, dt, J=11.0, 4.0 Hz, 3α-H). and 3.90 (3H, complex signal, 26-H_2 overlapping with the 16α-H dd), and this established that acetylation had occurred at 3β-, 15β- and 26-hydroxyls. Decoupling proved the interaction of the 3α-H signal with the 4α-proton resonating at δ 4.24 (bs, $W½=7$ Hz). In the electron impact mass spectrum of the acetate the highest molecular weight ion observed (*m/e* 576) corresponded to loss of water from the molecular formula $C_{32}H_{50}O_{10}$ ($C_{26}H_{44}O_7$ + three acetate); a series of peaks for stepwise water and acetic acid losses [*m/e* 558, 540; *m/e* 534 (M^+-CH_3CO_2H), 516 (intense), 498 (intense), 480 (intense); *m/e* 474 (M^+-$2CH_3CO_2H$); 456 (intense), 438 (base peak), 420; *m/e* 414 (M^+-$3CH_3CO_2H$), 396, 378] and peaks at *m/e* 285-283, and 267-265, corresponding to the loss of an acetoxylated C-7 side chain from M^+-$2CH_3CO_2H$-$2H_2O$ and M^+-$2CH_3CO_2H$-$3H_2O$, respectively, with and without 2H transfer [typical of steroids with a double bond in the side chain (10)], were observed.

The available quantity of this sterol was insufficient for further investigations;

nevertheless, we are confident that all the collected data permit assignment of the structure as 24ξ-methyl-26,27-bisnor-5α-cholest-22-ene-3β,4β,6β,8,15α,16β,25-heptol (**5**).

24ξ-Methyl-27-nor-5α-cholestane-3β,6β,8,15α,16β,26-hexol (**6**).—The electron impact mass spectrum showed a small molecular ion at *m/e* 484 corresponding to a fully saturated cholestane-hexol. The fragmentation pattern with ions for stepwise water loss [*m/e* 450 (base peak), 432, 414] and ions corresponding to the loss an hydroxylated C-8 side chain together with one, two, and three molecules of water (*m/e* 321, 303 and 285) and the intense ion at *m/e* 225 (70%; cleavage of the 12,13 and 8,14 bonds) was almost identical to that observed in the spectrum of the hexol **2** (3). The ^{1}H-nmr spectrum also contained several features, already observed in the spectrum of the hexol **2** (3); namely, two doublets of doublets at δ 4.00 (J=8 and 2.5 Hz) and 4.16 (J=10.5 and 2.5 Hz) and a seven-line multiplet with W½ of 20 Hz at δ 3.61 assigned to 16α-H, 15β-H and 3α-H. One methyl singlet at δ 1.15 was close to the value for CH_3-18 observed in the spectrum of the hexol **2**, while the C-19 methyl group gave rise to a signal at δ 1.205 shifted downfield by 0.18 ppm relative to the hexol **2**. This suggests a β-orientation for the hydroxyl group at C-6 in the new sterol. Present also was a one-proton signal at δ 3.89, which was quite narrow (W½=7 Hz), as would be expected for an equatorial proton (6α-H). This was coupled to an adjacent methylene pair (7α,7β), which appeared as two doublets of doublets at δ 2.46 (J=14 and 2.5 Hz) and 1.62 (J=14 and 3 Hz). These observations suggested that the fifth nuclear hydroxyl group was located at C-8. Two methyl doublets, at δ 0.96 and 0.93, and a multiplet at δ 3.43 (1H), coupled to another signal resonating under the methanol signal, completed the ^{1}H-nmr spectrum.

A ^{13}C-nmr spectrum in pyridine d_5 confirmed the presence of six carbons attached to oxygen (82.4, 80.52, 76.2, 73.8, 71.3, and 62.1 ppm) as well as the absence of carbon-carbon double bonds. Using sterols **1** and **2** as model compounds (2,3) and using the substituent effects for hydroxysteroids that have been published (11,12), the ^{13}C-nmr signals for the nuclear carbons attached to oxygen can be assigned easily (C-3: 71.2; C-6: 73.2; C-8: 76.2; C-15: 80.5; C-16: 82.4), thus leaving the 62.1 ppm signal for the side chain carbon attached to oxygen. The resonances associated with the hydroxymethylene carbon (C-26) in the sterols **1-4** have chemical shift values of 68.4-68.5 ppm, and this excluded a 26-hydroxycholestane side chain for the new sterol. Calculations (13) predict a chemical shift of 60.4 ppm for a hydroxyl substituent at C-26 of a 24-methyl-27-nor-cholestane side chain. Agreement between observed and calculated values is not very close, but we note that the chemical shift for C-29 in 29-hydroxy steroids recently described (14) is almost identical (62.01-62.06 ppm) with that for C-26 (62.1 ppm) in our sterol, which is similarly placed.

The ^{1}H-nmr spectrum of the derived 3β,15α,26-triacetate (Ac_2O and pyridine at room temperature; 3 CH_3CO- at δ 2.10, 2.05 and 2.04) provided evidence supporting the 24ξ-methyl-27-nor-5α-cholestane-3β,6β,8,15α,16β,26-hexol formulation for this novel steroid. It contained a double doublet at δ 4.86 (J=10.5 and 2.5 Hz) and a seven-line multiplet (W½=20 Hz) at δ 4.77 assigned to the acetoxymethine 15β- and 3α-protons, a complex three-proton signal centered at δ 3.94, and most significantly, a ddd at δ 3.86 with apparent couplings of 14.7 and 3.5 Hz, which had to be associated with one acetoxymethylene proton at C-26 (the other 26-H resonated under the complex signal at δ 3.94).

24ξ-Methyl-27-nor-5α-cholestane-3β,4β,6β,8,15α,16β,26-heptol (**7**).—The third polyhydroxylated minor sterol contains one more hydroxyl relative to the hexol **6**.

In the electron impact mass spectrum, the ion with the highest molecular weight observed (*m/e* 466) corresponded to loss of water from the molecular formula $C_{27}H_{48}O_7$

(fully saturated cholestane-heptol). The fragmentation pattern, with ions for stepwise water loss and ions corresponding to the loss of an hydroxylated C-8 side chain together with one, two, three, and four molecules of water (*m/e* 337, 319, 301, and 283), closely resembled that observed in the spectrum of the hexol **6**. The comparison of ^{1}H-nmr spectra of **6** and **7** indicated that the novel sterol **7** was related to **6** by introduction of the seventh hydroxyl group at 4β-position. The ^{1}H-nmr contained several features observed in the spectrum of **6**; namely, four doublets of doublets at δ 4.00 (J=8 and 2.5 Hz), 4.17 (J=10.5 and 2.5 Hz), 1.62 (J=14 and 3 Hz), and 2.47 (J=14 and 2.5 Hz) assigned to 16α-H, 15β-H. 7-H_2, respectively. The chemical shift of the methyl signals δ 0.93d, 0.96d and 1.14s are close to the values for CH_3-21, CH_3-28 and CH_3-18 in the hexol **6**. The fourth methyl group (CH_3-19) gave rise to a signal at δ 1.47 shifted downfield by 0.27 ppm relative to the hexol **6**, in agreement with a postulation of a 4β-OH in **7** [the additive substituent parameter for 4β-OH in a 5α-cholestane structure is reported by Zürker (9) to be 0.267 ppm downfield]. The resonance associated with 6α-H has moved downfield to δ 4.28 (narrow m, W½=7 Hz), whereas the signal for 4α-H was observed at δ 4.09 (narrow m, W½=7 Hz).

The remaining hydroxymethine (1H) and hydroxymethylene (2H) signals overlap, giving rise to a complex signal spread out between *ca.* δ 3.40 and 3.60, but when we measured the spectrum of the derived 3β,15α,26-triacetate (acetic anhydride and pyridine at room temperature, 3 *CH*$_3$CO- at δ2.12, 2.10, and 2.06) the resonance of the methine proton (3α-H) had moved downfield to δ 4.75 and appearerd as a dt with J of 12 and 3.5 Hz. Decoupling proved its interaction by 3.5 Hz with the 4α-proton at 4.24 (W½=7 Hz). The resonance of the hydroxymethylene had also moved downfield, shift of *ca.* 0.5 ppm, to δ 3.86 and δ 3.95 (overlapping with 16α-H); the shape of the isolated signal at δ 3.86 (ddd with J of 14, 7 and 3 Hz) was identical to that for the 26-H in the hexol **6**.

Attenuatoside A-II (**8**).—The minor glycoside, named attenuatoside A-II, [α]D, −15.7, was assigned the structure **8** by comparing its ^{1}H-nmr and ^{13}C-nmr spectra with those of the major attenuatoside A-I (**9**). The ^{13}C-nmr signals (pyridine-d_5) for the carbon atoms of the aglycone (C-1: 39.1, C-2: 32.0, C-3: 71.3, C-4: 33.1, C-5: 53.9, C-6: 66.5, C-7: 49.9, C-8: 76.6, C-9: 56.7, C-10: 37.4, C-11: 19.1, C-12: 42.6, C-13: 43.7, C-14: 61.7, C-15: 70.1, C-16: 42.2, C-17: 57.1, C-18: 16.5, C-19: 14.2, C-20: 35.5, C-21: 18.9, C-22: 32.0, C-23: 28.3, C-24: 83.4, C-25: 31.0, C-26: 18.1) were within 0.1 ppm from **9** (5). Assignment of the sugar carbon atoms have been made by comparing the spectrum (C-1′: 109.5, C-2′: 83.8, C-3′: 78.8, C-4′: 85.5, C-5′: 62.7) with that of methyl-α-L-arabinofuranoside (15).

The ^{1}H-nmr spectrum (CD_3OD) contained the following signals: aglycone moiety, δ 0.93 (3H, d, J=6.5 Hz), 0.94 (3H, d, J=6.5 Hz), 0.96 (3H, d, J=7 Hz), 1.01 (s, 19-H), 1.29 (s, 18-H), 3.50 (m, 3α-H), 3.70 (m, partially overlapped with 5-H′, 6β-H), 4.44 (bt, J=5.5 Hz, 15α-H), 24-H is under solvent signal; α-arabinofuranosyl moiety, δ 3.65 (dd, J=12 and 5 Hz, 5-H′), 3.75 (dd, J=12 and 3 Hz, 5-H′), 3.86 (dd, J=7 and 4 Hz, 3-H′), 4.00 (2H, complex, 2 and 4-H′, confirmed by decoupling), 4.95 (d, J=1 Hz, 1-H′).

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