Steroids from the Starfish *Euretaster insignis:* A Novel Group of Sulphated 3β,21-Dihydroxysteroids†

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The aqueous extracts of the starfish *Euretaster insignis* contain two groups of sterol sulphates. The more polar compounds are sulphated 3β,21-dihydroxysteroids; after solvolysis to remove the sulphate groups they were identified as (20*R*)-24-methylenecholestane-3β,21-diol (**3a**) (major), (20*R*)-24-methylenecholest-5-ene-3β,21-diol (**3b**) (minor), (20*R*,22*E*)-24-methylcholest-22-ene-3β,21-diol (**4a**) (major), (20*R*)-cholestane-3β,21-diol (**1a**) (minor), (20*R*,22*E*)-cholest-22-ene-3β,21-diol (**2a**) (minor), and (20*R*)-cholest-5-ene-3β,21-diol (**1b**) (minor). The less polar compounds are sterol sulphates which are 'normal' constituents of starfishes. Analysis of the free sterols mixture has revealed that it contains a low level of cholest-7-en-3β-ol (3% of the total sterol mixtures) and C_{26}, C_{27}, C_{28}, and C_{29} 5α-steroidal alcohols. This finding, in contrast with previous results indicating the preponderance of Δ^7-sterols in starfishes, could be related to the apparent absence of asterosaponins in this species.

In our continuing investigation of biologically active steroidal glycosides from *Echinoderms*,[1] we have been working on the extracts of the Pacific starfish *Euretaster insignis* with a view to isolating its 'asterosaponins'.[2] Column chromatography on Sephadex LH-20 of the methanol-soluble material from the lyophilysed aqueous extract, followed by droplet counter-current chromatography (DCCC), has shown that the starfish is apparently devoid of the above compounds and instead has led to the isolation of two steroidal sulphate fractions. Analysis of these two fractions and identification of their components is the subject of this paper. The lipid fraction has also been analysed for free sterols and the results are described herein.

Results and Discussion

The starfishes were collected at Nouméa, New Caledonia, and freshly collected specimens were extracted whole with water. The lyophilysed aqueous extracts were extracted with ethyl acetate, then with methanol–chloroform (1:9), followed by methanol.

Gel filtration of the methanol-soluble material on Sephadex LH-20 with methanol gave two steroidal sulphate fractions. The more polar fraction was further purified by DCCC to give a mixture of sulphated 3β,21-dihydroxysteroids. The less polar fraction contained the sulphated 3β-OH steroids.

The Sulphated 3β,21-*Dihydroxysteroids.*—The ^{1}H n.m.r. spectrum showed that this fraction is a mixture of steroids, with methyl singlets at δ_H 0.75, 0.76, and 0.78 for 18-H_3 protons and δ_H 0.87 and 1.07 for 19-H_3 protons. The secondary methyl group signals appeared as overlapping doublets at δ_H *ca.* 0.90 and as a separated doublet at δ_H 1.06. This latter signal, along with two olefinic signals at δ_H 4.75 and 4.71, is typical of a 24-methylenesteroid structure. The n.m.r. spectrum also included olefinic protons at δ_H 5.42 (m, 6-H) and 5.30 (m, 22- and 23-H) and sulphate methine and methylene protons at δ_H 4.03 and 4.22 (3α-H). The proton at C-3 of 5α-cholestan-3β-yl sulphate resonates at δ_H 4.22. Microanalysis showed the presence of sulphur and the analytical figures were in agreement with steroidal diol disulphate structures. The presence of sulphate groups were evidenced by a strong i.r. absorption at 1 250 cm^{-1}. Attempts at separation of the mixture failed. Solvolysis of this material afforded a mixture of dihydroxysteroids which was fractionated by h.p.l.c. on an ODS-1 column to give three fractions.

Fraction 1. This contained a single component (**3b**) which was crystallized from ethanol, m.p. 139—141 °C; $[\alpha]_D$ −23.8°. The high-resolution m.s. indicated a molecular formula $C_{28}H_{46}O_2$, corresponding to a C_{28} dihydroxylated steroid with two degrees of unsaturation. The mass spectrum showed loss of the side-chain (+ 2 H) (*m/z* 271), indicating[3] the presence of one oxygen function and one degree of unsaturation in the C_9 side-chain, the other unsaturation being in the nucleus. The strong peak at *m/z* 330 (McLafferty rearrangement) suggested a side-chain with a 24(28) double bond[4] and the 'extra' hydroxy group around C-20, C-21, or C-22. The ^{1}H n.m.r. spectrum included the expected olefinic protons at δ_H 4.75 and 4.70 and the isopropyl methyl doublet at δ_H 1.04. The conspicuous absence of the absorption of the C-21 methyl group, together with the presence of 2-H signal at δ_H 3.72, suggested that the compound possessed a primary hydroxy function at C-21. The spectrum displayed the usual Δ^5-3β-ol signals with a multiplet centred at δ_H *ca.* 3.52 and a doublet at δ_H 5.35. In the methyl region two singlets at δ_H 0.72 and 1.02 are in agreement with the expected values for the C-18 and C-19 angular methyl protons in Δ^5-3β-OH steroids. The compound's structure is thus 24-methylenecholest-5-ene-3β,21-diol (**3b**). ^{13}C N.m.r. spectrometry gave support to this formulation. The shifts of the nuclear carbons were within ±0.1 p.p.m. of those published for cholesterol[5] except for C-17 which in (**3b**) is shifted to higher field (51.1 *vs.* 56.2 p.p.m.) as expected for the presence of the 21-hydroxy group. The alcohol at C-21 also exerts a small deshielding effect on C-18 (12.3 *vs.* 11.9 p.p.m.) and C-16 (28.8 *vs.* 28.3 p.p.m.). The chemical shift assignments of the side-chain carbons (C-20 42.7, C-21 63.4, C-22 27.6, C-23 32.0, C-24 156.7, C-25 34.1, C-26 21.9, C-27 22.0, and C-28 106.5 p.p.m.) were made by comparison with those published for the side-chain of cycloeucalenol[6] (30-nor-24-methylenecycloartenol) and substituent parameter considerations. The remaining feature needed to establish the structure fully is the stereochemistry at C-20. (20*R*)- and (20*S*)-

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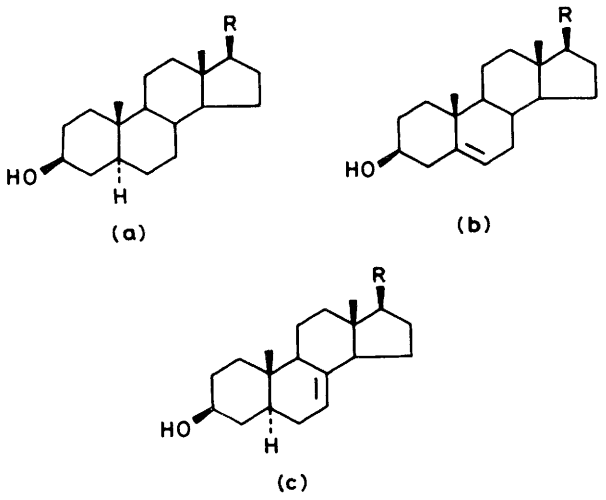

Figure 1. Structures of sterol ring systems. R = side-chain

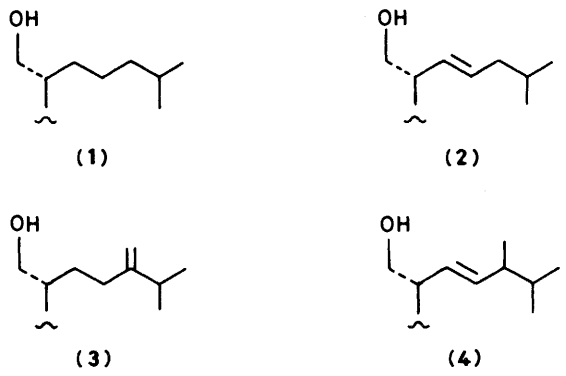

Figure 2. Structures of 3β,21-dihydroxysteroid side-chains

Cholest-5-ene-3β,21-diol have been synthesized[7,8] and the chemical shift for the 21-methylene protons is sensitive to the stereochemistry at C-20.[8] Our value of δ_H 3.72 (broad singlet) was in excellent agreement with the value δ_H 3.70 (singlet) reported for the 20*R* (natural configuration)-isomer as compared with δ_H 3.62 (multiplet) for the corresponding 20*S*-isomer. In confirmation compound (**3b**) was converted into a mixture of C-24 epimers of 24-methyl-5α-cholestan-3β-ol through (i) selective tritylation of the 21-hydroxy group, (ii) acetylation, (iii) removal of the trityl group upon hydrogenolysis, which also reduced the double bonds, (iv) tosylation, and (v) reduction with lithium aluminium hydride. The reduction product showed resonances at δ_H 0.905 [d, *J* 6.32 (24*S*-isomer)] and 0.896 [d, *J* 6.63 (24*R*-isomer)] for the C-21 methyl group, in total agreement with the 'common' 20*R* configuration; in the n.m.r. spectra of 20*S* compounds the C-21 methyl group is shifted *ca.* 0.1 p.p.m. upfield ($\Delta\delta_H$ *ca.* 0.80 p.p.m. in steroids with saturated side-chains).[9] Thus, the full structure of our steroid is (20*R*)-24-methylenecholest-5-ene-3β,21-diol (**3b**).

Fraction 2. Examination by m.s. showed that this was a mixture of at least two components with M^+ 416 and 402. The sterol of m.w. 416 (major component; a C_{28} dihydroxysteroid with one degree of unsaturation) had a 24(28) double bond, as indicated by a peak at *m/z* 332 (loss of the end of the side-chain by McLafferty rearrangement) in the mass spectrum of the mixture and by the expected two olefinic protons at δ_H 4.75 and 4.70 in the n.m.r. spectrum.

The n.m.r. spectrum also contained a resonance at δ_H 3.72, already observed in (**3b**) and assigned to 21-H_2. Thus, the major compound of the mixture is the 5,6-dihydro derivative of the previous compound (**3b**). In the olefinic region of the n.m.r. spectrum a doublet at δ_H 5.35 indicated a Δ^5-double bond and an AB pattern with lines centred at δ_H 5.48 (7-line multiplet with separations of 6.0, 8.5, and 16.0 Hz, 23-H) and 5.10 (dd, *J* 8.5 and 16.0 Hz, 22-H) indicated a Δ^{22}-*trans* double bond. Thus the compound of m.w. 402 (C_{27} dihydroxysteroid with one degree of unsaturation) turned out to be a mixture of two double-bond isomers. The ^{13}C n.m.r. spectrum of Fraction 2 showed intense signals expected for the major compound (**3a**) and small signals due to the isomeric C_{27} steroids. For example the intense signal at δ_C 51.1 p.p.m. (C-17) is accompanied by two smaller signals at δ_C 50.6 and 51.5 p.p.m. assignable to C-17 of (**2a**) and (**1b**), respectively; furthermore the C-3 signal at δ_C 71.4 p.p.m. is accompanied by a small signal at δ_C 71.9 p.p.m., identical with that observed in the spectrum of the Δ^5-compound (**3b**), while the C-21 signal at δ_C 63.4 p.p.m. is accompanied by a resonance shifted downfield to δ_C 66.5 p.p.m. and the C-20 signal at δ_C 42.7 p.p.m. is accompanied by a resonance shifted downfield to δ_C 48.7 p.p.m., which clearly indicates the presence of a Δ^{22}-double bond. In steroids the introduction of a *trans* Δ^{22}-double bond deshields C-21 by *ca.* 2.5 p.p.m. and C-20 by *ca.* 5.0 p.p.m.[5] The presence of steroids with Δ^5- and Δ^{22}-double bonds was further confirmed by small signals in the sp^2-C region at δ_C 121.6 and 141.1 p.p.m. (Δ^5) and 130.1 and 132.2 p.p.m. (Δ^{22}). Fraction 2 was then acetylated and the acetates (three peaks in g.l.c. with relative retention times of 2.73 (major), 2.14, and 2.12; cholesteryl acetate = 1) were fractionated over silver nitrate-impregnated silica gel. The first eluted fractions contained the acetates of (**1b**) and (**2a**) still in admixture. The m.s. showed a small molecular ion at *m/z* 486 and intense ions at *m/z* 426 (M^+ − AcOH) and 315 due to the loss of the side-chain from both (**1b**) and [(**2a**) + 2 H]. The ^1H n.m.r. spectrum showed the signals for (**1b**)-diacetate at 0.70 (18-H_3), 1.01 (19-H_3), 4.02 (dd, *J* 10.5 and 6.5 Hz)—4.21 (m, 21-H_2), 4.63 (m, 3α-H), and 5.37 (6-H), and signals for (**2a**)-diacetate at δ 0.70 (18-H_3), 0.82 (19-H_3), 3.90 (dd, *J* 10.5 and 8 Hz)—4.21 (m, 21-H_2), 4.70 (m, 3α-H), and 5.18 (m, 22- and 23-H). The isopropyl methyl signals of each compound overlap giving rise to a doublet (*J* 6.5 Hz) at δ_H 0.87.

The more polar fractions eluted from the argentic silica gel column contained (**3a**)-diacetate as the single component. The mass and n.m.r. spectra were fully consistent with the suggested structure. The assignment of the 20*R* configuration to (**3a**) is based on the shifts of the side-chain carbons identical with (**3b**) and on the shift of 21-H_2 (δ_H 3.72).[8] We tentatively assign the configuration at C-20 as 20*R* in the minor products (**1b**) and (**2a**) by analogy.

Fraction 3. This contained two compounds with M^+ 416 and 404. The sterol of m.w. 416 (major component, C_{28} dihydroxylated steroid with one unit of unsaturation) contains a 21-hydroxy-24-methyl-Δ^{22}-*trans*-side-chain, as indicated by two olefinic protons in the n.m.r. spectrum which appeared as well resolved 8-line patterns (ABxy system) at δ_H 5.08 (dd, *J* 16.0 and 8.7 Hz, 22-H) and 5.44 (dd, *J* 16.0 and 7.5 Hz, 23-H). The proton at C-20 is found at δ_H 2.20 and irradiation of this multiplet in a double-resonance experiment did indeed simplify the hydroxymethylene signal at δ_H 3.72 and also transformed the double doublet at δ_H 5.08 into a doublet with *J* 16 Hz. Several overlapping signals around 2.0 p.p.m., among which we suppose the allylic C-24 proton to be, were next irradiated; the olefinic double doublet at δ_H 5.44 collapsed into a doublet with *J* 16 Hz and the methyl doublet at δ_H 0.96 (28-H_3) collapsed into a singlet. Thus the major component of this mixture is (22*E*)-24-methylcholest-22-ene-3β,21-diol (**4a**) and the minor component could be 5α-cholestane-3β,21-diol (**1a**). Acetylation and fractionation over silver nitrate-impregnated silica gel gave the individual

Table. Sterol composition of *Euretaster insignis*

3β,21-Dihydroxysteroids	Mobility[a] h.p.l.c. (min)	Mobility[b] g.l.c. (r.r.t.)	(%)
(20*R*)-Cholestane-3β,21-diol (**1a**)[c]	31	2.22	6.6
(20*R*,22*E*)-Cholest-22-ene-3β,21-diol (**2a**)[c]	27	2.12	5.6
(20*R*)-Cholest-5-ene-3β,21-diol (**1b**)[c]	27	2.14	4.4
(20*R*)-24-Methylenecholestane-3β,21-diol (**3a**)[c]	27	2.73	40.3
(20*R*)-24-Methylenecholest-5-ene-3β,21-diol (**3b**)	25	2.67	20.0
(20*R*,22*E*)-24-Methylcholest-22-ene-3β,21-diol (**4a**)[c]	31	2.63	23.1

Monohydroxysteroids	(r.r.t.)[d]	Free sterols (%)	Sulphated sterols (%)
Δ⁷-Sterols			
Cholest-7-en-3β-ol (**7c**)		3.0	
5α-Steroidal alcohols			
(22*E*)-24-Methyl-26,27-dinorcholest-22-en-3β-ol (**5a**)	0.68	1.1	
(22*E*,24*S*)-27-Norcholest-22-en-3β-ol (**6a**)	0.80	4.2	
(22*E*)-Cholest-22-en-3β-ol (**8a**)	0.87	8.4	
(22*E*,24*S*)-24-Methylcholest-22-en-3β-ol (**9a**)[15,16]	0.92	26.7	
(22*E*,24*R*)-24-Methylcholest-22-en-3β-ol (**10a**)[15,16]	0.98	4.2	
[24(28)*E*]-24-Ethylidenecholestan-3β-ol (**11a**)	1.04	2.6	
Cholestan-3β-ol (**7a**)	1.10[e]	26.8	
(22*E*)-24-Ethylcholest-22-en-3β-ol (**12a**)	1.12[e]	17.8	
(24*R*)-24-Methylcholestan-3β-ol (**13a**)	1.19	Trace	
24-Ethylcholestan-3β-ol (**15a**)	1.28	5.1	
24-Methylenecholestan-3β-ol (**16a**)	0.94		Trace
Δ⁵-Sterols			
(22*E*)-Cholesta-5,22-dien-3β-ol (**8b**)	0.76		21.1
24-Methylenecholest-5-en-3β-ol (**16b**)	0.80		26.4
(22*E*,24*R*)-24-Methylcholesta-5,22-dien-3β-ol (**10b**)	0.94		6.8
Cholest-5-en-3β-ol (**7b**)	1.00		36.5
24-Methylcholest-5-en-3β-ol (**13b**) + (**14b**)[14,17]	1.10		2.6
24-Ethylcholest-5-en-3β-ol (**15b**)	1.16		6.3

[a] On ODS-1 column and methanol–acetonitrile–water (70:10:20) as eluant. [b] As acetates on SE-30 (25-m capillary column; 249 °C), standard cholesteryl acetate. [c] The C-20 stereochemistry is probable; the C-24 stereochemistry of (**4a**) was not assigned. [d] On ODS-2 column and pure methanol as eluant, standard cholesterol. [e] Insufficiently resolved; their relative ratios have been determined by integration of the n.m.r. signals for 18-H_3, δ_H 0.659 (cholestan-3β-ol) and 0.678 (24 ethylcholest-22-en-3β-ol).

components. The mass and ^1H n.m.r. spectra of both compounds were fully consistent with the suggested structures. The configuration 20*R* is assigned to (**1a**) on the basis of the shifts and the shape of the signal for proton 21-H in the spectrum of the acetate (δ 4.02, dd, *J* 8.5 and 6.5 Hz; 4.21, dd, *J* 10.5 and 3.5 Hz) which compare well with those of (**3a**)-diacetate. The assignment of the 20*R* configuration to the Δ^{22}-component (**4a**) is tentative. The Table lists the six 3β,21-dihydroxysteroids isolated from *Euretaster insignis* and the percentage of the sterol components.

The Sterol Sulphates.—Microanalysis showed the presence of sulphur and the n.m.r. spectrum clearly indicated a mixture of sterol sulphates (7-line multiplet at δ_H 4.20). Solvolysis of this material to remove sulphate groups gave a mixture of sterols which was partitioned by preparative high-pressure liquid chromatography (h.p.l.c.) into six main fractions which were each analysed by mass spectrometry and 250 MHz n.m.r. spectroscopy. All are known sterols and are listed in the Table together with their relative retention times (r.r.t.) in h.p.l.c. and percentage of the sterol components. Cholesterol was the major constituent of the mixture, accompanied by relative large proportions of (22*E*)-cholesta-5,22-dien-3β-ol (**8b**) and 24-methylenecholest-5-en-3β-ol (**16b**). This finding is in agreement with the results indicating cholesterol to be the major sulphated compound in starfishes.[10–13] The fraction with r.r.t. 1.10 turned out to be a mixture of C-24 epimers of 24-methylcholest-5-en-3β-ol (**13b**) + (**14b**).[14,17] The assignment of the configuration of the 24-methyl group in (22*E*)-24-methylcholesta-5,22-dien-3β-ol (**10b**) as 24*R* (β-H) is tentative and based on the shift of the 21-H_3 (δ_H 1.010) which is in agreement with the value δ_H 1.004 observed in (24*R*)-24-methylcholest-22-en-3β-ol (**10a**) as compared with δ_H 0.996 observed in the spectrum of the 24*S*-epimer (**9a**) and the shape of the ABxy system of the signal for the olefinic protons 22- and 23-H.[14]

The Free Sterols.—The Table lists the eleven sterols isolated from the starfish *Euretaster insignis*, together with their relative retention times (r.r.t.) in h.p.l.c. and percentage of the sterol components.

In (**9a**) (24*S*) and (**10a**) (24*R*) the stereochemistry at C-24 was deduced by comparison of the n.m.r. spectra of the two epimers. The spectra are very similar but the C-21 methyl doublet occurs at higher field (δ_H 0.996) in the spectrum of the more polar component (**9a**) than in the spectrum of the less polar component (**10a**) (δ_H 1.004) and this suggested the 24*S* (α-H) and 24*R* (β-H) stereochemistries for (**9a**) and (**10a**), respectively.[15,16] In confirmation, the major 24*S* (α-H)-Me-Δ^{22}-component (**9a**) was hydrogenated to give the saturated derivative. The shift for one of the C-26/C-27 methyl groups is sensitive to the stereochemistry at C-24, and our value of δ_H 0.805 was in agreement with the value of δ_H 0.801 reported for a 24*R* (α-H)-methyl group in a 5α-steroidal alkane as compared with δ_H 0.777 for the corresponding 24*S* (β-H) compound.[17] The ^{13}C n.m.r. spectra of

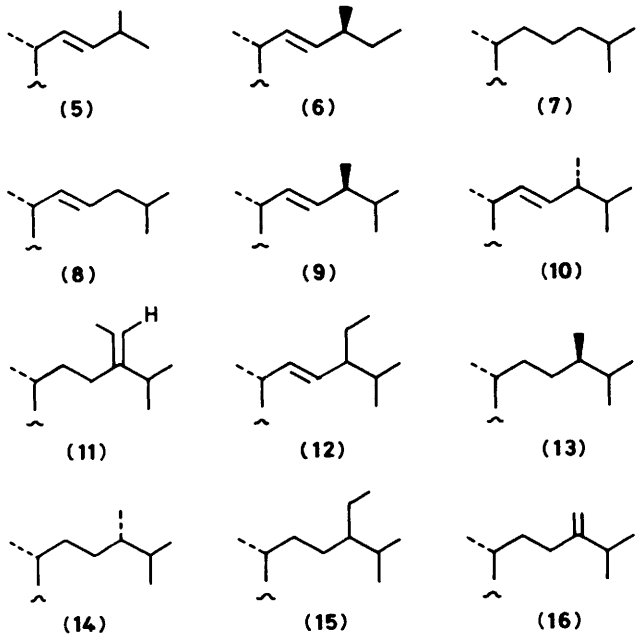

Figure 3. Structures of sterol side-chains

both epimeric 24-methylcholestanols have been described[18,19] and characteristic differences between 24*R*- and 24*S*-isomers were recognized expecially at C-25, C-26, and C-27. In the spectrum of our hydrogenated sample the shift values of δ_C 32.5, 20.7, and 18.4 p.p.m. for C-25, C-26, and C-27, respectively, were in close agreement with the values of δ_C 32.45, 20.22, and 18.28 p.p.m. reported for 24*R* (α-H)-methylcholestanol as compared with δ_C 31.58, 17.64, and 20.53 p.p.m. for the corresponding 24*S* (β-H)-compound.[18] The 24*S* configuration was tentatively assigned to the sterol (**6a**) because its C-21 methyl doublet (δ_H 0.998) is almost identical with that (δ_H 0.996) of compound (**9a**). The assignment of the configuration of the 24-ethyl group in C_{29} sterols by ^1H n.m.r. spectroscopy is not easy even if one has both epimers at C-24.[17] With ^{13}C n.m.r. the C-24 epimers are more readily distinguishable although the differences are small.[18,19] Insufficient amounts of compounds (**12a**) and (**15a**) did not permit their ^{13}C n.m.r. spectra to be measured.

Unlike in most animals, the major components of the sterol mixtures from starfishes are Δ^7-sterols, such as 5α-cholest-7-en-3β-ol, which usually is the most abundant sterol.[10] In this starfish cholest-7-en-3β-ol (**7c**) accounts for only 3% of the total sterol mixture and all the remaining sterols are 5α-cholestanol derivatives. It has been reported that cholesterol efficiently inhibited the haemolytic activity of *Marthasterias glacialis* saponins,[20,21] while lysis of sheep erythrocytes by *M. glacialis* saponins was only slightly inhibited by 5α-cholest-7-en-3β-ol.[21] This suggested that cytolysis results from removal of membrane cholesterol by the asterosaponins and the apparent immunity of starfishes to their own saponins is due to the presence of Δ^7-sterols instead of Δ^5-sterols. The small amount of Δ^7-sterols in *Euretaster insignis* could be related to the apparent absence of asterosaponins.

Experimental

General.—N.m.r. spectra were run on a Bruker WM-250 spectrometer; the spectra of the free sterols were run in $CDCl_3$, the spectra of the sulphated sterols were run in CD_3OD. High-resolution mass spectra were performed on a Kratos MS 902 spectrometer and low-resolution m.s. on an AEI MS 30 apparatus at 70 eV. H.p.l.c. was performed on a Associates HPLC system (6000 A pump equipped with a U6K injector and differential refractometer, model 401, as detector) using two different reverse-phase columns: Whatman Partisil M9 10/50 ODS-2 (50 cm × 9 mm i.d.) with absolute methanol as the mobile phase for separation of monohydroxy steroids, and Whatman Partisil M9 10/50 ODS-1 (50 cm × 9 mm i.d.) with methanol–acetonitrile–H_2O (70:10:20) for separation of dihydroxysteroids. Droplet counter-current (DCC) separation was made on a DCC-A apparatus manufactured by Tokyo Rikakikai Co. [250 tubes: solvent systems: chloroform–methanol–water (7:13:8), upper phase as mobile phase; and n-butanol–acetone–water (45:15:75), lower phase as mobile phase; flow rate 10—15 ml h^{-1}; the eluants were collected in 3 ml fractions and monitored by t.l.c. on silica-pre-coated glass sheets (Merck) with n-butanol–acetic acid–water (60:15:25); detection with cerium(IV) sulphate–H_2SO_4]. G.l.c. was performed on a Carlo Erba Fractovap 2900 capillary column (SE-30, 25 m) chromatograph.

Extraction, Sulphated Sterol Isolation, and Fractionation of the Desulphated Sterols.—*Euretaster insignis* starfishes (2.7 kg, fresh), collected off Nouméa, New Caledonia (1981 May), were extracted with water (5 l) for 5 h. The extracts were lyophilysed to give a residue (200 g), which was extracted with ethyl acetate (13.2 g of residue extracted), then chloroform–methanol (9:1) (10.4 g of residue extracted), and methanol (10.6 g of residue extracted).

The methanol-soluble material (2 g) was chromatographed on a column of Sephadex LH-20 (4 × 60 cm) to provide, in the first fractions, a mixture of sulphated 3β-21-dihydroxysteroids (0.3 g), R_F in SiO_2 t.l.c. 0.5, and in the subsequent fractions a mixture of sulphated monohydroxysteroids (0.13 g), R_F in SiO_2 t.l.c. 0.7.

The crude sulphated 3β,21-dihydroxysteroids were further purified by DCCC in n-butanol–acetone–water (45:15:75). In total, from methanol-soluble material (5 g) sulphated dihydroxysteroids (0.18 g) were obtained (Found: C, 53.9; H, 7.1; S, 10.5%); $\nu_{max.}$ 1 250 cm^{-1}; n.m.r. data of this fraction are given above; and sulphated monohydroxysteroids (0.26 g). Further amounts (0.25 g) of sulphated monohydroxysteroids were recovered from the chloroform–methanol-soluble material (3.2 g) by DCCC [chloroform–methanol–water (7:13:8)]. Each fraction was solvolysed in dioxane (0.5 ml)–pyridine (0.5 ml)/50 mg of sulphated material at 120 °C for 4 h and, after the mixture had cooled, water (5 ml) was added and the solution was extracted with diethyl ether (× 3). The organic layer was washed with water and evaporated under reduced pressure to give the free sterols mixture.

The 3β-OH steroids were fractionated by h.p.l.c. on an ODS-2 column into six fractions which were each analysed by mass spectrometry and n.m.r. spectroscopy. All fractions but one contained almost pure compounds, which were all known sterols (Table). The peak with r.r.t. 0.94 was a mixture of two sterols, the major (**10b**) and the minor (**16a**). The 24-methylenesteroid structure of (**16a**) was mainly based on the presence of a peak at *m/z* 316 (McLafferty rearrangement) in the mass spectrum and on the presence of two olefinic signals (broad singlets) at δ 4.72 and 4.67 in the n.m.r. spectrum of the mixture.

The 3β,21-dihydroxy steroids were fractionated by h.p.l.c. on an ODS-1 column into three fractions, peak 1 (9 mg, elution time 25 min), peak 2 (17 mg, elution time 27 min), and peak 3 (10 mg, elution time 31 min). G.l.c., m.s., and n.m.r. analyses of peaks 2 and 3 showed that they were still mixtures. They were then fractionated by argentic silica column chromatography [30% $AgNO_3$; gradient eluant light petroleum (b.p. 40—70 °C) and increasing amounts of diethyl ether] as the acetate

derivatives (pyridine–Ac_2O; room temperature). In the case of peak 2 one fraction still consisted of a mixture of two minor C_{27} components, (**2a**) and (**1b**), and their structures were established on the basis of m.s. and n.m.r. data taken on the mixture.

Physical Data of the 3β,21-*Dihydroxysteroids*.—For g.l.c. and h.p.l.c. retention times see the Table. The n.m.r. and m.s. data of the major components (**3a**) and (**4a**), which were each obtained in admixture with minor compounds after h.p.l.c. separation, are extracted from the spectra of the mixtures. For the minor C_{27} components we report below only the spectral data of their acetate derivatives. Multiplicities and *J* values (Hz) are shown in parentheses.

(20R)-24-*Methylenecholest*-5-*ene*-3β,21-*diol* (**3b**), m.p. 139—141 °C (from ethanol); $[\alpha]_D$ −23.8° (*c* 2 in MeOH); *m/z* 414.3395 (M^+, 16%. $C_{28}H_{46}O_2$ requires *M*, 414.3497), 396.3423 ($C_{28}H_{44}O$, 15), 330.2580 ($C_{21}H_{34}O_2$, 6.5), 285.2449 (10.5), 273.2228 ($C_{19}H_{29}O$, 50.2), 271.2048 ($C_{19}H_{27}O$, 100), 255.2130 ($C_{19}H_{27}$, 20), 253.2033 ($C_{19}H_{25}$, 16), 231.1803 ($C_{16}H_{23}O$, 6.5), 229.1776 ($C_{16}H_{21}O$, 18), 215.1776 ($C_{16}H_{23}$, 19), and 213.1659 ($C_{16}H_{21}$, 41); δ_H 0.72 (s, 18-H_3), 1.03 (s, 19-H_3) 1.04 (d, *J* 7, 26- and 27-H_3), 3.53 (m, $w_{\frac{1}{2}}$ 22, 3α-H), 3.72 (br s, 21-H_2), 4.70 and 4.75 (each s, together 28-H_2), and 5.36 (d, *J* 5, 6-H); δ_C (C-16) 28.8, (C-17) 51.1, (C-18) 28.8, (C-20) 42.7, (C-21) 63.4, (C-22) 27.6, (C-23) 32.1, (C-24) 156.7, (C-25) 34.1, (C-26) 21.9, (C-27) 22.0, and C(28) 106.5 p.p.m.; the remaining signals were within ±0.1 p.p.m. of those reported for cholesterol.[5]

(20R)-24-*Methylenecholestane*-3β,21-*diol* (**3a**), *m/z* 416 (M^+, 4.8%), 398 (5.9), 332 (4.6), 273 (100), 257 (12.1), 255 (19.6), 217 (8.2), and 215 (21.1); δ_H 0.68 (s, 18-H_3), 0.82 (s, 19-H_3), 1.04 (d, *J*, 7, 26- and 27-H_3), 3.60 (m, $w_{\frac{1}{2}}$ 22, 3α-H), 3.72 (br s, 21-H_2), and 4.70—4.74 (each s, together 28-H_2); δ_C (C-16) 28.8, (C-17) 51.1, (C-18) 12.4, (C-20) 42.7, (C-21) 63.4, (C-22) 27.6, (C-23) 32.2, (C-24) 156.7, (C-25) 34.1, (C-26) 21.9, (C-27) 21.9, and (C-28) 106.4 p.p.m.; the remaining signals were within ±0.1 p.p.m. of those reported for 5α-cholestane.[5]

(20R,22E)-24-*Methylcholest*-22-*ene*-3β,21-*diol* (**4a**), *m/z* 416 (M^+, 6.4%), 398 (4.6), 319 (1.4, 20–22-cleavage), 318 (2.6, 20–22-cleavage), 273 (100), 257 (11.6), 255 (15.3), 233 (15.3), 217 (7.8), and 215 (17.6); δ_H 0.69 (s, 18-H_3), 0.82 (s, 19-H_3), 0.84—0.86 (26- and 27-H_3), 0.96 (d, *J* 6.7, 28-H_3), 3.60 (m, $w_{\frac{1}{2}}$ 22, 3α-H), 3.76 (complex signal, 21-H_2), 5.08 (dd, *J* 16 and 8.7, 22-H), and 5.44 (dd, *J* 16 and 7.5, 23-H).

(20R)-24-*Methylenecholestane*-3β,21-*diyl diacetate* (**3a**)-*diacetate*, *m/z* 440 (M^+ − AcOH, 22%), 425 (8), 356 (10), 315 (100), 257 (5), and 255 (9); δ_H 0.68 (s, 18-H_3), 0.82 (s, 19-H_3), 1.02 (d, 26- and 27-H_3), 2.02 and 2.05 (each s, CH_2CO), 4.02 and 4.21 (each dd, *J* 10.5 and 6, and 10.5 and 3, together 21-H_2), 4.70 (m and s, 3α-H and 28-H), and 4.75 (s, 28-H).

(20R,22E)-24-*Methylcholest*-22-*ene*-3β,21-*diyl diacetate* (**4a**)-*diacetate*, *m/z* 440 (M^+ − AcOH, 41%), 425 (13), 397 (30), 337 (15), 315 (100), 257 (42), and 255 (43); δ_H 0.71 (s, 18-H_3), 0.82 (s, 19-H_3), 0.87 and 0.88 (overlapping doublets, 26-and 27-H_3), 0.93 (d, *J* 7, 28-H_3), 2.00 and 2.02 (each s, CH_3CO), 3.92 and 4.18 (each dd, *J* 10.5 and 8, and 10.5 and 3.5, together 21-H_2), 4.69 (m, 3α-H), 5.10 (dd, *J* 16 and 8.7, 22-H), and 5.26 (dd, *J* 16 and 8.4, 23-H).

(20R)-*Cholestane*-3β,21-*diyl diacetate* (**1a**)-*diacetate*, *m/z* 488 (M^+, 41%), 428 (95), 317 (95), and 215 (100); δ_H 0.68 (s, 18-H_3), 0.82 (s, 19-H_3), 0.86 (d, *J*, 7, 26- and 27-H_3), 2.02 and 2.05 (each s, CH_3CO), 4.02 and 4.19 (each dd, *J* 10.5 and 6, and 10.5 and 3, together 21-H_2) and 4.69 (m, 3α-H).

(20R)-*Cholest*-5-*ene*-3β,21-*diyl diacetate* (**1b**)-*diacetate and* (20R,22E)-*cholest*-22-*ene*-3β,21-*diyl diacetate* (**2a**)-*diacetate*. These were obtained in admixture after argentic silica gel chromatography and the data below are the m.s. and n.m.r. data of the mixture (*ca.* 1:1, g.l.c.); *m/z* 486 (M^+, 22), 426 (100), 315 (55), 255 (43), and 213 (31); δ_H (**1b**)-diacetate 1.01 (s, 19-H_3), 4.02 (dd, *J* 10.5 and 6.5, 21-H), 4.60 (m, 3α-H), and 5.37 (d, *J* 5, 6-H); δ_H (**2a**)-diacetate 0.82 (s, 18-H_3), 3.90 (dd, *J* 10.5 and 8, 21-H), 3.69 (m, 3α-H), and 5.18 (m, 22- and 23-H); signals for 18-H_3, 26- and 27-H_3, and one of the two 21-methylene protons of both compounds overlap giving rise to signals at δ_H 0.70 (s), 0.87 (d), and 4.20 (m), respectively; the acetate signals gave rise to well separated singlets at δ_H 2.024, 2.033, 2.037, and 2.057.

24ξ-*Methyl*-5α-*cholestan*-3β-*ol from* (**3b**).—A solution of compound (**3b**) (6 mg), trityl chloride (excess), and pyridine (0.5 ml) was heated at 90 °C for 24 h in a reacti vial. After being cooled, the yellow solution was poured into ice–water and extracted with dichloromethane. After removal of the solvents, the residue was chromatographed through a Pasteur pipette filled with a slurry of silica gel in dichloromethane to give the 21-*O*-trityl derivative (4 mg), characterized by n.m.r. spectrometry, δ_H CH_2OCPh_3, ABq with lines centred at δ_H 3.27 (*J* 10.5 and 5.0 Hz) and 3.33 (*J* 10.5 and 3.5 Hz), δ_H CHOH 3.52 (m). Acetylation with pyridine and acetic anhydride (room temperature) gave the 3β-OAc,21-*O*-trityl derivative, δ_H CHOAc 4.67 (m), which was hydrogenolysed on Pd–C in ethanol at room temperature overnight to give, after purification through a Pasteur pipette filled with silica gel in dichloromethane, a mixture of C-24 epimers of 21-hydroxy-24-methyl-5α-cholestan-3β-yl acetate, δ_H 3.68 (br s, 21-H_2) and 4.67 (m, 3α-H). Tosylation followed by lithium aluminium hydride reduction was carried out exactly as described by Bottin and Fetizon.[7] The product was purified by h.p.l.c. using an ODS-2 column and methanol as solvent to give a mixture of C-24 epimers of (20*R*)-24-methyl-5α-cholestan-3β-ol, *m/z* 402 (M^+); δ_H 0.656 (s, 18-H_3), 0.779 (d, *J* 6.4 Hz, 28-H_3), 0.785 [d, *J* 6.3, 27-H_3 (24*S*-isomer)], 0.809 [d, *J* 6.2 Hz, 27-H_3 (24*R*-isomer)], 0.810 (s, 19-H_3), 0.856 (d, *J* 6.8 Hz, 26-H_3), 0.860 (d, *J* 6.7 Hz, 26-H_3), 0.896 [d, *J* 6.6 Hz, 21-H_3 (24*R*-isomer)], and 0.905 [d, *J* 6.6 Hz, 21-H_3 (24*S*-isomer)].

Free Sterol Isolation and Fractionation.—The ethyl acetate-soluble material (*ca.* 8.5 g) was chromatographed on a silica gel (300 g, 70—230 mesh, kieselgel 60) column in dichloromethane to provide a crude sterol mixture which was acetylated (pyridine–Ac_2O; room temperature) and further purified by silica gel column chromatography [light petroleum (b.p. 40—70 °C), then benzene as eluant]. The mixed acetates (0.76 g) were fractionated by chromatography on a column filled with Lichroprep Si 60 (particle size 25—40 m) in light petroleum (b.p. 40—70 °C) and increasing amounts of benzene to provide the 5α-stanyl acetates mixture and cholest-7-en-3β-yl acetate, eluted in that order. The central fractions, which still consisted of a mixture, were further fractionated by h.p.l.c. on a μ-porasil column [hexane–benzene (7:3) as eluant]. In total cholest-7-en-3β-yl acetate (n.m.r. and m.s.) (11.8 mg), and mixed 5α-cholestanyl acetates (402 mg) were obtained. Fractionation was continued by h.p.l.c on an ODS-2 column (methanol as eluant), after hydrolysis (10% KOH in methanol–H_2O at reflux; 2 h), which gave nine fractions which were each analysed by mass spectrometry and n.m.r. spectroscopy. All fractions but one contained almost pure compounds (Table). The fraction with r.r.t. 1.10 consisted of two steroidal alcohols (**7a**) and (**12a**), in almost equal porportions and were identified on the basis of m.s. and n.m.r. data of the mixture and g.l.c. comparison with authentic samples.

Hydrogenation of compound (**9a**) was carried out in ethanol with Pd–C at room temperature and atmospheric pressure.

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