Starfish Saponins. Part 5.[1] Structure of Sepositoside A, a Novel Steroidal Cyclic Glycoside from the Starfish *Echinaster sepositus* †

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The toxic major saponin from the starfish *Echinaster sepositus*, has been completely characterized. The structure is unique having as it does both a Δ^7, 3β,6β-dioxygenated-23-oxosteroidal moiety and a sugar moiety [β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl] which bridges the C-3 and C-6 atoms of the steroid. The 3β-hydroxy-group of the steroid forms an *O*-acetal linkage with the glucuronate unit, while the HO–C(6‴) of the glucopyranosyl unit is attached to C-6 of the aglycone, forming a 6β-*O*-ethereal linkage.

HOLOTHUROIDS and asteroids are unique among marine animals because they contain toxic steroidal saponins known as holothurins and asterosaponins, respectively. These compounds, which have a wide variety of pharmacological activities, are apparently absent from the other three classes of echinoderms.[2]

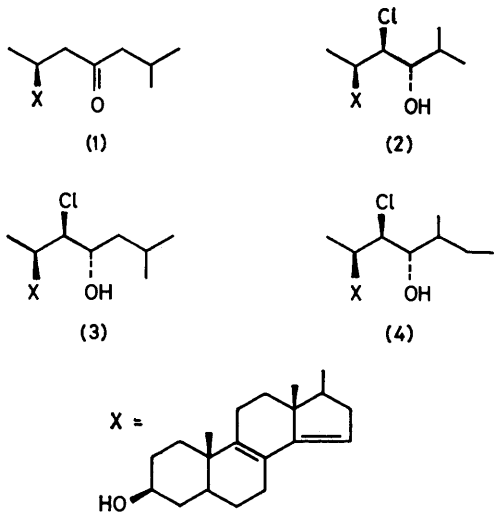
Asterosaponins exhibit haemolitic,[3] neurotoxic,[4] and antiviral [5] properties. They also induce escape-reactions in molluscs [6] and have been identified as the spawning inhibitor in the Japanese starfish *Asterias amurensis*.[7] More recently they have been reported to inhibit the production of 1-methyladenine in the follicle cell.[8]

From the chemical point of view asterosaponins are glycosides which, upon acid hydrolysis, give a steroidal aglycone (asterosapogenin), sugars, and a sulphate.[2,9] Most of the reported works have been concerned with the structure determination of the aglycones produced by acid hydrolysis, except for the structural studies of asterosaponin A and glycoside B_2 from *Asterias amurensis* [10,11] and thornasteroside A from *Acanthaster planci*.[12] The sapogenins possess a 3β,6α-dihydroxy-oxidation pattern and the biosynthetically unusual 9(11) double bond; [1,9,13] the polysaccharide moieties are attached by a glycoside linkage to C-6 while the sulphate ester is attached to C-3.[11,12,14]

Recently, we described the first examples of asterosapogenins lacking both the 6α-hydroxy-group and the 9(11) double bond, which were isolated from the hydrolysate of the *Echinaster sepositus* saponins mixture; the major genin was characterized as 3β-hydroxy-5α-cholesta-8,14-dien-23-one (1) [15] and the three minor ones were shown to be the chlorohydrins (2)—(4).[16] We also showed that the chlorohydrins originate from their corresponding 22,23-epoxides during hydrochloric acid hydrolysis.

We report herein the structure elucidation of the major saponin, sepositoside A, from *Echinaster sepositus*, which is of a novel type. It is devoid of the sulphate group and its structure includes a Δ^7, 3β,6β-dioxygenated steroidal nucleus and a cyclic trisaccharide moiety which bridges the C-3 and C-6 atoms of the steroid.

Fresh animals, collected at different periods of the year from the bay of Naples, were extracted by chopping and soaking them in water and the saponins were removed by Amberlite XAD-2 resin.[17] The crude saponins were chromatographed on silica gel to give, on elution with chloroform–methanol, a white solid (homogeneous in t.l.c.), which was separated into three fractions by reversed-phase h.p.l.c.



The central fraction, sepositoside A, which accounted for *ca.* 80% of the total saponin mixture, was shown to be homogeneous by spectroscopy (^{13}C and ^1H n.m.r.) and by degradative experiments (see below). Sepositoside A (5a) is toxic, LD_{50} = 43 mg kg^{-1} (i.p. injection, Litchfield and Wilcoxon method [18]), and, unlike other asterosaponins, is devoid of a sulphate group. Chemical analysis gave $C_{45}H_{69}O_{18}Na$; it is laevorotatory, $[\alpha]_D$ −68.5° (*c* 1, H_2O) and, on acid hydrolysis, it yielded the steroid (1) [15] as the sole sapogenin, and glucose, galactose, and glucuronic acid. Spot intensities in paper chromatography (p.c.) and integration of the g.l.c. peaks of the trimethylsilylated monosaccharide mixture showed that glucose and galactose were obtained in a 1 : 1 ratio, while glucuronic acid was present in much smaller amounts. It is well known that glucuronic acid decomposes during acid treatment,[19] but a 1 : 1 : 1 ratio of glucose, galactose, and glucuronic acid from sepositoside

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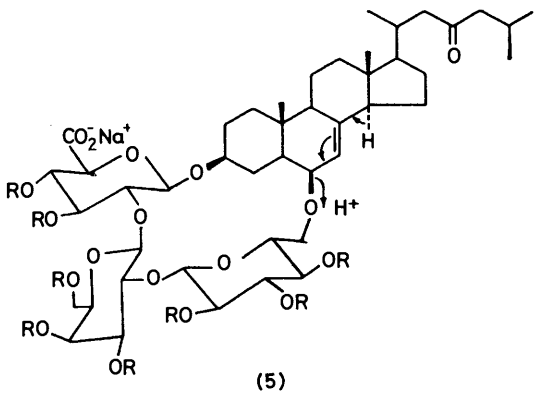
A was obtained by methanolysis and g.l.c. of the resulting methyl glycosides.

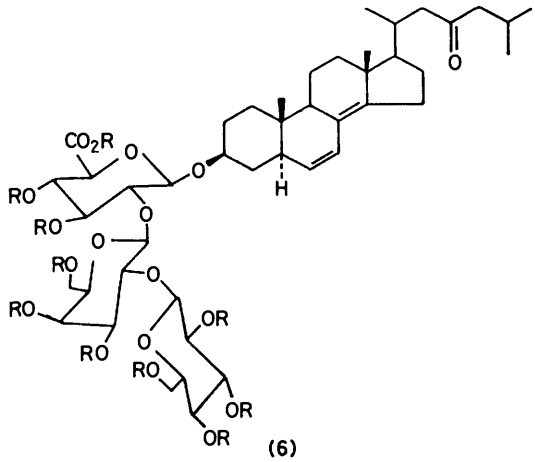
The u.v. spectrum of sepositoside A, which showed no absorption beyond λ 210 nm, established that the toxin does not possess the $\Delta^{8,14}$ conjugated diene system of (1); hence the structure of the asterosapogenin could not be the structure of the intact steroid glycoside prior to acid hydrolysis. The ^{13}C n.m.r. spectrum of the intact saponin showed the presence of only one trisubstituted double-bond, δ_C 143.0 (s) and 119.0 (d) p.p.m., which indicated that the $\Delta^{8,14}$ diene system in the aglycone (1) was formed by dehydration and possibly a double-bond migration during the acid hydrolysis. We note that the $\Delta^{8,14}$ diene system in a steroidal structure is particularly stable; it is formed, for example, from a $\Delta^{5,7}$ diene system by treatment with acid. It has been known for many years [20] that the reaction of ergosterol with hydrogen chloride in chloroform gives, successively, ergosterol-B_2 [$\Delta^{6,8(14)}$], -B_1 ($\Delta^{8,14}$), and -B_3 ($\Delta^{7,14}$).



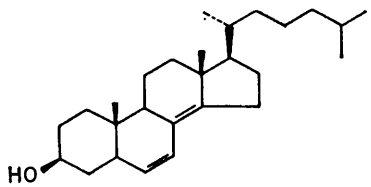
(5)
a; R = H
b; R = Me, $CO_2^-Na^+$ = CO_2Me

On very mild acid treatment (aqueous 1N HCl, room temp.), sepositoside A (5a) afforded a precipitate, $C_{54}H_{70}O_{18}$, soluble in methanol, $[\alpha]_D$ −27.4° (*c* 0.7, MeOH), u.v. active and still containing glucose, galactose, and glucuronic acid. The u.v. [$\lambda_{max.}$ 259 (s), 251 (ε 18 800), and 244 (s)] spectrum is consistent with a *transoid* conjugated diene system and is similar to that of ergosterol-B_2 [5α-ergosta-6,8(14),22-trien-3β-ol].[21] The ^1H n.m.r. spectrum (CD_3OD) of the opened saponin (6a) showed two 1-H olefinic signals at δ 6.12 (dd, *J* 10 and 3 Hz, 7-H) and 5.27 (br d, *J* 10 Hz, 6-H) in agreement with a steroidal $\Delta^{6,8(14)}$ diene system. Direct comparison of the spectra (u.v., ^1H n.m.r., and ^{13}C n.m.r.) of the opened saponin (6a) with those of the model 5α-cholesta-6,8(14)-dien-3β-ol (7), m.p. 112—115 °C, $[\alpha]_D$ −54.9° (*c* 0.4, $CHCl_3$); $\lambda_{max.}$ 259 (s), 251 (ε 21 096), and 244 nm (s) definitively established the 3β-*O*-glycosidated-5α-cholesta-6,8(14)-dien-23-one structure for (6a). ^1H and ^{13}C N.m.r. are given in Tables 1 and 2, respectively. Note that the best fit between the ^{13}C n.m.r. data is observed when the carbon shifts of the model (7) in $CDCl_3$ are compared with those of the permethylated opened saponin (6b) (see below), whose spectrum was also recorded using $CDCl_3$ as the solvent.

The sequence of sugar residues in (6a) was determined as follows. Permethylation of (6a) with methyl iodide–dimethylformamide (DMF)–sodium hydride [22] gave a



(6)
a; R = H
b; R = Me



(7)

deca-*O*-methyl derivative (6b), M^+ 1 038, (^1H and ^{13}C n.m.r. data are given in Tables 1 and 2) which, on acid hydrolysis, yielded 2,3,4,6-tetra-*O*-methylglucose (t.l.c. and g.l.c.). This established glucose as the terminal sugar and hence the sugar residue sequence is obvious from the mass-spectral fragmentation pattern (Figure). Thus, the fragment with *m/e* 423, accompanied by a series of peaks, derived by the elimination of methanol and/or the expulsion of C-6 (as ethylene oxide),[23] at *m/e* 391, 359, 327, 379, and 347 suggested the sequence glucose–galactose and the fragment with *m/e* 616—615 ($M^+ + H - 423$ and $M^+ - 423$), which represents the other half of the molecule, confirmed that glucuronic acid is linked directly to the aglycone unit. Further fragments are indicated in the Figure.

The interglycosidic linkage in the opened sepositoside A (6a) was determined using ^{13}C n.m.r. spectroscopy, which has recently been shown to be a powerful tool for elucidating structures of natural glycosides without using chemical degradation.[24] Assignments of the sugar carbon atoms (Table 3) have been made by comparing the spectrum with those of methyl-β-D-glucopyranoside,[25-27] -β-D-galactopyranoside,[28-30] and -β-D-glucuronopyranoside.

Whereas the anomeric carbon atoms appear in the region δ_C 104.9—105.9 p.p.m. in these glycosides, they

TABLE 1

^{1}H N.m.r. data for sepositoside A, derivatives, and model compounds [δ(*J* Hz)]

	Solvent	6-H	7-H	19-Me	18-Me	Anomeric H	OMe
(5a)	DMSO [a]		5.43 ($W_{\frac{1}{2}}$ 12)	0.82	0.55	4.43, 4.52, 5.03 (each d, *J* 8.0, 7.5, 7.5)	
(5b)	$CDCl_3$		5.45 (br d, 5; ($W_{\frac{1}{2}}$ 11)	0.94	0.62	4.54, 4.64, 4.72 (each d, *J* 8.0, 7.5, 6.5)	3.72, 3.61 (×2), 3.53, 3.49 (×4), 3.38
(6a)	CD_3OD	5.27 (d, 10)	6.12 (dd, 10.3)	0.66	0.96	obscured by H_2O signal	
(6b)	$CDCl_3$	5.26 (d, 10)	6.13 (dd, 10.3)	0.63	0.92	4.33 (2 H) and 4.7 (1 H) (each d, *J* 7)	3.72, 3.54, 3.47 (×7), 3.30, 3.32
(7)	$CDCl_3$	5.25 (d, 10)	6.12 (dd, 10.3)	0.64	0.91		
(8a)	$CDCl_3$	3.8 (m)	5.18 (br s, ($W_{\frac{1}{2}}$ 4)	0.85	0.55		
(9a)	$CDCl_3$	3.92 (m)	5.45 (br d, 5; $W_{\frac{1}{2}}$ 10)	0.94	0.60		

[a] DMSO = dimethyl sulphoxide.

TABLE 2

^{13}C N.m.r. shifts of sepositoside A, derivatives, and model compounds [a]

Aglycone carbon atoms	(5a) (D_2O) [b]	(5b) ($CDCl_3$) [c]	(6a) (CD_3OD) [d]	(6b) ($CDCl_3$) [e]	(7) ($CDCl_3$)	(8a) ($CDCl_3$) [f]	(9a) ($CDCl_3$)
1	38.8	37.8	38.1	36.8	37.0	37.2	38.4
2	28.8	28.6	30.4	29.0	31.7	30.8	31.5
3	*b*	*c*	80.5	80.1	71.6	71.0	71.6
4	31.1	29.7	34.0	32.6	36.1	33.7	34.3
5	45.3	44.9	46.0	44.8	45.0	48.5	45.0
6	*b*	*c*	126.8 [g]	125.7 [g]	126.0 [f]	69.7	68.5
7	119.0	118.3	131.1 [g]	130.1 [g]	129.6 [f]	122.1	120.5
8	143.0	141.7	127.7	125.6	125.5	141.2	143.6
9	49.9	49.2	49.0	48.3	48.5	49.1	49.5
10	34.4	34.0	37.1	36.0	36.0	35.4	33.9
11	23.5 [g]	21.9	20.9	19.8	19.9	21.3	22.0
12	40.4	39.4	36.3	35.3	35.4	39.3 [g]	39.6
13	44.2	43.6	45.0	43.9	43.9	43.5	43.5
14	55.8	55.0	147.6	147.8	147.7	54.9	54.9
15	23.7 [g]	23.1	25.8	24.9	25.1	22.8	23.0
16	28.8	28.0	28.6	27.6	27.5	27.9	28.0
17	57.0	56.1	57.5	56.2	56.3	56.1	56.1
18	12.9	12.1	19.8	19.4	19.4	11.8	12.0
19	15.1	14.4	11.9	11.3	11.5	13.9	16.0
20	33.5	32.8	32.6	31.5	34.9	36.0	36.2
21	20.8	19.9	20.6	20.2	19.0	18.8	18.8
22	50.8	50.4	51.1	50.3	36.9	36.0	36.2
23	211.7	210.6	213.8	210.9	23.9	23.8	23.9
24	53.3	52.5	53.5	52.6	39.7	39.4 [g]	39.6
25	25.1	24.5	25.8	24.6	28.1	27.9	27.9
26	23.4 [h]	22.6 [h]	22.9 [h]	22.5 [h]	22.8 [h]	22.5 [h]	22.6 [h]
27	23.5 [h]	22.7 [h]	23.0 [h]	22.5 [h]	22.6 [h]	22.8 [h]	22.8 [h]

[a] Chemical shifts are expressed as δ; the ^{13}C n.m.r. signals were assigned using ^{1}H single-frequency off-resonance decoupling and known chemical-shift rules, and by comparison with the literature data (J. W. Blunt and J. B. Stothers, *Org. Magn. Reson.*, 1977, **9**, 439) of each compound. [b] 1,4-Dioxan (δ 67.4 p.p.m.) was used as the internal standard in the calculation of chemical shifts; sugar carbon atoms δ_C 102.1 98.7, 96.7 (anomeric), 61.7 (CH_2–OH), 175.7 (–CO_2^-) and a broad group of overlapping signals between 69.7 and 77.6 for the remaining sugar carbons and C-3 and C-6 of the aglycone; the spectrum had a significant signal broadening. [c] The resonances of the sugar carbon atoms are δ_C 97.2, 99.8, and 102.3 (anomeric); 70.6 and 73.1 (C-6″-gal and C-6‴-gluc), 168.9 (*C*O_2Me); 72.8, 74.1 (×2), 74.4, 74.6, 74.9, 76.7, 76.9, 81.0, 81.7, 84.3, 85.9, and 86.6 (×2), for the remaining sugar carbons and C-3 and C-6 of the aglycone. The resonance of the methoxy-carbons are δ_C 52.25 (CO_2*Me*), 57.8 [C-3-gal (ref. 28)], 59.1, 59.7 [C-6 and C-4-gal (ref. 28)], 60.2, 60.3, 60.4, 60.6, and 60.8. [d] The resonances of the sugar carbon atoms are given in Table 3. [e] The resonances of the sugar carbon atoms are: δ_C 102.7, 84.5, 86.6, 79.7, 74.5, and 71.4 (C-1‴—C-6‴-gluc), 100.5, 79.7, 85.2, 72.74, 74.13, and 70.6 (C-1″—C-4″-gal), 102.2, 85.4, 86.6, 81.2, 74.0, and 168.9 (C-1′—C-6′-glucur); the resonances of the methoxy-carbon atoms are δ_C 52.4 (CO_2*Me*), 57.6 (C-3-gal), 59.1 (×2), 59.6, 60.1 (×2), 60.7 (×2), and 61.1. Assignments are tentative and based on the comparison of the spectrum with those of methyl 2,3,4,6-tetra-*O*-methyl-β-D-gluco- and -galacto-pyranosides (J. Haverkamp, J. P. C. M. van Dongen, and J. F. G. Vliegenthart, *Tetrahedron*, 1973, **29**, 3431 and ref. 28, respectively). [f] Differentiation of the pair C-5 and C-9 was made by comparing the spectrum with that of the di-*O*-methyl derivative (8b) in which the C-5 resonance was shifted to 45.9 p.p.m. while the C-9 remained unaffected. [g,h] Assignments in each vertical column can be reversed.

appear at δ_C 101.7 and 106.1 p.p.m. in (6a). At first these values are suggestive of β-glycopyranosyl linkages for all the monosaccharides; the C-1 resonances usually appear in the region δ_C 97.5—101.2 p.p.m. for the α-anomers; [30,31] moreover two doublets (2 H and 1 H) at

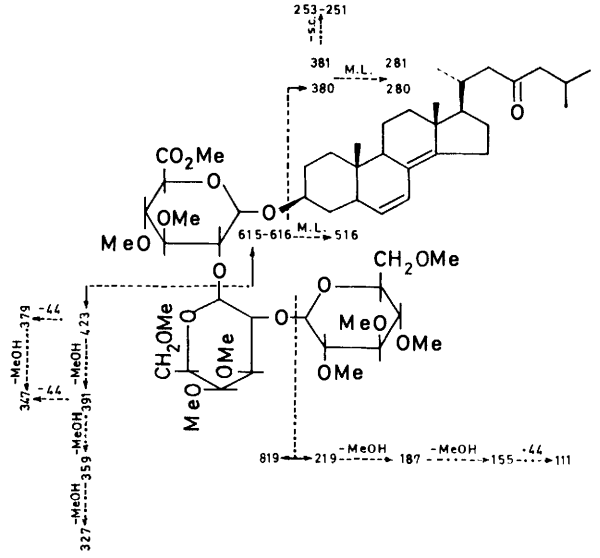


FIGURE Mass-spectral fragmentation pattern of (6b); M.L. = 20,22 cleavage and 17H transfer (McLafferty rearrangement); s.c. = side chain

δ 4.53 and 4.70 with J 7 Hz in the ^{1}H n.m.r. spectrum of the permethylated opened sepositoside A (6b) also indicate β-glycopyranosyl linkages [32] ($^4C_1$ conformation). The absolute D-configuration of glucose and galactose was established by isolating them from the hydrolysate of the saponin and determining their rotations, while the D-configuration of glucuronic acid was derived from the observed molecular rotation, $[M]_D$, for (6a) which was in good agreement with that calculated for the above β-D-configuration on the basis of Klyne's rule.[33]

Appearance of one anomeric carbon signal in the ^{13}C n.m.r. spectrum of (6a) at relatively high field (δ_C 101.7 p.p.m.) is explained in terms of shielding effects expected for the C-1 signal in secondary alcoholic β-D-glycopyranosides (δ_C 102.0—102.5 p.p.m.); so the signal at δ_C 101.7 p.p.m. is due to C-1′ of the glucuronate unit and its resonance at a slightly higher field than that reported by Tori *et al.*[34] for C-1 of 5α-cholestan-3β-yl-β-D-glucopyranoside seems to indicate a substitution at C-2′.[35] The presence of two signals at δ_C 62.1 and 62.6 p.p.m., which may be assigned to the HO–H_2C carbons, exclude a glycosidation at C-6″ of galactose. Glycosidation at C-4″ and C-3″ of the galactose unit can be also excluded by (i) the appearance of a signal at δ_C 69.9 p.p.m. which may only be assigned to HO–C-4″ of galactose. The possibility of *O*-substitution at C-4″ was also discarded because of the presence of two peaks at δ_C 83.0 and 85.5 p.p.m., which arose from glycosidated carbons. Recently, Cox *et al.*[36] have measured the ^{13}C n.m.r. spectrum of a trisaccharide containing a 4-*O*-substituted β-D-galactopyranosyl unit and reported, for C-4, a chemical shift of 78.31 p.p.m. (ii) The recent observation of Voelter *et al.*[30] of a strong upfield shift of δ_C 4.55 p.p.m. for C-4 in a 3-*O*-substituted galactopyranosyl residue; so it should be expected that a resonance line at *ca.* δ_C 65 p.p.m. (glycosidation at C-3 of the galactose moiety) and no line between δ_C 62.6 and 69.9 p.p.m. would appear in the spectrum of (6a). The C-2″ glycosidic linkage is also evident because the C-2″ carbon in the galactose residue is shifted downfield by *ca.* 10 p.p.m. (β-effect) to 83.0 p.p.m.; the C-3″ carbon atom is also shifted downfield by 2.5—3 p.p.m.,[29] whereas the other signals of the galactose moiety remain almost unaffected.

The C-3 carbon atom signal of the aglycone is shifted from δ_C 71.6 [free 5α-cholesta-6,8(14)-diene-3β-ol] to 80.5 p.p.m. in (6a) in agreement with the glycosidation shift reported by Tori *et al.*,[34] for 5α-cholestan-3β-yl-β-D-glucopyranoside and cholesteryl-β-D-glucopyranoside.

TABLE 3

^{13}C N.m.r. shifts of sugar carbons in (6a)

Sugar carbon atoms	(6a) [a]			Methyl α-D-glycopyranosides		
	Gluc	Gal	Glucur	Gluc [b]	Gal [c]	Glucur [d]
1	106.1	104.8	101.7	105.5	104.9	105.9
2	75.2	83.0	85.4	74.9	71.8	74.7
3	77.9	76.5 [e]	77.1 [e]	78.3	73.9	77.4
4	71.3	69.9	72.4	71.4	69.8	72.8
5	79.0	76.2 [e]	76.5	78.2	76.2	76.9
6	62.6	62.1	172.7	62.7	62.1	162.1 (CO_2Me)

[a] Measured in CD_3OD; the chemical shifts are expressed as δ; the peaks to the aglycone carbons are given in Table 2. [b] Ref. 25. [c] Refs. 28 and 30. [d] Measured in CD_3OD; assignments are based on the comparison of the spectrum with that of methyl β-D-glucopyranoside. [e] Signals in the same vertical and/or horizontal lines may be interchanged.

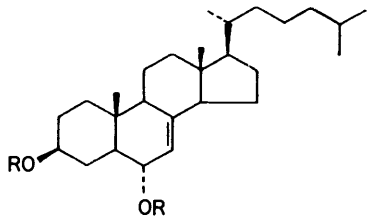
Thus, the remaining signals in the sugar carbon region of the ^{13}C n.m.r. spectrum are due to the glucuronate unit. The signal at δ_C 85.5 p.p.m. can be assigned to the glycosidated carbon atom and its appearance relatively downfield suggests a substitution at C-2′.

Indeed, the 1→2 linkage, galactose–glucuronate, has been confirmed by the classical chemical method of permethylation followed by acid hydrolysis and identification of the partially methylated sugars. The permethylated opened saponin (6b) was treated with sodium

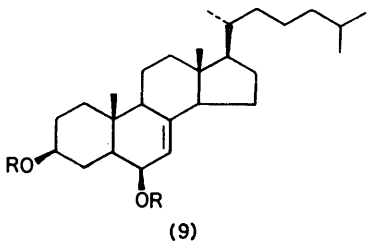
borohydride to reduce the methoxycarbonyl to a hydroxymethyl group.[37] The reduced sample was again methylated (methyl iodide–DMF–sodium hydride [22]) and then treated with a mixture of hydrochloric acid and acetic acid to afford a tri-*O*-methylglucose, which was identified as 3,4,6-tri-*O*-methylglucose. This definitively established that HO–C-2′ of glucuronate is the point of linkage for the galactosyl residue. The 1→2 linkage, glucose–galactose, was also confirmed from the mass spectra of the partially methylated galactitol acetate. After acid hydrolysis of the permethylated opened (6b), partially methylated sugars were separated by preparative t.l.c. and the tri-*O*-methylgalactose was treated with sodium borohydride and then acetylated with acetic anhydride–pyridine. Mass spectrometry of the resulting alditol acetate (*m/e* 233, 205, 189, 161, 129, 101, 87, 45, and 43) [38] confirmed it to be 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetylgalactitol. Thus the structure of (6a) is established as 3β-hydroxy-5α-cholesta-6,8(14)-dien-23-one-*O*-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside.

The easy formation, by very mild acid treatment, of a steroidal $\Delta^{6,8(14)}$ diene immediately suggested a Δ^7, 6-*O*-steroidal structure for the intact saponin. So we prepared both 5α-cholest-7-ene-3β,6α-diol and cholest-7-ene-3β,6β-diol model compounds. 5α-Cholest-7-ene-3β,6α-diol (8a), m.p. 189—191 °C, $[\alpha]_D$ +46°, was prepared by hydroboration of 7-dehydrocholesterol according to Caglioti *et al.*;[39] 5α-cholest-7-ene-3β,6β-diol (9a), m.p. 215—216 °C, $[\alpha]_D$ −35.0° (lit.,[40] m.p. 207—209 °C, $[\alpha]_D$ −31°) has been prepared by the manganese dioxide oxidation of (8a) followed by sodium borohydride reduction. Both epimeric 5α-cholest-7-ene-3β,6-diols, on very mild acid treatment (1N HCl in methanol, room temp.), afforded rapidly 5α-cholesta-6,8(14)-dien-3β-ol (7) paralleling the sepositoside A's chemical behaviour. Comparison of the ^1H and ^{13}C n.m.r. spectra (Tables 1 and 2) of the model compounds with those of the intact saponin (5a) and its permethylated derivative (5b), M^+ 1 024, supported the presence in the steroidal aglycone portion of a Δ^7, 6-*O*-structure and also indicated a 6β-stereochemistry. Particularly relevant in this respect were the chemical shift values for the 19- and 18-Me protons as well as the chemical shift and shape for the olefinic 7-H proton signal. In the spectrum of the 3β,6α-diol epimer (8a) these signals appeared at δ 0.85, 0.55 and 5.18 (br s, $W_{\frac{1}{2}}$ 4 Hz) and in that of 3β,6β-diol epimer (9a) they appeared at δ 0.94, 0.61, and 5.45 (br d, $W_{\frac{1}{2}}$ 10 Hz), respectively. In the spectrum of the permethylated saponin (5b), recorded in $CDCl_3$, the same signals were observed at δ 0.94, 0.62, and 5.45 (br d, $W_{\frac{1}{2}}$ 11 Hz). The significant difference observed for the 19-Me carbon signal in (9a) (δ_C 16.0 p.p.m.) and in (5b) (δ_C 14.4 p.p.m.) can be explained in terms of a substitution effect at the 6β-OH position in (5b). Indeed, in the spectrum of 5α-cholest-7-ene-3β,6β-diyl acetate, (9b) the 19-Me carbon is shifted upfield by *ca.* 1 to δ_C 15.1 p.p.m.

A more significant difference between the intact saponin (5a) and the opened saponin (6a), is the formation of a tri-*O*-methylglucose, identified as 2,3,4-tri-*O*-methylglucose, by acid hydrolysis of the permethylated intact saponin (5b) instead of the 2,3,4,6-tetra-*O*-methylglucose obtained from (6b).



(8)
a; R = H
b; R = Me



(9)
a; R = H
b; R = MeCO

This indicated a substitution at the HO–C-6‴ carbon of glucose in (5a), removable by very mild acid treatment. Moreover, in the mass spectrum of the permethylated sepositoside A (5b) (see Experimental section) the peaks at *m/e* 219 and 187 observed in the spectrum of the permethylated opened sepositoside A (6b) and due to the terminal permethylated glucose (Figure) are replaced by peaks at *m/e* 205, 187, and 173 indicative of a terminal trimethylated hexose. Further, the ^{13}C n.m.r. spectra of (5a) and (6a) contain marked differences in the sugar carbon region, especially for the chemical shifts of the anomeric carbons (see Tables 2 and 3), but much more significantly the spectrum of (5a) contained only one signal due to a primary hydroxy-bearing carbon atom at δ 61.7, whereas the spectrum of the opened glycoside (6a) showed two signals for HO–C-6 carbons at δ 62.6 and 62.1.

The above evidence gives the cyclic structure (5a) for the *E. sepositus* major saponin. This structure accounts for the peak at higher mass, *m/e* 1 024 (M^+), observed in the spectrum of the permethylated sepositoside A (5b), and its rearrangement to the opened saponin (6a) is straightforward [as shown by the arrows on (5)].

The cyclic structure (5a) of sepositoside A appears unique; the macrocyclic ring made up by the sugar moiety bridging C-3 and C-6 of steroid is unusual, but also the Δ^7, 6β-oxygenated steroidal structure has not previously been encountered among naturally occurring steroids.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. Semi-preparative h.p.l.c. separations were carried out on a μ-Bondapack C-18 column (7.8 mm × 30 cm) and on a carbohydrate analysis column (3.9 mm × 30 cm) using a differential refractometer, model 401, as the detector, a U6K injector, and a solvent delivery system, M6000, all from Waters Associates. G.l.c. was carried out on a Carlo Erba Fractovapor 2900 capillary column gas chromatograph fitted with a 20-m glass capillary column, OV-101 (gas flow, 2 ml min^{-1} hydrogen). T.l.c. was carried out on pre-coated silica-gel layers (2 mm, Merck F_{254}); descending paper chromatography (p.c.) was run on Whatman no. 1 filter paper using n-butanol–acetic acid–water (60 : 15 : 25, v/v/v, BAW) or ethyl acetate–pyridine–water (120 : 50 : 40 v/v/v, EPW) for the native monoses and the top layer of an n-butanol–ethanol–H_2O–NH_4OH (40 : 10 : 49 : 1) mixture for the methylated sugars.[41] Sugars were detected using standard aniline phosphate; saponins and other steroids with ceric sulphate–sulphuric acid.

Unless otherwise stated, u.v. spectra were taken for solutions in methanol, i.r. spectra for solutions in chloroform, and optical rotations for solutions in methanol. ^1H and ^{13}C N.m.r. spectra were taken on a Brücker apparatus WX-270 (tetramethylsilane as internal reference unless otherwise indicated). Mass spectra were measured with an A.E.I. MS 30 instrument at 70 eV; accurate mass measurements were performed with an A.E.I. MS902 instrument.

Standard Tri-O-methylglucoses.—These were obtained by permethylation (see below) of suitable disaccharides, subsequent acid hydrolysis (2N HCl–dioxan, 1 : 1, 4 h) and preparative t.l.c. in chloroform–acetone (2 : 1). 2,3,4-Tri-*O*-methylglucose [p.c. R_g (relative to 2,3,4,6-tetra-*O*-methylglucose) 0.89: g.l.c. (silylated sampler column temperature 100 °C) t_R 7.00 and 7.60 min] was prepared from rutin (Fluka AG).

2,3,6-Tri-*O*-methylglucose (R_g 0.86; t_r 9 min and 9 min 30 s) was prepared from maltose.

2,4,6-Tri-*O*-methylglucose (R_g 0.82; t_r 8 min and 9 min 2 s) was prepared from laminarin (U.S. Biochemical Corporation).

3,4,6-Tri-*O*-methylglucose (R_g 0.84; t_r 8 min and 8 min 43 s) was prepared from naringin (Fluka AG).

Isolation of Saponins.—*Echinaster sepositus* was collected in the bay of Naples at different periods of the year. Here we report a typical preparation. Fresh material (2.7 kg) was chopped into small pieces and soaked (3 h) in distilled water (*ca.* 1 l kg^{-1}). The aqueous extracts were clarified by centrifugation and passed through a column of Amberlite XAD-2 (B.D.H., 300 g) using the method of Gilgan *et al.*[17] The column was washed with water (1 bed vol) and methanol (2 bed vol). The methanol eluates were dried to give a syrup (2.7 g) which was then chromatographed on a column of silica gel (0.05—0.2 mm, Merck, 140 g) in chloroform–methanol, 7 : 3 (500 ml), 6 : 4 (500 ml), and finally 1 : 1 to give, in the chloroform–methanol 1 : 1 fractions, a crude saponin mixture as a pale yellow powder (1.52 g, single spot in t.l.c., R_F 0.41 in BAW). This material was subjected to semi-preparative h.p.l.c. (μ-Bondapack C-18 column; methanol–water 55 : 45; 3 000 lb in^{-2}; 5 ml min^{-1}; *ca.* 50 mg in 150 μl water) to give essentially three fractions. Removal of methanol under reduced pressure followed by lyophilization gave the saponins. Fractions 1 (t_r 8.08 min; 69 mg) and 3 (t 12.15 min; 100 mg) contained the minor saponins still in admixture with the major one; fraction 2 (t_r 9.67 min) yielded 440 mg of sepositoside A (5a) as a white amorphous powder, $[\alpha]_D$ −68.5° (*c* 1, H_2O); $\lambda_{max.}$ (H_2O) transparent above 210 nm; no molecular ion, fragments at *m/e* 416 (1%), 398 (8), 381 (9), 365 (20), 298 (14), 285 (6), 283 (18), 281 (16), 271 (12), 269 (8), and 85 (100%) corresponding to the aglycone (Found: C, 57.9; H, 7.4; Na, 2.5. $C_{45}H_{69}NaO_{18}$ requires C, 58.7; H, 7.5; Na, 2.5%; Na was detected and determined by flame emission); ^1H and ^{13}C n.m.r. data are given in Tables 1 and 2, respectively.

Hydrolysis of Sepositoside A (5a).—(a) *Aglycone.* Acid hydrolysis was with HCl in benzene–ethanol–water (100 mg saponin dissolved in 15 ml of 1 : 1 ethanol–H_2O, 2.5M in HCl), 15 ml benzene previously equilibrated with 1 : 1 ethanol–H_2O was added and the two layered mixture was refluxed for 16 h as in the method of Fleming *et al.*[42] to yield the 3β-hydroxy-5α-cholesta-8,14-dien-23-one (1) described in a previous paper.[15] Acid hydrolysis with aqueous 2N HCl in benzene (1 : 1) at 80 °C for 4 h gave the *3β-hydroxycholesta-6,8(14)-dien-23-one*, m.p. 120—122 °C, $[\alpha]_D$ −22° (*c* 1, $CHCl_3$) (Found: M^+ 398.3181. $C_{27}H_{42}O_2$ requires *M* 398.3184); $\lambda_{max.}$ 259 (s), 251 (ε 20 050), and 244 (s) nm; δ 0.64 (s, 19-Me), 0.94 (s, 18-Me), 0.97 (d, *J* 6 Hz, 21-Me), 0.92 (d, *J* 6 Hz, 26,27-Me), 3.65 (br m, 3-H), 5.28 (br d, *J* 10.5 Hz, 6-H), and 6.12 (dd, *J* 10.5 and 3 Hz, 7-H).

(b) *Sugars.* Samples of saponin (10 mg) were heated with 7% aqueous H_2SO_4 (2 ml) in stoppered tubes at 100 °C for 4 h. The aqueous medium was extracted several times with chloroform and then solid $BaCO_3$ was added and the precipitated $BaCO_3$ removed by centrifugation and washed with water. The supernatant solution and water washings were evaporated to dryness. The syrup (monoses mixture) was dissolved in water and analysed by p.c. in BAW and EPW; the presence of glucose, galactose, and glucuronic acid was established by comparison with authentic samples.

Methanolysis of Sepositoside A: Sugars Analysis.—A solution of sepositoside A (5a) (5 mg) in anhydrous 2.5N HCl–methanol (1 ml) was heated at 90 °C in a stoppered test tube for 24 h. After cooling, the reaction mixture was neutralized with Ag_2CO_3, filtered, and the filtrate evaporated to dryness under N_2. The residue was dissolved in TRISIL Z (0.1 ml; *N*-trimethylsilylimidazole in pyridine, Pierce Chemical C), left at room temperature for 15 min, and analysed by g.l.c. (122 °C). Standard glucose, galactose, glucuronic, and galacturonic acids were subjected respectively to methanolysis as above and silylated. The following carbohydrates were identified and their total areas were in the ratio 1 : 1 : 0 : 94; glucose t_r 12.30 (major) and 14.10, galactose t_r 8.40, 9.70 (major), and 11.40, and glucuronic acid t_r 4.40, 5.00 (major), 12.10, and 13.5 min (major).

Isolation of D-*Glucose and* D-*Galactose.*—The saponin (5a) (50 mg) was hydrolysed with 7% aqueous H_2SO_4 and the resulting sugar portion was subjected to semi-preparative h.p.l.c. (carbohydrate analysis column; acetonitile–H_2O–ethyl acetate, 60 : 10 : 30; 1 000 lb in^{-2}; 2 ml min^{-1}; *ca.* 3 mg in 100 μl water) to give the D-glucose {t_r 14 min, 5.1 mg; $[\alpha]_D$ +61° (H_2O) taken after 3 d; lit.,[43] D-glucose $[\alpha]_D$ +53° (final)} and D-galactose {t_r 16.80 min $[\alpha]_D$ +71° (H_2O), taken after 3 day; lit.,[44] D-galactose $[\alpha]_D$ +80° (final)}.

Mild Acid Treatment of Sepositoside A: Opened Glycoside (6a).—To a solution of sepositoside A (5a) (102 mg) in water (10 ml), aqueous 3N HCl (5 ml) was added. The solution

was placed in a water-bath at 50 °C and a white precipitate began to appear after *ca.* 1 min. Immediately, the solution was cooled in an ice-bath for 5 min and the precipitate, *opened glycoside* (6a), was collected by centrifugation, washed twice with water, and dried (89 mg). The *opened glycoside* (6a) had the following physical data $[\alpha]_D$ −27.4° (*c* 1.0) (Found: C, 59.5; H, 7.8; $C_{45}H_{70}O_{18}$ requires C, 60.1; H, 7.8%); $\lambda_{max.}$ 259 (s), 251 (ε 18 800), 244 (s) nm; R_F in t.l.c., BAW, 0.45; ^1H and ^{13}C n.m.r. spectra are given in Tables 1, 2, and 3. On acid hydrolysis (6a) gave glucose, galactose, glucuronic acid, and the steroid (1).

Configurations of Glucuronic Acid in Compound (6a).—$[M(D_{(6a)})] - [M(D_{aglycone})] - [M(D_{Me\text{-}\beta\text{-}D\text{-}glucopyranoside})] - [M(D_{Me\text{-}\beta\text{-}D\text{-}galactopyranoside})] = -246 + 88 + 66.4 + 33.0 = -58.5$ [glucuronic acid contribution in (6a)]; $[M(D)]$ of Me-β-D-glucuronopyranoside = −82.3.

Permethylation of the Saponins (5a) *and* (6a).—(a) *Permethylated sepositoside A* (5b). To a solution of (5a) (170 mg) in anhydrous dimethylformamide (DMF) (25 ml) was added sodium hydride (500 mg) under nitrogen and the solution was stirred in an ice-bath for 10 min. Methyl iodide (18 ml) was then added to the solution and the mixture was stirred for 10 min in an ice-bath and for a further 3 h at room temperature. The excess of sodium hydride was destroyed by adding methanol (4 ml), after which the mixture was poured into water and extracted with chloroform. The organic layer was washed with water, dried ($MgSO_4$), and evaporated. The residue (212 mg) was chromatographed on silica gel in chloroform–methanol (98 : 2) to give the *nona-O-methyl derivative* (5b) (203 mg) as a colourless oil (Found: C, 63.1; H, 8.5; $C_{54}H_{88}O_{18}$ requires C, 63.3; H, 8.6%); M^+ 1 024; $\nu_{max.}$ (liquid film) no OH absorption, 1 740, 1 710, 1 450, 1 370, and 760 cm^{-1}; $\lambda_{max.}$, transparent above 210 nm; *m/e* 1 024 (M^+, 2%), 380 (100%, aglycone), 281 (22%), 205 (45%), 187 (50%, and 173 (62%); ^1H and ^{13}C n.m.r. data are given in Tables 1 and 2, respectively.

(b) *Permethylated opened glycoside* (6b). The opened glycoside (6a) (66 mg) was permethylated under the same conditions as above to give an orange-yellow oil, which was purified by preparative t.l.c. [20 × 20 cm; chloroform–methanol (95 : 5); R_F 0.6; visible under u.v. light] to give the *deca-O-methyl derivative* (6b) (58 mg) as a colourless oil (Found: C, 63.8; H, 8.8. $C_{55}H_{90}O_{18}$ requires C, 63.6; H, 8.7%); M^+ 1 038; $\nu_{max.}$ (liquid film) no OH absorption, 1 740, 1 710, 1 440, 1 370, and 760 cm^{-1}; $\lambda_{max.}$ 259s, 251 (ε 17 700), 244s nm; *m/e* 1 038 (M^+, <1%), 819 (<1%), 616 (<1%), 615 (<1%), 516 (<1%), 423 (4), 391 (35), 381 (55), 380 (85), 379 (39), 359 (3), 347 (20), 327 (5), 281 (15), 280 (10), 253 (18), 251 (3), 219 (22), 187 (100), 155 (27), and 111 (55); the interpretation is given in the Figure; ^1H and ^{13}C n.m.r. spectra are given in Tables 1 and 2, respectively.

Hydrolysis of the Permethylated Saponins.—(a) Permethylated sepositoside A (5b) (20 mg) was hydrolysed with a glacial acetic acid–2N HCl (1 : 1) mixture in a sealed tube at 100 °C for 4 h. The solution was taken to dryness and analysed for methylated sugars by p.c. and g.l.c. (after silylation). P.c. showed three spots with R_g, relative to 2,3,4,6-tetra-*O*-methylglucose, of 0.89, 0.69, and 0.1. The spot of R_g 0.89 corresponded to 2,3,4-tri-*O*-methylglucose (direct comparison with an authentic specimen) as confirmed by g.l.c. (after silylation) and mixed injection with a silylated standard.

(b) Permethylated opened glycoside (6b) (30 mg) was hydrolysed as above. The hydrolysate contained 2,3,4,6-tetra-*O*-methylglucose (t.l.c., p.c., and g.l.c. with an authentic sample). In p.c., spots with R_g 0.69 (tri-*O*-methylgalactose) and 0.1, and in t.l.c. [methylene chloride–acetone (2 : 1)] spots with R_F 0.13 (tri-*O*-methylgalactose) and 0.01 were also observed (in t.l.c., 2,3,4,6-tetra-*O*-methylglucose had R_F 0.61 and 0.57). The partially methylated sugars were subjected to preparative t.l.c. in methylene chloride–acetone (2 : 1). The zone corresponding to the tri-*O*-methylgalactose (R_F 0.13) was extracted exhaustively with methanol. Evaporation of methanol gave a residue (checked for purity by p.c.), which was treated with an excess of sodium borohydride (20 mg) in water (2 ml) for 2 h at room temperature. Dowex-50W (H^+ form) was added and after 5 min the mixture was filtered and concentrated to dryness under reduced pressure. Boric acid was removed by co-distillation in the vacuum rotator with methanol. The residue was acetylated with acetic anhydride (1 ml) and pyridine (0.5 ml) at 100 °C for 10 min to give the 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol identified by mass spectrometry, no M^+, fragments at *m/e* 233 (3.3%), 205 (3.3), 189 (18), 161 (30), 129 (51), 101 (12), 87 (21), 45 (18), and 43 (100).

Determination of the Point of Linkage of Glucuronate.—Compound (6b) (34 mg) dissolved in ethanol (2 ml) was stirred with sodium borohydride (30 mg) at room temperature for 12 h, then treated with acetone and stirred for a further 20 min. The solution was poured into water and extracted with n-butanol. The organic layer was evaporated and applied to a preparative silica-gel layer (20 × 20 cm) developed with chloroform–methanol (95 : 5). The zone corresponding to the band with R_F 0.6 (visible under u.v. light) was extracted exhaustively with chloroform. Evaporation of the solvent gave a residue (no C=O i.r. absorption), which was permethylated and subsequently hydrolysed in the same way as before.

The hydrolysate was analysed for partially methylated sugars by p.c. [spots with R_g 1.00 (2,3,4,6-tetra-*O*-methylglucose), 0.84 (3,4,6-tri-*O*-methylglucose), and 0.69 (tri-*O*-methylgalactose)] and g.l.c. [after silylation, column temperature 100 °C, peaks with t_r 3 min 30 s and 3 min 48 s (2,3,4,6-tetra-*O*-methylglucose), 8 min and 8 min 43 s (3,4,6-tri-*O*-methylglucose), and 7 min 54 s and 9 min 25 s (tri-*O*-methylgalactose)]. The identity of 3,4,6-tri-*O*-methylglucose was checked by co-chromatography with authentic samples of all possible trimethylated glucoses.

5α-Cholest-7-ene-3β,6α-diol (8a).—5α-Cholest-7-ene-3β,6α-diol (8) was prepared by the hydroboration–oxidation of cholesta-5,7-dien-3β-ol using the method of Caglioti *et al.*,[39] m.p. 189—191 °C; $[\alpha]_D$ +47° (*c* 1, $CHCl_3$) (lit.,[39] m.p. 192 °C, $[\alpha]_D$ +48°); *m/e* 402 (M^+, 100%), 384 (50), 369 (20), 351 (32), 271 (40), and 253 (15); ^1H and ^{13}C n.m.r. spectra are given in Tables 1 and 2, respectively. Methylation with MeI–NaH–DMF (see above) of the diol (8a) and the usual work-up afforded 3β,6α-dimethoxy-5α-cholest-7-ene, [purified by preparative t.l.c. in methylene chloride–ethyl acetate (97 : 3) and crystallized from methanol], m.p. 70—71 °C; $[\alpha]_D$ +67.8° (*c*, 0.5, $CHCl_3$) (Found: M^+ 430.3813. $C_{29}H_{50}O_2$ requires *M*, 430.3811); *m/e* 430 (100%), 415 (6), 398 (5), 383 (9), 351 (4), 285 (3), 275 (3), and 253 (3); δ($CDCl_3$) 0.55 and 0.85 (18- and 19-Me), and 5.28 (br s, $W_{\frac{1}{2}}$ 4 Hz, 7-H); chemical shifts of carbon atoms are identical (±0.1 p.p.m.) to those for (8a) (Table 2) except for C-2 (δ_C 27.9), C-3 and C-6 (79.1 and 79.6), C-4 (28.9), C-5 (45.9), and C-7 (118.1 p.p.m.).

5α-Cholest-7-ene-3β,6β-diol (9a).—The diol (8a) (400 mg)

was stirred with excess of manganese dioxide in benzene for 12 h. Filtration and evaporation to dryness yielded a syrup which was purified by preparative t.l.c. in methylene chloride–ethyl acetate (1 : 1) to give 3β-hydroxy-5α-cholest-7-en-6-one (280 mg), $\nu_{max.}$ 1 658 cm^{-1} which, without further purification, was stirred with an excess of sodium borohydride in ethanol for 10 h. Acetone was added and the mixture was evaporated to dryness from chloroform. The extract was evaporated and the residue (220 mg) was crystallized from methanol to give the 5α-cholest-7-ene-3β,6β-diol (9a), m.p. 214—215 °C; $[\alpha]_D$ −34.0° (lit.,[40] m.p. 207—209 °C, $[\alpha]_D$ −31°); *m/e* 402 (M^+, 100%), 384 (35), 369 (20), 351 (20), 271 (30), 253 (10), and 247 (15); ^1H and ^{13}C n.m.r. spectra are given in Tables 1 and 2 respectively. The diol (9a) was acetylated with acetic anhydride–pyridine (10 : 1, 1 ml per 50 mg) at room temperature for 12 h to give the *diacetate* (9b), m.p. 138—141 °C, $[\alpha]_D$ −79° (*c* 1, $CHCl_3$) (Found: $M^+ - MeCO_2H$ 426.3496. $C_{29}H_{46}O_2$ requires 426.349 8); *m/e* 486 (M^+ 1%), 444 (1), 426 (55), 411 (9), 384 (27), 366 (100), 351 (45), 313 (50), 253 (50), and 211 (14); δ($CDCl_3$) 0.57, 0.97 (18- and 19-Me), 4.77 (m, 3-H), 5.1 (m, 6-H), and 5.38 (m, $W_{\frac{1}{2}}$ 11 Hz, 7-H); chemical shifts of carbon atoms are identical (±0.1 p.p.m.) to those reported in Table 2 for (9a) except C-1 (37.7), C-2 (27.4), C-3 (73.4), C-4 (29.7), C-5 (43.4), C-6 (69.9), C-7 (116.7), C-8 (142.1), and C-9 (15.1).

5α-*Cholesta*-6,8(14)-*dien*-3β-*ol* (7).—To a solution of the diol (8a) (200 mg) in chloroform (20 ml) was added 3N HCl–methanol (5 ml). After 10 min at room temperature the solution was washed with water, sodium hydrogencarbonate, water again, and then dried ($MgSO_4$) and evaporated to give the 5α-cholesta-6,8(14)-dien-3β-ol (7), which was purified by crystallization from methanol (160 mg), m.p. 113—115 °C, $[\alpha]_D$ −54° (*c* 1, $CHCl_3$) (Found: M^+ 384.339 5. $C_{27}H_{44}O$ requires *M* 384.3392); $\lambda_{max.}$ 259 (s), 251 (ε 21 096), and 244 (s); *m/e* 384 (M^+, 95%), 369 (40), 351 (18), 271 (100), 253 (27), and 211 (9); ^1H and ^{13}C n.m.r. spectra are given in Tables 1 and 2, respectively.

The diol (9a), when treated with HCl in the same way as before, also gave the diene (7).

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