MINOR AND TRACE STEROLS FROM MARINE INVERTEBRATES 56.† NOVEL COPROSTANOLS FROM THE MARINE SPONGE *PETROSIA FICIFORMIS*

Stefanie B. Seidel, John R. Proudfoot and Carl Djerassi
Department of Chemistry, Stanford University, Stanford, CA 94305
and
D. Sica and G. Sodano
Istituto per la Chimica di Molecole di Interesse Biologico,
Via Toiano 6, Arco Felice (Napoli), Italy

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ABSTRACT

Twelve stanols possessing the rare 5β-dihydro nucleus have been isolated from the marine sponge *Petrosia ficiformis*. These stanols have not previously been encountered in any samples of *P. ficiformis* which we have examined and appear to be the result of bacterial metabolism of the endogenous sponge sterols.

INTRODUCTION

A large number of new sterols with remarkable variations in side chain and nucleus have been isolated and characterized from marine invertebrates (1,2). As a member of the Porifera (sponges), *Petrosia ficiformis* (Class Demospongiae, Order Haplosclerida) contributed to the pool of new sterols with petrosterol (1) (3a,3b,4), ficisterol (2) (5), (24R,25R)-24,26-dimethylcholesta-5,26-dien-3β-ol (3) (6), 23(R),24(R)-methylenecholesterol (4) (7) and 23,24-dihydrocalysterol (5) (8). All hitherto reported sterols of *P. ficiformis* possess either the usual 3β-hydroxy-Δ^5 nucleus or, more rarely, the Δ^7- or 5α-dihydro nucleus. We now wish to report the isolation and characterization of twelve 5β-dihydro stanols from this same sponge.

Coprostanol (5β-cholestan-3β-ol) (6) is ubiquitous in the marine environment (mainly in sediments) and is used as indicator for fecal pollution (9,10). However, only a few coprostanols have been encountered in marine animals (11,12) as minor constituents. They are known bacterial metabolites and hence are not thought to play a functional

role in membranes because of their non-planar nucleus (cis configuration at ring A/B-junction) (13).

RESULTS AND DISCUSSION

Three specimens of the sponge *P. ficiformis* (collected in the Bay of Naples, Italy) were kept in an aquarium in running seawater with oxygenation in order to carry out an incorporation experiment. No special precautions were taken to prevent external bacterial contamination. After 20 days there were indications of deterioration and the sponges were extracted with 1:1 $CHCl_3$/MeOH. TLC examination (silica gel/CH_2Cl_2) of the total lipids showed two spots related to sterols: one corresponding to Δ^5-sterols (rf = 1.00 = cholesterol) and a second, less polar one, with rf = 1.40. Separation over a Florisil column with a hexane/dichloromethane gradient yielded 90 mg of free sterols and 178 mg of the less polar fraction.

Reverse phase HPLC separation of this less polar sterol mixture using absolute MeOH as eluent gave 9 fractions, which were further separated with CH_3CN/EtOAc 3:1 (v/v) into twelve pure compounds. Eight of these (Table 1) were identified as 5β-dihydro-3β-hydroxy stanols, the C18 (0.644-0.659 ppm) and C19 (0.957-0.959 ppm) chemical shifts (Table 2) being typical (9,11) of this nucleus (S). From 300 MHz NMR (Table 2) and low resolution GC-MS data (see Experimental) we could assign structures 6 -13. Four of these sterols (6, 7, 8 and 13) have been described previously (11, 12).

The remaining group of four sterols showed the C3-H multiplet at 3.625 ppm and the C19 methyl signal at 0.916 and were first believed to be 5α-dihydro stanols. However, comparison of one of the isolated

stanols (containing the petrosterol side chain) with an authentic sample of 5α-petrostan-3β-ol (18), obtained by catalytic hydrogenation of petrosterol, refuted this assumption. Here the C3-H occurred at 3.580 ppm and the C19-methyl signal appeared at 0.796 ppm (Table 3). As this sponge stanol (subsequently shown to be 16) had the C19-methyl shift at 0.916 ppm (+ 0.001 ppm), this eliminated the possibility of the 5α-dihydro-3β-hydroxy nucleus and also, from NMR additivity rules (14), the possibility of the 5α-dihydro-3β-hydroxy nucleus (C19 calculated to be ca. 0.763 ppm). That these compounds possessed the remaining 3α-hydroxy-5β-dihydro nucleus (epicoprostanol nucleus) was demonstrated by the epimerization procedure described in the following paragraph. We could assign the structures 14, 15, 16 and 17 on the basis of NMR (Table 4) and mass spectral data (see Experimental section). Molecular weights and HPLC and GC retention times are listed in Table 5.

To prove the assignment of the 5β-dihydro-3α-hydroxy nucleus, a sample of isolated 5β-petrostan-3β-ol (10) was oxidized to the 3-ketone (5β-petrostan-3-one) with pyridinium chlorochromate in CH_2Cl_2 and subsequently reduced using $LiAlH_4$ in THF. The reaction yielded 10% of the starting alcohol (10) and 90% of 5β-petrostan-3α-ol (16). The NMR data of the three isomeric petrostanols (10, 16, 18) are shown in Table 3. The relevant chemical shifts of the synthetic and natural specimen of 5β-petrostan-3α-ol (16) were identical.

The surprisingly high content of 5β-stanols in this sample of *P. ficiformis* strongly indicates bacterial conversion of the sterols which normally occur in this sponge. Moreover, the relative abundances of the various 5β-stanols shown in Tables 1 and 5 are comparable

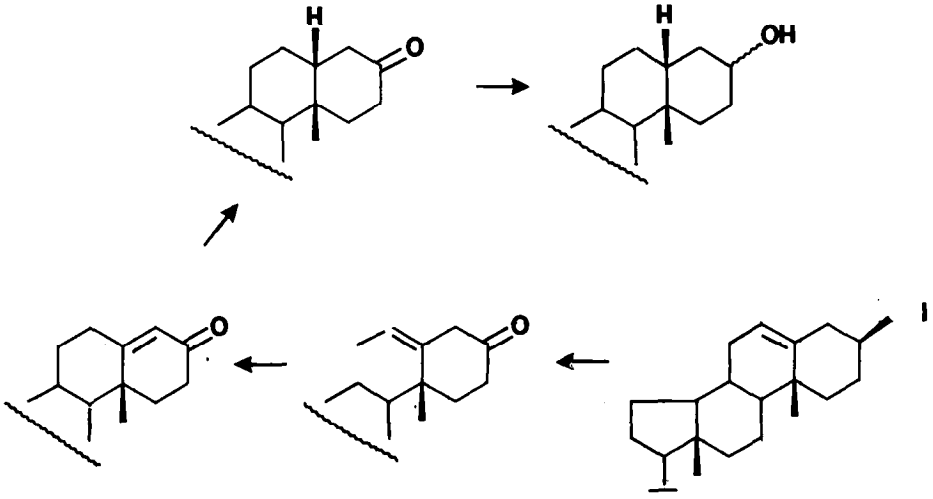
to those of the Δ^5-sterols found in the fresh sponge.

Microbial associations in sponges have been reported many times (15,16,17), and probably all demosponges possess bacterial symbionts, but no specific efforts were made to identify the bacterial population of *P. ficiformis*. There is a correlation between sponge density and the number of associated bacteria (16,17), but so far no final statement can be made about the specificity of this association. Wilkinson (17) found a distinctive population of facultative anaerobic bacteria (unlike those in the ambient water) specifically in three sponges, whereas the fourth sponge examined contained aerobic bacteria which were ubiquitous in the water. Only low numbers of anaerobic or autotrophic bacteria have been reported in sponges (18,19).

The microflora of *P. ficiformis* consists of a large number of bacteria and cyanobacteria, the latter occurring only in light-exposed animals (16). The most striking feature of this species is that the microorganisms are found intracellularly in so-called bacteriocytes. Nothing is known about the specificity or oxygen requirements and metabolism of these bacteria. On the other hand it is well known that the intestinal flora of humans and rats biotransforms cholesterol to coprostanol (20,21,22). The mechanism of this reduction was studied in growing cultures of isolated bacteria using stereospecifically labeled cholesterol (23). The proposed pathway is shown in Scheme 1. A similar pathway is probably operative in our case, the only difference being that we obtained a mixture of C3 epimers with a 4:1 ratio of coprostanols (S) to epicoprostanols (T). This can be due to a lack of stereospecificity of the enzyme performing the last step.

Attempts to induce other specimens of *P. ficiformis* to produce large amounts of 5β-stanols have so far been unsuccessful (Table 6). However, the 5β-stanol content of the second experiment was high enough to isolate 5β-petrostan-3β-ol (10), and comparison of the GC traces of the total 5β-stanol mixture showed them to be identical. It seems that the high proportion of 5β-stanols in the first experiment was due to a bacterial contaminant that is not a usual component of the sponge's microbiological flora. Otherwise, experiments 3 and 4 (Table 6) should also have produced large amounts of such 5β-stanols.

SCHEME 1



EXPERIMENTAL

Gas chromatography was performed at 260° C on a U-shaped column (1.8m x 2mm i.d.) packed with 3% OV17. The column was mounted in a Hewlett-Packard high efficiency gas chromatograph equipped with a flame ionization detector. Low resolution mass spectra were recorded with a Hewlett-Packard 5995 spectrometer. Fourier transform ^1H NMR spectra were recorded on a Nicolet Magnetics Corp. δ NMC-300 spectrometer operating at 300 MHz. The NMR spectra were recorded in $CDCl_3$ and referenced to the solvent peak $CHCl_3$ at 7.260 ppm. High performance liquid chromatography (HPLC) was carried out on a Waters Associates HPLC system (M6000 and 6000A pumps, R401 and 403 differential refractometers), using two Altex Ultrasphere 5 μ columns (10mm i.d. x

25cm) in series with methanol (solvent A) or acetonitrile/ethyl acetate 3:1 (solvent B) as mobile phases.

The total lipid extract (1.99g) was eluted from Florisil using a hexane/dichloromethane gradient. Along with the sterol fraction (90mg, rf = 1.00 = cholesterol), a less polar fraction was isolated (178mg, rf = 1.40 vs cholesterol). A portion of this less polar sample (30mg) was fractionated by HPLC (mobile phase methanol, 3mL/min, 4 injections). No identifiable compounds were eluted before 30 min. Fractions were then collected as described below and the stanols were identified from their NMR and mass spectral data.

FRACTION A (39-46 min) was reinjected using solvent B and contained 14, 15 and 7.

FRACTION B (46-49 min) was reinjected using solvent B and contained 8 and 16.

FRACTION C (49-51 min) was identical to the preceding fraction by GC.

FRACTION D (51-53 min) was reinjected in solvent B and contained 6 and 9.

FRACTION E (53-57 min) was reinjected in solvent B and contained 10, 11 and 17.

FRACTION F (57-61 min) was reinjected in solvent B and contained 10 and 12.

FRACTION G (60-67 min) contained 13.

Epimerization of 5β-petrostan-3β-ol (10) to 5β-petrostan-3α-ol (16). The 3β-hydroxy-5β-stanol 10 (7.9mg) in CH_2Cl_2 (1mL) with pyridinium chlorochromate (6mg) was stirred at room temperature until no starting material remained (TLC monitoring). The crude ketone, eluted from a short Florisil/silica gel column (CH_2Cl_2) and dried, was redissolved in THF and treated with excess lithium aluminum hydride. When no starting material remained (TLC monitoring), the reaction was quenched with ethyl acetate/water and the mixture filtered and evaporated. Fractionation by HPLC gave 16 and 10 in the ratio 9/1.

Catalytic hydrogenation of petrosterol (1). A mixture of petrosterol (1) (2.3mg) and PtO_2 (0.9mg) in ethyl acetate/water (1:1, 0.5mL) was stirred under H_2 for 2h. The reaction mixture was filtered and evaporated to give 5α-petrostan-3β-ol (18), m.p. 115-117° C (acetonitrile/dichloromethane) 300 MHz ^{1}H NMR 1.002 (3H, d, J = 5.9 Hz, C-21), 0.901 (3H, d, J = 6.1 Hz, C28 or C29), 0.884 (3H, d, J = 6.4 Hz, C28 or C29), 0.800 (3H, s, C19), 0.648 (3H, s, C18). Mass spectrum, m/z (relative intensity) 414 (M^+, 10), 399 (1), 357 (3), 316 (11), 301 (8), 273 (59), 255 (5), 233 (11), 215 (25), 55 (100).

ACKNOWLEDGMENTS

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TABLE 1. ABUNDANCE AND RELATIVE RETENTION TIMES (HPLC AND GC) OF THE STANOLS 6 - 13

Stanol	M^+	Abundance (%)	HPLC RRT[a]	GC RRT[a]
6	388	3.5	1.00	0.85
7	386	2.1	0.81	0.82
8	400	2.0	0.91	0.98
9	414	3.6	0.98	1.34
10	414	50.6	1.07	1.23
11	414	2.8	1.08	1.23
12	402	1.4	1.13	1.13
13	416	10.4	1.25	1.47
Total		76.4		

[a]Reference = cholesterol = 1.00.

TABLE 2. 300 MHz DATA ($CDCl_3$) OF THE STANOLS 6 - 13

	6	7	8	9	10	11	12	13
C3-H	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
C18-H	0.644	0.660	0.659	0.644	0.655	0.655	0.646	0.646
C19-H	0.957	0.959	0.959	0.957	0.959	0.959	0.958	0.958
C21-H	0.894 (6.7)	0.991 (6.7)	0.984 (6.6)	0.885 (6.3)	1.003 (6.0)	1.003 (6.3)	0.892 (6.6)	0.907 (6.5)
C22-H C23-H		5.230	5.170					
C23-H C24-H						0.40-0.50		
C25-H					0.50			
C26-H	0.883 (6.7)	0.854 (6.7)	0.831 (6.7)	5.730	0.10	0.946 (6.7)	0.846 (6.8)	0.829 (6.9)
C27-H	0.879 (6.4)	0.858 (6.6)	0.813 (6.7)	0.927 (6.9)	0.45	0.931 (6.7)	0.797 (6.8)	0.806 (6.9)
C28-H			0.906 (6.8)	0.789 (6.8)	0.903 (6.6)	-0.13	0.768 (6.4)	
C29-H				4.97 4.93	0.885 (6.6)	0.967 (6.7)		0.850 (7.8)

TABLE 3. SELECTED 300 MHz NMR DATA ($CDCl_3$) OF STANOLS 10, 16, 18

	10	16	18
C3-H	4.10(m, 1H)	3.625(m, 1H)	3.580(m, 1H)
C18-H	0.650(s, 3H)	0.640(s, 3H)	0.644(s, 3H)
C19-H	0.959(s, 3H)	0.916(s, 3H)	0.796(s, 3H)

TABLE 4. 300 MHz DATA ($CDCl_3$) OF THE EPI-STANOLS 14, 15, 16, 17

	14	15	16	17
C3-H	3.625	3.625	3.625	3.625
C18-H	0.652	0.637	0.640	0.638
C19-H	0.919	0.916	0.916	0.917
C21-H	0.984 (6.6)	0.895 (6.7)	1.004 (6.0)	0.886 (6.0)
C22-H C23-H	5.15			
C25-H			0.50	
C26-H	0.835 (6.5)	0.863 (6.7)	0.10	0.831 (6.8)
C27-H	0.815 (6.6)		0.45	0.809 (6.8)
C28-H	0.908 (6.5)		0.900 (6.6)	
C29-H			0.887 (6.7)	0.851 (n.a.)

TABLE 5. ABUNDANCE AND RELATIVE RETENTION TIMES (HPLC AND GC) OF THE EPI-COPROSTANOLS 14, 15, 16, 17

Stanol	M^+	Abundance (%)	HPLC RRT[a]	GC RRT[a]
14	400	1.2	0.78	0.98
15	388	1.2	0.83	0.85
16	414	16.6	0.90	1.37
17	416	0.4	1.08	1.38
Total		19.2		

[a]Reference = cholesterol = 1.00.

TABLE 6. ATTEMPTED INDUCTION OF 5β-STANOL FORMATION

Experimental Conditions	Decomposed after	% 5β-Stanols
1. Running seawater with oxygenation	20 days	66
2. Seawater without oxygenation	3 days	2
3. Seawater with oxygenation	5 days	Trace
4. Running seawater with oxygenation	No decomposition after 45 days	None

MASS SPECTRAL DATA

5β-Cholestan-3β-ol (6): 388 (M^+, 40), 374 (7), 373 (26), 370 (9), 355 (10), 331 (7), 234 (21), 233 (45), 217 (14), 216 (19), 215 (35), 43 (100).

5β-Cholest-22-en-3β-ol (7): 386 (M^+, 35), 371 (12), 303 (15), 302 (50), 287 (27), 274 (23), 273 (39), 257 (39), 55 (100).

5β-24-Methylcholest-22-en-3β-ol (8): 400 (M^+, 100), 385 (13), 339 (19), 303 (13), 302 (61), 287 (26), 274 (32), 273 (57), 272 (17), 257 (43), 255 (17), 229 (13), 217 (15), 215 (21), 213 (13), 202 (13).

5β-(24R,25R)-24,26-Dimethylcholest-26-en-3β-ol (9): 414 (M^+, 7), 316 (2), 301 (4), 274 (4), 273 (10), 272 (4), 260 (3), 257 (3), 255 (3), 234 (5), 233 (13), 231 (5), 229 (4), 219 (4), 217 (9), 216 (9), 215 (22), 201 (6), 55 (100).

5β-Petrostan-3β-ol (10): 414 (M^+, 14), 316 (10), 302 (4), 301 (10), 274 (19), 273 (67), 272 (15), 260 (5), 255 (9), 234 (6), 233 (12), 231 (4), 229 (5), 217 (7), 216 (10), 215 (26), 213 (4), 203 (4), 201 (6), 55 (100).

5β-Dihydrocalystanol (11): 414 (M^+, 11), 302 (15), 301 (9), 285 (9), 274 (24), 273 (63), 272 (15), 257 (30), 255 (17), 233 (13), 229 (11), 217 (11), 216 (9), 215 (26), 203 (9), 202 (9), 201 (11), 55 (100).

5β-24RS-Methylcholestan-3β-ol (12): 402 (M^+, 16), 387 (9), 384 (4), 369 (6), 248 (5), 235 (5), 233 (36), 231 (5), 218 (5), 217 (23), 216 (22), 215 (56), 203 (5), 201 (12).

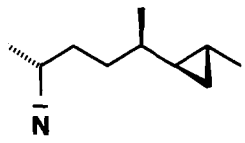
5β-24S-Ethylcholestan-β-ol (13): 416 (M^+, 73), 402 (12), 401 (36), 398 (24), 383 (21), 234 (46), 233 (76), 231 (15), 217 (39), 215 (100), 201 (21), 148 (100).

5β-24RS-Methylcholest-22-en-3α-ol (14): 400 (M^+, 36), 339 (7), 302 (22), 284 (11), 274 (14), 273 (27), 272 (9), 257 (30), 255 (9), 215 (16), 201 (16), 215 (100).

5β-Cholestan-3α-ol (15): 388 (M^+, 30), 371 (17), 370 (52), 355 (13), 233 (17), 217 (22), 216 (61), 215 (100).

5β-Petrostan-3α-ol (16): 414 (M^+, 46), 316 (24), 301 (18), 274 (27), 273 (95), 272 (79), 256 (10), 255 (24), 230 (10), 229 (16), 217 (14), 216 (35), 215 (70), 213 (13), 206 (13), 202 (11), 201 (19), 55 (100).

5β-24S-Ethylcholestan-3α-ol (17): 416 (M^+, 9), 401 (1), 399 (3), 398 (10), 383 (2), 344 (3), 257 (6), 234 (13), 233 (16), 231 (7), 230 (12), 229 (7), 218 (6), 217 (26), 216 (55), 215 (100), 203 (9), 201 (15).



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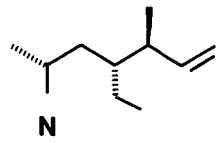

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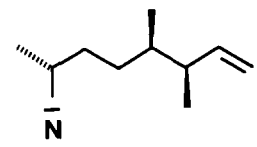

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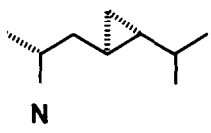

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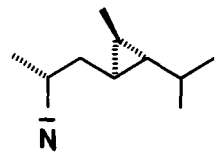

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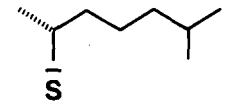

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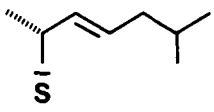

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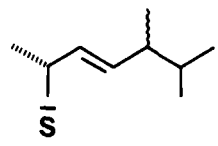

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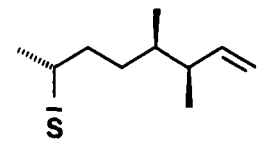

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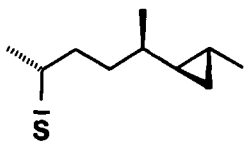

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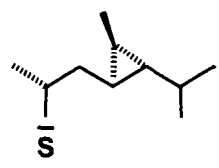

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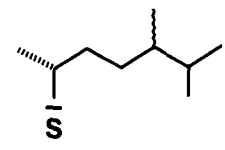

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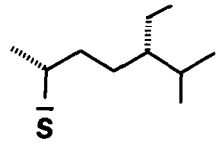

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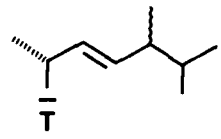

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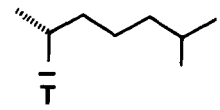

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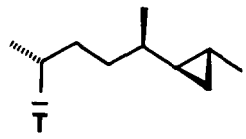

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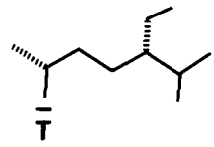

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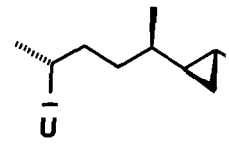

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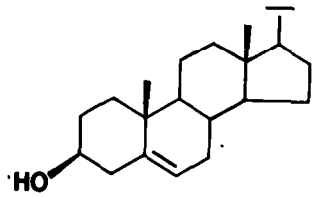

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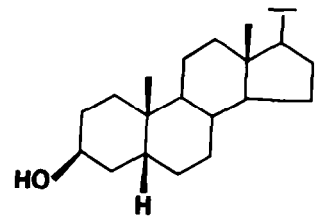

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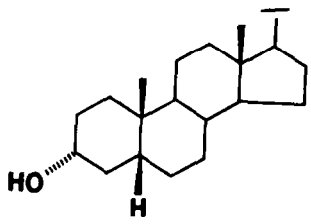

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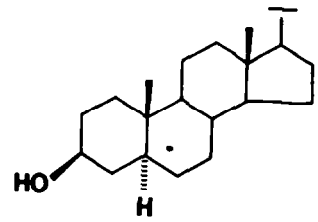

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