Minor and Trace Sterols in Marine Invertebrates. 26.[1] Isolation and Structure Elucidation of Nine New 5α,8α-Epidioxy Sterols from Four Marine Organisms

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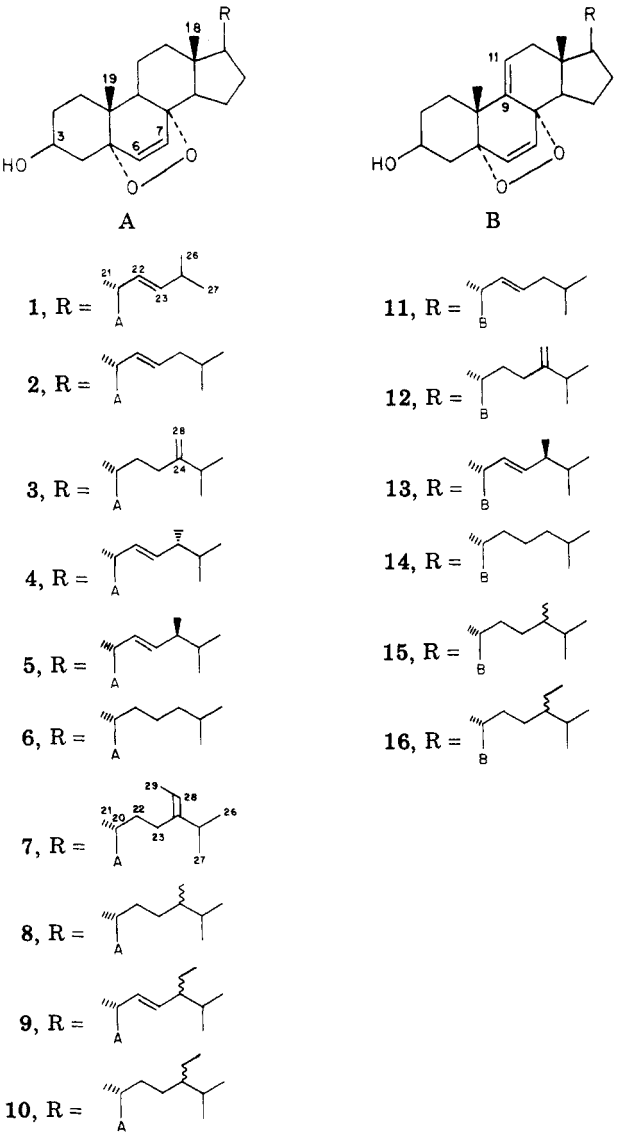
Sixteen 5α,8α-epidioxy Δ^6 and $\Delta^{6,9(11)}$ sterols, of which nine are new, have been isolated from the marine organisms *Ascidia nigra*, *Dendrogyra cylindrus*, *Thalysias juniperina*, and *Aplysia dactylomela* by reverse-phase high-performance liquid chromatography and characterized by high-resolution mass spectrometry and 360-MHz proton NMR spectroscopy. Attention is drawn to some unusual concentration-dependent NMR shifts of methyl signals. The probable biological significance of these epidioxy sterols is discussed with special reference to sterol biosynthesis.

Marine organisms have been the source of numerous 3β-hydroxy sterols and their oxygenated analogues.[3] Although more than 120 sterols—many of them with unusual side chains—are known from marine sources, until now only three reports[4-6] have appeared describing the occurrence of 5α,8α-epidioxy sterols and in all three occasions from sponges. However, these groups were unable to achieve a complete separation of the epidioxy sterol mixture.[7] Thus, the sponge *Axinella cannabina* contained 5α,8α-epidioxy-24 (*R*)-methylcholesta-6,22-dien-3β-ol (ergosterol peroxide, **4**) and 5α,8α-epidioxycholesta-6,22-dien-3β-ol (**2**).[4] Investigation of *Tethya aurantia*[5] has resulted in the isolation of **4** together with 5α,8α-epidioxy-24-methylcholesta-6,24(28)-dien-3β-ol (**3**), 5α,8α-epidioxycholest-6-en-3β-ol (**6**), and 5α,8α-epidioxy-24ξ-ethylcholest-6-en-3β-ol (**10**). More recently, the occurrence of **2**, **4**, and 5α,8α-epidioxy-24ξ-methylcholesta-6,22-dien-3β-ol (**9**) along with several steroidal 4,7,22-triene-3,6-diones in another sponge *Raphidostila incisa* has been reported.[6]

5α,8α-Epidioxy sterols have also been found to occur in lower terrestrial organisms such as fungi[8] and lichens,[8,9] but in contrast to the variety of sterol peroxides in sponges, only ergosterol peroxide (**4**) has been encountered in these terrestrial sources. The occurrence of the $\Delta^{9(11)}$ system along with the 5α,8α-epidioxy-Δ^6-nuclear moiety has been observed twice in nature: 5α,8α-epidioxy-24(*R*)-methylcholesta-6,9(11),22-trien-3β-ol (**13**) in *Rhizoctonia repens*[10] and 3β-hydroxy-5α,8α-epidioxyergosta-6,9(11),22-trien-12-one in *Fusarium monoliforme*.[11]

In continuing our current interest[3c,d] in sterols from marine sources, we have investigated the constitution of the epidioxy sterol fractions derived from *Ascidia nigra* (tunicate), *Dendrogyra cyclindrus* (common pillar coral), *Thalysias juniperina* (sponge, covered partly by the zoanthid, *Parazoanthus swifti*), and *Aplysia dactylomela* (sea hare). We now report the isolation and characterization of 16 epidioxy sterols from these four organisms. Of these epidioxy sterols nine are new. In previous studies[4,5,7] on epidioxy sterols referred to above, isolation of pure components was not considered, and consequently some epidioxy sterols were characterized as their diols (after sodium–ammonia reduction) where the epidioxy structure was no longer present. Therefore, we attempted to isolate all the epidioxy sterols including those which are epimeric at C-24 by reverse-phase HPLC and characterize them by careful elucidation of their mass and 360-MHz proton NMR spectra.



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(1) For part 25 in this series, see: Kokke, W. C. M. C.; Bohlin, L.; Fenical, W.; Djerassi, C. *Phytochemistry*, in press.
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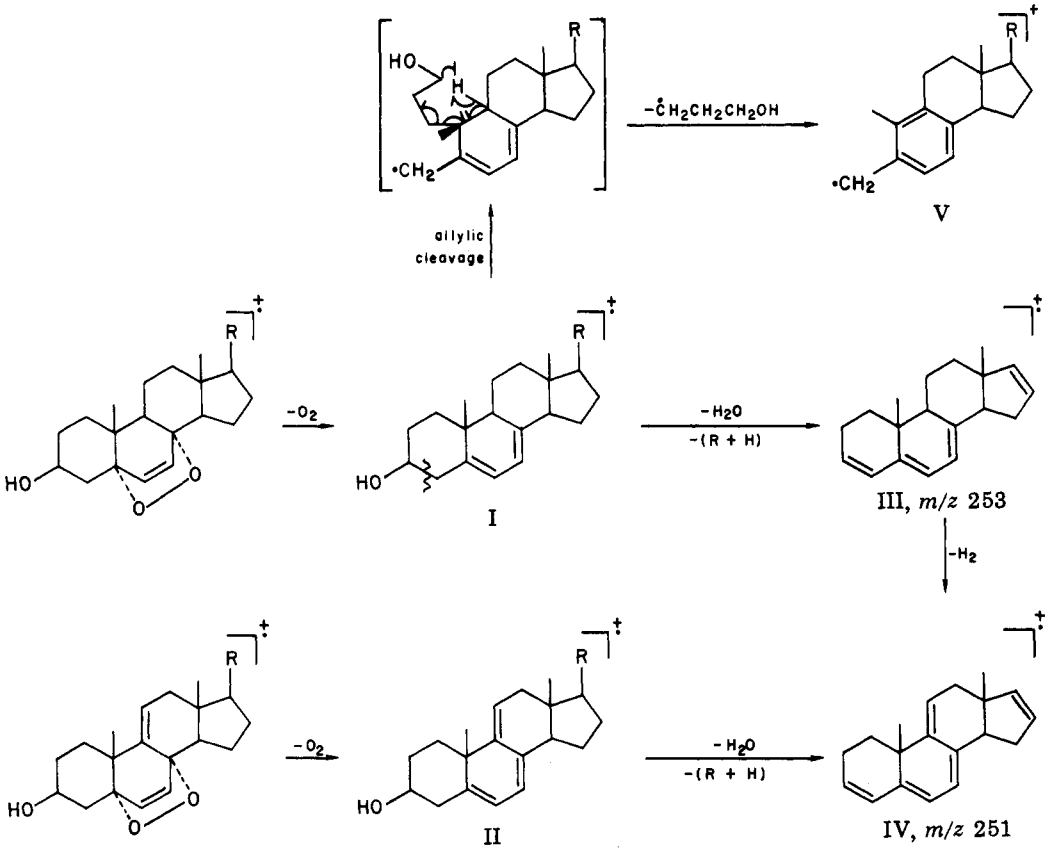
Scheme I



Mass Spectral and NMR Assignments

Initial separation of the epidioxy sterols of the four organisms was effected by TLC, where the peroxides display R_f values shorter than those of conventional sterols. Reverse-phase HPLC analysis of these fractions showed them to be complex mixtures (see Figure 1). However, repeated separation by reverse-phase HPLC employing different columns and mobile phases led to the isolation of 16 pure epidioxy sterols as judged by mass spectrometric and 360-MHz NMR analysis. The results of our analysis of the four organisms for epidioxy sterols are summarized in Table I along with their HPLC retention times.

In the mass spectra (MS) of all the isolated epidioxy sterols an intense peak was observed due to the loss of O_2 from the molecular ion, presumably by a retro-Diels–Alder-type fragmentation (fragments I and II, Scheme I). The resulting dienes, from the epidioxy sterols bearing the Δ^6 structure (nucleus A), then showed MS fragmentation characteristic of $\Delta^{5,7}$ sterols.[12] Of particular interest is the presence of a peak due to the fragment V, which was absent in $\Delta^{6,9(11)}$-epidioxy sterols (nucleus B). Further, the MS of the epidioxy sterols with nucleus B exhibited an

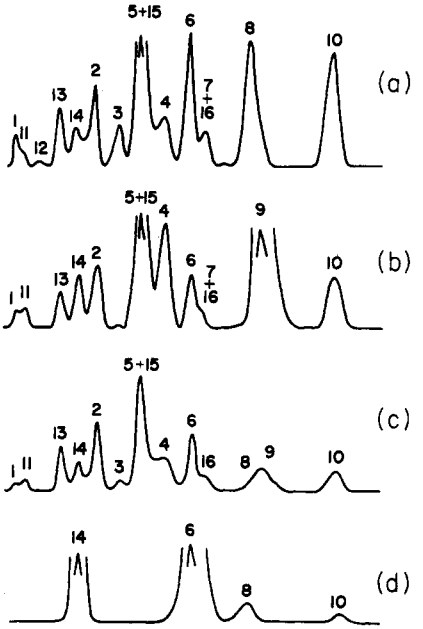


Figure 1. HPLC chromatograms of the epidioxy sterol mixtures from (a) *Ascidia nigra*, (b) *Dendrogyrus cylindrus*, (c) *Thalysias juniperina*, and (d) *Aplysia dactylomela*.

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Table I. Abundance (%) and HPLC Retentions Times of Epidioxy Sterols in Four Marine Organisms

epidioxy sterol	structure	% abundance in *Ascidia nigra*	*Dendrogyrus cylindrus*	*Thalysias juniperina*	*Aplysia dactylomela*	HPLC rel t_R[a] A[b]	B[c]	C[d]
5α,8α-epidioxy-24-norcholesta-6,22-dien-3β-ol	1	2.11	1.26	3.24		0.60		
5α,8α-epidioxycholesta-6,9(11),22-trien-3β-ol	11	0.67	1.05	4.32		0.62		
5α,8α-epidioxy-24-methylcholesta-6,9(11),24(28)-trien-3β-ol	12	0.84				0.66		
5α,8α-epidioxy-24(*S*)-methylcholesta-6,9(11),22-trien-3β-ol	13	4.38	2.44	6.70		0.71		
5α,8α-epidioxycholesta-6,9(11)-dien-3β-ol	14	2.61	3.49	5.40	24.85	0.74	0.58	0.73 (0.76)
5α,8α-epidioxycholesta-6,22-dien-3β-ol	2	5.81	4.12	13.40		0.77		
5α,8α-epidioxy-24-methylcholesta-6,24(28)-dien-3β-ol	3	3.04		2.16		0.84		
5α,8α-epidioxy-24ξ-methylcholesta-6,9(11)-dien-3β-ol	15	12.24	10.91	15.75		0.89	0.89	0.98 (0.96)
5α,8α-epidioxy-24(*S*)-methylcholesta-6,22-dien-3β-ol	5	8.91	7.94	11.46		0.89	0.89	0.95 (0.92)
5α,8α-epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol	4	4.47	9.15	8.00		0.94		(1.02)
5α,8α-epidioxycholesta-6-en-3β-ol	6	12.73	4.33	9.72	71.46	1.00	1.00	1.00 (1.00)
5α,8α-epidioxy-24-ethylcholesta-6,24(28)-dien-3β-ol	7	1.53	0.44			1.05	1.15	
5α,8α-epidioxy-24ξ-ethylcholesta-6,9(11)-dien-3β-ol	16	2.60	0.75	3.24		1.05	1.22	
5α,8α-epidioxy-24ξ-methylcholesta-6-en-3β-ol	8	trace	47.00	10.80	1.23	1.14		
5α,8α-epidioxy-24ξ-ethylcholesta-6,22-dien-3β-ol	9	21.33	trace	trace		1.16		
5α,8α-epidioxy-24ξ-ethylcholesta-6-en-3β-ol	10	15.94	7.12	5.83	0.82	1.36		

[a] Retention times are calculated relative to 5α,8α-epidioxycholesta-6-en-3β-ol (6). [b] Whatman Partisil M9 10/50 ODS-2 column (9mm × 50 cm) with 8% aqueous methanol as mobile phase. [c] Whatman Partisil M9 10/50 ODS-3 column (9 mm × 50 cm) with 15% aqueous methanol as mobile phase. [d] Altex Ultrasphere ODS column (10 mm × 35 mm) with (i) 8% aqueous methanol and (ii) 15% aqueous methanol as mobile phases (RRT in parentheses for mobile phase ii).

intense peak at m/z 251 (fragment IV), whereas in those with the nucleus A, the peak at m/z 253 (fragment III) was more prominent. Thus the MS served as a means of distinguishing epidioxy sterols bearing the $\Delta^{6,9(11)}$ structure from those with the Δ^6 structure. Supporting evidence for the above behavior came from the MS studies of synthetic **4**, **6**, and **14** prepared by the literature procedures.[13]

The 360-MHz NMR spectra (see Table II) of the natural epidioxy sterols were very informative and compatible with the proposed structures. In epidioxy sterols bearing the nucleus A, the C-6 and C-7 protons were found as a doublet of doublet (dd) around δ 5.95 and 6.28, respectively. The presence of $\Delta^{9(11)}$ unsaturation (nucleus B) causes a slight downfield shift of these signals. The C-11 proton in these appears as a dd at δ 5.23 (J = 5.9 and 1.9 Hz). Irradiation of this proton in **14** in a double resonance experiment showed it to be coupled to two protons whose signals then collapsed to an AB pattern at δ 2.11 and 1.89. The lack of any additional coupling of these protons requires the presence of $\Delta^{9(11)}$ unsaturation with two protons at C-12, as there is no other possibility for such an ABX system in the steroid skeleton or side chain. Further, in nucleus B epidioxy sterols, the C-19 protons resonated at a lower field (ca. δ 0.94) compared to those with nucleus A (ca. δ 0.67).

The NMR spectra were also useful in establishing the side chains present in these epidioxy sterols. Compared to the conventional Δ^5 sterols,[14] the methyl signals of epidioxy sterols fall within a smaller chemical shift region; in some sterols they were not resolved well enough to identify. However, good signal separations were observed in those having Δ^{22} or $\Delta^{24(28)}$ unsaturation. In the NMR spectrum of 5α,8α-epidioxy-24-norcholesta-6,22-dien-3β-ol (**1**), the C-22,23 olefinic proton signals appeared as two dd and the C-21, C-26, and C-27 methyl protons as doublets (see Table II). The signal due to the C-20 proton was present as a multiplet at δ 2.26, clearly separated from the methylene envelope. In a double resonance experiment, irradiation of this proton caused the dd at δ 5.305 due to the C-22 proton to collapse to a doublet (J = 15.48 Hz) and the C-21 proton doublet to a singlet (δ 0.999). In addition to establishing the side chain it also helped to recognize the signal due to the C-22 proton.

In the NMR spectrum of 5α,8α-epidioxy-24-ethylcholesta-6,24(28)-dien-3β-ol (**7**), in addition to the signals due to the C-6 and C-7 protons, a 1 H quartet (J = 6.62 Hz) centered at δ 5.317 was present in the olefinic region, and this was assigned to the C-28 proton by double resonance experiments. Irradiation of this proton caused the 3 H doublet due to the C-29 protons at 1.648 to collapse to a singlet. Further, the signal due to the C-25 proton appeared as a septet at 2.275 and irradiation of this caused the 6 H doublet at 1.093 due to the C-26 and C-27 protons to collapse to a singlet. The chemical shift of the C-25 proton in **7** was in agreement with the Z configuration for the $\Delta^{24(28)}$ unsaturation as in fucosterol.[15] It was also noted that the presence of the $\Delta^{24(28)}$ double bond causes an upfield shift of the C-18 and C-21 protons (Δ = 0.015 and 0.090, respectively) and a downfield shift of the C-26 and C-27 protons (Δ = 0.180) in the epidioxy sterols **3**, **7**, and **12**.

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Table II. 360-Mz ^{1}H NMR Data of 5α,8α-Epidioxy Sterols from the Marine Samples Investigated[a]

structure	C-3 H	C-6 H	C-7 H	C-11 H	C-18 H	C-19 H	C-21 H	C-22 H	C-23 H	C-26,27 H	C-28 H	C-29 H
1[c]	3.90 (m)	5.944 (d, J = 8.35)	6.282 (d, J = 8.27)		0.601 (s)	0.660 (s)	1.005 (d, J = 6.40)	5.305 (dd, J = 6.52, 15.20)	5.149 (dd, J = 9.20, 15.30)	1.005 (d, J = 6.40), 0.989 (d, J = 5.30)		
2	3.90 (m)	5.944 (d, J = 8.47)	6.283 (d, J = 8.42)		0.608 (s)	0.663 (s)	1.001 (d, J = 6.49)	5.316 (dd, J = 7.00, 15.20)	5.188 (dt, J = 8.50, 15.20)	0.920 (d, 6 H, J = 6.60)		
3	3.92 (m)	5.951 (d, J = 8.45)	6.286 (4, J = 8.48)		0.585 (s)	0.658 (s)	0.886 (d, J = 6.48)			1.085 (d, J = 6.83), 1.075 (d, J = 6.82)	4.918 (s) 4.871 (s)	
4	3.92 (m)	5.947 (d, J = 8.39)	6.286 (d, J = 8.47)		0.610 (s)	0.666 (s)	1.001[b] (d, J = 6.49)	5.251 (dd, J = 7.60, 15.27)	5.141 (dd, J = 8.36, 15.27)	0.913 (d, J = 6.71), 0.907 (d, J = 6.67)	0.991[b] (d, J = 6.78)	
	[3.92 (m)]	[5.950 (d, J = 8.40)]	[6.825 (d, J = 8.50)]		[0.609 (s)]	[0.668 (s)]	[1.002[b] (d, J = 6.58)]	[5.250 (dd, J = 7.50, 15.30)]	[5.140 (dd, J = 7.50, 15.30)]	[0.915 (d, J = 6.76), 0.908 (d, J = 6.76)]	[0.992[b] (d, J = 6.58)]	
5	3.95 (m)	5.944 (d, J = 8.43)	6.284 (d, J = 8.40)		0.610 (s)	0.664 (s)	1.002 (d, J = 6.77)	5.231 (dd, J = 7.97, 15.21)	5.134 (dd, J = 8.30, 15.15)	0.928 (d, J = 7.34), 0.858 (d, J = 6.70)	0.997 (d, J = 6.57)	
6	3.92 (m)	5.950 (d, J = 8.51)	6.294 (d, J = 8.48)		0.605 (s)	0.663 (s)	0.898 (d, J = 6.44)			0.939 (d, J = 6.63), 0.935 (d, J = 6.58)		
	[3.92 (m)]	[5.956 (d, J = 8.44)]	[6.291 (d, J = 8.47)]		[0.601 (s)]	[0.668 (s)]	[0.896 (d, J = 6.51)]			[0.939 (d, J = 6.63) 0.934 (d, J = 6.62)]		
7[d]	3.94 (m)	5.949 (d, J = 8.37)	6.287 (d, J = 8.54)		0.594 (s)	0.663 (s)	0.994 (d, J = 6.55)			1.093 (d, 6 H, J = 6.85)	5.317 (q, J = 6.62)	1.648 (d, J = 6.65)
8	3.92 (m)	5.953 (d, J = 8.45)	6.295 (d, J = 8.47)		0.603 (s)	0.665 (s)	0.931 (d, J = 6.84)			0.865 (d, J = 6.77), 0.862 (d, J = 6.83)	0.903 (d, J = 6.50)	
9	3.95 (m)	5.946 (d, J = 8.45)	6.285 (d, J = 8.50)		0.613 (s)	0.667 (s)	1.007 (d, J = 6.52)	5.128 (dd, J = 8.25, 15.13	5.038 (dd, J = 8.33, 14.88)	0.917 (d, J = 7.28), 0.887 (d, J = 6.20)		0.924 (t, J = 6.01)
10	3.90 (m)	5.951 (d, J = 8.44)	6.293 (d, J = 8.43)		0.606 (s)	0.665 (s)	0.920 (d, J = 7.14)			0.920 (d, 6 H, J = 7.14)		0.910 (t, J = 7.27)
11	3.92 (m)	6.000 (d, J = 3.35)	6.390 (d, J = 8.40)	5.230 (dd, J = 6.00, 1.85)	0.616 (s)	0.939 (s)	0.959 (d, J = 6.60)	5.239 (dd, J = 7.95, 15.20)	5.160 (dt, J = 8.30, 15.20)	0.921 (d, 6 H, J = 6.61)		
12	3.93 (m)	5.997 (d, J = 8.34)	6.383 (d, J = 8.41)	5.230 (dd, J = 5.52, 2.14)	0.595 (s)	0.932 (s)	0.842 (d, J = 6.42)			1.080 (d, J = 6.72), 1.070 (d, J = 6.85)	4.913 (s) 4.861 (s)	
13	3.93 (m)	5.995 (d, J = 8.49)	6.391 (d, J = 8.48)	5.239 (dd, J = 5.98, 1.85)	0.613 (s)	0.938 (s)	0.948[b] (d, J = 7.14)	5.218 (dd, J = 7.98, 15.13)	5.093 (dd, J = 8.78, 15.46)	0.906 (d, J = 6.74), 0.899 (d, J = 6.78)	0.996[b] (d, J = 6.81)	
14	3.95 (m)	6.002 (d, J = 8.49)	6.397 (4, J = 8.50)	5.232 (dd, J = 5.93, 1.85)	0.612 (s)	0.937 (s)	0.854 (d, J = 6.50)			0.934 (d, J = 6.50), 0.928 (d, J = 6.45)		
	[3.95 (m)]		[6.397 (d, J = 8.47)]	[5.233 (dd, J = 5.80), 1.57)]	[6.12 (s)]	[0.937 (s)]	[0.855 (d, J = 6.37)]			[0.935 (d, J = 6.59), 0.929 (d, J = 6.06)]		
15	3.92 (m)	6.002 (d, J = 8.51)	6.397 (d, J = 8.63)	5.232 (dd, J = 5.96, 1.89)	0.609 (s)	0.936 (s)	0.927 (d, J = 6.88)			0.857 (d, J = 6.88)	0.857 (d, J = 6.88)	
16	3.93 (m)	6.001 (d, J = 8.49)	6.395 (d, J = 8.51)	5.234 (dd, J = 5.92, 1.85)	0.611 (s)	0.937 (s)	0.894 (d, J = 7.35)			0.915 (d, 6 H, J = 8.09)		0.883 (t, J = 6.71)

[a] Spectral determinations made in C_6D_6, which is taken as the internal standard (δ 7.157); chemical shifts are in parts per million and J in hertz. Data for synthetic compounds are given in brackets. [b] These assignments may be reversed. [c] 1 also shows C20-H (2.260, multiplet). [d] 7 also shows C25-H (2.275, heptet).

Table III. Proton Chemical Shifts (ppm) in C_6D_6 (δ 7.157) Observed for Methyl Protons in 5α,8α-Epidioxycholesta-6,9(11)-dien-3β-ol (14) at Different Concentrations

concn, μg/μL	C-18 H	C-19 H	C-21 H	C-26,27 H
0.22	0.612	0.937	0.855	0.929, 0.935
0.40	0.612	0.938	0.855	0.929, 0.935
0.67	0.611	0.939	0.854	0.929, 0.934
1.11	0.610	0.942	0.854	0.927, 0.933
4.44	0.606	0.954	0.852	0.925, 0.930
induced shift[a] (Δ, ppm)	−0.006	+0.017	−0.003	−0.004, −0.005

[a] $\Delta = \delta_{4.44\mu g/\mu L} - \delta_{0.22\mu g/\mu L}$.

Although the structures of the natural epidioxy sterols were identified by their mass and 360-MHz NMR spectra (Table II), the identities of **4**, **6**, and **14** were further proven by comparision with synthetic samples.[13] As expected,[14] a considerable difference in the chemical shifts of the C-26 and C-27 protons was observed for the epimeric pair **4** and **5**. In **13** the configuration of the methyl group at C-24 was assumed to be *S* by comparison of the C-26, C-27, and C-28 proton chemical shifts with those in **4** and **5**. In the epidioxy sterols **8**, **9**, **10**, **15**, and **16** the methyl signals were not well enough separated to assign the structures of their side chains. Thus we cannot exclude with absolute certainty methyl or ethyl substitution at carbons other than C-24, although on grounds of analogy, alkylation at C-24 seems most likely.

An interesting observation was made when the NMR spectrum of **14** was recorded in C_6D_6 with varying concentrations. It was found that, with increasing concentration, the signal due to the C-19 methyl protons moves linearly upfield, whereas those due to the C-18, C-21, C-26, and C-27 methyl protons move downfield, the former effect being more significant (see Table III). Although pyridine-induced shifts in NMR have been observed for sterols,[16] to our knowledge this is the first report of concentration dependence of the methyl chemical shifts and may be attributable to the formation of a solute–solvent complex,[17] the overall shifts of the solute protons being influenced by the geometry of the resulting complex. It may be possible that the interaction occurs between the two electron deficient sites, namely C-5 and C-8 of the epidioxy sterol, and the π electrons of benzene. The operation of such a concentration effect should be taken into consideration when comparison of NMR spectra of epidioxy sterols is made.

Biosynthetic Considerations

It is interesting to note that the large variety of side chains which is found in general in marine 3β-hydroxy Δ^5 sterols[18] is also observed in the epidioxy sterols of the four organisms studied herewith. Until recently, reservation has been expressed[19] that naturally isolated ergosterol peroxide (**4**) may be an artefact. However, White and co-workers[20] investigated the conversion of ergosterol into its epidioxide (**4**) in two unrelated fungi, *Penicillium rubrum* and *Giberella fujikuori*, and demonstrated that both chemical (photooxidation) and enzymatic pathways are operative. It is pertinent to note that singlet oxygen ($^1\Delta O_2$) could be generated by activation of molecular oxygen by heme proteins, central constituents of mixed function oxygenases of animals, insects, and microorganisms,[21] and in that case the formation of epidioxides, even though carried out by $^1\Delta O_2$, would be a biological process. This point is substantiated by the recent isolation of prostaglandin endo peroxides on aerobic incubation[22] of arachidonic acid with a microsomal fraction of the vesicular gland of sheep.

In the present study, the artifactual origin of epidioxides can be excluded because in other marine extracts containing $\Delta^{5,7}$ sterols[23] obtained by the same experimental procedure we have not encountered corresponding 5α,8α-epidioxy sterols. Furthermore, the conventional sterol fractions of the four organisms did not contain any significant levels of $\Delta^{5,7}$ and $\Delta^{5,7,9(11)}$ sterols.[24] It is significant to note that none of the $\Delta^{5,7}$ analogues of the 5α,8α-epidioxy Δ^6 sterols **1**, **3**, **5**, **7**, and **8** and $\Delta^{5,7,9(11)}$ analogues of all the 5α,8α-epidioxy $\Delta^{6,9(11)}$ sterols **11**–**16** have thus far been encountered in nature.[3b,23]

5α,8α-Epidioxy sterols do not appear to be merely metabolic "dead ends", and it is probable that they may act as substrates for various enzyme systems.[20] Petzoldt and Kiestrich[25] had demonstrated that incubation of ergosterol peroxide (**4**) with *Mycobacterium crystallophagum* causes isomerization into epoxydiols, 5α,6α-epoxyergosta-8,22-dien-3β,7α-diol and its $\Delta^{8(14)}$ isomer. This leads back to Bergmanns' hypothesis[26] that epidioxides may be involved in the introduction of oxygen functions into terpenes. The cooccurrence of epidioxy sterols and $\Delta^{4,7}$ 3,6-diketones in the marine sponge *Raphidostila incisa*[6] has led to the proposal that epidioxy sterols may be biosynthetic precursors for the latter group of compounds. Furthermore, the recent isolation of several polyketide peroxides[27] from a number of marine sponges of the genus *Plakortis* suggests the possible occurrence of peroxidase enzymes in these organisms.

The in vivo transformation of Δ^8 to Δ^5 sterols is known to proceed via the intermediate $\Delta^{5,7}$ diene,[28] and it has been suggested that ergosterol peroxide (**4**) might be the precursor to ergosterol.[29] The question whether epidioxy

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(24) GC–MS analysis of the conventional sterol mixture from *A. nigra* had indicated the presence of only a trace amount of 24ξ-methyl-27-norcholesta-5,7-dien-3β-ol among a large variety (over 30) of Δ^5, Δ^7, and saturated sterols (Kokke, W. C. M. C.; Ha, T. B. T.; Djerassi, C., unpublished observations). GC–MS analysis of the conventional sterol mixtures from *A. dactylomela* and *T. juniperina* showed the absence of any $\Delta^{5,7}$ sterols. However, the following sterols were found: *A. dactylomela*, 22-dehydrocholesterol, desmosterol, 24-methylcholesta-5,24(28)-dien-3β-ol, 25-dehydroaplysterol, isofucosterol, 24ξ-methylcholesterol, and β-sitosterol; *T. juniperina*, 24-norcholesterol, 22-dehydrocholesterol, cholesterol, 24(*S*)-methylcholesta-5,22-dien-3β-ol, 24ξ-methylcholesterol, stigmasterol, and β-sitosterol (Gunatilaka, A. A. L.; Djerassi, C., unpublished observations).
(25) Petzold, K.; Kieslich, K. *Justus Liebigs Ann. Chem.* **1969**, *724*, 194–198.
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sterols play an intermediate role in the biosynthesis of $\Delta^{5,7}$ sterols in bakers yeast,[29b] or whether they arise from $\Delta^{5,7}$ sterols as in fungi,[20] is still not resolved.

It is interesting to note that *Ascidia nigra*, *Dendrogyrus cylindrus*, and *Thalysias juniperina* contain a variety of epidioxy sterols, whereas *Aplysia dactylomela* contains only four of them (Table I). This may be attributed to their food habits. The occurrence of epidioxy sterols with a wide variety of side chains with two common nuclei suggests, just like with *A*-nor sterols,[30] that dietary sterol precursors (e.g., $\Delta^{5,7}$ sterols) are very efficiently converted enzymatically by these organisms into epidioxides. Absence of $\Delta^{5,7}$ sterols in these organisms is analogous to the absence of Δ^5 sterols in several *A*-nor sterol producing sponges.

Experimental Section

General Methods. For separation of epidioxy sterol mixtures, a Waters high-pressure LC setup [M6000 pump, Waters Associates Model U6K septum type and Valco Model CV-6 HPax valve type injectors, R 401 differential refractometer, Whatman Partisil M9 10/50 ODS-2 (9 mm i.d. × 50 cm), ODS-3 (9 mm i.d. × 50 cm), and Altex Ultrasphere ODS (10 mm i.d. × 30 cm) columns, and mobile phases 8% and 15% aqueous methanol (see Table I)] was employed. ^{1}H NMR spectra were recorded in C_6D_6 on a Bruker HXS-360 (360 MHz) spectrometer at the Stanford Magnetic Resonance Laboratory. The chemical shifts are given in ppm with C_6D_6 as internal standard (δ 7.157), and the coupling constants are in hertz. The mass spectra were recorded at 70 eV on Varian MAT-44 (low-resolution) or Varian MAT-711 (high-resolution, double-focusing spectrometer equipped with a PDP-11/45 computer for data aquisition and reduction) mass spectrometers using a direct inlet system. Specific rotations were recorded on a Perkin-Elmer 141 polarimeter. The melting points (uncorrected) were determined on a Thomas-Hoover "Unimelt" capillary melting point apparatus.

Extraction and Purification of Epidioxy Sterol Mixtures.

a. *Ascidia nigra*. The frozen tunicate sample supplied by Professor Kenneth Kustin of Brandeis University was macerated with chloroform–methanol (1:1) in a blender. After standing overnight it was filtered and the residue was reextracted (2×) with the same solvent. The air-dried tunicate residue weighed 72.7 g. Evaporation of the combined filtrates afforded 4.4 g of a brown gum, which on chromatographic separation gave 538 mg of the conventional sterol fraction and 39 mg of the epidioxy sterol mixture. The latter was further purified by column chromatography on silica gel and elution with 20% ethyl acetate in methylene chloride.

b. *Aplysia dactylomela*. The digestive glands (8 kg wet weight) from 300 specimens collected off La Parguera, Puerto Rico, were stored in isopropyl alcohol for 1 month and then homogenized in a Waring blender with chloroform–methanol (2:1). The tissue was removed by filtration through cheesecloth, the extract was concentrated at reduced pressure, and the residue was suspended in water and extracted with methylene chloride (2 L). Most (308 g) of the organic solubles (314 g) were partitioned between hexane and 10% aqueous methanol. Chromatography of a portion (13 g) of the hexane solubles (232 g) over Silicar CC-7 (370 g) using a stepwise gradient of hexane (1 L), chloroform (1 L), and ethyl acetate (1 L) gave 4 g in the ethyl acetate fraction. Five grams of ethyl acetate eluted material (from combined runs) was chromatographed over Sephadex LH-20 with $CHCl_3$–CH_3OH (1:1). The fractions containing materials with R_f values similar to those of cholesterol on TLC were pooled (0.54 g), and most of this (0.52) was chromatographed over silica gel, using a stepwise gradient of ethyl acetate–hexane mixtures. Sterols (143 mg) were eluted with ethyl acetate–hexane (20:80) and the epidioxy sterol mixture (123 mg) with ethyl acetate–hexane (40:60).

c. *Thalysias juniperina*. Freshly collected sponge specimens from the U.S. Virgin Islands were frozen and later thawed and soaked with chloroform–methanol (1:1). Specimen weight after extraction and drying was 743 g. The concentrate of the chloroform–methanol extract was diluted with water and extracted continuously with methylene chloride to give 18.4 g of methylene chloride soluble material. Nine grams of this fraction afforded 358 mg of sterol fraction and 57 mg of epidioxy sterol fraction after chromatography over Sephadex LH-20 ($CHCl_3$–CH_3OH, 1:1) and silica gel (hexane–ethyl acetate gradient) similar to the procedure described above for *Aplysia dactylomela*.

d. *Dendrogyra cylindrus*. Approximately 50 lb of freshly collected pillar coral, *Dendrogyra cylindrus*, collected near St. Thomas, V.I., was soaked 2 times overnight in chloroform–methanol (1:2). The combined extracts were concentrated at reduced pressure and extracted and partitioned as described above for *T. juniperina* to give 24 g of methylene chloride solubles, which in turn yielded 14 g of hexane-soluble material. The latter was chromatographed over Sephadex LH-20 with chloroform–methanol (1:1), and fractions containing sterols (TLC analysis) were combined (approximately 5 g) and chromatographed over silica gel, using a gradient elution (hexane → hexane–ethyl acetate). The fractions containing sterols and epidioxy sterols were pooled to give 1.28 g of sterol mixture and 196 mg of epidioxy sterol mixture.

Separation of Epidioxy Sterols by Reverse-Phase HPLC. The TLC pure epidioxy sterol mixtures were subjected to preliminary reverse-phase high-performance LC separation, employing Whatman ODS-2 column with the mobile phase of 8% aqueous methanol (see Figure 1 and Table I). Further purification of the epidioxy sterols **1–4**, **6**, and **8–14** was achieved by repeated HPLC separation, using the same conditions. The separation of **7** and **16** was carried out with Whatman ODS-3 column and 15% aqueous methanol as the mobile phase. Complete separation of **5** from **15** was found to be extremely difficult. However, with Altex ODS column with 15% aqueous methanol as the mobile phase, a broad peak was observed and a reasonable separation was achieved by cutting it into several fractions.

Synthesis of the Epidioxy Sterols 4, 6, and 14. These epidioxy sterols were prepared by photosensitized oxygenation of the corresponding steroidal $\Delta^{5,7}$ dienes by the procedure described below. Cholesta-5,7,9(11)-trien-3β-ol required for the preparation of **14** was obtained from 7-dehydrocholesterol by the literature procedure.[13a]

The steroidal $\Delta^{5,7}$ diene (50 mg) dissolved in absolute ethanol (8 mL) containing 2 drops of a 10% solution of eosin in ethanol was refluxed while being irradiated with a 500-W tungsten lamp. Oxygen was passed through the irradiated solution. After 3 h (TLC control), the solution was evaporated and the resulting solid was chromatographed over silica gel. Elution with 15% ethyl acetate in dichloromethane gave the pure epidioxy sterol, which was recrystallized from methanol to yield colorless needles.

5α,8α-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol (4): mp 177–178 °C; $[\alpha]^{20}_D$ −25° ($CHCl_3$); for 360-MHz NMR, see Table II; the mass spectrum was found to be identical with that of the natural sample (see below).

5α,8α-Epidioxycholesta-6-en-3β-ol (6): mp 150–151 °C; $[\alpha]^{20}_D$ −6.0° ($CHCl_3$); for 360-MHz NMR, see Table II; the mass spectrum was found to be identical with that of the natural sample (see below).

5α,8α-Epidioxycholesta-6,9(11)-dien-3β-ol (14): mp 164–166 °C; $[\alpha]^{20}_D$ +95.3° ($CHCl_3$); for 360-MHz NMR, see Table II; the mass spectrum was found to be identical with that of the natural sample (see below).

Physical Data of Epidioxy Sterols. For HPLC relative retention times, see Table I. For 360-MHz proton NMR data, see Table II. The mass spectral data [*m*/*z* (assignment; relative intensity)] are given below.

5α,8α-Epidioxy-24-norcholesta-6,22-dien-3β-ol (1): 400.29546 (M^+, $C_{26}H_{40}O_3$, 2%; calcd 400.2977), 382.28753 ($C_{26}H_{38}O_2$, $M^+ - H_2O$; 3), 368.30916 ($C_{26}H_{40}O$, $M^+ - O_2$; 100), 353.28250 ($C_{25}H_{37}O$, $M^+ - O_2 - CH_3$; 1), 350.29961 ($C_{26}H_{38}$, $M^+ - O_2 - H_2O$; 2), 355.27458

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(31) Note Added in Proof: After submission of this paper, there has appeared a report on the presence of **12**, **13**, **17**, and **16** in two tunicates.[32]

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($C_{25}H_{35}$, $M^+ - O_2 - H_2O - CH_3$; 27), 330.22090 ($C_{21}H_{30}O_3$, $M^+ - C_5H_{10}$ by McLafferty fragmentation of side chain; 2), 309.25795 ($C_{23}H_{33}$, fragment V; 12), 301.17970 ($C_{19}H_{25}O_3$, $M^+ - O_2 - H_2O$ – side chain; 2), 253.19580 ($C_{19}H_{25}$, fragment III; 10), and 251.18041 ($C_{19}H_{23}$, fragment IV; 7).

5α,8α-Epidioxycholesta-6,22-dien-3β-ol (2): 414.31350 (M^+, $C_{27}H_{42}O_3$, 9%; calcd 414.3134), 396.30399 ($C_{27}H_{40}O_2$, $M^+ - H_2O$; 14), 382.32497 ($C_{27}H_{42}O$, $M^+ - O_2$; 100), 364.31076 ($C_{27}H_{40}$, $M^+ - O_2 - H_2O$; 19), 349.28860 ($C_{26}H_{37}$, $M^+ - O_2 - H_2O - CH_3$; 27), 330.21709 ($C_{21}H_{30}O_3$, $M^+ - C_6H_{12}$ by McLafferty fragmentation of side chain; 4), 323.27318 ($C_{24}H_{35}$, fragment V; 10), 301.18046 ($C_{19}H_{25}O_3$, $M^+ - O_2 - H_2O$ – side chain; 4), 253.19325 ($C_{19}H_{25}$, fragment III; 13), and 251.17921 ($C_{19}H_{23}$, fragment IV; 8).

5α,8α-Epidioxy-24-methylcholesta-6,24(28)-dien-3β-ol (3): 428.32943 (M^+, $C_{28}H_{44}O_3$, 3%; calcd 428.3290), 410.31827 ($C_{28}H_{42}O_2$, $M^+ - H_2O$; 4), 396.34091 ($C_{28}H_{44}O$, $M^+ - O_2$; 100), 363.30463 ($C_{27}H_{39}$, $M^+ - O_2 - H_2O - CH_3$; 22), 344.23534 ($C_{22}H_{32}O_3$, $M^+ - C_6H_{12}$ by McLafferty fragmentation of side chain; 1), 337.29121 ($C_{25}H_{37}$, fragment V; 10), 301.17971 ($C_{19}H_{25}O_3$, M^+ – side chain; 1), 253.19627 ($C_{19}H_{25}$, fragment III; 4), and 251.18052 ($C_{19}H_{23}$, fragment IV; 4).

5α,8α-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol (4) and Epimer 5: 428.32590 (M^+, $C_{28}H_{44}O_3$, 1%; calcd 428.3290), 410.31934 ($C_{28}H_{42}O_2$, $M^+ - H_2O$; 2), 396.33910 ($C_{28}H_{44}O$, $M^+ - O_2$; 100), 363.30689 ($C_{27}H_{39}$, $M^+ - O_2 - H_2O - CH_3$; 20), 337.28943 ($C_{25}H_{37}$, fragment V; 11), 271.20619 ($C_{19}H_{27}O$, $M^+ - O_2$ – side chain), 253.19508 ($C_{19}H_{25}$, fragment III; 10), and 251.17926 ($C_{19}H_{23}$, fragment IV; 2).

5α,8α-Epidioxycholesta-6-en-3β-ol (6): 416.32997 (M^+, $C_{27}H_{44}O_3$, 2%; calcd 416.3290), 398.32136 ($C_{27}H_{42}O_2$, $M^+ - H_2O$; 3), 384.33913 ($C_{27}H_{44}O$, $M^+ - O_2$; 100), 369.31307 ($C_{26}H_{41}O$, $M^+ - O_2 - CH_3$; 2), 366.32848 ($C_{27}H_{42}$, $M^+ - O_2 - H_2O$; 3), 351.30744 ($C_{26}H_{39}$, $M^+ - O_2 - H_2O - CH_3$; 31), 271.20545 ($C_{19}H_{27}O$, $M^+ - O_2$ – side chain; 4), 253.19547 ($C_{19}H_{25}$, fragment III; 3), and 251.17997 ($C_{19}H_{23}$, fragment IV; 2).

5α,8α-Epidioxy-24-ethylcholesta-6,24(28)-dien-3β-ol (7): 442.34253 (M^+, $C_{29}H_{46}O_3$, 1%; calcd 442.3447), 424.33450 ($C_{29}H_{44}O_2$, $M^+ - H_2O$; 2), 410.35940 ($C_{29}H_{46}O$, $M^+ - O_2$; 100), 395.33457 ($C_{28}H_{43}O$, $M^+ - O_2 - CH_3$; 2), 377.32292 ($C_{28}H_{41}$, $M^+ - O_2 - H_2O - CH_3$; 26), 351.30510 ($C_{26}H_{39}$, fragment V; 11), 326.21933 ($C_{22}H_{30}O_2$, $M^+ - H_2O - C_7H_{14}$ by McLafferty fragmentation of side chain; 1), 271.20701 ($C_{19}H_{27}O$, $M^+ - O_2$ – side chain; 4), 253.19602 ($C_{19}H_{25}$, fragment III; 2), and 251.17850 ($C_{19}H_{23}$; fragment IV; 3).

5α,8α-Epidioxy-24ξ-methylcholesta-6-en-3β-ol (8): 430.34538 (M^+, $C_{29}H_{46}O_3$, 2%; calcd 430.3447), 412.32769 ($C_{29}H_{44}O_2$, $M^+ - H_2O$; 4), 398.35325 ($C_{29}H_{46}O$, $M^+ - O_2$; 100), 383.33067 ($C_{27}H_{43}O$, $M^+ - O_2 - CH_3$; 2), 380.34366 ($C_{28}H_{44}$, $M^+ - O_2 - H_2O$; 4), 365.31428 ($C_{27}H_{41}$, $M^+ - O_2 - H_2O - CH_3$; 24), 339.30385 ($C_{25}H_{39}$, fragment V; 14), and 253.19479 ($C_{19}H_{25}$, fragment III; 6).

5α,8α-Epidioxy-24ξ-ethylcholesta-6,22-dien-3β-ol (9): 442.33864 (M^+, $C_{29}H_{46}O_3$, 1%; calcd 442.3447), 424.33615 ($C_{29}H_{44}O_2$, $M^+ - H_2O$; 3), 410.35741 ($C_{29}H_{46}O$, $M^+ - O_2$; 100), 392.34268 ($C_{29}H_{44}$, $M^+ - O_2 - H_2O$; 2), 377.31910 ($C_{28}H_{41}$, $M^+ - O_2 - H_2O - CH_3$; 24), 351.30623 ($C_{26}H_{39}$, fragment V; 10), 330.21916 ($C_{21}H_{30}O_3$, $M^+ - C_8H_{16}$ by McLafferty fragmentation of side chain; 1), 303.19311 ($C_{19}H_{27}O_3$, M^+ – side chain; 1), 253.19618 ($C_{19}H_{25}$, fragment III; 15), and 251.18081 ($C_{19}H_{23}$, fragment IV; 7).

5α,8α-Epidioxy-24ξ-ethylcholesta-6-en-3β-ol (10): 444.35930 (M^+, $C_{29}H_{48}O_3$, 2%; calcd 444.3603), 426.35042 ($C_{29}H_{46}O_2$, $M^+ - H_2O$; 5), 412.37266 ($C_{29}H_{48}O$, $M^+ - O_2$; 100), 394.35844 ($C_{29}H_{46}$, $M^+ - O_2 - H_2O$; 6), 379.33699 ($C_{28}H_{43}$, $M^+ - O_2 - H_2O - CH_3$; 27), 353.32144 ($C_{26}H_{41}$, fragment V; 12), and 253.19425 ($C_{19}H_{25}$, fragment III; 4).

5α,8α-Epidioxycholesta-6,9(11),22-trien-3β-ol (11): 412.29690 (M^+, $C_{27}H_{40}O_3$; calcd 412.2977), 394.28588 ($C_{27}H_{38}O_2$, $M^+ - H_2O$; 1), 380.30856 ($C_{27}H_{40}O$, $M^+ - O_2$; 70), 362.29870 ($C_{27}H_{38}$, $M^+ - O_2 - H_2O$; 66), 347.27489 ($C_{26}H_{35}$, $M^+ - O_2 - H_2O - CH_3$; 10), 328.20435 ($C_{21}H_{28}O_3$, $M^+ - C_6H_{12}$ by McLafferty fragmentation of side chain; 1), 253.19050 ($C_{19}H_{25}$, fragment III; 2), 251.17976 ($C_{19}H_{23}$, fragment IV; 100), and 249.16496 ($C_{19}H_{21}$, fragment IV – H_2; 16).

5α,8α-Epidioxy-24-methylcholesta-6,9(11),24(28)-trien-3β-ol (12): low-resolution mass spectra; 426 (M^+; 5%), 408 ($M^+ - H_2O$; 3), 394 ($M^+ - O_2$; 67), 376 ($M^+ - O_2 - H_2O$; 75), 361 ($M^+ - O_2 - H_2O - CH_3$; 22), 310 ($M^+ - O_2 - C_6H_{12}$ by McLafferty fragmentation of side chain; 9), 301 (M^+ – side chain; 4), 251 (fragment IV; 100), and 249 (fragment IV – H_2; 67).

5α,8α-Epidioxy-24(*S*)-methylcholesta-6,9(11),22-trien-3β-ol (13): 426.31488 (M^+, $C_{28}H_{42}O_3$, 4%; calcd 426.3134), 408.30244 ($C_{28}H_{40}O_2$, $M^+ - H_2O$; 3), 394.32203 ($C_{28}H_{42}O$, $M^+ - O_2$; 65), 376.31363 ($C_{28}H_{40}$, $M^+ - O_2 - H_2O$; 49), 361.28856 ($C_{27}H_{37}$, $M^+ - O_2 - H_2O - CH_3$; 6), 328.20577 ($C_{21}H_{28}O_3$, $M^+ - C_7H_{14}$ by McLafferty fragmentation of side chain; 2), 253.19212 ($C_{19}H_{25}$, fragment III; 2), 251.18154 ($C_{19}H_{23}$, fragment IV; 100), and 249.16545 ($C_{19}H_{21}$, fragment IV – H_2; 19).

5α,8α-Epidioxycholesta-6,9(11)-dien-3β-ol (14): 414.31295 (M^+, $C_{27}H_{42}O_3$, 7%; calcd 414.3134), 396.30320 ($C_{27}H_{40}O_2$, $M^+ - H_2O$; 2), 382.32223 ($C_{27}H_{42}O$, $M^+ - O_2$; 63), 367.30200 ($C_{26}H_{39}O$, $M^+ - O_2 - CH_3$; 13), 364.31399 ($C_{27}H_{40}$, $M^+ - O_2 - H_2O$; 100), 349.28936 ($C_{27}H_{37}$, $M^+ - O_2 - H_2O - CH_3$; 20), 253.19016 ($C_{19}H_{25}$, fragment III; 1), and 251.17902 ($C_{19}H_{23}$, fragment IV; 24).

5α,8α-Epidioxy-24ξ-methylcholesta-6,9(11)-dien-3β-ol (15): 428.32951 (M^+, $C_{28}H_{44}O_3$, 1%; calcd 428.3290), 410.32398 ($C_{28}H_{42}O_2 - H_2O$; 2), 396.33553 ($C_{28}H_{44}O$, $M^+ - O_2$; 100), 378.32873 ($C_{28}H_{42}$, $M^+ - O_2 - H_2O$; 5), 363.30710 ($C_{27}H_{39}$, $M^+ - O_2 - H_2O - CH_3$; 31), 253.19638 ($C_{19}H_{25}$, fragment III; 3), and 251.17972 ($C_{19}H_{23}$, fragment IV; 4).

5α,8α-Epidioxy-24ξ-ethylcholesta-6,9(11)-dien-3β-ol (16): 442.34373 (M^+, $C_{29}H_{46}O_3$, 5%; calcd 442.3447), 424.33887 ($C_{29}H_{44}O_2$, $M^+ - H_2O$; 3), 410.35099 ($C_{29}H_{46}O$, $M^+ - O_2$; 61), 395.33265 ($C_{28}H_{43}O$, $M^+ - O_2 - CH_3$; 12), 392.34740 ($C_{29}H_{44}$, $M^+ - O_2 - H_2O$; 100), 377.32330 ($C_{28}H_{41}$, $M^+ - O_2 - H_2O - CH_3$; 17), 251.17380 ($C_{19}H_{23}$, fragment IV; 27), and 249.16150 ($C_{19}H_{21}$, fragment IV – H_2; 3).

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