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# EPIDIOXY STEROLS FROM THE TUNICATES *DENDRODOA GROSSULARIA* AND *ASCIDIELLA ASPERSA* AND THE GASTROPODA *APLYSIA DEPILANS* AND *APLYSIA PUNCTATA*

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Steroidal endoperoxides constitute a long-recognized class of metabolites; the most characteristic being the 5α,8α-epidioxy sterol derived from ergosterol, isolated from certain terrestrial lichens and fungi (1,2). In recent years, the number of known endoperoxides has increased significantly as the result of their isolation from a number of marine animals (3-7). Marine endoperoxides, moreover, present all the structural variety known for steroids, and it has been clearly demonstrated (3,7) that they are true secondary metabolites and not artifacts.

In this paper we describe the isolation of endoperoxides **1-7** from the tunicates *Dendrodoa grossularia* Von Beneden (Ascidiidae) and *Ascidiella aspersa* Müller (Ascidiidae) and the gastropoda *Aplysia depilans*, Gmelin (Aplysiidae) and *Aplysia punctata* Cuvier (Aplysiidae). Compounds **2-7** have already been found in other marine animals, but this is the first report of the isolation and synthesis of **1**.

## RESULTS AND DISCUSSION

Silica-gel chromatography of methanolic extracts of *D. grossularia* (whole animal) produced a fraction that gave a $^1$H-nmr spectrum showing the vinylic AB system characteristic of steroidal endoperoxides. This mixture was separated by reverse phase hplc to give compounds **1** to **7** eluted in that order (Table 1).

The structures of **2-7** were deduced by comparison of their spectroscopic data (250 MHz $^1$H nmr and eims) with those of the literature (3).

Hrms of compound **1** established its molecular formula as $C_{27}H_{42}O_3$ and showed the expected loss of $O_2$. The lack of the M-127 fragment (7) and the presence of an *m/z* 323 peak (3) suggested the absence of a $\Delta^{9(11)}$ double bond. The $^1$H-nmr spectrum showed the AB system at δ=6.27 and δ=5.93 and a signal due to an olefinic proton at δ=5.27 identical to that of the C-24 proton of desmosterol. On the basis of these spectral data structure **1** was proposed and was further confirmed by synthesis from 25-hydroxycholesterol according to the scheme shown (see the Experimental section for full details).

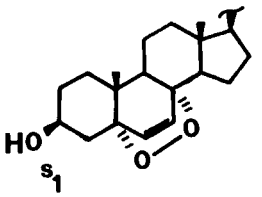
The other tunicate, *A. aspersa*, afforded the single endoperoxide **5**.

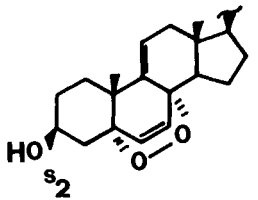
From the hepatopancreas (digestive gland) of the gastropod *A. punctata*, endoperoxides **1**, **2**, and **5** were isolated, whereas only **2** and **5** were found in *A. depilans*.

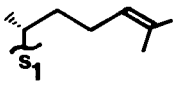
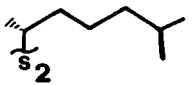
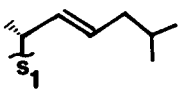
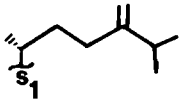
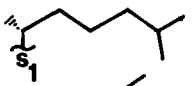
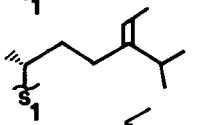
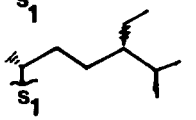
Our results complement those of Djerassi *et al.* (3), proving the wide distribution of these compounds among marine animals. The endoperoxide composition of the herbivorous *A. depilans* and *A. punctata* is shown to be quite similar to that of *Aplysia dactylomela* (3), **2** and **5** being the major components. In contrast, among the filter feeding tunicates greater diversity is found. *A. aspersa* contains the single epidioxy sterol **5**, while from *Ascidia nigra* (3) and *D. grossularia* fifteen and seven epidioxy sterols were isolated, respectively. The major component in *D. grossularia* is again **5**, with compound **7** being the only component having a 24-alkyl side chain, while in *A. nigra* half the peroxides isolated present this kind of side chain.

Analysis of the steroidal fraction of

TABLE 1. Relative Abundance and Hplc Relative Retention Times ($t_r$) of the Marine Epidioxy Sterols to Epidioxy Cholesterol




| Compound | $t_r$[a] | Relative Abundance (%) | | | |
|---|---|---|---|---|---|
| | | *Aplysia punctata* | *Aplysia depilans* | *Dendrodoa grossularia* | *Ascidiella aspersa* |
| 1  | 0.73 | 10.7 | | 9.4 | |
| 2  | 0.80 | 9.16 | 31.8 | 7.8 | |
| 3  | 0.81 | | | 5 | |
| 4  | 0.87 | | | | |
| 5  | 1 | 73 | 68.2 | 31.2 | 100 |
| 6  | 1.11 | | | 18.8 | |
| 7  | 1.29[b] | | | 15.6 | |

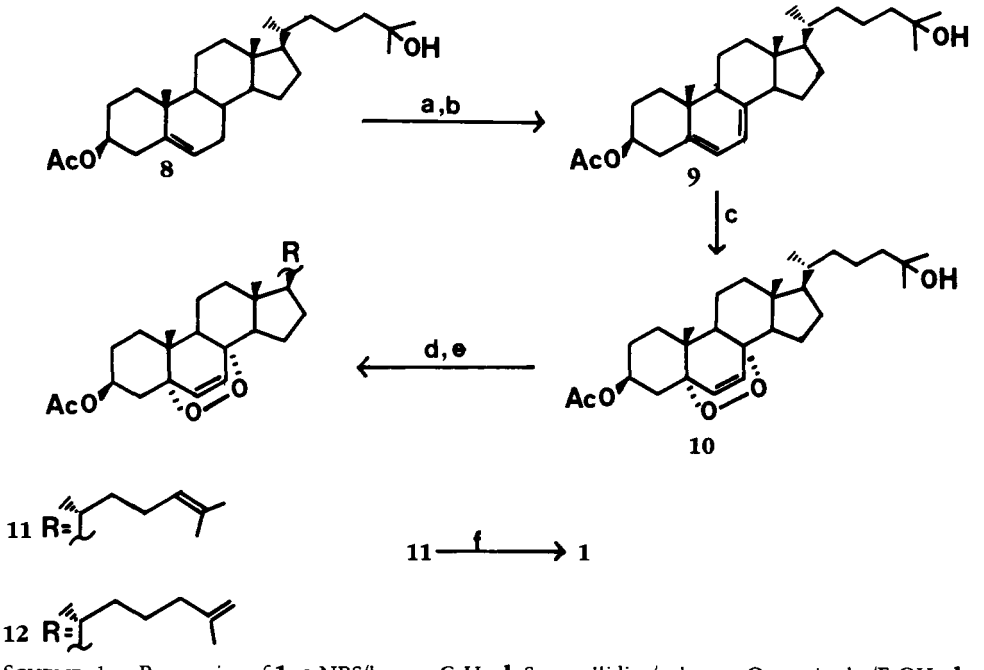
[a]15% $H_2O$/MeOH
[b]7.5% $H_2O$/MeOH

our four species[1] showed that no apparent relationship can be established between the structure of the steroids and the endoperoxides present in a given organism, which indicates the truly metabolic nature of these compounds whose derivations from dietary sterols has been suggested. In keeping with this hypothesis, we have found that the herbivorous gastropoda *A. depilans* and *A. punctata* have, in fact, quite different food habits, as is shown by their stomach contents. *A. punctata* consumes both red and green algae, but only green algae were found in our specimens of *A. depilans*. The difference in diet is directly reflected in the different polyhalogenated monoterpene contents of the two species[2] and may likewise be related to the presence of peroxide **1** in *A. punctata*, and its absence in *A. depilans*.

Interestingly, when the prostatic glands, the digestive glands, and the rest of the body of *A. punctata* and *A. depilans* were separately analyzed, they proved to have exactly the same endoperoxide composition, showing that these metabolites are distributed in the whole organism and not accumulated in a specific organ.

Finally, in contrast to what has been observed in certain sponges (8), we detected no steroidal ketones or epoxides that could have been the final products of the decomposition of peroxides.

The marine organisms *Doris verrucosa* and *Mytilus edulis* (both gastropoda), the bryozoan *Frustellida hispida*, and the sponges *Halichondria panicea* and *Hymeniacydon perleve*, in which steroidal peroxides were also sought, were found to be devoid of these compounds.




SCHEME 1. Preparation of **1**: **a** NBS/hexane-$C_6H_6$; **b** Sym-collidine/xylene; **c** $O_2$, eosin, hν/EtOH; **d** MsCl/THF; **e** $Bu_4NBr$; **f** KOH/MeOH

[1]*D. grossularia* was found to contain ergosterol, but no change in the epidioxy sterol composition was observed when the extraction was carried out very quickly in the dark and under argon atmosphere.

[2]Unpublished results.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—$^1$H-nmr spectra were taken in $C_6D_6$ and $CDCl_3$ solutions; $^{13}$C nmr was taken in $CDCl_3$; and chemical shifts are given in ppm with TMS as the internal standard; coupling constants are given in

Hz. The spectra were obtained with a Bruker WM 250 operating at 250.13 MHz for $^1$H nmr and at 63.25 MHz for $^{13}$C nmr. Mass spectra were run on a Kratos MS-25. A Waters Associates 6000A chromatograph was used for the hplc separations on a Whatman Partisil ODS column.

BIOLOGICAL MATERIAL.—*A. depilans* and *A. punctata* were identified by Dr. Victoriano Urgorri Carrasco; *D. grossularia* and *A. aspersa* were identified by Dr. Carmela García González, both of the Zoology Department, Faculty of Biology, University of Santiago de Compostela, Spain. Voucher specimens were deposited in the collection of the Zoology Department, Faculty of Biology, University of Santiago de Compostela, Spain.

EXTRACTION AND ISOLATION.—*D. grossularia* (4.4 kg) was collected intertidally at As Lagoas, La Coruña, Spain, and homogenized and extracted with MeOH and $Et_2O$ to give, after concentration in vacuo, 43.6 g of extract which was subjected to repeated silica gel column chromatography. Elution with $C_6H_6$/$Et_2O$ produced 170 mg of a mixture of the epidioxy sterols **1-7**, which were separated by reverse phase on a Partisil M9 10/50 ODS-3 column with 15% aqueous MeOH as the mobile phase (see Table 1).

*A. aspersa*.—Specimens of *A. aspersa* (20 kg), collected at Poba do Caramiñal, La Coruña, Spain, were extracted, after removal of the protective mantle, as described above for *D. grossularia*. The material (1.6 kg) gave 12.2 g of extract which afforded, after column chromatography and hplc, 9 mg of **5**.

*A. depilans*.—Four specimens of *A. depilans* (3 kg) collected by diving at La Coruña, Spain, afforded 8.96 g of extract which finally produced 150 mg of epidioxy sterol **5** and 70 mg of **2**.

*A. punctata*.—Specimens of *A. punctata* (13.8 kg) were stripped of the prostatic and the digestive glands. The digestive glands (1.9 kg) gave 18 g of extract which produced 75 mg of a mixture of epidioxy sterols **1** (8 mg), **2** (12 mg), and **5** (55 mg). Specimens of *A. punctata* were collected intertidally at Aguiño, La Coruña, Spain.

Compounds **2-7** showed $^1$H nmr (250 MHz, $C_6D_6$, $CDCl_6$) and eims data agreed with those reported in the literature (3).

Compound **1**: 5α, 8α-Epidioxycholesta-6,24-dien-3β-ol; $^1$H nmr ($C_6D_6$) δ 0.57 (s, 3H, 18-$CH_3$), 0.65 (s, 3H, 19-$CH_3$), 0.82 (d, $J$=6.46, 3H, 21-$CH_3$), 1.61 (s, 3H, 26-$CH_3$), 1.72 (s, 3H, 27-$CH_3$), 3.92 (m, 1H, 3-H), 5.25 (t, $J$=6.31, 24-H), 5.94 (d, $J$=8.35, 6-H), 6.27 (d, $J$=8.36, 7-H); $^{13}$C nmr ($CDCl_3$) δ 135.48, 131, 130.83, 125.02, 82.16, 79.44, 66.46, 56.39, 51.59, 51.12, 44.75, 39.45, 36.94, 35.83, 34.95, 34.70, 30.11, 29.65, 28.16, 25.64, 24.65, 23.38, 20.59, 18.46, 18.10, 17.57, 12.58; hrms obs $M^+$ *m/z* 414.3115 ($C_{27}H_{42}O_3$; cal. 414.3133); ms *m/z* (rel. int.) 414 ($M^+$, 8), 399 ($M^+$-$CH_3$, 14), 396 ($M^+$-$H_2O$, 16), 382 ($M^+$-$O_2$, 100), 367 (382-$CH_3$, 7), 349 (367-$H_2O$, 60), 323 (382-$C_3H_7O$, 30), 285 ($M^+$-$C_8H_{15}$-$H_2O$), 253 (382-$C_8H_{15}$-$H_2O$, 7), 251 (253-2H, 17).

PREPARATION OF **1**.—*3β-Acetoxycholesta-5,7-dien-25-ol* (**9**).—*N*-bromosuccinimide [230 mg (1.3 mmol)] in hexane (2.5 ml) was added in one lot to a refluxing solution of 3β-acetoxycholest-5-en-25-ol (**8**) (500 mg, 1.13 mmol prepared from 25-hydroxycholesterol) and dibenzoylperoxide (60 mg) in dry hexane-$C_6H_6$ (1:1) (20 ml). After refluxing (30 min, argon atmosphere), the mixture was ice-cooled, filtered, and the precipitate throughly washed, (3×6 ml) with hexane-$C_6H_6$ (1:1).

The combined filtrates were concentrated in vacuo at room temperature without exposure to light, and the resulting residue was then dissolved in dry xylene (30 ml) and added dropwise (15 min) to refluxing s-collidine (30 ml, stirring, argon atmosphere, darkness); reflux being continued for 30 min. The ice-cooled reaction was filtered into 100 ml $Et_2O$-$H_2O$ (1:1), and the organic phase was washed with cold 5% HCl, saturated $NaHCO_3$, and $H_2O$ until neutral pH was achieved.

After drying over $MgSO_4$, the solvent was distilled off under reduced pressure. The crude product was purified by tlc (silica gel, $Cl_2CH_2$/EtOH 5%) to give a mixture (248 mg) of dienes $\Delta^{4,6}$ and $\Delta^{5,7}$, which was immediately treated with $^1O_2$.

*3β-Acetoxy-5α,8α-epidioxycholest-6-en-25-ol* (**10**).—The mixture of steroidal dienes $\Delta^{4,6}$ and $\Delta^{5,7}$ dissolved in absolute EtOH (60 ml) containing 4 drops of a 10% solution of yellow eosin in EtOH was irradiated with a 300 W lamp (Philips, MLU 300 W) while oxygen was passed through the solution (3). After 3 h (tlc control), the solution was evaporated and the resulting solid chromatographed on silica gel plates ($Cl_2CH_2$/EtOH 5% as eluent), which furnished 149 mg of endoperoxide **10** (28% yield from **9**) and the diene $\Delta^{4,6}$: 42 mg, uv λ max (EtOH) 232, 242, 250, nm; ir (KBr) 3440, 1730 cm$^{-1}$; $^1$H nmr ($C_6D_6$) δ 0.61 (s, 3H, 18-$CH_3$), 0.64 (s, 3H, 19-$CH_3$), 0.92 (d, $J$=6.46, 3H, 21-$CH_3$), 1.12 (s, 6H, 26, 27-$CH_3$), 1.72 (s, 3H, 3β-acetoxy), 5.36 (m, 1H, 3α-H), 5.91 (d, $J$=8.38, 1H, 6-H), 6.31 (d, $J$=8.43, 1H, 7-H); ms *m/z* (rel. int.) 474 ($M^+$, 1), 414 ($M^+$-HOAc, 3), 396 (414-$H_2O$, 7), 382 (414-$O_2$, 100), 364 (382-$H_2O$, 10), 349 (364-Me, 6), 253 (382-$C_8H_{17}O$, 20), 251 (253-2H, 18); Anal. calcd for $C_{29}H_{46}O_5$: C, 73.38; H, 9.77. Found: C, 73.45; H, 9.52.

*3β-Acetoxy-5α,8α-epidioxycholesta-6,24-diene*

(**11**) and 3β-*Acetoxy*-5α,8α-*epidioxycholesta*-6,25-*diene* (**12**).—Triethylamine [47.5 mg (0.471 mmol)] and 54 mg (0.471 mmol) of MsCl were added to a solution of **10** (149 mg, 0.314 mmol) in dry THF (10 ml) for 10 min (stirring, −15°, argon atmosphere). After 30 min at that temperature, $Bu_4NBr$ (101 mg, 0.314 mmol) was added by means of dry THF (5 ml) (9).

The resulting solution, after stirring at room temperature for further 3 h, was added to ice-$H_2O$ (50 ml) and extracted with $Et_2O$. The organic layer was washed with 5% HCl, aqueous $NaHCO_3$, and $H_2O$ until the washings were neutral. The dried ($MgSO_4$) solution was evaporated in vacuo, and the residue was subjected to preparative tlc ($SiO_2/AgNO_3$, 5%, $CHCl_3$ as eluent, two developments). The plates were sprayed with 0.2% solution of fluorescein in absolute EtOH, furnishing **11** (79 mg), 55% yield, Rf 0.5) and the $\Delta^{25}$ isomer **12** (17 mg, 12% yield, Rf 0.3). Hydrolysis (5% methanolic KOH) of the epidioxyacetate **11** afforded **1** (61 mg, 85% yield) identical to the natural product.

*Compound* **11**.—$^1$H nmr ($C_6D_6$) δ 0.79-0.95 (Me groups), 2.01 (s, 1H, 3β-acetoxy), 4.98 (m, 1H, 3α-H), 5.08 (t, 1H, 24-H), 6.21 (d, $J$=8, 1H, 6-H), 6.51 (d, $J$=8.4, 1H, 7-H); ms *m/z* (rel. int.) 456 ($M^+$, 2), 396 ($M^+$-HOAc, 16), 378 (396-$H_2O$, 12), 364 (396-$O_2$, 100), 345 ($M^+$-$C_8H_{15}$, 40), 275 (35), 251 (50), 249 (55).

*Compound* **12**.—$^1$H nmr ($C_6D_6$) δ 0.58 (s, 3H, 18-$CH_3$), 0.63 (s, 3H, 19-$CH_3$), 0.87 (d, 3H, $J$=6.42, 3H, 21-$CH_3$), 1.70 (bs, 6H, 27-$CH_3$ and 3β-acetoxy), 4.86 (bs, 2H, 26-$CH_2$), 5.38 (m, 3H, 3α-H), 5.89 (d, $J$=8.27, 1H, 6-H), 6.29 (d, $J$=8.63, 1H, 7-H); ms *m/z* (rel. int.) 456 ($M^+$, 3), 396 ($M^+$-HOAc, 6), 378 (396-$H_2O$, 12), 364 (396-$O_2$, 100), 349 (364-$CH_3$, 8), 253 (364-$C_8H_{15}$, 10).

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