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Short side chain sterols from the tunicate *Polizoa opuntia*

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The sterol composition of the Patagonian tunicate Polizoa opuntia *was examined, and twenty-four compounds were identified as having either a Δ^5, Δ^7, or Δ^0 nucleus. Two of them, the rare 20-methylpregn-5-en-3β-ol and 20-ethylpregn-5-en-3β-ol, were previously unreported as natural products from tunicates. These compounds were present only as trace components; therefore, their structures were confirmed by synthesis from suitable precursors and by comparison of the spectral and chromatographic constants of the synthetic and natural compounds. (*Steroids* **61**:2–6, 1996)*

Keywords: sterols; tunicate; *Polizoa opuntia*

Introduction

The life cycle of marine tunicates includes both an invertebrate and a vertebrate stage. The first larval stage is motile and has a rudimentary notochord which, as in the case of ascidians, is eventually adsorbed when the hermaphrodytic tunicate settles on a surface to live most of its life in colonies or individually as an invertebrate. In this way, tunicates can be envisioned as an evolutionary bridge between vertebrates and invertebrates.

Although the sterol composition of marine tunicates has received considerably less attention than those of other phyla, the results to date reflect the high evolutionary niche occupied by these invertebrates. In general, tunicates contain Δ^5 sterols bearing conventional side-chains with cholesterol as the major component. Among the minor components, cholestanol and cholest-7-en-3β-ol have usually been detected.[1]

In some cases, 5,8-endoperoxides of several $\Delta^{5,7,9(11)}$ sterols have been reported[2] together with 24-hydroperoxy-24-vinylcholesterol and the corresponding 24-hydroxy compound.[3] *Ascidia nigra* was reported to contain4-methylsterols in addition to endoperoxides.[4,5] As these findings show, tunicates can produce sterols with quite unique features. Like those of other filter-feeders, the sterols produced by tunicates may have different origins, e.g., de novo biosynthesis, dietary sources, or symbionts. Feeding experiments have demonstrated that tunicates are capable of de novo biosynthesis of sterols.[6]

On the other hand, steroidal hormones have been reported in several classes of marine invertebrates. In particular, dehydroepiandrosterone, cortisone, and cortisol were found in the gonads of the tunicate *Ciona intestinalis*.[7] There are two proposed sequences for steroidal biosynthesis in marine invertebrates: one proceeds by the conventional route of desmosterol and cholesterol to pregnenolone, whereas the other route proceeds from mevalonic acid by a pathway that does not require the production of a C-27 sterol precursor. The latter route, which would predominantly lead to corticosteroids, requires a short-chain steroidal precursor, probably a C-22 sterol.[8]

Taking all of this into account as well as our ongoing research project in the field of marine sterols,[9] we decided to study the sterol composition of the tunicate *Polizoa opuntia* (family *Polizoidae*), which is quite abundant in the South Atlantic Ocean. The two main goals of this project were the search for novel sterols and, on the other hand, the possible detection of steroid precursors. As a result of this research, two short-chain sterols (C-22 and C-23 sterols) were conclusively identified for the first time as natural

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products
in tunicates, and their structures were confirmed by synthesis.

Experimental

General

HPLC was performed using a Thermo Separations pump (Spectra Series model P-100), a variable wavelength UV detector (model UV-100), and a Shodex RI-71 refractive index detector. High-performance liquid chromatography (HPLC) column: YMC-Pack ODS Rp-18 (20 × 250 mm; 5 μ; eluant: MeOH). For gas chromatography (GC), a Hewlett-Packard model 5890 gas chromatograph was used with a HP-5 capillary column (25 m × 0.32 mm i.d.; conditions: 180° → 280° rate: 10°C/min, then 280° isothermal). GC-mass spectrometry (MS) was performed on a VG Trio 2 (Fisons) mass spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC-200 NMR spectrometer.

Collection, extraction, and isolation

P. opuntia was collected by SCUBA at Punta Piedras, Rada Tilly, Chubut, Argentina. The animals (1 kg, wet weight) were kept frozen until worked up. The frozen animals were blended and extracted with EtOH (2 × 2L) and EtOAc (2 L). The combined extracts were evaporated, and the crude extract (12 g) was partitioned between hexane and MeOH/H_2O (9:1, v/v). The crude sterol mixture was detected exclusively in the hexane layer which was evaporated, leaving 1.6 g of a brownish oil. This oil was subjected to flash chromatography on silicagel (hexane/CH_2Cl_2 gradient). The crude sterol mixture (320 mg) was detected in the fraction eluted with CH_2Cl_2 and then separated by HPLC (see General) in 12 fractions. When possible, the main components of each fraction were purified by HPLC and identified by their NMR and MS spectra. This was not possible in the case of some of the minor components, in which case characterization was performed by GC-MS (see General) of the free sterol mixtures and the corresponding acetates (Ac_2O/py, room temperature, overnight).

Reduction of compound 4

Aldehyde 4 (100 mg) was dissolved in 10 mL of anhydrous THF and cooled to 0°C; 100 mg of $LiAlH_4$ was added, and the suspension was stirred for 30 min, after which EtOAc was added. The organic layer was washed successively with water and a saturated NaCl solution, and after evaporation, it yielded 72 mg of compound **5.**

(20R)-3α,5α-Cyclo-6β-methoxy-20-(hydroxymethyl)-pregnane (*5*). ^{1}H NMR (δ, $CDCl_3$) 3.50 (AB quartet [ABq], 2 H, H-22); 3.32 (s, 3 H, $-OCH_3$); 2.76 (t,1 H, J = 2.7 Hz, H-6); 1.05 (d, 3 H, J = 7 Hz, H-21); 1.02 (s, 3 H, H-19); 0.74 (s, 3 H, H-18); 0.65 (dd, 1 H, J = 8; 4 Hz, H-4); 0.43 (dd, 1 H, J = 8; 5 Hz, H-4).

Tosylation of compound 5

Compound **4** (70 mg) was dissolved in 5 mL of anhydrous pyridine; 85 mg of *p*-toluensulfonyl chloride (TsCl) was added, and the mixture was placed in the refrigerator for 48 h. The crude product was worked up in the usual way and purified by flash chromatography on silicagel to yield 56 mg of the tosylate **6.**

(20R)-3α,5α-Cyclo-6β-methoxy-20-(tosyloxymethyl)-pregnane (*6*). ^{1}H NMR (δ, $CDCl_3$): 7.78 (d, 2 H, Ar-H); 7.33 (d, 2 H, Ar-H); 3.88 (ABq, 2 H, H-22); 3.31 (s, 3 H, OCH_3); 2.76 (t, 1 H, J = 2.7 Hz, H-6); 2.45 (s, 3 H, Ar-CH_3); 1.01 (s, 3 H, H-19); 0.98 (d, 3 H, J = 6.6 Hz, H-21); 0.68 (s, 3 H, H-18); 0.64 (dd, 1 H, J = 8, 3 Hz, H-4); 0.43 (dd, 1 H, J = 8.5 Hz, H-4).

Reduction of compound 6

Compound **6** (55 mg) was dissolved in 2 mL of anhydrous THF; 50 mg of $LiAlH_4$ was added and the mixture was sonicated at room temperature for 3 h. The crude product was worked up as in the case of compound **4** and yielded 26 mg of compound **7.**

3α,5α-Cyclo-20-methyl-6β-methoxypregnane (*7*). ^{1}H NMR (δ, $CDCl_3$): 3.31 (s, 3 H, OCH_3); 2.76 (t, 1 H, J = 3 Hz, H-6); 1.02 (s, 3 H, H-19); 0.93 (d, 3 H, J = 6.5 Hz, H-21*); 0.83 (d, 3 H, J = 6.5 Hz, H-22*); 0.71 (s, 3 H, H-18); 0.65 (dd, 1 H, J = 8; 4 Hz, H-4) 0.43 (dd, 1 H, J = 8; 5 Hz, H-4). *Assignments may be interchanged.

Preparation of compound 1a

Compound **7** (25 mg) was dissolved in 7 mL of THF. Distilled water was added dropwise until saturation, and afterwards, 50 mg of *p*-toluensulfonic acid (pTsA) were added. The mixture was refluxed for 3 h and then extracted with CH_2Cl_2. The crude product was purified by prep TLC (silica; hexane/EtOAc, 3:1, v/v) and yielded 18 mg of the pure sterol **1a.**

20-Methylpregn-5-en-3β-ol (*1a*). ^{1}H NMR (δ, $CDCl_3$): 5.31 (bd, 1 H, J = 6 Hz, H-6); 3.55 (m, 1 H, H-3); 1.01 (s, 3 H, H-19); 0.94 (d, 3 H, J = 6.5 Hz, H-21*); 0.84 (d, 3 H, J = 6.5 Hz, H-22*); 0.68 (s, 3 H, H-18). *Assignments may be interchanged.

Preparation of compound 8

$PH_3P = CH_2$ (230 mg) was suspended in 10 mL of anhydrous THF, and a hexane solution of BuLi (2.5 M) was added dropwise until all of the solid was dissolved and the solution was brownish-orange in color. The solution was cooled to 0°C, and 109 mg of aldehyde **4** (dissolved in 1 mL THF) was added. The mixture was left for 1 h at 0°C and then for 2 h at room temperature. The resulting reaction mixture was poured over water and extracted with hexane to yield 290 mg of a brown oil after workup. Compound **8** (59 mg) was purified by flash chromatography on silica (hexane/ethyl ether mixtures).

(20R)-3α,5α-Cyclo-6β-methoxy-20-vinylpregnane (*8*). ^{1}H NMR (δ, $CDCl_3$): 5.25 (m, 3 H, H-22,23); 3.32 (s, 3 H, OCH_3); 2.77 (t, 1 H, J = 3 Hz, H-6); 1.02 (s, 3 H, H-19); 0.72 (s, 3 H, H-18); 0.64 (dd, 1 H, J = 5; 4 Hz, H-4); 0.42 (dd, 1 H, J = 8; 5 Hz, H-4).

Hydrogenation of compound 8

Compound **8** (55 mg) was dissolved in EtOAc, 50 mg of 10% Pd over charcoal was added, and the resulting mixture was hydrogenated for 36 h at 55 psig to yield 48 mg of compound **9** after workup.

(20R)-3α,5α-Cyclo-6β-methoxy-20-ethylpregnane (*9*). ^{1}H NMR (δ, $CDCl_3$): 3.32 (s, 3 H, OCH_3); 2.76 (t, 1 H, J = 3 Hz, H-6); 1.03 (s, 3 H, H-19); 0.72 (s, 3 H, H-18); 0.65 (dd, 1 H, J = 5; 4 Hz, H-4); 0.41 (dd, 1 H, J = 8; 5 Hz, H-4).

Preparation of compound ***1b***

Compound **9** (40 mg) was treated in the same way as compound **7** (see Preparation of compound **1a**). After workup and purification by preparative TLC, 28 mg of compound **1b** was obtained.

(20R)-20-Ethylpregn-5-en-3β-ol (*1b*). ^{1}H NMR (δ, $CDCl_3$): 5.35 (bd, 1 H, J = 5 Hz, H-6); 3.55 (m, 1 H, H-3); 1.01 (s, 3 H, H-19); 0.91 (d, 1 H, J = 6.5 Hz, H-21); 0.68 (s, 3 H, H-18).

Results

The crude sterol fraction of *P. opuntia* was obtained and fractionated by HPLC as outlined in the experimental section. Each fraction was analyzed by GC-MS, and when possible, the individual components were isolated by HPLC and identified by GC-MS and ^{1}H NMR. Results are listed in Table 1, and the structures of the identified sterols are depicted in Figure 1.

When the presence of Δ^7 sterols was suspected, the site of unsaturation was confirmed by ^{1}H NMR or by GC-MS of the corresponding acetates. Most of the sterols had conventional side chains with Δ^5, Δ^0, or Δ^7 nuclei. Two of the sterols received special attention, due to their unusual retention times in GC and their molecular weights, which suggested the presence of C-22 and C-23 compounds.

The mass spectra of **1a** and **1b** closely resembled previously reported data for short side-chain sterols.[10] GC-MS of compound **1a** showed a $M^{+\cdot}$ ion at m/z 316, together with losses of 15, 18, and 33 μ (m/z 301: $M^{+\cdot} - CH_3$, m/z 298: $M^{+\cdot} - H_2O$; m/z 283: $M^{+\cdot} - CH_3 - H_2O$). Other significant fragments were m/z 205, m/z 213, and m/z 231. In particular, the ions m/z 205 and m/z 231, differing in 26 μ, may correspond to the fragmentation of rings A and B, which is typical of Δ^5 sterols.[11] This fragmentation creates ions m/z 275 and m/z 301 in cholesterol. In compound **1a,** these fragments indicate the presence of a Δ^5 sterol with a C-3 side chain, namely a C-22 sterol.

Compound **1b** showed $M^{+\cdot}$ 330 and a fragmentation pattern which was similar to that of compound **1a.** Losses of CH_3· and H_2O were also observed (m/z 315, 312, and 297). The ions m/z 219 and m/z 245 (instead of m/z 205 and 231) suggested that, as in the case of **1a,** compound **1b** was also a Δ^5 sterol, but its side-chain was C-4 instead of C-3.

Since compounds **1a** and **1b** were present only as trace components, it was not possible to isolate them in sufficient amounts to obtain good quality NMR spectra. For that reason, we decided to synthesize both compounds in order to confirm the tentative structures. As part of a related research project on the biosynthesis of bufadienolides, we had the synthetic intermediate **4,** which was used to prepare compounds **1a** and **1b** as outlined in Figure 2. Compound **4** was easily prepared by reductive ozonolysis of the isomethyl ether of stigmasterol.

For the synthesis of **1a,** compound **4** was reduced to the alcohol **5** with $LiAlH_4$ in anhydrous THF. Compound **5** was treated with tosyl chloride in pyridine to yield compound **6,** which was in turn reduced to compound **7** by a second treatment with $LiAlH_4$ in anhydrous THF. Finally, the Δ^5 double bond was regenerated with *p*-toluenesulfonic acid in THF/H_2O to obtain compound **1a.**

On the other hand, for the synthesis of **1b,** compound **4** was transformed via a Wittig reaction with $Ph_3P = CH_2$ into the olefin **8,** which in turn was hydrogenated over Pd to obtain compound **9.** Treatment of **9** with *p*-toluenesulfonic acid in THF/H_2O yielded the Δ^5 sterol **1b.**

The synthetic compounds **1a** and **1b** had GC retention times identical to those of the natural compounds. This was verified by coinjection with the natural samples. Further-

Table 1 Sterol composition of *Polizoa opuntia*

n	Sterol	%	MS (m/z)
1a	20-Methylpregn-5-en-3β-ol	Trace	316 ($M^{+\cdot}$); 301; 298; 283; 255; 231; 213; 205
1b	20-Ethylpregn-5-en-3β-ol	Trace	330 ($M^{+\cdot}$); 315; 312, 297; 255; 245; 219; 213
1c	24-nor-cholesta-5,22-dien-3β-ol	4.28	370 ($M^{+\cdot}$); 355; 352; 337; 300; 271; 255; 213
1d	Cholest-5-en-3β-ol	18.33	386 ($M^{+\cdot}$); 371; 368; 353; 301; 275; 255; 213
1e	(22*E*)-cholesta-5,22-dien-3β-ol	4.22	384 ($M^{+\cdot}$); 369; 366; 351; 300; 271; 255; 213
1f	Cholesta-5,24-dien-3β-ol	7.94	384 ($M^{+\cdot}$); 369; 351; 300; 273; 271(100); 255
1g	24Ξ-methylcholest-5-en-3β-ol	2.31	400 ($M^{+\cdot}$); 385; 382; 367; 315; 289; 273; 255
1h	(22*E*)-24Ξ-methylcholesta-5,22-dien-3β-ol	10.19	398 ($M^{+\cdot}$); 380; 365; 300; 273; 271; 255; 213
1i	Ergosta-5,24(28)-dien-3β-ol	19.42	398 ($M^{+\cdot}$); 383; 365; 314(100); 299; 281; 271
1j	Stigmast-5-en-3β-ol	2.84	414 ($M^{+\cdot}$); 399; 396; 381; 329; 303; 273; 255
1k	(22*E*)-Stigmasta-5,22-dien-3β-ol	0.98	412 ($M^{+\cdot}$); 394; 379; 351; 300; 271; 255; 213
1l	(24*Z*)-Stigmasta-5,24(28)-dien-3β-ol	2.07	412 ($M^{+\cdot}$); 314(100); 299; 281; 271; 229; 213
1m	(24*E*)-Stigmasta-5,24(28)-dien-3β-ol	4.15	412 ($M^{+\cdot}$); 314(100); 299; 281; 271; 229; 213
2c	24-nor-5α-cholest-22-en-3β-ol	Trace	372 ($M^{+\cdot}$); 357; 302; 287; 273; 257; 215
2d	5α-Cholestan-3β-ol	13.14	388 ($M^{+\cdot}$); 373; 355; 234; 233; 215(100)
2e	(22*E*)-5α-cholest-22-en-3β-ol	0.36	386 ($M^{+\cdot}$); 371; 302; 287; 273; 257; 215
2g	5α-24Ξ-Methylcholestan-3β-ol	0.43	402 ($M^{+\cdot}$); 387; 234; 233; 215(100)
2i	5α-Ergost-24(28)-en-3β-ol	2.15	400 ($M^{+\cdot}$); 385; 316(100); 301; 273; 215
2j	5α-Stigmastan-3β-ol	0.70	416 ($M^{+\cdot}$); 401; 316; 287; 234; 233; 215
3d	5α-Cholest-7-en-3β-ol	2.48	386 ($M^{+\cdot}$); 371; 353; 300; 273; 255(100); 213
3e	(22*E*)-5α-Cholest-7,22-dien-3β-ol	0.08	384 ($M^{+\cdot}$); 369; 351; 300; 273; 271(100); 255
3i	5α-Ergosta-7,24(28)-dien-3β-ol	0.79	398 ($M^{+\cdot}$); 383; 314; 299; 271(100); 213
3j	5α-Stigmast-7-en-3β-ol	0.24	414 ($M^{+\cdot}$); 399; 381; 273; 255(100); 213
3k	(22*E*)-5α-Stigmasta-7,22-dien-3β-ol	0.05	412 ($M^{+\cdot}$); 397; 379; 314; 299; 271(100)

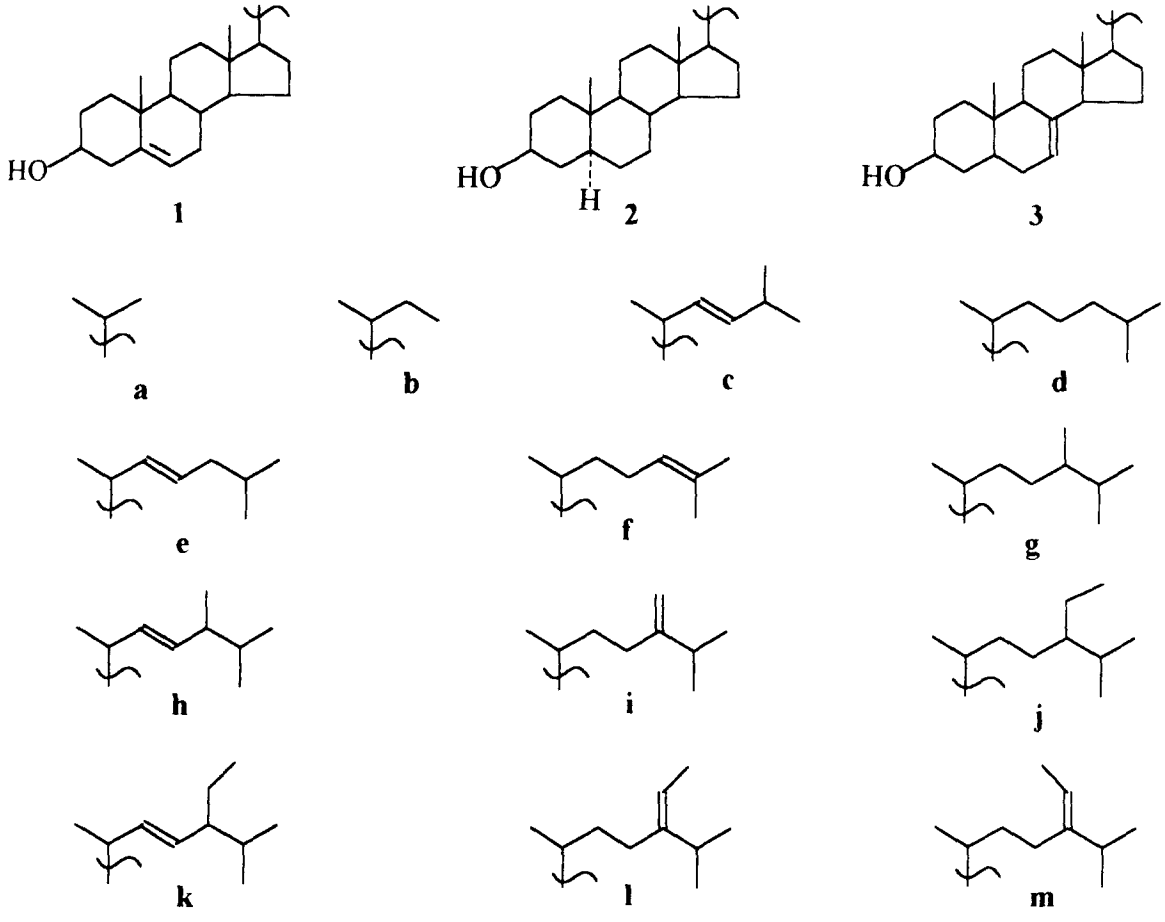

Figure 1 Sterols identified from *Polizoa opuntia.*

more, the GC-MS spectra of the natural and the synthetic compounds were identical. In this way, the structures of **1a** and **1b** were confirmed as 20-methylpregn-5en-3β-ol and 20-ethylpregn-5-en-3β-ol, respectively.

Discussion

As the results presented in Table 1 show, the sterol composition of *P. opuntia* is highly complex, with Δ^5 sterols

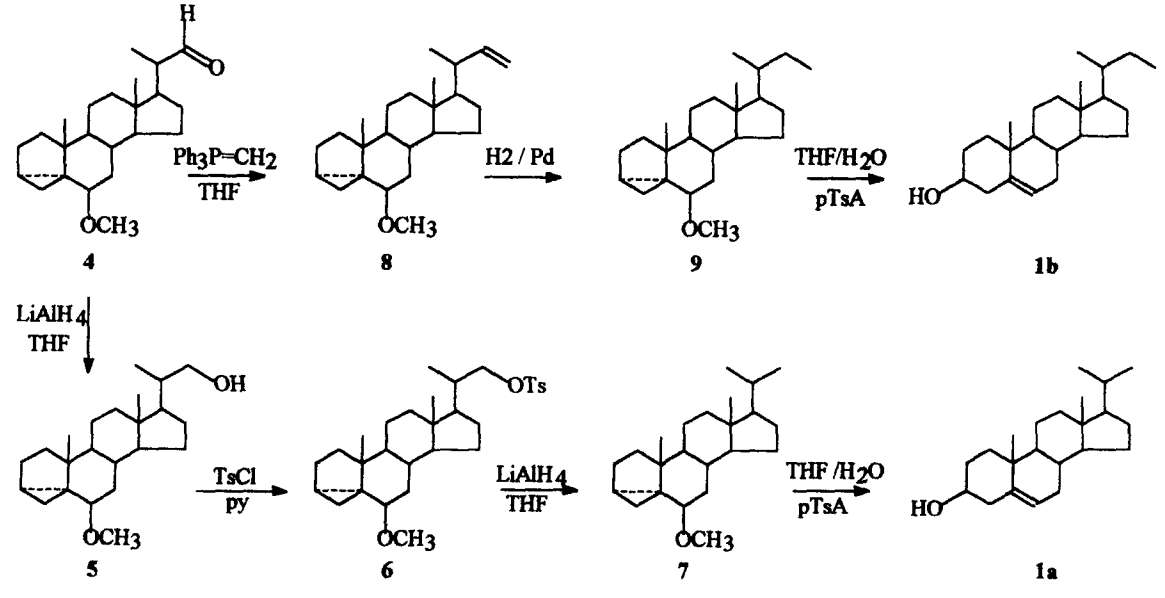

Figure 2 Synthesis of sterols **1a** and **1b.**

accounting for more than 70% of the mixture. Twenty-four compounds could be identified as having either Δ^5, Δ^0, or Δ^7 nuclei. The four major components are 24-methylenecholesterol, cholesterol, cholestanol, and brassicasterol. The presence of 24-methylenecholesterol as the major component is remarkable, although its percentage is quite similar to that of cholesterol. Also, the presence of cholestanol among the major components is uncommon in tunicates.

Sterols with short hydrocarbon side chains (namely C-0 to C-6 side chains) have been reported as trace constituents in marine *Coelenterata* and *Porifera*.[10,12] Also, Kanazawa tentatively characterized a C-22 sterol from the clam *Tapes philippinarum*.[13] To the best of our knowledge, the present paper represents the first conclusive identification of C-22 and C-23 sterols as trace components in tunicates.

Djerassi and co-workers suggest that an in vivo autoxidative process is the most likely origin for the short side-chain sterols, as opposed to biological degradative processes or biosynthesis from nonsqualene, nonpolyprenoid precursors.[10] These authors also provided evidence that these short side-chain sterols are not artifacts produced by sample handling or by laboratory work-up. As for the role of these compounds in living organisms, it is clear that they cannot be functional membrane constituents, a fact which supports the possibility of an autoxidative origin. However, a role for these short side-chain sterols in the biosyntheses of steroids, although highly improbable, cannot be definitely ruled out.

The finding of short side chain sterols in tunicates points towards the possibility of a more widespread distribution of these compounds among marine phylla. The diversity of sterols produced by *P. opuntia* shows that tunicates share some biosynthetic capabilities with lower marine invertebrates, and that they can still be good sources of novel sterols.

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