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MINI-REVIEW

STEROIDS IN PORIFERA. STEROLS FROM FRESHWATER SPONGES *EPHYDATIA FLUVIATILIS* (L.) AND *SPONGILLA LACUSTRIS* (L.)

R. MANCONI,* V. PICCIALLI,† R. PRONZATO* and D. SICA†‡

*Istituto di Zoologia dell'Università, Via Balbi 5, 16126 Genova, Italy; †Dipartimento di Chimica Organica e Biologica dell'Università, Via Mezzocannone 16, 80134 Napoli, Italy. (Tel: 20-64-11)

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Abstract—1. The sterols of the freshwater sponges *Ephydatia fluviatilis* and *Spongilla lacustris* were isolated and characterized by reverse phase HPLC, silver nitrate–silica gel TLC, mass spectrometry and ^{1}H NMR.

2. *Ephydatia fluviatilis* and *S. lacustris* have rather similar sterol composition and contain almost exclusively Δ^5-sterols. Cholesterol is the major sterol. In addition to the Δ^5-sterols *E. fluviatilis* contains a small amount of 24-methyl-5α-cholestan-3β-ol.

3. The C-24 configuration of 24-alkyl sterols was determined. Sterols with the 24β configuration predominate over the 24α epimers.

4. A summary on uncommon sterols in sponges is reported.

INTRODUCTION

In recent years much attention has been given to sterol content of sponges because these organisms contain the greatest variety of sterols of any animal group (Goad, 1978; Schmitz, 1978; Djerassi *et al.*, 1979; Djerassi, 1981).

Sterols with side chains containing cyclopropane or cyclopropene rings, and with highly branched side chains, have been found in several marine sponges. The cyclopropane sterol petrosterol (**1a**) is the principal sterol component (60%) of the sponge *Petrosia ficiformis* (order Haplosclerida, family Petrosiidae) collected in the Bay of Naples (Sica and Zollo, 1978; Mattia *et al.*, 1978; Ravi *et al.*, 1978) and is accompanied by ficisterol (**2a**) (Khalil *et al.*, 1980b), (24*R*,25*R*)-24,26-dimethylcholesta-5,26-dien-3β-ol (**3a**) (Khalil *et al.*, 1980a) and (23*R*,24*R*)-methylenecholesterol (**4a**) (Proudfoot and Djerassi, 1984). Since petrosterol is the major sterol in the sponge, it is likely that it is a membrane component.

Interesting sterols have been isolated from other members of the Petrosiidae family, *Strongylophora durissima*, *Xestospongia* sp. and *Xestospongia muta*.

The unusual sterol strongylosterol (**5a**) is the principal sterol (90%) of the sponge *Strongylophora durissima* from the Indian and Pacific Oceans (Bortolotto *et al.*, 1978) and is accompanied by the trace sterols **6a** and **7a** (Li and Djerassi, 1981a) arising from quadruple bioalkylation of the cholesterol side chain.

The Caribbean sponge of the genus *Xestospongia* (originally classified as *X. muta*) contains predominantly (71%) the C-30 sterol xestosterol (**8a**) (Kokke *et al.*, 1979) and the trace sterols **9a** (Li and Djerassi, 1981b) and **10a** (Li *et al.*, 1981b).

The true sponge *Xestospongia muta*, collected at Barbados, contains mutasterol (**11a**) as a minor component (3.2%) of the sterol fraction (Li *et al.*, 1981a).

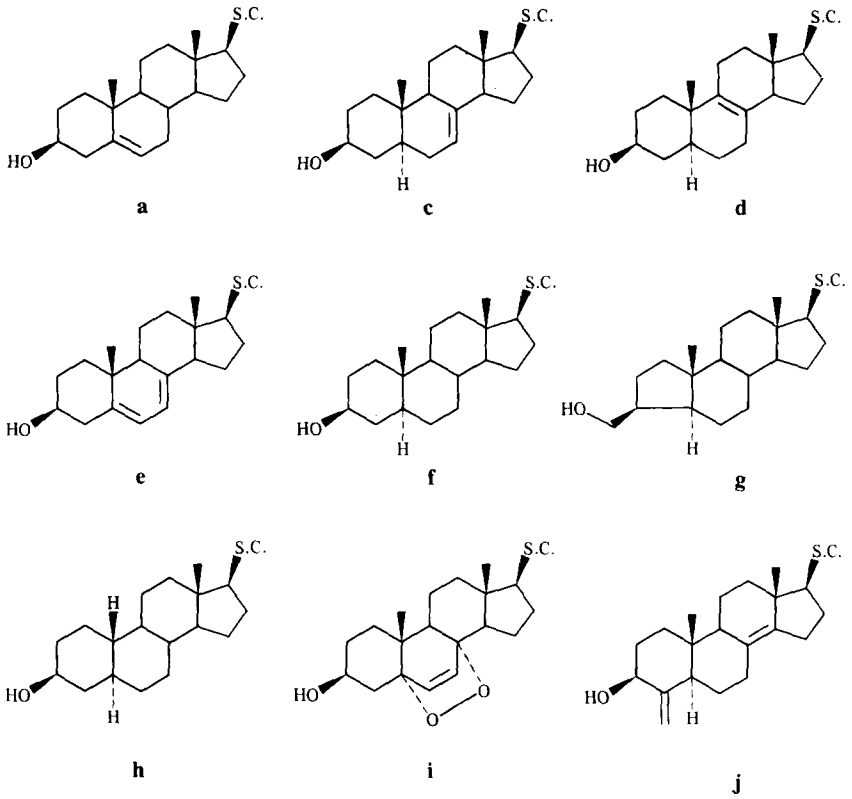
The cyclopropene sterol calysterol (**12a**) (Fattorusso *et al.*, 1975) and its isomers 23H-isocalysterol (**13a**) (Li *et al.*, 1982) and 24H-isocalysterol (**14a**) (Itoh *et al.*, 1983a) comprise 74% of the total sterol fraction of the sponge *Calyx nicaensis* (order Haplosclerida, family Ocenapiidae), collected in the Bay of Naples, and are accompanied by 23,24-dihydrocalysterol (**15a**) (Li *et al.*, 1982). The sponge also contains the only naturally occurring steroidal acetylenes, cholest-5-en-23-yn-3β-ol (**16a**) and 26,27-dinorcholest-5-en-23-yn-3β-ol (**17a**) (Steiner *et al.*, 1977) and a trace sterol, nicasterol (**18a**) (Proudfoot *et al.*, 1985).

Aplysterol, the 26-methylsterol (**19a**), was found to be the principal sterol of sponges of the genus *Aplysina* (= *Verongia*) (order Verongida, family Aplysinidae) (Minale and Sodano, 1977; Kokke *et al.*, 1978). *Aplysina fistularis* (= *Verongia thiona*), collected at La Jolla, California, contains sterols possessing side chains with one or two additional carbon atoms at C-26 (**19a** and **20a**), C-28 (**21a**), C-29 (**22a**) and C-26, C-27 (**23a**). In this sponge 25(26)-dehydroaplysterol (**20a**) and not aplysterol (**19a**) is the main sterol (Catalan *et al.*, 1985).

Pulchrasterol (**24c**) which is the main sterol component (74%) of the New Zealand sponge *Aciculites pulchra* (order Lithistida, family Scleritodermidae) is a product of double alkylation of the sterol side chain at position 26 (Crist *et al.*, 1983).

24-Isopropylcholesterol (**25a**) and 24-isopropyl-22-dehydrocholesterol (**26a**) are the major sterols (98%) of the Australian sponge of the genus *Pseudoaxinissa* (order Halicondrida, family Halicondridae) (Hofheinz and Oesterhelt, 1979). The same sponge contains also 24S,24-isopropenylcholesterol (**27a**) as

‡Author to whom correspondence should be addressed.



Structures of sterol ring systems.

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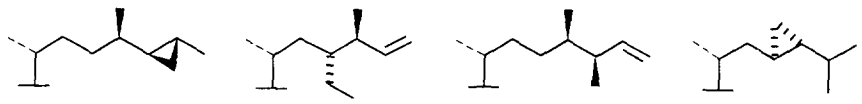

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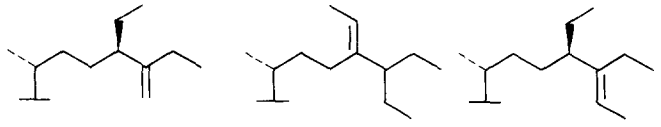
2. *Strongylophora durissima*



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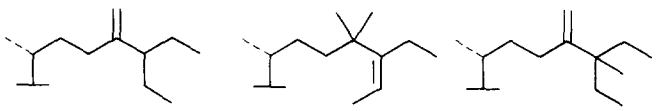
3. *Xestospongia* sp.



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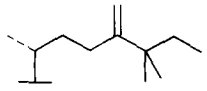
4. *Xestospongia muta*



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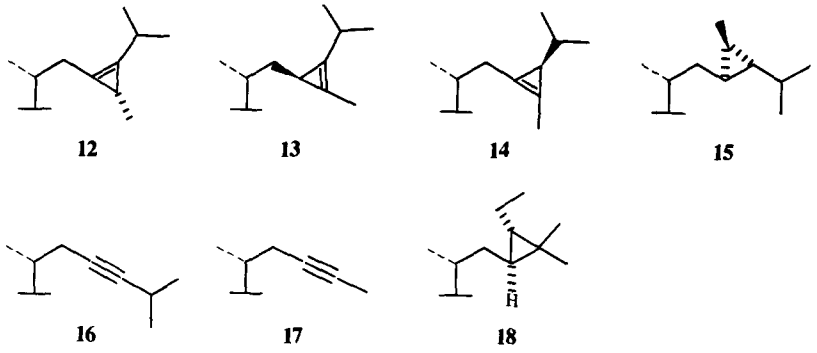
Uncommon sterols isolated from members of the Petrosiidae family. All sterols possess the nucleus **a**.



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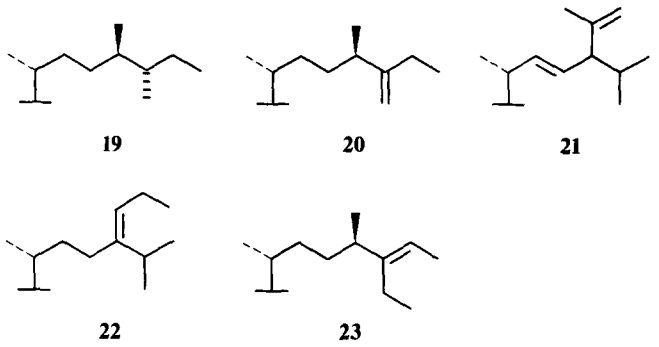
16 17 18

Unusual sterols of the sponge *Calyx nicaensis*. All sterols possess the nucleus **a**.



19 20 21

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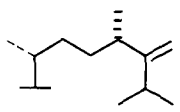
Non-conventional sterols from *Aplysina fistularis*. All sterols possess the nucleus **a**.

1. *Aciculites pulchra*



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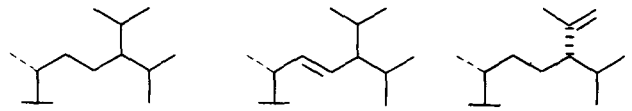
2. *Pseudoaxinissa* sp.



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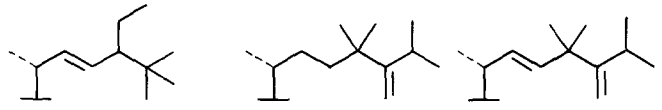
3. *Halichondriidae family*



28 29 30

Unusual sterols from some sponges. Sterol **24** has nucleus **c**, all others have nucleus **a**.

a minor sterol (1%) (Li and Djerassi, 1983; Stoilov *et al.*, 1986c) and trace sterols with highly branched side chains (Tam Ha *et al.*, 1985).

Tropical sponges of the Halichondridae family contain sterol **28a** with additional alkylation at C-25 (Shubina *et al.*, 1984) and sterols **29a** and **30a** with two additional methyl groups at C-26 and quaternary alkylation at C-24 (Shubina *et al.*, 1985).

Sterols with unsaturation at position 5 (**a**) occur frequently in sponges (Bergquist *et al.*, 1980) and in some cases 5α-stanols (**f**) are present (Dini *et al.*, 1985). Some sponges contain large amounts of $\Delta^{5,7}$-sterols (**e**) that are more labile (Sica and Piccialli, 1985; Sica *et al.*, 1987). During the extraction process without an antioxidant we found that they were converted easily into 5α,8α-epidioxysterols (**i**). Sterols

with the rare Δ^8-unsaturated nucleus (**d**) were found only in the sponge *Axinella cannabina* (order Axinellida, family Axinellidae) (Cafieri *et al.*, 1975; Itoh *et al.*, 1983b).

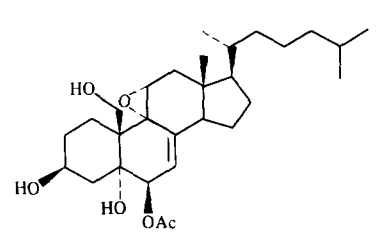
Two unusual sterols with unsaturation in the $\Delta^{8(14)}$ position and a 4-methylene nucleus (**56j** and **62j**) were isolated as the principal sterol constituents from the Red Sea sponges *Theonella conica* and *Theonella swinhoei* (order Lithistida, family Theonellidae), respectively (Kho *et al.*, 1981). They represent the first cases of 4-methylene sterols.

Sterols with modified ring structures have been isolated from some sponges. The first examples were found in the sponges *Axinella polypoides* and *Axinella verrucosa* (order Axinellida, family Axinellidae) collected in the Bay of Naples, which contain 19-norstanols (**h**) and 3β-hydroxymethyl-A-norsteranes (**g**), respectively (Minale and Sodano, 1977). A-norsteranes have been encountered in members of the Axinellidae and Hymeniacidonidae families (Bohlin *et al.*, 1982).

While polyhydroxylated steroids are common metabolites in marine invertebrates they are rarer in sponges. Sponges of the Dysideidae family have been shown to include uncommon steroid polyols. A tetrahydroxylated sterol with the unusual 9,11-epoxide group (**31**) was isolated from a species of the genus *Dysidea* (order Dictyoceratida, family Dysideidae) from Guam (Gunasekera and Schmitz, 1983). The polyhydroxylated 9,11-secosterol (**32**) with the unique 9,11-secocholestane system was isolated from the Australian sponge *Dysidea herbacea* (Capon and Faulkner, 1985). The polar fraction of the lipidic extract of the sponge *Spongionella gracilis* (order Dyctioceratida, family Dysideidae) contains dihydroxysterols (**33–35**) and trihydroxysterols (**36–43**) which could be biosynthetically related to the $\Delta^{5,7}$-sterols present in large amount in this organism (Piccialli and Sica, 1986, 1987).

Steroids having sulfated hydroxyl groups have been isolated from two sponges of the Halichondriidae family. Halistanol sulfate, a trisulfated derivative of 24,25-dimethylcholestane-2β,3α,6α-triol (**44**), has been isolated from the Okinawan sponge *Halicondria cf. moorei* (order Halicondrida, family Halichondriidae) (Fusetani *et al.*, 1981). Sokotrasterol sulfate (**45**), which in respect of **44** differs only in the side chain, was isolated from an unidentified sponge of the family Halichondridae collected in the Arabian Sea (Makarieva *et al.*, 1983). Another group of sterol sulfates (**46–48**) has been isolated from the sponge *Toxadocia zumi* (order Haplosclerida, family Adocidae) (Nakatsu *et al.*, 1983).

The other unusual steroids found in sponges are the steroidal alkaloids plakinamine A (**49**) and B (**50**) found in a species of the genus *Plakina* (order



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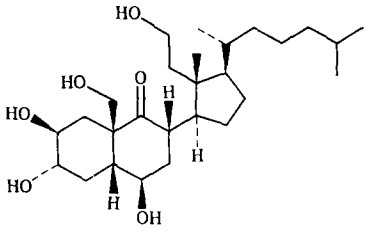

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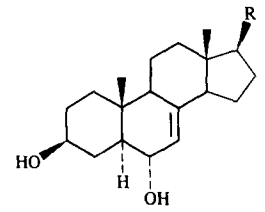
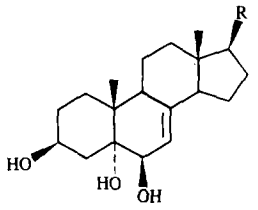
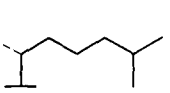
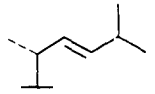
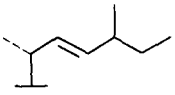
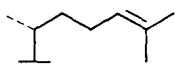


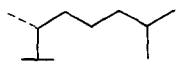


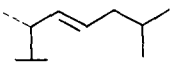
33 R = 

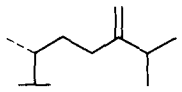
36 R = 

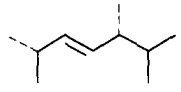
37 R = 

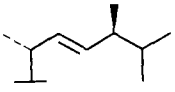
34 R = 

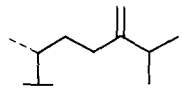
38 R = 

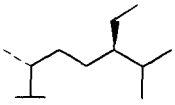
39 R = 

35 R = 

40 R = 

41 R = 

42 R = 

43 R = 

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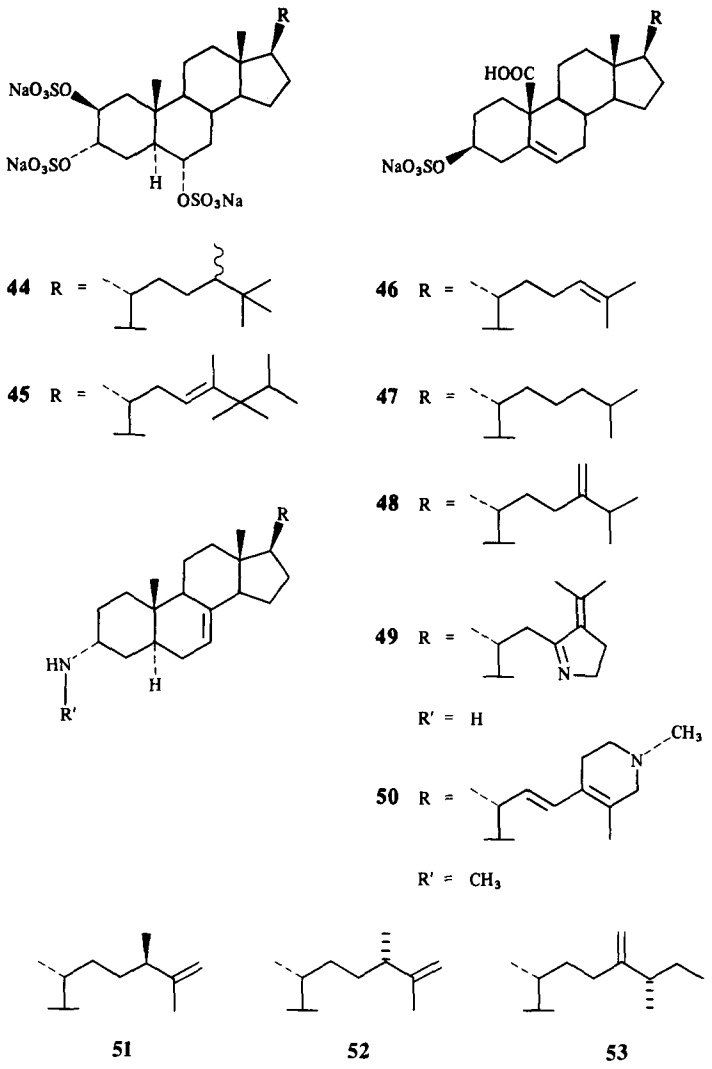
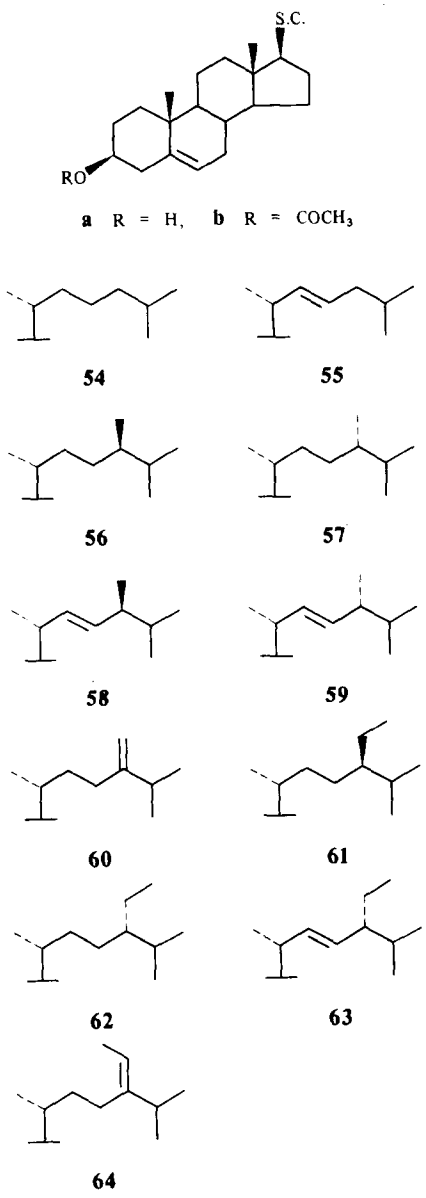


Sterols with additional oxygen functionalities in the nucleus and steroidal alkaloids.

Homosclerophorida, family Plakinidae) (Rosser and Faulkner, 1984).

The biosynthetic origin of sponge sterols is still unknown (Minale and Sodano, 1977). All the attempts performed to study the *de novo* sterol biosynthesis by incorporation of simple precursors, such as labelled acetate and mevalonate, gave very poor results (Minale and Sodano, 1977; Barrow, 1983; Stoilov *et al.*, 1986b). Nevertheless, significant incorporation of labeled mevalonate was recently encountered in *X. testudinaria* (Stoilov *et al.*, 1986d). Low incorporation seems to be due to difficulties in the uptake of low molecular weight, water-soluble compounds. Much more success has been obtained by Djerassi's group in experiments involving the transformation of exogenous sterols.

On the basis of direct incorporation experiments in the Pacific sponge *Aplysina fistularis* (= *Verongia thiona*) it was demonstrated that epicodisterol (**51a**), but not its C-24 epimer codisterol (**52a**), is efficiently converted into 25(26)-dehydroaplysterol (**20a**) (Catalan *et al.*, 1985). Petrosterol (**1a**), the major sterol of *Petrosia ficiformis*, was shown to derive by biomethylation of 24-methylenecholesterol (**60a**) via a complex rearrangement process (Proudfoot *et al.*, 1986). In a study on the biosynthesis of 24-isopropylcholesterols of the sponge *Pseudoaxinissa* sp. a very efficient side chain branching through double alkylation at C-28 was demonstrated, in contrast to relatively poor *de novo* biosynthesis (Stoilov *et al.*, 1986a,c). The biosynthesis of strongylosterol (**5a**), a product of triple bioalkylation of the cholesterol side chain, was studied in *Strongylophora durissima*, and it was shown that it proceeds via codisterol (**52a**) and 24(28)-dehydroaplysterol (**53a**) (Stoilov *et al.*, 1986b). Incorporation experiments carried out in the Australian sponge *Xestospongia testudinaria* demonstrated that codisterol (**52a**) or epicodisterol (**51a**) and 25(26)-dehydroaplysterol (**20a**) or its C-24 epimer are efficiently converted into xestosterol (**8a**) (Stoilov *et al.*, 1986d). Various radiolabeling experiments performed in the sponge *Calyx nicaensis* have demonstrated that 24-methylenecholesterol (**60a**) is converted into dihydrocalysterol (**15a**), which in turn undergoes a *cis*-dehydrogenation to 24H-isocalysterol (**14a**). This sterol might be



Sterol composition of sponges *E. fluviatilis* and *S. lacustris*.

the precursor of the other two cyclopropene sterols present in the sponge, calysterol (**12a**) and 23H-isocalysterol (**13a**) (Margot *et al.*, 1987).

Earlier, with radioactive tracer incorporation, it was shown that the sponge *Axinella verrucosa* and *Axinella polypoides* converted cholesterol (**54a**) to 3β-hydroxymethyl-A-nor-5α-cholestane (**54g**) and 19-nor-cholestanol (**54h**), respectively (Minale and Sodano, 1977).

Sterols from freshwater sponges

The colonization of freshwater by Porifera began during the Cambrian period (Paleozoic Era). During the process of diffusion in internal waters the Spongillidae (Porifera, Demospongiae) had to adapt to an environment in which the variations of the environmental parameters are more swift and frequent than those which occur in the marine habitats. The ecology and the physiology of these animals have been deeply influenced, and in fact freshwater sponges have a typical annual life cycle (Rader, 1985) characterized by the succession of diverse phases which are: development, gemmulation, degeneration, and quiescence. The Spongillidae are present in both the Boreal and Austral hemispheres with over 100 species (Penney and Racek, 1968).

Little work has been reported on the sterols of freshwater sponges (Mazur, 1941; Maquestiau *et al.*, 1978). *Ephydatia fluviatilis* and *Spongilla lacustris* are the most common species in Europe, where they are present both in the fluvial and lacustrine habitats. As part of our continuing studies on Porifera sterols this paper deals with a comparative study on the sterol composition of the freshwater sponges *E. fluviatilis* and *S. lacustris*.

MATERIALS AND METHODS

Collection of animals

The samples of *S. lacustris* come from the Scrivia Torrent (Po basin, Liguria), while *E. fluviatilis* has been collected in an artificial canal in Tagliavia (North-west Sicily).

Extraction and separation of sterol mixtures

Fresh tissues from *S. lacustris* and *E. fluviatilis* were cut into small pieces and extracted three times with 1:1 $CHCl_3$–MeOH at room temperature. Butylated hydroxytoluene was added to retard oxidation. The total lipid extracts were chromatographed with $CHCl_3$ over a silica gel column and fractions containing sterols (TLC analysis) were combined.

Fractionation of acetyl sterols by silver nitrate–silica gel TLC followed by HPLC

The sterols of *E. fluviatilis* and *S. lacustris* were acetylated overnight using acetic anhydride–pyridine (1:1) and the acetates were purified over a silica gel column eluted with increasing concentrations of diethyl ether in 40–70°C light petroleum. The steryl acetates were separated into four fractions by TLC on 0.5 mm thick $AgNO_3$–silica gel developed twice with hexane–benzene (40:60). Bands were located by spraying edge-strips with ceric sulfate in sulfuric acid, and the steryl acetates were recovered with diethyl ether. Each fraction was then subjected to reverse phase HPLC to obtain pure steryl acetates which were analysed by capillary GLC, MS and ^{1}H/NMR. In a representative TLC analysis of acetyl sterols of *S. lacustris* the slower moving fraction 1 (R_f 0.28) contained 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**60b**). The HPLC of fraction 2 (R_f 0.54) (MeOH, 1 ml/min) gave (22*E*)-cholesta-5,22-dien-3β-yl acetate (**55b**) and (24*E*)-24-ethylcholesta-5,24(28)-dien-3β-yl acetate (**64b**). Fraction 3 (R_f 0.62), subjected to reverse-phase HPLC, yielded **55b** (22*E*,24*R*)-24-methylcholesta-5,22-dien-3β-yl acetate (**59b**) and (22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-yl acetate (**58b**). Separation by HPLC of fraction 4 (R_f 0.77) gave cholesteryl acetate (**54b**), (22*E*,24*R*)-24-ethylcholesta-5,22-dien-3β-yl acetate (**63b**), (24*R*)-24-methylcholest-5-en-3β-yl acetate (**56b**), (24*S*)-24-methylcholest-5-en-3β-yl acetate (**57b**), and (24*S*)-24-ethylcholest-5-en-3β-yl acetate (**62b**). The results are given in Table 1.

Separation of total free sterols by HPLC analysis

The crude sterol mixtures of *E. fluviatilis* and *S. lacustris* were purified by crystallization from methanol and fractionated by reverse phase HPLC on a Hibar Supersphere column (3μ) eluted with MeOH–H_2O (96:4; 1 ml/min) with 1 mg of sterol mixture per injection dissolved in a minimum

Table 1. Sterol composition of sponges (%)

Sterol	RRT* HPLC	RRT* HPLC	*E. fluviatilis*	*S. lacustris*
54a	1.00	1.00	74.5	65.7
55a	0.77	0.91	2.6	1.8
56a	1.06	1.29	2.6	1.5
57a	1.06	1.29	0.7	6.1
58a	0.81	1.11	2.2	1.2
59a	0.89	1.11	1.5	2.8
60a	0.79	1.26	0.6	4.1
61a	1.12	1.61	4.2	—
62a	1.12	1.61	6.3	9.0
63a	1.02	1.40	2.6	6.0
64a	0.88	1.61	1.5	1.3
†	1.21	1.31	0.6	—

*Retention time of acetate derivatives relative to cholesteryl acetate used as the standard (1.00) for both GC-RRT and HPLC-RRT.
†24-Methyl-5α-cholestan-3β-ol.

volume of methanol. Eight fractions were collected. The constituents, after further purification by HPLC, were identified by their mass and ^{1}H/NMR spectra.

From the sponge *S. lacustris* were isolated cholesterol (**54a**), 22-dehydrocholesterol (**55a**), campesterol (**56a**), 22,23-dihydrobrassicasterol (**57a**), 24-epibrassicasterol (**58a**), brassicasterol (**59a**), clionasterol (**62a**) and poriferasterol (**63a**). The same sterols were isolated from the sponge *E. fluviatilis* and, in addition, sitosterol (**61a**) and the stanol 24-methyl-5α-cholestan-3β-ol were found.

Analytical methods

Capillary gas chromatography was carried out on a Carlo Erba Fractovap 4160 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (30 m × 0.32 mm), at 240°C with H_2 as the carrier gas.

High performance liquid chromatography (HPLC) was performed on a Hibar Supersphere 3μ column (4 × 250 mm), using a Varian 2010 pump, a Waters R 401 differential refractometer and a Rheodine model 7125 injector. ^{1}H/NMR spectra were recorded on a Bruker WM-270 spectrometer in $CDCl_3$ solutions with Me_4Si as internal standard. Low resolution mass spectra were recorded at 70 eV on an AEI 30 instrument.

RESULTS AND DISCUSSION

The total lipids were extracted from the sponges *E. fluviatilis* and *S. lacustris* and chromatographed on a silica gel column to yield the sterol fractions. Separation of acetyl sterols by argentic thin layer chromatography followed by reverse phase HPLC separation of fractions gave pure compounds. This method is especially useful for the separation of sterols with a 24(28) double bond (**60a** and **64a**).

The free total sterols of sponges were also fractionated by HPLC. This method revealed the presence in the sponge *E. fluviatilis* of a saturated ring sterol identified as 24-methyl-5α-cholestan-3β-ol. The isolated sterols were identified by comparison of their mass and ^{1}H/NMR spectra with those of previously identified compounds. The C-24 configurations of 24-alkylsterols were determined by comparison of the ^{1}H/NMR spectra of the isolated sterols with the ^{1}H/NMR spectra of authentic samples. The determination of this configuration has biosynthetic and taxonomic implications (Goad, 1978). The sponge *S. lacustris* contains 24β-ethyl sterols (**62a,63a**) while the 24β-methyl sterols (**57a,59a**) are accompanied by the 24α-epimers (**56a,58a**). These sterols are also present in *E. fluviatilis* which contains in addition 24α-ethylcholesterol (**61a**).

Table 1 lists the sterols isolated together with the relative retention times (RRT) in HPLC and gas chromatography. From these results it may be seen that *E. fluviatilis* and *S. lacustris* contained very similar sterol profiles and cholesterol is the principal sterol present. Sterol mixtures of *E. fluviatilis* do not contain C_{26} sterols or 27-norsterols which are ubiquitous in the marine environment and have been encountered in many marine sponges (Schmitz, 1978). They are present in trace amounts in the sponge *S. lacustris*.

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