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New cytotoxic steroids from the marine sponge *Dysidea fragilis* coming from the lagoon of Venice

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The sterol composition of the sponge Dysidea fragilis, *coming from the lagoon of Venice, has been investigated; our results confirmed the variability of* D. fragilis *biochemistry. The sponge elaborates, in addition to eight usual 3β-hydroxy sterols, thirteen polyhydroxysterols, eight of them **(6–13)** were novel compounds. Their structures were established by spectroscopic data. New compounds 3β,5α,6β,7α-tetrahydroxy-cholest-8(9)-en-11-one **(8)**, 3β,5α,6α-trihydroxy-9,11-secocholest-7-en-9-one **(9)** and 3β,5α,6α,9α-tetrahydroxy-cholest-7-ene-6-sulfate **(11)** were proved to be cytotoxic on two different tumor cell lines in vitro.* (Steroids **60**:666–673, 1995)

Keywords: dictyoceratida; *Dysidea fragilis;* polyhydroxysteroids; sulfate steroids; NMR spectra; cytotoxic activity

Introduction

In the frame of a study on the filtering organisms of the lagoon of Venice, aiming to ascertain the possible influence of this peculiar environment on the metabolism of the benthic filter-feeders, we examined specimens of a Dictyoceratida sponge, *Dysidea fragilis,* inhabiting the northern part of the lagoon, which has been reported to possess a secondary metabolism strongly dependent on the environmental conditions.[1–5]

Recently, the analysis of the sterol composition of a specimen of *D. fragilis* from the Black Sea has been reported.[6] This study showed that cholesterol and 7-dehydrocholesterol were the main sterols in this specimen and led to the isolation of two new Δ^7-2α,3α,5α,6β,9α,11α-hexahydroxysterols. More recently, a new $\Delta^{8(14),24(28)}$-3β,6α-dihydroxysterol has been isolated from a *D. fragilis* sample coming from the South China Sea.[7]

In this paper we report the analysis of the sterol composition of *D. fragilis,* collected in the lagoon of Venice. Our results, remarkably differing from those obtained from the samples previously analyzed, confirm the variability of *D. fragilis* biochemistry; the sponge contained large amounts of a polar sterol fraction, comprising thirteen polyhydroxysterols; eight of them **(6–13)** were novel compounds whose structural determination is here reported. None of the remaining five known sterols were previously found in *D. fragilis* samples. The sponge elaborates also eight usual 3β-hydroxysterols (see Table 1), but they were present in much lesser quantities than the polyoxygenated ones. The cytotoxic activity of compounds **8, 9,** and **11,** estimated on WEHI 164 (murine fibrosarcoma) and J774 (murine monocyte/macrophages) cell-line, is also reported (Table 2).

Experimental

General methods

Medium pressure liquid chromatography (MPLC) was performed on a Buchi apparatus. HPLC was performed on a Varian HPLC Model 5000 with a Merck Si60 Lichrospher 5 mm using a dual cell refractometer detector. Combined GLC-MS analysis was performed on a Hewlett-Packard 5890 chromatograph with a mass selective detector MSD HP 5970 MS and a split/splitness injector for capillary columns using a fused silica column, 25 m × 0.2 mm HP5 (cross-linked 5% PhMeSilicone, 0.33 mm film thickness). UV spectra (EtOH) were recorded on a Beckman DU 70 instrument. Fourier transform IR spectra were recorded on a Bruker IFS-48 spectrophotometer. High resolution mass spectra (HRMS) were obtained by electron impact at 70 eV on a Kratos MS 50 mass spectrometer. FABMS spectra were obtained in a glycerol matrix on a VG ZAB mass spectrometer. ^1H and ^{13}C NMR spectra were run on a Bruker AMX-500 spectrometer in CD_3OD and pyridine-d_5. The nature of each carbon resonance was deduced from dis-

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Table 1 Sterol composition of the lagoonal specimen of *Dysidea fragilis*

Common 3β-hydroxysteroids	mg/kg dry wt.	Poly-hydroxy-steroids	mg/kg dry wt.
cholesta-5,22-dien-3β-ol	24.6	1	25.0
cholesterol	376.2	2	25.0
24-methylcholesta-5,22-dien-3β-ol	50.0	3	31.2
24-methylcholest-5-en-3β-ol	7.5	4	18.7
24-ethylcholesta-5,22-dien-3β-ol	15.0	5	18.7
24-ethylcholest-5-en-3β-ol	15.0	6	31.2
cholestanol	108.7	7	18.7
24-ethyl-3β-cholestanol	2.5	8	187.5
		9	125.0
		10	62.5
		11	1500
		12	1000
		13	562.5

tortionless enhancement by polarization transfer (DEPT) experiments performed using polarization transfer pulses of 90°C and 135°. Homonuclear ^1H connectivities were determined by using the COSY experiments. One bond heteronuclear ^1H-^{13}C connectivities were determined with a HETCOR pulse sequence optimized for a $^1J_{C\text{-}H}$ of 125-Hz.

Extraction and isolation of the sterols

Specimens of *D. fragilis,* identified by Prof. Maurizio Pansini (Istituto di Zoologia dell'Università di Genova), were collected by scraping the solid surfaces (particularly the submerged portions of "briccole", the wood markers present along the canals) present in the northern part of the lagoon of Venice (Canale di Mazzorbo, Burano). They were frozen immediately after collection; a voucher specimen is deposited at the Dipartimento di Chimica Delle Sostanze Naturali-Università di Napoli "Federico II".

The sponge (80 g dry weight after extraction), fresh thawed, was homogenized and extracted with methanol at room temperature. The extracts were concentrated in vacuo to give an aqueous suspension that was extracted with ethyl acetate. Separation of the ethyl acetate soluble materials (2 g) by gradient silica gel MPLC chromatography (hexane → ethyl acetate → methanol) gave two crude sterol bands, A and B.

The less polar fraction A (0.06% dry wt), eluted with hexane/EtOAc 8:2, v/v, contained conventional sterols. They were characterized by GLC-MS analysis of their acetate derivatives prepared by treating with acetic anhydride and pyridine (1:1, v/v) for 18 h at room temperature. The sterol acetates were purified on a silica gel column eluting with hexane containing increasing amounts of Et_2O and analyzed by GLC-MS. The identification of the sterol acetates was based on comparison of the GC-MS spectra with those of authentic specimens. The quantitation of sterols, reported in Table 1, was performed by a programmable integrator using 5α-cholestane as an internal standard.

Fraction B (0.7% dry wt), eluted from the MPLC column with methanol, was rechromatographed on a silica gel column (70–230 mesh, Merck, 200 g) eluting with a linear gradient of methanol (2% to 50%) in $CHCl_3$ to give three polyhydroxysterol mixtures. They were further fractionated and purified by HPLC on a Lichrospher RP-18 column thus obtaining compounds **1** (2 mg), **2** (2 mg), **3** (2.5 mg), **4** (1.5 mg), **5** (1.5 mg), **6** (2.5 mg), and **7** (1.5 mg); using CH_3OH/H_2O 9:1, v/v, as eluent), compounds **8** (15 mg), **9** (10 mg) and **10** (5 mg) (CH_3OH/H_2O 8:2, v/v), and the most polar compounds **11** (120 mg), **12** (80 mg), and **13** (45 mg).

Compound 6. HREIMS: m/z 430.3075 (430.3072 calculated for $C_{27}H_{42}O_4$); ^1H NMR values ($CDCl_3$) with ^{a} superscript are submerged by other signals: δ 1.68^a (H-2ax), 2.10^a (H-2eq), 3.99^a (m, H-3ax), 2.20^a (H-4ax), 1.59^a (H-4eq), 3.41 (d, J = 4.6 Hz, H-6), 6.80 (dd, J = 4.6, 0.9 Hz, H-7), 3.68 (m, H-11a), 3.80 (m, H-11b), 1.15^a (H-12a), 1.63^a (H-12b), 3.38 (ddd, J = 11.3, 8.1, 0.9 Hz, H-14), 1.62^a (H-15a), 1.74^a (H-15b), 1.73^a (H-17), 0.68 (s, 3H-18), 1.24 (s 3H-19), 2.10^a (H-20), 1.02 (d, J = 6.8 Hz, 3H-21), 5.26 dd, J = 15, 7.5 Hz, H-22), 5.30 (ddd, J = 15, 7.0, 6 Hz, H-23), 1.58^a (H-25), 0.86 (d, J = 7 Hz, 3H-26 and 3H-27); ^{13}C NMR ($CDCl_3$): δ 16.1 (q), 17.3 (q), 21.8 (q), 22.6 (q), 22.7 (q), 37.8 (t), 40.5 (t), 53.6 (d), 60.2 (t), 63.9 (d), 69.0 (s), 131.9 (d), 136.0 (d), 139.8 (d), 134.8 (s), 203.0 (s).

Compound 7. HREIMS: m/z 444.3231 (444.3228 calculated for $C_{28}H_{44}O_4$); ^1H NMR values ($CDCl_3$) with ^{a} superscript are submerged by other signals: δ 1.68^a (H-2ax), 2.10^a (H-2eq), 3.99^a (m, H-3ax), 2.21^a (H-4ax), 1.60^a (H-4eq), 3.41 (d, J = 4.6 Hz, H-6), 6.80 (dd, J = 4.6, 0.9 Hz, H-7), 3.69 (m, H-11a), 3.80 (m, H-11b), 1.16^a (H-12a), 1.61^a (H-12b), 3.39 (ddd, J = 11.5, 8.2, 0.9 Hz, H-14), 1.62^a (H-15a), 1.74^a (H-15b), 1.73^a (H-17), 0.68 (s, 3H-18), 1.24 (s, 3H-19), 2.13^a (H-20), 1.03 (d, J = 6.5 Hz, 3H-21), 5.18 (m, H-22), 5.23 (m, H-23), 1.62^a (H-24), 0.84 (d, J = 7 Hz, 3H-26), 0.87 (d, J = 7 Hz, 3H-27), 0.92 (d, J = 7 Hz, 3H-28); ^{13}C NMR ($CDCl_3$): δ 15.5 (q), 17.0 (q), 18.5 (q), 21.4 (q), 20.6 (q), 20.9 (q), 37.5 (t), 40.5 (t), 53.6 (d), 60.2 (t), 63.9 (d), 69.0 (s), 132.2 (d), 136.4 (d), 139.8 (d), 134.8 (s), 202.0 (s).

Compound 8. HREIMS: m/z 448.3180 (448.3177 calculated for $C_{27}H_{44}O_5$); IR: ν_{max} 3320, 1664 cm^{-1}; UV: λ_{max} 254 nm (ε 8020); ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 9. HREIMS: m/z 432.3230 (432.3228 calculated for $C_{27}H_{44}O_4$ [$M^+ - H_2O$]); IR: ν_{max} 3325, 1666 cm^{-1}; UV: λ_{max} 251 nm (ε 11000); ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 10. HREIMS: m/z 430.3075 (430.3072 calculated for $C_{27}H_{42}O_4$ [$M^+ - H_2O$]); IR: ν_{max} 3325, 1665 cm^{-1}; UV: λ_{max} 251 nm (ε 11000); ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 11. FABMS negative ion mode: m/z 513; IR: ν_{max} 1250 cm^{-1}; ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 12. FABMS negative ion mode: m/z 525; ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 13. FABMS negative ion mode: m/z 527 ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Compound 14. HREIMS: m/z 434.3387 (434.3384 calculated for $C_{27}H_{46}O_4$); ^1H and ^{13}C NMR data are reported in Tables 3 and 4.

Table 2 IC_{50} (μg/ml) of new compounds **8, 9,** and **11**[a]

Cell-line	8	9	11	6-MP
WEHI 164	36.0 ± 1.4	39.2 ± 0.5	50.4 ± 0.6	1.29 ± 0
J 774	1.68 ± 1.0	8.64 ± 0.3	17.12 ± 0.5	0.001 ± 0

[a]In vitro cytotoxic activity (IC_{50} in μg/ml) of compounds **8, 9, 11,** and **6-MP** (6-mercaptopurine) on WEHI 164 (murine fibrosarcoma cell-line) and J 774 (murine monocyte/macrophages cell-line). The results are expressed as mean ± S.E.M. of three experiments in triplicate.

Table 3 Selected ^{1}H-NMR data (CD_3OD) of sterols 8–14^a

Compound	8	9	10	11	12	13	14
Proton							
1ax	1.74^{b,c}	206, ddd (14, 13.8, 4)	2.06, ddd (14, 13.8, 4)	2.25^{b,c}	2.22^{b,c}	2.22^{b,c}	2.25^{b,c}
1eq	2.65, ddd (13, 3.5, 3)	1.58^{b,c}	1.58^{b,c}	1.42^{b,c}	1.42^{b,c}	1.42^{b,c}	1.45^{b,c}
2ax	1.56^{b,c}	1.49^{b,c}	1.49^{b,c}	1.50^{b,c}	1.50^{b,c}	1.50^{b,c}	1.52^{b,c}
2eq	1.80^{b,c}	1.88^{b,c}	1.89^{b,c}	1.89^{b,c}	1.89^{b,c}	1.89^{b,c}	1.90^{b,c}
3	4.05, m	3.91, m	3.91, m	3.92, m	3.92, m	3.92, m	3.95, m
4ax	2.11^{b,c}	1.55^{b,c}	1.55^{b,c}	1.62^{b,c}	1.61^{b,c}	1.62^{b,c}	1.51^{b,c}
4eq	1.73^{b,c}	2.23, dd (13, 4)	2.23, dd (13, 4)	2.27^{b,c}	2.29^{b,c}	2.29^{b,c}	2.15^{b,c}
6	3.67 bs	4.44, bd (2)	4.44, bd (2)	4.68, bs	4.68, bs	4.67, bs	3.92, bs
7	3.99, bs	6.31, bd (2)	6.30, bd (2)	5.41, bs	5.41, bs	5.41, bs	5.12, bs
11		a: 3.65, m b: 3.77, m	a: 3.65, m b: 3.77, m	a: 1.39^{c,d} b: 2.02^{d}	a: 1.38^{c,d} b: 2.01^{d}	a: 1.39^{c,d} b: 2.02^{d}	
12	ax: 2.76, d (14) eq: 2.48, d (14)	a: 1.17^{b,c} b: 1.56^{b,c}	a: 1.18^{b,c} b: 1.58^{b,c}	a: 1.65^{c,d} b: 1.88^{c,d}	a: 1.65^{c,d} b: 1.89^{c,d}	a: 1.63^{c,d} b: 1.87^{c,d}	
14	2.92, dd (13, 7)	3.31, bdd (11,9)	3.31, bdd (11, 9)	2.52, bdd (10, 7.5)	2.51, bdd (10, 7.5)	2.52, bdd (10, 7.5)	2.53, bdd (10,7)
15	a: 2.21^{b,c} b: 1.67^{b,c}	a: 1.65^{b,c} b: 1.70^{b,c}	a: 1.65^{b,c} b: 1.70^{b,c}	a: 1.61^{b,c} b: 1.49^{b,c}	a: 1.60^{b,c} b: 1.49^{b,c}	a: 1.60^{b,c} b: 1.49^{b,c}	a: 1.62^{b,c} b: 1.52^{b,c}
16	a: 2.07^{b,c} b: 1.45^{b,c}	a: 1.45^{b,c} b: 1.85^{b,c}	a: 1.46^{b,c} b: 1.85^{b,c}	a: 1.62^{c,d} b: 1.82^{c,d}			
17	1.50^{b,c}	1.72^{b,c}	1.72^{b,c}	1.31^{c,d}			
18	0.78, s	0.76, s	0.76, s	0.62, s	0.65, s	0.65, s	0.63, s
19	1.41, s	1.28, s	1.26, s	1.12, s	1.12, s	1.12, s	1.10, s
20	1.46^{b,c}	1.47^{b,c}	2.16^{b,c}	1.43^{b,c}	2.06^{b,c}	1.40^{b,c}	1.44^{b,c}
21	0.96, d (6.5)	1.05, d (6.5)	1.10, d (6.5)	0.98, d (6.5)	1.02, d (6.5)	0.98, d (6.5)	0.98, d (6.5)
22	a: 1.08^{b,c} b: 1.44^{b,c}	a: 1.04^{b,c} b: 1.45^{b,c}	5.25, dd (14.5, 8)		5.20, dd (14.5, 7.9)		
23	a: 1.23^{b,c} b: 1.41^{b,c}	a: 1.21, m b: 1.41, m	5.29, m		5.25, dd (14.5, 6.5)		
24	1.18, m	1.16, m		1.18^{b,c}	1.65^{b,c}	1.05^{b,c}	
25	1.56^{b,c}	1.56^{b,c}	1.58^{b,c}	1.55^{b,c}	1.49^{b,c}	1.72^{b,c}	1.53^{b,c}
26	0.92, d (7)	0.92, d (7)	0.95, d (7)	0.91, d (7)	0.87, d (7)	0.85, d (7)	0.91, d (7)
27	0.92, d (7)	0.92, d (7)	0.95, d (7)	0.91, d (7)	0.85, d (7)	0.88, d (7)	0.91, d (7)
28					0.95, d (7)	0.91, d (7)	

^{a}J values (Hz) are given in parentheses.
^{b}Assignments based on ^{1}H-^{1}H COSY experiment.
^{c}Submerged by other signals.
^{d}Assignments based on HETCOR experiment.

Determination of biological activity

Cells. WHEI 164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) heat-inactivated, 25 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). J 774 cells (murine monocyte/macrophages cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm and incubated at 37°C, in DMEM medium supplemented with 10% FCS, 25 mM HEPES, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Cytotoxicity assay. WEHI 164 (2×10^4 cells) or J 774 (1×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter the medium was replaced with 50 μL of fresh medium and 50 μL aliquot of 1:2 v/v serial dilution of each test compound **8**, **9**, and **11** was added and then the cells were incubated for 72 h. In some experiments 6-mercaptopurine (6-MP) was added. The cells viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] conversion assay.[8] Briefly, 25 mL of MTT (5 mg/mL) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5.[9]

The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with compounds **8**, **9**, **11**, and 6-MP was calculated as:

$$\% \text{ dead cells} = 100 - (\text{OD treated/OD control}) \times 100$$

The results (Table 2 are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%)

Materials. All reagents for cell culture, except FCS (Flow laboratories) were from Gibco; MTT and 6-mercaptopurine from Sigma.

Results and discussion

The methanol extract of *D. fragilis* was evaporated and the residue was partitioned between ethyl acetate and water. To determine the sterol profile of the sponge, the ethyl acetate extract was chromatographed by MPLC on a Si gel column thus obtaining two crude sterol bands, A and B.

The less polar fraction A (0.06% dry wt), containing conventional sterols, was acetylated and subjected to further chromatographic separation on a Silica gel column. Analysis of each fraction by GC-MS allowed the identification and quantitation of the individual sterol acetates. The results, listed in Table 1, revealed that 3β-hydroxy- and Δ^5-3β-hydroxy cholestanols were the constituents of the minor fraction A.

Fraction B, by far the most abundant steroidal material,

Table 4 ^{13}C-NMR data[a] (CD_3OD) of sterols 8-14

Pos.	8	9	10	11	12	13	14
1	29.6	28.5	28.5	28.1	28.0	28.1	28.0
2	31.6	30.9	31.0	31.5	31.5	31.6	30.7
3	67.5	67.4	67.5	68.0	68.0	67.8	67.8
4	41.0	39.3	39.3	41.0	41.0	41.0	40.7
5	76.9	80.8	80.8	76.0	76.0	76.0	75.8
6	79.1	71.5	71.5	79.8	79.8	79.8	71.2
7	73.7	144.0	144.0	119.6	119.1	119.1	122.0
8	138.7[b]	137.7	137.5	144.5	144.5	144.7	143.3
9	154.2[b]	206.0	206.0	78.1	78.1	77.8	78.6
10	41.6	47.1	47.1	42.6	42.6	42.6	42.2
11	203.9	59.2	59.2	29.0	28.8	28.9	29.0
12	58.7	42.1	42.0	36.0	36.1	36.3	36.5
13	48.1	50.5	50.5	45.0	45.0	45.2	44.9
14	52.6	43.6	43.6	51.8	52.0	51.5	51.6
15	24.6	27.7	27.8	23.8	23.5	24.1	23.8
16	28.6	27.2	27.2	28.3	28.3	28.3	28.2
17	56.5	51.4	51.4	56.8	56.6	56.6	57.4
18	12.2	17.8	18.0	12.0	12.3	11.8	11.8
19	23.3	20.2	20.2	21.3	21.3	21.3	20.8
20	37.3	35.9	39.3	37.2	40.4	36.9	37.4
21	19.0	19.4	21.4	19.9	21.5	19.1	19.3
22	37.0	36.6	138.0	36.5	133.6	34.0	37.2
23	24.9	25.6	127.1	25.0	130.0	30.1	24.9
24	40.6	40.6	41.9	40.8	43.9	41.8	40.7
25	29.6	29.1	28.6	29.2	34.1	32.5	29.2
26	23.2[c]	23.0[d]	22.8[e]	23.5[f]	19.9[g]	18.8[h]	22.9[i]
27	22.9[c]	23.2[d]	23.1[e]	23.9[f]	20.5[g]	20.3[h]	23.1[i]
28	—	—	—	—	18.5	14.8	—

[a]δ values are in ppm from the residual solvent signal (δ 49.0). ^{13}C Assignments were based on DEPT and HETCOR experiments, and substituent parameter effects[19–21].
[b–i]The resonances with identical superscript may be reversed.

(0.7% dry wt), was a very complex mixture of polar steroids. It was separated into individual compounds by combination of a further Silica gel column chromatography (eluting with a linear gradient of methanol—2% to 50%—in chloroform) and repeated reverse-phase HPLCs using several different solvent systems (methanol/water 9:1, v/v, to give compounds **1–7;** methanol/water 8:2 (v/v) to give compounds **8–13**). The obtained products **1–13**, which appeared to be pure compounds on the basis of a preliminary spectroscopic analysis (NMR, MS), were subjected to an extensive structural analysis.

Compounds **1–5** were identified by comparison of their spectral properties with those reported in literature. Sterols **1–4** were previously isolated from *Spongionella gracilis,*[10] a marine sponge belonging to the family Dysideidae (order Dictyoceratida), while compound **5** was shown to be elaborated by the sponge *Aplysilla glacialis*[11] (family Aplysillidae, order Dendroceratida).

The remaining compounds **6** and **7**, present in very small quantities, had molecular formula $C_{27}H_{42}O_4$ and $C_{28}H_{44}O_4$, respectively, as deduced by HREIMS. ^1H and ^{13}C NMR data (see Experimental) indicated that they possessed the same nuclear structure of **5.** The nature of the side chains was established by their ^1H NMR spectra, which included the resonances of the side chains assigned to **6** and **7**, respectively on the basis of ^1H-^1H COSY experiment (see Experimental).

The molecular formula of **8** was established by HREIMS providing a molecular ion at m/z 448.3180 appropriate for the molecular formula $C_{27}H_{44}O_5$. ^{13}C NMR spectrum confirmed the presence of 27 carbon atoms, including one ketone carbonyl (s, δ 203.9), three CHOH (doublets at δ 67.5, 79.4 and 73.7) and one -C-OH (s, δ 76.9) functions, which accounted for all the oxygen atoms of the molecule.

The infrared spectrum contained bands at ν_{max} 3320 (OH) and at ν_{max} 1664, which indicated that the C=O functionality was α,β-unsatured. This was confirmed by the UV absorbtion at λ_{max} 254 nm (ε 8020) and by ^{13}C NMR spectrum, which in the sp^2 region exhibited, in addition to the carbonyl signal, only two resonances, at δ 138.7 (s) and 154.2 (s).

The sterol structure of compound **8** was evident from its ^1H NMR spectrum, which showed methyl signals at δ 0.78 (3H, s), 1.41 (3H, s), 0.96 (3H, d, J = 6.5 Hz), and 0.92 (6H, d, J = 7 Hz) assigned to methyls 18, 19, 21, and 26/27 of a cholestane skeleton, respectively.

Although the high field region of the ^1H NMR spectrum in CD_3OD contained several overlapping signals, the ^1H-^1H COSY experiment provided useful informations enough to assign all the proton resonances, delineating three spin systems. The first one comprised the protons linked to C_1-C_2-C_3-C_4 of ring A; the second spin system was the hydroxymethine proton at C-6, coupled with H-7 resonating at δ 3.99 and the third sequence extended from H-14 to the side chain protons through H_2-15, H_2-16, and H-17. A short-range ^1H-^{13}C chemical shift correlation NMR experiment (HETCOSY) permitted unambiguous associations of the signals of the protons belonging to the above segments with

the signals of the carbons to what they were attached (Table 3). The above data delineated most of the structural features of sterol **8.** Additional information, sufficient to build up the sterol structure of **8,** was obtained from further ^{1}H and ^{13}C NMR data, which evidenced the presence of an isolated methylene group [^{1}H-NMR: δ 2.76 (1H, d, J = 14 Hz, H_{ax}-12), 2.48 (1H, d, J = 14 Hz, H_{eq}-12); ^{13}C NMR: δ 58.7] and two quaternary carbon atoms [C-10 (d 41.6) and C-13 (δ 48.1)]. Further support for the proposed structure was obtained from the ^{1}H-^{1}H COSY spectrum which exhibited four-bond coupling between H-14/H-12_{eq} and H-12_{ax}/H_3-18, respectively.

Consideration on the chemical shifts and coupling constants of some key signals in the ^{1}H NMR spectrum allowed establishment of the orientation of the hydroxy groups at C-3, C-5, C-6, and C-7 in **8.** The multiplet at δ 4.03, due to H-3, had a complexity normally seen in a 3β-hydroxy sterol. The downfield position (~0.4 ppm) of this signal relative to that of a normal 5α-cholestan-3β-ol is clearly indicative of an α-oriented OH at position 5.[12–14] This was further supported by the downfield shift of the 3-H signal to δ 4.31 in pyridine-d_5, indicating its 1,3-diaxial interaction with an OH group.[15]

The OH group at C-6 was β-positioned on account of the chemical shift value observed for H-4_{ax}. According to Fujimoto et al.,[16] the resonances of H-4 protons in 3β,5α,6-cholestanetriols show a diagnostic dependence on the configuration at C-6. Their resonances are strongly influenced by a deshielding effect of the 6-OH group through a 1,3-diaxial (with the 6β isomer) or 1,3-diequatorial (with the 6α isomer) interaction, which is intensified in pyridine solution. The low-field chemical shift values observed for the 4-H_{ax} of **8** both in CD_3OD (δ 2.11) and pyridine-d_5 (δ 2.95) unequivocally established the β orientation of 6-OH.

The stereochemistry at C-7 was established on the basis of the coupling constant H-6/H-7 (J = 1.5 Hz) in the light of a conformational analysis on the two epimers at C-7 by MM2 calculations. The lowest energy conformer of the 7*S* epimer of **8,** in which the OH group is α orientated, showed the B ring in a distorted conformation with a dihedral angle between H-6 and H-7 of 81.7° (Figure 1); this value is in a good agreement with the observed coupling constant (J = 1.5 Hz). On the contrary, the preferential conformation of the 7*R* epimer had the dihedral angle of 36° (Figure 2), which would require a higher coupling constant value (J = 5.5 Hz) by a Karplus generalized equation.

All the above data allowed to assign the stereostructure of compound **8** as reported in Figure 3.

Negative ion FAB mass spectrum of compound **11** exhibited an intense molecular anion at m/z 513; combined analysis of the FABMS and ^{1}H and ^{13}C NMR spectra suggested the molecular formula $[C_{27}H_{45}O_7S]^-$. The IR spectrum contained an absorption band at 1250 cm^{-1} consistent with the presence of a sulfate group; this was confirmed by solvolysis of **11** in dioxane-pyridine solution at 120° (3 h) to give the desulfated **14** which had the molecular formula $C_{27}H_{46}O_4$ as deduced from its HREIMS (m/z 434.3387).

The high field region of the ^{1}H NMR spectrum of **11** in CD_3OD contained signals for five methyl protons of a cholestane structure: singlets at δ 0.62 (CH_3-18) and 1.12 (CH_3-19) and doublets at δ 0.98 (d, J = 6.5 Hz, CH_3-21) and 0.91 (d, J = 7 Hz, CH_3-26 and CH_3-27). Additional signals were present at δ 3.92 (m) and 4.68 (bs) attributable to two oxygenated methines and δ 5.41 (bs) due to an olefinic proton.

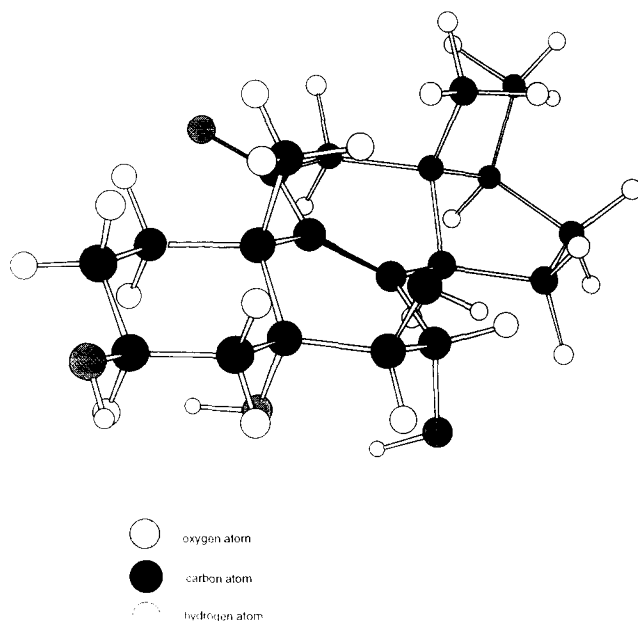

Figure 1 Lowest energy conformation of the 7S epimer of **8** (MM2 calculations).

Inspection of its molecular formula revealed that **11** had an additional unsaturation besides those due to the steroidal nucleus. This was assigned to a trisubstituted double bond on the basis of the ^{13}C NMR spectrum which, in the olefinic region, showed only two signals at δ 144.5 (s) and 119.6 (d).

Useful information on the structure of **11** were obtained by the ^{1}H ^{1}H connectivities observed through a COSY ex-

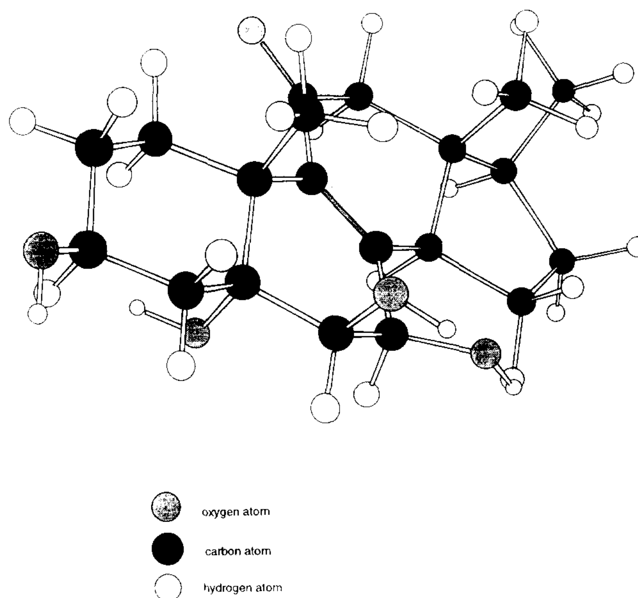

Figure 2 The preferential conformation of the 7R epimer of **8** (MM2 calculations).

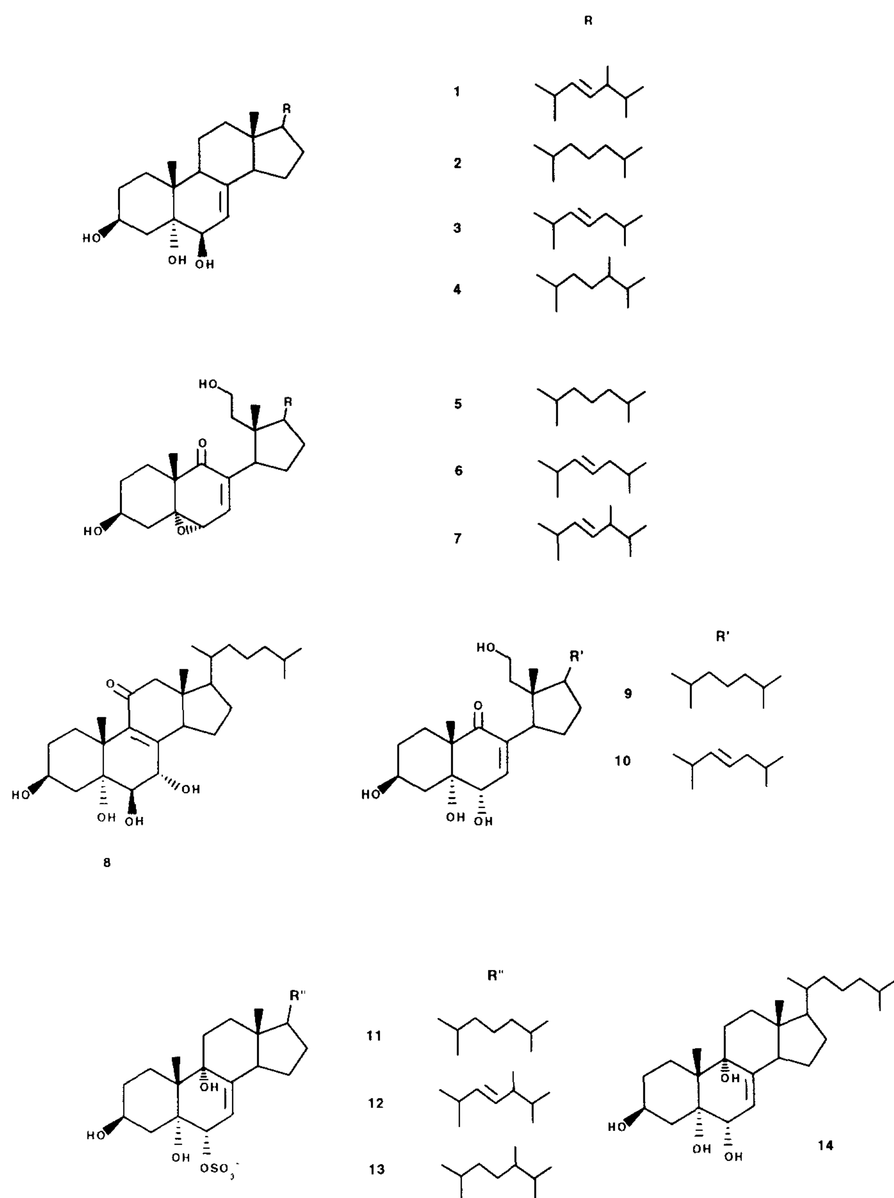

Figure 3 Polyhydroxysterols isolated from the sponge *Dysidea fragilis.*

periment which permitted to assign the C-1–C-2–C-3–C-4 part structure and the large segment C-6–C-7–C-14–C-15–C-16–C-17–C-20–C-21 (H-7 appeared to be correlated to H-14 by a long range coupling). A severe overlapping of the signals prevented the extension of the segment from C-21 to C-27.

On the basis of the above structural information and considering the structural requirements of a sterol framework, the double bond was positioned at C-7/C-8, while the remaining oxygenated functions (three hydroxyl groups and one sulfate group) were linked to the two secondary carbons C-3 and C-6 and to the two tertiary carbons C-5 and C-9. The location of the sulfate group at C-6 was indicated by the upfield shift of the H-6 signal to δ 3.92 in the ^{1}H NMR spectrum of the desulfated derivative **14** (see Table 3). This conclusion was corroborated by comparison of the ^{13}C NMR spectra of **11** and **14**: the resonance of C-6 at 79.8 ppm in **11** was shifted upfield to 71.2 ppm in **14** as expected upon removal of the 6-sulfate group.

The stereochemistry of molecule **11** was based on the following arguments. The seven-line multiplet at δ 3.92 had the normal complexity of the 3α proton of an A/B trans steroid; its low-field chemical shift is typical of 3β-sterols bearing an α-oriented hydroxyl group at C-5.

When the ^{1}H NMR spectrum of **11** was run in pyridine-d_5 a remarkable downfield shift of the H-4_{eq} signal (δ 3.21) was observed in comparison with the spectrum recorded in CD_3OD (δ 2.27). The solvent shifts pointed unequivocally to an α-orientation of the OSO_3^- group at C-6. The α orientation of the OH at C-9 was suggested by the downfield position of the H-1_{ax} signal in the ^{1}H NMR spectrum of **11** (δ 2.25) in CD_3OD, which was further shifted downfield in pyridine solution (δ 2.81), indicating a 1,3-diaxial interaction.

Thus, structure **11** was established as 3β,5α,6α,9α-tetrahydroxy/cholest-7-ene-6-sulfate; it should be noted that the epimer at C-6 of the desulfated product was previously isolated from *Spongia officinalis.*[17]

The FAB-MS (negative ion mode) of compound **12** showed a molecular ion species at m/z 525. Comparison of its spectral data (^1H and ^{13}C NMR) with those of **11** clearly indicated that they differ only in the nature of the side chains. Particularly, ^1H and ^{13}C NMR spectra suggested the presence in **12** of a C_9-unsaturated side chain (Tables 2 and 3). A $^1H-^1H$ COSY experiment in CD_3OD of **12** delineated the connectivities among the vicinal protons in the side chain and permitted positioning of the CH_3 group at C-24 and the double bond at C-22/C-23. The large value (14.5 Hz) of the coupling constant between H-22 and H-23 was indicative of the E configuration of the 22/23 double bond.

Analogously, sterol **13** (FABMS negative ion mode: m/z 527) was found to be clearly related to compounds **11** and **12** on the basis of its ^1H and ^{13}C NMR data. It was identified as a 24-methyl derivative of **11**, as deduced from the presence of a 3H doublet at δ 0.91 in the ^1H NMR spectrum, and a methyl signal at δ 14.8 in the ^{13}C NMR spectrum. It was located at C-24 on the basis of a ^1H ^1H COSY experiment which allowed assignment of the side-chain resonances in the ^1H NMR spectrum (Table 3).

In the HREIMS of compound **9** the highest peak at m/z 432.3230 (calculated 432.3228 for $C_{27}H_{44}O_4$) corresponded to loss of H_2O from the molecular formula $C_{27}H_{46}O_5$, which was determined by considering the ^{13}C NMR spectrum and DEPT measurements.

The IR spectrum showed hydroxyl absorption at 3325 cm^{-1} and a band at ν_{max} 1666 cm^{-1} which suggested an α,β-unsaturated carbonyl functionality confirmed by the UV absorption at λ_{max} 251 nm (ϵ 11,000). The ^1H NMR spectrum in CD_3OD contained signals for an olefinic proton [δ 6.31, bd (J = 2 Hz), H-7], two protons belonging to hydroxy-methine groups [δ 3.91, m, H-3; δ 4.44, bd (J = 2 Hz), H-6] and five methyls [δ 0.76 (s), CH_3-18; δ 1.28 (s), CH_3-19; δ 1.05 (d, J = 6.5 Hz), CH_3-21; δ 0.92 (d, J = 7 Hz), CH_3-26 and CH_3-27] characteristic of a steroid structure. In addition the ^1H NMR spectrum exhibited signals for two mutually coupled methylene protons [δ 3.65 (m, H-11α), 3.77 (m, H-11β), 1.17 (overlapped, H-12α), and 1.56 (overlapped, H-12β)] attributable to the methylene protons at C-11 and C-12 of a 9-hydroxy-9,11-secosteroid.

Information useful in assigning the proposed structure for **9**, was obtained from a detailed analysis of the ^1H, ^{13}C, and ^{13}C DEPT-NMR spectra in combination with ^1H-^1H and ^1H-^{13}C COSY experiments which provided evidence for the presence in the molecule of the segments C-1–C-2–C-3–C-4, and C-6–C-7–C-14–C-15–C-16–C-17–C-20(C-21)–C-22–C-23–C-24–C-25–C-26/C-27 (Tables 3 and 4).

The stereostructure **9** was further confirmed by comparison of our data with those previously reported for the 3,9-diacetyl derivative of **9**, synthesized by Pika et al.[11] from glaciasterol, and for the strictly related sterol 24-nor-9,11-seco-11-acetoxy-3β,6α-dihydroxycholesta-7,22(*E*)-dien-9-one very recently isolated from the soft coral *Gersemia fruticosa*.[18]

In the EIHRMS of compound **10** the highest peak at m/z 430.3075 (calculated 430.3072 for $C_{27}H_{42}O_4$) corresponded to loss of H_2O from the molecular formula $C_{27}H_{44}O_5$, which was calculated by considering the ^{13}C NMR spectrum and DEPT measurements. The ^1H and ^{13}C NMR spectra clearly indicated that **10** had the same nucleus as **9**, the only differences being confined in the side chain. The presence in **10** of a double bond at C-22/C-23 was indicated by its mass spectrum, which showed a significant peak at m/z 301 [M-2H_2O-side chain]$^+$, and by a ^1H-^1H COSY experiment which showed the proton connectivities of the side chain (Tables 2 and 3). The configuration of the Δ^{22} double bond was established to be *E* on the basis of the large value (14.5 Hz) of the H-22/H-23 coupling constant.

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