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New Cytotoxic Sterols from the Soft Coral *Gersemia fruticosa*

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Abstract: Six new polyoxygenated sterols **1-6** were isolated from the soft coral *Gersemia fruticosa*. The structures of these compounds were determined by MS, ^{1}H- and ^{13}C- 1D and 2D FT NMR spectroscopy. Compounds **1-6** showed a moderate cytotoxic activity against human erythroleukemia K-562 cells and other leukemia cell lines. © 1997 Elsevier Science Ltd. All rights reserved.

Although a variety of highly oxygenated unconventional sterols, including a small number of 9,11-secosterols[1] and 5,6-epoxy sterols[2] have been isolated from corals, relatively little work has been reported on the composition of sterols of cold-water corals. In the course of a search for bioactive fatty acid metabolites in the White Sea (the Arctic Ocean) soft coral *Gersemia fruticosa*[3] we found that some fractions from silica gel chromatographic purification of the coral ethyl acetate extract exhibited a considerable antiproliferative activity on tumor cells *in vitro*. Further chromatographic separation of the most active fractions guided by bioassay resulted in the isolation and characterization of a new cytotoxic 9,11-secosterol, 24-nor-9,11-seco-11-acetoxy-3β,6α-dihydroxycholesta-7,22(E)-dien-9-one (7)[4]. Herein we describe the isolation of six new cytotoxic minor sterols **1-6** from *G.fruticosa* and elucidation their structures.

RESULTS AND DISCUSSION

A lyophilized sample of the coral was extracted with EtOAc and the extract was partitionated between n-hexane and methanol/water (1:1). The aqueous phase was extracted with n-hexane and EtOAc. The combined solubles were subjected to silica gel column chromatography followed by repeated normal and reversed-phase HPLC to afford six new sterols **1-6**.

The structures and configurations of substituents of the separated compounds were determined by ^{1}H- and ^{13}C-1D, and 2D FT NMR spectroscopy on a Bruker AMX-500 instrument. ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and proton-

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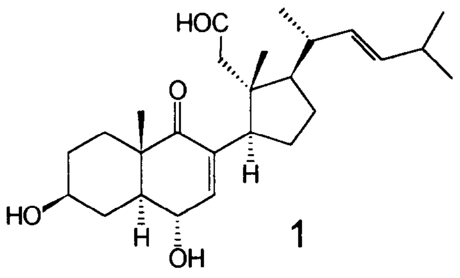
detected long range ^{1}H-^{13}C 2D correlations were measured using standard pulse programs. The proton-detected long-range correlations were especially useful to complete the structures around quaternary carbon atoms and to assign unambiguously these atoms and angular methyl groups connected with them. The compounds were studied in $CDCl_3$ and in most cases also in C_6D_6 solutions. Different solvent effects change overlap patterns in crowded proton spectra and enable in this way an additional assignment of several cross peaks on 2D FT diagrams. The assignment of carbon chemical shifts of **1** to **6** is given in the Table. A comparison of the data obtained with those reported previously on the related compounds provided a valuable additional confirmation on the reliability of present results. Compounds **1** and **2** are closely related to the 11-acetoxy derivative of **1** (compound 7) isolated by us, for which a detailed assignment is given in [4a]. Compound **2** differs from the reported sterol derivative 7 only by having a regular number of carbon atoms in the steroid side chain.



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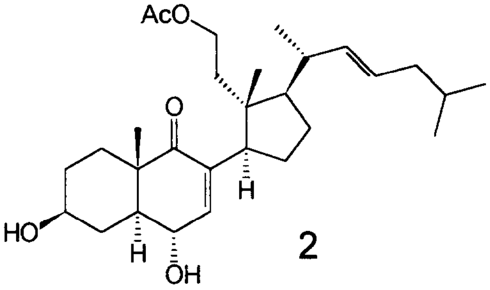

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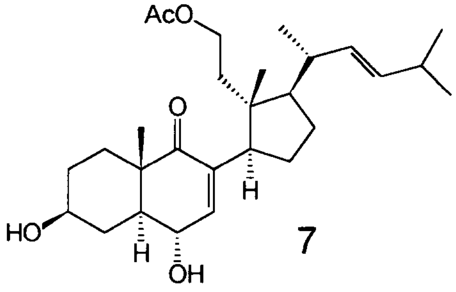

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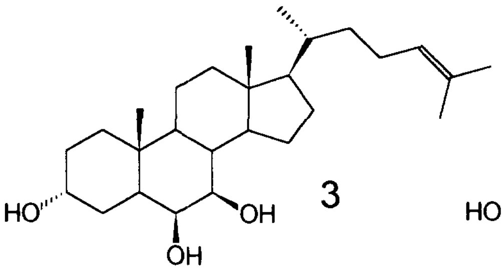
Configurations of the OH groups in **3** were confirmed by ^{1}H-^{1}H coupling patterns of carbinol protons, equatorial H-3 and H-6 having only weak coupling with vicinal protons (H-2 and H-4, and H-5 and H-7, all approx. 2.5 Hz), and a diaxial coupling between H-7 and H-8 (9.1 Hz). The chemical shift values of the side chain in **3** are similar to those of the other reported steroids, such as desmosterol[5].

For **4** the presence of the bridgehead connected oxirane ring was confirmed by observing a single large one bond H-C coupling (174 Hz) for C-6. Orientations of oxirane and 3-OH groups are determined by comparing chemical shifts of the latter with those of isomeric 5β,6β- and 5α,6α-epoxycholesterols[6]. The OH group at C-7 is β-oriented due to a low field shift of C-15 (27.2 ppm) from its 1,3-diaxial interaction with the OH group as compared to the chemical shift of C-15 in cholesterol (24.3 ppm)[7] and a low γ-effect of the OH group(-1.7 ppm) on C-9 of the model 5α,6α-epoxycholesterol[6]. The side chain structure of **4** is the same as in **3**.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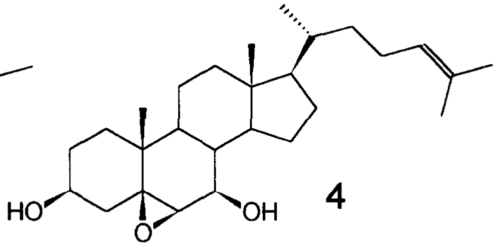
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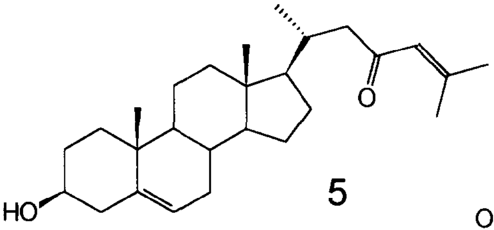
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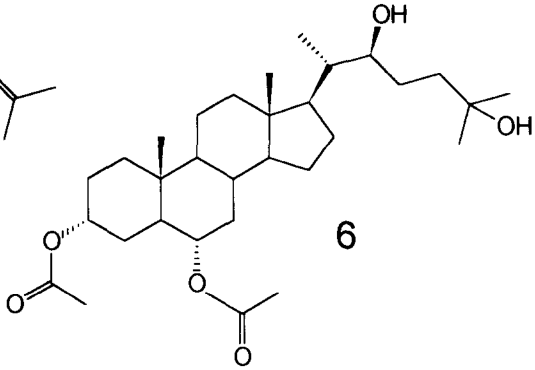
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The structure of compound **5** is quite close to that of cholesterol, the difference being in the side chain structure with end isopropylidene group and the carbonyl group conjugated with it. Correspondingly, the chemical shifts of C-1 to C-19 are practically the same as in cholesterol and carbonyl group influences in its characteristic way the shieldings of unsaturated carbon atoms C-24 and C-25 conjugated with it.

In **6** configurations at C-3 and C-6 are determined simply by the help of model 5α-cholestan-3α-yl and 5α-cholestan-6α-yl acetates[8]. The side chain structure is similar to that observed in ecdysone[9,10]. However, carbon chemical shifts of the side chain in **6** indicate a different mutual configuration of C-20 and C-22. In order to determine an absolute configuration at C-22, R- and S-α-methoxy-α-trifluoromethylphenylacetic (MTPA) acid esters of **6** were prepared and investigated. A comparison of ^{1}H and ^{13}C chemical shifts[11] points to S-configuration at C-22. On the other hand, it is known that the mutual configuration of diastereomeric secondary alcohols with a vicinal methyl group can be determined by a comparison of carbon chemical shifts of diastereoisomers[12]. In the *syn* configuration the methyl carbon and the chiral carbon connected to it are shifted to high fields, whereas other carbon atoms around chiral centers are shifted to low fields. As compared with ecdysone, in the present case C-20 and C-21 are shifted to high fields and C-17 and C-23 to low fields, evidencing of the *syn* configuration of OH and methyl groups. This gives support to the configuration of the methyl group at C-20 as in ecdysone or cholesterol.

Table. ^{13}C Chemical Shifts of Compounds **1** to **6** in $CDCl_3$ solution (δ_C=77.0 ppm)

Carbon	**1**	**2**	**3**	**4**	**5**	**6**
1	31.78	31.80	34.02	36.89	37.23	32.87
2	30.52	30.49	29.04	30.88	31.63	25.63
3	69.98	70.01	66.47	69.03	71.75	69.03
4	32.74	32.79	32.91	41.67	42.27	27.29
5	48.57	48.66	40.09	67.25	140.78	44.04
6	69.39	69.47	74.76	67.53	121.58	72.69
7	147.14	146.05	76.78	74.73	31.84	37.52
8	136.25	136.89	38.14	38.25	31.87	34.09
9	203.83	203.57	52.39	49.70	50.05	53.48
10	44.64	44.72	35.35	34.17	36.47	36.79
11	203.71	61.46	20.47	21.92	21.02	20.67
12	52.48	37.05	39.81	39.52	39.65	39.73
13	46.12	45.96	43.60	43.00	42.44	42.51
14	43.69	42.77	55.37	55.40	56.78	56.09
15	25.64	25.77	27.21	27.23	24.24	23.94
16	26.48	26.90	28.67	28.51	28.42	27.62
17	51.25	50.73	55.14	55.16	56.27	52.60
18	16.83	17.30	12.12	11.74	11.89	11.92
19	15.95	16.08	14.95	16.79	19.37	12.42
20	38.47	38.58	35.47	35.44	33.14	40.77
21	21.69	21.55	18.70	18.67	19.73	11.62
22	131.82	136.43	36.06	36.03	51.50	74.13
23	136.25	127.60	24.70	24.67	201.56	30.20
24	-	42.03	125.11	125.09	124.33	40.77
25	30.99	28.54	130.94	130.97	154.63	70.69
26	22.54	22.35	17.61	17.61	20.64	28.93
27	22.63	22.30	25.70	25.70	27.64	29.94

Sterols **1-6** inhibited the *in vitro* growth of human leukemia K562 cells with IC_{50} values of 16, 21, 21, 14, >60, and 29 μM, respectively. Also, compounds **3**,**4** and **6** tested on the other leukemia cell lines exhibited a similar antiproliferative activity with IC_{50} values of 18, 14 and 29 μM for HL60 cells and 21,18 and 21 μM for P388.D1 cells, respectively. Compound **4** induced an apoptosis of K562 cells with a characteristic internucleosomal DNA degradation (unpublished data).

EXPERIMENTAL

General Experimental Procedures

HREIMS of compounds **3-6** were obtained at on a Hitachi M 80-A mass spectrometer at 70 eV. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer in $CDCl_3$ and C_6D_6 solutions. The ^1H chemical shifts were referenced to the residual $CHCl_3$ (7.27 ppm) and ^{13}C chemical shifts to the solvent ($CDCl_3$, 77.0 ppm). HPLC was performed on a LKB HPLC apparatus equipped with a LKB 2140 rapid spectral and Beckman 156 refractive index detectors.

Biological Material

The coral *G. fruticosa* Sars 1860 (order Alcyonacea, family Nephtheidae) was collected in September 1991 and in August 1993 in the Gulf of Kandalaksha (White Sea) near the Kartesh foreland at a depth of 25-30 m. A voucher specimen is on file at our laboratories.

Extraction and Isolation

The crude EtOAc extract of the freeze-dried coral (980 g) was evaporated in vacuo to give dark orange oil (48.5 g). The oil was partitioned four times between n-hexane and methanol/water (1:1, v/v). The aqueous phase was extracted twice with n-hexane and EtOAc. The hexane and EtOAc solubles were combined and concentrated at reduced pressure to give 8.3 g of residue. The residue was chromatographed over silica gel (200g) with petroleum ether-ethyl acetate in the order of increasing polarity to afford fractions A (EtOAc-petroleum ether 1:2, 1200 ml; 927 mg) and B (EtOAc-petroleum ether 1:1, 900 ml and 3:1, 900 ml; 749 mg). Fraction A was purified over silica gel (50g) with 3.5% i-PrOH/hexane. The active fraction (123 mg) was further purified by HPLC (300 x 20 mm) using the same eluent to give **5** (19.3 mg). The final purification on a RP-HPLC column (250 x 4.8 mm) eluted with CH_3OH/H_2O (87:13) gave 15.8 mg of **5**. The purification of fraction B over silica gel (40 g) eluting with a step gradient of acetone/$CHCl_3$ yielded active fractions B1 (1% of acetone, 205 mg), B2 (3% of acetone, 115 mg), and B3 (5% of acetone, 183 mg). Fraction B1 was repeatedly purified by HPLC on a Silasorb SGX column (300 x 20 mm) using iPrOH/hexane (1:9) as mobile phase, and by reversed-phase HPLC on a Serva Octadecyl-Si100-Polyol column eluted with AcN/H_2O (1:1) to give 20.7 mg of **6**. Further separation of fraction B2 on the same columns using 16% iPrOH/hexane for normal phase HPLC and 50% AcN/H_2O for reversed phase HPLC led to three pure secosterols **1** (1.5 mg), 7 (3.5 mg), and **2** (1.9 mg). Similar HPLC purification of fraction B3 gave 24.3 mg of **3** and 33.6 mg of **4**.

1: Colorless oil; UV λ_{max} (EtOH) 239 nm; ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.47 (m, ax) and 1.86 (m, eq); *H-2* 1.46 (m, ax) and 1.94 (m, eq); *H-3* 3.61 (m, ax); *H-4* 1.46 (m, ax) 2.31 (m, eq); *H-5* 1.78 (m, ax); *H-6* 4.30 (d9.9, m, ax); *H-7* 6.60 (d1.7, m); *H-11* 9.91 (d1.7, d3.7); *H-12* 2.04 (d1.7, d15.7) and 2.25 (d3.7, d15.7); *H-14* 3.56 (t10, m); *H-15* 1.50 (m) and 1.77 (m); *H-16* 1.66 (m); *H-17* 1.90 (m); *H-18* 0.73 (s); *H-19* 1.11 (s); *H-20* 2.15 (m); *H-21* 1.00 (d6.5); *H-22* 5.29 (d 6.3, d 15.4); *H-23* 5.25 (d 8.1, d 15.4, m); *H-25* 2.22 (m); *H-26,27* 0.95 (d 6.8); ^{13}C NMR see Table.

2: Colorless oil; UV λ_{max} (EtOH) 239 nm; ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.48 (m, ax) and 1.89 (m, eq); *H-2* 1.49 (m, ax) and 1.94 (m, eq); *H-3* 3.61 (m, ax); *H-4* 1.45 (m, ax) and 2.31 (dm, eq); *H-5* 1.78 (m, ax); *H-6* 4.27 (d1.9, d 9.8, ax); *H-7* 6.52 (d2.1); *H-11* 4.18 (m) and 4.19 (m); *H-12* 1.27 (m) and 1.63 (m); *H-14* 3.26 (d 8.7, d 11.0); *H-15* 1.49 (m) and 1.72 (m); *H-16* 1.59 (m); *H-17* 1.73 (m); *H-18* 0.69(s); *H-19* 1.13 (s); *H-20* 2.20 (m); *H-21* 1.06 (d 6.8); *H-22* 5.31 (m); *H-23* 5.32 (m); *H-24* 1.85 (m); H-25 1.59 (m); *H-26* 0.871 (d 6.6); *H-27* 0.873 (d 6.6); *Ac* at 2.02 (s); ^{13}C NMR see Table.

3: White amorphous powder; $[\alpha]_D = +47.4°$ (*c* 2.1, MeOH); HREIMS: Obsd m/z 418.3439, $C_{27}H_{46}O_3$ requires m/z 418.3447; ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.28 (m, ax) and 1.41 (m, eq); *H-2* 1.74 (m, ax) and 1.59 (m, eq); *H-3* 4.17 (p2.5, eq); *H-4* 1.44 (m, eq) and 2.08 (d 2.3 t 13.5, ax); *H-5* 1.63 (m, ax); *H-6* 3.62 (t 2.5, eq); *H-7* 3.32 (d 9.0, m, ax); *H-8* 1.64 (m); *H-9* 0.81 (d 2.5, t 9.0); *H-11* 1.35 (m, ax) and 1.57 (m, eq); *H-12* 1.12 (m, ax) and 2.01 (m, eq); *H-14* 1.21 (m); *H-15* 1.43 (m) and 1.86 (m); *H-16* 1.30 (m) and 1.88 (m); *H-17* 1.08 (m); *H-18* 0.70(s); *H-19* 0.98 (s); *H-20* 1.39 (m); *H-21* 0.94 (d 6.8); *H-22* 1.04 (m) and 1.41 (m); *H-23* 1.88 (m) and 1.99 (m); *H-24* 5.09 (t 6.5, m); *H-26* 1.60 (s); *H-27* 1.68 (d 0.8); ^{13}C NMR see Table.

4: White plates; m.p. 122-123°C; $[\alpha]_D = +49.5°$ (*c* 2.87, MeOH); HREIMS: Obsd m/z 401.3059; Calcd for $C_{26}H_{41}O_3$ m/z 401.3056 (M^+-CH_3); ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.21 (m, ax) and 1.89 (m, eq); *H-2* 1.42 (m, ax) and 1.89 (m, eq); *H-3* 3.72 (m, ax); *H-4* 1.48 (d 13.2 t 4.0, eq) and 2.04 (t 13.2, m, ax); *H-6* 3.14 (d 1.2); *H-7* 3.51 (m); *H-8* 1.43 (m); *H-9* 0.72 (m); *H-11* 1.23 (m, ax) and 1.29 (m, eq); *H-12* 0.91 (m, ax) and 1.87 (m, eq); *H-14* 1.04 (m); *H-15* 1.36 (m) and 1.92 (m); *H-16* 1.27 (m) and 1.85 (m); *H-17* 1.03 (m); *H-18* 0.65 (s); *H-19* 1.01 (s); *H-20* 1.37 (m); *H-21* 0.92 (d 6.8); *H-22* 1.03 (m) and 1.40 (m); *H-23* 1.84 (m) and 2.02 (m); *H-24* 5.08 (t 7.0, m); *H-26* 1.60 (s); *H-27* 1.68 (d 0.6); ^{13}C NMR see Table.

5: White plates; m.p. 103-104°C; UV λ_{max} (EtOH) 237 nm; $[\alpha]_D$ = +12.8° (*c* 1.92, MeOH); HREIMS: Obsd m/z 398.3192. Calcd for $C_{27}H_{42}O_2$ m/z 398.3185; ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.07 (m, ax) and 1.84 (m, eq); *H-2* 1.50 (m, ax) and 1.84 (m, eq); *H-3* 3.53 (m, ax); *H-4* 2.25 (d 11.0, d 13.0, m , ax) and 2.30 (d 1.7, d 4.8, d 13.0, eq); *H-6* 5.35 (t 1.7, d 5.4); *H-7* 1.47 (m, ax) and 1.94 (m, eq); *H-8* 1.46 (m); *H-9* 0.93 (m); *H-11* 1.43 (m) and 1.50 (m); *H-12* 1.17 (m, ax) and 2.00 (m, eq); *H-14* 1.01 (m); *H-15* 1.09 (m) and 1.59 (m); *H-16* 1.29 (m) and 1.80 (m); *H-17* 1.13 (m); *H-18* 0.73 (s); *H-19* 1.01 (s); *H-20* 2.00 (m); *H-21* 0.93 (d 6.3); *H-22* 2.09 (m) and 2.49 (d 2.8, d 15.0); *H-24* 6.06 (m); *H-26* 2.14 (d 1.2); *H-27* 1.88 (s); ^{13}C NMR see Table.

6: Colorless oil; $[\alpha]_D$ = +37.3° (*c* 0.67, MeOH); HREIMS: Obsd m/z 502.3655 (M^+-H_2O); Calcd for $C_{31}H_{50}O_5$ m/z 502.3658; ^{1}H NMR (500.17 MHz, $CDCl_3$), δ *H-1* 1.29 (m, ax) and 1.50 (m, eq); *H-2* 1.60 (m) and 1.75 (m); *H-3* 5.05 (p 2.5, eq); *H-4* 1.37 (m) and 1.75 (m); *H-5* 1.60 (m); *H-6* 4.64 (d 4.5, t 11.2, ax); *H-7* 0.95 (m, ax) and 1.98 (m, eq); *H-8* 1.52 (m); *H-9* 0.80 (d 3.9, t 11.0); *H-11* 1.25 (m) and 1.53 (m); *H-12* 1.18 (m, ax) and 1.98 (m, eq); *H-14* 1.15 (m); *H-15* 1.09 (m) and 1.57 (m); *H-16* 1.30 (m) and 1.90 (m); *H-17* 1.45 (m); *H-18* 0.66 (s); *H-19* 0.85 (s); *H-20* 1.41 (m); *H-21* 0.91 (d 6.4); *H-22* 3.63 (d 1.2, d 4.2, d 8.6); *H-23* 1.40 (m) and 1.64 (m); *H-24* 1.41 (m); *H-26* 1.22 (s); *H-27* 1.23 (s); *C-3 Ac* 2.06 (s); *C-6 Ac* 2.02 (s); ^{13}C NMR see Table.

Biological Assays

The fractions obtained from chromatographic purification were dissolved in ethanol at a concentration of 10 mg/ml for *in vitro* cytotoxicity screening. Assays based on a viable cell count were performed on K-562 cells, routinely at concentrations of 10 and 20 µg/ml using a 48 h drug exposure protocol.

The cell growth inhibition of pure individual compounds were performed as described in 4(b).

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