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IMMUNOSUPPRESSIVE COMPOUNDS FROM A DEEP WATER MARINE SPONGE, *AGELAS FLABELLIFORMIS*

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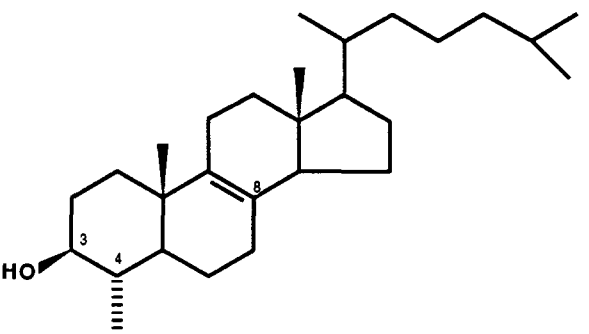
ABSTRACT.—Two immunosuppressive compounds, 4α-methyl-5α-cholest-8-en-3β-ol [**1**] and 4,5-dibromo-2-pyrrolic acid [**2**] were isolated from a deep water marine sponge, *Agelas flabelliformis*. Their structures were determined by comparison of their spectral data with those of samples isolated from other organisms. Both compounds were highly active in suppression of the response of murine splenocytes in the two-way mixed lymphocyte reaction (MLR) with little to no demonstrable cytotoxicity at lower doses. In addition, 4,5-dibromo-2-pyrrolic acid suppressed the proliferative response of splenocytes to suboptimal concentrations of the mitogen, concanavalin A (Con A). These results describe for the first time compounds isolated from the marine sponge *A. flabelliformis* that possess potent in vitro immunosuppressive activity.

Sterols (1) and pyrrole metabolites (2) are common metabolites in marine sponges. Cholesterol, the most common sterol in nature, is the main component of the marine organism's sterol mixture. 4α-Methylsterols are not common among sponges but many 4α-methylsterols have been isolated from dinoflagellates (3). 4α-Methyl-5α-cholest-8-en-3β-ol [**1**] is not a common metabolite, but it has been previously isolated from a unicellular marine red alga, *Porphyridium cruentum* (4), and animal (5) and bacterial sources (6). The genus *Agelas* is known to contain many brominated pyrrole metabolites. Their structures vary from simple pyrroles to the complicated metabolite sceptrin (7). 4,5-Dibromo-2-pyrrolic acid [**2**] has been previously isolated from *Agelas oroides* (8). No immunosuppressive activities have been reported previously from either **1** or **2** or related compounds.

RESULTS AND DISCUSSION

The sterol **1** was isolated from *Agelas flabelliformis* Carter (Agelasidae) collected in the Bahamas. Repeated Si gel chromatography of the immunosuppressive EtOAc-soluble fraction yielded a crystalline solid: mp 136°, $[\alpha]^{26}D$ 44.8° ($c = 0.14$, $CHCl_3$). The formula $C_{28}H_{48}O$ was established by hreims *m/z* 400.3714 (Δ 0.9 mmu for $C_{28}H_{48}O$). Ir data indicated the presence of a hydroxyl group (3610 cm^{-1}), and the typical 3β-hydroxyl group was indicated by the methine signal at δ 3.07. Appearance of the methine signal as a double triplet with coupling constants of 10.0, 10.0, and 4.2 Hz indicated that the methine proton is axial and that it is coupled to three adjacent hydrogens as in other 4α-methylsterols. The two axial-axial couplings of $J = 10.0$ and 10.0 Hz con-



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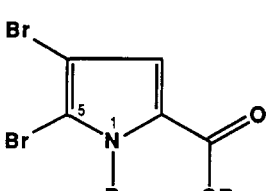

2 R=H
3 R=Me

firmed the α-equatorial nature of the 4-methyl group. The ^{13}C-nmr spectrum revealed only two singlets in the olefinic region (δ 128.16, 135.06), indicating the presence of a tetrasubstituted double bond. Three-bond proton-carbon coupling experiment (XHCORR with suitable delay values) (9) showed coupling between the methyl doublet protons at δ 0.97 and the carbon signal at δ 76.54 assigned to C-3. Similarly there was coupling between the methyl singlet protons at δ 0.95 assigned to the methyl attached to C-10 and the carbon signal at δ 135.06. These data confirmed the presence of a 4-methyl group and an 8,9 double bond in the molecule. The mass spectrum showed a characteristic peak at *m/z* 287 for the loss of the side chain expected for a monounsaturated 4-methylsterol (10). The structure of **1** was confirmed by comparing its spectral data with the available data reported for the same compound isolated from a unicellular marine alga (4).

Repeated chromatography of the same immunosuppressive EtOAc-soluble fraction on Si gel with polar solvent combinations yielded a white solid mp (sublimes) 148°. The formula $C_5H_3Br_2NO_2$ was established by eims. The structure was identified by comparison of its physical and spectral properties with 4,5-dibromo-2-pyrrolic acid [**2**] isolated from the sponge *A. oroides* (8) and also by converting **2** to 4,5-dibromo-1-methylpyrrole-2-carboxylic acid methyl ester [**3**] (11).

4,5-Dibromo-2-pyrrolic acid [**2**] suppressed the two-way murine mixed lymphocyte reaction in a dose responsive manner (Table 1). Suppression was accompanied by high levels of toxicity in the range of 31.3 μg/ml to 500 μg/ml. Complete suppression (100%) was still evident at a concentration of 15.6 μg/ml, with a corresponding borderline toxicity (66%). Further dilutions resulted in significant levels of decreasing

TABLE 1. Immunosuppressive Effect of 4,5-Dibromo-2-pyrrolic Acid on the Two-Way Murine Mixed Lymphocyte Response.

Concentration (μg/ml)	Cpm[a]	% Suppression[b]	% Viability (LCV)
500	1766 ± 1818	100	10
250	285 ± 56	100	0
125	235 ± 49	100	12
62.5	1035 ± 992	100	27
31.3	278 ± 122	100	27
15.6	3154 ± 1296	100	66
7.8	150977 ± 17634	40	99
3.9	111230 ± 23461	59	80
2.0	168414 ± 27544	31	99
1.0	206190 ± 5703	13	95
0.5	235823 ± 35464	0	113

[a]Counts per minute ± standard error of the mean. Mean of triplicate determinations.

[b]$\%\ \text{Suppression} = 100 - \frac{(\text{cpm of test}) - [\text{cpm of cells alone (24468)}]}{[\text{cpm of Positive MLR (234586)}] - [\text{cpm of cells alone (24468)}]} \times 100.$

suppression with no associated toxicity (% viability > 70%). This compound also suppressed the mitogenic response of murine splenocytes to submitogenic concentrations of concanvalin A (Con A) (LCON, Table 2) with kinetics similar to those described

TABLE 2. Immunosuppressive Effect of 4,5-Dibromo-2-pyrrolic Acid on the Mitogenic Response of Murine Splenocytes to Concanavalin A (LCON).

Concentration (μg/ml)	Cpm[a]	% Suppression[b]	% Viability (LCV)
500	512 ± 33	83	10
250	256 ± 24	99	0
125	339 ± 138	83	12
62.5	282 ± 65	83	27
31.3	312 ± 117	83	27
15.6	5371 ± 472	84	66
7.8	9620 ± 4000	64	99
3.9	8678 ± 1000	70	80
2.0	20954 ± 5421	29	99
1.0	16642 ± 2936	43	95
0.5	17927 ± 4028	39	113
0.3	14644 ± 2376	50	100

[a]Counts per minute ± standard error of the mean. Mean of triplicate determinations.

[b]$\%\ \text{Suppression} = 100 - \frac{(\text{cpm of test}) - [\text{cpm of cells alone (171)}]}{[\text{cpm of Positive Control (240654)}] - [\text{cpm of cells alone (171)}]} \times 100.$

above. 4α-Methyl-5α-cholest-8-en-3β-ol [**1**] was strongly suppressive (100% suppression) with no associated toxicity when tested in a concentration range of 2.0 to 62.5 μg/ml (Table 3). Concentrations greater than 62.5 μg/ml were toxic to splenocytes. Suppression was still evident at 1.0 (81%) and 0.5 (46%) μg/ml, decreasing to 0% at 0.25 μg/ml. The compound was not tested in the LCON assay.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were taken on a Perkin-Elmer 1310 spectrophotometer. ^{1}H-nmr spectra were recorded on a Bruker 360 MHz instrument. ^{13}C-nmr spectra were

TABLE 3. Immunosuppressive Effect of 4α-Methyl-5α-cholest-8-en-3β-ol on the Two-Way Murine Mixed Lymphocyte Response.

Concentration (μg/ml)	Cpm[a]	% Suppression[b]	% Viability (LCV)
500	261 ± 50	100	6
250	299 ± 43	100	10
125	775 ± 374	100	28
62.5	2974 ± 58	100	72
31.3	3750 ± 683	100	85
15.6	5056 ± 643	100	92
7.8	6042 ± 221	100	80
3.9	12319 ± 4580	100	99
2.0	20454 ± 3987	100	77
1.0	57926 ± 8914	81	97
0.5	122864 ± 3431	46	118
0.25	239102 ± 21253	0	104

[a]Counts per minute ± standard error of the mean. Mean of triplicate determinations.

[b]$\%\ \text{Suppression} = 100 - \frac{(\text{cpm of test}) - [\text{cpm of cells alone (21723)}]}{[\text{cpm of Positive MLR (210726)}] - [\text{cpm of cells alone (21723)}]} \times 100.$

obtained on the same instrument operating at 90.5 MHz. The low resolution eims were performed by using a VG 70 SE mass spectrometer operating at 70 eV. Optical rotations were measured with a Jasco DIP 360 digital polarimeter.

EXTRACTION AND ISOLATION.—The marine sponge *A. flabelliformis* was collected in the Bahamas at a depth of 155 m using an untethered deep sea submersible. A voucher specimen is deposited in the Indian River Coastal Museum at Harbor Branch Oceanographic Institution. The freshly thawed sponge (749 g, wet wt) was extracted repeatedly with MeOH-toluene (3:1). The concentrated extract was then partitioned between EtOAc and H_2O. The immunosuppressive EtOAc-soluble fraction (2.0 g) was chromatographed over Si gel [Kieselgel 60] using a $CHCl_3$-MeOH step gradient (0→20% MeOH) and monitored by immunomodulatory assay. The active fraction (600 mg) was rechromatographed over Si gel [Kieselgel 60H] with hexane-CH_2Cl_2 (1:1) followed by increasing amounts of CH_2Cl_2 to give 10 fractions. Fraction 6, which eluted with 75% CH_2Cl_2/hexane, furnished pure steroid **1**.

4α-Methyl-5α-cholesta-8-en-3β-ol.—Steroid **1** (14 mg): mp 136° [lit. (5) 136–137°]; $[\alpha]^{26}D = 44.8°$ ($c = 0.14$, $CHCl_3$); ir ($CHCl_3$) 3610, 2920, 2863, 1460, 1375, 1330, 1035, 1022, 1005 cm^{-1}; ^1H nmr (360 MHz in $CDCl_3$) δ 0.58 (3H, s, H-18), 0.841, 0.846 (3H each, d, $J = 6.9$ Hz, H-26, -27), 0.902 (3H, d, $J = 6.5$ Hz, H-21), 0.952 (3H, s, H-19), 0.971 (3H, d, $J = 6.3$ Hz, H-28), 3.082 (1H, ddd, $J =$ 10.0, 10.0, 4.2 Hz, H-3α); ^{13}C-nmr (90 MHz in $CDCl_3$) δ 11.25 (q, C-18), 15.05 (q, C-28), 18.75 (q, C-21), 18.88 (q, C-19), 20.90 (t), 22.55, 22.77 (2q, C-26, -27), 22.77 (t), 23.74 (t), 23.96 (t), 27.49 (t), 28.01 (d, C-25), 28.80 (t), 31.24 (t), 35.12 (t), 36.19 (t), 36.29 (s, C-10), 36.29 (d, C-20), 37.06 (t), 39.25 (d, C-5), 39.53 (t, C-24), 46.94 (d, C-4), 51.88 (d, C-14), 54.96 (d, C-17), 76.54 (d, C-3), 128.16 (s, C-8), 135.06 (s, C-9); hreims *m/z* 400.3714, Δ 0.9 mmu for $C_{28}H_{48}O$; eims *m/z* (rel. int.) 400 (100%), 385 (15), 367 (9), 287 (13), 269 (8), 261 (6), 245 (12), 243 (12), 226 (14), 199 (2), 187 (5), 173 (8), 161 (15), 159 (10), 149 (8), 148 (4), 147 (18), 134 (15), 133 (13), 121 (18), 119 (14), 109 (13), 107 (17), 105 (18), 95 (21), 47 (16).

Another portion of the EtOAc-soluble fraction (3 g) was chromatographed on Si gel with a CH_2Cl_2/MeOH step gradient, and the biologically active fractions were monitored by immunomodulatory assay. Rechromatography of the active fraction (460 mg) that eluted with a mixture of 20% MeOH in CH_2Cl_2 on RP C-18 with 50% H_2O/MeOH yielded the immunosuppressive compound 4,5-dibromo-2-pyrrolic acid [**2**].

4,5-Dibromo-2-pyrrolic acid.—Compound **2** (18 mg): mp sublimes 148° [lit. (8) mp sublimes 148°]. Methylation of the compound **2** (10 mg) with excess of CH_2N_2 in Et_2O afforded the dimethylated compound **3** (10 mg): mp 90° [lit. (11) mp 87–90°].

IN VITRO TESTING.—The immunomodulatory properties of the two compounds were determined using murine splenocytes in the two-way mixed lymphocyte reaction (MLR) and the mitogenic response to Con A (LCON). Whole splenocyte suspensions were prepared from C57BL/6J and BALB/c mice as follows: Spleens were aseptically removed and disassociated in cold 4° RPMI 1640 tissue culture medium (TCM) (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 60 μg/ml l-glutamine, and 5×10^{-5} M 2-mercaptoethanol). The resulting cell suspensions were incubated at 0° for 5 min to allow large debris to settle. The cells remaining in the supernatant were removed and washed once with TCM. Viable cell counts were determined by trypan blue exclusion, and each cell suspension was adjusted to a concentration of 2×10^6 cells/ml. For all assays, serial twofold dilutions of the compounds were prepared in absolute EtOH, and a volume of 0.010 ml was added to empty wells of microtiter plates and allowed to evaporate to dryness. Wells containing EtOH alone and similarly evaporated served as controls. For the MLR, a volume of 0.10 ml of each cell suspension was added together to test and control wells of microtiter plates. Wells containing 0.20 ml of each cell suspension alone served as controls. Plates were incubated at 37° in a 5% CO_2/95% air atmosphere for 88 h. Following the incubation, 1.0 μCi of ^3H-thymidine was added to each well, and the plates were returned to the incubator for 5 h. The contents of each well were harvested onto glass fiber filter strips, and the incorporation of ^3H-thymidine was determined using a liquid scintillation counter. Toxicity of the compounds for mouse splenocytes was similarly determined using a colorimetric assay, based on the ability of viable cells to cleave the colorless tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (M.T.T.), to a colored formazan product (12). Briefly, a volume of 0.075 ml of an M.T.T. solution (550 μg/ml of M.T.T. in TCM) was added to test and control wells containing one of the populations of splenocytes and incubated for 5 h after the initial 88-h incubation period. Microtiter plates were centrifuged at $100 \times g$, supernatants were carefully removed, and a volume of 0.20 ml of iPrOH was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was determined for each well using a plate reader.

Toxicity was indicated for those cultures with dilutions of compounds which resulted in less than 70% of the control (no compound) response. For the LCON assay, 0.10 ml of either splenocyte suspension was added to triplicate wells of microtiter plates. Con A was diluted to a concentration of 0.6 μg/ml, and

volume of 0.10 ml was added to each well. Plates were incubated at 37° for 72 h as described above. Following the incubation period, 1.0 μCi of ^{3}H-thymidine was added to each well, and the plates were returned to the incubator for 5 h. The plates were harvested and processed as described above. Results for the MLR and LCON assays (see Discussion) were expressed as absolute counts per min and percent of suppression. The viability of cultures with dilution of test compounds was expressed as a percentage of the positive control (no compound).

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