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HIV-Inhibitory Natural Products. 11. Comparative Studies of Sulfated Sterols from Marine Invertebrates⊥,[1]

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A total of 22 sulfated sterols isolated from marine sponges, ophiuroids (brittle stars), and asteroids (sea stars) were comparatively evaluated for their antiviral activity against HIV-1 and HIV-2. In general, sterols with sulfate groups at position 2, 3, or 6 were the most active, with EC_{50} values of 3–13 μM against HIV-1 (RF) and 2–8 μM against HIV-2 (CBL20). Those compounds which were sulfated on the sterol D ring were completely inactive against both HIV-1 and HIV-2. Overall, sulfated sterols active against HIV-1 were also active against HIV-2.

The National Cancer Institute is actively acquiring and screening extracts from diverse plant, marine, and microbial sources for anti-HIV activity.[2] Initial anti-HIV screening[2,3] of the aqueous extracts from marine organisms[3] revealed an unexpectedly large number of active extracts (e.g., ~15% of the organisms tested).[4] A chemical screening protocol has been developed to facilitate the preliminary dereplication and prioritization of these extracts for subsequent study.[5] We have recently described the isolation of ibisterol sulfate and halistanol sulfate from extracts of several marine sponges which were selected for investigation on the basis of their characteristic activity elution pattern in the chemical screening protocol.[5,6]

Sulfated sterols have been described from a wide variety of marine organisms, particularly sponges and echinoderms.[7] Several of these sterols reportedly have a broad spectrum of biological activities. For example, hymenosulfate, from the microalga *Hymenomonas* sp., potently induced Ca^{2+} release from the sarcoplasmic reticulum.[8] Halistanol sulfate from *Halichondria* cf. *moorei* was antimicrobial, hemolytic, and ichthyotoxic;[9] sulfated sterols from *Toxadocia zuma* were antimicrobial, cytotoxic, and antifouling.[10] More recently, sulfated sterols with antiviral activity have also been reported. Examples include the weinbersterol disulfates from the sponge *Petrosia weinbergi*,[11] which were active against both feline leukemia virus and HIV-1, as well as ibisterol sulfate and halistanol sulfate, which were active against HIV-1.[6]

As part of our continuing exploration of the anti-HIV activity of sulfated sterols, we have comparatively evaluated 22 sulfated sterols from marine sponges, ophiuroids (brittle stars, Echinodermata, class Ophiuroidea), and asteroids (sea stars, Echinodermata, class Asteroidea) for activity against HIV-1 and HIV-2. The results revealed a distinct structure–activity relationship among these compounds.

Table 1. Cytoprotective Effects of Sulfated Sterols against HIV-1 and HIV-2, As Measured by XTT

	HIV-1		HIV-2	
no.	EC_{50} (μM)[b]	IC_{50}[a] (μM)[b]	EC_{50} (μM)[b]	IC_{50} (μM)[b]
1	13	>128	8[c]	51
2	6	41	3	41
3	3	17	7	94
4	6	56	2[c]	56
5	19	>161	128[c]	>161
6	48	>160		
7	18	>150	3	>150
8	13	>149		
9	NP[d]	>125	13[c]	>125
10	NP	>126	NP	>126
11	NP	>123	NP	69
12	NP	>174	NP	>174
13	NP	>164	NP	>164
14	NP	64	NP	64
15	97	>161	~161	>161
16	86	>157	39[c]	>157
17	122	>152	107[c]	>152
18	164	>312	63	>156
19	97	>161	NP	>161
20	241	>322	80	>161
21	157	>313	110	>157
22	NP	<0.02		

[a] Upper limit for anti-HIV-1 testing was 100 μg/mL. [b] Assumes sterols present as sodium salts (see diagrams for structural details). [c] Does not reach 100% protection. [d] NP is no protection of cells to HIV-induced killing.

Results and Discussion

Sponge Sterol Activity against HIV-1. Initial anti-HIV screening of four sponge sterols, ibisterol sulfate (**1**), halistanol sulfate (**2**), 26-methylhalistanol sulfate (**3**), and 25-demethylhalistanol sulfate (**4**), originally isolated from *Topsentia* sp. (**1**),[6] *Halichondria* cf. *moorei* (**2**),[9] and *Pseudaxinyssa digitata* (**3**, **4**),[12] respectively, showed essentially complete protection against the cytopathic effects of HIV-1 infection in the NCI primary screen[2,3] (EC_{50} values of 13, 6, 3, and 6 μM, respectively; see Table 1). All four of these sterols share a common 2α,3β,6α-trisulfate substitution pattern. For a more definitive comparison of the anti-HIV-1 activity of these compounds, a battery of interrelated assays was performed in individual wells of 96-well microtiter plates.[13]

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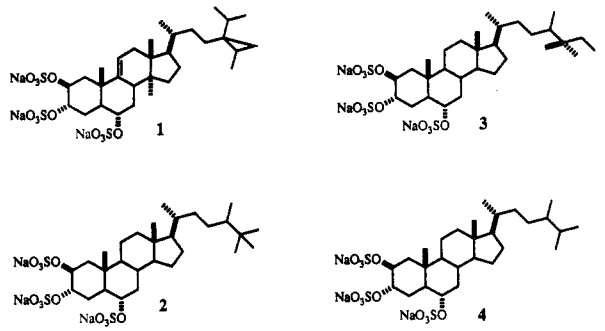


Cellular viability, in the presence and absence of 1–4, was estimated in uninfected and HIV-1 infected cells, both by an adaptation[13] of the published XTT-tetrazolium method,[3] and by means of the fluorescent probe 2′,7′-bis-(carboxyethyl)-5-carboxyfluorescein 6-acetoxymethyl ester (BCECF),[14] a nonfluorescent molecule which enters viable cells where it is hydrolyzed by cellular esterases to a fluorescent compound. Total cellular DNA content was measured with the dye, 4′,6′-diamidino-2-phenylindole (DAPI),[15] which fluoresces when intercalated at A-T specific sites in chromatin. Virus replication indices, including supernatant viral reverse transcriptase (RT), viral p24 antigen (p24), and syncytium-forming units (SFU), were assayed as described.[13]

As exemplified in Figure 1, halistanol sulfate (2) prevented the cytopathic effects of HIV-1 in CEM-SS human lymphoblastoid target cells (EC_{50} 1.1–5.4 μM) but was directly cytotoxic to the target cells at 10-fold higher concentrations (IC_{50} 54–61 μM). Compound 3 also inhibited the viral replicative indices RT, p24, and SFU, giving IC_{50} values of 12.2, 12.2, and 24.4 μM, respectively. Similar results (data not shown) as those depicted for 2 were obtained for compounds 1, 3, and 4, which were consistent with earlier results from comparative testing in the primary screen.

Results of Asteroid Sterols against HIV-1. Ten sulfated sterols were isolated from a diverse collection of sea stars: *Tremaster novaecaledonia*[16,17] (5–9), *Asterias amurensis*[18] (10–11), *Styracaster caroli*[19] (12–13), and *Echinaster brasiliensis*[20] (14). These sterols fall into two broad structural classes: (1) sterols with sulfates present only on the A and B rings and with rather simple side chains (5–8) and (2) those which have oxygen or sulfate substitution on the sterol D ring (9–14). These latter sterols' side chains vary from simple lanosterol types, which may be substituted at C-24 (9–11), to complex branched side chains which have polar OH or sulfate groups (12–14). All of these sterols were tested against HIV-1 at an upper concentration limit of 100 μg/mL (123–164 μM).

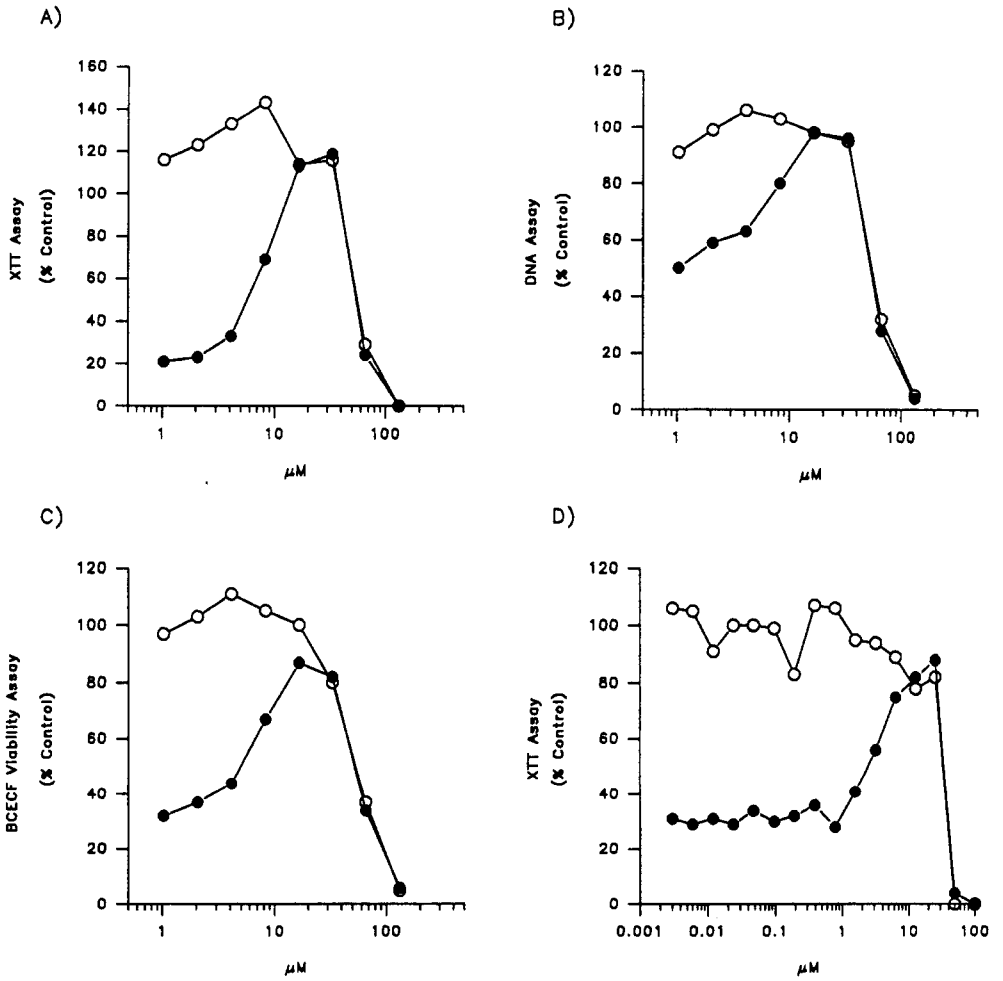
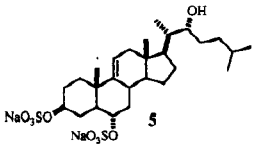
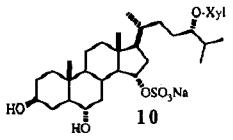
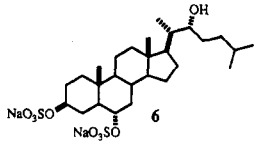
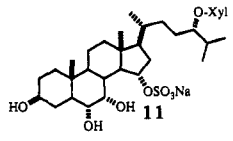


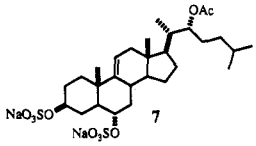
Figure 1. Graphs A–C show the effects of a range of concentrations of sterol 2 (halistanol sulfate) upon uninfected CEM-SS cells (○) and upon HIV-1 (RF) infected CEM-SS cells (●), as determined after 6 days in culture. Graph A depicts the relative numbers of viable CEM-SS cells as assessed by the XTT assay; graph B depicts the relative DNA content of the respective culture; graph C depicts the relative numbers of viable CEM-SS cells as assessed by the BCECF assay. Graph D shows the effects of a range of concentrations of 2 upon uninfected CEM-SS cells (○) and upon HIV-2 (CBL20) infected cells (●), as assessed by the XTT assay after 6 days in culture. In all graphs, data points are represented as the percent of the respective uninfected, nondrug treated control values; each point was derived from the mean of at least duplicate determinations; standard errors averaged ≤10% of the respective means.

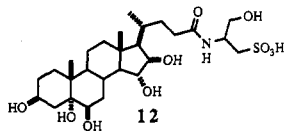


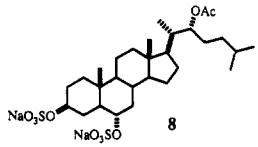


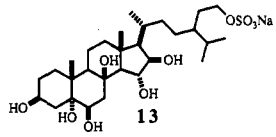


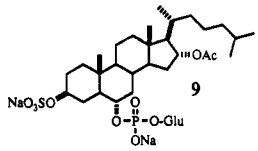


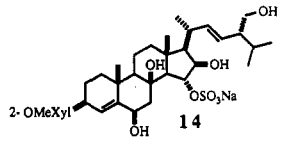


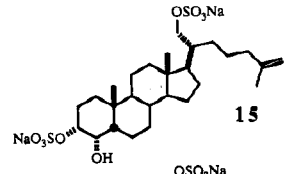


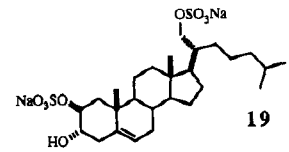


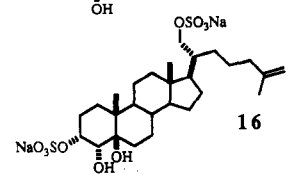


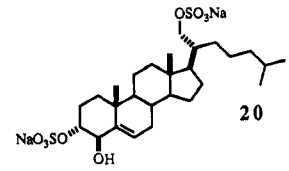


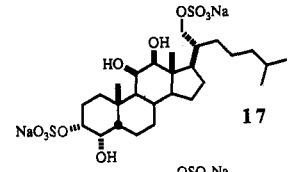


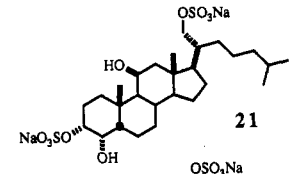


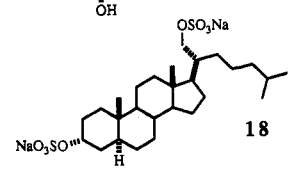


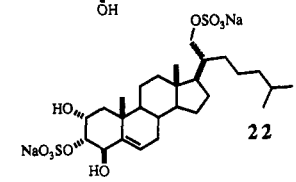












As with the sponge sterols 1–4, those sterols with sulfates exclusively on the A and B rings (5–8) inhibited the cytopathicity of HIV-1 in the primary screen (EC_{50} 13–48 μM). The more detailed multiparameter analysis of the *in vitro* activity of these compounds indicated that they had very similar activity profiles to those of the sponge sterols (data not shown). For example, sterol 5 could prevent the cytopathic effects of HIV-1 (EC_{50} 30.5–41.8 μM) and abort HIV-1 replication (IC_{50} 80, 101, and 90 μM, respectively, for RT, p24, and SFU in CEM-SS target cells). Results similar to those obtained with 5 were also observed for 6–8 (Table 1).

The second group of asteroid sterols (9–14) were all inactive against HIV at the highest tested concentrations (100 μg/mL). The unifying structural feature of these sterols is the presence of oxygen substituents on the sterol D ring.

Results of Ophiuroid Sterols against HIV-1. The final group of sulfated sterols (15–22) tested against HIV-1 were isolated from the ophiuroids (brittle stars) *Ophilepis superba*[21] (15–16), *Ophioderma longicaudum*[22] (17–18), *Ophiotrix fragilis*[23] (20), *Ophiosphate gigaes*[24] (19, 22), and *Ophiocoma dentata*, *Ophiartrum elegans*, and *Ophiaracna incrassata*[25] (21). These compounds are all sulfated at position 21 on the sterol side chain and, with the exception of 22, inhibited the cytopathic effects of HIV-1 infection in CEM-SS cells with EC_{50} values from 86–241 μM (Table 1).

Several of these sterols were also studied in the multiparameter assays (data not shown), further confirming the inhibitory effects of the sterols on the cytopathic effects and replication of HIV-1. As with the other active compounds, the sterol concentrations required to inhibit viral replication were somewhat greater (e.g., 2-fold) than those required to produce a comparable level of cytoprotection.

Discussion of HIV-1 Results. From these data it was apparent that sterols sulfated exclusively on the A and B rings were the most effective in preventing HIV-1-induced cytopathicity. This activity was not significantly altered by the presence of a Δ9(11) olefin (1, 5, 7) or by additional sites of oxygenation located on the sterol side chain, so long as these were not sulfated (5–8). Sterols which were sulfated at C-21 on the side chain (15–22) or those with a *cis* A/B ring juncture (15–17 and 21) retained modest activity, but their EC_{50} values were nearly 1 order of magnitude higher. HIV-1-inhibitory activity was completely eliminated by oxygenation of the D ring (10–14). Results with the viral replication indices showed similar overall SAR patterns; however, these data also indicated that inhibition of viral replication required somewhat higher sterol concentrations than required for cytoprotection yet were still below the cytotoxic concentrations.

Results of HIV-2 (CB120) Studies. Studies were undertaken using the CBL20 strain of HIV-2 in CEM cells.[26] Those sterols which were protective against HIV-2 (1–5, 7, 9, 16, 17, 20; see Table 1) generally showed up to 70 or 80% protection, at which point there was also observable toxicity of the sterol toward the uninfected control CEM-SS cells (e.g., see Figure 1D). In general, those sterols active against HIV-1 were also active against the CBL20 strain of HIV-2. As was the case with HIV-1, sterols with sulfate groups on the A and B rings were the most active; oxygenation on the side chain did not diminish their potency, unless a sulfate was present; however, those sterols which were sulfated on the D ring are completely inactive.

Results of HIV-2 (ROD) Studies. Anticytopathicity studies of the sterols against the ROD strain of HIV-2 in CEM cells indicated that only two of the sterols tested showed any protection. Compounds 2 and 3 produced a maximum of 30% protection at 67 μM; however, both were substantially cytotoxic to the host cells at less than the antiviral cytoprotection concentrations (not shown).

Experimental Section

Compounds 1 and 2 were isolated from *Topsentia* sp. and *Pseudoaxinyssa* sp., respectively, collected under contract for the National Cancer Institute. Their purification and spectroscopic characteristics have been detailed elsewhere.[6,27] The remaining sterols were isolated and purified to homogeneity as described in the text and cited references. The sea stars *T. novaecaledoniae* and *S. caroli* and the ophiuroids *O. dentata*, *O. elegans*, and *O. incrassata* were collected by ORSTOM, Noumea, New Caledonia, under the project SMIB "Substances Marine d'Interest Biologique" ORSTOM-CNRS. The sea star *Asterias amurensis* was collected along the Pacific coast of Okkaido, Japan, by the Faculty of Agriculture, Tohoku University, Sendai. The sea star *E. brasiliensis* was collected at the Grand Bahama Island during the 1990 Columbus Iselin expedition. The ophiuroids *O. longicaudum* and *O. fragilis* were collected in the Bay of Naples; the ophiuroid *O. superba* was collected at Zampa, Okinawa, Japan, by the Department of Marine Sciences, University of Ryukyus, and the ophiuroid *O. gigas* was collected at Gondwana, during the 1989–90 Italian Antarctic Expedition.

Antiviral Assays. The primary anti-HIV screen and confirmatory assays were performed as described elsewhere.[2,3,13] The CEM-SS human lymphocytic target cell line used in the antiviral assays was maintained in RMPI 1640 medium (Gibco, Grand Island, NY) without phenol red and supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 50 μg/mL gentamicin (complete medium). Exponentially growing cells were pelleted and resuspended at a concentration of 1×10^5 cells/mL in complete medium. For the HIV-1 studies, the Haitian variant of HIV, HTLV-III_{RF} (1.4×10^6 syncytium-forming unit/mL (SFU/mL)) was used throughout. For HIV-2 studies, either the CBL20 strain or ROD strain was used. Frozen virus stock solutions were thawed immediately before use and resuspended in complete medium to yield a multiplicity of infection (MOI) previously determined to give complete cell killing or maximal levels of virus production at 6 days postinfection (MOI of 0.01–0.05). The appropriate amounts of the sterols for anti-HIV evaluations were dissolved in 100% DMSO and then diluted in complete medium to the desired initial concentration (and with final DMSO content not exceeding 1%). All serial drug dilutions, reagent additions, and plate-to-plate transfers were carried out with an automated Biomek 1000 Workstation (Beckman Instruments, Palo Alto, CA).

For the multiparameter anti-HIV analysis experiments yielding the data depicted in Figure 1, additional details are as follows. Uninfected CEM-SS cells were plated at a density of 1×10^4 cells in 50 μL of complete medium. Diluted HIV-1 was then added to appropriate wells in a volume of 50 μL to yield a multiplicity of infection of 0.8. Appropriate cell, virus, and drug controls were incorporated in each experiment; the final volume in each microtiter well was 200 μL. Quadruplicate wells were used for virus-infected cells, and duplicates were used for uninfected cells. Plates were incubated at 37 °C in an atmosphere containing 5% CO_2 for 6 days. Subsequently, aliquots of cell-free supernatant were removed from each well and analyzed for reverse transcriptase activity, p24 antigen production, and syncytia as described.[13] Cellular growth or viability then was estimated on the remaining contents of each well using the XTT,[3,13] BCECF,[14] and DAPI[15] assays as described.[13]

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