Halichondria sulfonic acid, a new HIV-1 inhibitory guanidino-sulfonic acid, and halistanol sulfate isolated from the marine sponge Halichondria rugosa Ridley & Dendy

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A new sulfur-containing guanidine derivative, halichondria sulfonic acid (1) showing anti-HIV-1 activity, and halistanol trisulfate (2) with anti-tumor activity have been isolated from the marine sponge Halichondria rugosa Ridley & Dendy collected in the Chinese Southern Sea. The structure of 1 was elucidated by analysis of spectroscopic and crystal data.

Keywords: Sponge; Halichondria rugosa; Halichondria sulfonic Acid; anti-HIV-1

1. Introduction

Sulfur is one of the most abundant elements in seawater. Many sulfur compounds have been isolated from marine organisms [1], and especially Porifera possess a broad variety of chemical structures. The types of sulfonic acids and sulfates isolated from sponges include terpenoids [2], aromatics [3], sterols [4] and other complex structures [5]. Most of the sulfated compounds have a broad spectrum of biological activities [1]. Some exhibit activity against human immunodeficiency virus type 1 (HIV-1) [6]. Clathsterol, isolated from Clathria sp., was shown to inhibit HIV-1 reverse transcriptase [7].

In a similar way, natural products bearing a guanidine function attracted attention because of their wide range of bioactive activities, and the important feature of guanidinium compounds is their ability to interact with anionic species [8–10]. Palau’amine A and related metabolites, guanidine derivatives isolated from sponges,
possess cytotoxic activity against P388, L1210 and KB as well as antifungal and antiviral activity [8].

Compounds including both a sulfonic acid and a guanidine functionality are very rare in nature, some peptides, the dicodermins, halicyndramides and microspinosamides being rare examples. In this study, the investigation of the sponge Halichondria rugosa Ridley & Dendy (Halichondriidae) led to the isolation of the guanidine compound, halichondria sulfonic acid (I) (figure 2), and the sulfated sterol halistanol trisulfate (2), which has been isolated previously from different sponges [11–14]. In bioactivity assays, halichondria sulfonic acid (I) exhibited anti-HIV-1 activity, while halistanol trisulfate (2) showed additional anti-tumor activity on the cell line level. Details of the isolation and structure elucidation of the halichondria sulfonic acid (I) are presented subsequently.

The freshly thawed sponge, collected in the South Chinese Sea, was extracted three times with methanol. The organic solvent was evaporated, and the obtained concentrate dissolved in water, and successively partitioned between cyclohexane, ethyl acetate and n-butanol. The n-butanol fraction was concentrated and chromatographed on silica gel, and compound 1 and halistanol trisulfate (2) were separated by RP-18 column chromatography.

Compound 1 was obtained as colorless crystals, with ESI mass spectra displaying signals at m/z 204 [M + Na]+, 385 [2M + Na]+ in the positive ion mode. In the negative ion mode, m/z 180 [M − H]− and 361 [2M − H]− were visible suggesting a molecular weight of 181 Da. The molecular formula C4H12N3O3S was determined by HR EIMS ([M+]+ 182.05946; Calcd 182.05939). The IR spectrum exhibited strong absorptions bands at 1205 cm−1, consistent with the presence of sulfate groups. Strong bands at 1638, 3220 and 3177 cm−1 were indicative of NH groups.

The final structure of compound 1 was established using a combination of heteronuclear two-dimensional NMR and crystal structure analysis. The 1H NMR spectrum contained only a 3H singlet at δ 3.12 assigned to a methyl connected to a heteroatom, and two methylene triplets at δ 3.83 and 3.29, respectively. The 13C APT NMR spectrum indicated a quaternary carbon signal at δ 158.0, two methylene (48.2, 47.2) and a methyl signal (36.7). The HMBC and HMQC spectra delivered the partial structure of figure 1 which includes all carbon atoms, however, a complete structure could not be assigned due to the lack of further information. The exact structure of compound 1 was finally obtained by crystal structure analysis.

The structure of 1 is not mentioned in the Chemical Abstracts. Surprisingly, however, 1 was obtained by Dittrich [15] more than 100 years ago by reaction of cyanamide with methyltaurin and was named methyltaurocyamin or methylguanidine ethyl sulfonic acid, respectively. The corresponding demethyl derivative (taurocyamine, guanidinotaurine) has been isolated from urine [16].

Figure 1. HMBC couplings in 1.
Compound 1 crystallized with two molecules in the asymmetric unit as shown in figure 3. The sulfonic acid moiety forms hydrogen bonds with the symmetry equivalent guanidinium groups and water molecules (data not shown).

Compound 2 displayed in the $^1$H NMR spectrum the characteristic signals of a steroid with at least four methyl signals at $\delta$ 0.79 and 0.85, and methylene and methine signals in the range of $\delta$ 1.18 and 2.28. The $^{13}$C/APT NMR spectra indicated the presence of 29 carbon signals. The (+)-ESI mass spectrum indicated a quasi molecular ion peak of [M – Na + 2H]$^+$, and ESI HRMS delivered the molecular formula C$_{29}$H$_{51}$O$_{12}$S$_3$Na$_2$ (found 733.23297, Calcd 733.23325). With these data, compound 2 was identified as halistanol trisulfate, which had been previously isolated from the sponge *Halichondria* cf Moorei Bergquist [11].

Compound 1 exhibited activity against HIV-1 with EC$_{50}$ 29 µg mL$^{-1}$ and IC$_{50}$ > 200 µg mL$^{-1}$. Halistanol trisulfate (2) was also active against HIV-1 [6] and
possessed additional activity against tumor cell lines, the human hepatoma QGY-7701 and chronic myelogenous leukemia K562 with IC\textsubscript{50} values of 26 and 52 \(\mu\)g mL\textsuperscript{-1}, respectively.

2. Experimental section

2.1. General experimental procedures

IR spectra were obtained with Perkin-Elmer 297 infrared spectrometer. \(^1\)H, \(^{13}\)C and 2D NMR spectra were recorded on a Varian Unity 300 spectrometer. All chemical shifts are reported with respect to TMS (\(\delta_H = 0\)). HPLC ESI MS: LCQ (Finnigan). ESI HR mass spectra were recorded on an APEX IV, 7T, FT-ICR mass spectrometer (Bruker Daltonik). Microtiter reader M-550, Bio-Rad.

2.2. Crystal structure analysis

Compound I crystallizes in the monoclinic space group \(P2_1/c\) with two molecules in the asymmetric unit and two water molecules. The needle-like crystals were grown by evaporation from water solution. For data collection, the crystal was shock frozen in a cold nitrogen stream using perflouropolyether oil as cryoprotectant.

The data reduction was performed using SAINT [17] and the data were corrected semi-empirically for absorption and other effects with SADABS [18]. The phase problem was solved by conventional direct methods using SHELXS [19] and the model was refined against \(F^2\) on all data by full-matrix least-squares with SHELXL [20]. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were included at geometrically calculated positions.

2.3. Crystal data

Empirical formula: C\textsubscript{4}H\textsubscript{11}N\textsubscript{3}O\textsubscript{3}S + OH, crystal size: 0.1 \times 0.1 \times 0.1 mm\textsuperscript{3}, space group \(P2_1/c\), unit cell dimensions: \(a = 16.669(10)\) \(\AA\), \(b = 7.844(6)\) \(\AA\), \(c = 14.128(10)\) \(\AA\), \(\alpha = \beta = 90^\circ\), \(\gamma = 111.199(3)^\circ\), non-hydrogen atomic volume = 1722.36(2) \(\AA^3\), \(\rho_{\text{Calcd}} = 1.529 \text{ mg cm}^{-3}\), 15521 reflections were measured, 2447 unique reflections, completeness (%) = 98.7, \(R(\text{int})\) (%) = 0.0407, \(I/\sigma = 12.36\), \(R_{\text{int}} = \Sigma |I - \langle I \rangle| / \Sigma I\). The refinement converged to \(R_1 = \Sigma |F_o| - |F_c| / \Sigma |F_o| = 0.0406\) for 2747 reflections \(F_o > 4\sigma\) and 0.0418 for all 14865 data, \(wR_2 = \Sigma w(F_o^2 - F_c^2)^2 / \Sigma w(F_o^2)^2\) \(1/2 = 0.1101\) for 14865 data and 12/228 parameters. The crystal data have been deposited in the Cambridge crystallographic database with the CCDC number 293576.

2.4. Animal material

The sponge was collected at a depth of 10 m in the Southern Chinese Sea during December 2002. Prof. Jinhe Li identified the sponge as \(H. \text{rugosa}\) Ridley & Dendy. A voucher sample was deposited in the Specimen Depository of the Institute of Oceanology, Chinese Academy of Sciences, Qingdao.
2.5. Extraction and isolation

The collected sponge was immediately frozen and kept at -20°C. The frozen sponge was thawed, cut into pieces (1.5 kg, wet wt.) and extracted with methanol by stirring for 3 h. The concentrated methanolic extract was dissolved in water, and successively partitioned between cyclohexane, EtOAc and n-butanol. The n-butanol fraction was concentrated and chromatographed on silica gel with CH₂Cl₂/MeOH (10:0–0:10). The 100% MeOH fraction was separated on silica gel column again and the fraction 1 (CH₂Cl₂/MeOH 4:6) and fraction 2 (MeOH) were obtained. Fraction 1 was separated on RP-18 using water as eluent to give 1 (9 mg) as crystals. Fraction 2 delivered RP-18 312 mg of compound 2.

2.5.1. Halichondria sulfonic acid (1).

Colorless needles; IR (KBr): νmax 3363, 3220, 3177, 2926, 2853, 1661, 1638, 1543, 1462, 1376, 1264, 1205, 1108, 1053, 104,10, 1002 cm⁻¹; ¹H NMR (D₂O, 300 MHz): δ 3.83 (t, J = 6.6 Hz, 2H), 3.29 (t, J = 6.6 Hz, 2H), 3.12 (s, 3H); ¹³C NMR (D₂O/CD₃OD, 300 MHz): δ 158.0, 48.2, 47.2, 36.7; (+)-ESI MS: m/z 566 ([3M + Na]⁺, 100), 204 ([M + Na]⁺, 76), 385 ([2M + Na]⁺, 50); (-)-ESI MS: m/z 180 ([M – H]⁻, 94), 361 ([2M – H]⁻, 100); (+)-ESI HRMS: m/z 182.05946 (Calcd 182.05939 for C₄H₁₂N₃O₃S, [M + H]⁺).

2.5.2. Halistanol trisulfate (2).

White solid; assignments are based on literature values [4–6]; ¹³C NMR (D₂O, 300 MHz): δ 79.4 (C-6), 75.1 (C-2), 74.7 (C-3), 56.1 (C-17), 56.0 (C-14), 54.1 (C-9), 44.1 (C-5, C-24), 43.8 (C-24, C-13), 42.6 (C-12), 39.7 (C-1), 37.9 (C-10), 36.5 (C-20), 35.5 (C-22), 33.7 (C-8), 33.0 (C-25), 28.3 (C-6), 28.1 (C-23), 27.3 (C-26, C-27, C-28), 23.8 (C-4), 23.6 (C-15), 20.7 (C-11), 19.1 (C-21), 14.6 (C-19), 14.4 (C-28), 11.9 (C-18); HR ESI MS: m/z = 733.23297 (Calcd 733.23325 for C₂₉H₅₁O₁₂S₃Na₂, [M – Na + 2H]⁺).

2.6. Anti-tumor assay

The cytotoxicity was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) cell viability assay [21]. Exponentially growing cells were seeded into 96-well plates at a density of 10⁵ cells well and incubated for 4 h at 37°C prior to drug addition. The concentrations of the fraction were between 0.75 and 100 µg mL⁻¹. Triplicate wells were used for each treatment. The cells were incubated with the sample for 48 h at 37°C in a humidified 5% CO₂ atmosphere. To each well, 10 µL of MTT (5 mg mL⁻¹ final concentration) was added, and the plates were incubated at 37°C for 4 h to allow MTT to form the formazan by reacting with metabolically active cells. The formazan crystals were solubilized for 4 h (or overnight) at 37°C with a solution containing digestion buffer (20% sodium dodecyl sulfate (SDS), 50% dimethylformamide (DMF) pH 4.7). The absorbance of each well was measured in a microtiter reader (M-550, Bio-Rad) at 595 nm and a reference wavelength of 655 nm. Cisplatin was used as a positive control at a concentration of 0.1 µg mL⁻¹. 0.9% NaCl was used as a negative control. The IC₅₀ values were performed using LD₅₀ software.
2.7. Cells and virus

C8166 and HIV-1IIIB strains were kindly donated by Medical Research Council (MRC), AIDS Reagent Project, UK. The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco), and HIV-1IIIB was obtained from the culture supernatant of H9/HIV-1IIIB cells. The 50% HIV-1 tissue culture infectious dose (TCID₅₀) in C8166 cells was determined and calculated by Reed and Muench method. Virus stocks were stored in small aliquots at −70°C. The titer of virus stock was $9 \times 10^5$ TCID₅₀ mL⁻¹.

The human hepatoma (Qgy7710), Burkitt’s lymphoma (Raji), chronic myelogenous leukemia (K562) cell lines, which were kindly donated by Dalian Medical University, were maintained as exponentially growing cultures in RPMI 1640 culture medium, supplemented with 10% fetal bovine serum, pH 7.2–7.4. All cell lines were cultured at 37°C in an atmosphere of 5% CO₂ in air (100% humidity).

2.8. Cytotoxicity assay

The cellular toxicity of compounds on HIV-1 host cells C8166 was assessed by MTT colorimetric assay as described previously [22]. Briefly, 100 µL of $3 \times 10^4$ cells was seeded on a microtiter plate, 100 µL of various concentrations of compounds were added and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 72 h. Of the supernatant, 100 µL were discarded, MTT reagent was added and incubated for 4 h and 100 µL 50% DMF-10% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 595/630 nm. The results were shown by absorbance values. The minimum cytotoxic concentration that caused the reduction of viable cells by 50% concentration (CC₅₀) was determined from dose–response curve.

2.9. Anti-HIV-1 activity assay

In the presence of 100 µL various concentrations of compounds, C8166 cells ($4 \times 10^5$ mL⁻¹) were infected with HIV-1IIIB at a multiplicity of infection (MOI) of 0.06. They were then cultured in final volume of 200 µL. The plates were incubated in a humidified incubator at 37°C and 5% CO₂. Azidothymidine (AZT) was used for drug control. After 3 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope. The percentage inhibition of syncytial cell formation was calculated by percentage of syncytial cell number in compounds-treated culture to that in infected control culture. The minimum inhibitory concentration that reduced CPE by 50% (ECₕ₀) was interpolated from plots generated from the data. The therapeutic index (TI) was calculated from the ratio of CC₅₀/ECₕ₀ [23].

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Halistanol sulfate isolated from the marine sponge *H. rugosa* Ridley & Dendy

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