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Isolation and Biological Evaluation of the isolated Compounds from the Red Sea Marine Tunicate *Styela clava*

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Abstract

Three new compounds, clavaside A [methyl 2-O-(1,3-dihydroxypropyl)-xylopyranoside] (**1**), 3*β*,8-dimethylnonan-1-ol (**2**) and 1-(2-methylpropoxy)-13-methyltetradeca-3,6-diene (**3**) along with the ten known compounds, namely 3-decen-1-ol (**4**), methoxy *α*-D-glucose (**5**), thymidine (**6**), uridine (**7**), palmitic acid (**8**), 9-hexadecenoic acid (**9**), 1,3-di(9-hexadecenoyl)-2-tetradecanoylglycerol (**10**), 1,3-ditetradecanoyl-2-(9-hexadecenoyl)-glycerol (**11**), cholesterol (**12**) and 24-methylene cholesterol (**13**) have been isolated from the Marine Tunicate *Styela clava* collected from the Red Sea. Acetylation of compounds **6**, **7**, **12** and **13** yielded thymidine 3`,5`-diacetate (**6-Ac**), uridine 2`,3`,5`-triacetate (**7-Ac**), cholesterol-3-acetate (**12-Ac**) and 24-methylene cholesterol-3-acetate (**13-Ac**), respectively. All compounds were tested for their antiprotozoal, antimicrobial and cytotoxic activities.

Keywords- Antimicrobial, Antiprotozoal, Clavaside A, Cytotoxicity, *Styela clava*, Tunicate.

INTRODUCTION

Red Sea has one of the highest levels of marine biodiversity and has great seasonal fluctuations of air and water temperatures [1]. More than 15,000 natural products have been isolated from marine organisms in the last three decades [2]. The ascidians are commonly found in waters all over the world, along the coasts and deep to the bottoms, which is one of the important marine sources for active metabolites. In the early 1970s, it was reported that ascidians extracts possess a variety of bioactivities such as cytotoxicity, antitumor and immune regulatory properties [3]. Since 1980s, a greater variety of bioactive substances with antitumor, antiviral, antimicrobial, immune regulatory, and biocatalytic activities were extracted from the ascidians [3]. *Styela clava* is a highly invasive ascidian species that is now found in all corners of the globe [4]. The research dealing with the isolation and biological evaluation of active compounds from marine origin, the chemistry and the biological properties of the tunicate *Styela clava* collected from the Red Sea was investigated, which led to the isolation of three new compounds (**1-3**).

MATERIALS AND METHODS

General experimental procedures:

^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on a Bruker BioSpin Gm bH 400 and 500 spectrometers (Bruker, Rheinstetten, Germany). High resolution mass spectrometry were measured using a Bruker BioApex FT mass spectrometer (Bruker, Rheinstetten, Germany). GC/MS analysis was carried out using an HP 6890 series GC (Agilent Technologies, Santa Clara, CA, USA), equipped with a split/splitless capillary injector, an HP 6890 series injector autosampler and an Agliant DB-5ms column (30 m x 0.25 mm x 0.25 µm), interfaced to a HP 5973 mass selective detector (MSD). The injector temperature was 250 °C, and 1 µL of sample was injected in the splitless mode, with the splitless time set at 60 s, the split flow set at 50 mL/min and the septum purge valve set to close 60 s after the injection occurred. The oven temperature was raised from 70 to 270 °C (held for 20 min) at a rate of 5 °C/min, for a total run time of 60 min; the transfer line temperature was 280 °C. Chemicals for pharmacological studies were purchased from Sigma-Aldrich (St. Louis, MO, USA) except the fetal bovine serum (Midwest Scientific, Valley Park, MO, USA) and the Bio-rad Bradford dye (Fisher Scientific, Pittsburg, PA, USA).

Tunicate material, Collection and Identification:

The tunicate *Styela clava* (coll. no. SAA-116) was collected at depths between 10 and 20 m from the Egyptian Red Sea coast at Safaga. The sample was directly frozen after collection and stored at -20 °C. A voucher specimen was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt, under a registration no. SAA-116.

Extraction and isolation:

The frozen tunicate was chopped while into small pieces and extracted with MeOH (4 x 2 L) at room temperature for 6 hrs. The crude extract was concentrated under reduced

pressure to give a dry dark green viscous crude extract (4.503 g). Four grams of the residue was subjected to fractionation using vacuum liquid chromatography over a silica gel column (100 g, 160-200 mesh) eluting with each 500 mL of hexanes-EtOAc (75:25 , 0:100) followed by each 500 mL EtOAc-MeOH (50:50, 0:100) yielding 4 fractions. Fraction 2 (650 mg) was fractionated on silica gel column (30 g, 2.0 cm x 34.0 cm) and eluted with hexanes/EtOAc in a gradient manner from 5 to 20% to yield four fractions (2A-2D). Subraction 2A (50.6 mg) was further purified on a silica gel column (3 g, 0.8 cm x 25.0 cm) using hexanes/EtOAc (95:5), yielding compounds **10** (15.6 mg) and **11** (19.1 mg). Subraction 2B (102.4 mg) was further purified on a RP column (8 g, 0.8 cm x 25.0 cm) using MeOH/H_2 O (97.5:2.5) to yield compounds **12** (27.8 mg) and **13** (15.7 mg). Compounds **8** (10.7 mg) and **9** (8.5 mg) were isolated from subraction 2C (80.3 mg) using a silica gel column (4 g, 0.8 cm x 25.0 cm) eluted with hexanes/EtOAc (90:10). Fraction 3 (1.2 g) was subjected to silica gel column (60 g, 2.5 cm x 46.0 cm) (CH_2Cl_2/MeOH in gradient manner from 2 to 10%) to give three subfractions (3A-3C). Subfraction 3A (227.4 mg) was subjected to silica gel column (12 g, 34 cm x 2 cm) and eluted with (CH_2Cl_2/MeOH, 95:5) yielding 2 fractions (3A-1 and 3A-2). Fraction 3A-1 (51.6) was purified on a SPE-C_{18} (5 g) column (MeOH/H_2O, 55.5:44.5) to yield compounds **2** (9.6 mg), **3** (2.4 mg) and **4** (4.2 mg). Fraction 3A-2 (103.2) was subjected to silica gel column (3 g, 25 cm x 0.8 cm) and eluted with (CH_2Cl_2/MeOH, 90:10) to yield compound **6** (60.6 mg, R_f = 0.33). Subfraction 3B (157.9) was purified on a SPE-C_{18} (10 g) column (MeOH/H_2O, 50:50) followed by sephadex LH-20 column (100 g, 2.5 cm x 46.0 cm) using MeOH as a mobile phase to yield compounds **5** (10.8) and **7** (20.9). Subfraction 3C (70.2 mg) was subjected to a SPE-C_{18} (10 g) column (MeOH/H_2O, 50:50) followed by silica gel column (2 g, 0.8 cm x 25.0 cm) and eluted with (CH_2Cl_2/MeOH, 90:10) and sephadex LH-20 column (100 g, 2.5 cm x 46.0 cm) using MeOH as a mobile phase to give compound **1** (27.1 mg).

Acetylation of compounds 6, 7, 12 and 13:
Ten mg of each compound were dissolved in 1 mL of pyridine and then two mL of acetic anhydride was added. The reaction mixture was kept at room temperature for 12 hrs [5]. The solvent was evaporated using vacuum concentrator to give compounds **6-Ac**, **7-Ac**, **12-Ac** and **13-Ac**.

Esterfication of compound 8 and 9:
Compounds **8** and **9** (five mg each) were dissolved in one mL of chloroform. One mL of 20 mM cupric acetate monohydrate in methanol and 5 mL of 0.5 N HC1 in methanol were added. The mixture was stored for 60 min at room temperature, and then extracted with ten mL of chloroform after addition of ten mL of water [6]. The pooled chloroformic extract was washed with water and then evaporated to dryness under a flow of nitrogen to give the corresponding fatty acid methyl ester of compounds **8** and **9**.

Acid hydrolysis of compound 10 and 11:
HCl/MeOH (1.2%, two mL) was added to two mg of compounds **10** and **11** and then stored for 20 min at 45 °C. The solution was extracted with hexanes (four mL). The fatty acid methyl esters in the hexanes layer were identified by GC/MS [7].

In vitro antiprotozoal assay:
All compounds were tested for their antiprotozoal activities against *Leishmania donovani* Promastigote*, L. donovani* Amastigote*, L. donovani* Amastigote/THP1 cells and *Trypanosoma brucei* (Manda, et al., 2014). The assays have been adapted to 384 well microplate format. In a 384 well micro-plate, the samples with appropriate dilution were added to the three protozoal cultures (2 × 106 cell/mL). The plates were incubated at 26 °C for 72 hrs (37 °C for axenic amastigotes and *T. brucei* trypomastigotes) and growth of the parasites in cultures were determined by Alamar Blue assay [8, 9]. The compounds were tested at concentrations ranging from 10-0.4 µg/mL. The compounds were also tested against *L. donovani* intracellular amastigotes in THP1 cells employing a parasite-rescue and transformation assay [10]. Pentamidine and Amphoterecin B were used as the standard antileishmanial agents as well as DFMO (*α*-difluoromethylornithine) was used as a positive control for antitrypanosomal activity. IC_{50} and IC_{90} values were computed from dose-response curves using XLfit software.

In vitro antimicrobial assay:
All organisms used for the biological evaluation were obtained from the American Type Culture Collection (Manassas, VA, USA) and including the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods as previusly described [11, 12] and references therein. *M. intracellulare* was tested using a modified method according to method described in literature [13].

Cytotoxicity evaluation:
The cytotoxicity of the isolates was determined towards a panel of selected cell lines according to a method described earlier [14].

RESULTS

Chemistry:
*Clavaside A (methyl 2-O-(1,3-dihydroxypropyl)-xylopyranoside) (**1**):*Yellow amorphous powder; $[\alpha]^{25}_D$ + 43.9° (*c* = 0.87, MeOH); ^{1}H NMR (400 MHz, MeOD-d_6): 3.33 (1H, m, H-4), 3.42 (3H, s, OCH_3), 3.45 (1H, m, H-2), 3.54 (1H, m, H-2`), 3.62 (4H, d, *J* = 5.2 Hz, H-1` and 3`), 3.70 (1H, m, H-3), 3.82 (2H, dd, *J* = 2.6 Hz, 11.8 Hz, H-5), 4.70 (1H, d, *J* = 4 Hz, H-1); $^{13}$C NMR (100MHz, MeOD-$d_6$): 54.3 ($OCH_3$), 61.2 (C-5), 62.3 (C-1` and 3`), 70.3 (C-

4), 72.1 (C-2`), 72.3 (C-2)73.7 (C-3), 99.7 (C-1); HRESIMS (positive ion mode) *m/z* 239.1067 ($[M+H]^+$, calcd for $C_9H_{19}O_7$, 239.1131).

*3β,8-dimethylnonan-1-ol (**2**)*:Yellow oil; $[\alpha]^{25}_D$ + 24.0° (*c* = 0.05, $CDCl_3$); ^{1}H NMR (400 MHz, pyridine-d_6): 0.77 (3H, d, *J* = 6.4 Hz, H-11), 0.85 (6H, d, *J* = 6.4 Hz, H-9 and 10), 1.01 (2H, m, H-5), 1.06 (2H, m, H-6), 1.08 (2H, m, H-7), 1.22 (2H, m, H-4), 1.36 (1H, m, H-3), 1.44 (1H, m, H-8), 1.71 (2H, m, H-2), 4.46 (2H, t, *J* = 7 Hz, H-1); ^{13}C NMR (100MHz, pyridine-d_6): 19.8 (C-11), 22.9 (C-10), 23.0 (C-9), 25.5 (C-6), 28.1 (C-2), 28.3 (C-8), 33.3 (C-3), 33.7 (C-5), 37.7 (C-4), 39.7 (C-7), 68.7 (C-1); HRESIMS (positive ion mode) *m/z* 195.1756 ($[M+Na]^+$, calcd for $C_{11}H_{24}NaO$, 195.1725).

*1-(2-methylpropoxy)-13-methyltetradeca-3,6-diene (**3**)*: Brown amorphous powder; ^{1}H NMR (400 MHz, pyridine-d_6): 0.80 (6H, d, *J* = 6.4 Hz, H-14 and 15), 0.91 (3H, d, *J* = 7.6 Hz, H-3`), 0.92 (3H, d, *J* = 6.4 Hz, H-4`), 1.19, 1.21 (2H, m, H-9), 1.38 (2H, m, H-10), 1.39 (2H, m, H-5), 1.60 (2H, m, H-12), 1.84 (1H, m, 2`), 1.97 (1H, m, H-13), 1.98 (2H, m, H-8), 1.99 (2H, m, H-11), 2.45 (2H, m, H-2), 4.04 (2H, t, *J* = 7.2 Hz, H-1), 4.17 (1H, dd, *J* = 2, 9.0 Hz, H-1`a), 4.27 (1H, dd, *J* = 3.2, 9.2 Hz, H-1`b), 5.35 (1H, m, H-6), 5.37 (1H, m, H-7), 5.43 (1H, m, H-3), 5.46 (1H, m, H-4); $^{13}$C NMR (100MHz, pyridine-$d_6$) data: 19.8 (C-3`, 4`), 21.3 (C-11), 22.3 (C-15), 23.2 (C-14), 25.4 (C-9), 28.6 (C-13), 33.5 (C-8), 33.6 (C-2), 34.3 (C-2`), 34.5 (C-5), 39.9 (C-12), 68.3 (C-1), 73.5 (C-1`), 129.3 (C-6), 129.9 (C-3), 132.6 (C-7), 133.0 (C-4); HRESIMS (positive ion mode) *m/z* 281.1881 ($[M+H]^+$, calcd for $C_{11}H_{24}O$, 281.2884).

Antiprotozoal activity:

Antiprotozoal activity of all compounds were tested against *Leishmania donovani* Promastigote, *Leishmania donovani* Amastigote, *Leishmania donovani* Amastigote/THP1 and *Trypanosoma brucei*. Compound **13** showed good antitrypanosomal activity with IC_{50} and IC_{90} values of 3.45, 6.75 µg/mL, respectively, compared to 3.62, 8.41 µg/mL of DFMO (α-difluoromethylornithine), the positive control.

Antimicrobial Activity:

Antimicrobial activity of all compounds were determined against bacterial strains, Methicillin-resistant *Staphylococcus aureus* (MRSa), *Escherichia coli, Pseudomonas aeruginosa* and *Mycobacterium intracellulare*, as well as against pathogenic fungi including *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* with no significant activity.

Cytotoxic Activity:

For searching new cytotoxic agents. The isolated compounds were tested against four cancer cell lines (SK-MEL, KB, BT-549, SK-OV-3) and one noncancer kidney epithelial cell line (LLC-PK_1). None of the compounds was cytotoxic to all the cancer cell lines up to the highest tested concentration of 25 µg/mL (data not shown). However, compound **13** showed cytotoxicity to kidney cells with IC_{50} value of 23 µg/mL.

DISCUSSION

Chemistry:

Three compounds (**1**-**3**) were isolated and spectroscopically analyzed.

Compound 1 was isolated as a yellow amorphous powder, with specific rotation $[\alpha]^{25}_D$ + 43.9° (c = 0.87, MeOH). Its molecular formula was $C_9H_{18}O_7$ based on the positive-mode HRESIMS ion at *m/z* 239.1067 $[M+H]^+$. The ^{1}H and ^{13}C NMR spectroscopic data of **1** displayed five oxygenated methines, including C-1 (δ_H 4.70 / δ_C 99.7), C-2 (δ_H 3.45 / δ_C 72.3), C-3 (δ_H 3.70 / δ_C 73.7), C-4 (δ_H 3.33 / δ_C 70.3) and C-2` ($\delta_H$ 3.54 / $\delta_C$ 72.1) and three oxygenated methylene groups C-5 ($\delta_H$ 3.82 / $\delta_C$ 61.2), C-1` (δ_H 3.62 / δ_C 62.3) and C-3` (δ_H 3.62 / δ_C 62.3) along with one methoxy group (δ_H 3.42 / δ_C 54.3) suggesting the presence of one sugar moiety. Compound **1** was considered as glycosylglycerol derivative based on comparing its NMR spectroscopic data with those reported in literature [15-18]. The sugar moiety was determined as α-D-xylopyranosyl with a methyl group attached at C-1 and 1,3-dihydroxypropyl group at C-2. The ^{1}H-^{1}H COSY between H-1 (δ_H 4.70) and H-2 (δ_H 4.45) along with the HMBC correlation between OCH_3 protons (δ_H 3.42) and C-1 (δ_C 99.7) confirmed the location of the methoxyl and 1,3-dihydroxypropyl groups at C-1 and C-2 respectively. From the above, compound **1** was elucidated as methyl 2-O-(1,3-dihydroxypropyl)-xylopyranoside named clavaside A.

Compound 2 was isolated as a yellow oil. Its molecular formula was established as $C_{11}H_{24}O$ from the positive-mode HRESIMS ion at *m/z* 195.1756 $[M+H]^+$. ^{1}H NMR data exhibited isopropyl moiety [δ_H 0.85 (6H, d, *J* = 6.4) and δ_H 1.44 (1H, m)], a methine proton at δ_H 1.36 (1H, m), cluster from 6 methylene protons [δ_H 4.46 (2H, t, *J* = 7), δ_H 1.71 (2H, m), δ_H 1.22 (2H, m), δ_H 1.08 (2H, m), δ_H 1.06 (2H, m) and δ_H 1.01 (2H, m)] and a methyl group [δ_H 0.77 (3H, d, *J* = 6.4)] indicating a branched fatty alcohol. ^{13}C NMR spectrum displayed 11 carbon resonances including two methines [C-3 (δ_C 33.3) and C-8 (δ_C 28.3)], six methylene carbons [C-1 (δ_C 68.7), C-7 (δ_C 39.7), C-4 (δ_C 37.7), C-5 (δ_C 33.7), C-2)δ_C 28.1) and C-6 (δ_C 25.5)] and three methyls, [C-9 (δ_C 23.0), C-10 (δ_C 22.9) and C-11 (δ_C 19.8)]. The attachment of the methyl group at C-3 (δ_C 33.3) was inferred from HMBC correlations between H_2-1 and C-3 as well as between the methyl group protons (δ_H 0.77) and C-3. The β-orientation of the methyl group was determined from comparing the ^{13}C NMR data and specific rotation $[\alpha]^{25}_D$ + 24.0° (c = 0.05, $CDCl_3$) with literature [19]. Therefore, compound **2** was defined as 3β,8-dimethylnonan-1-ol.

Compound 3 was isolated as a brown amorphous powder. Its molecular formula, $C_{19}H_{36}O$ identified based on the positive-mode HRESIMS ion at *m/z* 281.1881 $[M+H]^+$. NMR spectral data showed two isopropyl moieties, nine methylene groups, and four unsaturated methines. HMBC correlations [H-1 (δ_H 4.40)/C-3 (δ_C 126.9); H-4 (δ_H 5.46)/C-2 (δ_C 33.6); H-8 (δ_H 1.98)/C-6 (δ_C δ_C 129.3), C-7 (δ_C 132.6)] indicating the position of the two double bonds (C-3/C-4 and C-6/C-7). The presence of an isopropoxy moiety was confirmed using the ^{1}H-^{1}H COSY between H-1` ($\delta_H$ 4.17, 4.27) and H-2` (δ_H 1.84), along with HMBC

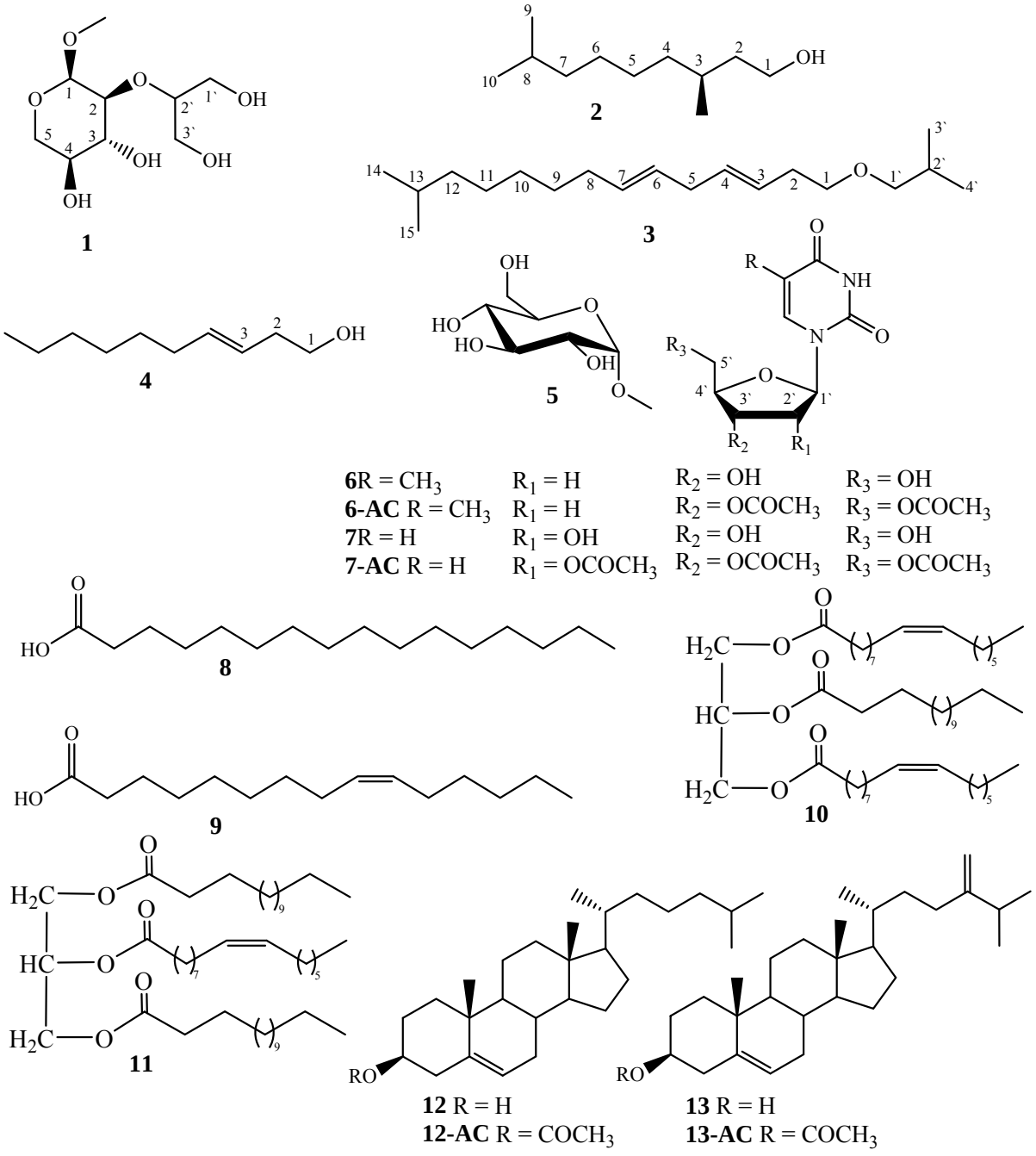

Figure 1. Chemical structures of the isolated compounds (**1-13**).

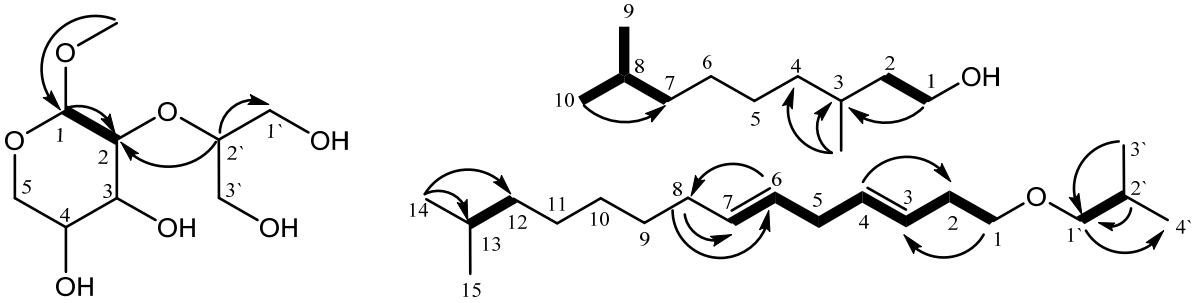

Figure 2. Some important COSY (▬)and HMBC (↷) correlations of (**1-3)**.

correlations between [H-1` (4.17, 4.27)/C-3` (δ_C 17.5); H-3` (0.91)/C-3` (δ_C 73.5). From the above, compound **3** was elucidated as 1-(2-methylpropoxy)-13-methyltetradeca-3,6-diene.

Compounds 4-13 were identified by comparison of their spectroscopic characteristics with those previously reported in the literature as 3-decen-1-ol (**4**) [20], methoxy *α*-D-glucose (**5**) [21], thymidine (**6**) [22], uridine (**7**) [23], palmitic acid (**8**) [24], 9-hexadecenoic acid (**9**) [25], 1,3-di(9-hexadecenoyl)-2-tetradecanoylglycerol (**10**) [26, 27], 1,3-ditetradecanoyl-2-(9-hexadecenoyl)-glycerol (**11**) [26, 28], cholesterol (**12**) [29] and 24-methylene cholesterol (**13**) [30, 31]. Acetylation of compounds **6**, **7**, **12** and **13** yielded thymidine 3`,5`-diacetate (**6-Ac**), Uridine 2`,3`,5`-triacetate (**7-Ac**), cholesterol-3-acetate (**12-Ac**) and 24-methylene cholesterol-3-acetate (**13-Ac**), respectively.

CONCLUSIONS

Three new and ten known compounds were isolated from the Red Sea tunicate *Styela clava*. The structures were elucidated by spectroscopic analysis. The antimicrobial and cytotoxic activities of the isolated compounds were evaluated. None was cytotoxic to cancer cells and no antimicrobial activity was observed. However, compound **13** displayed good antitrypanocidal activity, and cytotoxic against LLC-PK_1.

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