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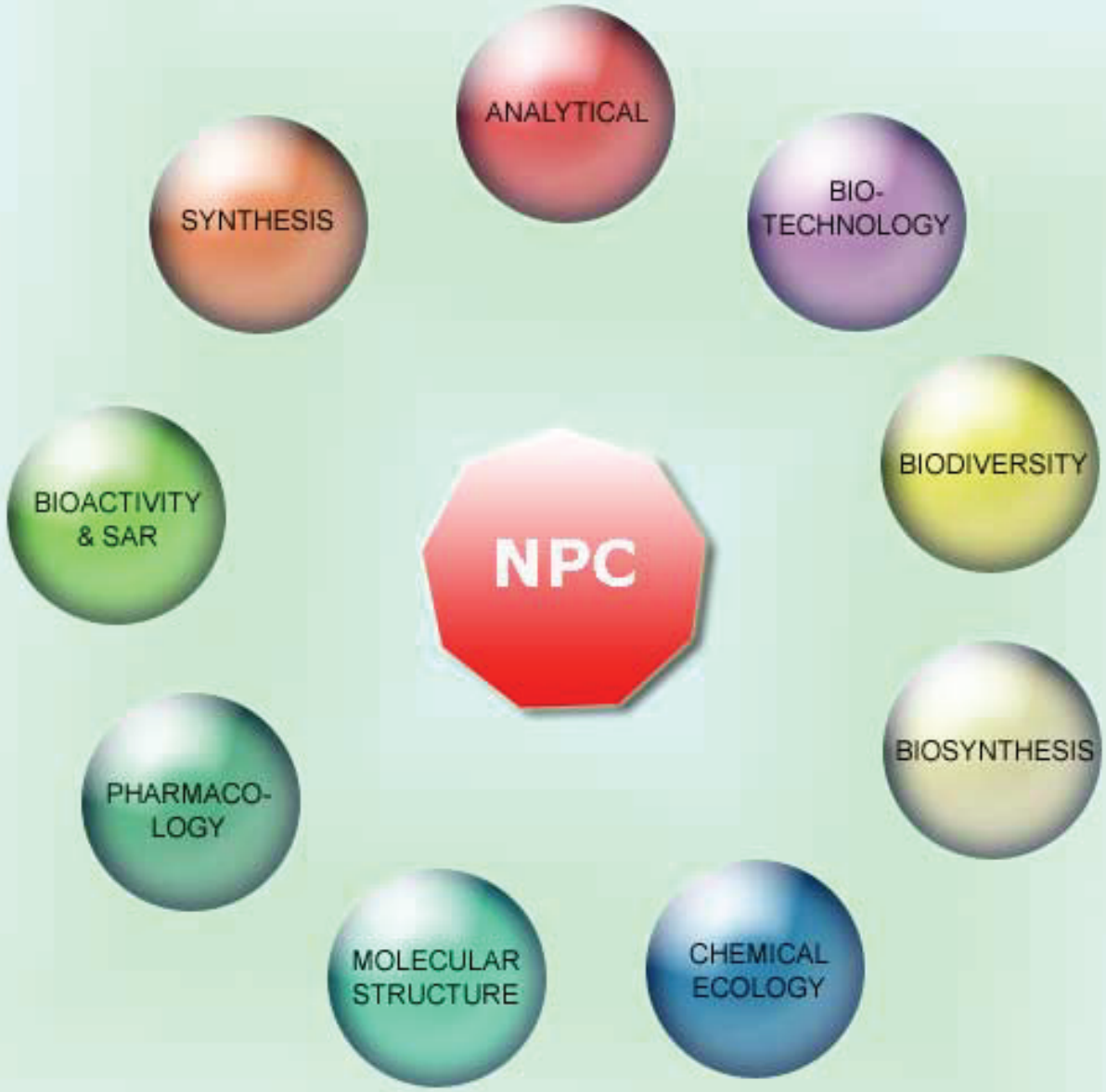

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Polyhydroxy Sterols Isolated from the Red Sea Soft Coral *Lobophytum crassum* and their Cytotoxic Activity

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One new (**1**) together with four known sterols (**2 - 5**) and a sesquiterpene (**6**) were isolated from a polar extract of the Red Sea soft coral *Lobophytum crassum*. The compounds were identified as 24-methylenecholest-5-ene-1α,3β,11α-triol 1-acetate (**1**), 24-methylenecholest-5-ene-1α,3β,11α-triol (**2**), 24-methylenecholest-5-ene-3β-ol (**3**), 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**4**), 24-methylenecholestane-3β,5α,6β-triol (**5**) and alismoxide (**6**) based on extensive NMR analysis. The cytotoxicity of compounds **1** – **6** was evaluated *in vitro* using three human cancer cell lines *viz*., HepG2, Hep-2 and HCT-116. Compound **1** showed selective cytotoxic activity against HepG2, while **3** exhibited cytotoxicity against all tested cell lines.

Keywords: *Lobophytum crassum*, Red Sea, Polyhydroxy sterols, Cytotoxicity.

Alcyonaceans (soft corals, Phylum: Coelenterata) belonging to the genus *Lobophytum* form a rich source of cemberanoids having diversified macrocyclic skeletons [1]. *L. crassum* is distributed in the indo-Pacific and Red Sea regions. A few cemberanoids have been identified from the nonpolar extract of a sample collected previously from the Gulf of Suez in the Red Sea [2], while samples collected from the Indo-Pacific region contained cemberane diterpenes [3-5]. Other metabolites *viz*., glycolipids [6] and polyhydroxy sterols have also been reported [7a, b]. Different biological activities for these isolates e.g. HIV-inhibitory [8], cytotoxic [9-11], and anti-inflammatory [12, 13] have also been reported.

The Red Sea has a unique ecological nature containing diverse flora and fauna considered to be potentially one of the most important sources of bioactive compounds. Exploration of this untapped natural resource would certainly be regarded as a high priority venture due to the universally expected impending climatic and environmental changes which will no doubt also affect the ocean flora and fauna. The ongoing search by our group for bioactive metabolites from Red Sea marine organisms [14] directed our attention to the soft coral *L. crassum* collected near Hurghada, Red Sea. We now report on the isolation, purification, structural elucidation and biological evaluation of compounds found in a *L. crissum* extract.

The concentrated crude MeOH extract of *L. crassum* was partitioned between EtOAc and water. The water extract was evaporated and the residue re-extracted with methanol and added to the ethyl acetate fraction. The combined EtOAc fractions were chromatographed repeatedly to afford the pure metabolites **1-6** (see Experimental Section).

Compound **1**, obtained as an amorphous powder, had the molecular formula $C_{30}H_{48}O_4$ established by HRESIMS {*m/z* 473.6605 (M^++1),

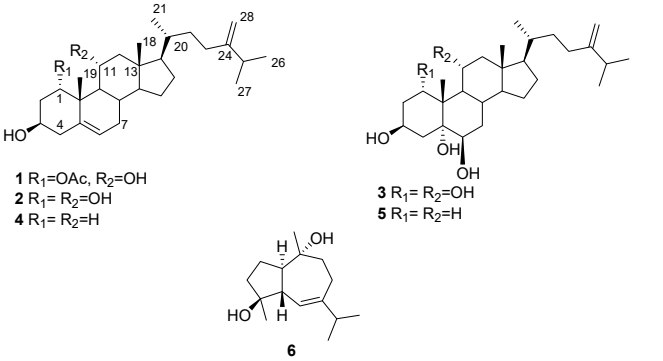

Figure **1**.Chemical structures of compounds **1-6**.

EIMS *m/z* 472 (M^+)}. Successive losses of 18 and 60 mass units {*m/z*, 454 (M^+– H_2O), 394 (M^+– OAc – H_2O)} suggested the presence of free hydroxyl group(s), while the fragment peak at *m/z* 412 (M^+– OAc – H^+) supported the presence of an acetyl group. The IR spectrum demonstrated the presence of hydroxyl (3500 cm^{-1}), carbonyl (1725 cm^{-1}) and a terminal methylene double bond (1646, 1250 and 880 cm^{-1}). The ^1H NMR spectrum ($CDCl_3$, 600 MHz) confirmed the presence of a terminal methylene group at δ 4.62 and 4.68 [each a singlet], an olefinic proton at δ 5.56 (d, *J* = 6.2), five methyl signals at δ 0.68 (s), 0.92 (d, *J* = 6.1), 1.00 (d, *J* = 6.8), 0.98 (d, *J* = 6.8), and 1.22 (s), and an acetoxy at δ 2.02 (s). Signals at δ 5.66 (1H, br s), 3.88 (1H, m), and 3.92 (1H, m) are assigned to protons attached to hydroxylated carbons based on HSQC spectra, the first one attached to an acetoxy group. ^{13}C and DEPT spectral analysis indicated the presence of 30 carbon atoms which are attributed to six methyls (including an acetoxy), nine methylenes (including an olefinic), ten methines (including an olefinic and three oxygenated), and five quaternary carbons (including two olefinic and a carbonyl groups). The above data suggested that this was a tri-hydroxylated 24-methylenecholest-5-ene derivative.

Furthermore, the NMR spectral data of **1** were remarkably similar to those for compound **2** [15] with the only difference being the presence of an additional acetoxy group in **1**. The large deshielding of H-1 at δ 5.66 vs that of δ 4.21 for compound **2** [15] suggested that the acetoxy group was situated at C-1, while the multiplet centered at δ 3.92 had a similar complexity normally seen for a 3 β-carbinol proton in a steroid skeleton [16]. The oxygenated methine signals at δ_C 78.3 (C-1) 66.7 (C-3) and 68.9 (C-11) are in similar positions as the 1α,3β,11α-triol system in the known compound **2**. Further confirmation for the position of the acetoxy group at C-1 was provided by HMBC correlations which showed cross-peaks (among others) between H-1/C-5, CO (carbonyl carbon); H-19/C-1, C-11, C-10; H-3/C-1, C-5. The relative configuration of the trihydroxylated position (C-1, C-3, and C-11) was established using NOESY, which showed correlations (among others) between Me-19/H-1, H-11; Me-18/H-11, acetyl group/H-3. The above data confirmed the chemical structure of **1** as 24-methylenecholest-5-ene-1α,3β,11α-triol 1-acetate.

The methanol extract also afforded five known compounds *viz*., **2**-**6**. Compound **2** (24-methylenecholest-5-ene-1α,3β,11α-triol) was previously isolated from both *Sinularia dissecta* [17] and *Palythoa tuberculosa* [15], while 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**4**) was isolated from both the Formosan soft coral *Sinularia gibberosa* [18] and the South China Sea soft coral *L. crassum* [19]. Compound **3** was only recently isolated from *Sinularia polydactyla* collected from the Red Sea [20], while 24-methylenecholestane-3β,5α,6β-triol (**5**) was reported from *Sinularia* sp. [21, 22]. Alismoxide (**6**) is a rare metabolite, identified previously from *Alisma rhizomes* [23], and was recently isolated from *Lithophyton arborium* [24].

The cytotoxic activity investigations against the growth of the human cancer cell lines HepG2, Hep2 and HCT-116 (Table 1) illustrated that compound **3** exhibited strong cytotoxicity toward the growth of HepG2, Hep-2 and HCT-116, with IC_{50} values of 1.90, 5.82 and 6.46 μM, respectively. HepG2 cell lines on the other hand showed a greater sensitivity towards compounds **1**, **5** and **6** than other cell lines (IC_{50} 1.90, 3.00 and 3.77 μM respectively).

Table 1: Cytotoxicity of compounds **1-6**.

Compound/extract	cancer cell line (IC_{50}, μM)		
	HepG2	Hep-2	HCT-116
EtOAc extract*	2.1	2.0	0.9
1	1.90	10.15	10.15
2	19.52	19.05	21.39
3	1.94	5.82	6.46
4	6.74	11.99	15.24
5	3.00	8.08	11.31
6	3.77	11.33	17.20
Doxorubicin	2.21		
Vinblastine		3.20	5.67

*μg/mL

The Red Sea is well known for its high salinity and low nutrient content. Such conditions generate different environments which are able to affect the secondary metabolites profile of the growing species in the same area. Earlier studies on the non-polar fraction of the same species by Kashman [2] demonstrated the presence of a cembrane diterpene. The difference between Kashman's work and this study could most likely be due to the extraction methods employed as well as the collection site. The chemical contents of *Lobophytum* species vary considerably depending on the geographical location and season of collection [11, 12, 25-26]. Generally, sterol patterns in marine invertebrates have a more complex profile than that of terrestrial organisms. The symbiotic relationships between organisms also complicates the sterol composition [27]. Fleshy soft corals, particularly the Alcyonarian corals of the genera *Lobophytum, Sinularia*, and *Sarcophyton* produce 3β,5α,6β−trihydroxy sterols. The isolation of Δ^5 steroid **4** together with 5,6-diol derivative **5** supports the assumption that the unoxidized Δ^5 sterol is the starting precursor [28].

Experimental

General experimental procedures: IR spectra were recorded on a JASCO FT/IR-8400S infrared spectrophotometer, UV spectra on a Shimadzu-265 spectrophotometer, and NMR spectra on a Jeol spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C using $CDCl_3$ with TMS as internal standard. Chemical shifts are given in δ (ppm) and coupling constants in Hertz (Hz). EIMS were recorded on a Shimadzu Qp-2010 (Tokyo, Japan) and Triple Quadropole TQD mass spectrometer (Waters, Milford, MA, USA) for ESI-MS. Perkin-Elmer model 343 plus polarimeter using a Na lamp at 25ºC (Shelton, CT, USA) used for optical rotation. Si gel 60 (Merck, 230-400 mesh) was used for column chromatography, and precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) for TLC analyses.

Animal materials: The soft coral *L. crassum* Marenzeller, 1886, was collected using scuba technology at Hurghada (Red Sea, Egypt) during May 2013 at a depth of 2-3 m. Upon collection the material was kept in MeOH. The organism was identified by Dr Abdel-Hamid Abdel-Rahman Mohamed Ali, co-author of this paper. A voucher sample (AB8-2013) is kept at the National Institute of Oceanography and Fisheries, Suez Branch, Egypt.

Extraction and isolation: Sliced bodies of *L. crassum* (2 kg fresh material) were exhaustively extracted with MeOH (4 L X 3). The organic layer was filtered and concentrated under vacuum and then partitioned between EtOAc and H_2O. The water layer was dried and washed with methanol (X3) and combined with the EtOAc fraction. The residue thus obtained (32.5 g) was subjected to CC on Si gel and eluted with a gradient of EtOAc in *n*-hexane in order of increasing polarity (0-100%) to yield 16 fractions. Fraction 9, eluted with *n*-hexane-EtOAc (8:2) yielded **1** (10 mg) and **6** (15 mg). Fraction 13, eluted with *n*-hexane-EtOAc (1:1), yielded 5 sub-fractions. The fourth and fifth sub-fractions were combined and subjected to preparative TLC using DCM-MeOH (9:1) to yield **2** (5 mg) and **5** (10 mg). Fraction 5 eluted with *n*-hexane-EtOAc (98:2) yielded **4** (23 mg), while fraction 15 eluted with DCM- MeOH (1 to 10%), gave 8 sub-fractions, of which 6 and 7 were combined and subjected to prep.-TLC using DCM-MeOH (9:1) to afford **3** (10 mg)

24-Methylenecholest-5-ene-1α,3β,11α-triol 1-acetate (1)
Amorphous white powder.
$[\alpha]_D^{25}$: 38.3 (*c* 0.28, MeOH).
IR (KBr) cm^{-1}: 3500, 1725, 1646, 1600, 1250, 880.
^{1}H NMR ($CDCl_3$ 600 MHz) δ_H: 5.66, (br s H-1); 1.75, 2.08 (m, each, H-2); 3.96 (m, H-3); 2.22, 2.37 (m each, H-4); 5.56, (d, *J* = 6.2 Hz, H-6); 1.99, 1.65 (m, each, H-7); 1.53 (m, H-8); 1.63 (m, H-9); 3.88 (m, H-11); 1.24, 2.31 (m, each, H-12); 1.13 (m, H-14); 1.24, 1.63 (m, each, H-15); 1.24, 2.00 (m, each, H-16); 1.14 (m, H-17); 0.68 (s, H-18); 1.22 (s, H-19); 1.76 (m, H-20); 0.92 (d, *J* = 6.1, H-21); 1.41, 1.05 (m, each, H-22); 1.24, 1.39 (m, each, H-23); 1.94 (m, H-25); 1.00 (d, *J* = 6.8, H-26); 0.98 (d, *J* = 6.8, H-27); 4.62, 4.68 (s, each, H-28); 2.02 (s, OAc).
^{13}C NMR (150 MHz): δ_C: 78.3, (CH, C-1); 35.6 (CH_2, C-2); 66.7 (CH, C-3); 41.8 (CH_2, C-4); 137.0 (C, C-5); 124.5 (CH, C-6); 31.3 (CH_2, C-7); 31.5 (CH, C-8); 48.9 (CH, C-9); 41.6 (C, C-10); 68.9 (CH, C-11); 51.1 (CH_2, C-12); 43.1 (C, C-13); 56.3 (CH, C-14); 24.0 (CH_2, C-15); 28.3 (CH_2, C-16); 55.7 (CH, C-17); 13.1 (CH_3, C-

18); 18.6 (CH_3, C-19); 35.6 (CH, C-20); 18.7 (CH_3, C-21); 34.5 (CH_2, C-22); 30.8 (CH_2, C-23); 156.8 C, C-24); 33.8 (CH, C-25); 21.9 (CH_3, C-26); 22.0 (CH_3, C-27); 106.0 (CH_2, C-28); 21.6,171.9 (CH_3, C respectively, OAc).
EIMS *m/z*: 472 $[M]^+$, 454, 394, 412; HRESIMS *m/z*: 473.6605 $[M^+ + 1]$.

Cytotoxicity assay: Human liver tumor cell lines (HepG-2), human colon tumor cells (HCT-116) and human epidermoid larynx carcinoma (Hep2) were obtained from the American Type Culture Collection and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin and 100 IU/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2. Metabolites **1**-**3** were dissolved in DMSO at a concentration of 1mg/mL, which were then diluted to appropriate concentrations with culture medium when used. Tumor cells ($5x10^4$-10^5 cells/ well) were incubated with serial dilutions of metabolites **1**-**6** in 96-well culture plates for 48 h, and their cytotoxicity was measured spectrophotometrically at 564 nm with an ELISA microplate reader (Meter tech. Σ 960, USA). All assays were performed in triplicate. The results were expressed as percentages, and the effective dose required to inhibit cell growth by 50% (IC_{50}) was determined. This work was carried at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

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