Research Article

^{13}C-NMR of Steroids from the Soft Coral *Litophyton arboreum*

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ABSTRACT

From the soft coral *Litophyton arboreum*, five compounds were isolated **(1-5)** and identified by different spectral techniques. The isolated compounds included 24-methyl-cholesta-5,24(28)-diene-3β,19-diol **(1)**, 24-methylcholesta-5,24(28)-diene-3β,7β, 19-triol **(2)**, 4α,24-Dimethyl-cholest-22-Z-en-3β-ol **(3)**, 24-methylencholesterol **(4)**, *N*-acyl-sphinga-4 (*E*), 8(*E*)-dienine **(5)**. The structures of the isolated compounds were elucidated by mass spectrometric and 1D and 2D-NMR spectroscopic data. It is the first time to report the complete ^{13}C signal assignments of steroid **(3)**.

Keywords: *Litophyton arboreum*; ceramanoids; steroids; ^{13}C-NMR.

INTRODUCTION

Marine organisms as a source of natural product delivered numerous novel compound with sensational multiple pharmacological properties. Soft corals are Coelenterates (class Anthozoa, subclass Octocorallia, order Alcyonacea, family Alcyoniidae), of which some are symbiotic associations of coral animals (polyps) with their algal partners (Zooxanthellae)[1]. They contain fairly large variety of secondary metabolites of terpenoids, mainly cembranoidditerpenes, and steroids, of which many were reported to have various biological activities[2,3]. The previous chemical investigation for the genus *Litophyton* revealed the presence of steroid compounds with antileukemic activity[4,5] and cembrane and furanocembranoidditerpen, sesquiterpene and bicyclogermacren[6,7]. The soft coral *Litophyton arboreum* showed very strong cytotoxicity and selectivity in leukaemia cancer cell lines U937 and moderate cytotoxicity in Hela cell lines and strong HIV-1PR inhibitory activity[8,9] as well as moderate cytotoxicity against HUVEC, K-562, L-929[7]. In our searching for bioactive compounds from marine organisms, a sample of the soft coral *Litophyton arboreum* (Nephtheiddae) from the Red Sea, we have isolated four steroids **1**- **4**, with one ceramide **5**.

MATERIALS AND METHODS

Experimental

General

Column chromatography (CC): silica gel 60 (40-63μm, *Merck*). Chromatographic fractions were monitored by TLC on silica gel 60 F_{254} plates (*Merck*) and visualized by heating after spraying with phosphomolybdic acid. IR spectra were recorded on Equinax 55 spectrometer (*Bruker GmbH*, Leipzig, Germany).

NMR spectra were recorded on a *BrukerAC-300* spectrometer, at 300.0 MHz for ^{1}H and 75 MHz for ^{13}C, respectively, in $CDCl_3$ using the solvent signals as internal standard (^{1}H 7.24; ^{13}C 77.00 for $CDCl_3$). EI-MS with a Finnigan MAT 8500.

Animal Material

The fresh soft coral of *L. arboreum* was collected from Red Sea, Hurghada, Egypt in December 2010 at a depth of 10 m. It was identified by Mr. Mohamed Abdel Ghany, Environmental Researcher, Red Sea Protectorates, Egypt.

Isolation and Purification of Compounds 1-5

The fresh soft coral of *L. arboreum* (250 g) were sliced, homogenized with EtOH. The EtOH extract (15 g) was partitioned between Hexane and ethyl acetate.

The ethyl acetate extract (1.0 g) was subjected to column chromatography on silica gel and eluted with chloroform, chloroform/methanol (10:1, 10:3) to afford crude steroid fractions (400 mg).

Purification of the crude fractions was performed on CC on silica gel then PTLC (Chloroform/methanol, 21:1.75) to give compound **1** (40.0 mg), **2** (15.40 mg), **3** (7.5 mg), **4** (25.6 mg)and **5** (5.3 mg).

Analytical Data

Compound 1: 24-methyl-cholesta-5,24(28)-diene-3β,19-diol, EIMS m/z (%): 414.0 $[M]^+$ (10), 384 (75), 366 (100), 300 (29), 283 (27), 257 (42), IR (MeOH), film: 3600, 1640, 890 cm^{-1}, ^{13}C-NMR: Table 1.

Compound 2: 24-methylcholesta-5,24(28)-diene-3β,7β, 19-triol, EIMS m/z (%): 430 $[M]^+$ (4), 412 (10), 382 (21), 363 (10), ^{13}C-NMR: Table 1.

Compound 3: 4α,24-Dimethyl-cholest-22-Z-en-3β-ol, EIMS m/z (%): 414 $[M]^+$ (75), 396 (40), 381 (30), 353 (20), 316 (20), 287(70), 271 (95); ^{13}C-NMR: Table 1.

Compound 4: 24-methylencholesterol, EIMS m/z (%): 398 $[M]^+$ (10), 380 (58), 365 (27), 314 (100); ^{13}C-NMR: Table 1.

Compound 5: N-acyl-sphinga-4 (E), 8(E)-dienine, EIMS m/z (%): 535 $[M]^+$ (4), 298 (21), 281 (17), 273 (5); IR (MeOH), film: 3395, 3300, 2950, 1625, 1040, 960 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ 3.93 (1H, brdd, 11.1, 3.2 Hz, H-1), 3.68 (1H, brdd 11.1, 3.1 Hz, H-1), 3.90 (1H, m, H-2), 4.30 (1H, m, H-3), 5.51 (1H, dd, 15.2, 6.6 Hz, H-4), 5.76 (1H, dt, 15.2, 6.0 Hz, H-5), 2.15 (2H, m, H-6), 2.07 (2H, m, H-7), 1.96 (2H, m, H-10), 6.26 (1H, d, 7.2 Hz, NH), 2.21 (2H, t, 7.5 Hz, H-2'), 1.60 (2H, m, H-3'), 0.86 (6H, t, 6.5 Hz, H-18, H-16'); ^{13}C-NMR($CDCl_3$) δ 62.5 (C-1), 54.4 (C-2), 74.6 (C-3), 129.0 (C-4), 133.5 (C-5), 36.8 (C-2'), 25.75 (C-3'), 22.67 (C-17, C-15'), 31.89 ~ 32.57 (X CH_2), 29.19 ~ 29.68 (X CH_2), 14.1 (C-18, C-16').

RESULTS AND DISCUSSION

Isolation of Compounds (1-5)

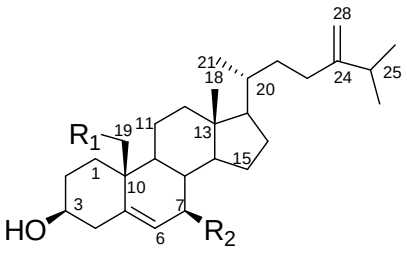
The ethanol extract of the fresh soft coral of *Litophyton arboreum* were concentrated under vacuum and partition between hexane/ethyl acetate and water. The ethyl acetate fraction was subjected to column chromatography on silica gel and eluted with chloroform and an increasing proportion of methanol during the course of elution to afford the crude compound **1**-**5**. The purification for the crude compounds was done by successive chromatography on silica gel, then PTLC using chloroform-methanol to afford pure compounds **1**-**5**.

Structure Determination

The IR spectrum of compound **1** showed the presence of a terminal methylene group at (1640, 890 cm^{-1}) and a hydroxyl group (3600 cm^{-1}). The ^{1}H-NMR showed the presence of primary hydroxyl group by two signals at δ 3.60 and 3.80 (d, J = 11.4 Hz) and secondary hydroxyl group at δ 3.57 (m) with ^{13}C-NMR at δ 62.7 and 71.3 respectively. The presence of trisubstituted double bond appeared at δ 5.76 (d, J = 4.0 Hz), terminal methylene group at δ 4.66 (brs) while the four methyl signals appeared at δ 0.73 (H-18, s), 0.94 (d, J = 6.5 Hz, H-21), 1.02 (d, J = 6.9 Hz, H-26), 1.03 (d, J = 6.9 Hz, H-27). A complete agreement of ^{13}C-NMR data of **1** (Table 1) with those of the previously isolated steroid: 24-methyl-cholesta-5,24(28)-diene-3β,19-diol from *Nephtheaerecta*[10].

The comparison of R_f values for **2** (0.23) and **1** (0.43) using chloroform/methanol (21:1.75) suggested that compound **2** is more polar to **1**. The TOFMS of compound **2** showed the molecular ion peak at m/z 453.38 with 16 mass units more to **1** which suggested the presence of an additional hydroxyl group of compound **2**. The ^{1}H-NMR showed the presence of the additional hydroxyl group at δ 3.81 (t, 8.1 Hz) with ^{13}C-NMR at δ 72.50.

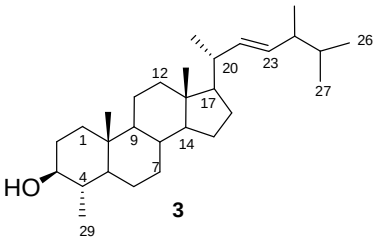
By using ^{1}H-^{1}HCOSY, HMQC and HMBC experiments, the position of hydroxyl group was determined at C-7. The presence of hydroxyl group at C-7 causes the downfield shift at C-5/C-6 double bond and C-8 by (Δδ +2.70 for C5/C-6 and Δδ + 2.3 for C-8) as compared to **1**. The ^{13}C-NMR data of **2** (Table 1) showed a close similarity for ring A, C and D as compared with **1**. From the above finding the structure of compound **2** is 24-methylcholesta-5,24(28)-diene-3β,7β, 19-triol which was previously isolated from *Litophyton viridis*[11]. It was reported that compound **2** possessed strong cytotoxicity activity against different human cancer cell lines, human prostate cancer cell lines LNCaP with IC50 of (15.5 and 4.9 mg/ml), and A549 cancer cell lines (0.81, 0.69 mg/ml) as well as HIV activity[8].



1: R_1 = OH R_2 = H

2: R_1 = OH R_2 = OH

4: R_1 = H R_2 = H



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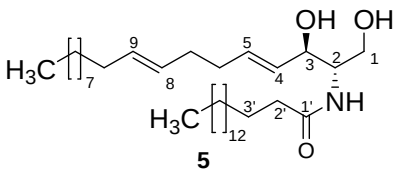

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Figure 1: Compounds (1-5) Isolated from *L.arboreum*

Table 1: ^{13}C-NMR (125 MHz) data of compounds 1- 4 in CD_3Cl

Position	1	2	3	4
	δ_C	δ_C	δ_C	δ_C
1	33.3	33.2	36.8	37.2
2	31.2	31.9	31.1	31.7
3	71.3	71.1	76.6	71.8
4	42.3	42.2	39.3	42.4
5	135.5	138.2	50.9	140.8
6	127.3	130	28.6	121.7
7	32.0	72.5	29.3	31.9
8	33.4	35.7	34.8	31.9
9	50.4	48.6	54.6	50.1
10	41.5	41.4	36.0	36.5
11	22.0	21.7	21.1	21.1
12	40.0	39.9	40.0	39.8
13	42.5	43.1	42.7	42.3
14	57.6	56.9	56.2	56.8
15	24.0	26.1	24.1	24.3
16	28.2	28.5	28.3	28.2
17	56.0	55.3	56.6	56.0
18	12.2	12.2	12.3	11.9
19	62.7	62.7	13.4	19.4
20	35.7	35.7	40.2	35.7
21	18.7	18.7	20.9	18..7
22	34.7	34.7	135.9	34.7
23	31.0	31.0	131.7	31.0
24	156.9	156.9	42.8	156.9
25	33.8	33.8	33.1	34.0
26	21.8	21.9	19.64	21.9
27	21.9	21.9	19.95	22.0
28	105.9	106.0	17.8	105.9
29			15.1	

The ^{1}H-NMR of compound **3** showed a characteristic signal for 4α-methyl sterols of H-3α proton at 3.1 ($CDCl_3$) which appears as a predictable sextet[12] and this was a method used to demonstrate the presence of 4α-methyl sterols.

The methyl signals appeared at δ 0.94 (d, *J* = 6.5, 3H-21), 0.82 (d, *J* = 6.7, 3H-26), 0.78 (d, *J* = 6.7, 3H-27), 0.97 (d, *J* = 6.4, 3H-29), 0.87 (d, *J* = 6.7, 3H-28), 0.64 (3H-18), 0.82 (3H-19).

The olefinic double bond appeared at δ 5.15 (pseudo-triplet, *J* = 5.5), the value of coupling constant suggested the *cis* configuration of the C-22/C-23 double bond with ^{13}C-NMR at δ 131.64 and 135.90 (Table 1). The GC-MS fragmentation pattern of compound **3** showed a close similarity to the 4α,24-Dimethyl-cholest-22*E*-en-3β-ol[13]. By analysis of ^{1}H-^{1}H- COSY, HMQC and HMBC the steroid skeleton was determined as 4α,24-Dimethyl-cholest-22-*Z*-en-3β-ol. To the best of our knowledge, it is the first time to report the complete ^{13}C signal assignments of steroid **3.**

The MS of compound **4** exhibited to the molecular ion peak at 398.0. ^{1}H-NMR showed the exomethylen group at δ 4.64 (1H, br s) and 4.70 (1H, br s), the trisubstituted double bond at δ 5.31 (d, *J* = 5.2 Hz) and the oxymethine at δ 3.5 (m) with the ^{13}C-NMR signals at δ 156.9, 105.9, 140.7, 121.7 and 71.8 respectively.

Comparison of ^{13}C-NMR data of **4** with those previously reported of 24-methylencholesterol[14] showed a complete agreement. Compound **4** showed activity for treat various diseases associated with free radicals induced damage like cancer, diabetes[15].

The EIMS of compound **5** showed a molecular ion peak at m/z 535, $[M]^+$. The ^{1}H- and ^{13}C-NMR spectral data of **5** showed two methyl signals at δ 0.85 (t, J = 7.0 Hz), a strong methylen signal at δ 1.23 with ^{13}C- signals around 29.2~29.65, four olefin methine signals at δ 5.36, 5.37, 5.54 and 5.74 with ^{13}C- signals at δ 129.0, 129.2, 131.4, 133.5 in addition to, primary and secondary hydroxyl groups at δ 62.5 and 74.6. The strong IR absorption at 1625 cm^{-1} and a carbonyl signal at δ 174.0 with abroad signal at δ 6.25 (*J* = 7.2 Hz) revealed the presence of secondary amide group. By using ^{1}H-^{1}HCOSY, HMQC experiments, the connectivity between functional groups were determined, where the amide proton was coupled to the methine proton at δ 3.90 which was coupled to the primary and secondary hydroxyl groups at 3.68/3.92 and 4.3 respectively. The oxymethine proton at 4.3 was

coupled to the olefinic protons at δ 5.54 and 5.74 for H-4 and H-5 respectively which was connected to the H-8/H-9 double bond through two methylene protons at 2.1 and 2.08. The large values of the coupling constants for both double bond (J = 15.0 Hz) indicated to the *trans* configuration. HMBC experiments confirmed the connectivity for the ceramide skeleton. The long chain methylene groups were determined by two characteristic fragments ions at m/z 281 and 298. Therefore, the structure of compound **5** is N-acyl-sphinga-4 (E), 8(E)-dienine. The optical rotation of compound **5**: $[\alpha]^{23}_{D}$ = - 6.5 confirmed the structure of compound **5** is identical with the previously isolated ceramide from the gorgonian *Acabaria undulate*[16].

CONCLUSION

Careful study for ethyl acetate fraction of the soft coral *Litophyton arboreum* leads to isolation of four steroids and ceramide compounds. it is the first time to report the complete ^{13}C data assignments of steroid **3**.

The compounds isolated from marine organisms exhibited to interesting cytotoxicity for cancer cell lines (leukaemia and hela) as well as strong HIV-1PR inhibitory activity.

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