

Article

New 19-Oxygenated Steroids from the Soft Coral *Nephthea chabrolii*

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Received: 3 May 2012; in revised form: 30 May 2012 / Accepted: 30 May 2012 /

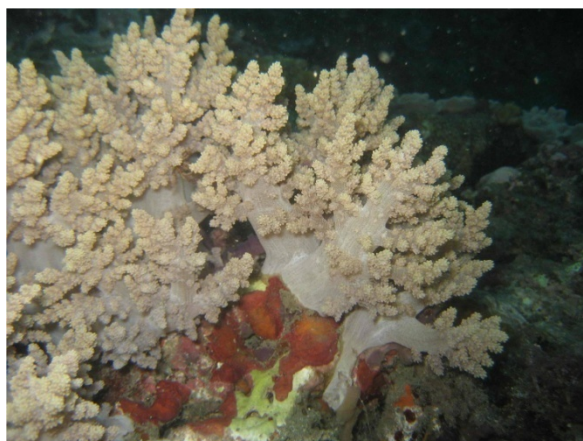
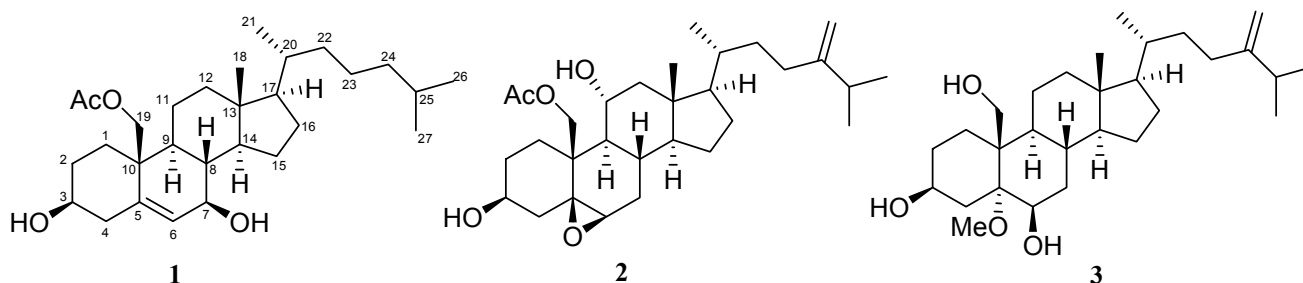
Published: 6 June 2012

Abstract: In order to search for novel bioactive substances from marine organisms, we investigated the acetone extract of the soft coral *Nephthea chabrolii* collected at San-Hsian-Tai, Taitong County, Taiwan. From this extract three new 19-oxygenated steroids, nebrosteroids N–P (1–3) were isolated. The structures of these compounds were elucidated by extensive spectroscopic analyses.

Keywords: *Nephthea Chabrolii*; 19-oxygenated steroids; cytotoxicity; anti-HCMV

1. Introduction

Numerous secondary metabolites including sesquiterpenoids, diterpenoids, meroditerpenoids, and steroids have been isolated from soft corals of the genus *Nephthea* [1–26]. Previous bioassay results on these materials showed them to exhibit diverse biological properties including cytotoxic [4–7,18,20], anti-inflammatory [13,14,23,26] and antimicrobial activities [19]. The acetone extract of the soft coral *Nephthea chabrolii* (Figure 1) collected off the San-Hsian-Tai coast, Taiwan, in July 2008 was found to be cytotoxic towards P-388 mouse lymphocytic leukemia cell lines. Chromatographic fractionation led to the isolation of three new compounds, nebrosteroids N–P (1–3) (Figure 2).

Figure 1. Soft coral *Nephthea chabrolii*.**Figure 2.** Structures of compounds 1–3.

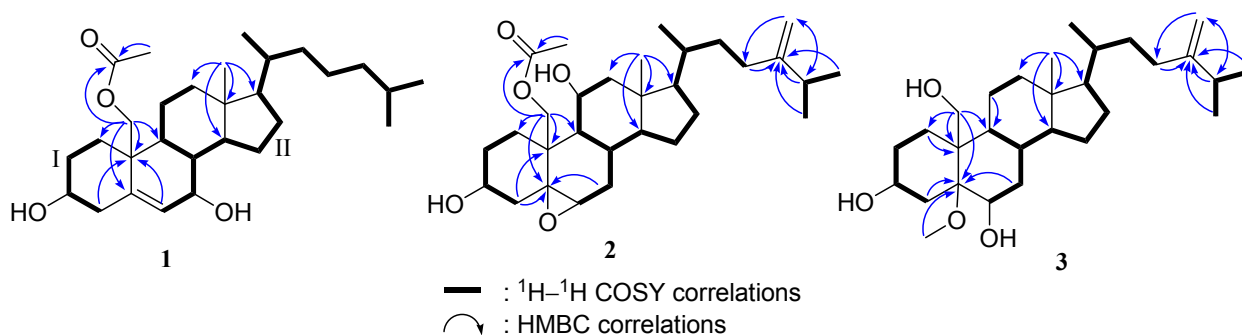
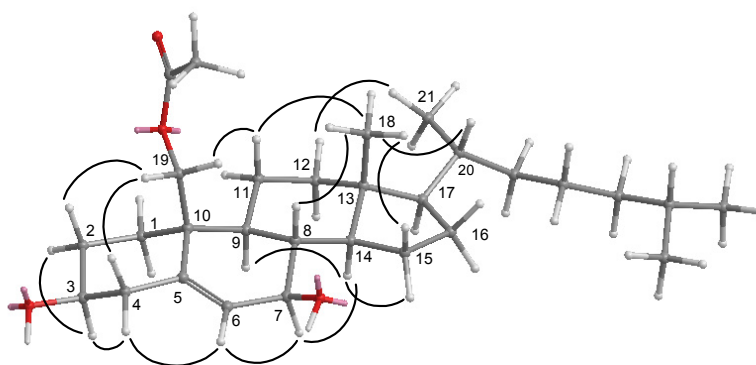
2. Results and Discussion

Nebrosteroid N (**1**) had a molecular formula of $C_{29}H_{48}O_4$ as established by interpretation of its HRESIMS and NMR data. The IR spectrum of **1** indicated the presence of hydroxyl(s) ($\nu_{\max}$ 3393 cm^{-1}) and an ester group ($\nu_{\max}$ 1738 cm^{-1}). Further, the ^1H NMR spectrum revealed the presence of a tertiary methyl (δ_{H} 0.71), three secondary methyls (δ_{H} 0.92, 0.87, and 0.86), and two oxymethines [δ_{H} 3.59 (1H, m), 3.82 (1H, d, $J = 7.6$ Hz)], and an oxymethylene [δ_{H} 3.99, 4.50 ($J_{\text{AB}} = 11.8$ Hz)]. The presence of a trisubstituted double bond was revealed by NMR signals [δ_{H} 5.54 (1H, br s), δ_{C} 129.6 (CH), 138.1 (C_q)] (Table 1). NMR data of **1** exhibited the presence of an acetoxyl group [δ_{H} 2.07 (3H, s), δ_{C} 21.1 (CH_3), 170.7 (C_q)]. The ^{13}C NMR and DEPT spectra of **1** contained resonances for eleven sp^3 methylenes, eight sp^3 methines, two quaternary sp^3 carbons, one sp^2 methine, one quaternary sp^2 carbon, and one carbonyl. Comparison of NMR chemical shift values of **1** with those of cholest-5-en-3 β ,7 β ,19-triol [27] reported from the black coral *Antipathes subpinnata* as well as its HMBC cross-peaks of H_2 -19/ C -1, C -5, C -9, C -10, carbonyl carbon at C -19 suggested that **1** may be a 19-acetyl analogue of cholest-5-en-3 β ,7 β ,19-triol. Interpretation of the ^1H - ^1H COSY spectrum led to partial structures I and II (Figure 3). Rings A and B were elucidated on the basis of HMBC cross-peaks (Figure 3) between H_2 -19/ C -1, C -5, C -9, C -10 and H_2 -4, H -6/ C -10, whereas rings C and D were completed based on HMBC correlations between H_3 -18/ C -12, C -13, C -14, C -17. The NOESY correlations (Figure 4) observed between H -11 β and H_3 -18, H -11 β and H -19, H -19 and H -4 β , H_3 -18 and H -8, H_3 -18 and H -20, H -3 and H -4 α , H -6 and H -7, H -9 and H -14, and H -7 and H -14 in **1** confirmed that nebrosteroid N (**1**) was cholest-5-en-3 β ,7 β ,19-triol 19-acetate.

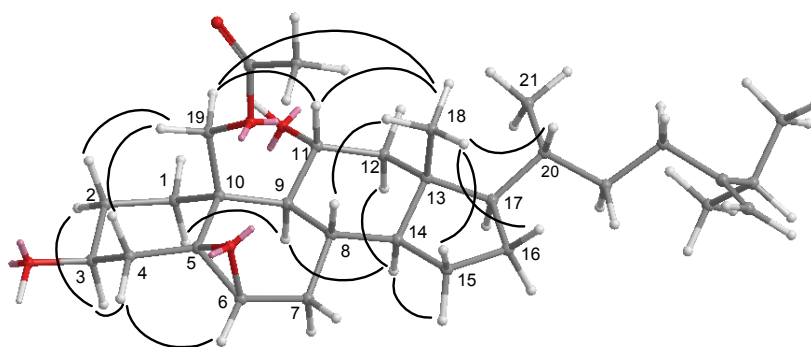
Table 1. ^1H and ^{13}C NMR data for compounds 1–3 measured in CDCl_3 .

Position	1		2		3	
	δ_{H}^a (J in Hz)	δ_{C}^b	δ_{H}^c (J in Hz)	δ_{C}^d	δ_{H}^a (J in Hz)	δ_{C}^b
1	α : 1.07 m β : 2.04 m	33.6	α : 1.34 m β : 2.65 m	35.4	α : 1.72 m β : 1.48 m	29.4
2	α : 1.87 m β : 1.44 m	31.7	α : 1.80 m β : 1.34 m	31.4	α : 1.86 m β : 1.33 m	31.0
3	3.59 m	71.1	3.72 m	69.1	3.88 m	67.5
4	α : 2.41 m β : 2.31 d (11.6)	41.8	α : 1.40 m β : 2.30 d (12.0)	42.7	α : 2.04 m β : 2.00 m	33.4
5		138.1		61.3		79.3
6	5.54 br s	129.5	2.96 d (2.5)	59.8	3.85 m	69.4
7	3.82 d (7.6)	72.4	α : 1.27 m β : 2.09 m	31.9	α : 1.52 m β : 1.48 m	33.4
8	1.65 m	41.9	1.70 m	29.6	2.17 m	31.4
9	1.04 m	48.3	0.80 m	57.3	1.42 m	45.0
10		39.5		38.9		43.3
11	α : 1.57 m β : 1.47 m	21.6	4.08 m	68.9	α : 1.02 m β : 1.46 m	21.5
12	α : 1.12 m β : 2.04 m	39.7	α : 1.16 m β : 2.24 m	50.8	α : 1.16 m β : 2.01 m	40.4
13		43.0		43.1		43.2
14	1.07 m	56.6	0.97 m	55.5	1.03 m	57.1
15	α : 1.79 m β : 1.42 m	26.1	α : 1.59 m β : 1.07 m	24.0	α : 1.54 m β : 1.03 m	24.0
16	α : 1.87 m β : 1.31 m	28.5	α : 1.90 m β : 1.30 m	28.2	α : 1.84 m β : 1.26 m	28.3
17	1.07 m	55.4	1.17 m	55.8	1.06 m	56.0
18	0.71 s	11.9	0.71 s	12.7	0.72 s	12.5
19	3.99 d (11.8) 4.50 d (11.8)	64.3	4.30 d (12.0) 4.93 d (12.0)	64.8	3.70 d (12.0) 4.26 d (12.0)	65.9
20	1.37 m	35.7	1.40 m	35.6	1.40 m	35.8
21	0.92 d (6.0)	18.7	0.95 d (6.5)	18.6	0.94 d (6.8)	18.7
22	0.99 m 1.33 m	36.2	1.13 m 1.52 m	34.4	1.14 m 1.53 m	34.7
23	1.14 m 1.32 m	23.8	1.87 m 2.09 m	30.9	1.86 m 2.10 m	31.0
24	1.12 m	39.5		156.7		156.9
25	1.50 m	28.0	2.22 m	33.8	2.23 m	33.8
26	0.86 d (6.4)	22.8	1.02 d (7.0)	22.0	1.02 d (6.8)	22.0
27	0.87 d (6.4)	22.5	1.03 d (7.0)	21.8	1.03 d (6.8)	21.9
28			4.65 s 4.72 s	106.0	4.66 s 4.71 s	105.9
OAc	2.07 s	21.1 170.7	2.11 s	21.2 170.7		
OMe					3.17 s	48.3

^a Spectra were measured at 400 MHz; ^b Spectra were measured at 100 MHz; ^c Spectra were measured at 500 MHz; ^d Spectra were measured at 125 MHz.

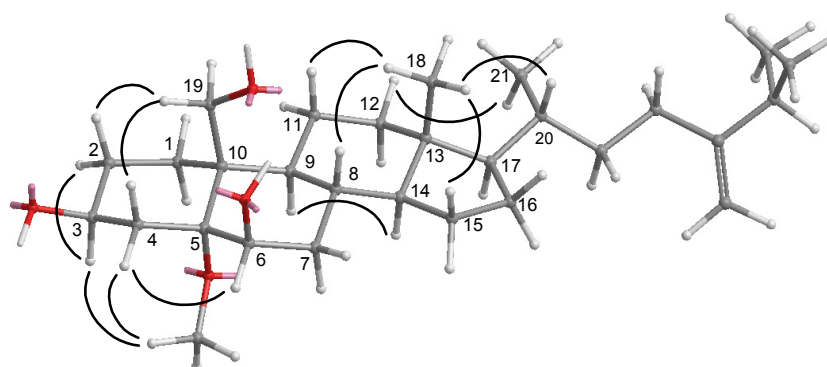
Figure 3. COSY and HMBC correlations of compounds 1–3.**Figure 4.** NOESY correlations of compound 1.

Nebrosteroid O (**2**) was assigned the molecular formula of $\text{C}_{30}\text{H}_{48}\text{O}_5$ based on its HRESIMS and ^{13}C NMR data. ^{13}C NMR spectra of **2** showed the presence of five methyls, ten sp^3 methylenes, nine sp^3 methines, one sp^2 methylene, three quaternary sp^3 carbons, and one quaternary sp^2 carbons. Analysis of the 1D and 2D NMR data (Table 1 and Figure 3) showed that **2** contains one primary acetox group [δ_{H} 2.11 (3H, s); δ_{C} 21.2 (q), 170.7 (s)], two secondary hydroxy groups at δ_{H} 3.72 (1H, m), 4.08 (1H, m) and δ_{C} 69.1 (d), 68.9 (d), one trisubstituted epoxy ring [δ_{H} 2.96 (1H, d, $J = 2.5$ Hz); δ_{C} 59.8 (d), 61.3 (s)] and one terminal methylene group [δ_{H} 4.65 (1H, s), 4.72 (1H, s); δ_{C} 156.7 (s), 106.0 (t)]. These spectral data resembled those for the armatinol A [16] except that **2** contained an additional hydroxyl function at C-11. The placement of this moiety was made on the basis of COSY (Figure 3) correlations between H-9, H-11 and H₂-12. The secondary hydroxyl was deduced to be α oriented based on H₂-19 and H₃-18 showing correlations to H-11 in the NOE spectrum (Figure 5).

Figure 5. NOESY correlations of compound 2.

Nebrosteroid P (**3**) had the formula of $C_{29}H_{50}O_4$ as determined by HRESIMS and NMR data thus required five double bond equivalents. The IR spectrum of **3** showed the absorptions for hydroxyl ($\nu_{\max}$ 3432 cm^{-1}) and terminal methylene ($\nu_{\max}$ 1639, 886 cm^{-1}). Its NMR data (Table 2) contain four methyls [δ_H 0.72 (3H, s), 0.94 (3H, d, $J = 6.8$ Hz), 1.02 (3H, d, $J = 6.8$ Hz), 1.03 (6H, d, $J = 6.8$ Hz); δ_C 12.5, 18.7, 21.9, 22.0], two oxymethines [δ_H 3.88 (1H, m), 3.85 (1H, m); δ_C 67.5, 69.4], one oxymethylene [δ_H 4.30 (1H, d, $J = 12.0$ Hz), 4.93 (1H, d, $J = 12.0$ Hz); δ_C 65.9], a terminal methylene signal [δ_H 4.66 (s), 4.71 (s); δ_C 105.9 (CH_2), 156.9 (qC)], and a methoxy group [δ_H 3.17 (3H, s); δ_C 48.3]. The ^{13}C NMR and DEPT spectra of **3** contained ten sp^3 methylenes, eight sp^3 methines, three quaternary sp^3 carbons, one sp^2 methine, and one quaternary sp^2 carbon. These spectra data resembled those of armatinol B [16] except that **3** contained a tertiary methoxy instead of a tertiary hydroxyl at C-5. Based on the HMBC correlation from methoxyl protons to C-5 (Figure 3) and NOESY correlations from methoxyl protons to H-3 (Figure 6), nebrosteroid P was elucidated as 5 α -methoxy-24-methylene cholestan-3 β ,6 β ,19-triol.

Figure 6. NOESY correlations of compound **3**.



Nebrosteroids N–P (**1–3**) exhibited cytotoxicity against P-388 cell line with ED_{50} of 0.9, 1.2, and 1.7 $\mu\text{g/mL}$, respectively (see Table 2). Nebrosteroids N–P (**1–3**) were also examined for their antiviral activity towards human cytomegalovirus (HCMV) using a human embryonic lung (HEL) cell line; all compounds were found to be inactive.

Table 2. Cytotoxicity and Anti-HCMV Activity of **1–3**.

Compounds	ED_{50} ($\mu\text{g/mL}$)				
	A549	HT-29	P-388	HEL	Anti-HCMV
1	6.7	9.5	0.9	23.5	>100
2	5.9	5.9	1.2	15.4	>100
3	7.2	9.5	1.7	16.1	>100
mithramycin	0.18	0.21	0.15	NT	NT

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were determined with a JASCO P1020 digital polarimeter. UV and IR spectra were obtained on JASCO V-650 and JASCO FT/IR-4100 spectrophotometers, respectively. NMR

spectra were recorded on a Varian MR 400 NMR spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C or on a Varian Unity INOVA 500 FT-NMR spectrometer at 500 MHz for ^1H and 125 MHz for ^{13}C , respectively. ^1H NMR chemical shifts are expressed in δ referring to the solvent peak δ_{H} 7.27 for CDCl_3 , and coupling constants are expressed in Hz. ^{13}C NMR chemical shifts are expressed in δ referring to the solvent peak δ_{C} 77.0 for CDCl_3 . MS were recorded by a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, Darmstadt, Germany, 230–400 mesh) and LiChroprep RP-18 (Merck, 40–63 μm) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254} , 0.25 mm) and precoated RP-18 $\text{F}_{254\text{s}}$ plates (Merck) were used for thin-layer chromatography (TLC) analysis. High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV detector at 220 nm together with a semi-preparative reversed-phase column (Merck, Hibar LiChrospher RP-18e, 5 μm , 250 $\times$ 25 mm).

3.2. Biological Material

The soft coral *N. chabroliei* was collected by hand using scuba at San-Hsian-Tai, Taitong County, Taiwan, in July 2008 at a depth of 12 m and stored in a freezer until extraction. The voucher specimen (SST-22) was identified by Chang-Feng Dai, National Taiwan University and deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Taiwan.

3.3. Extraction and Isolation

A specimen of soft coral *N. chabroliei* (2.0 kg) was minced and extracted with acetone (3 L $\times$ 4) at room temperature. The combined acetone extracts were then partitioned between H_2O and EtOAc. The resulting EtOAc extract (24.9 g) was subjected to gravity silica gel 60 column chromatography (Si 60 CC) using *n*-hexane and *n*-hexane/EtOAc of increasing polarity, to give 20 fractions. The fraction 13 (0.65 g), eluted with EtOAc, was further subjected to Si 60 CC (EtOAc) to give 7 subfractions. A subfraction 13-6 (299 mg), was separated by a RP-18 flash column (MeOH/ H_2O , 45:55 to 100% MeOH) to give 12 fractions. The subfraction 13-6-11, eluted with MeOH/ H_2O (90:10), was purified by RP-18 HPLC (MeOH/ H_2O , 95:5) to afford **3** (2.4 mg). Likewise, the subfraction 13-7 (177 mg), was separated by a RP-18 flash column (MeOH/ H_2O , 45:55 to 100% MeOH) to give 6 fractions. In turn, a subfraction 13-7-6, eluted with MeOH, was further purified by RP-18 HPLC (MeOH/ H_2O , 90:10) to afford **1** (1.9 mg) and **2** (0.7 mg).

Nebrosteroid N (**1**): White amorphous powder; $[\alpha]_{\text{D}}^{25} +12.8$ (*c* 0.1, CHCl_3); IR (neat) ν_{max} 3393, 2930, 2867, 1738, 1466, 1383, 1237, 1042, 970 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) and ^{13}C NMR (CDCl_3 , 100 MHz) data in Table 1; HRESIMS m/z 483.3448 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{29}\text{H}_{48}\text{O}_4\text{Na}$, 483.3450).

Nebrosteroid O (**2**): White amorphous powder; $[\alpha]_{\text{D}}^{25} -32.2$ (*c* 0.1, CHCl_3); IR (neat) ν_{max} 3394, 2926, 2861, 1737, 1644, 1549, 1461, 1371, 1242, 1044, 889 cm^{-1} ; ^1H NMR (CDCl_3 , 500 MHz) and ^{13}C NMR (CDCl_3 , 125 MHz) data in Table 1; HRESIMS m/z 511.3398 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{48}\text{O}_5\text{Na}$, 511.3399).

Nebrosteroid P (**3**): White amorphous powder; $[\alpha]_{\text{D}}^{25} -44.0$ (*c* 0.1, CHCl_3); IR (neat) ν_{max} 3432, 2950, 2871, 1639, 1458, 1375, 1062, 1028, 886 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) and ^{13}C NMR (CDCl_3 , 100 MHz) data in Table 2; HRESIMS m/z 485.3604 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{29}\text{H}_{50}\text{O}_4\text{Na}$, 485.3607).

3.4. Cytotoxicity Assay

Cytotoxicity was determined on P-388 (mouse lymphocytic leukemia), HT-29 (human colon adenocarcinoma), and A-549 (human lung epithelial carcinoma) tumor cells using a modification of the MTT colorimetric method according to a previously described procedure [28,29]. The provision of the P-388 cell line was supported by J.M. Pezzuto, formerly of the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago. HT-29 and A-549 cell lines were purchased from the American Type Culture Collection. To measure the cytotoxic activities of tested compounds, five concentrations with three replications were performed on each cell line. Mithramycin was used as a positive control.

3.5. Anti-HCMV Assay

To determine the effects of natural products upon HCMV cytopathic effect (CPE), confluent human embryonic lung (HEL) cells grown in 24-well plates were incubated for 1 h in the presence or absence of various concentrations of tested natural products with three replications. Ganciclovir was used as a positive control. Then, cells were infected with HCMV at an input of 1000 pfu (plaque forming units) per well of a 24-well dish. Antiviral activity was expressed as IC_{50} (50% inhibitory concentration), or compound concentration required to reduce virus induced CPE by 50% after 7 days as compared with the untreated control. To monitor the cell growth upon treating with natural products, an MTT-colorimetric assay was employed [30].

4. Conclusion

The first investigation of soft coral *N. chabroliei* collected at San-Hsian-Tai (Taitong County, Taiwan) has led to the isolation of three new 19-oxygenated steroids, nebrosteroids N–P (**1–3**). Nebrosteroids N–P (**1–3**) exhibited cytotoxicity against P-388 cell line with ED_{50} of 0.9, 1.2, and 1.7 $\mu\text{g/mL}$, respectively. However, previously isolated cholestene derivatives, nebrosteroids I–K [13] did not show cytotoxicity. In order to rule out the possibility of **3** as an isolation artifact, a solution of **2** was kept at room temperature for three days in the presence of Si-60 or RP-18 gel in MeOH. However, the formation of **3** was not observed.

Acknowledgments

This research was financially supported by grants from the National Science Council (NSC99-2628-B-110-002-MY3) and Ministry of Education of Taiwan awarded to C.-Y.D.

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Samples Availability: Not available.

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