New Cytotoxic Sesquiterpenes from the Gorgonian *Isis hippuris*

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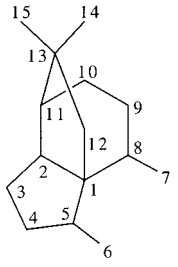
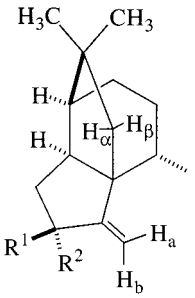
Received May 31, 2000

Five new suberosane sesquiterpenes, suberosenol A (**1**), suberosenol B (**2**), suberosanone (**3**), suberosenol A acetate (**4**), and suberosenol B acetate (**5**), along with the known sesquiterpene subergorgic acid (**6**), have been isolated from the gorgonian *Isis hippuris*. The structures of these metabolites were established by spectroscopic and chemical methods. Metabolites **1** and **3**−**5** were found to exhibit potent cytotoxicity toward P-388, A549, and HT-29 cancer cell lines.

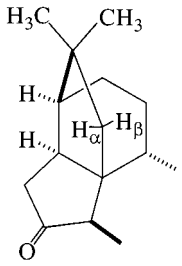
Previous studies on chemical constituents of the gorgonian *Isis hippuris* L. (phylum Cnidaria, order Gorgonacea, family Isididae)[1] had led to the isolation of several highly oxygenated steroids, which possess a special acetal functionality in their structures.[2−5] As part of our continuing study on discovery of bioactive metabolites from the Taiwanese gorgonacea octocorals,[6−10] the gorgonian *I. hippuris* has been the subject of an investigation due to the impressive cytotoxicity of its organic extract toward P-388 (mouse lymphocytic leukemia) tumor cells (ED_{50} = 5×10^{-6} μg/mL). This study has led to the isolation of five new suberosane sesquiterpenes, suberosenol A (**1**), suberosenol B (**2**), suberosanone (**3**), suberosenol A acetate (**4**), and suberosenol B acetate (**5**), along with the known sesquiterpene subergorgic acid (**6**).[11] It was found that only two suberosane-related compounds, suberosenone (**7**)[12,13] and its dimer, alertenone,[13] have been isolated previously from marine organisms. Subergorgic acid has been found to be cardiotoxic,[11] whereas suberosenone has been shown to exhibit potent cytotoxicity toward several cancer cell lines.[12,14] In the cytotoxicity testing of metabolites **1**−**6**, we observed that **1** and **3**−**5** exhibited potent cytotoxicity toward P-388, A549 (human lung adenocarcinoma), and HT-29 (human colon adenocarcinoma) cancer cell lines.



suberosane



1: R^1 = OH, R^2 = H
2: R^1 = H, R^2 = OH
4: R^1 = OAc, R^2 = H
5: R^1 = H, R^2 = OAc
7: R^1, R^2 = O



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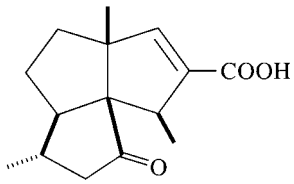

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Results and Discussion

The gorgonian *I. hippuris* was frozen immediately after collection and subsequently freeze-dried. The freeze-dried organism was extracted with *n*-hexane to afford a crude extract. The crude extract was separated by extensive column chromatography on Si gel and afforded sesquiterpenes **1**−**6**, see Experimental Section.

Suberosenol A (**1**) was obtained as a white powder. Its HREIMS established the molecular formula $C_{15}H_{24}O$. Thus, four degrees of unsaturation were determined for the molecule of **1**. The IR spectrum of **1** showed the presence of a hydroxyl group (ν_{max} 3260 cm^{-1}). From the ^{13}C spectral data of **1** (Table 1), an exocyclic olefin (δ 162.6, s; 104.1, t) and three methyls (δ 17.5, q; 26.9, q; 35.1, q) were deduced. Furthermore, signals for two olefinic protons (δ 5.06, 1H, d, J = 2.5 Hz; 4.79, 1H, d, J = 2.5 Hz) and three methyls (δ 1.20, 3H, s; 1.11, 3H, s; 0.88, 3H, d, J = 7.0 Hz) were also observed in the ^1H NMR spectrum of **1** (Table 1). From the above data, metabolite **1** should be a sesquiterpene containing three rings. The gross structure of **1** and all of the ^1H and ^{13}C chemical shifts associated with the molecule were determined by a series of 2D NMR studies [HMBC, $^1H-^1H$ COSY (Table 1 and Figure 1), and HMQC]. From the $^1H-^1H$ COSY spectrum of **1**, it was possible to establish the proton sequences from H-2 to H_2-3, H_2-3 to H-4, H_3-7 to H-8, H-8 to H_2-9, H_2-9 to H_2-10, H_2-10 to H-11, and H-11 to H-2. These data, together with the $^1H-^{13}C$ long-range correlations observed in an HMBC experiment on **1**, established the molecular framework of sesquiterpene **1**.

The relative stereochemistry of **1** was deduced from a 2D NOE experiment (Table 1 and Figure 2). It was found that H-2 shows NOE correlations with H_3-7, and H-11 and does not exhibit correlation with H_2-12, indicating that H-2, H_3-7 and H-11 are situated on the same face of the six-membered ring and were assigned as the α-protons because the C-12 methylene is the β-substituent at C-1. H-4 was found to exhibit strong NOE response with H-2, revealing the α-orientation of this proton. Based on the above observations, the structure of **1**, including the relative stereochemistry, was elucidated unambiguously.

Suberosenol B (**2**) was isolated as a white powder, and a molecular formula of $C_{15}H_{24}O$ was established by HREIMS. The spectral data (IR, MS, 1D and 2D NMR) of **2** were very

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10.1021/np000271n CCC: $19.00
Published on Web 10/24/2000

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts, HMBC, ^{1}H−^{1}H COSY, and NOESY Correlations for **1**

C/H	^{1}H[a]	^{13}C[b]	HMBC	^{1}H−^{1}H COSY	NOESY
1		58.2 (s)[d]	H-2, H_2-3, H_2-6, H-8, H_3-7, H_2-9, H_2-12		
2	1.97 dt (14.0; 7.0)[c]	46.0 (d)	H_2-3, H-8, H_2-10, H-11, H_2-12	H_2-3, H-11	H-3α, H-4, H_3-7, H-11
3α	2.14 dt (11.0; 7.0)	39.0 (t)	H-2, H-4, H-11	H-2, H-3*β*, H-4	H-2, H-4
β	1.80 m			H-2, H-3α, H-4	H_3-15
4	4.30 m	77.5 (d)	H_2-3, H_2-6	H_2-3	H-2, H-13α
5		162.6 (s)	H_2-3, H_2-6		
6a	4.79 d (2.5)	104.1 (t)		H-6b	H-6b, H-8
b	5.06 d (2.5)			H-6a	H-6a
7	0.88 d (7.0)	17.5 (q)	H-8, H_2-9	H-8	H-2, H-9α
8	1.96 m	38.2 (d)	H-2, H_3-7, H_2-9, H_2-10, H_2-12	H_3-7, H_2-9	H-6a
9α	1.23 m	26.3 (t)	H_3-7, H-8, H_2-10,	H-8, H-9*β*, H_2-10	H-10α, H_3-7
β	2.02 m		H-11	H-8, H-9α, H_2-10	H_3-14
10α	1.49 dt (14.0; 2.5)	28.1 (t)	H-2, H-8, H_2-9, H-11	H_2-9, H-10*β*, H-11	H-9α
β	1.61 br d (14.0)			H_2-9, H-10α, H-11	H-9*β*, H_3-14
11	1.78 m	49.3 (d)	H-2, H_2-3, H_2-9, H_2-10, H_3-14, H_3-15	H-2, H_2-10	H-2
12α	1.77 d (14.0)	55.5 (t)	H-2, H-8, H_3-14,	H-12*β*	
β	1.82 d (14.0)		H_3-15	H-12α	H_3-14
13		39.1 (s)	H-2, H_2-10, H-11, H_3-14, H_3-15		
14	1.11 s	26.9 (q)	H-11, H_2-12, H_3-15		H-9*β*, H-10*β*, H-12*β*
15	1.20 s	35.1 (q)	H-11, H_2-12, H_3-14		H-3*β*

[a] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. [b] 125 MHz in $CDCl_3$ at 25 °C. [c] *J* values (in Hz) in parentheses. [d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are in ppm downfield from TMS.

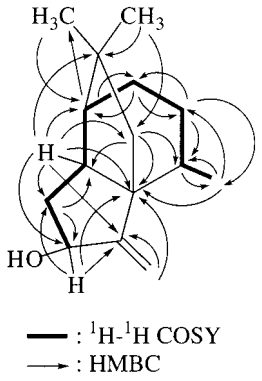


Figure 1. ^{1}H−^{1}H COSY and selective HMBC correlations for **1**.

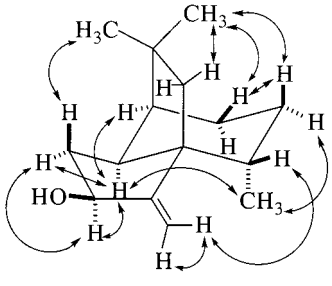


Figure 2. Selective NOE correlations of **1**.

similar to those of **1**. However, the melting point (74−75 °C) and optical rotation ([α]$^{25}_D$ −10°, *c* 0.1, $CHCl_3$) of **2** were substantially different from those of **1** (mp 106−108 °C; [α]$^{25}_D$ −232°, *c* 0.1, $CHCl_3$), indicating that these two compounds are isomers. Furthermore, by comparison of the related ^{1}H and ^{13}C NMR spectral data of **2** (Tables 2 and 3) with those of **1**, it was revealed that the hydroxyl group at the C-4 position in **2** should be α-oriented. In the NOESY experiment of **2**, H-4 correlated with H-3*β* but not with H-2, also confirmed that suberosenol B (**2**) was the C-4 epimer of suberosenol A (**1**).

Suberosanone (**3**) had the same molecular formula as those of metabolites **1** and **2**, $C_{15}H_{24}O$, as determined by HREIMS. The IR spectrum of **3** revealed the presence of a ketone carbonyl group (ν_{max} 1738 cm^{-1}) and absence of the hydroxyl group. From the ^{13}C NMR spectral data of **3**, a ketone carbonyl group could be identified by the signal of a carbon that appeared at δ 220.5 (s). Therefore, compound **3** was tricyclic. ^{13}C and DEPT spectra showed the presence of four methyls, four methylenes, four methines, and three quaternary carbons. The gross structure of **3** was determined by the assistance of 2D NMR experiments, including ^{1}H−^{1}H COSY and HMBC. C-4 was identified as the carbonyl carbon based on the connectivities between the carbonyl carbon at δ 220.5 (s) and H-2 (δ 2.38), H_2-3 (δ 2.48, 2.35), H-5 (δ 2.39), and H_3-6 (δ 0.90) in the HMBC spectrum. In the NOESY experiment of **3**, H-5 was found to exhibit strong NOE responses with H-2 and H_3-7, revealing the α-orientation of this proton. Furthermore, H_3-6 was found to exhibit NOE response with H-8, but not with H_3-7, indicating the *β*-orientation of the C-6 methyl. Based on the above observations, the structure of **3**, including the relative configuration, was established unambiguously.

Suberosenol A acetate (**4**) had the molecular formula $C_{17}H_{26}O_2$, as determined by HREIMS. Thus, **4** contained five degrees of unsaturation. The IR absorption of **4** showed the presence of a carbonyl group (ν_{max} 1739 cm^{-1}). A carbonyl resonance in the ^{13}C NMR spectrum of **4** at δ 171.0 (s) (Table 3) confirmed the presence of an ester in the molecule. In the ^{1}H NMR spectrum of **4** (Table 2), an acetate methyl signal (δ 2.12, 3H, s) was observed. It was found that the NMR spectra (^{1}H and ^{13}C) of **4** were very similar to those of **1**, except that **4** showed signals corresponding to an additional acetoxyl group. Also, the ^{1}H NMR spectra revealed that the signals corresponding to the hydroxy-bearing C-4 methine group in **1** (δ 4.30) were shifted downfield in **4** (δ 5.35), indicating that metabolite **4** is the 4-acetyl derivative of sesquiterpene **1**. Acetylation of **1** yielded a less polar product, which was found to be identical with compound **4** by comparison of the physical (optical rotation) and spectral (MS, IR, ^{1}H and ^{13}C NMR)

Table 2. ^{1}H Chemical Shifts of Sesquiterpenes **2**−**5**[a]

proton	2	3	4	5
2	2.39 dd (11.5; 7.5)[b]	2.38 m	1.97 dt (14.0; 6.5)	2.35 dd (12.0; 7.5)
3α	1.79 ddd (13.5; 7.5; 4.5)	2.48 dd (7.0; 1.5)	2.22 dt (11.0; 6.5)	1.82 dd (13.5; 7.5)
β	2.08 ddd (13.5; 6.5; 4.5)	2.35 dd (7.0; 3.0)	1.90 m	2.14 dd (13.5; 5.5)
4	4.57 dd (5.5; 4.5)		5.35 m	5.57 d (5.5)
5		2.39 q (7.0)		
6a	4.79 s	0.90 d (7.0)	4.79 d (2.5)	4.86 s
b	5.06 s		4.96 d (2.5)	5.28 s
7	0.96 d (7.0)	1.04 d (7.0)	0.90 d (7.0)	0.92 d (7.0)
8	2.01 m	1.89 q (7.0)	1.99 m	2.00 m
9α	1.29 m	1.33 dd (7.0; 7.0)	1.26 m	1.28 m
β	1.99 m	2.06 m	1.97 m	2.05 m
10α	1.51 m	1.62 ddd (13.5, 6.5, 2.5)	1.49 m	1.52 m
β	1.62 m	1.68 m	1.62 m	1.61 m
11	1.79 m	1.79 br t (3.0)	1.78 m	1.79 m
12α	1.59 d (14.0)	1.34 d (14.5)	1.77 d (14.0)	1.62 d (14.5)
β	1.67 d (14.0)	1.40 d (14.5)	1.87 d (14.0)	1.69 d (14.5)
14	1.11 s	1.04 s	1.11 s	1.11 s
15	1.17 s	1.09 s	1.20 s	1.17 s
acetate methyl			2.12 s	2.01 s

[a] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. [b] *J* values (in Hz) in parentheses. The values are ppm downfield from TMS.

Table 3. ^{13}C Chemical Shifts of Sesquiterpenes **2**−**5**[a]

C	2	3	4	5
1	59.1 (s)[b]	56.9 (s)	58.1 (s)	59.0 (s)
2	49.0 (d)	43.8 (d)	45.6 (d)	49.3 (d)
3	37.8 (t)	40.7 (t)	35.0 (t)	35.8 (t)
4	76.4 (d)	220.5 (s)	77.8 (d)	78.5 (d)
5	162.2 (s)	50.0 (s)	157.1 (s)	157.5 (s)
6	107.5 (t)	8.1 (q)	105.7 (t)	110.7 (t)
7	17.8 (q)	17.0 (q)	17.4 (q)	17.5 (q)
8	36.9 (d)	35.6 (d)	37.8 (d)	37.4 (d)
9	26.5 (t)	26.9 (t)	26.2 (t)	26.5 (t)
10	27.9 (t)	28.3 (t)	27.9 (t)	27.8 (t)
11	49.7 (d)	49.6 (d)	49.4 (d)	49.5 (d)
12	54.9 (t)	47.7 (t)	55.1 (t)	55.0 (t)
13	39.6 (s)	39.2 (s)	39.1 (s)	39.6 (s)
14	26.9 (q)	27.0 (q)	26.9 (q)	26.9 (q)
15	34.8 (q)	34.3 (q)	35.1 (q)	34.9 (q)
acetate methyl			21.3 (q)	21.5 (q)
acetate carbonyl			171.0 (s)	170.7 (s)

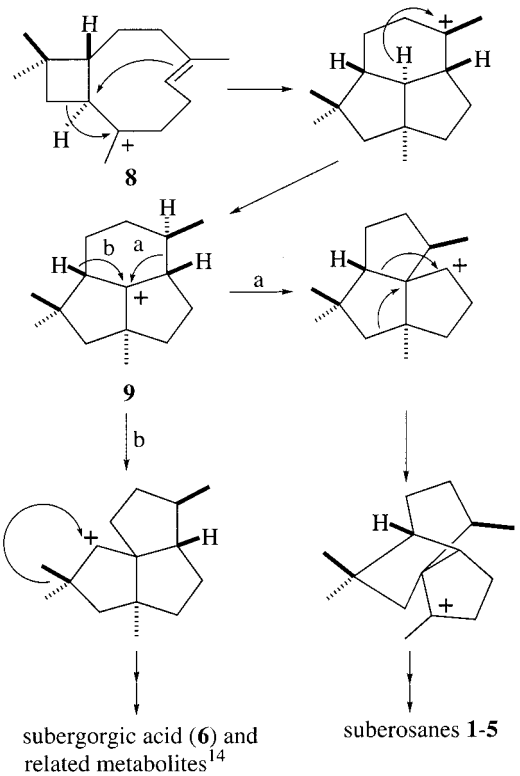
[a] Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C. [b] Multiplicity deduced by DEPT and indicated by usual symbols. The values are ppm downfield from TMS.

data. Thus, the structure of **4**, including the relative stereochemisty, was established.

Suberosenol B acetate (**5**) had the same molecular formula as that of **4**. The spectral data [IR, MS, ^{1}H and ^{13}C NMR (Tables 2 and 3)] of **5** were very similar to those of **4**, indicating that these two compounds are isomers. In the ^{1}H NMR spectrum of **5**, an acetate methyl was observed at *δ* 2.01 (3H, s). An HMBC experiment on **5** further revealed the connectivity between H-4 (*δ* 5.57) and the carbonyl carbon (*δ* 170.7) of the acetate and demonstrated the location of the acetate to be at C-4. In the NOESY experiment of **5**, H-4 (*δ* 5.57) showed correlations with H-3*β*, but not with H-2, which confirmed the *β*-orientation of H-4. Thus, suberosenol B acetate (**5**) was assigned as the C-4 epimer of suberosenol A acetate (**4**). Furthermore, acetylation of suberosenol B (**2**) yielded a compound that was found to be identical with sesquiterpene **5** by comparison of the physical (optical rotation) and spectral (IR, MS, ^{1}H and ^{13}C NMR) data.

Metabolite **6** was identified as a known sesquiterpene, subergorgic acid, which had been isolated from a Pacific gorgonian, *Subergorgia suberosa*. Its physical (mp) and spectral (IR, MS, ^{1}H, and ^{13}C NMR) data are in full agreement with those reported previously.[11] Compound **6** and its analogues were isolated later from an Indian gorgonian *S. suberosa*.[14]

Scheme 1. The Plausible Biogenetic Pathways of Suberosanes (**1**−**5**) and Subergorgic Acid (**6**), Based on the Proposals of Coates and Bohlmann[15,16]



The co-occurrence of the two different skeletons, suberosanes (**1**−**5**) and subergorgic acid (**6**) supports the fascinating suggestions of Coates[15] and Bohlmann[16] that compounds of these types are biogenetically derived from ring-expansion of the caryophyllenyl cation (**8**), following by *π*-cyclization, hydride 1,3-shift to a key branchpoint cationic intermediate **9**, and the following series of carbonium ion rearrangements *via* two different pathways (Scheme 1).

The cytotoxicity of compounds **1**−**6** toward P-388, A549, and HT-29 cancer cell lines was evaluated, and the results are shown in Table 4. These data show that suberosane sesquiterpenes **1**−**5** were significantly cytotoxic against the proliferation of the above cells, whereas subergorgic acid (**6**) was inactive. The most active compound is **1**, which

Table 4. Cytotoxic Data of Sesquiterpenes **1**−**6**[a]

compound	cell lines ED_{50} (μg/mL)		
	P-388	A549	HT-29
1	$< 5.0 \times 10^{-6}$	5.1×10^{-3}	$< 5.0 \times 10^{-6}$
2	3.4	0.2	2.1
3	$< 5.0 \times 10^{-6}$	3.6×10^{-2}	$< 5.0 \times 10^{-6}$
4	7.6×10^{-3}	8.0×10^{-2}	3.6×10^{-4}
5	7.4×10^{-2}	3.6×10^{-1}	5.0×10^{-3}
6	13.3	> 50	> 50

[a] For significant activity of pure compounds, an ED_{50} value of ≤ 4.0 μg/mL is required. See Geran et al.[19]

contains a β-hydroxyl group at the allylic position of the 5,6-double bond and exhibited potent cytotoxicity toward the above three cancer cells. Compound **3**, which lacks the α,β-unsaturated carbonyl functionality as that in the molecule of suberosenone, also displayed potent activity against the growth of P-388 and HT-29 cells, suggesting that the molecular skeleton, not the functionalities, is the main factor for the potent cytotoxicity of these suberosane terpenoids. Furthermore, compound **4**, which is the acetyl derivative of **1**, was found to be less cytotoxic than **1**, revealing that the substitution may also influence the cytotoxicity of these compounds. The above results indicate that the suberosane-related compounds, both natural and synthetic,[15] may warrant further biological studies for the discovery of the useful anticancer drugs in the future.

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Hitachi I-2001 infrared spectrophotometer. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C, in $CDCl_3$ using TMS as an internal standard. EIMS spectra were obtained with a VG Quattro GC/MS spectrometer operating at 70 eV. HREIMS spectra were recorded on a JMX-HX 110 mass spectrometer. Si gel (Merck, 230−400 mesh) was used for column chromatography.

Animal Material. The gorgonian *I. hippuris* was collected by hand using scuba at Green Island, located on the southeast coast of Taiwan, in February 1999, at depths of 25 m, and was stored in a freezer until extraction. Colonies of *I. hippuris* are branched, with thick coenenchymas and articulated axes. The axis consists of brown horny nodes and white calcareous internodes. The internodes are symmetrically sculptured, with ridges and furrows alternating around the whole circumference. The sclerites are diverse in shape and size. The colony morphology and the shapes of sclerites indicate that the sample is *I. hippuris*.[1,17] A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University (specimen no. GISC-102).

Extraction and Isolation. The gorgonian (1.0 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with *n*-hexane (5 L × 3). The *n*-hexane-soluble fraction exhibited potent cytotoxicity toward P-388 tumor cells ($ED_{50} = 5 \times 10^{-6}$ μg/mL). The organic extract was evaporated to give a dark green residue (8.7 g). The mixture was separated by Si gel column chromatography, using *n*-hexane and *n*-hexane−EtOAc mixtures of increasing polarity. Compound **5** was eluted with *n*-hexane−EtOAc (40:1), **4** with *n*-hexane−EtOAc (35:1), **3** with *n*-hexane−EtOAc (30:1), **2** with *n*-hexane−EtOAc (15:1), **1** with *n*-hexane−EtOAc (12:1), **6** with *n*-hexane−EtOAc (7:1).

Suberosenol A (1): white powder (14.6 mg); mp 106−108 °C; $[\alpha]^{25}_D$ −232° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3260 and 883 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* 220 $[M]^+$ (11), 205 (12), 202 (3), 187 (6), 163 (100), and 149 (24); HREIMS *m/z* 220.1827 (calcd for $C_{15}H_{24}O$, 220.1821).

Suberosenol B (2): white powder (8.6 mg); mp 74−75 °C; $[\alpha]^{25}_D$ −10° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3370 and 890 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 220 $[M]^+$ (14), 205 (20), 202 (9), 187 (16), 163 (100), and 149 (54); HREIMS *m/z* 220.1828 (calcd for $C_{15}H_{24}O$, 220.1821).

Suberosanone (3): colorless oil (4.6 mg); $[\alpha]^{25}_D$ −60° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 1738 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 220 $[M]^+$ (57), 205 (11), 191 (2), 164 (16), 163 (21), and 137 (100); HREIMS *m/z* 220.1828 (calcd for $C_{15}H_{24}O$, 220.1821).

Suberosenol A acetate (4): colorless oil (2.2 mg); $[\alpha]^{25}_D$ −110° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 1739, 1238, and 892 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 262 $[M]^+$ (0.4), 220 (3), 205 (11), 202 (8), 187 (4), 163 (60), 149 (3), 145 (100), and 43 (70); HREIMS *m/z* 262.1933 (calcd for $C_{17}H_{26}O_2$, 262.1926).

Suberosenol B acetate (5): colorless oil (0.6 mg); $[\alpha]^{25}_D$ −8° (*c* 0.03, $CHCl_3$); IR (KBr) ν_{max} 1740, 1239, and 890 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 262 $[M]^+$ (0.4), 220 (5), 205 (10), 202 (10), 187 (5), 163 (67), 149 (6), 145 (100), and 43 (59); HREIMS *m/z* 262.1928 (calcd for $C_{17}H_{26}O_2$, 262.1926).

Acetylation of Suberosenol A (1). Suberosenol A (**1**) (4.6 mg) was stirred with 1 mL of Ac_2O in 1 mL of pyridine for 48 h at room temperature. After evaporation of excess reagent, the residue was separated by column chromatography on Si gel to give pure suberosenol A acetate (**4**) (*n*-hexane−EtOAc, 35:1; 4.3 mg, 78%). Physical and spectral data were in full agreement with those of the natural product **4**.

Acetylation of Suberosenol B (1). Following the above procedure, suberosenol B (**2**) (1.7 mg) was acetylated to produce suberosenol B acetate (**5**) (*n*-hexane−EtOAc, 40:1; 1.6 mg, 84%). Physical and spectral data were in full agreement with those of the natural product **5**.

Cytotoxicity Testing. P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A549 and HT-29 were purchased from the American Type Culture Collection. Cytotoxicity assays of the tested compounds **1**−**6** were carried out by a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[18] The cultured cells were treated at eight concentrations of pure test compounds ranging from 50 to 5×10^{-6} μg/mL. All assays were performed in triplicate. The results were expressed as a percentage, relative to control incubations, and the effective dose required to inhibit cell growth by 50% (ED_{50}) was determined.

Acknowledgment. We thank Prof. Chang-Feng Dai (Institute of Oceanography, National Taiwan University) for identification of the specimen.

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NP000271N