# Excavatolides F−M, New Briarane Diterpenes from the Gorgonian *Briareum excavatum*

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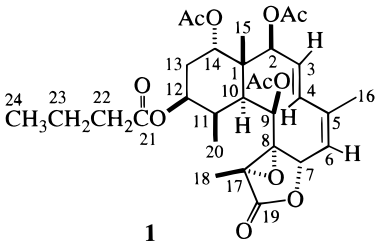
Eight new briarane-type diterpenes, excavatolides F−M **(1**−**8)**, have been isolated from the gorgonian *Briareum excavatum*. The structures and relative stereochemistry of these compounds were established by spectral analysis and chemical methods. The cytotoxicity of these compounds toward various cancer cell lines has also been determined.

Studies on the chemical constituents of the gorgonian corals of the genus *Briareum* have led to the isolation of a series of novel oxygenated diterpenes including briaranes and asbestinins.[1] Among these metabolites, briarane diterpenoids continue to be the target of many investigations because of their structural complexity and interesting biological activities (e.g., cytotoxic,[2−6] antiinflammatory,[7−9] antiviral,[2,9] insecticidal,[10,11] antifouling,[12] and immunomodulatory effects[13]). The genus *Briareum* is placed taxonomically within the order Gorgonacea or Alcyonacea[4,14,15] and is known under the synonym *Solenopodium*.[16] In a continuing survey of Formosan marine organisms with promising cytotoxicity against the growth of various cancer cell lines, the gorgonian *Briareum excavatum* (Nutting) (family Briareidae, order Gorgonacea, phylum Cnidaria) has been the subject of an investigation and was selected due to the cytotoxicity of its organic extract toward P-388 (mouse lymphocytic leukemia) and KB (human nasopharyngeal carcinoma) tumor cells. A previous investigation on the chemical constituents of *B. excavatum* has led to the isolation of five briarane derivatives, excavatolides A−E.[17] Several of these metabolites were shown to exhibit cytotoxicity toward various cancer cell lines. In this paper, we report the isolation of seven new briarane diterpenes, excavatolides F−K and M **(1**−**6** and **8)**, from the further investigation of the chemical constituents of the gorgonian *B. excavatum*. In addition, excavatolide L **(7)** was isolated for the first time as a natural product. The structures and relative stereochemistry of these compounds were determined by spectroscopic and chemical methods.

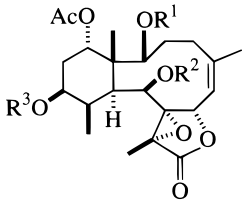
## Results and Discussion

Specimens of *B. excavatum* were frozen immediately after collection and subsequently freeze-dried. The freeze-dried organism was extracted with EtOAc to afford a crude extract that was found to exhibit cytotoxicity against the P-388 cell line with an $ED_{50}$ of 0.5 $\mu$g/mL and the KB cell line with an $ED_{50}$ of 0.6 $\mu$g/mL. The extract was separated by extensive column chromatography on silica gel and afforded eight new briarane-type metabolites, excavatolides F−M **(1**−**8)**.

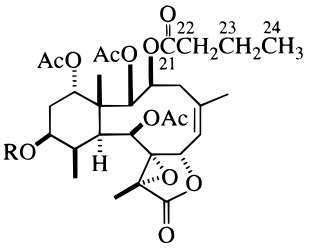
Excavatolide F **(1)** was obtained as a white powder. Its HREIMS established the molecular formula $C_{30}H_{40}O_{11}$. Thus, 11 degrees of unsaturation were determined for the molecule of **1**. The IR spectrum of **1** showed the presence of a carbonyl group of a $\gamma$-lactone ($\nu_{max}$ 1774 cm$^{-1}$) and ester carbonyl groups ($\nu_{max}$ 1732 cm$^{-1}$). A strong UV absorption at 225 nm suggested the presence of a conjugated diene that existed in a *S-trans* conformation in the structure of **1**.[18] The gross structure of **1** and all of the $^1$H and $^{13}$C



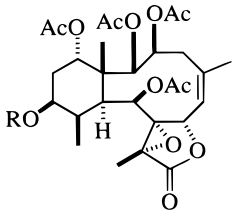
**1**



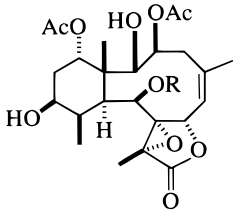
**2** : $R^1 = R^2 = R^3 = Ac$
**7** : $R^1 = R^3 = Ac$, $R^2 = H$
**9** : $R^1 = n$-PrCO, $R^2 = R^3 = Ac$
**10** : $R^1 = Ac$, $R^2 = R^3 = H$



**3** : R = $COCH_2CH_2CH_3$
**4** : R = Ac
**11** : R = H



**5** : R = $COCH_2CH_2CH_3$
**6** : R = Ac
**12** : R = H



**8** : R = H
**13** : R = Ac

chemical shifts associated with the molecule were determined by a series of 2D NMR experiments ($^1$H−$^1$H, $^1$H−$^{13}$C COSY, and HMBC) (Table 1). From the $^1$H−$^1$H COSY spectrum of **1**, it was possible to establish the proton sequences from H-2 to H-4 and H-6 to H-7. These data, together with the $^1$H−$^{13}$C long-range correlations between

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

| position | $^1$H[a] | $^{13}$C[b] | HMBC | $^1$H−$^1$H COSY |
|---|---|---|---|---|
| 1 | | 45.6 (s)[d] | H-2, H-3, H-9, H-10, H-11, H-14, $H_3$-15 | |
| 2 | 5.42 d (10.0)[c] | 75.5 (d) | H-4, $H_3$-15 | H-3 |
| 3 | 5.77 dd (15.6; 10.0) | 126.6 (d) | H-2, H-4 | H-2, H-4 |
| 4 | 6.63 d (15.6) | 137.5 (d) | H-2, H-6, $H_3$-16 | H-3, $H_3$-16 |
| 5 | | 141.7 (s) | H-3, H-4, H-7, $H_3$-16 | |
| 6 | 5.29 d (4.4) | 116.8 (d) | H-4, H-7, $H_3$-16 | H-7, $H_3$-16 |
| 7 | 4.58 d (4.4) | 76.6 (d) | | H-6, |
| 8 | | 68.1 (s) | H-6, H-9, H-10, $H_3$-18 | |
| 9 | 5.55 br s | 73.3 (d) | H-10, H-11 | |
| 10 | 2.29 br s | 37.6 (d) | H-9, H-12, H-14, $H_3$-15 | H-11 |
| 11 | 2.24 m | 39.0 (d) | H-9, $H_3$-20 | H-10, $H_3$-20 |
| 12 | 4.97 m | 69.9 (d) | H-10, $H_2$-13, H-14, $H_3$-20 | $H_2$-13 |
| 13 | 1.80 m | 26.2 (t) | | H-12, H-14 |
| 14 | 4.94 br s | 74.1 (d) | H-2, H-10, $H_3$-15 | $H_2$-13 |
| 15 | 1.36 s | 16.1 (q) | H-2, H-10, H-14 | |
| 16 | 1.85 s | 23.6 (q) | H-6 | H-4, H-6 |
| 17 | | 63.2 (s) | H-9, $H_3$-18 | |
| 18 | 1.58 s | 9.9 (q) | | |
| 19 | | 170.6 (s) | $H_3$-18 | |
| 20 | 0.94 d (7.2) | 10.0 (q) | H-10, H-11 | H-11 |
| acetate methyls | 1.96 s | 20.8 (q) | | |
| | 2.09 s | 21.1 (q) | | |
| | 2.17 s | 21.2 (q) | | |
| ester carbonyls | | 169.9 (s) | | |
| | | 170.0 (s) | | |
| | | 170.2 (s) | | |
| butyrate | | 172.9 (s) | H-12, $H_2$-22, $H_2$-23 | |
| | $CH_3$ 0.91 t (7.6) | 13.6 (q) | $H_2$-22, $H_2$-23 | |
| | $CH_2$ 1.64 m | 18.3 (t) | $H_2$-22, $H_3$-24 | |
| | $CH_2$ 2.26 t (7.6) | 36.2 (t) | $H_2$-23, $H_3$-24 | |

[a] Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C. [b] 100 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.

H-2 and C-1, C-3, and C-4; H-3 and C-1, C-5; H-4 and C-2, C-3, C-5, and C-6; H-6 and C-4, C-8; H-7 and C-5, C-6; H-9 and C-1, C-8, and C-10; and H-10 and C-1, C-8, and C-9, observed in an HMBC experiment, established the connectivities from C-1 to C-10 in a 10-membered ring. A vinyl methyl group attached at the C-5 position was confirmed by the HMBC correlations between $H_3$-16 and C-4, C-5, and C-6. The methylcyclohexane ring, which is fused to the 10-membered ring at C-1 and C-10, was elucidated by $^1$H−$^1$H correlations from H-10 to H-11, H-11 to $H_3$-20, and H-12 to H-14, and by the key HMBC correlations between H-2 and C-14; H-9 and C-11; H-10 and C-12, C-14, and C-20; and H-11 and C-1, C-9, and C-20. The ring juncture C-15 methyl group was positioned at C-1 from the key HMBC correlations between $H_3$-15 and C-1, C-2, C-10, and C-14. The *n*-butyrate positioned at C-12 was confirmed from the connectivity between H-12 ($\delta$ 4.97) and the carbonyl carbon ($\delta$ 172.9) of the *n*-butyryloxyl group. Furthermore, the HMBC correlations also revealed the positions of three acetates attached to C-2, C-9, and C-14. These data, together with the HMBC correlations between H-9 and C-17, and $H_3$-18 and C-8, C-17, and C-19, unambiguously established the molecular framework of **1**.

The relative stereochemistry of **1** was deduced from vicinal $^1$H−$^1$H coupling constants and from a ROESY experiment (Figure 1). The *trans* geometry of the C-3/C-4 double bond was indicated by a 15.6 Hz coupling constant between H-3 ($\delta$ 5.77) and H-4 ($\delta$ 6.63). Furthermore, the NOE correlations of H-10 with H-11 and H-12 indicated that these three protons are situated on the same face of the six-membered ring and were assigned as the α protons since the C-15 methyl is the $\beta$-substituent at C-1. Thus, the *n*-butyrate at C-12 was at the $\beta$ face and is *cis* to C-20 methyl. H-14 was found to exhibit NOE responses with H-2 and $H_3$-15 but not with H-10, revealing the $\beta$-orientation of this proton. The H-7 signal showed a correlation with H-9. Consideration of molecular models showed that H-7 should be placed at the $\beta$ face. Furthermore, $H_3$-18 was found to exhibit NOE responses with H-11 and $H_3$-20, indicating the $\beta$-orientation of the C-18 methyl. On the basis of the above observations, the structure of **1**, including the relative stereochemistry, was elucidated unambiguously. To the best of our knowledge, a briarane derivative containing a 3(*E*),5(*Z*)-diene moiety in a *S-trans* conformation has not been reported to date.

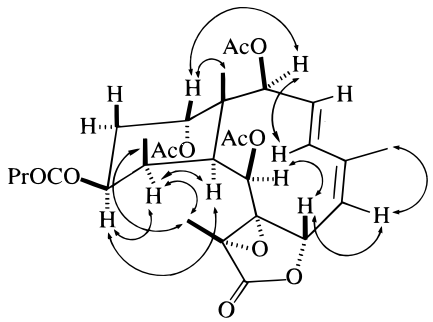


**Figure 1.** Selective NOE correlations of **1**.

Excavatolide G **(2)** was isolated as a white powder. The IR absorptions of **2** showed the presence of a $\gamma$-lactone ($\nu_{max}$ 1778 cm$^{-1}$) and ester carbonyl groups ($\nu_{max}$ 1732 cm$^{-1}$) in **2**. The EIMS of **2** exhibited peaks at *m*/*z* 490 ($M^+$ − AcOH), 430 ($M^+$ − 2AcOH), 370 ($M^+$ − 3AcOH), and 310 ($M^+$ − 4AcOH), suggesting the presence of four acetoxyl groups in the molecule. In the $^1$H NMR spectrum of **2**, four acetate methyls were observed at $\delta$ 2.00 (3H, s), 2.02 (3H, s), 2.05 (3H, s), and 2.22 (3H, s). Carbonyl resonances in the $^{13}$C NMR spectrum of **2** at $\delta$ 168.1 (s), 170.1 (s), 170.3 (s), 170.6 (s), and 170.6 (s) confirmed the presence of a $\gamma$-lactone and four acetoxyl groups. From the above data, the structure

**Table 2.** $^1$H NMR Chemical Shifts for Compounds **2**−**6** and **8**

| | compound | | | | | |
|---|---|---|---|---|---|---|
| proton | **2**[a] | **3**[b] | **4**[b] | **5**[b] | **6**[b] | **8**[c] |
| 2 | 5.07 d (7.8)[d] | 5.14 br s | 5.14 br s | 5.13 br s | 5.13 br s | 3.47 br s |
| 3α | 2.58 dt (15.0; 3.3) | 5.86 d (6.0) | 5.79 d (6.0) | 5.82 d (6.0) | 5.78 d (6.3) | 5.80 d (8.0) |
| 3*β* | 1.69 m | | | | | |
| 4α | 2.00 m | 2.11 m | 2.12 m | 2.02 m | 2.05 m | 1.91 m |
| 4*β* | 2.40 br d (13.2) | 3.99 dd (15.9; 7.5) | 3.96 dd (15.6; 7.5) | 3.99 dd (15.6; 7.5) | 3.99 dd (14.7; 7.5) | 3.67 dd (15.6; 8.0) |
| 6 | 5.26 d (9.0) | 5.37 d (7.2) | 5.36 d (7.2) | 5.37 d (7.5) | 5.37 d (7.2) | 5.35 d (8.0) |
| 7 | 5.18 d (9.0) | 5.67 d (7.2) | 5.66 d (7.2) | 5.67 d (7.5) | 5.67 d (7.2) | 5.79 d (8.0) |
| 9 | 4.94 br s | 5.54 d (10.5) | 5.52 d (10.2) | 5.53 d (10.2) | 5.53 d (10.2) | 4.21 d (9.6) |
| 10 | 2.38 m | 3.14 dd (10.5; 4.8) | 3.14 dd (10.2; 4.8) | 3.13 dd (10.2; 5.1) | 3.13 dd (10.2; 4.8) | 2.98 dd (9.6; 5.2) |
| 11 | 2.18 m | 2.59 m | 2.59 m | 2.60 m | 2.59 m | 2.38 m |
| 12 | 5.10 m | 4.94 br d (11.7) | 4.95 m | 4.91 m | 4.90 br d (11.7) | 3.86 m |
| 13 | 1.65 m | 1.84 m | 1.82 m | 1.80 m | 2.02 m | 1.66 m |
| | 1.87 m | 2.14 br d (15.0) | 2.02 br d (15.6) | 2.11 br d (15.3) | 2.10 br d (13.2) | 1.89 m |
| 14 | 4.83 dd (3.3; 3.0) | 4.65 br s | 4.64 br s | 4.65 br s | 4.63 br s | 4.76 dd (3.2; 2.8) |
| 15 | 1.21 s | 0.81 s | 0.82 s | 0.82 s | 0.81 s | 1.23 s |
| 16 | 1.99 s | 1.93 s | 1.85 s | 1.93 s | 1.86 s | 1.98 s |
| 18 | 1.64 s | 1.48 s | 1.47 s | 1.48 s | 1.47 s | 1.53 s |
| 20 | 1.04 d (7.5) | 1.07 d (7.2) | 1.06 d (6.9) | 1.07 d (6.6) | 1.06 d (6.9) | 0.97 d (6.8) |
| OH-2 | | | | | | 6.32 br s |
| OH-9 | | | | | | 7.24 br s |
| OH-12 | | | | | | 3.82 br s |
| acetate methyls | 2.00 s | 2.22 s | 1.93 s | 1.98 s | 1.92 s | 2.06 s |
| | 2.02 s | 2.24 s | 2.20 s | 2.21 s | 1.98 s | 2.11 s |
| | 2.05 s | 2.38 s | 2.22 s | 2.23 s | 2.20 s | |
| | 2.22 s | | 2.38 s | 2.38 s | 2.23 s | |
| | | | | | 2.38 s | |
| butyrate | | 2 × $CH_3$ 0.82 t (7.5) | $CH_3$ 0.83 t (7.5) | $CH_3$ 0.82 t (7.5) | | |
| | | 2 × $CH_2$ 1.50 m | $CH_2$ 1.53 m | $CH_2$ 1.51 m | | |
| | | 2 × $CH_2$ 2.26 t (7.5) | $CH_2$ 2.24 t (7.5) | $CH_2$ 2.20 t (7.5) | | |

[a] The $^1$H NMR spectra were recorded at 300 MHz in $CDCl_3$ at 25 °C. [b] 300 MHz in $Me_2CO$-$d_6$ at −70 °C. [c] 400 MHz in $Me_2CO$-$d_6$ at 25 °C. [d] *J* values (in Hz) in parentheses. The values are downfield from TMS.

of diterpene **2** could be seen to be very similar to those of the two known compounds, 11,12-deoxy-12-acetoxystecholide E acetate **(9)**[3] and excavatolide E **(10)**.[17] Also, it was found that the *n*-butyryloxyl group at the C-2 position of compound **9** was replaced by an acetoxyl group by comparing the related spectral data of **2** with those of compound **9**. Furthermore, acetylation of **10** gave a less polar product that was found to be identical with diterpene **2** by comparison of the physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data. Thus, the molecular structure of **2** was confirmed.

Excavatolide H **(3)** was isolated as a white powder, and a molecular formula of $C_{34}H_{48}O_{13}$ was established by HRFABMS. Accordingly, 11 degrees of unsaturation were determined for the molecule of **3**. It was found that the $^1$H and $^{13}$C NMR spectra of **3** in $CDCl_3$ revealed mostly broad peaks when measured at room temperature. These observations suggested the existence of slowly interconverting conformers of this compound in solution at ambient temperature. To reduce the rate of conversion of these various conformers and to make more reliable assignments of the NMR signals of the stablized conformers, the $^1$H and $^{13}$C spectra of **3** were measured at −70 °C in $Me_2CO$-$d_6$. It was found that at this temperature mainly one conformer existed and the signals for each proton (Table 2) and carbon (Table 3) of the molecule were sharpened and could be assigned by the assistance of DEPT and 2D NMR ($^1$H−$^1$H, $^1$H−$^{13}$C COSY, and HMBC) spectra. From the above spectral data, a trisubstituted olefin was deduced from the signals of two carbons at *δ* 140.0 (s) and 122.5 (d). An 8,-17-epoxide group was confirmed from the signals of two quaternary oxygenated carbons at *δ* 69.0 (s) and 60.5 (s) and from the chemical shift of $H_3$-18 (*δ* 1.48, 3H, s). In the $^{13}$C NMR spectrum of **3**, six carbonyl resonances appeared at *δ* 170.2 (s), 170.8 (s), 172.0 (s), 172.3 (s), 172.4 (s), and 172.8 (s) and confirmed the presence of a *γ*-lactone and five other ester groups. On the basis of the above data, metabolite **3** was found to be a tetracyclic compound. In the $^1$H NMR spectrum of **3**, three acetate methyls (*δ* 2.22, 3H, s; 2.24, 3H, s; 2.38, 3H, s) and two *n*-butyryloxyl groups [*δ* 0.82 (6H, t, *J* = 7.5 Hz), 1.50 (4H, m), and 2.26 (4H, t, *J* = 7.5 Hz)] were also observed. The carbon signals at *δ* 172.3 (s) and 172.4 (s) were correlated with the signals of the methylene protons of the *n*-butyrates at *δ* 2.26 in the HMBC spectrum of **3** (Table 4) and were consequently assigned as the carbon atoms of the two *n*-butyrate carbonyl groups. Furthermore, the two *n*-butyrate esters could be positioned at C-3 and C-12 as confirmed from the connectivities between H-3 (*δ* 5.86) and the carbonyl carbon at *δ* 172.3 (s) and H-12 (*δ* 4.94) and the carbonyl carbon at *δ* 172.4 (s), in an HMBC experiment of **3**. It was observed that the spectral data ($^1$H and $^{13}$C NMR) of **3** were very similar to those of a known diterpene, excavatolide B **(11)**.[17] However, the chemical shifts for H-12 and C-12 of **3** ($\delta_H$ 4.94, br d, *J* = 11.7 Hz; $\delta_C$ 69.9, d) were found to be shifted downfield, in comparison with the analogous data of **11** ($\delta_H$ 3.88, m; $\delta_C$ 65.8, d), which suggested that the 12*β*-hydroxyl group of **11** was replaced by a *n*-butyryloxyl group in diterpene **3**. On the basis of the above observations, excavatolide H **(3)** was assigned as the 12-*n*-butyryl derivative of excavatolide B **(11)**. Butyrylation of excavatolide B **(11)** gave a less polar product identical to diterpene **3** by comparison of the physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data and confirmed the structure proposed for **3**.

Excavatolide I **(4)** had the molecular formula of $C_{32}H_{44}O_{13}$ as determined by HRFABMS, with 11 degrees of unsaturation thereby being determined for the molecule. The IR absorptions of **4** showed the presence of a *γ*-lactone ($\nu_{max}$ 1790 cm$^{-1}$) and ester carbonyl groups ($\nu_{max}$ 1736 cm$^{-1}$). Like **3**, the sharpened NMR ($^1$H and $^{13}$C) signals of **4** (Tables 2 and 3) were also obtained in $Me_2CO$-$d_6$ at −70 °C. Carbonyl

**Table 3.** $^{13}C$ NMR Chemical Shifts of Compounds **2**−**6** and **8**

| | compound | | | | | |
|---|---|---|---|---|---|---|
| carbon | **2**[a] | **3**[b] | **4**[b] | **5**[b] | **6**[b] | **8**[c] |
| C-1 | 45.8 (s)[d] | 44.0 (s) | 43.9 (s) | 44.0 (s) | 44.0 (s) | 45.3 (s) |
| C-2 | 75.0 (d) | 81.1 (d) | 80.8 (d) | 81.1 (d) | 80.9 (d) | 83.4 (d) |
| C-3 | 28.6 (t) | 73.4 (d) | 73.5 (d) | 73.4 (d) | 73.6 (d) | 76.1 (d) |
| C-4 | 26.0 (t) | 34.1 (t) | 33.8 (t) | 34.1 (t) | 33.9 (t) | 35.0 (t) |
| C-5 | 144.9 (s) | 140.0 (s) | 139.9 (s) | 139.9 (s) | 139.9 (s) | 140.8 (s) |
| C-6 | 118.4 (d) | 122.5 (d) | 122.4 (d) | 122.5 (d) | 122.5 (d) | 123.3 (d) |
| C-7 | 74.7 (d) | 74.2 (d) | 74.1 (d) | 74.2 (d) | 74.2 (d) | 74.4 (d) |
| C-8 | 70.8 (s) | 69.0 (s) | 69.9 (s) | 69.1 (s) | 69.0 (s) | 71.1 (s) |
| C-9 | 73.6 (d) | 64.9 (d) | 64.7 (d) | 64.8 (d) | 64.9 (d) | 66.9 (d) |
| C-10 | 41.1 (d) | 40.1 (d) | 40.0 (d) | 40.1 (d) | 40.1 (d) | 42.3 (d) |
| C-11 | 29.7 (d) | 32.9 (d) | 32.8 (d) | 32.8 (d) | 32.8 (d) | 35.7 (d) |
| C-12 | 69.7 (d) | 69.9 (d) | 69.8 (d) | 70.1 (d) | 70.1 (d) | 67.2 (d) |
| C-13 | 31.7 (t) | 27.0 (t) | 26.9 (t) | 26.9 (t) | 27.0 (t) | 31.5 (t) |
| C-14 | 75.6 (d) | 81.5 (d) | 81.4 (d) | 81.4 (d) | 81.5 (d) | 80.6 (d) |
| C-15 | 15.5 (q) | 18.0 (q) | 17.9 (q) | 18.0 (q) | 18.1 (q) | 21.3 (q) |
| C-16 | 27.3 (q) | 22.7 (q) | 22.0 (q) | 22.2 (q) | 20.8 (q) | 21.3 (q) |
| C-17 | 64.9 (s) | 60.5 (s) | 60.4 (s) | 60.5 (s) | 60.6 (s) | 59.6 (s) |
| C-18 | 9.9 (q) | 10.1 (q) | 10.0 (q) | 10.1 (q) | 10.3 (q) | 9.4 (q) |
| C-19 | 170.6 (s) | 172.8 (s) | 172.7 (s) | 172.3 (s) | 172.4 (s) | 173.1 (s) |
| C-20 | 11.1 (q) | 10.3 (q) | 10.2 (q) | 10.3 (q) | 10.1 (q) | 9.1 (q) |
| acetate methyls | 21.1 (q) | 21.5 (q) | 20.7 (q) | 21.0 (q) | 21.0 (q) | 22.0 (q) |
| | 21.3 (q) | 22.2 (q) | 21.6 (q) | 21.5 (q) | 21.4 (q) | 23.0 (q) |
| | 21.4 (q) | 22.3 (q) | 22.2 (q) | 22.3 (q) | 22.1 (q) | |
| | 21.5 (q) | | 22.6 (q) | 22.7 (q) | 22.4 (q) | |
| | | | | | 22.8 (q) | |
| ester carbonyls | 168.1 (s) | 170.2 (s) | 169.7 (s) | 170.1 (s) | 169.8 (s) | 170.1 (s) |
| | 170.1 (s) | 170.8 (s) | 170.1 (s) | 170.4 (s) | 170.2 (s) | 170.3 (s) |
| | 170.3 (s) | 172.0 (s) | 170.7 (s) | 170.7 (s) | 170.4 (s) | |
| | 170.6 (s) | | 171.9 (s) | 171.9 (s) | 170.5 (s) | |
| | | | | | 170.8 (s) | |
| butyrate | | 172.3 (s) | 172.4 (s) | 172.2 (s) | | |
| | | 172.4 (s) | $CH_3$ 13.6 (q) | $CH_3$ 13.7 (q) | | |
| | | 2 × $CH_3$ 13.7 (q) | $CH_2$ 18.3 (t) | $CH_2$ 18.3 (t) | | |
| | | 2 × $CH_2$ 18.4 (t) | $CH_2$ 35.7 (t) | $CH_2$ 35.7 (t) | | |
| | | 2 × $CH_2$ 35.8 (t) | | | | |

[a] The $^{13}C$ NMR spectra were recorded at 75 MHz in $CDCl_3$ at 25 °C. [b] 75 MHz in $Me_2CO$-$d_6$ at −70 °C. [c] 100 MHz in $Me_2CO$-$d_6$ at 25 °C. [d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are downfield from TMS.

**Table 4.** Protons to Which Long-Range Correlations Were Observed in the HMBC Experiments on Diterpenes **3**, **5**, and **8**

| carbon | **3** | **5** | **8** |
|---|---|---|---|
| 1 | H-2, H-3, H-10, $H_3$-15 | H-2, H-10, H-11, $H_3$-15 | H-10, H-13, H-14, $H_3$-15 |
| 2 | H-4$\beta$, $H_3$-15 | H-4$\beta$, $H_3$-15 | $H_2$-4, H-14, $H_3$-15 |
| 3 | H-2, $H_2$-4, | H-2, $H_2$-4, | H-2, $H_2$-4 |
| 4 | H-2, H-6, $H_3$-16 | H-2, H-6, $H_3$-16 | |
| 5 | H-3, $H_2$-4, H-7, $H_3$-16 | H-3, $H_2$-4, H-7, $H_3$-16 | H-3, $H_2$-4, H-7, $H_3$-16 |
| 6 | $H_2$-4, H-7, $H_3$-16 | H-4$\beta$, H-7, $H_3$-16 | $H_2$-4, H-7, $H_3$-16 |
| 7 | H-9 | H-9 | H-9 |
| 8 | H-9, H-10, $H_3$-18 | H-9, $H_3$-18 | H-9, H-10, $H_3$-18 |
| 9 | H-10 | H-10 | H-10, $H_3$-20 |
| 10 | H-2, H-9, H-14, $H_3$-15, $H_3$-20 | H-2, H-9, H-11, H-14, $H_3$-15, $H_3$-20 | H-2, H-9, H-14, $H_3$-15, $H_3$-20 |
| 11 | H-9, $H_3$-20 | H-9, $H_3$-20 | H-9, H-10, H-13, $H_3$-20 |
| 12 | H-10, H-11, H-14, $H_3$-20 | H-11, H-14, $H_3$-20 | H-10, H-13, H-14, $H_3$-20 |
| 13 | H-11 | H-11 | H-14 |
| 14 | H-2, $H_3$-15 | H-2, $H_3$-15 | H-13, $H_3$-15 |
| 15 | H-10 | H-10 | H-10, H-14 |
| 16 | $H_2$-4, H-6 | $H_2$-4, H-6 | $H_2$-4 |
| 17 | H-9, $H_3$-18 | H-9, $H_3$-18 | H-9, $H_3$-18 |
| 19 | $H_3$-18 | $H_3$-18 | H-10, $H_3$-18 |
| 20 | H-11 | H-11 | H-10 |
| 3-*n*-PrCO | H-3, $H_{2-22}$ | | |
| 12-*n*-PrCO | H-12, $H_2$-26 | H-12, $H_2$-22 | |

resonances in the $^{13}C$ NMR spectrum of **4** at $\delta$ 169.7 (s), 170.1 (s), 170.7 (s), 171.9 (s), 172.4 (s), and 172.7 (s) confirmed the presence of a $\gamma$-lactone and five other esters in the molecule. In the $^1H$ NMR spectrum, four acetate methyls ($\delta$ 1.93, 3H, s; 2.20, 3H, s; 2.22, 3H, s; 2.38, 3H, s) and an *n*-butyryloxyl group [$\delta$ 0.83 (3H, t, $J$ = 7.5 Hz), 1.53 (2H, m), and 2.24 (2H, t, $J$ = 7.5 Hz)] were observed. It was found that the spectral data ($^1H$ and $^{13}C$ NMR) of **4** were very similar to those of the known diterpene excavatolide B **(11)**,[17] except that **4** showed signals corresponding to an additional acetoxyl substituent. Also, the $^1H$ and $^{13}C$ NMR spectra revealed that the signals corresponding to the hydroxyl-bearing C-12 methine group in **11** ($\delta_H$ 3.88, m; $\delta_C$ 65.8, d) were shifted downfield in **4** ($\delta_H$ 4.95, m; $\delta_C$ 69.8, d), indicating that excavatolide I **(4)** is the 12-acetyl derivative of compound **11**. Acetylation of excavatolide B **(11)** gave a less polar product that was found to be identical with diterpene **4** by comparison of the physical (mp and

optical rotation) and spectral (MS, IR, $^1$H and $^{13}$C NMR) data. Thus, the structure of diterpene **4** was confirmed.

Excavatolide J **(5)** had the same molecular formula as that of **4**, $C_{32}H_{44}O_{13}$, as determined by HRFABMS, with 11 degrees of unsaturation. The sharpened NMR ($^1$H and $^{13}$C) signals of **5** (Tables 2 and 3) were obtained in $Me_2$-CO-$d_6$ at −70 °C, and the spectral data (IR, MS, $^1$H and $^{13}$C NMR) of **5** were very similar to those of **4**. However, the melting point (203−205 °C) and optical rotation ($[\alpha]^{26}_D$ +38°, *c* 0.6, $CHCl_3$) of **5** were substantially different from those of **4** (mp 225−227 °C; $[\alpha]^{26}_D$ +23°, *c* 1.0, $CHCl_3$), indicating that these compounds are isomers. In the $^1$H NMR spectrum of **5**, four acetate methyls were observed at $\delta$ 1.98 (3H, s), 2.21 (3H, s), 2.23 (3H, s), and 2.38 (3H, s). The additional acyl group was found to be an *n*-butanoyl group, which showed seven contiguous protons [$\delta$ 0.82 (3H, t, $J$ = 7.5 Hz), 1.51 (2H, m), and 2.20 (2H, t, $J$ = 7.5 Hz)]. From the $^1$H−$^1$H COSY spectrum, it was possible to establish the proton sequences from H-2 to H-4; H-6 to H-7 and $H_3$-16; and H-9 to H-14. The $^{13}$C NMR signal at $\delta$ 172.2 correlated with the signal of the methylene protons at $\delta$ 2.20 in the HMBC spectrum (Table 4) and was consequently assigned as the carbon atom of the *n*-butyrate carbonyl. The $^1$H−$^{13}$C long-range correlations observed in an HMBC experiment of **5** further revealed the connectivity between H-12 ($\delta$ 4.91) and the carbonyl carbon ($\delta$ 172.2) of the *n*-butyrate unit and demonstrated the location of the *n*-butyrate to be at C-12. The positions of the other four acetoxyl groups at the C-2, C-3, C-9, and C-14 positions were also confirmed by the connectivities between the four methine protons at $\delta$ 5.13 (H-2), 5.82 (H-3), 5.53 (H-9), and 4.65 (H-14) and the ester carbonyls at $\delta$ 170.1 (s), 170.4 (s), 170.7 (s), and 171.9 (s), respectively, in the HMBC spectrum of **5**. On the basis of the above data, excavatolide J **(5)** was assigned as the 12-*n*-butyryl derivative of excavatolide C **(12)**. Furthermore, butyrylation of excavatolide C **(12)**[17] yielded a compound that was found to be identical with diterpene **5** by physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data comparison.

Excavatolide K **(6)** had the molecular formula of $C_{30}H_{40}O_{13}$ as determined by HRFABMS. Thus, 11 degrees of unsaturation were determined for the molecule of **6**. Both the $^1$H and $^{13}$C NMR spectra of **6** were measured at −70 °C in $Me_2CO$-$d_6$. It was observed that the spectral data ($^1$H and $^{13}$C NMR) of **6** were similar to those of the known diterpene, excavatolide C **(12)**.[17] However, the chemical shifts for H-12 and C-12 of **6** ($\delta_H$ 4.90 and $\delta_C$ 70.1) were found to be shifted downfield by comparison of these data with those of **12** ($\delta_H$ 3.89 and $\delta_C$ 65.8), indicating that the 12-hydroxyl group in compound **12** was replaced by an acetoxyl group. In the $^1$H NMR spectrum of **6**, five acetate methyls appeared at $\delta$ 1.92 (3H, s), 1.98 (3H, s), 2.20 (3H, s), 2.23 (3H, s), and 2.38 (3H, s). Carbonyl resonances in the $^{13}$C NMR spectrum of **6** at $\delta$ 169.8 (s), 170.2 (s), 170.4 (s), 170.5 (s), 170.8 (s), and 172.4 (s) revealed the presence of a $\gamma$-lactone and five acetoxyl groups. Furthermore, by comparison of the $^1$H and $^{13}$C NMR spectral data of **4** and **6**, it was seen that the signals corresponding to the *n*-butyryloxyl group in **4** were absent and replaced by those of an additional acetoxyl group in **6**. On the basis of the above observations, the structure of excavatolide K **(6)** was established as the 12-acetyl derivative of excavatolide C **(12)**. Acetylation of **12** gave diterpene **6**, by comparison of their physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data.

Excavatolide L **(7)** had the molecular formula $C_{26}H_{36}O_{10}$ as determined by HRFABMS, implying nine degrees of unsaturation. By comparison of the physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data with those of a known semisynthetic diterpene, compound **7** was found to be 12-acetylexcavatolide E.[17] Acetylation of excavatolide E **(10)**[17] gave two products, which were found to be diterpene **7** and excavatolide G **(2)**. Furthermore, acetylation of **7** gave a less polar product, which was identical with excavatolide G **(2)**, and further confirmed the structure of **7**. Diterpene **7** was isolated for the first time from a natural source.

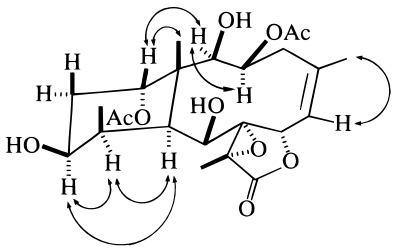


**Figure 2.** Selective NOE correlations of **8**.

Excavatolide M **(8)** gave a molecular formula $C_{24}H_{34}O_{10}$ by HRFABMS, indicating eight degrees of unsaturation. The IR spectrum revealed the presence of a $\gamma$-lactone ($\nu_{max}$ 1778 cm$^{-1}$), ester carbonyl groups ($\nu_{max}$ 1734 cm$^{-1}$), and hydroxyl groups ($\nu_{max}$ 3284 cm$^{-1}$). The $^1$H and $^{13}$C NMR spectra of **8** (Tables 2 and 3) were recorded at 25 °C in $Me_2$-CO-$d_6$. From the $^{13}$C NMR spectral data of **8**, a trisubstituted olefin could be identified by signals of two carbons that appeared at $\delta$ 123.3 (d) and 140.8 (s). An 8,17-epoxide group was confirmed from signals of two oxygenated quaternary carbons that appeared at $\delta$ 71.1 (s) and 59.6 (s) and from the chemical shift of $H_3$-18 ($\delta$ 1.53, 3H, s). Carbonyl resonances in the $^{13}$C NMR spectrum at $\delta$ 170.1 (s), 170.3 (s), and 173.1 (s) confirmed the presence of a $\gamma$-lactone and two other esters. From the above data, metabolite **8** was found to be a tetracyclic compound. In the $^1$H NMR spectrum of **8**, two acetate methyl signals were observed at $\delta$ 2.06 (3H, s) and 2.11 (3H, s). The briarane framework of **8** was determined from its HMBC spectrum (Table 4). It was found that the spectral data (IR, $^1$H and $^{13}$C NMR) of **8** were very similar to those of excavatolide D **(13)**,[17] except that **13** contains one more acetyl group in comparison with **8**. The chemical shift of H-9 in compound **13** ($\delta$ 5.51) was shifted upfield to $\delta$ 4.21 in **8**, suggesting that **8** is the 9-deacetyl product of **13**. Also, three hydroxyl protons appeared as singlets at $\delta$ 3.82, 6.32, and 7.24 and correlated in the $^1$H−$^1$H COSY spectrum with the H-12, H-2, and H-9 signals, respectively. Thus, these hydroxyl groups were positioned in turn at C-12, C-2, and C-9, and the chemical shifts of these three hydroxyl protons were assigned unambiguously. The relative stereochemistry of **8** was established by a NOESY experiment (Figure 2). On the basis of the analysis of all of its spectral data, diterpene **8** was found to be the 9-deacetyl derivative of excavatolide D **(13)**. Acetylation of **8** gave a less polar product, which was found to be identical with excavatolide K **(6)** by comparison of the physical (mp and optical rotation) and spectral (IR, MS, $^1$H and $^{13}$C NMR) data, and confirmed the structure of excavatolide M **(8)**. It is noted that **8** is the only briarane-type diterpenoid known to possess $\beta$-hydroxyl groups at all three of the C-2, C-9, and C-12 positions.

The absolute configurations of excavatolides B−E have been determined previously, as shown by structures **11**, **12**, **13**, and **10**, respectively.[17] As excavatolides F−M were isolated along with excavatolides A−E from the same

**Table 5.** Cytotoxicity Data for Compounds **1**−**6** and **8**[a]

| compound | cell lines $ED_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | P-388 | KB | A-549 | HT-29 |
| **1** | 6.2 | 7.0 | 5.2 | 5.5 |
| **2** | 15.7 | >50 | 22.8 | >50 |
| **3** | >50 | >50 | >50 | >50 |
| **4** | >50 | >50 | >50 | >50 |
| **5** | 3.8 | 6.5 | 5.2 | 5.2 |
| **6** | 0.9 | 3.3 | 3.0 | 1.3 |
| **8** | 0.001 | 1.0 | 0.1 | 2.2 |

[a] For significant activity of pure compounds, an $ED_{50}$ value of ≤4.0 μg/mL is required. See Geran et al.[20]

organism, it is reasonable on biogenetic grounds to assume that diterpenes **1**−**8** have the same absolute configurations as **10**−**13**. The acylation of excavatolides B **(11)**, C **(12)**, and E **(10)** gave related compounds identical with excavatolides H **(3)** and I **(4)**, J **(5)** and K **(6)**, and G **(2)** and L **(7)**, respectively, and further confirmed the above assumption. Therefore, excavatolides F−M were assumed to possess the absolute configurations as represented by structures **1**−**8**.

The cytotoxicity of compounds **1**−**6** and **8** against the growth of P-388, KB, A-549, and HT-29 cancer cell lines was evaluated and the results are shown in Table 5. These data show that **6** and **8** were broadly cytotoxic for the cells in the limited panel. By comparison of these results with the cytotoxicity of the related diterpenes that had been reported previously,[17] it was found that functional group variations at the C-2, C-3, C-9, and C-12 positions in the molecules of the excavatolide diterpenes can influence the cytotoxicity of the compounds of this type significantly. Compound **8** may warrant further biological studies in the future. The cytotoxicity of compound **7** has been reported previously.[17] As all the metabolites, excavatolides A−M, isolated from *B. excavatum* to date exhibited weaker cytotoxicity toward KB cells in comparison with the crude extract, further investigation on the metabolites of this organism seems worthwhile in order to isolate other bioactive substances.

## Experimental Section

**General Experimental Procedures.** UV spectra (in MeOH) were recorded on a Hitachi U-3210 UV spectrophotometer. The NMR spectra were recorded on a Varian VXR-300/5 FT-NMR at 300 MHz for $^1$H and 75 MHz for $^{13}$C or on a Bruker AMX-400 FT-NMR at 400 MHz for $^1$H and 100 MHz for $^{13}$C, respectively, in $Me_2CO$-$d_6$ using TMS as internal standard, unless otherwise indicated. Other general experimental procedures followed those reported previously.[17]

**Animal Material.** The gorgonian *B. excavatum* was collected by hand using scuba at South Bay, Kenting, located at the southernmost tip of Taiwan, in July 1995, at depths of 4−5 m, and was stored in a freezer until extraction. A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University (specimen no. KTSC-103).

**Extraction and Isolation.** The marine organism (3.6 kg fresh wt) was collected and freeze-dried. The freeze-dried material (1.9 kg) was minced and extracted exhaustively with EtOAc (15 L × 5). The organic extract was evaporated to give a dark green residue (96.1 g). The residue was dissolved in ethyl acetate (800 mL), and the solution was stored at 0 °C to give a solid (5.5 g) that was found to be a mixture of long-chain esters arising from saturated fatty acids and alcohols and was discarded. The remaining mixture was separated by Si gel column chromatography, using hexanes and hexanes−EtOAc mixtures of increasing polarity. Diterpenoid **1** was eluted with hexanes−EtOAc (6:1), **2** with hexanes−EtOAc (5:1), **3** with hexanes−EtOAc (5:1−4:1), **4** with hexanes−EtOAc (4:1), **5** with hexanes−EtOAc (4:1−3:1), **6** with hexanes−EtOAc (3:1), **7** with hexanes−EtOAc (3:1−2:1), and **8** with hexanes−EtOAc (1:1).

**Excavatolide F (1):** white powder (40.2 mg); mp 79−80 °C; $[\alpha]^{27}_D$ −28° (*c* 1.0, $CHCl_3$); UV (MeOH) $\lambda_{max}$ 225 nm (ε 6610); IR (KBr) $\nu_{max}$ 1774, 1732, 1372, 1246, 1222, 1016, 972 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; EIMS *m/z* 576 $[M]^+$ (0.1), 516 (0.1), 456 (0.2), 368 (0.4), 326 (2), 43 (100); HREIMS *m/z* 576.2565 (calcd for $C_{30}H_{40}O_{11}$, 576.2559).

**Excavatolide G (2):** white powder (5.5 mg); mp 219−220 °C; $[\alpha]^{27}_D$ +40° (*c* 0.2, $CHCl_3$); IR (KBr) $\nu_{max}$ 1778, 1732, 1436, 1380, 1254, 1230, cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; EIMS *m/z* 490 (0.6, $M^+$ − AcOH), 430 (0.7), 370 (3), 310 (4), 43 (100); HREIMS *m/z* 490.2187 (calcd for $C_{26}H_{34}O_9$, $M^+$ − AcOH, 490.2193).

**Excavatolide H (3):** white solid (12.0 mg); mp 189−190 °C; $[\alpha]^{27}_D$ +27° (*c* 0.3, $CHCl_3$); IR (KBr) $\nu_{max}$ 1790, 1740, 1440, 1376, 1268, 1232 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; FABMS *m/z* 665 [0.3, $(M + H)^+$], 605 (0.7), 577 (0.3), 517 (0.8); HRFABMS *m/z* 665.3170 (calcd for $C_{34}H_{49}O_{13}$, 665.3159).

**Excavatolide I (4):** white solid (74.5 mg); mp 225−227 °C; $[\alpha]^{26}_D$ +23° (*c* 1.0 $CHCl_3$); IR (KBr) $\nu_{max}$ 1790, 1736, 1448, 1372, 1266, 1240 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; FABMS *m/z* 637 [1, $(M + H)^+$], 577 (2), 517 (1), 489 (4), 457 (0.3); HRFABMS *m/z* 637.2857 (calcd for $C_{32}H_{45}O_{13}$, 637.2847).

**Excavatolide J (5):** white solid (47.2 mg); mp 203−205 °C; $[\alpha]^{26}_D$ +38° (*c* 0.6, $CHCl_3$); IR (KBr) $\nu_{max}$ 1786, 1732, 1436, 1374, 1262, 1228 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; FABMS *m/z* 637 [2, $(M + H)^+$], 577 (5), 517 (1), 489 (4), 457 (0.7); HRFABMS *m/z* 637.2880 (calcd for $C_{32}H_{45}O_{13}$, 637.2847).

**Excavatolide K (6):** white solid (39.6 mg); mp 178−180 °C; $[\alpha]^{26}_D$ +35° (*c* 0.7, $CHCl_3$); IR (KBr) $\nu_{max}$ 1788, 1744, 1438, 1380, 1277, 1236 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; FABMS *m/z* 609 [0.3, $(M + H)^+$], 549 (1), 489 (0.8), 429 (0.4); HRFABMS *m/z* 609.2563 (calcd for $C_{30}H_{41}O_{13}$, 609.2535).

**Excavatolide L (7):** white solid (6.5 mg); mp 269−270 °C (lit.[17] mp 268−270 °C); $[\alpha]^{26}_D$ +28° (*c* 0.5, $CHCl_3$) (lit.[17] $[\alpha]^{26}_D$ +28° (*c* 0.18, $CHCl_3$)). The physical and spectral data (IR, $^1$H and $^{13}$C NMR, and MS) are in full agreement with those reported previously.[17]

**Excavatolide M (8):** white solid (27.4 mg); mp 219−221 °C; $[\alpha]^{26}_D$ +74° (*c* 0.6, $CHCl_3$); IR (KBr) $\nu_{max}$ 3284, 1778, 1734, 1446, 1374, 1280, 1234 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 2 and 3; FABMS *m/z* 483 [4, $(M + H)^+$], 465 (0.2), 423 (1), 405 (0.5), 363 (0.4), 345 (0.9); HRFABMS *m/z* 483.2232 (calcd for $C_{24}H_{35}O_{10}$, 483.2220).

**Acetylation of Excavatolide E (10).** Excavatolide E **(10)** (30.0 mg) was stirred with 3 mL of $Ac_2O$ in 3 mL of pyridine for 72 h at room temperature. After evaporation of excess reagent, the residue was separated by column chromatography on Si gel to give pure excavatolide G **(2)** (hexanes−EtOAc, 5:1; 7.5 mg, 21%) and excavatolide L **(7)** (hexanes−EtOAc, 3:1; 22.1 mg, 68%); physical and spectral data were in full agreement with those of the natural products **2** and **7**.

**Acetylation of Excavatolide B (11).** According to the above procedure, excavatolide B **(11)** (20.4 mg) was acetylated to produce excavatolide I **(4)** (hexanes−EtOAc, 4:1; 18.9 mg, 87%); physical and spectral data were in full agreement with those of the natural product **4**.

**Acetylation of Excavatolide C (12).** According to the above procedure, excavatolide C **(12)** (19.0 mg) was acetylated to the product excavatolide K **(6)** (hexanes−EtOAc, 3:1; 16.9 mg, 83%); physical and spectral data were in full agreement with those of the natural product **6**.

**Acetylation of Excavatolide M (8).** According to the above procedure, excavatolide M **(8)** (5.2 mg) was acetylated to the product excavatolide K **(6)** (hexanes−EtOAc, 3:1; 3.8 mg, 58%); physical and spectral data were in full agreement with those of the natural product **6**.

**Butyrylation of Excavatolide B (11).** Excavatolide B **(11)** (21.2 mg) was stirred with 4 mL of *n*-butyric anhydride in 4 mL of pyridine for 72 h at room temperature. After evaporation of excess reagent, the residue was separated by column

chromatography on Si gel to give pure excavatolide H **(3)** (hexanes−EtOAc, 5:1; 19.1 mg, 81%); physical and spectral data were in full agreement with those of the natural product **3**.

**Butyrylation of Excavatolide C (12).** According to the above procedure, excavatolide C **(12)** (22.4 mg) was butyrylated to the product excavatolide J **(5)** (hexanes−EtOAc, 4:1; 21.9 mg, 87%); physical and spectral data were in full agreement with those of the natural product **5**.

**Cytotoxicity Testing.** KB and P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A-549 (human lung adenocarcinoma) and HT-29 (human colon adenocarcinoma) were purchased from the American Type Culture Collection. The cytotoxic activities of tested compounds against the above four cancer cells were assayed with a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[19]

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