Pachyclavulariaenones D−G, New Diterpenoids from the Soft Coral *Pachyclavularia violacea*

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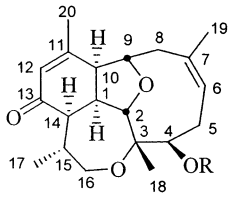
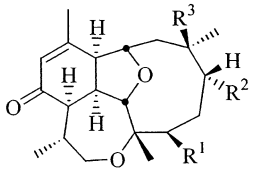
Four new diterpenoids, pachyclavulariaenones D−G (**1**−**4**), have been isolated from the soft coral *Pachyclavularia violacea*. The structures and relative stereochemistry of metabolites **1**−**4** were established on the basis of extensive NMR studies and chemical methods. The structure, including the relative configuration of pachyclavulariaenone F (**3**), was further confirmed by a single-crystal X-ray analysis. Pachyclavulariaenone G (**4**) has been shown to exhibit significant cytotoxicity toward P-388 and HT-29 cancer cells.

During the past several years, we have been devoted to the discovery of new bioactive metabolites from gorgonian corals. The investigation has resulted in the isolation of several cytotoxic suberosane-related sesquiterpenes from the gorgonian *Isis hippuris*[1] and a series of cytotoxic briarane diterpenes from the organisms *Briareum* spp.,[2] *Briareum excavatum*,[3−8] and *Junceella fragilis*.[9] In our continuing search for bioactive compounds from marine invertebrates, we found that an octacoral, *Pachyclavularia violacea* (Quey & Gaimard, 1833), which was collected along the coast of Kenting, located in the southernmost tip of Taiwan, has a colonial morphology very similar to those of *Briareum* spp.[10,11] As several structurally novel diterpenoids have been isolated from *P. violacea*[12−14] and because the organic extract of this organism showed significant cytotoxicity against the growth of P-388 (mouse lymphocytic leukemia) cells (ED_{50} = 0.3 μg/mL), we then carried out the investigation on searching for bioactive metabolites from this organism. Our previous studies have led to the isolation of nine novel diterpenoids from *P. violacea*, namely, pachyclavulariaenones A−C (**5**−**7**)[15] and pachycalvulariales G−L.[16] Our continuing investigation has further afforded the isolation of four new compounds, pachyclavulariaenones D−G (**1**−**4**). The molecular structures of metabolites **1**−**4** were determined by spectroscopic and chemical methods, and the structure of **3** was unambiguously established by a single-crystal X-ray analysis. In the cytotoxicity testing, we observed that pachyclavulariaenone F (**4**) exhibited significant cytotoxicity toward P-388 and HT-29 cancer cell lines.



1 : R = H
5 : R = $COCH_2CH_2CH_3$
6 : R = Ac



2 : R^1 = OAc, R^2 = OAc, R^3 = OH
3 : R^1 = OH, R^2 = OAc, R^3 = OH
4 : R^1 = OH, R^2 = OH, R^3 = OH
7 : R^1 = OAc, R^2 = OH, R^3 = OH

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Results and Discussion

Pachyclavulariaenone D (**1**) was isolated as a white powder with a molecular formula of $C_{20}H_{28}O_4$ requiring seven degrees of unsaturation, as established by HR-FABMS. The FABMS of **1** exhibited peaks at *m/z* 333 [M + H]$^+$, 315 [M + H − H_2O]$^+$, suggesting the presence of a hydroxy group in **1**. The IR spectrum exhibited a broad absorption at ν_{max} 3345 cm^{-1} and an intense absorption at 1672 cm^{-1}, indicating the presence of the hydroxy and enone structural moieties in **1**. Inspection of the ^{13}C NMR spectral data (Table 1) for **1** with the assistance of the DEPT spectrum showed the presence of four methyls, three methylenes, seven sp^3-hybridized methines, one carbonyl and one quaternary sp^3-carbon, and two trisubstituted double bonds. The ^{1}H NMR spectrum (Table 2) also showed the presence of four methyl groups, including a methyl attached to a methine carbon (δ 1.05, 3H, d, J = 7.1 Hz), a methyl attached to an oxygen-bearing quaternary carbon (δ 1.25, 3H, s), and two olefinic methyls (δ 1.86, 3H, s and 1.93, 3H, s). Two protons showed absorption peaks at δ 3.81 (1H, d, J = 10.6 Hz) and 4.32 ppm (1H, dd, J = 8.0, 2.2 Hz), respectively, suggesting the presence of a 2,9-disubstituted ether ring as those appearing in pachyclavulariaenone-based compounds.[14] The second ether ring in the molecules of **1** was further characterized by the signals of an oxymethylene group (δ 3.58, 2H, m). Also, three signals appearing at δ 3.12 (1H, m), 2.82 (1H, brs), and 2.38 ppm (1H, m) were found to be very similar to signals of the ring-junctured protons (H-1, H-10, and H-14) of pachyclavulariaenones A−C.[15] The signals of the remaining sp^3-methine proton H-15 (δ 2.61, 1H, m) and two olefinic protons, H-6 (δ 5.61, 1H, t, J = 7.8 Hz) and H-12 (δ 5.92, 1H, s), were further identified. On the basis of the above observations, metabolite **1** was assumed to be a pachyclavulariaenone-derived natural product. It was further found that the spectral data (^{1}H and ^{13}C NMR) of **1** were very similar to those of pachyclavlariaenone B (**6**).[15] However, the chemical shift for H-4 (δ_H 4.06, 1H, d, J = 7.9 Hz) of **1** was found to be upfield in comparison with that of **6** (δ_H 5.28, 1H, d, J = 7.8 Hz). Thus, the acetoxy group of **6** should be replaced by a hydroxy group in **1**. Connectivities from ^{1}H−^{1}H COSY and HMBC spectral analyses further confirmed the molecular framework of **1** (Figure 1).

The relative stereochemistry of **1** was determined by a NOESY spectrum (Figure 2). It was found that H-1 showed NOE interactions with H-10 and H-14, and H-14 exhibited an NOE interaction with H_3-17 but not with H-15, sug-

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Table 1. ^{1}H NMR Chemical Shifts for Compounds **1**−**4**

proton	**1**[a]	**2**[b]	**3**[b]	**4**[c]
1	3.12 m	3.61 ddd (7.6, 4.4, 3.6)	3.48 m	3.46 m
2	3.81 d (10.6)[d]	4.05 d (8.0)	4.04 d (9.9)	3.61 m
4	4.06 d (7.9)	5.90 dd (4.8, 3.2)	4.35 dd (8.2, 4.3)	3.96 m
5α	2.90 m	2.10 m	2.23 m	1.67 m
β	1.97 m			
6	5.61 t (7.8)	5.60 br	5.95 br	4.47 m
8α	2.80 br s	2.39 br t (13.7)	2.22 d (3.4)	1.93 m
β	2.04 m	1.84 m	1.80 t (12.2)	1.60 m
9	4.32 dd (8.0, 2.2)	5.05 dd (9.6, 2.8)	5.02 dd (12.2, 3.4)	4.62 m
10	2.82 br s	2.78 d (4.8)	2.74 br d (5.8)	2.84 d (4.5)
12	5.92 s	6.06 s	6.03 s	5.87 br s
14	2.38 m	2.64 d (3.6)	2.59 m	2.56 br s
15	2.61 m	2.84 m	2.83 m	2.46 br s
16α	3.58 m	3.47 dd (10.8, 2.0)	3.49 dd (13.3, 3.0)	3.34 m
β		3.75 d (10.8)	3.81 d (13.3)	
17	1.05 d (7.1)	1.25 d (6.0)	1.13 d (7.4)	1.01 d (7.0)
18	1.25 s	1.44 s	1.42 s	1.06 s
19	1.86 s	1.57s	1.54s	1.10 s
20	1.93 s	1.80 s	1.84 s	1.98 s
acetate methyls		2.13 s	2.03 s	
		2.13 s		

[a] The ^{1}H NMR spectra were recorded at 300 MHz in $CDCl_3$ at 25 °C. [b] 400 MHz in pyridine-d_5 at 70 °C. [c] 400 MHz in acetone-d_6 at −68 °C. [d] *J* values (in Hz) in parentheses. The values are downfield from TMS.

Table 2. ^{13}C NMR Chemical Shifts for Compounds **1**−**4**

carbon	**1**[a]	**2**[b]	**3**[b]	**4**[c]
C-1	39.1 d[d]	38.6 d	38.0 d	37.2 d
C-2	85.4 d	85.2 d	84.6 d	83.3 d
C-3	78.6 s	76.8 s	77.9 s	77.5 s
C-4	72.5 d	71.7 d	69.7 d	69.8 d
C-5	33.1 t	*e*	35.7 t[g]	40.0 t
C-6	127.3 d	77.3 d	77.4 d	71.6 d
C-7	129.9 s	73.4 s	73.6 s	75.0 s
C-8	38.6 t	44.9 t	45.5 t	44.8 t
C-9	81.6 d	79.1 d	79.0 d	79.0 d
C-10	48.7 d	51.7 d	51.7 d	51.4 d
C-11	156.6 s	156.9 s	158.6 s	158.6 s
C-12	126.9 d	128.3 d	128.2 d	127.6 d
C-13	198.0 s	197.3 s	197.1 s	198.0 s
C-14	48.4 d	49.4 d	49.3 d	48.3 d
C-15	31.9 d	31.2 d	30.8 d	30.6 d
C-16	65.1 t	64.7 t	64.7 t	64.0 t
C-17	18.1 q	17.3 q	17.3 q	16.9 q
C-18	19.0 q	18.3 q	17.1 q	16.9 q
C-19	29.7 q	29.7 q	26.6 q	22.9 q
C-20	21.9 q	21.2 q	21.1 q	21.5 q
acetate methyls		21.1 s	21.1 q	
		170.1 s[f]	170.2 s	
		21.1 s		
		170.2 s[f]		

[a] The ^{13}C NMR spectra were recorded at 75 MHz in $CDCl_3$ at 25 °C. [b] 100 MHz in pyridine-d_5 at 70 °C. [c] 100 MHz in acetone-d_6 at −68 °C. [d] Multiplicity deduced by DEPT and indicated by the usual symbols. [e] Signal not observed. [f] Assignment may be interchanged in each column. [g] Signal broadened and assigned by DEPT and HMQC spectral. The values are downfield from TMS.

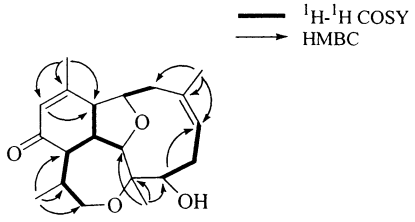

Figure 1. Selective ^{1}H−^{1}H COSY and HMBC correlations of pachyclavulariaenone D (**1**).

gesting that H-1, H-10, H-14, and H_3-17 should be positioned on the same side and was assigned as α-oriented protons. Furthermore, H-2 exhibited an interaction with H_3-18, but not with H-1; H_3-18 showed NOE responses with

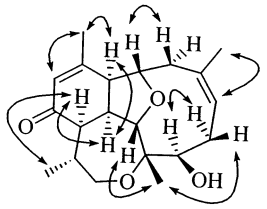

Figure 2. Selective NOE correlations of pachyclavulariaenone D (**1**).

H_β-5, but not with H-4. Thus, H-2 and H_3-18 should be located on the *β*-face. Also, H-4 showed NOE responses with H_α-5, but not with H_3-18, revealing the α-orientation of H-4. The *trans* orientation of the 6,7-double bond was determined by the NOE interaction between H-6 and H_3-19. Hence, the structure of pachyclavulariaenone D could be established as described by formula **1**.

Pachyclavulariaenone E (**2**) was isolated as a white solid, and its molecular formula, $C_{24}H_{34}O_8$, was established from HREIMS. Thus, eight degrees of unsaturation were determined for the molecule of **2**. The IR spectrum contained a broad absorption at ν_{max} 3470 (hydroxy) and intense absorptions at ν_{max} 1732 (ester carbonyl group) and 1672 cm^{-1} (enone carbonyl). The EIMS of **2** exhibited peaks at *m/z* 450 $[M]^+$, 432 $[M - H_2O]^+$, 390 $[M - HOAc]^+$, 372 $[M - H_2O - HOAc]^+$, 330 $[M - 2\ HOAc]^+$, and 312 $[M - H_2O - 2\ HOAc]^+$, also suggesting the presence of a hydroxy and two acetoxy groups in **2**. The ^{1}H NMR ($CDCl_3$) spectrum was found to be very similar to that of **1**, but the ^{13}C NMR spectrum of **2** in $CDCl_3$ gave mostly very weak or broad signals, suggesting the existence of slowly interconverting conformers in $CDCl_3$ solution. It was found that the signals of both ^{1}H and ^{13}C NMR spectra became well-resolved in pyridine-d_5 at 70 °C. The ^{13}C NMR spectrum of **2** is similar to that of **1**, except that the signals for carbons of the 6,7-double bond disappeared and were replaced by signals of two oxygenated carbons. Furthermore, it was found that the spectral data (IR, ^{1}H and ^{13}C NMR) of **2** are very similar to those of a known diterpene, pachycalvulariaenone C (**7**),[14] suggesting that pachyclavulariaenone E (**2**) is the acetyl derivative of compound **7**. We observed further that acetylation of **7** gave a diacetoxyl diterpenoid which was found to be identical to **2**, by comparison of the physical and spectral data. Thus, the structure of pachyclavulariaenone E (**2**) was determined unambiguously.

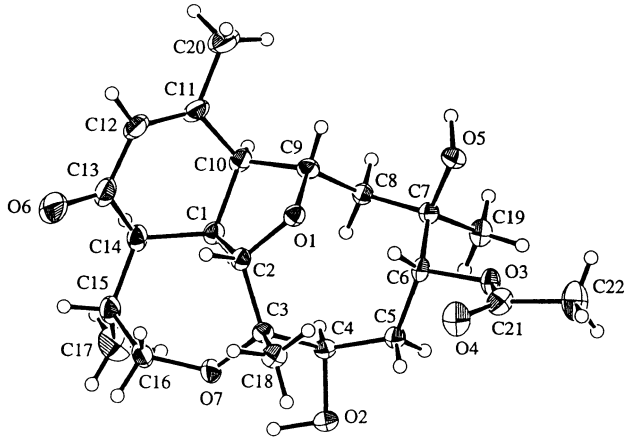

Figure 3. Computer-generated ORTEP plot of **3** showing the relative configurations.

Pachyclavulariaenone F (**3**) was purified as colorless crystals and had a molecular formula of $C_{22}H_{32}O_7$, as determined by HREIMS. Thus, seven degrees of unsaturation were determined for **3**. The IR spectrum exhibited absorption bands of hydroxy (ν_{max} 3340 cm^{-1}), the enone carbonyl (ν_{max} 1663 cm^{-1}), and ester carbonyl (ν_{max} 1736 cm^{-1}) groups. The EIMS exhibited peaks at *m/z* 408 $[M]^+$, 390 $[M - H_2O]^+$, 372 $[M - 2\ H_2O]^+$, 348 $[M - HOAc]^+$, 330 $[M - H_2O - HOAc]^+$, and 312 $[M - 2\ H_2O - HOAc]^+$, indicating the presence of an acetoxy and two hydroxy groups in **3**. Like compound **2**, well-resolved NMR spectra (^{1}H and ^{13}C NMR) of **3** could be obtained in pyridine-d_5 at 70 °C. The NMR spectra were very similar to those of **2** except NMR data indicated that only one acetoxy group was present in **3**. From this information, the planar structure of pachyclavulariaenone F (**3**) was established. The COSY and HMBC spectra correlations of this metabolite also were in full agreement with the structure **3**. The relative stereochemistry of **3** was also deduced using a NOESY spectrum, which showed that the relative configuration of **3** should be similar to that of **2**. Acetylation of **3** was found to give a product that is identical to **2**, suggesting **3** is the acetyl derivative of **2**. A single-crystal X-ray structure analysis was further carried out in order to confirm the molecular structure of **3**. The X-ray structure of **3** (Figure 3) demonstrated the location of two hydroxy groups and one acetoxy group at C-4, C-7, and C-6, respectively. Therefore, the structure of **3**, including the relative configuration, was determined unambiguously.

Pachyclavulariaenone G (**4**) was obtained as a white powder. Its molecular formula, $C_{20}H_{30}O_6$, was established by mass spectral data and extensive NMR studies. Thus, six degrees of unsaturation were determined for **4**. The IR absorption of **4** also showed the presence of the hydroxy (ν_{max} 3420 cm^{-1}) and the enone carbonyl groups (ν_{max} 1668 cm^{-1}) in **4**. Unlike **2** and **3**, the well-resolved NMR spectra of **4** was obtained in acetone-d_6 at −68 °C. However, it was found that the spectral data (1D and 2D NMR) of **4** were very similar to those of **2** and **3**. The olefinic and carbonyl resonances in the ^{13}C NMR spectrum of **4** appeared at δ 127.6 (d, C-12), 158.6 (s, C-11), and 198.0 (s, C-13), respectively, again confirming the presence of a conjugated enone moiety. Furthermore, acetylation of **4** also gave a less polar product, which was found to be identical to compound **2**, after comparison of physical and spectral data. On the basis of the above observations, the structure of **4**, including the relative stereochemistry, was elucidated unambiguously.

The cytotoxicity of pachyclavulariaenones D−G (**1**−**4**) against the growth of P-388, A549, and HT-29 cancer cell lines also has been studied. The results showed that compounds **1**−**3** are not cytotoxic against the above cells. However, compound **4** was shown to exhibit significant cytotoxicity toward P-388 (ED_{50} = 0.2 μg/mL) and HT-29 (ED_{50} = 3.2 μg/mL) tumor cells.[17]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. Melting points were determined using a Fisher-Johns melting points apparatus and were uncorrected. IR spectra were measured on Hitachi I-2001 and Jasco FT/IR-5300 infrared spectrophotometers. Ultraviolet spectra 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 $CDCl_3$ or pyridine-d_5 or acetone-d_6 using TMS as an internal standard, unless otherwise indicated. MS spectra were obtained with a VG QUATTRO GC/MS spectrometer. HRMS spectra were recorded on a JEOL JMX-HX 110 mass spectrometer. High-performance liquid chromatography (HPLC) was conducted with a Hibar Si-60 column (7 μm, 250 mm × 25 mm i.d.) for normal phase. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytic TLC.

Animal Material. The soft coral *P. violacea* (3.0 kg fresh wt) was collected by hand using scuba on reefs at depths of 10−15 m, along the coast of Kenting, located in the southernmost tip of Taiwan in September 1995, and was stored in a freezer until used. A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University.

Extraction and Isolation. The freeze-dried organism (711 g) was minced and extracted exhaustively with EtOAc. The organic extract was evaporated to give a dark oily residue (60.0 g). The EtOAc layer was found to exhibit significant cytotoxity against the P-388 cell line with an ED_{50} of 0.3 μg/mL and the KB cell line with an ED_{50} of 4.3 μg/mL. The organic extract was chromatographed on silica gel column chromatography, using EtOAc−hexane (stepwise, 0−100% EtOAc) to afford 47 fractions. Further separation and purification of these fractions were carried out by silica gel column chromatography and normal-phase HPLC. Fraction 15 was further separated by silica gel column chromatography eluting with hexane−EtOAc (5:1) to obtain compound **1**. Fraction 21 was further separated by normal-phase HPLC eluting with hexane−EtOAc (2:1) to afford compound **2**. Fraction 26 was separated by normal-phase HPLC eluting with hexane−EtOAc (1:1) to afford compound **3**. Fraction 28 was further purified by silica gel chromatography eluting with hexane−EtOAc (1:1 to 1:4) to obtain compound **4**.

Pachyclavulariaenone D (1): white solid (1.7 mg); mp 94−96 °C; $[\alpha]^{29}_D$ +67° (*c* 0.09, $CHCl_3$); IR (neat) ν_{max} 3445, 2930, 1672, 1381, 1242, and 1078 cm^{-1}; UV (95% EtOH) λ_{max} 229 nm (ϵ 11 950); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 333 [5, $(M + H)^+$], 318 [1], 315 [8], and 300 [1]; HRFABMS *m/z* 333.2066 (calcd for $C_{20}H_{29}O_4$, 333.2067).

Pachyclavulariaenone E (2): white solid (6.6 mg); mp 97−99 °C; $[\alpha]^{29}_D$ +31° (*c* 0.33, $CHCl_3$); IR (neat) ν_{max} 3470, 2926, 1732, 1672, 1373, 1251, and 1033 cm^{-1}; UV (95% EtOH) λ_{max} 229 nm (ϵ 10 880); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 450 [0.2, $(M)^+$], 432 [0.1, $(M - H_2O)^+$], 390 [0.4, $(M - HOAc)^+$], 372 [0.2, $(M - H_2O - HOAc)^+$], 330 [0.4, $(M - 2\ HOAc)^+$], and 312 [0.2, $(M - H_2O - 2\ HOAc)^+$]; HREIMS *m/z* 450.2251 (calcd for $C_{24}H_{34}O_8$, 450.2254).

Pachyclavulariaenone F (3): colorless crystals (43.8 mg); mp 87−90 °C; $[\alpha]^{29}_D$ +41° (*c* 1.74, $CHCl_3$); IR (neat) ν_{max} 3440, 2928, 1736, 1663, 1380, 1248, and 1034 cm^{-1}; UV (95% EtOH) λ_{max} 229 nm (ϵ 11 028); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 408 [0.2, $(M)^+$], 390 [1, $(M - H_2O)^+$], 372

[0.2, $(M - 2\ H_2O)^+$], 349 [2, $(M - OAc)^+$], 330 [2, $(M - H_2O - HOAc)^+$], and 312 [0.3, $(M - 2\ H_2O - HOAc)^+$]; HREIMS *m*/*z* 408.2148 (calcd for $C_{22}H_{32}O_7$, 408.2149).

Pachyclavulariaenone G (4): white solid (8.6 mg); mp 189−192 °C; $[\alpha]^{29}_D$ +14° (*c* 0.43, $CHCl_3$); IR (neat) ν_{max} 3420, 2926, 1732, 1668, 1383, 1259, and 1028 cm^{-1}; UV (95% EtOH) λ_{max} 229 nm (ϵ 10 834); ^1H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m*/*z* 349 [12, $(M + H - H_2O)$], 331 [8, $(M + H - 2\ H_2O)$]; HRFABMS *m*/*z* 349.2015 [$C_{20}H_{30}O_6 + H - H_2O$] (calcd for $C_{20}H_{29}O_5$, 349.2016).

Acetylation of Pachyclavulariaenone F (3). A solution of pachyclavulariolide F (**3**) (4.2 mg) in pyridine (2.0 mL) was added with acetic anhydride (1.0 mL), and the mixture was stirred at room temperature for 48 h. After evaporation of excess reagent, the residue was separated by column chromatography on silica gel to give compound **2** (2.5 mg).

Acetylation of Pachyclavulariaenone G (4). According to the above procedure, pachyclavulariaenone G (**4**) (3.9 mg) was acetylated to the compound **2** (2.8 mg); physical and spectral data were in full agreement with those of the natural product **2**.

Single-Crystal X-ray Crystallography of 3.[18] Suitable colorless prisms of **3** were obtained from a solution in EtOAc. The crystal (0.60 × 0.78 × 0.86 mm) belongs to the orthorhombic system, space group $P2_12_12_1$ (# 19) with $a = 8.438(2)$ Å, $b = 10.677(4)$ Å, $c = 23.949(3)$ Å, $V = 2169.0(9)$ Å^3, $Z = 4$, $D_{calcd} = 1.073$ g/cm^3, μ(Mo Kα) = 0.71 cm^{-1}. Intensity data were measured on a Rigaku AFC7S diffractometer up to $2\theta_{max}$ of 50.0°. Of the 2223 reflections collected only 1941 unique reflections with $I > 3.00\sigma(I)$ were used in the calculations. The structure was solved by direct methods and refined by a full-matrix least-squares procedure. The non-hydrogen atoms were given anisotropic thermal parameters. All hydrogen atoms were put at their idealized positions. The refinement converged to a final $R = 0.047$, $R_w = 0.081$.

Cytotoxicity Testing. P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A549 and HT-29 cells were purchased from the American Type Culture Collection. Cytotoxicity assays were carried out according to the procedure described previously.[19]

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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