Cytotoxic Clerodane Diterpenoids from *Casearia membranacea*

Ya-Ching Shen,*,† Yuan-Bin Cheng,† Atallah F. Ahmed,† Cheng Lin Lee,† Shun-Ying Chen,‡ Ching-Te Chien,‡ Yao-Haur Kuo,§ and Guo-Lin Tzeng†

Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung 804, Taiwan, Republic of China, Division of Silviculture, Taiwan Forestry Research Institute, Taipei, Taiwan, Republic of China, and National Research Institute of Chinese Medicine, Taipei, Taiwan, Republic of China

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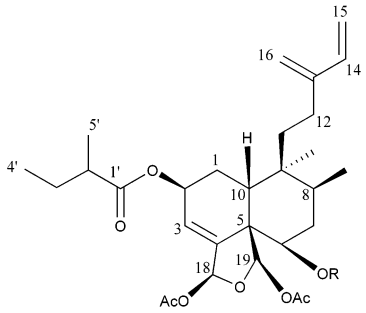
Bioassay-guided fractionation of the EtOAc extract of *Casearia membranacea* leaves and twigs afforded three new clerodane diterpenes, caseamembrins M−O (**1**−**3**), and the known *rel*-(2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,-18*S*,19*R*)-2-(2-methylbutyryloxy)-6-hydroxy-18,19-di-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene (**4**) and caseamembrin D (**5**). The structures of **1**−**3**, including the relative configurations, were established by extensive NMR spectroscopic analyses. The cytotoxic activities of the isolated diterpenoids against human oral epidermoid (KB), medulla (Med), and colon (DLD-1) cancer cell lines were evaluated.

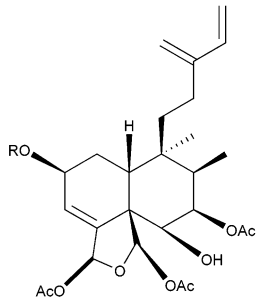
The genus *Casearia* is a rich source of clerodane-type diterpenoids.[1−5] Many of these diterpenoids possess cytotoxic, immunomodulatory,[6−9] trypanocidal,[10] and antimalarial[11] activities. *C. membranacea* Hance (Flacourtiaceae) is a tree growing wildly in the northern region of Taiwan.[12] Previously, a cytotoxic butenolide, two dolabellanes, a chroman, and a benzoquinol derivative were reported from this species.[13] In a continuation of our phytochemical research on this tree,[14−16] a bioassay-guided fractionation of the EtOAc extract of the leaves and twigs has led to the isolation of three new clerodane-type diterpenoids, caseamembrins M−O (**1**−**3**), along with the known *rel*-(2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(2-methylbutyryloxy)-6-hydroxy-18,19-di-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene (**4**)[8] and caseamembrin D (**5**).[14] The structures of the new compounds, including their relative configuration, were determined on the basis of extensive spectroscopic analyses. The cytotoxic activities of the isolated diterpenoids against a limited panel of human cancer cell lines (KB, Med, and DLD-1) have been evaluated.

The powdered leaves and twigs of *C. membranacea* were extracted with acetone, and the concentrated organic layer was then partitioned between EtOAc and H_2O (1:1) to produce the EtOAc extract. Separation of the EtOAc extract, using different chromatographic methods, including CC, HPLC, and preparative TLC, yielded **1**−**5** (see Experimental Section).

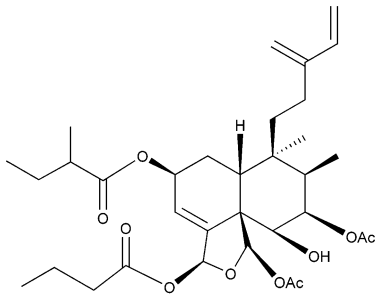
The molecular formula of **1** was established as $C_{33}H_{48}O_9$ from HRESIMS, implying 10 degrees of unsatauration. The UV absorption (λ 224 nm) and IR bands (1742 and 1635 cm^{-1}) indicated functionalities of conjugated diene and esters. The ^1H and ^{13}C NMR data of **1** (Tables 1 and 2) revealed the presence of two acetal methines (δ_H/δ_C 6.53/98.2; 6.51/95.1), one trisubstituted olefinic bond (δ_H/δ_C 5.97/123.1; 144.3), two oxymethines (δ_H/δ_C 5.43/66.0; 4.99/73.5), one tertiary methyl (δ_H/δ_C 0.96/25.5), and one secondary methyl (δ_H/δ_C 0.92/15.5), which are diagnostic for clerodane diterpenes.[1−5,10,11,14−16] The olefinic region of the ^1H NMR spectrum exhibited three proton signals with characteristic *cis*/*trans* coupling at δ 5.19 (d, J = 16.5 Hz, H_a-15), 5.03 (d, J = 10.6 Hz, H_b-15), and 6.44 (dd, J = 16.5, 10.6 Hz, H-14) and two proton singlets at δ 4.95 and 5.06 (H_2-16) indicative of respectively monosubstituted and 1,1-disubstituted double bonds of the clerodane side chain at C-9. The carbon signals including those of ester carbonyl



1 R = $CO(CH_2)_2CH_3$
4 R = H



2 R = $CO(CH_2)_2CH_3$
3 R = $COCH_2CH(CH_3)_2$



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* To whom correspondence should be addressed. Tel: 886-7-525-2000, ext. 5058. Fax: 886-7-525-5020. E-mail: ycshen@mail.nsysu.edu.tw.
† National Sun Yat-Sen University.
‡ Division of Silviculture, Taiwan Forestry Research Institute.
§ National Research Institute of Chinese Medicine.

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Table 1. ^{1}H NMR Data ($CDCl_3$, 300 MHz)[a] of Compounds **1**–**3**

no.	1	2	3[b]
1	2.02 m (β), 1.96 m	2.05 m (β), 1.97 m	2.04 m (β), 1.96 m
2	5.43 brs	5.45 brs	5.43 brs
3	5.97 brs	5.99 brd (3.2)	6.02 brs
6	4.99 dd (4.5, 10.0)	3.67 d (10.2)	3.67 d (10.5)
7	1.70 m	5.01 m	5.01 m
8	2.40 m	1.85 m	1.85 m
10	2.30 m	2.36 m	2.37 m
11	1.26 m	1.25 m	1.24 m
		1.68 m	1.68 m
12	2.10 m	2.12 m	2.10 m
14	6.44 dd (10.6, 16.5)	6.43 dd (10.8, 17.6)	6.43 dd (10.8, 17.5)
15	5.19 d (16.5)	5.17 d (17.6)	5.17 d (17.5)
	5.03 d (10.6)	5.04 d (10.8)	5.02 d (10.8)
16	5.06 s, 4.95 s	5.02 s, 4.98 s	5.01 s, 4.98 s
17	0.92 d (6.7)	0.90 d (6.6)	0.90 d (6.9)
18	6.51 s	6.71 s	6.71 s
19	6.53 s	6.52 s	6.52 s
20	0.96 s	0.99 s	0.99 s
2′	2.46 m	2.35 m	2.24 m
3′	1.70 m	1.70 m	2.10 m
4′	0.98 t (7.0).	0.98 t[c]	1.00 d[c]
5′	1.18 d (7.0).		1.00 d[c]
2″	2.36 t (7.5)		
3″	1.66 m		
4″	0.96 m		
OAc	1.89 s	1.88 s	1.86 s
	2.06 s	2.04 s	2.01 s
		2.10 s	2.16 s

[a] Assignments were made using COSY and HMBC techniques. [b] Measured at 500 MHz. [c] Overlapped signals.

carbons (δ 170.0, 169.9, 173.0, 175.8) were attributable to two *O*-acetyl, one *O*-*n*-butyryl, and one 2′-methylbutyryloxyl ester substituents. The tricyclic structure of **1** with its four ester substituents was further established by detailed analyses of ^{1}H–^{1}H COSY (H-1/H-2/H-3; H-6/H-7/H-8; H-11/H-12) and HMBC (H-12/C-9; H-14/C-16; H-15/C-13; H-18/C-3,C-19; H-19/C-4,C-5,C-6,C-18; H-3/C-1,C-5,C-18; H-6/C-4,C-19,C-1″). These findings confirmed the location of a six-carbon diene side chain at C-9 and the *n*-butyryloxy group at C-6. Similarly, the attachments of the two acetate esters at C-18 and C-19 were proved by HMBC with the corresponding acetate carbonyl signal. Finally, the remaining H-2 (δ 5.43) provided the assignment of the 2′-methylbutyrate moiety at C-2.

The β-orientation of the 2′-methylbutanoyloxy group at C-2 was deduced from the splitting pattern of H-2 (br s) and the characteristic chemical shift of C-2 (δ 66.0).[8,17] The NOESY correlations between H-2/H_α-1; H_α-1/H-6, H-8, and H_3-20; and H-19/H-6 and H-18 (Figure 1) further established the α-orientation of H-6, H-8, H-18, H-19, and H_3-20. Other NOESY interactions between H-1β/H-10 and H_3-17 disclosed the β-orientation of H-10 and H_3-17. The *n*-butyration of *rel*-(2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(2-methylbutyryloxy)-6-hydroxy-18,19-acetyloxy-18,19-epoxycleroda-3,13(16),14-triene (**4**) yielded a product identical with **1**, confirming not only the structural assignment but also the configuration of **1**. On the basis of the above findings, the structure of caseamembrin M (**1**) was fully determined as 2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(2-methylbutyryloxy)-6-*n*-butyryloxy-18,19-di-*O*-acetyl-18,19-epoxycleroda-3,13-(16),14-triene.

The molecular formula of **2** was established as $C_{30}H_{42}O_{10}$ from HRESIMS and NMR spectra. The UV and IR spectra of **2** revealed functionalities similar to those of **1**. The ^{1}H NMR and ^{13}C NMR data (Tables 1 and 2) revealed another esterified clerodane diterpene skeleton. Inspection of ^{13}C NMR data and 2D NMR (^{1}H–^{1}H COSY, HMQC, and HMBC) correlations of **2** indicated three acetate groups and one *n*-butyrate group as four esters linked to the skeleton of **2**. The oxymethine proton at δ 5.01 (H-7) and the two acetal protons at δ 6.71 (H-18) and at δ 6.52 (H-19) exhibited HMBC correlations to the carbonyl carbons (δ_C 173.1, 170.3 and δ_C 169.4) of one *n*-butyryloxy and two *O*-acetyl groups, respectively. The remaining two oxymethines at δ 3.67 and 5.01 were assigned to the protons at H-6 and H-7, respectively, as a result of HMQC correlations to methine carbons at δ_C 75.2 and 75.3. This was supported by a COSY correlation between H-6/H-7 as well as HMBC correlations between H-6 and C-4 and between H-7 and C-9. The third *O*-acetyl functionality was assigned to be at C-7 by the HMBC correlation between H-7 and the *O*-acetyl carbonyl carbon at δ 171.7. The *O*-acetyl groups at C-18 and C-19 and *O*-*n*-butyryl group at C-2 were

Table 2. ^{13}C NMR Data ($CDCl_3$, 75 MHz)[a] of Compounds **1**–**3**

no.	1	2	3[b]
1	26.7 (CH_2)	26.9 (CH_2)	26.8 (CH_2)
2	66.0 (CH)	66.0 (CH)	66.1 (CH)
3	123.1 (CH)	122.2 (CH)	122.2 (CH)
4	144.3 (C)	144.6 (C)[c]	144.6 (C)
5	52.1 (C)	53.8 (C)	53.8 (C)
6	73.5 (CH)	75.2 (CH)[d]	75.2 (CH)[c]
7	33.0 (CH_2)	75.3 (CH)[d]	75.3 (CH)[c]
8	37.1 (CH)	41.8 (CH)	41.8 (CH)
9	37.3 (C)	39.1 (C)	39.1 (C)
10	36.8 (CH)	35.9 (CH)	35.9 (CH)
11	27.9 (CH_2)	29.2 (CH_2)	29.1 (CH_2)
12	23.7 (CH_2)	23.8 (CH_2)	23.8 (CH_2)
13	145.0 (C)	144.7 (C)[c]	144.7 (C)
14	140.4 (CH)	140.4 (CH)	140.4 (CH)
15	112.2 (CH_2)	112.3 (CH_2)	112.2 (CH_2)
16	115.5 (CH_2)	115.9 (CH_2)	115.9 (CH_2)
17	15.5 (CH_3)	11.2 (CH_3)	11.2 (CH_3)
18	95.1 (CH)	95.5 (CH)	95.5 (CH)
19	98.2 (CH)	98.0 (CH)	98.0 (CH)
20	25.5 (CH_3)	25.8 (CH_3)	22.4 (CH_3)
1′	175.8 (CO)	173.1 (CO)	172.4 (CO)
2′	41.1 (CH)	36.5 (CH_2)	43.7 (CH_2)
3′	27.0 (CH_2)	18.7 (CH_2)	25.8 (CH)
4′	11.6 (CH_3)	13.6 (CH_3)	22.4 (CH_3)
5′	16.6 (CH_3)		25.7 (CH_3)
1″	173.0 (CO)		
2″	36.6 (CH_2)		
3″	18.2 (CH_2)		
4″	13.6 (CH_3)		
OAc	21.2 (CH_3)	21.0 (CH_3)	21.0 (CH_3)
	169.9 (CO)	169.4 (C)	169.4 (C)
OAc	21.5 (CH_3)	21.2 (CH_3)	21.2 (CH_3)
	170.0 (CO)	170.3 (CO)	170.3 (CO)
OAc		21.4 (CH_3)	21.4 (CH_3)
		171.7 (CO)	171.6 (CO)

[a] Multiplicities were assigned from DEPT and HMQC spectra. [b] Measured at 125 MHz. [c,d] Data are interchangeable.

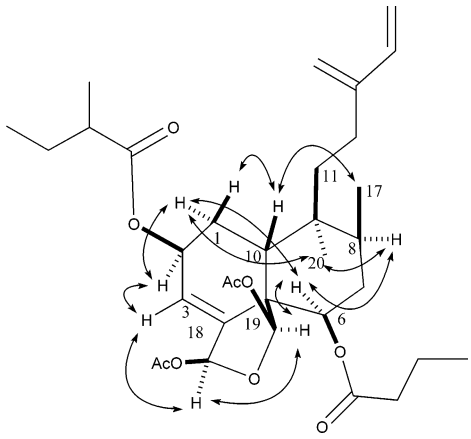

Figure 1. Selected NOESY correlations of **1**.

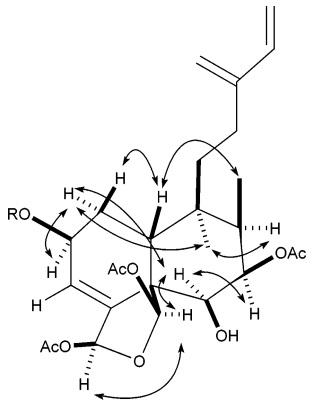

Figure 2. Selected NOESY correlations of **2** and **3**.

Table 3. Cytotoxicity of Compounds **1**−**5** against Human Tumor Cells (ED_{50}, μg/mL)[a]

compound	KB[b]	DLD-1[c]	Med[d]
1	18.4	14.2	14.4
2	8.94	4.37	3.27
3	6.94	2.54	1.94
4	3.60	1.18	1.93
5	5.57	1.81	2.42
mitomycin	0.13	0.10	0.10

[a] The concentration that inhibits 50% of the growth of human tumor cell lines after 72 h exposure according to the method described in the Experimental Section. [b] Oral epidermoid carcinoma. [c] Human colon adenocarcinoma. [d] Human medulloblatoma.

β-oriented, utilizing the same NOE correlations as those observed in **1**. Furthermore, the NOESY spectrum of **2** showed interactions between H-1α/H-6; H-6/H-19; H-6/H-7; and H-7/H-20, which indicated the β-orientation of the hydroxyl at C-6 and the *O*-acetyl at C-7 (Figure 2). On the basis of the above findings, caseamembrin N (**2**) was defined as 2*S*,5*R*,6*R*,7*S*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(*n*-butyryloxy)-6-hydroxy-7,18,19-tri-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene.

The molecular formula of **3** was determined as $C_{31}H_{44}O_{10}$ as concluded from its HRESIMS and NMR spectroscopic data. The ^{1}H and ^{13}C NMR data of **3** were similar to those of **2**, designating **3** as a clerodane diterpene differing in one of its ester substituents. Compound **3** was esterified by three acetyl groups and one 3-methylbutyryloxy group, as inferred from its collective spectroscopic data, including correlations observed in the ^{1}H−^{1}H COSY and HMBC spectra. The oxymethine proton H-2 (δ 5.43) exhibited an HMBC correlation to the carbonyl of the 3-methylbutyrate carbonyl carbon at δ_C 172.4, whereas the acetal protons H-18 (δ 6.71) and H-19 (δ 6.52) showed long-range correlations with the carbonyl carbons at δ_C 170.3 and 169.4, respectively. The signals of the oxymethines C-6 (δ_H 3.67 and δ_C 75.2) and C-7 (δ_H 5.01 and δ_C 75.3) were superimposable to those of compound **2**, indicating the same substitution pattern. The NOESY spectrum displayed interactions between H-18/H-19; H-19/H-6; H-6/H-7; H-6/Hα-1; and Hα-1/H-2, disclosing the β-orientation of the hydroxyl at C-6, the *O*-acetyl at C-7, and the 3-methylbutyryloxyl at C-2. The above findings and other detailed analyses of the NOESY spectrum (Figure 2) defined caseamembrin O (**3**) as 2*S*,5*R*,6*R*,7*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-[3-methylbutyryloxy]-6-hydroxy-7,18,19-tri-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene.

Compounds **1**−**5** were evaluated for their cytotoxic activity against human oral epidermoid (KB), colon (DLD-1), and medulla (Med) cancer cell lines, and the results are shown in Table 3. Compounds **2**−**5** showed significant activity (1−9 μg/mL, 2.5−16 μM) against the three tumor cell lines, while **1** was nearly inactive. Compounds **3**−**5** showed selective activity against DLD-1 and Med tumor cells (ED_{50}'s 1.18−4.37 μg/mL, 2.7−7.8 μM). A SAR study revealed that acylation of the C-6 hydroxyl group resulted in a reduction of activity. These data agree with the previous report that the free hydroxyl group at C-6 is critical for biological function.[14]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR and UV spectra were measured on Hitachi T-2001 and Hitachi U-3210 spectrophotometers, respectively. Low-resolution EIMS and FABMS spectra were recorded on a VG Quattro 5022 mass spectrometer. High-resolution ESIMS spectra were measured on a JEOL HX 110 mass spectrometer. The ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC, and NOESY spectra were recorded on a Bruker FT-300 spectrometer or on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively, using TMS as an internal standard. The chemical shifts are given in δ (ppm) and coupling constants in Hz. Silica gel 60 (Merck) was used for column chromatography (CC), and precoated silica gel plates (Merck, Kieselgel 60 F-254, 1 mm) were used for preparative TLC.

Plant Material. Leaves and twigs of *C. membranacea* were collected during May 2002 in Taipei County, Taiwan. This species was identified by one of the authors (C.-T.C.). A voucher specimen (TP207-1) has been deposited in the Institute of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan.

Extraction and Isolation. The powdered leaves and twigs of *C. membranacea* (6 kg) were extracted thrice with acetone, then the acetone extract was filtered and concentrated under vacuum. The resulting residue was partitioned between EtOAc/H_2O to produce an EtOAc-soluble part (270 g), which was partitioned with *n*-hexane/MeOH/H_2O (4:3:1) to yield *n*-hexane- and MeOH-soluble fractions. The latter (110 g) was column chromatographed on silica gel and eluted with *n*-hexane/EtOAc (100:1 to 1:5) to afford 25 fractions (F1 to F25). F15 (1.76 g) was separated on a Sephadex LH-20 column to yield six fractions. F15-3 (518 mg) was chromatographed on a silica gel column eluting with a gradient mixture of *n*-hexane/EtOAc (200:1 to 5:1) to produce 15 fractions. F15-3-1 (89 mg) was subjected to preparative TLC (Si gel) using a mixture of *n*-hexane/CH_2Cl_2/MeOH (50:50:3) to afford caseamembrin M (**1**, 5.8 mg) and *rel*-(2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(2-methylbutyryloxy)-6-hydroxy-18,19-di-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene (**4**, 49 mg).[8] F-20 (7.5 g) was subjected to a Sephadex LH-20 column and eluted with MeOH to yield caseamembrin D (**5**, 4.97 g). F21 (8.1 g) was fractionated over a silica gel column using *n*-hexane/CH_2Cl_2/EtOAc (100:2:3 to 1:2:3) for elution to afford 13 fractions (F21-1 to F21-13). F21-5 (1.34 g) was separated on a Sephadex LH-20 using MeOH for elution to give a residue (cas21-52, 523 mg). Part (100 mg) of the residue was separated by reversed-phase HPLC (C_{18}) using MeOH/H_2O (3:1) to yield caseamenbrins N (**2**, 42 mg) and O (**3**, 42.5 mg).

Caseamembrin M (1): colorless powder; $[\alpha]_D^{25}$ +17.9° (*c* 0.125, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.23) nm; IR (KBr) ν_{max} 2976, 1742, 1635 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz), Table 2; FABMS *m/z* 469 (M^+ − 2HOAc + H); EIMS *m/z* (70 eV) 528 (M^+ − HOAc), 486, 444, 426, 384, 327, 314, 297, 225, 187, 85, 71; HRESIMS *m/z* 611.3199 (calcd for $C_{33}H_{48}O_9Na$, 611.3196).

Butyration of 4. *rel*-(2*S*,5*R*,6*R*,8*S*,9*S*,10*R*,18*S*,19*R*)-2-(2-Methylbutyryloxy)-6-hydroxy-18,19-di-*O*-acetyl-18,19-epoxycleroda-3,13(16),14-triene (**4**, 15 mg)[8] was treated with *n*-butyric anhydride/pyridine (each 0.5 mL) at 50 °C for 3 h to produce, after usual workup, 12 mg of reaction product, whose physical data (^{1}H NMR, ^{13}C NMR, specific rotation, and MS) are identical to those of caseamembrin M (**1**).

Caseamembrin N (2): colorless powder; $[\alpha]_D^{25}$ +9.8° (*c* 0.7, MeOH); UV (MeOH) λ_{max} (log ϵ) 222 (4.25) nm; IR (KBr) ν_{max} 3445, 2972, 1735, 1633 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz), Table 2; FABMS *m/z* 585 (M^+ + Na); EIMS *m/z* (70 eV) 444, 427, 384, 373, 327, 292, 267, 253, 237, 187, 159, 149, 135, 107, 95, 81, 71; HRESIMS *m/z* 585.2672 (calcd for $C_{30}H_{42}O_{10}Na$, 585.2676).

Caseamembrin O (3): colorless powder; $[\alpha]_D^{25}$ +38.8° (*c* 1.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.21) nm; IR (KBr) ν_{max} 3450, 2968, 1736, 1636 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), Table 2; FABMS *m/z* 599 (M^+ + Na); EIMS *m/z* (70 eV) 239, 201, 185, 173, 157, 135, 119, 105, 95, 81; HRESIMS *m/z* 599.2834 (calcd for $C_{31}H_{44}O_{10}Na$, 599.2382).

Cytotoxicity Assay. The cells for assay were cultured in a RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. The cytotoxicity assay depends on the binding of methylene blue to fixed monolayers of cells at pH 8.5, washing the monolayer, and releasing the dye by lowering the pH value. Samples and control standard drugs were prepared at a concentration of 1, 10, 40, and 100 μg/mL. After seeding 2880 cells/well in a 96-well microplate for 3 h, 20 μL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After removing the medium from the microplates, the cells were fixed with 10% formaldehyde in 0.9% saline for 30 min, then dyed with 1% (w/v) methylene blue in 0.01 M borate-buffer (100 μL/well) for 30 min. The 96-well plate was dipped into a 0.01 M borate-buffer solution four times in order to remove the dye. Then, 100 μL/well of EtOH/0.1 M HCl (1:1) was added as a dye-eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The ED_{50} value was defined by a comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

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