New Bioactive Clerodane Diterpenoids from the Roots of *Casearia membranacea*

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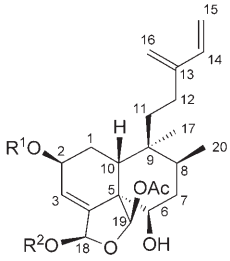
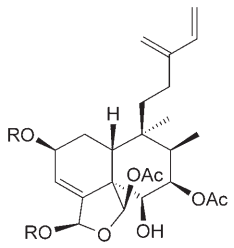
Bioassay-guided fractionation of the acetone extract of the roots of *Casearia membranacea* furnished three new clerodane diterpenes, caseamembrins S–U (**1**–**3**) and the known caseamembrin Q (**4**). Their structures were established by extensive spectroscopic analyses, especially 2D-NMR. Compounds **1**–**4** were tested against human tumor cells, including HeLa (cervical epitheloid carcinoma), DLD-1 (colon carcinoma), Daoy (medulloblastoma), and KB (oral epidermoid carcinoma) cell lines. Caseamembrin T (**2**) exhibited the most potent activity against Daoy cells (ED_{50} = 10 ng/ml), superior to that of the standard drug mitomycin.

Introduction. – Plants of the genus *Casearia* are reported to be a rich source of clerodane diterpenes with interesting biological functions such as antimycobacterial, antimalarial, and potent cytotoxic activities [1–5]. The tropical tree *Casearia membranacea* HANCE (Flacourtiaceae) grows wildly in the northern part of Taiwan [6]. Despite the numerous clerodane diterpenoids isolated from the leaves and stems of different collections of this species [7–11], the constituents of its roots have been scarcely investigated so far [11]. In a preliminary investigation, a fraction from the root extract of this plant showed promising cytotoxicity against human medulloblastoma cancer cells, with an ED_{50} value of 0.06 μg/ml. Bioassay-guided fractionation of the acetone extract of the roots now led to the isolation of three new clerodane diterpenoids, caseamembrins S–U (**1**–**3**), along with the known compound caseamembrin Q (**4**) [12]. Herein, we report the isolation, structural elucidation, and the biological evaluation of the isolates **1**–**4**, which were tested against a panel of human cancer cell lines.

Results and Discussion. – 1. *Structure Elucidation.* Compound **1** was isolated as a colorless, amorphous, optically active solid ($[\alpha]_D^{25} = +30.9$ (MeOH)). Its molecular formula was established as $C_{27}H_{38}O_8$ by HR-ESI-MS, indicating nine degrees of unsaturation. Characteristic UV (223 nm) and IR (1731 cm^{-1}) absorption bands pointed to the presence of a conjugated diene and ester groups, respectively. The ^{1}H-



	R^1	R^2
1	EtCO	Ac
2	$EtCH_2CO$	Ac
3	$EtCH_2CO$	Me



4 R = $EtCH_2CO$

and ^{13}C-NMR data of **1** (*Tables 1* and *2*, resp.), revealed an esterified clerodane-diterpene skeleton [13].

Inspection of the NMR data of **1** indicated two Ac and one propanoyl groups. In the olefinic region, three signals with characteristic *cis*/*trans* couplings were found at δ(H) 5.20 (*d*, J = 18 Hz, H_a–C(15)), 5.03 (*d*, J = 10.6 Hz, H_b–C(15)), and 6.44 (*dd*, J = 18, 11 Hz, H–C(14)), which indicated the presence of a vinyl group. Two *singlet*s at δ(H) 4.93 and 5.06 (CH_2(16)) further pointed to a 1,1-disubstituted C=C bond, making up the clerodane side chain at C(9) [14]. Two acetal H-atoms at δ(H) 6.51 (H–C(18)) and 6.53 (H–C(19)) exhibited HMBC correlations to the C=O resonances at δ(C) 170.1 and 169.9 of the two AcO groups (*Fig. 1*). The oxymethine signal at δ(H) 3.78 (H–C(6)) was correlated to a quaternary C-atom at δ(C) 53.6 (C(5)), and a second oxymethine at δ(H) 5.44–5.50 was assigned to H–C(2), based on HMBC correlations of H–C(10) (δ(H) 2.23–2.29) with C(2), C(6), and C(19). The propanoyloxy group was positioned at C(2) due to a correlation of H–C(2) to the carboxy group at δ(C) 173.9. These assignments were supported by HMQC, HMBC, and COSY correlations; the latter indicated connectivities of H–C(10)/CH_2(1)/H–C(2)/H–C(3), of H–C(6)/CH_2(7)/H–C(8)/Me(20), and of CH_2(11)/CH_2(12) (*Fig. 1*).

The relative configuration of **1** was determined on the basis of NOESY correlations (*Fig. 2*). NOEs of H–C(2)/H–C(3), of H–C(18)/H–C(3), of H–C(18)/H–C(19), and of H–C(19)/H–C(6) indicated β-orientation of the AcO groups at C(2), C(18), and C(19), as well as of 6-OH. In addition, the NOE interactions between Me(17)/H–C(8) and H–C(10)/Me(20) were in agreement with β-orientated Me(20) and CH_2(11) groups. Thus, from the above data, the structure of compound **1** was established as (2*S**,5*R**,6*R**,8*S**,18*R**,19*S**)-18,19-diacetoxy-18,19-epoxy-6-hydroxycleroda-3,13(16),14-trien-2-yl propanoate, and named *caseamembrin S*.

Compound **2** was isolated as a colorless, amorphous, optically active solid ($[\alpha]_D^{25}$ = +32.6 (MeOH)). The molecular formula of **2** was determined by HR-ESI-MS as $C_{28}H_{40}O_8$, which is 14 mass units higher than in the case of **1**. The ^{1}H- and ^{13}C-NMR data of **2** (*Tables 1* and *2*, resp.) were similar to those of **1**, indicating that **2** was a clerodane-diterpene analogue of **1** with essentially the same substitution pattern [15].

Table 1. *^{1}H-NMR Data of* **1**–**3**. At 500 MHz in $CDCl_3$; δ in ppm J in Hz. Assignments were confirmed by COSY and HMBC techniques. Arbitrary atom numbering.

Position	**1**	**2**	**3**
1	1.88–1.94 (*m*, H_β), 1.80–1.86 (*m*, H_α)	2.02–2.07 (*m*, H_β), 1.95–1.99 (*m*, H_α)	1.90–1.95 (*m*, H_β), 1.84–1.88 (*m*, H_α)
2	5.44–5.50 (*m*)	5.45 (br. *s*)	5.45–5.50 (*m*)
3	5.98 (*d*, *J* = 3.5)	5.99 (br. *d*, *J* = 3.2)	6.05 (*d*, *J* = 3.0)
6	3.78 (*dd*, *J* = 11.5, 4.0)	3.67 (*d*, *J* = 10.2)	3.75 (*dd*, *J* = 11, 3.5)
7	1.69–1.75 (*m*)	1.68–1.75 (*m*, 2 H)	1.68–1.73 (*m*, 2 H)
8	1.67–1.73 (*m*)	1.82–1.87 (*m*)	1.66–1.74 (*m*)
10	2.23–2.29 (*m*)	2.32–2.38 (*m*)	2.27–2.33 (*m*)
11	1.50–1.55 (*m*, H_β), 1.20–1.23 (*m*, H_α)	1.23–1.28 (*m*, H_β), ,1.21–1.27 (*m*, H_α)	1.21–1.25 (*m*, H_β), 1.21–1.26 (*m*, H_α)
12	1.15–1.20 (*m*, H_β), 2.06–2.12 (*m*, H_α)	2.10–2.15 (*m*, 2 H)	2.05–2.10 (*m*, 2 H)
14	6.44 (*dd*, *J* = 11, 18)	6.43 (*dd*, *J* = 10.8, 17.6)	6.40–6.47 (*m*)
15	5.20 (*d*, *J* = 18), 5.03 (*d*, *J* = 10.6)	5.17 (*d*, *J* = 17.6), 5.04 (*d*, *J* = 10.8)	5.18 (*d*, *J* = 18), 5.03 (*d*, *J* = 10.5)
16	5.06, 4.93 (2*s*)	5.02, 4.98 (2*s*)	5.05 (*s*, 2 H)
17	0.96 (*s*)	0.99 (*s*)	0.97 (*s*)
18	6.51 (*s*)	6.71 (*s*)	5.48 (*s*)
19	6.53 (*s*)	6.52 (*s*)	6.44 (*s*)
20	0.92 (*d*, *J* = 6.7)	0.90 (*d*, *J* = 6.6)	0.91 (*d*, *J* = 6.5)
2′	2.43–2.49 (*m*)	2.31–2.38 (*m*)	2.30–2.35 (*m*)
3′	1.66–1.74 (*m*)	1.67–1.74 (*m*)	1.60–1.68 (*m*)
4′	–	0.95–1.00 (*m*)	0.94–1.01 (*m*)
AcO	1.89 (*s*)	1.88 (*s*)	1.90 (*s*)
AcO	2.06 (*s*)	2.04 (*s*)	–
MeO	–	–	3.43 (*s*)

Compound **2** had two AcO groups (δ(H) 1.88, 2.04 (2*s*)) and a butanoyloxy group [δ(H) 2.35 (*m*); 1.70 (*m*); 0.98 (*t*, *J* = 6.9 Hz)], as inferred from correlations observed in the ^{1}H,^{1}H-COSY, HMQC, and HMBC spectra. The oxymethine signal for H–C(2) (δ(H) 5.45) exhibited an HMBC correlation to the carboxy group at δ(C) 173.1, and the acetal signals H–C(18) (δ(H) 6.71) and H–C(19) (δ(H) 6.52) showed correlations with the carboxy C-atoms at δ(C) 170.1 and 169.8 ppm, respectively. Comparison of the chemical shifts and coupling constants of **1** and **2** allowed us to identify the latter as the butanoyl analogue of **1**, and was named *caseamembrin T*.

Caseamembrin U (**3**) was isolated as a colorless, amorphous, optically active solid ($[\alpha]_D^{25} = +19.5$ (MeOH)). It's molecular formula was determined as $C_{27}H_{40}O_7$ by HR-ESI-MS and NMR analyses (*Tables 1* and *2*). The ^{1}H- and ^{13}C-NMR data of **3** were very similar to those of **2**, differing only in the signals of one ester substituent. An AcO, a butanoyloxy, and a MeO group were identified, with typical correlations in the ^{1}H,^{1}H-COSY, HMQC, and HMBC spectra. The MeO group at δ(H) 3.43 showed an HMBC correlation with the acetal resonance at δ(C) 104.6. The signals of H–C(2) [δ(H) 5.45–5.50; δ(C) 66.2] and H–C(6) [δ(H) 3.75; δ(C) 73.3] of **3** were superimposable to those of **2**, indicating the same substitution pattern and configuration at these positions, which

Table 2. *^{13}C-NMR (DEPT) Data of* **1**–**3**. Recorded at 125 MHz in $CDCl_3$; δ in ppm. Arbitrary atom numbering.

Position	**1**	**2**	**3**	Position	**1**	**2**	**3**
1	26.9 (*t*)	26.9 (*t*)	27.0 (*t*)	16	115.5 (*t*)	115.4 (*t*)	115.3 (*t*)
2	66.1 (*d*)	66.2 (*d*)	66.2 (*d*)	17	25.4 (*q*)	25.3 (*q*)	25.4 (*q*)
3	121.8 (*d*)	121.8 (*d*)	121.7 (*d*)	18	95.6 (*d*)	95.6 (*d*)	104.6 (*d*)
4	145.2 (*s*)	145.1 (*s*)	146.3 (*s*)	19	97.8 (*d*)	97.9 (*d*)	95.4 (*d*)
5	53.6 (*s*)	53.7 (*s*)	53.6 (*s*)	20	15.7 (*q*)	15.7 (*q*)	15.7 (*q*)
6	73.1 (*d*)	73.1 (*d*)	73.3 (*d*)	1′	173.9 (*s*)	173.1 (*s*)	173.2 (*s*)
7	37.1 (*t*)	36.5 (*t*)	36.5 (*t*)	2′	27.9 (*t*)	36.5 (*t*)	36.2 (*t*)
8	36.5 (*d*)	37.1 (*d*)	37.4 (*d*)	3′	9.2 (*q*)	18.7 (*t*)	18.6 (*t*)
9	36.2 (*s*)	36.5 (*s*)	37.8 (*s*)	4′	–	13.6 (*q*)	13.7 (*q*)
10	37.3 (*d*)	37.4 (*d*)	37.6 (*d*)	AcO	21.5 (*q*)	21.2 (*q*)	21.6 (*q*)
11	28.0 (*t*)	28.1 (*t*)	29.7 (*t*)		169.9 (*s*)	169.8 (*s*)	169.4 (*s*)
12	23.7 (*t*)	23.8 (*t*)	23.8 (*t*)	AcO	21.2 (*q*)	21.4 (*q*)	–
13	145.1 (*s*)	145.3 (*s*)	145.3 (*s*)		170.1 (*s*)	170.1 (*s*)	
14	140.3 (*d*)	140.4 (*d*)	140.4 (*d*)	18-MeO	–	–	56.2 (*q*)
15	112.4 (*t*)	112.3 (*t*)	112.3 (*t*)				

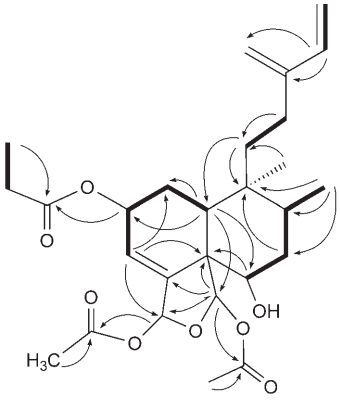


Fig. 1. *Key COSY* (—) *and HMBC* (H→C) *correlations of* **1**

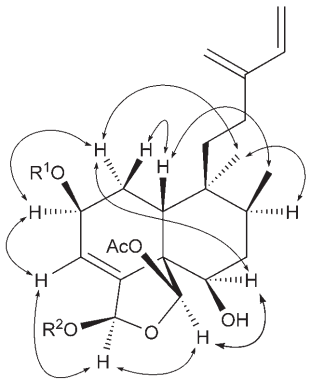


Fig. 2. *Key NOESY Interactions of* **1** *and* **3**

was further confirmed by NOESY correlations. The above findings, thus, helped us to elucidate the structure of **3** as (2*S**,5*R**,6*R**,8*S**,18*R**,19*S**)-19-acetoxy-18,19-epoxy-6-hydroxy-18-methoxycleroda-3,13(16),14-trien-2-yl butanoate, which was named *caseamembrin U*.

2. *Biological Studies.* Compounds **1**–**4** were tested for their cytotoxic activities against human cervical epitheloid (HeLa), colon (DLD-1), medullablastoma (Daoy), and oral epidermoid (KB) cancer cell lines. As indicated in *Table* 3, compounds **1**–**3** exhibited significant activity against the four tumor cell lines, whereas **4** was nearly inactive. Among them, compound **2** showed the highest activity against all four tested cell lines, its potency being superior to that of the standard compound minomycin, when tested against Daoy cells (ED_{50} = 10 ng/ml).

Table 3. *Cytotoxicities of Clerodane Diterpenes against Different Human Tumor Cells.* For details and abbreviations, see *Exper. Part*.

Compound	ED_{50} [μg/ml][a])			
	HeLa	DLD-1	Daoy	KB
1	6.74	3.34	3.50	10.41
2	0.62	0.52	0.01	2.50
3	7.92	3.28	4.72	9.39
4	14.4	19.1	5.44	21.84
Mitomycin[b])	0.10	0.23	0.13	0.17

[a]) The concentration inhibiting 50% of tumor cell growth after 72 h at 37°. [b]) Positive control.

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Experimental Part

General. Column Chromatography (CC): silica gel *60* (200–300 mesh; *Merck*). Thin-layer chromatography (TLC): silica gel GF_{254} (*Merck*). UV Spectra: *Hitachi U-3210* spectrophotometer; λ_{max} (log ε) in nm. Optical rotations: *Jasco DIP-1000* spectropolarimeter. IR Spectra: *Hitachi T-2001* spectrophotometer, in CH_2Cl_2. NMR Spectra: *Varian Unity-INOVA-500* FT-NMR spectrometer; δ in ppm rel. to Me_4Si, J in Hz. HR-ESI-MS: *JEOL JMS-HX-110* mass spectrometer; in *m*/*z*.

Plant Material. The roots of *Caseria membranacea* HANCE were collected in May 2003 from Taipei County, Taiwan. Identification was carried out by one of the authors (*C.-T. C.*). A voucher specimen (TP207–2) was deposited at the School of Pharmacy, National Taiwan University, Taipei, Taiwan.

Extraction and Isolation. The dried roots of *C. membranacea* (1.9 kg) were extracted with acetone (3 × 10 l). After filtration and solvent removal, the resulting residue was extracted with AcOEt/H_2O 1:1. The AcOEt-soluble part (23 g) was purified by CC (SiO_2; hexane/AcOEt 100:1 → 1:10, then AcOEt/MeOH 50:1 → 3:1) to afford 45 fractions. *Fr. 23* (0.32 g) was separated by RP-HPLC (MeOH/MeCN/H_2O 75:5:20) to yield **1** (5 mg), **2** (12 mg), **3** (5 mg), and **4** (5 mg).

Caseamembrin S (=*(2*S*,*5*R*,*6*R*,*8*S*,*18*R*,*19*S*)-18,19-Diacetoxy-18,19-epoxy-6-hydroxycleroda-3,13(16),14-trien-2-yl Propanoate*; =*(1*R*,*3*S*,*5*S*,*6a*R*,*7*S*,*8*S*,*10*R*,*10a*R*)-1,3-Diacetoxy-3,5,6,6a,7,8,9,10-octahydro-10-hydroxy-7,8-dimethyl-7-(3-methylidenepent-4-en-1-yl)naphtho[1,8a-c]furan-5-yl Propanoate*; **1**). Colorless, amorphous powder. $[\alpha]_D^{25} = +30.9$ (c = 0.2, MeOH). UV (MeOH): 223 (4.05). IR (neat): 3441, 2926, 1731, 1455, 1231, 736. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. ESI-MS: 513 ($[M+Na]^+$). HR-ESI-MS: 513.2466 ($[M+Na]^+$, $C_{27}H_{38}NaO_8^+$; calc. 513.2464).

Caseamembrin T (=*(2*S*,*5*R*,*6*R*,*8*S*,*18*R*,*19*S*)-18,19-Diacetoxy-18,19-epoxy-6-hydroxycleroda-3,13(16),14-trien-2-yl Butanoate*; =*(1*R*,*3*S*,*5*S*,*6a*R*,*7*S*,*8*S*,*10*R*,*10a*R*)-1,3-Diacetoxy-3,5,6,6a,

7,8,9,10-octahydro-10-hydroxy-7,8-dimethyl-7-(3-methylidenepent-4-en-1-yl)naphtho[1,8a-c]furan-5-yl Butanoate; **2**). Colorless, amorphous powder. $[\alpha]_D^{25} = +32.6$ ($c = 0.2$, MeOH). UV (MeOH): 221 (4.12). IR (KBr): 3456, 2965, 1730, 1454, 1373, 1231, 736. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. ESI-MS: 527 ($[M+Na]^+$). HR-ESI-MS: 527.2619 ($[M+Na]^+$, $C_{28}H_{40}NaO_8^+$; calc. 527.2621).

Caseamembrin U (=*(2S*,5R*,6R*,8S*,18R*,19S*)-19-Acetoxy-18,19-epoxy-6-hydroxy-18-methoxycleroda-3,13(16),14-trien-2-yl Butanoate*; =*(1R*,3S*,5S*,6aR*,7S*,8S*,10R*,10aR*)-1-Acetoxy-3,5,6,6a,7,8,9,10-octahydro-10-hydroxy-3-methoxy-7,8-dimethyl-7-(3-methylidenepent-4-en-1-yl)naphtho[1,8a-c]furan-5-yl Butanoate*; **3**). Colorless, amorphous powder. $[\alpha]_D^{25} = +19.5$ ($c = 0.2$, MeOH). UV (MeOH): 224 (4.02). IR (KBr): 3453, 2962, 1730, 1596, 1454, 1373, 1227, 736. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. ESI-MS: 499 ($[M+Na]^+$). HR-ESI-MS: 499.2673 ($[M+Na]^+$, $C_{27}H_{40}NaO_7^+$; calc. 499.2672).

Cytotoxicity Assay. The cells (HeLa, DLD-1, Daoy, or KB) were cultured in *RPMI-1640* medium under a 5% CO_2 atmosphere in an incubator at 37°. The cytotoxicity assay was based on the binding of Methylene Blue to fixed cell monolayers at pH 8.5, washing, and releasing the dye by lowering the pH. Samples and positive controls were prepared at concentrations of 1, 10, 40, and 100 μg/ml. After seeding 2,880 cells/well in a 96-well microplate for 3 h, 20 μl of sample or standard agent was placed in each well, which was incubated at 37° for 3 d. After removing the medium from the microplates, the cells were fixed with 10% formaldehyde in 0.9% saline for 30 min, and dyed with 1% (*w*/*v*) Methylene Blue in 0.01M borate buffer (100 μl/well) for 30 min. The 96-well plate was dipped into a 0.01M borate-buffer soln. (4×) to remove excess dye. Then, 100 μl/well of EtOH/0.1M HCl 1:1 was added as a dye-eluting solvent, and the VIS absorbance was measured with a microtiter plate reader (*Dynatech MR-7000*) at 650 nm. The ED_{50} value was determined, by comparison with the untreated cells, as the concentration of test sample resulting in 50% reduction of absorbance.

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