Two New Cytotoxic Clerodane Diterpenoids from *Casearia membranacea*

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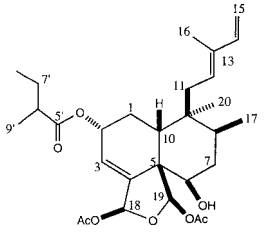
In addition to casearlucin A (3), two new clerodane diterpenes, caseamembrols A (1) and B (2) have been isolated from the leaves and twigs of *Casearia membranacea* by bioassay-guided fractionation. The structures of the new compounds were established on the basis of extensive 1D- and 2D-NMR spectroscopic analysis. Compounds 1—3 exhibited significant cytotoxicity against human prostate (PC-3) cancer cells.

Key words Flacourtiaceae; *Casearia membranacea*; clerodanes; cytotoxicity

Clerodanes are a group of novel diterpenoids with interesting biological effects such as antitumor-related activities.[1—5] *Casearia membranacea* HANCE (Flacourtiaceae) is the only species that belongs to the genus *Casearia* in Taiwan.[6] During the course of searching for novel antitumor agents from the terrestrial plants in Taiwan,[7—9] an ethanolic extract of the leaves and twigs of *C. membranacea* collected in Ken-ting showed significant cytotoxicity against the human prostate tumor cells (PC-3). To explore the origin of biological activity, bioassay-guided fractionation of the extract of the leaves and twigs of this species has resulted in the isolation of two new clerodane diterpenes, designated as caseamenbrols A (**1**) and B (**2**), together with the known casearlucin A (**3**). Herein, we wish to report the isolation, structural elucidation and cytotoxicity of these novel compounds.



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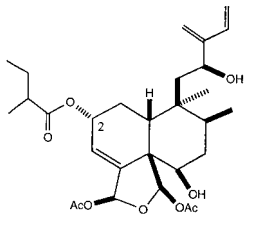

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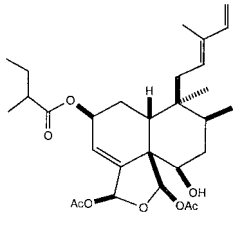

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Results and Discussion

The ethanolic extract of *C. membranacea* was fractionated by repeated column (Si gel and Sephadex LH-20) chromatography and preparative TLC to furnish caseamenbrols A (**1**, 0.012%) and B (**2**, 0.024%) in addition to casearlucin A (0.0023%).[10]

The FAB-MS and ^{13}C-NMR spectra of **1** revealed its molecular weight as 518 and molecular formula $C_{29}H_{42}O_8$. The UV spectrum showed absorption due to conjugated diene at λ 225 nm. The IR spectrum displayed absorption bands specific for a hydroxyl group (3445 cm^{-1}), ester carbonyl (1735 cm^{-1}) and olefinic bonds (1639 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectral values (Table 1) revealed the presence of a hydroxyl (δ_H 3.99) along with two acetyloxy (δ_C 170.2, 169.5, 21.3, 21.7 and δ_H 2.08, 1.93) and 2-methylbutanoyloxy substituents (Table 1). These findings were further supported by COSY and NOESY spectra as well as FAB-MS fragments at *m*/*z* 500 $[M-H_2O]^+$, 458 $[M-AcOH]^+$ and 416 [M−methylbutyric acid]$^+$.[10] Subtracting the signals of carbon atoms assignable to the three esters afforded a diterpene with twenty carbon atoms. The olefinic region of the ^{1}H-NMR spectrum revealed proton signals with characteristic *cis*/*trans* coupling at δ 5.08 (d, J=17.3 Hz, H-15a), 4.92 (d, J=10.7 Hz, H-15b) and 6.29 (dd, J=17.3, 10.7 Hz, H-14) indicating a terminal monosubstituted olefin. In addition, two olefinic proton signals were detected at δ 5.35 (H-12) and 5.88 (H-3) correlated to carbon signals at δ 128.9 and 124.2 respectively. Two deshielded acetal proton singlets were observed at δ 6.68 (H-18) and 6.46 (H-19) which were correlated to methine signals at δ 95.2 and 96.8 respectively.[10] Furthermore, ^{1}H-NMR spectrum revealed a methyl singlet at δ 0.83 (H-20), a methyl doublet at δ 0.91 (J=6.2 Hz, H-17) and a deshielded oxymethines at δ 5.61 (H-2) along with the oxymethine at δ 3.99 (H-6). Taking into account three olefinic bonds and three ester carbonyls, the presence of three rings is justified to satisfy nine degrees of unsaturation. The spectral data of **1** are in conformity with the basic skeleton of clerodane diterpenes previously isolated from *Casearia*.[11,12] The presence of a six-carbon diene side chain at C-9 was confirmed by the long range correlations between H-12/C-9; H-14/C-12, C-13, C-16; H-15/C-13/C-14, C-16. The position of the acylated acetal at C-18/C-19 was proved by long range correlation between H-18/C-5, C-19 and H-19/C-5, C-6, C-18. The proton signal at δ 5.88 (H-3) exhibited a long range correlation with carbon signals at δ 26.2 (C-1), 53.5 (C-5) and 95.2 (C-18) demonstrating that a dou-

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Table 1. ^{1}H-NMR (300 MHz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) Data of **1**

C atom No.	δ_C	δ_H (HMQC)[a]	HMBC[b]	COSY[c]
1	26.2	2.18 m		H-2, H-10
2	70.5	5.61 m	C-4, C-5′	H-1, H-3
3	124.2	5.88 br s	C-1, C-5	H-2, H-18
4	144.3	—		
5	53.5	—		
6	74.1	3.99 dd (12.2, 6.6)	C-4, C-5, C-7, C-8	H-7
7	37.6	1.64 m		H-6, H-8
8	36.8	1.74 m		H-7, H-17
9	38.4	—		
10	41.5	2.42 dd (14.2, 2.4)		H-1
11	30.1	2.25 m		H-12
		1.68 m		
12	128.9	5.33 d (5.1)	C-9	H-11
13	135.9	—		
14	141.3	6.29 dd (17.3, 10.7)	C-13, C-12, C-16	H-15
15	111.1	5.08 d (17.3)	C-13, C-14, C-16	H-14
		4.92 d (10.7)		
16	12.0	1.64 s		
17	15.6	0.91 d (6.2)	C-9	
18	95.2	6.68 br s	C-1′, C-5, C-19	H-3
19	96.8	6.46 s	C-3′, C-5, C-6	H-6
20	25.1	0.83 s		H-12
1′	170.2	—		
2′	21.3	2.08 s		
3′	169.5	—		
4′	21.7	1.93 s		
5′	176.6	—		
6′	41.2	2.37 m	C-5′, C-8	H-7′, H-9′
7′	26.8	1.52 m		H-6′, H-8′
		1.23 m		
8′	11.7	0.95 t (7.0)	C-6′	H-7′
9′	16.6	1.14 d (7.0)		H-6′

a) Values in parentheses are coupling constants in Hz. *b*) Correlations H→C. *c*) Correlations H→H.

Table 2. ^{1}H-NMR (300 MHz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) Data of **2**

C atom No.	δ_C	δ_H (HMQC)[a]	HMBC[b]	COSY[c]
1	26.2	2.18 m		H-2, H-10
2	70.4	5.53 br t (7.4)	C-4, C-5′	H-1, H-3
3	124.0	5.85 br s	C-1, C-2, C-5, C-10	H-2, H-18
4	143.9	—		
5	53.7	—		
6	74.6	3.95 dd (12.0, 4.2)	C-5, C-8	H-7
7	37.3	1.25 m		H-6, H-8
		1.73 m		
8	36.8	1.71 m		H-7, H-17
9	39.1	—		
10	46.6	2.01 m		H-1
11	37.1	1.81 m	C-9, C-12	H-12
12	82.5	4.71 br d (4.0)	C-11	H-11
13	145.8	—		
14	135.3	6.27 dd (17.6, 11.2)	C-13, C-15	H-15
15	115.8	5.46 d (17.6)		H-14
		5.14 d (11.2)		
16	116.9	5.25 s	C-12, C-13, C-14	—
		5.16 s		—
17	15.8	1.06 d (6.6)	C-8	H-8
18	95.1	6.69 br s	C-1′, C-5, C-19	H-3
19	97.5	6.40 s	C-3′, C-5, C-6, C-18	—
20	23.8	1.03 s		—
1′	170.2	—		
2′	21.2	2.07 s	C-18	—
3′	169.8	—		
4′	21.7	1.98 s	C-19	—
5′	176.5	—		
6′	41.1	2.37 m	C-5′, C-8′, C-9′	H-7′, H-9′
7′	26.7	1.51 m	C-6′, C-5′	H-6′, H-8′
		1.68 m		
8′	11.7	0.91 t (7.0)		H-7′
9′	16.5	1.14 d (7.0)	C-5′	H-6′

a) Values in parentheses are coupling constants in Hz. *b*) Correlations H→C. *c*) Correlations H→H.

ble bond is located between C-3 and C-4. The oxymethine signal at δ 3.99 (H-6), correlated to a carbon signal at δ 74.1, revealed long-range correlations to carbon signals at δ 144.3 (C-4), 53.5 (C-5), 37.6 (C-7) and 36.8 (C-8) demonstrating that a hydroxyl group is located at C-6. The relative downfield shift of signalat δ 5.61 (H-2) as well as its long range correlations to carbon signals at δ 144.3 (C-4) and δ 175.0 (C-5′) established the point of attachment of the 2-methylbutanoyl ester to C-2. Likewise, the attachment of the two acetate ester to C-18 and C-19 of the acetal ring was inferred by long range correlations between H-18/C-1′ and H-19/C-3′. The diene side chain at C-9 was evidenced to be in the *E*-form as proved by the strong NOE correlation between H-12/H-14 as well as the relative upfield signal of C-16 at δ 12.0.[13]

The relative stereochemistry at the chiral centers was determined primarily through the NOESY spectrum as well as comparison with reported NMR data of analogous clerodane diterpenes.[1,10] The coupling constant of the signal at δ 2.42 (1H, dd, J=14.2, 2.4 Hz, H-10) suggested the β-axial orientation of H-10 while the NOE correlations between H-2/βH-10 proved that H-2 is β-oriented. On the other hand, NOE correlations between αH-8/αH-6, αH-6/αH-19, αH-19/αH-18 finalized the proposed structure of **1**. Hence, the structure

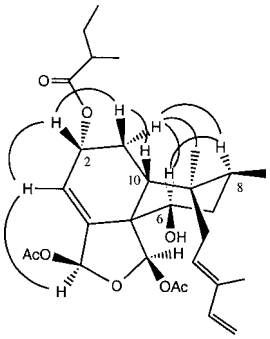

Fig. 1. Key NOESY Correlations for **1**

of **1** was established as 2*R**,5*R**,6*R**,8*S**,9*S**,10*R**,18*S**,19*R**)-18,19-diacetoxy-18,19-epoxy-6-hydroxy-2-(2-methylbutanoyloxy)cleroda-3,12,14-triene. It was named caseamembrol A. The authors believe beyond any doubt that the published structure of a similar compound is erroneous and that the diene side chain of that compound should be in a *Z*-form.[1]

The molecular formula of **2** was established as $C_{29}H_{42}O_9$ from FAB-MS and ^{13}C-NMR spectra. It displayed almost identical NMR spectral data (Table 2) to those of **1** regarding

rings A and B of the clerodane nucleus as well as the diacylated acetal ring and the same ester at C-2. The only difference between **2** and **1** resides in the signals attributable to the diene side chain at C-9. The olefinic region of the ^{1}H-NMR spectrum of **2** revealed, in addition to a signal at δ 5.85 (H-3), proton signals with characteristic *cis*/*trans* coupling at δ 5.46 (d, J=17.6 Hz, H-15a), 5.14 (d, J=11.2 Hz, H-15b) and 6.27 (dd, J=17.6, 11.2 Hz, H-14) indicating a terminal monosubstituted olefin. Conversely, another terminal methylene, identified by two proton singlets at δ 5.25 (H-16a) and 5.16 (H-16b), did not display any splitting implying that they are connected to a quaternary carbon (C-13). In addition to signals assigned to C-2, C-6, C-18 and C-19 (Table 2), the DEPT spectra revealed a deshielded oxymethine signal at δ 82.5 (C-12) which was correlated to a proton signal at δ 4.71 (H-12). The relative deshielding of the last signals suggested its proximity to a double bond. Furthermore, the deshielding of C-11 in **2** (δ 7.0), compared to **1**, indicated that a hydroxyl group is most probably attached to C-12. The postulated structure of the diene side chain was established through HMBC experiment that showed correlations between H-14/C-15, C-13; H-16/C-12, C-13, C-14; H-12/C-11, C-9. These results unambiguously located the hydroxyl group at C-12 of the diene side chain and were in accordance of the proposed structure of **2**. Comparison of the chemical shifts of H-12 and C-12 in **2** with those data in casearlucins H (δ 4.47, 67.5) and I (δ 4.73, 80.8) suggested that the hydroxyl group in **2** was β-orientated.[10] The stereochemistry of C-12 was tentatively assigned as S because the configuration of C-12 in casearlucins H and I have been established as R and S, respectively, by Mosher method. NOE data were consistent with those previously mentioned for **1**. Thus, **2** was identified as 2*R**,5*R**,6*R**,8*S**,9*S**,10*R**,12*S**,18*S**,19*R**)-18,19-diacetoxy-18,19-epoxy-6-hydroxy-2-(2-methylbutanoyloxy)-cleroda-12-hydroxy-3,13(16),14-triene. It was named caseamembrol B.

In conclusion, the structure of casearvestrin B should be revised to a *Z*-form for C12–13 double bond.[1] The isolated clerodanes **1**—**3** were tested *in vitro* against human prostate (PC-3) tumor cells. All compounds exhibited significant cytotoxicity against human PC tumor cells with IC_{50} at 2.45, 5.66 and 6.65 μM, respectively. Taxol gave an IC_{50} value of 0.16 μM under the same conditions.

Experimental

General Experimental Procedures Optical rotation was measured on a JASCO DIP-1000 polarimeter. IR spectra were recorded with a HORIBA FT-720 spectrophotometer. ^{1}H- and ^{13}C-NMR, COSY, HMQC, HMBC and NOESY spectra were recorded using a Bruker FT-300 (AVANCE) or a Varian FT-500 (INOVA) NMR instrument. EI-MS and FAB-MS were measured with a VG Quattro 5022 and JEOL JMS-SX 102 mass spectrometers.

Plant Material *Casearia membrancea* Hance was collected in ken-ting, Ping-tong county in March, 2001. A voucher specimen (TP207-1) was deposited in the Institute of Marine Resources, National Sun Yat-sen University, Kaohsiung, Taiwan.

Extraction and Isolation The leaves and twigs of *Casearia membrancea* (dry, 250 g) was extracted with EtOH and the extract (31 g) was partitioned between EtOAc and H_2O (each 300 ml). The EtOAc-soluble residue (21 g) was chromatographed on a Si gel column and eluted with *n*-hexane (300 ml) and solvent mixtures of *n*-hexane/EtOAc (60 : 1, 50 : 1, 40 : 1 to 1 : 1, each 300 ml) to afford 14 fractions. Cytotoxicity screening tests of these fractions revealed that both fractions 11 and 12 were active (3.99 μg/ml and 5.52 μg/ml respectively). Fraction 11 (2.01 g) was rechromatographed on a silica gel column and eluted with *n*-hexane/EtOAc (5 : 1, 4 : 1, 3 : 1 to 1 : 3) to give fractions A (0.69 g) and B (0.86 g). Fraction A was applied on a Sephadex LH-20 column and developed with MeOH to yield caseamembrol A (**1**, 29 mg) and caseamembrol B (**2**, 60 mg). Fraction 12 (1.29 g) was chromatographed on a silica gel column and eluted with *n*-hexane/EtOAc (5 : 1, 4 : 1, 3 : 1 to 1 : 3) to give a residue, which was fractionated using a Sephadex LH-20 column and developed with MeOH to yield a fraction (62 mg). This fraction was applied on a preparative TLC plate (Si gel, 1 mm thickness) and developed with *n*-hexane/EtOAc (3 : 1) to yield casealucin (**3**, 5.7 mg).

Caseamembrol A (**1**): Isolated as an amorphous solid: $[\alpha]_D^{25}$ −8.3° (c=0.38, MeOH); UV (MeOH) λ_{max} (log ε) 234 nm (shouder); IR (neat) ν_{max} 3433, 2967, 2934, 1735, 1720, 1645 cm^{-1}; ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectral data: Table 1; FAB-MS m/z 541 $[M+Na]^+$; HR-FAB-MS m/z 541.2765 ($[M+Na]^+$, Calcd for $C_{29}H_{42}O_8Na$, 541.2777); EI-MS (70 eV) m/z 213, 203, 185, 171, 153, 131, 128, 115, 91, 74, 57, 41.

Caseamembrol B (**2**): Isolated as an amorphous solid: $[\alpha]_D^{25}$ −11° (c=0.38, MeOH); UV (MeOH) λ_{max} (log ε) 229 nm (shouder); IR (neat) ν_{max} 3430, 2965, 2935, 1734, 1722, 1643 cm^{-1}; ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectral data: Table 2; FAB-MS m/z 557 $[M+Na]^+$; HR-FAB-MS m/z 557.2719 ($[M+Na]^+$, Calcd for $C_{29}H_{42}O_9Na$, 557.2726); EI-MS (70 eV) m/z 186, 171, 149, 143, 135, 115, 95, 91, 87, 74, 60, 57, 41.

Cytotoxicity Assay A bioassay against PC-3 (human prostate carcinoma) tumor cells was based on sulforhodamine B (SRB) assay method.[14] Experimental drugs were solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or 1/2 log serial dilutions are made to provide a total of five drug concentrations plus control. SRB solution (100 μl) at 0.4% w/v in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 10% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. The IC_{50} value was defined, by a comparison with the untreated cells, as the concentration of test sample resulting in a 50% reduction of absorbance. Taxol was used as a standard compound.

Acknowledgments The authors thank the National Science Council, Republic of China (grant number NSC 92-2320-B-110-011) for the financial support. We acknowledge C.-L. Ho and S.-C. Yu of the NSC Southern NMR and MS Instrument Center for the measurement of high-resolution NMR (500 MHz) and MS spectra.

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