Cytotoxic Clerodane Diterpenoids from *Casearia obliqua*

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A crude bioactive EtOH extract of the twigs of *Casearia obliqua* afforded two new clerodane diterpenes, caseobliquins
A (**1**) and B (**2**). Additionally, bioactivity-directed fractionation on a bioactive hexane extract of the leaves from this
species led to the isolation of the known clerodane diterpenes *rel*-6β-hydroxyzuelanin-2β-benzoate and *rel*-2α-
hydroxyzuelanin-6β-benzoate (**3** and **4**) as a mixture and 2β-hydroxyzuelanin-6β-cinnamate (**5**). The structures of the
new clerodanes **1** and **2** were established on the basis of their 1D and 2D NMR spectroscopic data, and the new compound
1 and the known substance **5** had their absolute configurations determined by circular dichroism spectroscopy. The
cytotoxicity of several of the compounds isolated was evaluated against a small panel of human tumor cell lines.

The plant genus *Casearia* (Salicaceae *sensu lato*) contains about
180 species distributed in the tropics[1] and is known as a rich source
of clerodane-type diterpenes.[2−9] This genus is widely distributed
in Brazil, where *Casearia sylvestris* is the most frequent species,
occurring from the Amazon basin throughout the Cerrado to the
Atlantic forest and the south of Brazil.[10]

In a continuing investigation on bioactive clerodane diterpenes
from *Casearia* species, phytochemical studies were carried out for
the first time on *Casearia obliqua* Spreng, a tree that grows
commonly in the southern part of Brazil, where it is popularly
known as "guaçatonga-vermelha".[11] Antimalarial, cytotoxic, im-
munomodulatory, and trypanocidal effects have been shown by
several clerodane diterpenoids from plants of the genus *Casearia*.[2−9]

Results and Discussion

A bioactive ethanol extract from the twigs of *C. obliqua* was
partitioned with hexane, Et₂O, EtOAc, and H₂O. The cytotoxic Et₂O
fraction was chromatographed on Sephadex LH-20, followed by
preparative RP-HPLC on a C₁₈ column, affording the new clerodane
diterpenes caseobliquin A (**1**) and caseobliquin B (**2**). The bioactive
hexane extract from leaves of *C. obliqua* was chromatographed on
silica gel, followed by preparative RP-HPLC on a C₁₈ column,
yielding three known compounds, *rel*-6β-hydroxyzuelanin-2β-
benzoate (**3**) and *rel*-2α-hydroxyzuelanin-6β-benzoate (**4**) (which
were obtained as a mixture) and 2β-hydroxyzuelanin-6β-cinnamate
(**5**). The structures of compounds **1** and **2** were deduced on the
basis of their spectroscopic data (UV, IR, CD, 1D and 2D NMR,
MS) and by comparison with values reported in the literature. The
cytotoxic activity of several of the diterpenes isolated was evaluated
against a small panel of human cancer cell lines.

Caseobliquin A (**1**) was obtained as a yellow amorphous solid
and showed a cationized ion at *m/z* 619.2513 [M + Na]⁺ (calcd
619.2513), corresponding to a molecular formula of C₃₃H₄₀O₁₀,
determined by HRTOFESIMS. The IR spectrum showed the
presence of hydroxy group (3436 cm⁻¹) and carbonyl ester (1749,
1735, 1717 cm⁻¹) functionalities. The ¹³C and DEPT NMR data
(Table 2) revealed 33 signals attributable to nine quaternary carbons
(including four carbonyls), 14 methines, four methylenes, and six

1, **2**, **3**, **4**, **5**
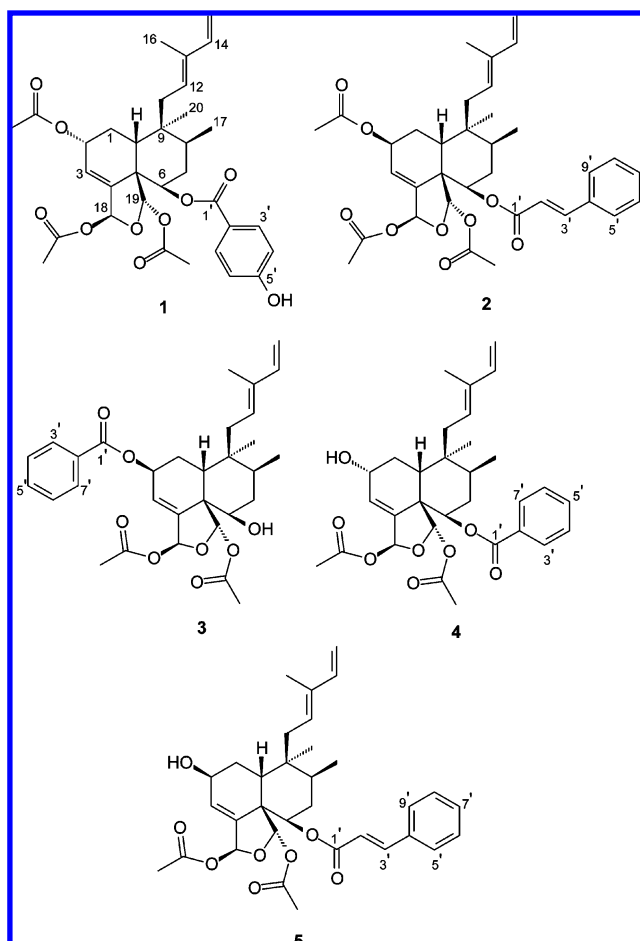
methyls. The ¹H NMR spectrum (Table 1) showed the presence of
two methyl groups at δ 0.97 (d, *J* = 7.0 Hz, H-17) and 0.92 (s,
H-20), an oxymethine proton at δ 5.54 (m, H-2), two acetal-acyloxy
methine protons at δ 6.39 (t, *J* = 1.5 Hz, H-18) and 6.67 (s, H-19),
and a trisubstituted olefinic proton at δ 5.95 (sl, H-3), consistent
with a casearin clerodane skeleton as isolated from *Casearia*
species.[10,12−14] The six-carbon diene side chain (C-11−C-16)
indicated the presence of a terminal unsaturated methylene group,
supported by the resonances at δ 5.09 (d, *J* = 17.5 Hz, H-15) and

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Table 1. ^{1}H NMR Spectroscopic Data (500 MHz, CD$_3$OD) for Compounds **1** and **2**

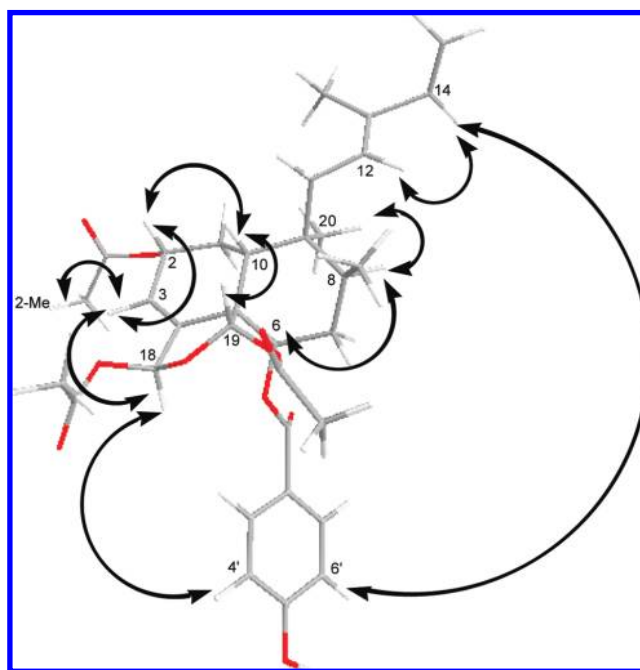|	caseobliquin A (**1**)	caseobliquin B (**2**)		caseobliquin A (**1**)	caseobliquin B (**2**)
position	δ_H (*J* in Hz)	δ_H (*J* in Hz)	position	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1	1.84 m	1.99 m	17	0.97 d (7.0)	0.97 d (7.0)
	1.89 m	2.09 m			
2	5.54 m	4.41 m	18	6.39 t (1.5)	6.49 t (1.5)
3	5.95 brs	6.06 d (4.0)	19	6.67 s	6.67 s
4			20	0.92 s	0.91 s
5			1′		
6	5.34 dd (4.5, 12.0)	5,11 dd (4.5, 12.0)	2′		6.48 d (16.0)
7	1.78 m	1.78 m	3′	6.83 d (9.0)	7.80 d (16.0)
	1.89 m	1.93 m			
8	2.04 m		4′	7.97 d (9.0)	
9			5′		7.63 m
10	2.58 dd (3.0, 14.0)	2.57 dd (3.5, 14.4)	6′	7.97 d (9.0)	7.40 m
11	1.80 m 2.29 dd (8.0, 17.0)	2.33 dd (8.8, 17.5)	7′	6.83 d (9.0)	7.40 m
12	5.54 m	5.58 m	8′		7.40 m
13			9′		7.63 m
14	6.43 dd (17.5, 11.0)	6.41 dd (10.5, 17.5)	O$_2$CCH$_3$-2	2.07 s	1.90 s
15	4.93 d (11.0)	4.92 d (10.5)	O$_2$CCH$_3$-18	2.03 s	2.05 s
	5.09 d (17.5)	5.10 d (17.5)		1.95 s	1.94 s
16	1.67 s	1.67 s	O$_2$CCH$_3$-19		

Table 2. ^{13}C NMR Spectroscopic Data (125 MHz, δ_C, mult.) for Compounds **1−4**

position	**1**[a]	**1**[b]	**2**[b]	**3**[a]	**4**[a]
1	29.6	27.2, CH$_2$	30.2	37.4, CH$_2$	29.4, CH$_2$
2	70.5	72.2, CH	64.4	67.1, CH	63.8, CH
3	125.3	126.5, CH	128.1	121.7, CH	126.5, CH
4	143.1	144.5, qC	142.5	145.6, qC	142.3, qC
5	52.6	53.3, qC	57.8	53.7, qC	51.9, qC
6	75.0	76.5, CH	75.5	72.8, CH	74.7, CH
7	34.9	34.5, CH$_2$	34.1	27.0, CH$_2$	33.1, CH$_2$
8	38.1	37.3, CH	37.6	37.1, CH	36.4, CH
9	39.2	39.5, qC	37.6	37.6, qC	37.7, qC
10	44.9	43.3, CH	38.0	36.7, CH	36.1, CH
11	31.0	31.1, CH$_2$	31.0	30.4, CH$_2$	30.4, CH$_2$
12	128.8	130.4, CH	130.8	128.9, CH	129.0, CH
13	137.0	136.9, CH	136.3	135.7, qC	135.7, qC
14	141.0	142.7, CH	142.5	141.2, CH	141.2, CH
15	111.3	111.1, CH$_2$	111.0	111.0, CH$_2$	111.0, CH$_2$
16	13.8	12.0, CH$_3$	12.2	11.9, CH$_3$	11.9, CH$_3$
17	15.4	15.8, CH$_3$	15.7	15.5, CH$_3$	15.4, CH$_3$
18	94.9	96.0, CH	94.0	95.6, CH	95.4, CH
19	97.2	98.4, CH	98.2	97.0, CH	97.7, CH
20	24.8	25.3, CH$_3$	25.4	25.0, CH$_3$	25.0, CH$_3$
1′	165.1	167.1, qC	167.7	165.8, qC	166.0, qC
2′	121.9	121.8, qC	118.2	130.5, qC	130.5, qC
3′	115.6	116.3, CH	147.1	129.6, CH	129.8, CH
4′	132.3	133.1, CH	135.0	128.4, CH	128.6, CH
5′	160.0	163.9, qC	129.1	133.1, CH	133.4, CH
6′	132.3	133.1, CH	130.0	128.4, CH	128.6, CH
7′	115.6	116.3, CH	130.5	129.6, CH	129.8, CH
8′			130.0		
9′			129.1		
O$_2$CCH$_3$-2	170.8	172.5, qC	179.0		
	21.0	21.0, CH$_3$	23.1		
O$_2$CCH$_3$-18	170.0	171.8, qC	171.9	170.0, qC	170.1, qC
	21.0	20.9, CH$_3$	21.0	21.2, CH$_3$	21.1, CH$_3$
O$_2$CCH$_3$-19	169.8	171.1, qC	171.1	169.4, qC	169.6, qC
	21.9	21.9, CH$_3$	21.8	21.5, CH$_3$	21.7, CH$_3$

[a] CDCl$_3$. [b] CD$_3$OD; multiplicities were determined by analyses of the DEPT 90° and 135°.

Figure 1. Selected NOE enhancements observed for **1**.

4.93 (d, *J* = 11.0 Hz, H-15), which were coupled to a methine proton signal at δ 6.43 (dd, *J* = 11.0, 17.5 Hz, H-14). The other methine proton signal was observed as a multiplet at δ 5.54 (H-12). This side chain was also confirmed by gCOSY correlations of H-11 to H-12 and H-14 to H-15. The gHMBC spectrum exhibited correlations of H-11 to C-9, C-10, C-12, and C-13; H-14 to C-12 and C-16; and H-15 to C-13, revealing that this side chain is located at the C-9 position. The configuration of the C-12,13 double bond was found to be *E* on the basis of the NOESY correlations between H-12 and H-14 (Figure 1). The *p*-hydroxybenzoate group was

deduced from the resonances at δ 6.83 (2H, d, *J* = 9.0 Hz, H-3′ and H-7′) and 7.97 (2H, d, *J* = 9.0 Hz, H-4′ and H-6′). The gHMBC spectrum showed a correlation of the oxymethine proton, H-6, to the carbonyl carbon (δ 167.1, C-1′) of the *p*-hydroxybenzoate group, which indicated that the *p*-hydroxybenzoate is located at C-6 (δ 76.5). The H-18 (δ 6.39) and H-19 (δ 6.67) protons were correlated to carbonyl groups (δ 171.8 and 171.1), respectively, establishing these as sites of acetylation. By elimination, the third acetate group (δ 172.5 and 21.0) could be located at C-2.

On comparing compound **1** with other clerodane diterpenoids, this compound showed differences in the configurations of the carbons C-5, C-6, C-8, C-9, C-10, and C-18, as indicated by chemical shift data for compound **1** in relation to literature values.[15] The relative configuration of all eight chiral centers was assigned on the basis of their coupling constants and by NOESY correlations (Table 1 and Figure 2). A 1,3-diaxial NOESY correlation was observed for H-6 and H-8, which indicated that the C-8 methyl group (C-17) is in an equatorial orientation. The *trans* configuration between the C-17 and C-20 methyl groups was proposed because of the chemical shift at δ 25.3 for C-20, which also supported a
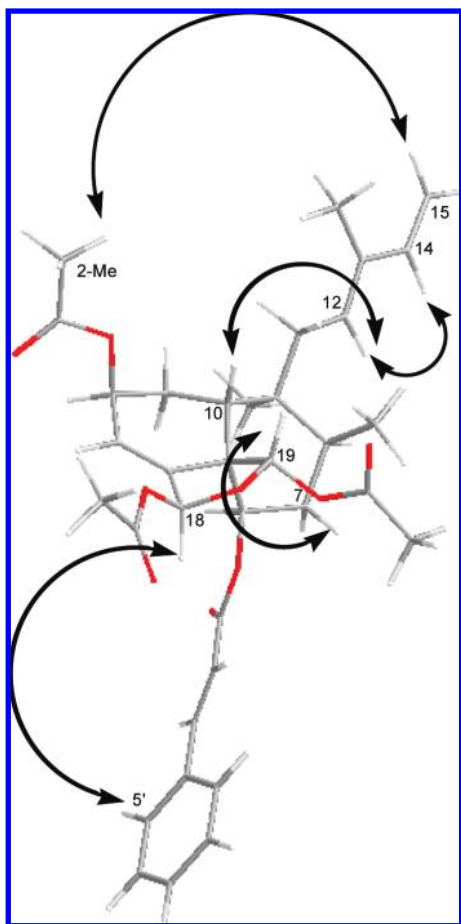
Figure 2. Selected NOE enhancements observed for **2**.

cis-A/B ring junction.[10,15,17] The *J* values of 3.0 and 14.0 Hz for the coupling of H-10 to H-1 protons revealed that H-10 is in an axial position, while the NOESY correlations between H-2/H$_\beta$-10 showed that H-2 is β-oriented. The chemical shift at δ_C 70.5 in CDCl$_3$ for the C-2 oxymethine carbon was consistent with the C-2 substituent having an α-orientation, as reported for related compounds.[1,18] The acetal proton, H-19, was also assigned with a β-orientation on the basis of the NOESY correlation of H-19 to H-10, and to the other acetal proton, H-18, an α-orientation was assigned because of the NOE enhancement observed for H-18 with the protons H-4′/H-6′ from the *p*-hydroxybenzoate moiety (δ 7.97). This could occur only if H-18 is on the opposite face of the diacetal furan ring in relation to H-19 (Figure 1).[16]

X-ray crystallographic data were used earlier to assign the absolute configuration for a series of clerodane diterpenes similar to **1**.[15] The absolute stereochemistry of **1** was determined by circular dichroism spectroscopy, in which a positive Cotton effect was exhibited at 241 nm. The C-9 carbon of the clerodane diterpenes occurs with a *R* orientation when a negative Cotton curve is evident,[12] so **1** was assigned with an *S* orientation at C-9. Following this argument, it was possible to determine the complete absolute configuration of compound **1**, as follows: 2*R*, 5*R*, 6*R*, 8*S*, 9*S*, 10*R*, 18*S*, 19*S*. This new compound, named caseobliquin A, is a diastereoisomer of casearborin E.[15]

Caseobliquin B (**2**) was isolated as a yellow amorphous solid, with a molecular formula of C$_{35}$H$_{41}$O$_9$ as determined by HRTOFES-IMS (observed *m/z* 629.2747 [M + Na]$^+$). The complete ^{1}H and ^{13}C NMR data for **2** are shown in Tables 1 and 2. The entire data set was similar to that for **1** except for the modification from the substituent at C-6, with that in **1** a *p*-hydroxybenzoate moiety and that for **2** a cinnamoyl moiety.

Table 3. Cytotoxic Activity of Compounds Isolated from *Casearia obliqua* against Human Tumor Cell Lines[a]

	cell line			
compound	HL-60 (leukemia)	HCT-8 (colon)	MDA/MB-435 (melanoma)	SF-295 (glioblastoma)
1	>5	>5	>5	>5
3 and **4**[b]	1.0	0.13	0.33	0.14
5	>5	>5	>5	>5
doxorubicin	0.07	1.5	0.12	0.75

[a] Data are presented as IC$_{50}$ values given in μM obtained by nonlinear regression. [b] Tested as a mixture.

gCOSY, gHMQC, and gHMBC NMR experiments performed supported the same connectivities for **2** as in **1**. The cinnamoyl group was deduced from the resonances at δ 6.48 (1H, d, *J* = 16.0 Hz, H-2′), 7.80 (1H, d, *J* = 16.0 Hz, H-3′), 7.40 (3H, m, H-6′, H-7′ and H-8′), and 7.63 (2H, m, H-5′ and H-9′). The gHMBC spectrum showed a correlation of the oxymethine proton, H-19 (δ 6.67), linked to the acetate carbonyl at δ 171.1. The ester groups were located by comparison with appropriate values reported in the literature,[16] the cinnamoyl was placed at C-6 (δ 75.5), and the acetate groups were placed at carbons C-2 (δ 64.4) and C-18 (δ 94.0). These assignments were corroborated by NOE enhancements between H-12 and the methyl group (δ 1.90) of the acetate moiety at position 2 and between H-15 and the methyl group (δ 2.05) of the acetate moiety at position 18 (Figure 2).

Khan et al.[16] used NOESY data to assign the relative configuration for a series of clerodane diterpenoids that have a two-dimensional structure identical to that of **2**, differing only in the stereochemistry of C-2. The chemical shift at δ 64.4 for the C-2 oxymethine carbon was consistent with the C-2 substituent having a β-orientation, as reported for related compounds,[9,17] whereas from the literature this value is δ 70.2 (α-orientation).[16] On the basis of the evidence above, compound **2** could be determined to have the relative configuration 2*S*, 5*R*, 6*R*, 8*S*, 9*S*, 10*R*, 18*S*, 19*S*. This is a diastereoisomer of *rel*-2α-acetoxyzuelanin-6β-cinnamate[16] and has been named caseobliquin B.

The three known clerodane diterpenes (**3**−**5**) were identified on the basis of their spectroscopy data (UV, IR, and NMR) and comparison with appropriate values reported in the literature. The ^{13}C NMR data of compounds **3** and **4** and the absolute configuration of **5** are included in this paper, in order to complement the lack of this information in previous reports of these compounds.[16,19]

Four compounds were evaluated for their cytotoxic activity, using the MTT assay, corroborating the well-established cytotoxicity of this compound class. The cytotoxic activities of compound **1**, a mixture **3** and **4**, and **5** were evaluated in vitro against HL-60 (human myeloblastic leukemia), HCT-8 (human colon carcinoma), MDA/MB-435 (human melanoma), and SF-295 (human glioblastoma) cells. Inhibitory concentration (IC$_{50}$) values were determined, and the results are shown in Table 3. The mixture of compounds **3** and **4** showed significant cytotoxicity against four cancer cell lines (HL-60, HCT-8, MDA/MB-435, and SF-295), with IC$_{50}$ values ranging from 0.13 to 1.0 μM, close to data obtained for the positive control, doxorubicin (Table 3).

Experimental Section

CH$_3$OH as internal standard. Positive-ion HRMS were recorded on a UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer using an internal standard. Analytical HPLC was performed on a Varian Pro Star 230 with a UV–vis detector (model 330) using a Phenomenex C$_{18}$ column (250 mm × 4.6 mm, 5 μm), in order to develop the reversed-phase conditions, which were scaled up for preparative work. Preparative HPLC was performed on a Varian Prep-Star 400 system using a Phenomenex C$_{18}$ (250 mm × 21.2 mm, 10 μm) preparative column. Column chromatography was performed over silica gel 230–400 mesh (Merck) or on Sephadex LH-20 (Pharmacia Biotech). TLC was performed using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 PF$_{254}$ plates. Spots on TLC plates were visualized under UV light and by spraying with anisaldehyde-H$_2$SO$_4$ reagent followed by heating at 120 °C.

Plant Material. *Casearia obliqua* leaves and twigs were collected in May 2007 at Campinas municipality, São Paulo, Brazil. The plant material was identified by one of the authors (R.B.T.). A voucher specimen (IAC46529) has been deposited in the herbarium of the Instituto Agronômico de Campinas (São Paulo State, Brazil). Recent studies on the phylogeny of the Flacourtiaceae, based on plastid rbcL DNA sequences, have shown that the family is polyphyletic and recognized by two major clades, one with close affinities to the Salicaceae and the other with the Achariaceae. Among those genera of Flacourtiaceae now assigned to Salicaceae *sensu lato* is the genus *Casearia*.[20]

Extraction and Isolation. The twigs (534.0 g) of *C. obliqua* were extracted exhaustively by maceration with hexane, ethanol, and water (3.0 L × 3), successively, at room temperature. The ethanol extract was concentrated under reduced pressure (≤40 °C) to yield 9.3 g of a crude extract. This ethanol extract was then diluted with MeOH–H$_2$O (3:1) and partitioned successively with hexane (1.0 L × 3), Et$_2$O (1.0 L × 3), and EtOAc (1.0 L × 3). After removal of the solvent, each extract yielded 2.9, 1.0, 1.1, and 4.0 g of residue, respectively. Part of the cytotoxic Et$_2$O fraction (1.0 g) was subjected to gel permeation chromatography on Sephadex LH-20 (78.0 × 3.0 cm), eluted with methanol, to afford 95 fractions, which were combined on the basis of TLC visualized with anisaldehyde-H$_2$SO$_4$. Cytotoxic fraction 50 (54.0 mg) was submitted to preparative RP-HPLC on a C$_{18}$ column (Phenomenex Luna 250 mm × 21.2 mm, 10 μm), eluted with MeOH–H$_2$O–AcOH (85:15:0.01) at a flow rate of 15 mL/min and UV detection at 254 nm, affording the clerodane diterpenes **1** (6.0 mg) and **2** (1.3 mg).

The leaves (94.7 g) of *C. obliqua* were extracted exhaustively by maceration successively with hexane, ethanol, and water (3.0 L × 3) at room temperature. The hexane extract was concentrated under reduced pressure (≤40 °C) to yield 1.5 g of a crude extract. This extract exhibited cytotoxicity against four human cancer cell lines and was subjected to column chromatography over silica gel (15.0 × 4.5 cm), eluted in a gradient step mode from hexane to EtOAc, yielding 15 fractions. Cytotoxic fraction 8 (114.0 mg) was subjected to preparative RP-HPLC on a C$_{18}$ column, eluted with MeOH–H$_2$O (78:22) at a flow rate 14 mL/min and UV detection at 254 nm, affording the clerodane diterpenes **5** (6.2 mg) and a mixture of **3** and **4** (1.7:1.0; 10.0 mg).

Caseobliquin A (1): yellow, amorphous solid; [α]^{25}$_D$ +52 (*c* 1.0, MeOH); UV (CHCl$_3$) λ_{max} (log ε) 241 (3.48), 280 (3.50) nm; CD (*c* 0.03, MeOH) $\Delta\varepsilon$ (nm) +4.5 (241), +2.4 (280); IR (KBr) ν_{max} 3436, 2950, 1735, 1250 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRTOFESIMS *m/z* 619.2513 [M + Na]$^+$ (calcd for C$_{33}$H$_{40}$O$_{10}$Na, 619.2513).

Caseobliquin B (2): yellow, amorphous solid; [α]^{29}$_D$ +51 (*c* 0.5, CHCl$_3$); UV (CHCl$_3$) λ_{max} (log ε) 243 (4.58) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRTOFESIMS *m/z* 629.2747 [M + Na]$^+$ (calcd for C$_{35}$H$_{42}$O$_9$Na, 629.2721).

rel-6β-Hydroxyzuelanin-2β-benzoate and rel-2α-Hydroxyzuelanin-6β-benzoate (3 + 4, 1.7:1 mixture): yellow, amorphous solid; [α]^{30}$_D$ +70 (*c* 1.0, CHCl$_3$); UV (CHCl$_3$) λ_{max} (log ε) 243 (4.12) nm; IR (KBr) ν_{max} 3448, 2925, 1756, 1714, 1272, 715 cm^{-1}; ^{13}C NMR data, see Table 2; HRTOFESIMS *m/z* 577.2199 [M + K]$^+$ (calcd for C$_{31}$H$_{38}$O$_8$K, 577.2198).

2β-Hydroxyzuelanin-6β-cinnamate (5): yellow, amorphous solid; [α]^{30}$_D$ +66 (*c* 1.0, CHCl$_3$); UV (CHCl$_3$) λ_{max} (log ε) 239 (3.80), 282 (4.00) nm; CD (*c* 0.03, MeOH) $\Delta\varepsilon$ (nm) +20.0 (239), +9.6 (282); IR (KBr) ν_{max} 3440, 1712, 1637 cm^{-1}; ^{13}C NMR data, see Table 2; HRTOFESIMS *m/z* 587.2616 [M + Na]$^+$ (calcd for C$_{33}$H$_{40}$O$_8$Na, 587.2615).

Cytotoxicity Assay. The cytotoxicity of the clerodane diterpenes (**1**, **3** and **4**, **5**) was tested against four tumor cell lines: SF-295 (human glioblastoma), HCT-8 (human colon carcinoma), HL-60 (human leukemia), and MDA/MB-435 (human melanoma). Cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin, at 37 °C with 5% CO$_2$. Cells were seeded in 96-well plates (10^5 cells/well for adherent cells or 0.3 × 10^6 cells/well for suspended cells in 100 μL of medium). After 24 h, the compounds (0.39–25 μg/mL) dissolved in DMSO were added to each well. Doxorubicin (0.01–0.58 μg/mL) was used as positive control. The proliferation of tumor cells was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product. At the end of 69 h incubation, the medium was replaced by fresh medium containing 0.5 mg/mL of MTT. Three hours later, the formazan product was dissolved in DMSO, and absorbance was measured using a multiplate reader at 595 nm (DTX-880, Beckman Coulter). The IC$_{50}$ values were obtained by nonlinear regression using the Graphpad program (Institutive Software for Science, San Diego, CA).[21]

Supporting Information Available: 1D and 2D NMR data for compounds **1**–**4** (Figures S1–S30). This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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