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Cytotoxic Clerodane Diterpenes from *Casearia rupestris*

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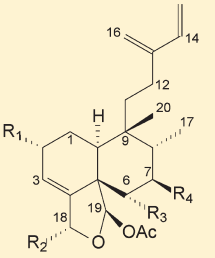
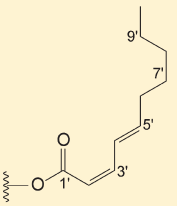
Supporting Information

ABSTRACT: Four new clerodane diterpenes, casearupestrins A−D (**1**−**4**), were isolated from the leaves of *Casearia rupestris*. Compounds **1** and **4** were acetylated to yield 2,7-di-*O*-acetylcasearupestrin A (**5**) and 2,6-di-*O*-acetylcasearupestrin D (**6**). All compounds were evaluated for cytotoxicity against a small panel of human cancer cell lines. Casearupestrin A (**1**) exhibited the most potent activity against MDA/MB-435 (human melanoma) and SF-295 (human glioblastoma) cells, superior to that of the standard drug doxorubicin.





2Z,4E-decadienoate

	R_1	R_2	R_3	R_4
1	OH	OAc	2Z,4E-decadienoate	OH
2	OH	OAc	OH	2Z,4E-decadienoate
3	OH	OCH_3	OH	2Z,4E-decadienoate
4	OH	OAc	OAc	2Z,4E-decadienoate
5	OAc	OAc	2Z,4E-decadienoate	OAc
6	OAc	OAc	OAc	2Z,4E-decadienoate

The tropical genus *Casearia* (*Salicaceae sensu lato*) contains ca. 180 species,[1] and it is a rich source of clerodane-type diterpenes.[2−13] The genus occurs widely in Brazil, where *C. sylvestris* is the most frequent species, occurring from the Amazon basin throughout the Cerrado (savanna) to the Atlantic Forest and Southern Brazil. The clerodanes are a group of diterpenoids with interesting biological effects such as antifungal,[14] antileishmanial and antiplasmodial,[15] antimalarial and antimycobacterial,[16] antitumor,[17,18] cytotoxic,[1,14,16,19−31] immunomodulatory,[32] and trypanocidal activities.[33] In our continuing investigation of the bioactive clerodane diterpenes from *Casearia* species, we undertook the first phytochemical investigation of *Casearia rupestris* Eichler. This shrub or small tree grows up to 6.0 m in height and is found in Brazil (Mato Grosso and Minas Gerais States) and Bolivia (Sta. Cruz State) in the Cerrado, grasslands, and sometimes pastures, at altitudes of 350−500 m.[34] Investigation of the cytotoxic Et_2O fraction yielded four new clerodane diterpenes (**1**−**4**) (Figure 1). Compounds **1** and **4** were acetylated, resulting in two new derivatives (**5**, **6**) (Figure 1). The cytotoxic activity of the diterpenes (**1**−**6**) against a panel of human cancer cell lines (HL-60, MDA/MB-435, HCT-8, and SF-295) has been evaluated.

RESULTS AND DISCUSSION

Investigation of the Et_2O phase of the EtOH extract of the leaves of *C. rupestris* led to the isolation of four new clerodane diterpenoids, casearupestrins A−D (**1**−**4**). 2,7-Di-*O*-acetylcasearupestrin A (**5**) and 2,6-di-*O*-acetylcasearupestrin D (**6**) were obtained by acetylation of compounds **1** and **4**, respectively. The structures (Figure 1) of the compounds were elucidated on the basis of spectroscopic and spectrometric data (IR, UV, NMR, and MS) and by comparison with NMR data of reported compounds.[6]

Casearupestrin A (**1**) was obtained as a white, amorphous solid ($[\alpha]^{24}_D$ +4.3 (*c* 1.0, MeOH)). The molecular formula

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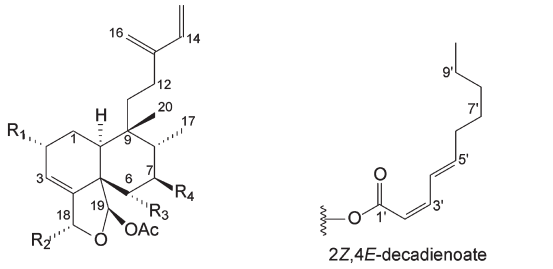

	R_1	R_2	R_3	R_4
1	OH	OAc	2*Z*,4*E*-decadienoate	OH
2	OH	OAc	OH	2*Z*,4*E*-decadienoate
3	OH	OCH_3	OH	2*Z*,4*E*-decadienoate
4	OH	OAc	OAc	2*Z*,4*E*-decadienoate
5	OAc	OAc	2*Z*,4*E*-decadienoate	OAc
6	OAc	OAc	OAc	2*Z*,4*E*-decadienoate

Figure 1. Structure of the clerodane diterpenes from *Casearia rupestris*.

$C_{34}H_{48}O_9$ was determined by HRESIMS, based on the sodiated ion peak at *m*/*z* 623.3190 $[M + Na]^+$ (calcd 623.3190), which implies 11 degrees of unsaturation. The IR spectrum showed the presence of carbonyl esters (1746 and 1712 cm^{-1}). The UV spectrum displayed absorptions at λ_{max} 223 nm (log ε 1.40) and 268 nm (log ε 1.50), corresponding to diene and dienone chromophores, respectively. The ^{13}C and DEPT 135° NMR data showed the presence of 34 carbons divided into seven quaternary (including three carbonyl carbons), 13 methine, nine methylene, and five methyl carbons. The olefinic region of the ^1H NMR spectrum of **1** (Table 1) revealed proton signals with characteristic *cis/trans* coupling at δ_H 5.06 (1H, d, *J* = 10.5 Hz, H-15), 5.30 (1H, d, *J* = 17.5 Hz, H-15), and 6.47 (1H, dd, *J* = 10.5, 17.5 Hz, H-14), indicating a terminal monosubstituted olefinic moiety. Another terminal methylene, identified by two singlets at δ_H 4.94 and 5.03 (2H, H-16), was connected to a quaternary carbon (C-13). An additional olefinic doublet was observed at δ_H 6.04 (1H, d, *J* = 4.0 Hz, H-3) and correlated to a methine carbon at δ_C 128.6 (C-3) in the gHMQC spectrum. Two deshielded acetal protons were observed at δ_H 6.35 (1H, t, *J* = 1.5 Hz, H-18) and 6.49 (1H, s, H-19), which correlated to methine signals at δ_C 96.3 and 99.8, respectively. The ^1H NMR spectrum also revealed the presence of two methyl groups at δ_H 1.06 (1H, brd, *J* = 4.0 Hz, H-17) and 1.07 (1H, s, H-20) and three oxymethines at δ_H 4.45 (1H, brt, *J* = 4.0 Hz, H-2), 5.18 (1H, d, *J* = 10.0 Hz, H-6), and 3.66 (1H, t, *J* = 10.0 Hz, H-7). The spectroscopic data of **1** (Tables 1 and 2) are in accord with the basic skeleton of clerodane diterpenoids previously isolated from *Casearia* species.[10,18,35] The acyclic moiety at C-9, including the diene group, was deduced from the correlations of H-12 with C-11, H-14 with C-13 and C-16, H-15 with C-13 and C-14, and H-16 with C-14 (Figure 2). The position of the hydroxy group at C-7 was deduced by the long-range correlation of the oxymethine signal at δ_H 3.66 (H-7) with δ_C 77.5 (C-6), 44.8 (C-8), and 11.7 (C-17) (Figure 2). A decadienoate moiety, identified by gHMBC and gCOSY correlations (Figure 2), was characterized by a *Z*- (δ_H 5.68, *J* = 11.5 Hz; 6.69, *J* = 11.5 Hz) and an *E*-double bond (δ_H 7.37, *J* = 14.0, 11.5 Hz; 6.16, *J* = 14.0, 7.0 Hz) and a triplet methyl (δ_H 0.98, *J* = 7.0 Hz). This same 2*Z*,4*E*-decadienoate unit has been verified in other clerodane diterpenes isolated from *C. pitumba* and *C. arguta*.[6,36] Its position was determined at C-6 (δ_C 77.5), on the basis of the long-range correlation observed in the gHMBC spectrum between the oxymethine proton (H-6) and the carbonyl carbon (δ_C 167.3, C-1′). Finally, since H-19 (δ_C 6.49) was correlated with the carbonyl group (δ_C 171.4) of one acetate unit, the second acetate unit (δ_C 171.8 and 21.0) must be located at C-18 (Figure 2).

The relative configuration of **1** was determined by using coupling constants, 1D-NOE data, and ^{13}C NMR chemical shifts (Table 1 and Figure 3). The *trans* configuration between the C-17 and C-20 methyl groups was shown by the chemical shift at δ_C 26.4 for C-20, which also supported a *cis*-A/B ring junction.[3,5,10] The *J* values of 3.5 and 13.5 Hz for the coupling of H-10 and H-1 revealed that H-10 (*rel*-α) is in the axial position. The NOE enhancement between H-19 and H-10, and H-19 and H-7, indicated the same α-orientation to H-19 and H-7 (Figure 2). The large coupling constant between H-6 (δ_H 5.18 — 1H, d, *J* = 10.0 Hz) and H-7 (δ_H 3.66 — 1H, t, *J* = 10.0 Hz) supported their 1,2-diaxial relationship. The NOE interaction between H-6 (*rel*-β) and H-8 implied that H-8 and Me-17 were at axial and equatorial positions, respectively. A small coupling constant of 4.0 Hz for H-2/H-1 suggested an equatorial orientation for H-2 (*rel*-β), which was confirmed by the broad triplet at δ_H 4.45 and the chemical shift of C-2 at δ_C 64.2. From the allylic coupling between H-3 and H-18 (*J* = 1.5 Hz) and NOE enhancement observed between H-18 and H-6 it can be deduced that H-18 also has a β-orientation. Therefore, casearupestrun A (**1**), a new clerodane diterpenoid, was assigned as *rel*-(2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*R*)-18,19-diacetoxy-18,19-epoxy-6-[(2′*Z*,4′*E*)-decadienoyloxy]-2,7-dihydroxyclero-3,13(16),14-triene.

The MS, UV, and IR data and the specific rotation value of casearupestrin B (**2**) were comparable with those of **1**. The ^1H and ^{13}C NMR spectroscopic data (Tables 1 and 2) revealed the same substituents and substitution patterns. However, changes in the chemical shift values related to H-6 (δ_H 3.75 — 1H, d, *J* = 10.5 Hz) and H-7 (δ_H 5.15 — 1H, t, *J* = 10.5 Hz) required placement of the decadienoate ester at C-7. The structure **2** was corroborated by the same correlations observed for **1** in the gHMQC, gHMBC, and gCOSY (Figure 2). The observed NOESY interactions and coupling constants for casearupestrin B (**2**) indicate the same relative configuration as **1** (Figure 3 and Table 1). Hence, casearupestrin B (**2**) was assigned as *rel*-(2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*R*)-18,19-diacetoxy-18,19-epoxy-7-[(2′*Z*,4′*E*)-decadienoyloxy]-2,6-dihydroxyclero-3,13(16),14-triene.

The structures of the remaining two compounds can be rationalized by comparison with the previous ones. Analysis of the NMR data of compound **3** revealed a strong similarity to compounds **1** and **2**. However, the molecular formula $C_{33}H_{48}O_8$, determined by the HRESIMS, revealed a sodiated ion peak at *m*/*z* 595.3241 $[M + Na]^+$, which implied 10 degrees of unsaturation. The main difference observed in the ^1H and ^{13}C NMR spectra of compound **3** in relation to compounds **1** and **2** (Tables 1 and 2) was the presence of signals at δ_H 3.36 (1H, s) and δ_C 55.4. These signals indicated that an acetoxy group at C-18 was replaced by a methoxy group, which was confirmed by the gHMBC correlation between H-18 and the methoxy group. The relative configuration of **3** was identical to **1** on the basis of the same NOESY interactions (Figure 3) and coupling constants. Thus, casearupestrin C (**3**) was assigned as *rel*-(2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*R*)-19-acetoxy-18,

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Table 1. ^{1}H NMR Data for Compounds 1−6 (methanol-d_4, 500 and 300 MHz, J in Hz)

position	1	2	3	4	5	6
1	2.00 m	1.34 m	1.96 m	2.10 m	2.19 m	2.08 m
	2.06 m	1.91 m				2.17 m
2	4.45 brt (4.0)	4.39 brt (4.0)	4.37 brt (4.0)	4.35 brt (4.0)	5.55 m	5.52 m
3	6.04 brd (4.0)	6.02 brd (4.0)	6.05 brd (4.0)	6.07 brd (4.0)	6.07 brd (5.5)	6.07 brd (4.0)
6	5.18 d (10.0)	3.75 d (10.5)	3.74 d (10.0)	5.28 brs	5.31 m	5.29 brd
7	3.66 t (10.0)	5.15 t (10.5)	5.16 t (10.0)	5.30 d (12.0)	5.37 m	5.32 brd
8	1.84 m	1.91 m	1.92 m	2.05 m	2.16 m	2.10 m
10	2.47 dd (3.5, 13.5)	2.46 dd (4.0, 13.5)	2.44 dd (4.5, 13.0)	2.51 dd (3.5, 13.5)	2.40 dd (5.0, 22.5)	2.40 dd (5.0, 22.0)
11	1.47 m	1.46 m	1.32 m	1.97 m	1.35 m	1.38 m
12	2.16 m	2.25 m	2.24 m	2.24 m	2.21 m	2.21 m
	2.23 m					
14	6.47 dd (10.5, 17.5)	6.46 m	6.51 brdd (8.5, 17.5)	6.47 dd (7.0, 11.0)	6.49 brdd (11.0, 18.5)	6.50 brdd (11.0, 18.0)
15	5.06 d (10.5)	5.04 d (10.5)	5.06 d (8.5)	5.03 d (7.0)	5.10 m	5.10 m
	5.30 d (17.5)	5.29 d (17.5)	5.30 d (17.5)	5.30 d (11.0)	5.33 m	5.38 m
16	4.94 s	5.04 s	5.01 s	5.06 s	5.07 s	5.06 s
	5.03 s	5.00 s	5.05 brs	5.40 brs		
17	1.06 brd (4.0)	0.89 d (7.0)	0.91 brd (6.5)	0.93 brd (7.0)	0.92 brd (6.5)	0.95 brd (7.0)
18	6.35 t (1.5)	6.65 brt (2.0)	5.48 t (1.5)	6.33 t (1.5)	6.33 t (2.5)	6.36 t (3.0)
19	6.49 s	6.51 s	6.48 s	6.53 s	6.58 s	6.58 s
20	1.07 s	1.04 s	1.04 brs	0.98 s	1.08 s	1.08 s
2′	5.68 d (11.5)	5.63 d (11.5)	5.64 d (11.0)	5.40 d (11.0)	5.55 brd (19.0)	5.54 brd (19.0)
3′	6.69 t (11.5)	6.65 t (11.5)	6.67 t (11.0)	6.70 t (11.0)	6.74 t (19.0)	6.71 t (19.0)
4′	7.37 brdd (11.5, 14.0)	7.38 brdd (11.5, 14.0)	7.39 brdd (11.0, 15.5)	7.32 brdd (11.0, 15.5)	7.31 brdd (4.5, 19.0)	7.34 brdd (4.0, 19.0)
5′	6.16 dd (7.0, 14.0)	6.14 dd (7.0, 14.0)	6.15 dd (7.0, 15.5)	6.18 dd (7.5, 15.5)	6.24 m	6.20 m
6′	2.20 m	2.17 m	2.22 m	2.22 m	2.24 m	2.24 m
7′	1.27 m	1.91 m	1.48 m	1.46 m	1.49 m	1.49 m
	1.69 m	1.34 m				
8′	1.34 m	1.34 m	1.35 m	1.35 m	1.35 m	1.36 m
9′	1.34 m	1.34 m	1.35 m	1.35 m	1.35 m	1.36 m
10′	0.98 t (7.0)	0.92 t (7.0)	0.93 m	0.92 brt	0.92 m	0.93 brt
2-O_2CCH_3					2.12 s	2.12 s
6-O_2CCH_3				1.95 s	1.95 s	1.97 s
18-OCH_3			3.36 s			
18-O_2CCH_3	2.02 s	2.05 s		2.04 s	2.04 s	2.07 s
19-O_2CCH_3	1.86 s	1.87 s	1.87 s	1.87 s	1.89 s	1.90 s

19-epoxy-7-[(2′*Z*,4′*E*)-decadienoyloxy]-2,6-dihydroxy-18-methoxyclero-3,13(16),14-triene.

Compound **4** had the molecular formula $C_{36}H_{50}O_{10}$, determined by HRESIMS (observed m/z 665.3297 $[M + Na]^+$). The ^{1}H and ^{13}C NMR data of **4** (casearupestrin D) (Tables 1 and 2) were similar to those of **2** except for replacement of a hydroxy group at C-6 by an acetoxy group [δ_H 1.95 (3H, s), δ_C 171.5, δ_C 20.9]. The ^{1}H and ^{13}C NMR assignments of compound **4** were confirmed by 2D NMR correlations, and its configuration was established by the sign of the specific rotation, NOESY interactions, and coupling constants (Figure 3). Thus, casearupestrin D (**4**) was assigned as *rel*-(2*R*,5*S*,6*S*,8*R*,9*R*,10*S*,18*R*,19*R*)-6,18,19-triacetoxy-18,19-epoxy-7-[(2′*Z*,4′*E*)-decadienoyloxy]-2-hydroxyclero-3,13(16),14-triene.

Acetylation of **1** and **4** with Ac_2O−pyridine yielded two new clerodane diterpenes derivatives, **5** and **6**, respectively (Tables 1 and 2, NMR data). The products were named 2,7-di-*O*-acetylcasearupestrin A (**5**) and 2,6-di-*O*-acetylcasearupestrin D (**6**).

The results of the biological tests of compounds **1**−**6** are shown in Table 3. Casearupestrins A (**1**), B (**2**), and D (**4**) showed significant cytotoxicity against four cell lines (HL-60, HCT-8, MDA/MB-435, and SF-295), with IC_{50} values ranging from 0.10 to 1.3 μM. Compound **1** had an IC_{50} value of 0.36 μM against MDA/MB-435 and SF-295 cancer cell lines, superior to that of the standard drug doxorubicin (Table 3). Acetylation of compounds **1** and **4** resulted in decreased cytotoxicity (Table 3), indicating that the C-2 OH group is essential for the cytotoxicity of these compounds.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 LC polarimeter. UV spectra were measured on a Hewlett-Packard 8453 A spectrometer. The circular dichroism spectra were obtained on a JASCO PU-2089 HPLC system configured with a UV−vis (MD-2010) and a circular dichroism (CD-2095) detector. IR spectra were measured on a Nicolet Impact 400 spectrometer, using KBr disks. The 1D (^{1}H, ^{13}C, DEPT, and NOE)

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Table 2. ^{13}C NMR Data for Compounds 1−6 (methanol-d_4, 125 and 75 MHz, δ)[a]

position	1	2	3	4	5	6
1	30.6, CH_2	30.6, CH_2	30.6, CH_2	30.5, CH_2	27.8, CH_2	27.8, CH_2
2	64.2, CH	64.2, CH	64.3, CH	64.0, CH	67.5, CH	67.4, CH
3	128.6, CH	127.0, CH	126.5, CH	129.3, CH	125.2, CH	125.3, CH
4	141.9, C	143.5, C	144.0, C	141.1, C	144.2, C	144.1, C
5	54.3, C	55.2, C	55.1, C	54.3, C	54.3, C	54.2, C
6	77.5, CH	75.2, CH	75.3, CH	75.8, CH	74.2, CH	75.6, CH
7	72.2, CH	76.1, CH	76.1, CH	73.5, CH	74.7, CH	73.4, CH
8	44.8, CH	43.1, CH	43.0, CH	42.7, CH	42.5, CH	42.5, CH
9	39.8, C	40.2, C	40.2, C	40.2, C	40.2, C	40.2, C
10	37.2, CH	36.5, CH	36.3, CH	37.3, CH	38.3, CH	38.3, CH
11	29.4, CH_2	29.5, CH_2	30.5, CH_2	30.5, CH_2	30.6, CH_2	30.6, CH_2
12	25.0, CH_2	25.1, CH_2	25.0, CH_2	25.0, CH_2	25.1, CH_2	25.1, CH_2
13	147.0, C	146.9, C	147.0, C	146.8, C	146.6, C	146.6, C
14	141.5, CH	141.6, CH	141.6, CH	141.5, CH	141.4, CH	141.4, CH
15	113.2, CH_2	113.1, CH_2	113.1, CH_2	113.2, CH_2	113.2, CH_2	113.2, CH_2
16	115.5, CH_2	115.7, CH_2	115.6, CH_2	115.1, CH_2	115.9, CH_2	115.9, CH_2
17	11.7, CH_3	11.5, CH_3	11.5, CH_3	11.3, CH_3	11.3, CH_3	11.3, CH_3
18	96.3, CH	97.2, CH	105.7, CH	96.0, CH	96.0, CH	95.9, CH
19	99.8, CH	100.1, CH	99.7, CH	99.5, CH	99.5, CH	99.5, CH
20	26.4, CH_3	26.2, CH_3	26.3, CH_3	26.0, CH_3	25.8, CH_3	25.8, CH_3
1′	167.3, C	168.0, C	168.0, C	167.4, C	166.8, C	167.4, C
2′	115.9, CH	116.3, CH	116.3, CH	115.7, CH	114.6, CH	115.1, CH
3′	147.7, CH	147.1, CH	147.1, CH	148.1, CH	148.9, CH	148.2, CH
4′	128.3, CH	128.3, CH	128.2, CH	128.0, CH	128.1, CH	128.0, CH
5′	147.2, CH	146.9, CH	146.8, CH	148.0, CH	148.5, CH	148.0, CH
6′	33.9, CH_2	33.9, CH_2	33.9, CH_2	33.9, CH_2	33.9, CH_2	33.9, CH_2
7′	30.5, CH_2	30.6, CH_2	29.5, CH_2	29.4, CH_2	29.3, CH_2	29.4, CH_2
8′	32.4, CH_2	32.5, CH_2	32.5, CH_2	32.5, CH_2	32.5, CH_2	32.5, CH_2
9′	23.4, CH_2	23.5, CH_2	23.5, CH_2	23.5, CH_2	23.4, CH_2	23.5, CH_2
10′	14.3, CH_3	14.3, CH_3	14.3, CH_3	14.3, CH_3	14.3, CH_3	14.3, CH_3
2-O_2CCH_3					172.1, C 21.0, CH_3	172.1, C 21.0, CH_3
6-O_2CCH_3				171.5, C 20.9, CH_3	171.6, C 20.6, CH_3	171.5, C 20.9, CH_3
18-OCH_3			55.4, CH_3			
18-O_2CCH_3	171.8, C 21.0, CH_3	172.0, C 21.0, CH_3		171.9, C 20.9, CH_3	172.0, C 20.9, CH_3	171.8, C 20.9, CH_3
19-O_2CCH_3	171.4, C 21.8, CH_3	171.5, C 21.8, CH_3	171.9, C 21.9, CH_3	171.9, C 21.9, CH_3	170.9, C 21.7, CH_3	171.0, C 21.7, CH_3

[a] ^{13}C NMR data were assigned by DEPT-135° and gHMQC experiments; multiplicities were determined with the assistance of DEPT-135° and 90° experiments.

and 2D ($^1H-^1H$ gCOSY, $^1H-^{13}C$ gHMQC, gHMBC, and gNOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^1H) and 125 MHz (^{13}C) with pulse field gradient and a Varian INOVA 300 spectrometer (7.4 T) at 300 MHz (^1H) and 75 MHz (^{13}C), using methanol-d_4 as the internal standard for ^{13}C NMR chemical shifts and residual undeuterated solvent (methanol) as the internal standard for ^1H NMR, with the δ scale reported relative to TMS. Positive-ion HRMS spectra were recorded on an UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer, using TFANa as the internal standard. Analytical HPLC was performed on a Varian Pro Star 230 with UV−vis detector (model 330), using a Phenomenex C_{18} column (250 mm × 4.6 mm, 5 μm). Open column chromatography was performed over silica gel (40−63 μm, Merck) or silica C18 (40 μm, J. T. Baker) 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 observed under UV light and by spraying the plates with anisaldehyde−H_2SO_4 reagent, followed by heating at 120 °C.

Plant Material. *Casearia rupestris* leaves were collected in May 2007 in Campinas municipality, São Paulo State, Brazil. The plant material was identified by one of the authors (R.B.T.). A voucher specimen (IAC41542) has been deposited in the herbarium of the Instituto Agronômico de Campinas (São Paulo State).

Extraction, Isolation, and Chemical Transformation. The dried and ground leaves (0.46 kg) were exhaustively extracted by successive maceration with hexanes, EtOH, and H_2O (3.0 L × 3) at room temperature. The EtOH extract was concentrated under reduced pressure at 40 °C to yield 30.0 g of a crude extract. This extract was then diluted with MeOH−H_2O (3:1) and successively partitioned with hexanes (1.0 L × 3), Et_2O (1.0 L × 3), and EtOAc (1.0 L × 3). After removal of the solvent, each extract yielded 10.8, 3.2, and 1.2 g, respectively. Part of the cytotoxic Et_2O (3.0 g) fraction was subjected to gel permeation chromatography on Sephadex LH-20 (78.0 × 3.0 cm) eluted with MeOH, to afford 38 fractions. These fractions were combined on the basis of their TLC profiles. Fraction 12 (1.7 g) was subjected to column chromatography (silica gel-C_{18}, 78.0 × 3.0 cm,

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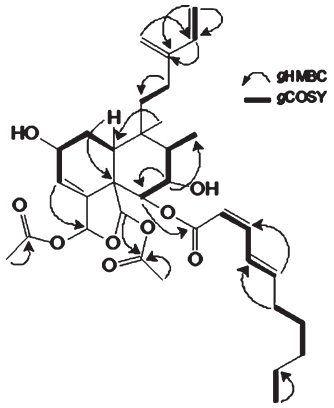

Figure 2. Important gHMBC and gCOSY correlations observed for **1**.

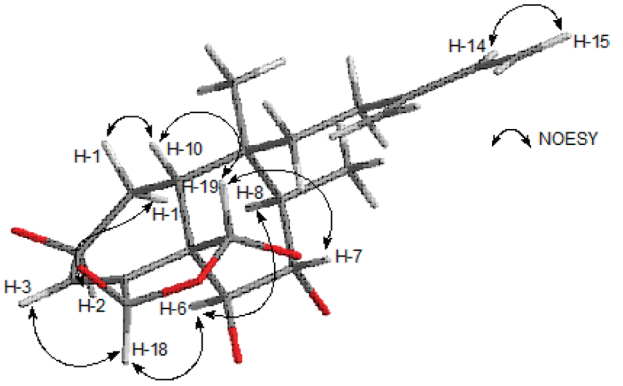

Figure 3. Important NOE enhancements observed for compounds **1**−**4**.

Table 3. Cytotoxic Activity of Compounds 1−4 from the Leaves of *Casearia rupestris* and Derivatives 5 and 6 on Human Tumor Cancer Cell Lines after 72 h Exposure

	cell lines (IC_{50}, μM)[a]			
compound	HL-60	MDA/MB-435	HCT-8	SF-295
1	0.10	0.36	0.13	0.36
2	0.85	1.3	0.28	0.80
3	>5	>5	>5	>5
4	0.25	0.93	0.25	0.43
5	4.3	>5	>5	>5
6	>5	>5	>5	>5
doxorubicin	0.04	0.83	0.02	0.40

[a] Data are presented as IC_{50} values and 95% confidence intervals obtained by nonlinear regression for leukemia (HL-60), melanoma (MDA-MB-453), colon (HCT-8), and glioblastoma (SF-295) cells from two independent experiments. Doxorubicin was used as positive control.

40 μm) using a step gradient from H_2O−MeOH 23:77 to 00:100, yielding 55 fractions. Fraction 14 (175.0 mg) was subjected to silica gel column chromatography (21.8 × 2 cm, 230−400 mesh) eluted with hexanes−EtOAc (6:4), to yield compound **1** (79.0 mg). Fraction 30 (88.0 mg) was subjected to silica gel column chromatography (21.8 × 2 cm, 40−63 μm) eluted with hexanes−EtOAc (4:6) to yield compound **2** (13.0 mg). Fraction 34 (150.5 mg) was subjected to silica gel column chromatography (21.8 × 2 cm, 40−63 μm) eluted with hexanes−EtOAc (1:1) to yield compound **3** (10.0 mg). Finally, fraction 36 (356.0 mg) was subjected to silica gel column chromatography (21.8 × 2 cm, 40−63 μm) eluted with hexanes−EtOAc (1:1) to yield compound **4** (212.0 mg). Compounds **1** and **4** (20 mg each) were dissolved in pyridine (4.0 mL) and treated with Ac_2O (3.0 mL) at room temperature for 24 h. The solvents were removed by evaporation under reduced pressure, resulting in compounds **5** (18.0 mg) and **6** (18.0 mg).

Casearupestrin A (***1***): white, amorphous solid; $[\alpha]_D^{24}$ +4.3 (*c* 0.1, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 223 (1.40), 268 (1.50); CD (*c* 0.02, MeOH) $\Delta\varepsilon$ (nm) +6.5 (223), +6.6 (268); IR (KBr) ν_{max} cm^{-1} 3434, 2959, 2932, 1746, 1712, 1378, 1230, 1028; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 623.3190 $[M + Na]^+$ (calcd for $C_{34}H_{48}O_9Na$, 623.3190).

Casearupestrin B (***2***): white, amorphous solid; $[\alpha]_D^{24}$ +76 (*c* 0.1, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 223 (1.48), 268 (1.55); CD (*c* 0.03, MeOH) $\Delta\varepsilon$ (nm) +5.4 (223), +6.0 (268); IR ν_{max} cm^{-1} 3436, 1746, 1712, 1636, 1177, 1000, 900; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 623.3206 $[M + Na]^+$ (calcd for $C_{34}H_{48}O_9Na$, 623.3190).

Casearupestrin C (***3***): white, amorphous solid; $[\alpha]_D^{20}$ +72 (*c* 0.1, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 223 (1.43), 268 (1.48); CD (*c* 0.03, MeOH) $\Delta\varepsilon$ (nm) +5.2 (223), +5.7 (268); IR ν_{max} cm^{-1} 3436, 1714, 1636, 1178, 1001, 897; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 595.3241 $[M + Na]^+$ (calcd for $C_{33}H_{48}O_8Na$, 595.3241).

Casearupestrin D (***4***): white, amorphous solid; $[\alpha]_D^{25}$ +81 (*c* 0.1, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 223 (1.70), 268 (1.80); CD (*c* 0.04, MeOH) $\Delta\varepsilon$ (nm) +5.2 (223), +5.0 (268); IR ν_{max} cm^{-1} 3446, 1746, 1225, 1029, 905; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 665.3297 $[M + Na]^+$ (calcd for $C_{36}H_{50}O_{10}Na$, 665.3296).

2,7-Di-O-acetylcasearupestrin A (***5***): colorless oil; $[\alpha]_D^{25}$ +56 (*c* 0.1, MeOH); UV λ_{max}^{MeOH} nm (log ε) 223 (1.43), 268 (1.60); IR ν_{max} cm^{-1} 3454, 2953, 1752, 1374, 1227, 1026; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 707.3401 $[M + Na]^+$ (calcd for $C_{38}H_{52}O_{11}Na$, 707.3401).

2,6-Di-O-acetylcasearupestrin D (***6***): colorless oil; $[\alpha]_D^{25}$ +99 (*c* 0.1, MeOH); UV λ_{max}^{MeOH} nm (log ε) 223 (1.40), 268 (1.55); IR ν_{max} cm^{-1} 3455, 2953, 1750, 1374, 1227, 1026; ^1H and ^{13}C NMR (methanol-d_4), see Tables 1 and 2, respectively; HRTOF-ESIMS *m*/*z* 707.3401 $[M + Na]^+$ (calcd for $C_{38}H_{52}O_{11}Na$, 707.3401).

Cytotoxicity Assay. The cytotoxicity of the clerodane diterpenes (**1**−**6**) 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 (105 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 the 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 (Intuitive Software for Science, San Diego, CA).[37]

■ ASSOCIATED CONTENT

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

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