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Potent New Cell Adhesion Inhibitory Compounds from the Root of *Trichilia rubra*

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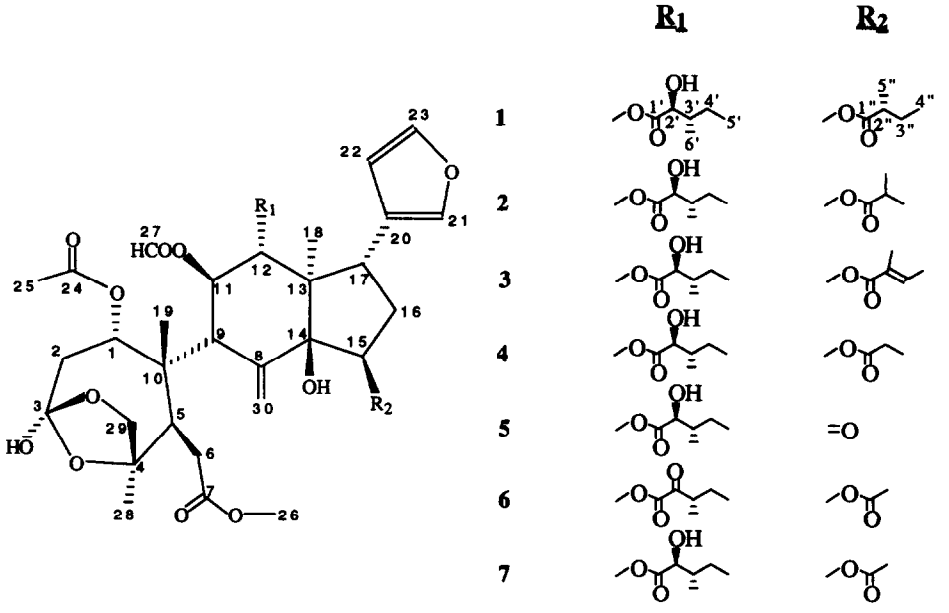
Abstract: A series of seco-limonoids, with uncommon hemi ortho ester A-rings, was isolated from the root of *Trichilia rubra*. Their structures were determined by extensive spectroscopic studies. The five new and two previously known compounds were all found to be potent inhibitors of LFA-1:ICAM-1 mediated cell adhesion.

Cell adhesion processes play significant roles in pathological conditions, such as chronic inflammation, cancer metastases and viral infections. Interactions between a number of cell surface glycoproteins mediate various stages of cell adhesion.[1,2] One family of adhesion molecules consists of the heterodimeric integrins. There are three known leukocyte-specific integrins with a common β_2 chain. The binding of one of these, LFA-1 (lymphocyte function associated antigen-1), to ICAM-1 (intercellular adhesion molecule-1) mediates leukocyte adhesion to endothelial layers when leukocytes leave the bloodstream at sites of inflammation.[3] Antibodies against β_2 integrins attenuate inflammation in animal models.[4-7] Specific inhibitors of integrin mediated cell adhesion may have therapeutic potential as antiinflammatory and antimetastatic agents. In the course of our search for natural products that inhibit cell adhesion, one extract from the root of *Trichilia rubra* was identified as having potent inhibitory activity in a bioassay for LFA-1:ICAM-1 mediated adhesion of JY and HeLa cells, developed and automated in-house for large throughput screening.

Trichilia rubra, a small tree indigenous to tropical South America, belongs to the Meliaceae family. It is widely distributed in the Amazon basin, preferring periodically or permanently flooded land.[8] There are no literature reports to date on the chemical constituents of *Trichilia rubra* , but a number of other *Trichilia* species, from Africa or South America, were found to contain lignans, steroids, phytosterols, triterpenoids and limonoids. The plant sample was collected in Peru and taxonomically identified by professor Sidney McDaniel (Mississippi State University). Voucher specimens are deposited in the herbarium of the Mississippi State University.

The dichloromethane extract of the root material was fractionated under a bioassay directed isolation procedure using silica flash chromatography and semipreparative normal and reverse phase HPLC. Seven bioactive compounds were obtained as pure white noncrystalline solids. They are named rubrin A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**) and G (**7**), in the order of their elution in normal phase HPLC. The weight of the major component **1**, 15 mg, corresponds to an 0.005% overall yield.



For compound **1**, FAB-HRMS data established the molecular formula as $C_{41}H_{58}O_{16}$. The field desorption mass spectrum exhibited peaks at m/z 806, 774, 714, 686 and 668 daltons, corresponding to the molecular ion and the subsequent loss of one molecule of CH_3OH, CH_3COOH, CO, and H_2O, respectively. The ^1H NMR spectrum showed characteristic low-field signals for a formate group (7.93 ppm), a monosubstituted furan ring (7.32 ppm, 7.14 ppm, and 6.26 ppm), four peaks of methine protons on oxygen-bearing carbons (6.03 ppm, 5.56 ppm, 5.26 ppm, and 5.24 ppm), and signals for an exocyclic methylene group (5.83 ppm and 5.31 ppm). At higher field, two methyl triplets, two methyl doublets, three aliphatic methyl singlets, an acetyl methyl, a methoxy, and three exchangeable proton signals were observed.

In agreement with the MS results, 41 carbon signals were present in the proton-decoupled ^{13}C NMR spectrum. Five ester carbonyl peaks (including the formate carbon at 161.0 ppm), the resonances of the four furan carbons, and two olefinic carbons were displayed at low field. A quaternary carbon signal at 119.4 ppm indicated a high degree of oxygenation. With the aid of a DEPT experiment four more quaternary carbons, ten methines, six methylenes, and nine methyl carbons were assigned. An HMQC spectrum provided direct proton-carbon correlation information for all protonated carbons.

Nine sets of directly coupled proton spin systems were found in the DQF COSY spectrum. These spin systems were connected through quaternary carbons or oxygen atoms as determined with the help of long-range proton-carbon connectivity data from the HMBC experiment (Figure 1). The resulting modified tetracyclic triterpenoid skeleton of **1**, with an opened B-ring, a hemi ortho ester A-ring and the furan

Table 1: Proton NMR Assignments for Compounds **1-7**

	1	2	3	4	5	6	7
H1	5.24 (m)	5.23 (m)	5.22 (m)	5.25 (m)	5.25 (dd, 3.2, 12.4)	5.21 (dd, 3.4, 12.3)	5.21 (m)
H2α	2.04 (m)	2.01 (m)	2.06 (m)	2.04 (m)	2.03 (dd, 3.2, 13.9)	2.02 (m)	2.06 (m)
H2β	2.57 (t, 13.2)	2.58 (t, 13.2)	2.59 (t, 13.2)	2.58 (t, 13.2)	2.57 (t, 13.2)	2.53 (t, 13.5)	2.59 (t, 13.4)
H5	2.98 (d, 9.6)	2.97 (d, 9.6)	2.98 (d, 9.3)	2.95 (d, 9.4)	2.95 (d, 10.7)	2.93 (d, 9.6)	2.94 (d, 9.5)
H6α	1.65 (d, 17.2)	1.66 (d, 17.0)	1.67 (d, 16.6)	1.66 (d, 17.3)	1.71 (d, 17.8)	1.64 (d, 17.1)	1.65 (d, 17.1)
H6β	2.73 (dd, 9.9, 17.3)	2.74 (dd, 10.0, 17.2)	2.75 (dd, 10.0, 17.4)	2.73 (dd, 9.9, 17.1)	2.73 (dd, 10.0, 17.9)	2.71 (dd, 9.8, 17.1)	2.73 (dd, 9.7, 17.2)
H9	4.15 (d, 8.1)	4.15 (d, 8.2)	4.16 (d, 9.2)	4.15 (d, 8.2)	4.19 (d, 8.2)	4.14 (d, 8.2)	4.17 (d, 7.9)
H11	5.26 (m)	5.26 (m)	5.26 (m)	5.24 (m)	5.37 (dd, 8.5, 10.7)	5.28 (dd, 8.7, 10.0)	5.24 (m)
H12	6.03 (d, 11.2)	6.04 (d, 11.2)	6.06 (d, 11.2)	6.03 (d, 11.2)	6.05 (m)	6.02 (d, 11.0)	6.03 (d, 11.1)
H15	5.56 (dd, 5.1, 9.3)	5.58 (dd, 5.2, 9.4)	5.59 (dd, 5.5, 9.5)	5.65 (dd, 5.2, 9.4)		5.66 (dd, 5.2, 9.4)	5.67 (dd, 5.3, 9.4)
H16α	2.44 (m)	2.42 (m)	2.44 (m)	2.38 (m)	2.97 (dd, 8.5, 18.8)	2.34 (m)	2.35 (m)
H16β	2.05 (m)	2.03 (m)	2.12 (m)	2.12 (m)	2.30 (dd, 9.4, 19.2)	2.14 (m)	2.12 (m)
H17	3.94 (dd, 8.9, 9.8)	3.95 (t, 9.3)	3.96 (t, 9.1)	3.96 (t, 10.0)	3.97 (dd, 7.4, 9.3)	3.98 (dd, 8.7, 10.0)	3.97 (t, 8.9)
H18	0.99 (s)	0.99 (s)	1.00 (s)	0.99 (s)	1.00 (s)	1.00 (s)	0.98 (s)
H19	1.34 (s)	1.38 (s)	1.32 (s)	1.34 (s)	1.29 (s)	1.34 (s)	1.37 (s)
H21	7.14 (s)	7.15 (s)	7.16 (s)	7.16 (s)	7.20 (s)	7.12 (s)	7.15 (s)
H22	6.26 (s)	6.27 (s)	6.28 (s)	6.27 (s)	6.25 (s)	6.23 (s)	6.26 (s)
H23	7.32 (s)	7.33 (s)	7.34 (s)	7.34 (s)	7.37 (s)	7.27 (s)	7.34 (s)
H25	2.01 (s)	2.02 (s)	2.03 (s)	2.03 (s)	2.02 (s)	2.00 (s)	2.02 (s)
H26	3.74 (s)	3.76 (s)	3.76 (s)	3.76 (s)	3.72 (s)	3.74 (s)	3.76 (s)
H27	7.93 (s)	7.94 (s)	7.95 (s)	7.94 (s)	7.93 (s)	7.97 (s)	7.94 (s)
H28	1.37 (s)	1.34 (s)	1.39 (s)	1.37 (s)	1.42 (s)	1.32 (s)	1.34 (s)
H29α	3.62 (d, 8.4)	3.63 (d, 8.4)	3.62 (d, 8.4)	3.63 (d, 8.4)	3.64 (d, 8.6)	3.61 (d, 8.5)	3.63 (d, 8.4)
H29β	4.12 (d, 8.5)	4.13 (d, 8.6)	4.13 (d, 8.8)	4.12 (d, 8.6)	4.11 (d, 8.6)	4.10 (d, 8.5)	4.12 (d, 8.6)
H30(Z to C9)	5.83 (s)	5.85 (s)	5.85 (s)	5.84 (s)	5.98 (d, 1.2)	5.83 (s)	5.84 (s)
H30(E to C9)	5.31 (s)	5.32 (s)	5.31 (s)	5.36 (s)	6.06 (d, 1.4)	5.36 (s)	5.36 (s)
H2'	3.24 (d, 3.2)	3.26 (m)	3.28 (d, 3.1)	3.26 (m)	3.20 (m)		3.27 (m)
H3'	1.50 (m)	1.51 (m)	1.48 (m)	1.53 (m)	1.52 (m)	2.52 (m)	1.49 (m)
H4'	1.16 (m)	1.19 (m)	1.17 (m)	1.18 (m)	1.16 (m)	1.49 (m)	1.18 (m)
	1.10 (m)	1.10 (m)	1.13 (m)	1.10 (m)	1.08 (m)	1.17 (m)	1.12 (m)
H5'	0.77 (t, 7.5)	0.78 (t, 7.4)	0.79 (t, 7.4)	0.78 (t, 7.4)	0.78 (t, 7.4)	0.76 (t, 7.4)	0.78 (t, 7.3)
H6'	0.85 (d, 7.0)	0.86 (d, 6.8)	0.87 (d, 7.0)	0.86 (d, 6.8)	0.86 (d, 4.7)	0.86 (d, 6.8)	0.86 (d, 6.8)
H2"	2.42 (m)	2.66 (m)		2.42 (m)		2.10 (s)	2.13 (s)
H3"	1.70 (m)	1.20 (dd, 7.1)	7.10 (q, 7.0)	1.17 (t, 7.6)			
	1.50 (m)	1.20 (dd, 7.1)	1.75 (d, 7.1)				
H4"	0.89 (t, 7.5)		1.86 (s)				
H5"	1.16 (d, 7.1)						

substitution at C17 of the D-ring, was described previously in hispidin A by Jolad et al.[10], and in nymania I by McLachlan and Taylor.[11]

Table 2: COSY and NOESY Correlations in the Spectra of Compound **1**.

Position	COSY crosspeak	NOESY crosspeak	Position	COSY crosspeak	NOESY crosspeak
H1	H2α, H2β	H19	H25		H27
H2α	H1, H2β		H26		
H2β	H1, H2α		H27		H5', H6'
H5	H6α, H6β	H28	H28		H5, H6β
H6α	H5, H6β	H29β	H29α	H29β	H28
H6β	H5, H6α	H1, H19, H29β	H29β	H29α	H6α, H6β
H9	H11	H30(Z)	H30(Z to C9)	H30(E)	H9, H28
H11	H9, H12	H18	H30(E to C9)	H30(Z)	
H12	H11	H17, H19	H2'	H3'	H21, H22, H6'
H15	H16α, H16β	H18	H3'	H2', H4', H6'	
H16α	H15, H16β, H17	H18, H21, H22	H4'	H3', H5'	
H16β	H15, H16α, H17		H5'	H4'	H27
H17	H16α, H16β	H12, H21	H6'	H3'	H27, H2'
H18		H11, H15, H22	H2"	H3", H5"	
H19		H1, H12	H3"	H2", H4"	
H21	H22, H23	H17, H18, H2'	H4"	H3"	
H22	H21, H23	H16α, H18, H2'	H5"	H2"	
H23	H21, H22				

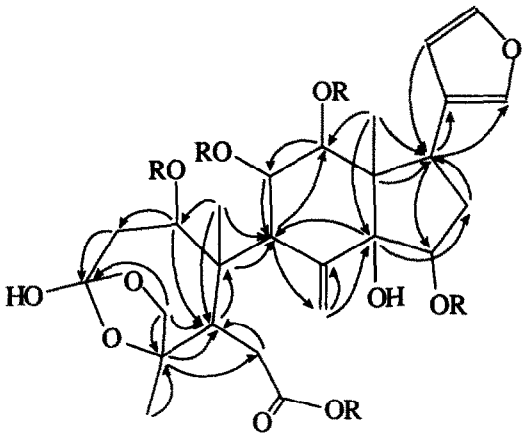


Figure 1. Observed HMBC correlations of the tetranortriterpenoid skeleton.

Unambiguous assignments of the ester group positions in **1** were also determined on the basis of HMBC data. Correlations were observed from the ring methine protons and the ester sidechain protons to the corresponding ester carbonyl carbons (Figure 2). The relative stereochemistry of the 15 chiral carbons of **1** was determined using coupling constant data (Table 1) and proton-proton through-space correlations from NOESY experiments (Table 2). These stereochemical assignments are in good agreement with data reported from X-ray crystallography of a biosynthetically related compound, prieurianin (**8**).[12,13]

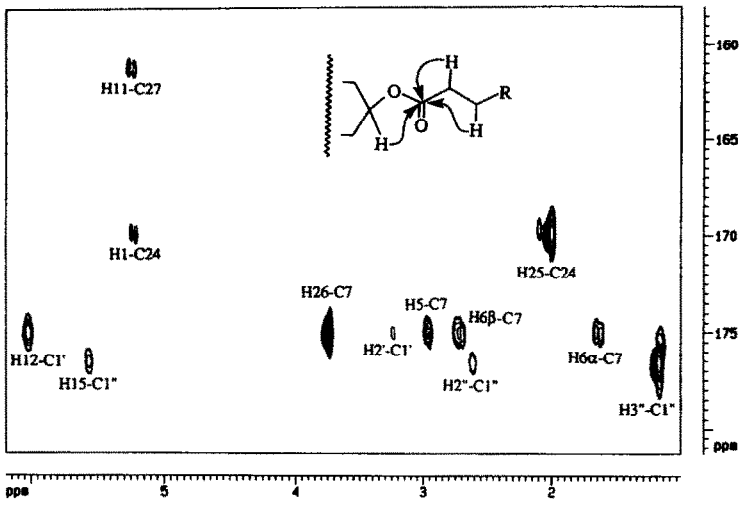
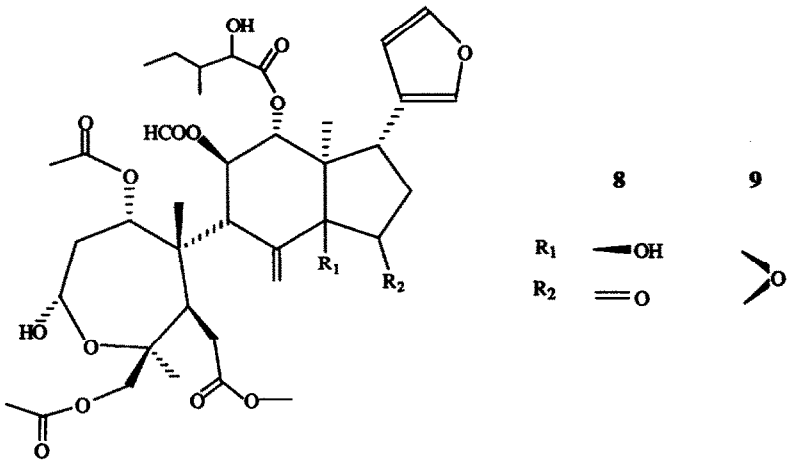


Figure 2. 500 MHz ^{1}H-^{13}C HMBC correlations from the ring methine protons and ester sidechain protons to the carbonyl carbons.

The structures of compounds **2-7** were elucidated following the strategy described for **1**. In comparison to **1**, only the ester substitution on C15 is different in **2**, **3**, **4** and **7**. The D-ring in compound **5** has a ketone at position 15, while compound **6** has an α-ketoester group on C12. Compounds **1**, **2**, **4**, **6** and **7** are novel. Compound **3** appears to be identical to hispidin A, isolated previously from *Trichilia hispida*.[10] Compound **5** has the same proposed structure as nymania I, from *Nymania capensis*, although the spectral characterization of nymania I was not fully described.[11]



Compounds **1-7** all exhibited potent inhibitory activity in the LFA-1:ICAM-1 mediated cell adhesion assay with IC_{50} values of 10-25 nM. The results were reproducible and the dose-response behavior was characteristic of the cell adhesion assay. None of the compounds showed cytotoxicity at concentrations up to 20 μM in either a 5-hour or a 24-hour tritiated thymidine uptake assay or an MTT assay. To obtain information regarding the significance of the hemi ortho ester system to biological activity, we isolated prieurianin-type limonoids, the closest known structurally related class, from a *Guarea* species.[14] The inhibitory activity of prieurianin (**8**) and 14, 15β-epoxyprieurianin (**9**) were orders of magnitude less (IC_{50} values of 10-20 μM). More distantly related limonoids displayed no activity up to 20 μM concentrations.[14] The results suggest that the unique hemi ortho ester A-ring is crucial to potency.

Table 3: Carbon-13 Assignments for Compounds **1-7**

	1	**2**	**3**	**4**	**5**	**6**	**7**
C1	70.9	70.9	71.0	71.0	70.6	71.0	71.1
C2	39.8	39.8	39.7	39.7	39.8	39.8	39.7
C3	119.4	119.4	119.4	119.3	119.4	119.4	119.4
C4	82.6	82.6	82.6	82.5	82.7	82.6	82.6
C5	48.8	48.9	49.1	48.9	48.8	49.0	49.0
C6	33.8	33.9	34.0	33.9	33.6	33.9	33.9
C7	174.8	174.8	174.8	174.9	175.6	175.1	174.9
C8	143.0	142.5	142.4	142.6	142.8	142.3	142.4
C9	49.6	49.6	49.6	49.5	48.6	49.6	49.7
C10	49.1	49.1	49.2	49.1	49.4	49.0	49.1
C11	71.3	71.4	71.6	71.5	71.2	71.7	71.6
C12	74.5	74.6	74.6	74.5	74.6	75.9	74.6
C13	50.4	50.5	50.3	50.4	50.6	50.2	50.5
C14	84.5	84.5	84.6	84.6	80.8	84.8	84.7
C15	72.9	72.9	73.4	72.7	207.3	72.7	72.8
C16	36.7	36.6	36.5	36.3	41.6	36.1	36.2
C17	39.4	39.4	39.5	39.4	35.1	39.8	39.6
C18	13.4	13.5	13.5	13.4	13.1	13.4	13.4
C19	17.0	17.0	17.0	17.4	16.6	17.0	17.2
C20	124.3	124.3	124.5	124.3	126.3	124.3	124.3
C21	140.2	140.2	140.2	140.2	140.6	140.1	140.2
C22	110.8	110.9	110.9	110.8	110.6	111.0	110.8
C23	142.6	142.6	142.6	142.4	143.0	142.6	142.7
C24	169.7	169.7	169.7	169.7	169.7	169.9	169.8
C25	21.1	21.1	21.1	21.1	21.1	21.2	21.1
C26	52.6	52.7	52.7	52.7	53.0	52.8	52.8
C27	161.0	161.0	161.0	161.0	160.9	160.7	161.0
C28	28.7	28.7	28.4	28.4	28.8	28.4	28.4
C29	73.6	73.6	73.5	73.5	73.7	73.5	73.5
C30	122.0	122.1	122.3	122.2	123.2	122.3	122.2
C1'	174.9	174.9	174.9	174.9	174.9	160.7	175.0
C2'	74.5	74.5	74.6	74.5	74.1	197.1	74.5
C3'	38.2	38.2	38.3	38.2	38.2	43.5	38.3
C4'	23.2	23.2	23.3	23.2	23.2	24.9	23.3
C5'	11.6	11.6	11.6	11.6	11.6	11.3	11.6
C6'	15.1	15.1	15.1	15.1	15.2	14.3	15.1
C1"	175.9	176.4	167.4	173.7		170.3	169.7
C2"	41.4	34.3	128.1	27.8		20.8	20.8
C3"	26.6	18.9	138.6	9.1			
C4"	11.7	18.7	11.9				
C5"	16.5		14.4				

EXPERIMENTAL

General Methods: Optical rotations were determined using the sodium D line on a Perkin-Elmer 241 polarimeter in methanol at 25°C. Infrared spectra were recorded on a Nicolet IBM IR/3X spectrometer. The samples were prepared and mounted as KBr micropellets. Ultraviolet spectra were obtained on a Shimadzu 160U uv-visible spectrophotometer in methanol. Field desorption mass spectroscopy was carried out on a VG Analytical ZAB 2-SE spectrometer. High resolution mass spectral data were obtained on a VG Autospec-Q spectrometer in positive fast atom bombardment (FAB) mode using a Cs^+ ion gun at 30 kV. The samples were dissolved in methanol and meta-nitrobenzyl alcohol (mNBA) was used as the matrix. Nuclear magnetic resonance (NMR) spectra were recorded on Varian Gemini 300, Bruker AMX-360 or Bruker AMX-500 spectrometers at 27°C. The samples were dissolved in $CDCl_3$. The chemical shifts are reported as δ values in ppm relative to the signal of tetramethylsilane (TMS), solvent peaks were used as reference. COSY[15,16], DQF-COSY[17], NOESY[18], HMQC[19] and HMBC[20] spectra were obtained using standard pulse sequences. NOESY and HMQC spectra were recorded in phase-sensitive mode using time proportional phase incrementation (TPPI)[21,22]. The NOESY mixing times were 300 msec, 400 msec or 500 msec. The HMQC and HMBC experiments were optimized for one-bond proton-carbon couplings of about 140 Hz and for long range proton-carbon couplings of about 6.5 Hz, respectively. Typically 1024 x 512 data points were acquired and zero-filling was used in the t1 domain to 1024 points. The time domain data was Fourier transformed after multiplication with appropriate window functions in both dimensions.

Isolation Procedure: The dry root material was ground and subjected to Soxhlet extraction with dichloromethane for 24 hours. Silica flash column chromatography (Si Gel 60, EM Science) was used with a hexane:isopropanol gradient to fractionate the extract. 25 fractions were collected and, based on normal and reverse phase thin layer chromatography, 7 fractions were pooled and monitored for activity by the cell adhesion assay. The components of the single active fraction were separated by semipreparative normal phase high performance liquid chromatography (HPLC) on a Waters system using a YMC A-023 silica column with hexane:isopropanol gradient and photodiode array detection (Waters 990). The active compounds were purified by semipreparative reverse phase HPLC (Waters system, YMC ODS A-323 column) with a methanol:water gradient and with photodiode array detection. All seven compounds were obtained as white noncrystalline solids.

Rubrin A (1): $[\alpha]_D^{25}$ -27.7° (*c* =4.4, MeOH); UV λ_{max} (MeOH) 215 nm (ε 4,700); FTIR (KBr) 3525, 2995, 1728, 1379, 1250, 1180, 1005 cm^{-1}; HRFABMS observed (MNa^+) m/z 829.3662, $C_{41}H_{58}O_{16}Na$ requires 829.3623; ^1H NMR in Table 1; ^{13}C NMR in Table 3.

Rubrin B (2): $[\alpha]_D^{25}$ -26.2° (*c* =2.9, MeOH); UV λ_{max} (MeOH) 215 nm (ε 4,500); FTIR (KBr) 3520, 3000, 1726, 1376, 1250, 1185, 1015 cm^{-1}; HRFABMS observed (MNa^+) m/z 815.3471, $C_{40}H_{56}O_{16}Na$ requires 815.3466; ^1H NMR in Table 1; ^{13}C NMR in Table 3.

Hispidin A (3): $[\alpha]_D^{25}$ -25.9° (*c* =1.9, MeOH); UV λ_{max} (MeOH) 215 nm (ε 12,800); FTIR (KBr) 3520,

3005, 1729, 1652, 1376, 1244, 1005 cm^{-1}; HRFABMS observed (MNa+) m/z 827.3501, $C_{41}H_{56}O_{16}Na$ requires 827.3466; ^{1}H NMR in Table 2; ^{13}C NMR in Table 1.

Rubrin D (4): $[\alpha]_D^{25}$ -26.2° (*c* =4.5, MeOH); UV λ_{max} (MeOH) 215 nm (ε 4,500); FTIR (KBr) 3520, 2995, 1726, 1375, 1248, 1002 cm^{-1}; HRFABMS observed (MNa+) m/z 801.3294, $C_{39}H_{54}O_{16}Na$ requires 801.3309; ^{1}H NMR in Table 1; ^{13}C NMR in Table 3.

Nymania I (5): $[\alpha]_D^{25}$ -83.9° (*c* =4.6, MeOH); UV λ_{max} (MeOH) 215 nm (ε 4,100); FTIR (KBr) 3480, 2980, 1730, 1640, 1275, 1198, 1020 cm^{-1}; HRFABMS observed m/z 743.2873 for MNa+ and m/z 721.3039 for MH+, $C_{36}H_{48}O_{15}Na$ requires 743.2891 and $C_{36}H_{49}O_{15}$ requires 721.3071; ^{1}H NMR in Table 1; ^{13}C NMR in Table 3.

Rubrin F (6): $[\alpha]_D^{25}$ -19.8° (*c* =1.2, MeOH); UV λ_{max} (MeOH) 215 nm (ε 5,500); FTIR (KBr) 3520, 2995, 1730, 1640, 1260, 1188, 1015 cm^{-1}; HRFABMS observed m/z 785.3014 for MNa+ and m/z 763.3194 for MH+, $C_{38}H_{50}O_{16}Na$ requires 785.3000 and $C_{38}H_{51}O_{16}$ requires 763.3177; ^{1}H NMR in Table 1; ^{13}C NMR in Table 3.

Rubrin G (7): $[\alpha]_D^{25}$ -22.9° (*c* =4.9, MeOH); UV λ_{max} (MeOH) 215 nm (ε 4,600); FTIR (KBr) 3510, 2990, 1730, 1640, 1265, 1190, 1020 cm^{-1}; HRFABMS observed m/z 787.3167 for MNa+ and m/z 765.3307 for MH+, $C_{38}H_{52}O_{16}Na$ requires 787.3153 and $C_{38}H_{53}O_{16}$ requires 765.3334; ^{1}H NMR in Table 1; ^{13}C NMR in Table 3.

14,15β-epoxyprieurianin (9): The dichloromethane extract of the root of a *Guarea* sp., collected and identified by professor Sidney McDaniel, was fractionated and **9** was purified with the same methods as described above. After repeated normal and reverse phase HPLC chromatographic steps 18 mg pure compound was obtained, which was identified on the basis of one- and two-dimensional NMR spectroscopy at 50°C in $CDCl_3$ and low and high resolution FAB mass spectroscopy. The spectral data are consistent with the data reported earlier.[23]

Prieurianin (8): Prieurianin was obtained by stirring **9** (3 mg) in DMSO at 75°C for 18 hours.[24] Compound **8** was then isolated by a reverse phase HPLC procedure as mentioned above. The proton NMR data recorded at 50°C in $CDCl_3$ was identical to reported data.[12]

Cell Adhesion Assay: JY cells, an EBV transformed B cell line which expresses LFA-1, were stained with a fluorescent, lipophilic membrane dye (PKH26, Zynaxis Cell Sciences, Malvern, PA). Labeled JY cells and potential inhibitors were added to the wells of 96 well microtiter plates which contained confluent monolayers of HeLa cells, a carcinoma cell line which expresses ICAM-1. 50 ng/mL phorbol myristate acetate (PMA) was then added to stimulate the JY cells to convert LFA-1 to its high avidity binding state.[3] The cultures were incubated for 45 minutes at 37°C. Nonadherent JY cells were washed off, the remaining cells were solubilized with 1% Triton X-100 (Sigma, St.Louis, MO) and fluorescence quantitated using an ICN/Flow Fluoroskan II microplate reader (LabSystems Instr., Raleigh, NC) with excitation wavelength of 544 nm and emission at 590 nm. Anti-CD18 monoclonal antibody was used as a positive control. Percent inhibition was calculated and statistical analysis was performed.

Cytotoxicity Assays: Tritiated thymidine uptake/proliferation assays were performed with PKH26 stained JY cells. The cells were incubated at 37°C with the test or control compounds and PMA for either 5 or 24 hours,

and pulsed with tritiated thymidine during the last 5 hours. In some assays the cells were treated with the compounds for 45 minutes (the length of the adhesion assay), washed extensively, then incubated for 24 hours and pulsed as described. The thymidine uptake was quantitated by scintillation counting.

The MTT assay was performed with PKH26 stained JY cells. The cells were incubated at 37°C with the test compounds and PMA for 48 hours. The tetrazolium dye, which is cleaved metabolically by active mitochondria, was added during the last 4 hours. The cells and the crystals formed by cleavage the dye were solubilized, and the color reaction quantitated on an ELISA plate reader (Bio-Tek Instruments Inc., Winooski, VT) at 570 nm with a reference wavelength of 630 nm. The 45 minutes long washout experiments described above were also carried out.

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