Calmodulin Inhibitors from *Leucophyllum ambiguum*[1]

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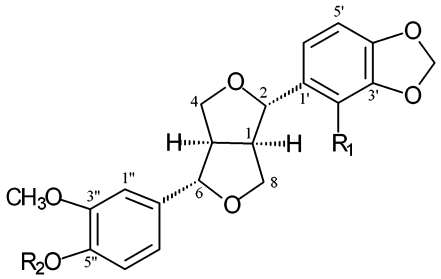
Activity-directed fractionation of a CH_2Cl_2−MeOH (1:1) extract of *Leucophyllum ambiguum* led to the isolation of two new lignans designated with the trivial names of 2′-methoxykobusin (**1**) and 2′-methoxy-4″-hydroxydemethoxykobusin (**2**). In addition, the known compounds kobusin (**3**), 2′,2″-dimethoxysesamin (**4**), *trans*-cinnamic acid, apigenin, and apigetrin were obtained. The identification of the novel analogues **1** and **2** was accomplished by spectral methods. The structure of **1** was unequivocally confirmed by X-ray analysis. Compounds **1**−**4** interacted with bovine-brain calmodulin and inhibited the activation of the calmodulin-dependent enzyme cAMP phosphodiesterase.

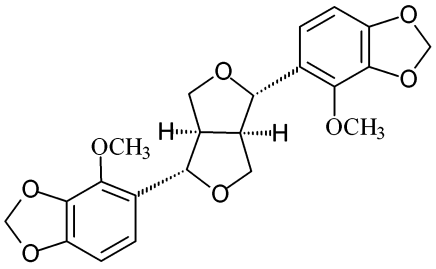
Leucophyllum ambiguum Humb. & Bonpl. (Scrophulariaceae) is a perennial medicinal herb, which grows scattered along the Sierra Madre Oriental in North Mexico, from Nuevo Leon to Hidalgo. It is commonly known as "cenizo de Monterrey", "chamizo", and "raguno". A decoction prepared from the whole plant is employed for treating hepatic ailments.[2] As part of our search for biologically active compounds from dryland plants of Latin America, we describe in this investigation the isolation and characterization of the major calmodulin (CaM) inhibitors from *L. ambiguum*. Calmodulin is a major Ca^{2+}-binding protein, highly active, implicated in a variety of cell functions through the activation of CaM-dependent enzymes, such as phosphodiesterase, protein phosphatase, and nitric oxide (NO) synthase. Calmodulin influences a number of important biological events. Accordingly, agents that inhibit the activity of CaM should have profound pharmacological effects. Indeed certain antipsychotic drugs, smooth muscle relaxants, α-adrenergic blocking agents, cytoprotective compounds, and neuropeptides inhibit CaM.[3]

Results and Discussion

A crude CH_2Cl_2−MeOH (1:1) extract prepared from the whole plant of *L. ambiguum* showed an inhibitory effect (IC_{50} = 250 μg/mL) on the activity of CaM-sensitive cyclic AMP phosphodiesterase (cAMP) and accordingly was subjected to bioassay-guided fractionation. The active extract was initially fractionated by column chromatography over silica gel to yield nine primary fractions (LA_1−LA_9). Extensive chromatography of the active fraction LA_4 (see Experimental Section) resulted in the isolation of the new lignans of 2′-methoxykobusin (**1**) and 2′-methoxy-4″-hydroxydemethoxykobusin (**2**). In addition, kobusin (**3**),[4] 2′,2″-dimethoxysesamin (**4**),[5] *trans*-cinnamic acid,[6] apigenin,[7] and apigetrin[8] were isolated from the active and inactive primary fractions. The spectral properties of the five known compounds, including IR and ^{1}H NMR and ^{13}C NMR data, were identical to those previously described in the literature.



	R_1	R_2
1	CH_3	OCH_3
2	H	OCH_3
3	CH_3	H



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Compound **1** was assigned a molecular formula of $C_{22}H_{24}O_7$ as inferred from its HREIMS and ^{13}C NMR spectral data. The NMR spectra (Table 1) showed signals corresponding to a furofuran type of lignan[8] and were very similar to those of kobusin (**3**).[4] However, the ^{1}H NMR data (Table 1) indicated that compound **1** has an additional methoxyl group and one less aromatic methine proton than kobusin (**3**). The NMR data together with data from a standard series of 2D NMR (NOESY, COSY, and HMBC) experiments indicated that one aromatic methine (δ_C 106.6) in **3** is replaced by a quaternary carbon (δ_C 140.6) in **1**. Another notable difference in the ^{1}H NMR spectrum of **1** when compared to that of **3** was the absence of the

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Table 1. ^{1}H and ^{13}C NMR Spectral Data for Compounds **1** and **2**[a,b]

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
1	3.00 m	54.5[c]	3.00 m	54.5[c]
2	5.03 d (4.0)	82.4	5.03 d (4.2)	82.3
4eq	4.20 dd (6.0, 9.0)	71.3[c]	4.20 dd (6.3, 9.0)	71.2
4ax	3.92 dd (3.5, 9.0)		3.92 dd (3.6, 9.0)	
5	3.0 m	54.0[c]	3.0 m	54.0[c]
6	4.68 d (6.0)	85.4	4.66 d (5.7)	85.5
8eq	4.33 dd (7.5, 9.0)	73.2	4.33 dd (7.2, 9.0)	73.1
8ax	4.00 dd (4.0, 9.0)		3.99 dd (4.5, 9.0)	
1′		127.2		127.2
2′		140.6		140.6
3′		136.3		136.2
4′		148.8		148.8
5′	6.51 d (8.0)	102.1	6.51 d (8.0)	102.1
6′	6.85 br d (8.0)	118.3	6.85 br d (8.0)	119.1
1″		133.6		133.0
2″	6.91 d (1.5)	109.2	6.90 d (1.8)	108.6
3″		149.2		146.6
4″		148.6		145.2
5″	6.83 d (8.5)	111.0	6.82 br d (8.1)	114.2
6″	6.88 dd (2.0, 8.5)	118.3	6.89 dd (1.5, 8.1)	118.3
CH_3O-2′	4.01 s	59.4	4.01 s	59.4
CH_3O-3″	3.89	55.9	3.90	56.0
CH_3O-4″	3.87 s	55.9		
O-CH_2-O	5.92 br s	101.0	5.92 br s	100.1

[a] ^{1}H NMR: ppm in $CDCl_3$, 500 MHz; *J* in Hz. [b] ^{13}C NMR: ppm in $CDCl_3$, 125 MHz. [c] Interchangeable values in same vertical column.

multiplet due to the aromatic protons (δ_H 6.8−6.9) of the piperonyl ring.[4] These signals were now replaced by an AB system at δ_H 6.51 (d, J = 8.0 Hz, H-5′) and 6.85 (brd, J = 8.0 Hz, H-6′). In addition, a new singlet appearing at δ_H 4.00 due to an extra methoxyl group was observed in the ^{1}H NMR spectrum; this methoxyl group signal showed a NOESY correlation with the resonance at δ_H 5.92 (OCH_2O), thus indicating attachment of the third methoxyl at C-2′. The diequatorial orientation of the aromatic rings was supported by the chemical shifts of C-1/C-1′, H-1/H-5, and H-2/H-6.[9−11] The *cis* arrangement of the fused tetrahydrofurofuran rings in all natural lignans restricts the configurations of the bridge atoms C-1 and C-5 to be either (*R*)/(*R*) or (*S*)/(*S*).[11] According to literature data, all (+)-sesamin and (+)-kobusin type of lignans have the absolute configuration *R* at the bridge carbons C-1 and C-5.[13,14] In the present work, the novel lignans showed positive optical rotations implying the C-5 (*R*)/C-1 (*R*) absolute configuration. Accordingly, the configuration at C-2 and C-6 could be assigned as *S*. These observations suggested that **1** was the C-2′ methoxy derivative of **3**.

The structure of **1** was unequivocally assigned by X-ray crystallography. The molecular structure is illustrated in Figure 1, showing the atom numbering used in the X-ray crystal structure determination. The crystal structure of compound **1** (Figure 1) clearly shows the *cis* fusion of the two five-membered rings of the furo[3,4-*c*]furan moiety with the essentially planar aromatic substituents displaying a *syn* orientation. The five-membered rings have an envelope conformation with the oxygen O-2, O-5 atoms as a flap. The van der Waals forces seem to be responsible for the crystal cohesion.

2′-Methoxy-4″-hydroxydemethoxykobusin (**2**) was assigned the elemental composition $C_{21}H_{22}O_7$, as determined by HREIMS, indicating that **2** had one methyl group less than **1**. The NMR characteristics of **2** were very similar to those of **1** (Table 1). Close inspection of the NMR data of **2** indicated the presence of a hydroxyl group (δ_H 3.66) instead of the methoxyl group (δ_H 3.87) found at C-4″ in **1**. In addition, the chemical shift value for the C-4″ signal was shifted diamagnetically in comparison to that of **1**. Treatment of compound **2** with an ethereal solution of CH_2N_2 yielded **1**. These observations led us to assign the structure of **2** as depicted.

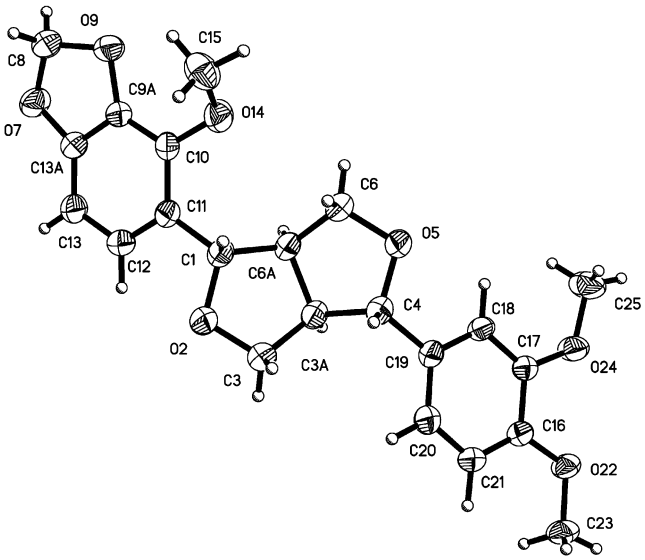

Figure 1. ORTEP diagram of compound **1** (thermal ellipsoids at 30% probability level).

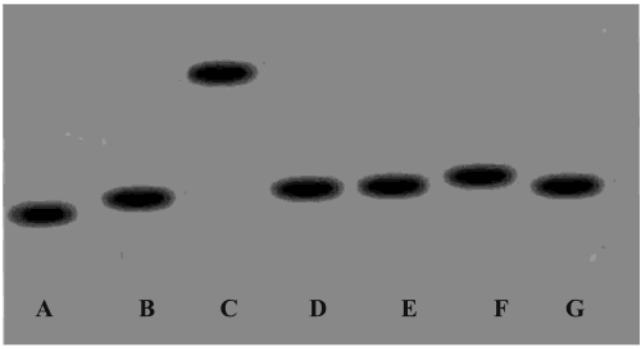

Figure 2. SDS-PAGE of bovine-brain CaM after treatment with compounds **1**−**4**. Electrophoresis of 2 μg samples of CaM in the presence of 1 mM $CaCl_2$. Pretreatment of the calmodulin samples, for 1.5 h at 30 °C in the presence of $CaCl_2$: (A) no additions; (B) DMSO; (C) 0.033 μg/mL of chlorpromazine in DMSO; (D) **1**; E, **2**; F, **3**; G, **4**; H, extract. In all cases 0.033 μg/mL of **1**−**4** in DMSO were applied.

Compounds **1**−**4** interacted with bovine brain-calmodulin as detected in a SDS-PAGE electrophoresis procedure[15] (Figure 2). CaM treated with the lignans had lower electrophoretic mobility than untreated CaM. The effect was comparable to that of chlorpromazine, a well-known CaM inhibitor.[3] In addition, the activation of the calmodulin-dependent enzyme cAMP was inhibited in the presence of **1**−**4** and CaM (Figure 3; IC_{50}= 14.4, 26.4, 7.4, and 5.6 μM, respectively). The inhibitory activity of compounds **3** and **4** was higher than that of chlorpromazine, which was used again as positive control (IC_{50} = 10.2 μM). The activity of the enzyme was determined according to the procedure of Sharma and Wang.[16] The activity of the phosphodiesterase was determined on the basis of the inorganic phosphate released. The phosphate produced in the assay was measured by the method of Sumner.[17] Thus, lignans **1**−**4** are CaM inhibitors and should have physiological effects of medicinal and agrochemical interest.[3,15]

Experimental Section

General Experimental Procedures. Melting point determinations were performed using a Fisher-Johns apparatus

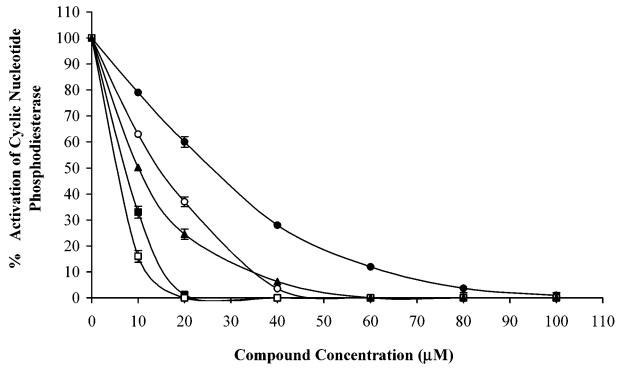

Figure 3. Effect of compounds **1** (○), **2** (●), **3** (■), **4** (□), and chlorpromazine (▲) on CaM-dependent cAMP. Enzyme activity was measured as a function of compound concentration at saturating concentrations of bovine-brain CaM (0.2 μg). The values are expressed as a percentage of maximum activity obtained with each compound. Each data point represents the mean of analysis of three independent biological samples. Vertical bars represent maximum standard deviations.

and are uncorrected. Optical rotations were recorded on a JASCO DIP 360 digital polarimeter. IR spectra were obtained using KBr disks on a Perkin-Elmer 599B spectrophotometer. UV spectra were recorded on a Shimadzu 160 UV spectrometer in MeOH solution. NMR spectra including COSY, NOESY, HMBC, and HMQC experiments were recorded in $CDCl_3$ on a Varian Unity Plus 500 spectrometer or on a Bruker DMX500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C) NMR, using tetramethylsilane (TMS) as an internal standard. MS were obtained on a JEOL JMS-AX505HA mass spectrometer. Column chromatography: Si gel 60 (70−230 mesh, Merck). TLC: Si gel 60 F254 (Merck).

Plant Material. The whole plant of *L. ambiguum* was collected in July 1995 in San Luis Potosí, Mexico. A voucher specimen (Bye 20021) has been deposited in the National Herbarium (MEXU), Instituto de Biología, UNAM.

Extraction and Isolation. The air-dried plant material (2.8 kg) was ground into a powder and extracted by maceration with CH_2Cl_2−MeOH (1:1) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 752 g of residue, which was subjected to column chromatography over silica gel (700 g) and eluted with a gradient of hexane−CH_2Cl_2 (8:2 → 0:10) and CH_2Cl_2−MeOH (8:2 → 0:10). Fractions of 1 L each were collected and pooled based on TLC profiles to yield nine major fractions. Fraction LA_4 was active (IC_{50} = 250 μg/mL) according to the cAMP enzymatic assay.[16] Column chromatography of active fraction LA_4 (60 g) on Si gel (900 g), eluting with a concentration gradient of hexane−CH_2Cl_2−MeOH starting with hexane, afforded nine secondary fractions (10−18). According to the enzymatic assay, the activity was concentrated in secondary fractions 12 and 14 (IC_{50} = 200 and 2015 μg/mL, respectively). Active fraction 12 [10.5 g, eluted with hexane−CH_2Cl_2 (7:3)] was further resolved on a Si gel column (200 g), eluting with a concentration gradient of hexane−CH_2Cl_2−MeOH, starting with hexane, to afford six tertiary fractions (12-A−12-F). From fraction 12-D, 60 mg of compound **4** was obtained in crystalline form. Further column chromatography of active fraction 14 (12.5 g) on Si gel (250 g), eluting with a concentration gradient of hexane−CH_2Cl_2−MeOH, starting with hexane, afforded six further tertiary fractions (14-A−14-F), of which fraction 14-C was active. Preparative TLC of fraction 14-C using CH_2Cl_2−MeOH (98:2) as eluant, runing three developments, yielded **1** (54 mg), **2** (30 mg), and **3** (40 mg). From primary fraction LA_3, eluted with hexane−CH_2Cl_2 (1:1), crystallized *trans*-cinamic acid (80 mg). From fractions LA_5 and LA_7 apigetrin (88 mg) and apigenin (1.5 g), respectively, spontaneously crystallized.

2′-Methoxykobusin (1): colorless crystalline needles, mp 90−92 °C; $[\alpha]_D$ +104° (*c* 1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 229 (4.1), 278 (4.0) nm; IR ν_{max} ($CHCl_3$) 2936, 2871, 1629, 1607, 1515, 1468, 1258 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; EIMS *m*/*z* 400 [M$^+$ (94)], 219 (17), 233 (9), 191 (80), 179 (44), 165 (100), 151 (33); HRMS *m*/*z* 400.4115 (calcd for $C_{22}H_{24}O_7$, 400.4120).

2′-Methoxy-4″-hydroxydemethoxykobusin (2): colorless oil; $[\alpha]_D$ +74° (*c* 1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 240 (4.2), 279 (4.0) nm; IR ν_{max} ($CHCl_3$) 3370, 2935, 2872, 1629, 1609, 1515, 1467, 1258 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; EIMS *m*/*z* 386 [M$^+$ (60)], 233 (12), 205 (28), 191 (82), 179 (65), 165 (68), 163 (28), 151 (100); HRMS *m*/*z* 386.3857 (calcd for $C_{21}H_{22}O_7$, 386.3860).

X-ray Crystal Structure Determination of Compounds 1.[18] Appropriate crystals were mounted, in air, on glass fibers. Accurate cell parameters were determined by refinement from the setting of 25 reflections and diffraction intensities measured at 293 K using an $\omega-\theta$ scan method on a Siemens P4/PC diffractometer equipped with graphite-monochromated Cu Kα radiation (λ 1.54178 Å). Crystal size 0.60 × 0.40 × 0.20 mm; colorless prisms; orthorhombic system; space group $P2_12_12_1$ with unit cell parameters (at 25 °C) a = 7.2162(2) Å, b = 8.9211(2) Å, c = 30.918(1) Å, V = 1990.4(1) Å^3, Z = 4 $F(000)$ = 848, D_{calc} = 1.336 g cm^{-3}, μ/mm^{-1} = 0.861. The intensities of three standard reflections, recorded every 100 collected reflections, showed no changes. All data sets were corrected for Lorentz−polarization effects, but no absorption corrections were applied. The structure of each compound was determined by direct methods (SIR92)[19] and refined by full-matrix least-squares methods using SHELXL97.[20] Hydrogen atoms attached to C atoms were set to ride on the parent C atoms. An isotropic temperature factor 1.2 times the U_{eq} of the parent atom was used. The non-hydrogen atoms were refined with anisotropic thermal parameters.

Evaluation of the Interaction of Lignans 1−4 with Bovine Brain Calmodulin. The interaction of lignans **1**−**4** with bovine-brain CaM (Sigma) was performed using a denaturing homogeneous electrophoresis (SDS-PAGE) procedure. SDS-PAGE was carried out according to a previously described procedure[15] using a 15% polyacrylamide gel. The interaction of the compounds with CaM was evaluated by observing the difference in electrophoretic mobility in the presence of Ca^{2+}. Each electrophoretic run was done in triplicate, and chlorpromazine was used as positive control. The experimental conditions are described briefly in the legends of Figure 2.

Cyclic Nucleotide Phosphodiesterase Assay. A cyclic nucleotide phosphodiesterase and bovine-brain calmodulin assay was performed by a modification of the method described by Sharma and Wang.[16] Bovine brain CaM (0.2 μg) as enzyme activator was incubated with 0.015 units of calmodulin-deficient-calmodulin-dependent cAMP from bovine brain (Sigma) for 3 min in 800 μL of assay solution containing 0.3 units of 5′-nucleotidase, 45 mM Tris-HCl, 5.6 mM magnesium acetate, 45 mM imidazole, and 2.5 mM calcium chloride, pH 7.0. Compounds were then added to the assay medium at 10, 20, 40, 60, 80, and 100 μM in DMSO, and the samples were incubated for 30 min. Then, 100 μL of 10.8 mM cAMP, pH 7.0, was added to start the assay. After 30 min, the assay was stopped by the addition of 100 μL of 55% trichloroacetic acid solution. All of the above steps were carried out at 30 °C. The phosphodiesterase reaction was coupled to the 5′-nucleotidase (*Crotalus atrox* venom from Sigma) reaction, and the amount of inorganic phosphate released represented the activity of the phosphodiesterase. The phosphate produced in the assay was measured by the method of Sumner.[17] The wavelength used for the phosphate assay was 660 nm. Once more, chlorpromazine was used as a positive control.

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

References and Notes

(1) This work represents part of the Ph.D. thesis of S. Rojas.
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