Chemical Constituents from *Lippia sidoides* and Cytotoxic Activity

Sônia Maria O. Costa,† Telma Leda G. Lemos,*,‡ Otília Deusdênial L. Pessoa,‡ Claudia Pessoa,‡ Raquel C. Montenegro,‡ and Raimundo Braz-Filho§

Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará, CP 12200 60451-970 Fortaleza, Ceará, Brazil, Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, CP 3157 60430-270 Fortaleza, Ceará, Brazil, and Setor de Química de Produtos Naturais-LCQUI-CCT, Universidade Estadual do Norte Fluminense, 28015-620 Campos, Rio de Janeiro, Brazil

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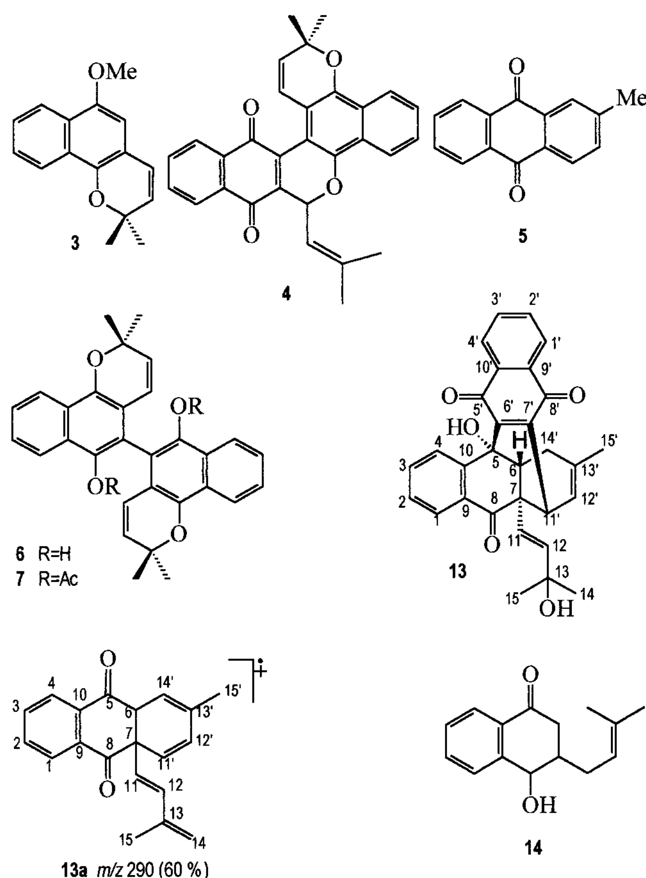
Eleven known compounds and a new prenylated naphthoquinone, lippsidoquinone (**13**), were isolated from ethanol extracts of *Lippia sidoides*. Their structures were established by a combination of 1D and 2D NMR, IR, and EIMS spectral data analysis. The cytotoxic properties of compounds **3**−**13** were evaluated against HL60, SW1573, and CEM cell lines. Only tectol (**6**) and lippsidoquinone (**13**) exhibited significant activity against human leukemia cell lines HL60 and CEM.

The genus *Lippia* (Verbenaceae) comprises a great number of medicinal and economically important species, including several that are used in traditional Brazilian medicine.[1,2] *Lippia sidoides* Cham., popularly known as "alecrim pimenta", is an odoriferous plant that grows wild in the northeastern region of Brazil. In the traditional medicine the aerial parts of this species are used as an anti-infective agent, particularly as an antiseptic. The efficacy of this traditionally used plant as an antiseptic was demonstrated by the leaves' essential oil, which contains thymol and carvacrol as major constituents, and showed bactericidal and fungicidal activities.[3,4] The hydrolate shows molluscicidal activity against *Biomphalaria glabrata* and larvicidal activity against *Aedes aegypti*.[5]

As a response to the great interest generated by this plant and its widespread medicinal use, it was introduced in the state government phytotherapy programs in many counties of several northeastern states of Brazil, and it is now cultivated in several medicinal gardens to produce natural antiseptic pharmaceutical preparations.[5]

There have been numerous chemical studies of *Lippia* spp. which have mostly focused on essential oils. As a result of our search for novel bioactive natural products, in this paper we report the results of an investigation of *L. sidoides.* In addition to the known compounds [3-*O*-acetyloleanolic acid (**1**),[6] methyl 3,4-dihydroxybenzoate (**2**),[7] lapachenol (**3**),[8] tecomaquinone (**4**),[9] tectoquinone (**5**),[10] tectol (**6**),[9] acetylated tectol (**7**),[9] quercetin (**8**),[11] luteolin (**9**),[11] glucoluteolin (**10**),[11] taxifolin (**11**),[11] and isolariciresinol (**12**)[12]], one novel prenylated naphthoquinone, lippsidoquinone (**13**), was isolated. The structures of these natural products were established by a combination of 1D and 2D NMR, IR, and EIMS spectral data analysis and comparison with values described in the literature. The cytotoxic effects of some of the compounds against three cancer cell lines (HL60, SW1573, and CEM) were evaluated. The nomenclature and numbering of the new natural product **13** follow the two prenylated naphthoquinone moieties as biogenetic precursors.The known compounds (**1**−**12**) were identified on the basis of spectral analysis involving comparison with literature data. In a previous paper we reported the results of extensive application of 1D and 2D NMR techniques to establish the complete ^{1}H and ^{13}C resonance assignments of tecomaquinone (**4**), tectol (**6**), and the acetyl derivative of tectol (**7**).[9]

Chart 1



Lippisidoquinone (**13**) was obtained as a yellow material, mp 80−82 °C, $[\alpha]_D^{25}$ −28° (*c* 0.0005, $CHCl_3$). The EIMS showed the $[M]^+$ peak at *m*/*z* 466 (21%), compatible with the molecular formula $C_{30}H_{26}O_5$ (18 degrees of unsaturation) deduced in combination with ^{1}H NMR, ^{13}C NMR, and DEPT analysis. The ^{13}C NMR spectrum revealed 30 signals, sorted through DEPT experiments into 13 quaternary carbon atoms (10 sp^2, including three carbonyl groups at δ_C 200.7, 187.1, and 183.2; and three sp^3, including two

* To whom correspondence should be addressed. Tel: 55-85-2889954. Fax: 55-85-2889782. E-mail: tlemos@dqoi.ufc.br.
† Universidade Estadual Vale do Acaraú (UVA).
‡ Universidade Federal do Ceará (UFCE).
§ Universidade Estadual do Norte Fluminense (UENF).

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Table 1. ^{1}H, ^{13}C, HMQC, and HMBC NMR Spectral Data for Compound **13** ($CDCl_3$)[a]

	^{1}H−^{13}C HMQC, $^{1}J_{CH}$		^{1}H−^{13}C HMBC, $^{n}J_{CH}$		^{1}H−^{1}H NOESY
	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	
C					
5	75.4		H-6, HO-5	H-4, 2H-14′	
7	51.4		H-6, H-11′	H-12, H-14′β	
8	200.7			H-1	
9	130.8			H-2, H-4	
10	149.4			H-1, H-3, HO-5, H-6	
13	70.9		H-12, 3H-14/3H-15	H-11	
5′	187.4			H-4′	
6′	141.6			H-6, HO-5, H-11′	
7′	147.0		H-11′		
8′	183.2			H-1′	
9′	131.7			H-4′	
10′	131.8			H-1′, H-3′	
13′	139.1		2H-14′, 3H-15′	H-6, H-11′	
CH					
1	127.9	7.74 (dd, 8.0, 1.2)			
2	127.9	7.32 (br, t, 8.0)	H-3	H-4	
3	133.5	7.59 (dt, 8.0, 1.2)	H-2, H-4	H-1	
4	123.7	7.85 (br d, 8.0)	H-3	H-2	HO-5
6	43.7	2.92 (dd, 1.7, 8.2)	2H-14′	HO-5, H-11, H-11′	H-12, H-11, H-14′β
11	127.7	5.93 (d, 16.1)	H-12		
12	138.2	5.70 (d, 16.1)	H-11	3H-14, 3H-15	
1′	126.4	7.98 (dd, 2.2, 7.3)		H-3′	
2′	134.3	7.77−7.60 (m)		H-4′	
3′	133.9	7.77−7.60 (m)		H-1′	
4′	126.4	7.92 (dd, 2.2, 7.0)		H-2′	
11′	38.8	3.96 (dd, 1.7, 7.2)	H-12′	H-6, H-11	H-11, H-12′
12′	120.6	5.70 (d, 7.2)	H-11′	2H-14′, 3H-15′	H-11′, 3H-15′, H-11
CH_2					
14′	28.9	2.98 (br d, 20.0), H-14′α	H-6	3H-15′	HO-5
		2.17 (dd, 20.0, 8.2), H-14′β			H-6
CH_3					
14	29.7	1.25 (s)			H-12
15	29.5	1.28 (s)			H-11
15′	22.4	1.58 (s)			
HO-5		6.45 (s)			H-4, H-14′α

[a] Chemical shifts are indicated in δ (ppm) and coupling constants (J in Hz) are in parentheses. ^{1}H NMR: 500 MHz. ^{13}C NMR: 75 MHz.

oxygenated at δ_C 75.4 and 70.9), 13 methines (11 sp^2 and two sp^3 at δ_C 43.7 and 38.8), one methylene, and three methyl groups (Table 1), which allowed the determination of the expanded molecular formula $(C)_8(C{=}O)_3(C{-}OH)_2(CH)_{13}(CH_2)(CH_3)_3 = C_{30}H_{26}O_5$. The presence of two hydroxyl groups was suggested by peaks at m/z 448 (100%, M − H_2O) and 430 (15%, m/z 448 − H_2O) observed in the mass spectrum, which were attributed to sequential loss of two H_2O molecules. The IR spectrum revealed absorptions corresponding to hydroxyl groups (ν_{max} 3446, 3222 cm^{-1}), a conjugated carbonyl function (ν_{max} 1674 cm^{-1}), and an aromatic ring (ν_{max} 1597 and 1458 cm^{-1}). The presence of an anthracenone moiety **I** was recognized through analysis of the homonuclear ^{1}H−^{1}H COSY and ^{13}C (HBBD and DEPT), heteronuclear HMQC, HMBC, and mass spectra by the following data (Table 1). (a) The ^{1}H NMR spectra showed signals of an AA′BB′ system corresponding to an *ortho*-disubstituted aromatic ring [δ_H 7.74 (dd, J = 8.0 and 1.2 Hz, H-1), 7.32 (br t, J = 8.0 Hz, H-2), 7.59 (dt, J = 8.0 and 1.2 Hz, H-3), and 7.85 (br d, J = 8.0 Hz, H-4)], one ABC system attributed to H-6 [δ_H 2.92 (dd, J = 8.2 and 1.7 Hz)] and 2H-14′ [δ_H 2.98 (br d, J = 20.0 Hz, geminal coupling, H-14′α) and 2.17 (dd, J = 20.0 and 8.2 Hz, geminal coupling and vicinal spin−spin interaction with H-6, H-14′β)], two vicinal hydrogens H-11′ [δ_H 3.96 (dd, J = 7.2 and 1.7 Hz)], and the olefinic H-12′ [δ_H 5.70 (d, J = 7.2 Hz)] and AB-type signals at δ_H 5.93 (H-11, d) and 5.70 (H-12, d) with a large coupling constant (J = 16.1 Hz), consistent with two hydrogens of an (E)-disubstituted double bond located between two quaternary carbon atoms (C-7 and C-13), along with two singlets corresponding to three methyl groups at δ_H 1.58 (3H-15′, bound to sp^2 carbon C-13′), 1.25 (3H-14), and 1.28 (3H-15). (b) The HMBC spectrum revealed cross-peaks corresponding to heteronuclear long-range couplings ($^{2}J_{CH}$ and $^{3}J_{CH}$) between carbon and hydrogen atoms as follows: C-5 (δ_C 75.4) with H-6 (δ_H 2.92, $^{2}J_{CH}$), HO-5 (δ_H 6.45, $^{2}J_{CH}$), H-4 (δ_H 7.85, $^{3}J_{CH}$), and 2H-14′ (δ_H 2.98 and 2.17, $^{3}J_{CH}$); C-7 (δ_C 51.4) with H-6 (δ_H 2.92, $^{2}J_{CH}$), H-11′ (δ_H 3.96, $^{2}J_{CH}$), H-12 (δ_H 5.70, $^{3}J_{CH}$), and H-14′α (δ_H 2.98, $^{3}J_{CH}$); C-8 (δ_C 200.7) with H-1 (δ_H 7.74, $^{3}J_{CH}$); C-13 (δ_C 70.9) with H-12 (δ_H 5.70, $^{2}J_{CH}$), 3H-14 (δ_H 1.25, $^{2}J_{CH}$), 3H-15 (δ_H 1.28, $^{2}J_{CH}$), and H-11 (δ_H 5.93, $^{3}J_{CH}$), and C-13′ (δ_C 139.1) with 2H-14′ (δ_H 2.98 and 2.17, $^{2}J_{CH}$), 3H-15′ (δ_H 1.58, $^{2}J_{CH}$), H-6 (δ_H 2.92, $^{3}J_{CH}$), and H-11′ (δ_H 3.96, $^{3}J_{CH}$), along with others summarized in Table 1. (c) The peak at m/z 290 (60%) was observed in the mass spectrum, which was attributed to fragment **13a**. The remaining signals observed in the 1D ^{1}H and ^{13}C NMR and cross-peaks revealed by 2D shift-correlated spectra were analogously used to postulate the presence of a 1,4-naphthoquinone moiety containing an unsubstituted aromatic ring (**II**), as summarized in Table 1. The junction of the two **I** and **II** (Figure 1) moieties by two bonds between C-5 and C-6′ and C-7′ and CH-11′ was clearly revealed by the HMBC spectrum through heteronuclear long-range couplings of the carbon and hydrogen atoms C-6′ (δ_C 141.6) with H-6 (δ_H 2.92, $^{3}J_{CH}$), HO-5 (δ_H 6.45, $^{3}J_{CH}$), and H-11′ (δ_H 3.96, $^{3}J_{CH}$) and C-7′ (δ_C 147.1) with H-11′ (δ_H 3.96, $^{2}J_{CH}$).

The NOESY spectrum of **13** furnished information about spatial proximity (dipolar coupling) between hydrogen atoms (Table 1), which allowed the relative configuration to be established as shown in Figure 1. $^{1}J_{HH}$ values and

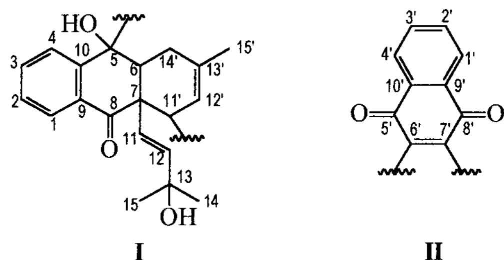

Figure 1. Partial structures of lippisodoquinone **13**.

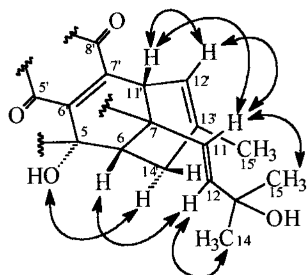

Figure 2. Important NOESY correlations of **13**.

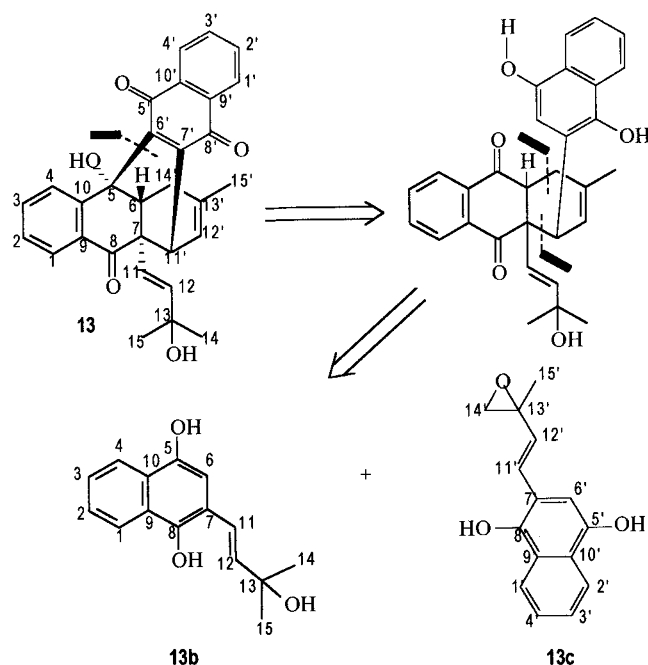

Figure 3. Retroanalysis and a possible biosynthetic pathway of lippisidoquinone (**13**) from prenylated naphthaquinone.

the cross-peaks observed in the $^1H-^1H$ NOESY spectrum of **13** (Table 1 and Figure 1) were used to assign the hydrogens H-14′α and H-14′β (δ_H 2.17, dd, $J = 20.0$ and 8.2 Hz), since only the later revealed NOE with H-6β (δ_H 2.92, dd, $J = 1.7$ and 8.2 Hz).

Thus, the structure of the new dimer **13** containing two prenylated naphthoquinoid moieties (1 to 15 and 1′ to 15′), named lippisidoquinone, has been characterized. This is the second report of the isolation of a naphthoquinone from *L. sidoides,* although flavonoids[13] and naphthoquinoids[14] are common metabolites in plants of this genus.

Structural examination of the dimer **13** in view of using biosynthetic arguments and application of a retroanalysis led us to suggest that the biogenetic route proceeds by enzymatic cyclization of moieties **13b** and **13c** (Figure 3). The isolation of **14** described in previous phytochemical investigation of this species[14] is in accordance with suggested biosynthetic precursors.

Table 2. Cytotoxicity of Compounds **3**−**13** Isolated from *Lippia sidoides*[a]

compound	cell lines IC_{50} (μg/mL) HL60	SW1573	CEM
6	1.79 ± 0.60	*b*	0.78 ± 0.8
13	2.38 ± 0.13	*b*	2.58 ± 0.14
3−**5**, **7**−**12**	>10	>10	>10

[a] For significant activity of pure compounds, an IC_{50} value of ≤4.0 μg/mL is required.[15] [b] Not determined

The cytotoxic effects of the constituents **3**−**13** were evaluated against human promyelocytic leukemia (HL60), small lung cell carcinoma (SW1573), and human acute lymphoblastic leukemia (CEM) cells (Table 2). Of the 11 tested compounds, only tectol (**6**) and lippsidoquinone (**13**) exhibited consistent in vitro activity against HL60 and CEM cells.

Experimental Section

General Experimental Procedures. Melting points were determined on a Mettler (model FP-52) melting point apparatus. Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR spectra were recorded on a FT-IR Spectrum 1000 spectrometer, using KBr pellets. EIMS data were obtained at 70 eV on a VG-auto spec mass spectrometer. The ^1H and ^{13}C NMR were obtained on either a Bruker DPX 300 FT spectrometer operating at 300 and 75 MHz, respectively, or a DRX 500 spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C. For the ^{13}C NMR data, the number of attached protons was determined by the distortionless enhancement through a polarization transfer experiment. Two-dimensional NMR data were obtained using the standard pulse sequences of either the DRX 300 for homonuclear correlated spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY) or the DPX 500 heteronuclear multiple quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond correlation spectroscopy (HMBC). Column chromatography was carried out on Si gel (230−400 mesh, Aldrich) and over Sephadex LH-20. TLC analyses were performed on a precoated Si gel W/UV indicator (Aldrich) and visualized by exposure to short-wavelength UV (λ_{max} = 254) and/or by spraying with a mixture of vanillin−perchoric acid−EtOH followed by heating.

Plant Material. *Lippia sidoides* was collected at ESAM, Mossoró, Rio Grande do Norte, Brazil, in August 1997 and identified by Professor Afrânio Gomes Fernandes. A voucher specimen (No. 25149) is deposited in the Herbarium Prisco Bezerra of the Departamento de Biologia of Universidade Federal do Ceará.

Extraction and Isolation. Dried, ground heartwood (3.3 kg), stems (3.0 kg), leaves (500 g), and bark (850 g) were extracted with EtOH at room temperature, and the extracts taken to dryness under reduced pressure. The EtOH extract of trunk (31 g), stem (51 g), and leaves (25 g) was submitted to the same chromatography procedure. Each extract was chromatographed over Si gel and eluted with hexane, $CHCl_3$, EtOAc, and MeOH. The EtOH extract of bark (34 g) was diluted with MeOH/H_2O (1:1) and then extracted with $CHCl_3$, EtOAc, and *n*-BuOH. The hexane fraction obtained from the heartwood led to the isolation of quinone **3** (25.0 mg), while the $CHCl_3$ fraction yielded five quinines, **4** (15.0 mg), **5** (25.0 mg), **6** (249 mg), and **7** (15.0 mg), including the new compound **13** (10.8 mg). The hexane fraction from the stems afforded compounds **1** (15.2 mg) and **2** (12.0 mg), while the $CHCl_3$ fraction yielded by chromatogaphy on Sephadex LH-20 three flavonoids, **8** (168.0 mg), **9** (19.0 mg), and **11** (40.0 mg). Both EtOAc and MeOH fractions from the leaves afforded the flavonoid **10** (490.0 mg), and finally the substance **12** (20.8 mg) was isolated from the bark $CHCl_3$ layer.

Lippisidoquinone 1: yellow film; mp 80−82 °C; $[\alpha]^{25}_D$ −28° (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3446, 3222, 2924, 2858,

1674, 1597, 1458, 1290, 756 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; EIMS (70 eV) m/z 466 ($[M]^+$, 21), 448 (100), 390 (70), 375 (27), 347 (13), 290 (59).

Tumor Cell Growth Inhibition Assays. A microassay for cytotoxicity was performed using a MTT assay, described by Mosman.[15] Briefly, 0.2×10^5 cell/100 μL (adherent cells) and 0.5×10^6 cells/100 μL (suspension cells) were seeded in 96-well microplates (Nunck) and preincubated for 24 h in order to allow cell attachment. For the suspension cells this is not necessary. After plating the cells, fresh medium (100 μL) containing various concentrations (0.39−25 μg/mL) of test drug was added to the cultures. The cells were incubated with each drug for 3 days. Cell survival was evaluated by adding MTT tetrazolium salt solution with fresh medium (no serum). After 3 h incubation at 37 °C, 150 μL of DMSO was added to dissolve the precipitate of reduced MTT. Microplates were then shaken for 15 min, and the absorbance was determined at 550 nm with a multiwell scanning spectrophotometer. HL60 (human promyelocytic leukemia) SW1573 (small lung cell carcinoma), and CEM (human acute lymphoblastic leukemia) were maintained in RPMI1640 (Gibco BRL), containing 10% fetal bovine serum (FBS; Gibco BRL), 1% penicillin, and streptomycin solution (Life Technologies), and incubated at 37 °C in a atmosphere of 5% CO_2.

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