Cytotoxic Naphthoquinones from Roots of *Lippia microphylla*

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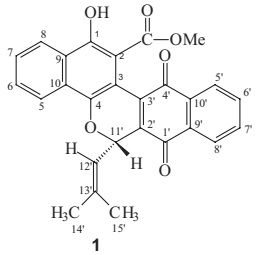
A new prenylated naphthoquinone dimer named microphyllaquinone (**1**), a mixture of 6-methoxy- and 7-methoxy-naphtho[2,3-*b*]-furan-4,9-quinones (**2a**/**2b**) and tecomaquinone I (**3**), were isolated from roots of *Lippia microphylla*. The structures were elucidated by spectroscopic methods, including detailed 1D and 2D (COSY, NOESY, HMQC, HMBC) NMR data. Unpublished ^{13}C NMR data of **2a** and **2b** are reported. The *in vitro* cytotoxic activity of the isolated compounds was tested against five types of tumor cells.

Key words: Lippia microphylla, Naphthoquinones, Cytotoxicity Activity

Introduction

The genus *Lippia* (Verbenaceae) constituted of herbs, shrubs and small trees, includes approximately 200 species, distributed mainly in South and Central America and Tropical Africa territories. Most of them are traditionally utilized in folk medicine for the treatment of several diseases (Lemos *et al*., 1990; Valentin *et al*., 1995; Forestiere *et al*. 1996; Pascual *et al*., 2001). As a continuation of our program on the evaluation of plants used in Brazilian traditional medicine, we have investigated *L. sidoides* (Lemos *et al*., 1999; Costa *et al*., 2001) and volatile components of the aerial parts of *L. microphylla* (Lemos *et al*., 1992). In the present paper we report the isolation and characterization of a new prenylated naphthoquinone dimer (**1**), a mixture of isomers 6-methoxy- and 7-methoxy-naphtho[2,3-*b*]-furan-4,9-quinones (**2a** and **2b**, respectively) and tecomaquinone I (**3**), from the roots of *L. microphylla*, popularly known as "alecrim-de-tabuleiro". Since **2a**/**2b** had been previously isolated from *Lantana camara*, only the ^{1}H NMR data were provided (Abeygunawardena *et al*., 1991). Here the complete ^{1}H and ^{13}C NMR spectral data are reported for the first time.



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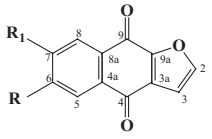

2a **R** = OMe, **R$_1$** = H
2b **R** = H, **R$_1$** = OMe

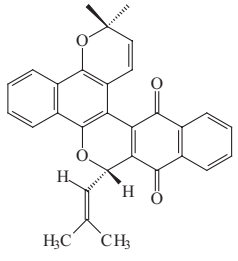

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Fig. 1. Structures of microphyllaquinone (**1**); 6-methoxy/7-methoxy-naphtho[2,3-*b*]-furan-4,9-quinones, (**2a**/**2b**); tecomaquinone I (**3**).

Results and Discussion

After chromatography over silica gel investigation of the ethanol extract from the roots of *L.*

microphylla, led to the isolation of four naphthoquinones. Three of them were the known furanonaphthoquinones **2a**/**2b** and tecomaquinone I (**3**). Compound **1**, was isolated as red crystals, m.p. 197–200° C. Its IR spectrum showed absorption bands for hydroxyl (3403 cm^{-1}) and conjugated carbonyl groups (1665 and 1640 cm^{-1}). The molecular ion peak with *m/z* 440 daltons, in conjunction with the ^{1}H and ^{13}C NMR spectra (Table I) showing 27 signals, allowed the determination of its molecular formula as $C_{27}H_{20}O_6$. The DEPT 135° spectrum revealed three methyl groups, one as methoxy (δ 52.17), one methine, nine monohydrogenated sp^2 carbons, and by comparison with the ^{13}C NMR spectrum fourteen non-hydrogenated sp^2 carbons, including three carbonyls (δ 184.8, 182.5 and 171.2) and two oxygenated (δ 154.8 and 147.8). ^{1}H and ^{13}C NMR data comparison of **1** and tecomaquinone I (**3**) (Lemos *et al.*, 1999), revealed a striking similarity, except for the missing cyclic isoprene moiety for **1** and in addition, the presence of a methyl ester for **1**, Fig. 1. Even though a more deshielded absorption for C-3 (conjugated with the methyl ester) was expected, initially the complete ^{13}C data assignment was a difficult task because the absence of any hydrogen long-range correlation with C-3 or C-2 (this one is *ortho* to the hydroxy, but is conjugated to the quinone C-1′) did not allow an unambiguous assignment of those carbons. Fortunately, the HMBC data obtained in dry DMSO-d_6 revealed long-range correlations of the hydroxy hydrogen (δ 10.88) with C-1 (δ 154.8), C-9 (δ 127.7) and C-2 (δ 104.4). In addition, the distinction between C-2′(δ133.4) and C-3′ (δ 137.4), both conjugated to the quinone carbonyls, was done by the correlations of the carbinolic like, and doubly allylic H-11′(δ 6.51) with C-4 (δ 147.8), C-2′ (δ 133.4), C-3′ (δ 137.4) and C-1′ (δ 182.5), as well as by the correlations of the vinyl hydrogen H-12′ (δ 5.6) with C-14′(δ 26.4) and C-15′(δ 19.3). Finally, the relative stereochemistry suggested for **1**, similarly to tecomaquinone I (**3**), was confirmed by the NOESY analysis. Thus the structure of **1**, a new prenylated naphthoquinone

Table I. ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) data for **1**.

C	HMQC		HMBC	
	δ_C	δ_H, *J* (Hz)	$^{2}J_{CH}$	$^{3}J_{CH}$
1	154.8			H-8
2	104.4			
3	109.8			
4	147.8			H-11′, H-5
5	123.5	8.27 d (*J* = 9,5)		H-7
6	129.8	7.69 m		H-8
7	129.0	7.69 m		H-5
8	124.7	8.44 d (*J* = 9.5)		H-6
9	127.7			H-5, H-7
10	128.6			H-6, H-8
11	171.2			MeO-11′
1′	182.5			H-11′, H-8′
2′	133.4			H-11′
3′	137.4			H-11′
4′	184.8			H-5′
5′	126.8	8.11 d (*J* = 8.7)		H-7′
6′	134.0	7.78 m		H-8′
7′	134.1	7.78 m		H-5′
8′	126.5	8.16 d (*J* = 7.5)		H-6′
9′	132.6			
10′	133.5			
11′	68.3	6.51 d (*J* = 9.3)	H-12′	
12′	117.9	5.60 d (*J* = 9.3)	H-11′	3H-14′, 3H-15′
13′	142.8		3H-14′, 3H-15′	H-11′
14′	26.4	1.68 s		H-12′, 3H-15′
15′	19.3	2.10 s		H-12′, 3H-14′
MeO-11	52.2	3.72 s		
HO-1		11.28 s		

dimer named microphyllaquinone was established.

Compounds **2a**/**2b**, were obtained as orange crystals, m.p. 162–165° C. Its IR spectrum showed strong absorption bands consistent with the presence of a conjugated carbonyl (1675 cm^{-1}) and aromatic ring (1591, 1580 and 1480 cm^{-1}). The molecular formula $C_{13}H_8O_4$ was deduced from its ^1H and ^{13}C NMR spectra. All proton and carbon signals appeared in pairs (except to the OMe group) with light chemical shift differences. This clearly suggested that **2** should be a mixture of isomers. Integration of the ^1H NMR signals allowed to establish a 2:1 relative ratio of **2a**/**2b**. Full assignments of ^1H and ^{13}C NMR spectra of the major isomer were assisted by COSY, HMQC and HMBC experiments and are consistent with the structure of 6-methoxynaphtho[2,3-*b*]-furan-4,9-quinone (**2a**). Therefore, the minor compound must be 7-methoxy-naphtho[2,3-*b*]-furan-4,9-quinone (**2b**). Direct comparison of the ^1H NMR data of these compounds with the literature values showed to be identical (Abeygunawardena *et al*., 1991).

Compounds **1** and **2a**/**2b** were tested against five cultured tumor cell lines *in vitro*. These substances display cytotoxicity values ranging from 0.8 to 3.0 μg/ml when tested against HL-60, CEM, HCT8 and MCF-7 human cancer cell lines obtained from the American Type Culture Collection (ATCC) and B16 (murine melanome) from Universidade Federal do Rio de Janeiro. Compound **1** was slightly less active than the furanonaphthoquinones **2a**/**2b** which showed higher cytotoxicity against resistant cell lines B16 and HCT-8. The IC_{50} values of each component for the proliferation of five tumor cells are summarized in Table II. The cytotoxic activity of tecomaquinone I (**3**) was previously reported (Costa *et al*., 2001).

Experimental

General procedures

Melting points were measured on a Mettler Toledo FP90 apparatus and were uncorrected. The optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1000 FT-IR spectrometer. Mass spectral data were acquired on a VG Auto Spec spectrometer. The NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 on a Bruker Avance DRX-500 (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometer. Proton and carbon chemical shifts were referenced relative to the corresponding solvents signals, δ_H 7.24 and δ_C 77.0 ($CHCl_3$) and δ_H 2.49 and δ_C 39.5 (DMSO-d_6). Si gel 60 (Merck, 70–230 mesh) was used for column chromatography. Precoated Si gel plates (Merck, kieselgel 60 F_{254}, 0.20 mm) were used for analytical TLC. Chromatographic fractions were visualized by TLC by spraying with vanillin-perchloric acid-EtOH followed by heating.

Plant material

The roots of *L. microphylla* were collected in Varzea Alegre, Ceará State – Brazil, in April, 1999. The identity of plant was verified by Dr. Edson P. Nunes, and a voucher specimen (No. 2287) was deposited in the Herbarium Prisco Bezerra of the Departamento de Biologia, Universidade Federal do Ceará.

Extraction and isolation

The dried, ground biomass (940 g) from the roots of *L. microphylla* was extracted at room temperature with EtOH. After solvent evaporation under reduced pressure, a brown residue

Table II. IC_{50} values in μg/ml from cytotoxicity assay[a] of naphthoquinones from *L. microphylla*.

Cell Lines	IC_{50} (mean ± SE) (**1**)	(**2a**/**2b**)
B16 (murine melanoma)	3.13 ± 0.12	0.77 ± 0.05
CEM (human limphocyte leukemia)	2.57 ± 0.33	1.61 ± 0.25
HL60 (human promyelocyte leukemia)	2.92 ± 0.13	1.62 ± 0.09
HCT8 (human colon adenocarcinoma)	2.33 ± 0.29	0.69 ± 0.06
MCF7 (human breast adenocarcinoma)	2.44 ± 0.13	1.30 ± 0.05

[a] Taxol (0.14 μg/ml) was used as the positive control (100% death of all cell lines).

(30 g) was obtained. This material was fractioned over a Si gel column with hexane/$CHCl_3$ (1:1 v/v), $CHCl_3$, EtOAc and MeOH. The $CHCl_3$ fraction (8.2 g) was chromatographed over Si gel and eluted with 0–100% EtOAc–hexane mixtures. The residue obtained from the 5% EtOAc–hexane fraction was subjected to repeated column chromatography to yield **1** (100 mg), **3** (15 mg, m.p. 188–189° C, according previous reported data), and, **2a**/**2b** (8 mg) as an isomer mixture.

Cytotoxicity bioassays

For CEM and HL-60 (leukemia), B16 (melanoma), HCT8 (colon) and MCF-7 (breast) cancer cell lines, a microassay for cytotoxicity was performed using the microtitre MTT (tetrazonium [3-(4′-5′-dimethylthiazol-2′-yl)-2,5-diphenyl-tetrazoliumbromide]) dye assay under conditions of continuous drug exposure as previously described (Mosmann, 1983), by the following procedure. Adherent cancer cell lines at the concentration of 0.3×10^6 cells/ml and suspended cells at 0.5×10^6 cells/ml were seeded in 96 well microplates. The adherent cells were incubated during twenty four hours to allow cell attachment. The compounds were added to the cell cultures at concentrations of 0.39–25 μg/ml, and the cells incubated for three days. The MMT solution was added three hours before the end of the incubation time. Cell survival was evaluated with a multiwell scanning spectrophotometer at 540 nm. All compounds were tested in three replicate wells in each plate, and experiments were repeated at least two times.

Microphyllaquinone (**1**): $C_{27}H_{20}O_6$, red crystals, m.p. 197–200° C; $[\alpha]_D^{25}$ –130.2 (*c* 0.004, $CHCl_3$); IR (KBr): ν_{max} = 3403, 2913, 1665, 1640, 1595, 1439, 1372, 1335, 1241, 1034, 850, 769,704 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table I; EIMS (70 eV) *m/z* 440 ($[M]^+$, 47), 425 (19), 408 (13), 393 (100), 381 (6).

Methoxynaphtho[2,3-b]furan-4,9-quinones (**2**): $C_{13}H_8O_4$, orange crystals, m.p. 162–165° C; IR (KBr): ν_{max} = 2923, 1675, 1591, 1480, 1439, 1363, 1268, 1233, 1098, 969, 883, 774 cm^{-1}. ^1H NMR for **2a** (500 MHz, $CDCl_3$): δ = 3.99 (s, OMe), 7.00 (d, *J* = 1.5, H-3), 7.22 (dd, *J* = 8.7, 2.5, H-7), 7.69 (d, *J* = 2.5, H-5), 7.77 (d, *J* = 1.5, H-2), 8.15 (d, *J* = 8.7, H-8); ^{13}C NMR for **2a** (125 MHz, $CDCl_3$): 56.4 (s, OMe), 109.2 (C-3), 111.5 (C-5), 119.9 (C-7), 126.9 (C-8a), 129.9 (C-8), 131.2 (C-3a), 135.1 (C-4a), 149.0 (C-2), 153.1 (C-9a), 164.7 (C-6), 173.9 (C-4), 180.2 (C-9). ^1H NMR for **2b** (500 MHz, $CDCl_3$): δ = 3.99 (s, OMe), 6.99 (d, *J* = 1.5, H-3), 7.20 (dd, *J* = 8.7, 2.5, H-6), 7.66 (d, *J* = 2.5, H-8), 7.75 (d, *J* = 1.5, H-2), 8.18 (d, *J* = 8.7, H-5); ^{13}C NMR for **2b** (125 MHz, $CDCl_3$): 56.4 (s, OMe), 108.9 (C-3), 111.8 (C-8), 119.9 (C-6), 126.1 (C-4a), 129.8 (C-5), 130.5 (C-3a), 135.9 (C-8a), 148.5 (C-2), 153.5 (C-9a), 164.7 (C-7), 173.4 (C-4), 180.9 (C-9).

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Abeygunawardena C., Kumar V., Marshall D. S., Thomson R. H., and Wickramaratne D. B. M. (1991), Furanonaphthoquinones from two *Lantana* species. Phytochemistry **30**, 941–45.

Costa S. M. O., Lemos T. L. G., Pessoa O. D. L., Pessoa C., Montenegro R. C., and Braz-Filho R. (2001), Chemical constituents from *Lippia sidoides* and cytotoxic activity. J. Nat. Prod. **64**, 792–795.

Forestiere A. M., Monfort M. T., Ragusa S., and Trovato A. (1996), Antiinflamatory, analgesic and antipyretic activity in rodents of plants extracts used in Africa medicine. Phytother. Res. **10**, 100–106.

Lemos T. L. G., Matos F. J. A., Alencar J. W., and Craveiro A. A. (1990), Antimicrobial activity of essential oils of Brazilian plants. Phytother. Res. **4**, 82–84.

Lemos T. L. G., Monte F. J. Q. , Matos F. J. A., Alencar J. W., and Craveiro A. A. (1992), Chemical composition and antimicrobial activity of essential oil from Brazilian plants. Fitoterapia **63**, 266–268.

Lemos T. L. G., Costa S. M. O., Pessoa O. D. L., and Braz-Filho R. (1999), Total assignments of ^1H and ^{13}C NMR spectra of tectol and tecomaquinone I. Magn. Reson. Chem. **37**, 908–911.

Mosmann T. (1983), Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J. Immunol. Methods **65**, 55–63.

Pascual M. E., Slowing K., Carretero E., Sánchez Mata D., and Villar A. (2001), *Lippia*: traditional uses, chemistry and pharmacology: a review. J. Ethnopharmacol. **76**, 201–214.

Valentin A., Pelissier Y., Benoit F., Marion C., Kone D., Mallie M., Bastide J. M., and Bessiere J. M. (1995), Composition and antimalarial activity *in vitro* of volatile components of *Lippia multiflora*. Phytochemistry **40**, 1439–1442.