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## Antifungal Amide from Leaves of *Piper hispidum*

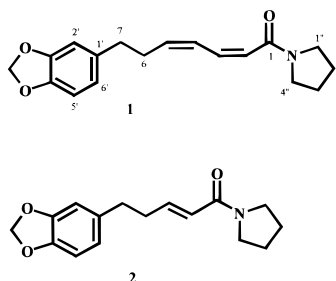
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Bioactivity-guided fractionation of a CH<sub>2</sub>Cl<sub>2</sub> extract from leaves of *Piper hispidum* (Piperaceae) yielded a new pyrrolidine amide, *N*-[7-(3',4'-methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine **1**, in addition to two known amides *N*-[5-(3',4'-methylenedioxyphenyl)-2(*E*)-pentadienoyl]pyrrolidine and *N*-[2-(3',4'-methylenedioxy-6'-methoxyphenyl)-2(*Z*)-propenoyl]pyrrolidine. The structure of compound **1** was elucidated by interpretation of spectral data, including ES–MS. Compound **1** showed antifungal activity against *Cladosporium sphaerospermum*.

Chemical studies carried out on Piperaceae species have revealed the occurrence of essential oils, pyrones, lignoids, and unsaturated amides.<sup>1</sup> Some of these compounds have generated interest as a result of their potent insecticidal properties.<sup>2</sup> In the course of our ongoing search for bioactive compounds from Brazilian flora,<sup>3</sup> we have investigated the CH<sub>2</sub>Cl<sub>2</sub> extract from leaves of *Piper hispidum* H. B. K. (Piperaceae) employing two preliminary bioassays: anticancer activity based on growth evaluation of genetically engineered mutants of the yeast *Saccharomyces cerevisiae*<sup>4,5</sup> and antifungal effect utilizing direct bioautography with the fungus *Cladosporium sphaerospermum*.<sup>6</sup> The extract exhibited moderate and nonselective activity on all the mutant strains tested, suggesting the presence of antifungal agents rather than potential anticancer agents. In fact, it did show activity against the fungus *C. sphaerospermum* (MIC 1096 µg). Subsequent bioactive-guided fractionation led to the isolation of a new amide *N*-[7-(3',4'-methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine (**1**) and the known amides *N*-[5-(3',4'-methylenedioxyphenyl)-2(*E*)-pentadienoyl]pyrrolidine and *N*-[2-(3',4'-methylenedioxy-6-methoxyphenyl)-2(*Z*)-propenoyl]pyrrolidine. Of these, the amide **1** exhibited antifungal activity.



Compound **1** has a molecular formula of C<sub>18</sub>H<sub>21</sub>NO<sub>3</sub> as determined by electrospray mass spectral analysis

(ES–MS) and <sup>13</sup>C NMR data. The UV spectrum displayed an absorption at 262 nm, indicating the presence of a conjugated dienamide system. The IR spectrum exhibited bands at 1640 (conjugated carbonyl group), 1620 (conjugated double bond), and 925 (methylenedioxyphenyl group) cm<sup>−1</sup>. The <sup>1</sup>H NMR spectrum (Table 1) of **1** revealed signals for 3',4'-methylenedioxyphenyl protons (δ 5.86) and signals related to four olefinic protons. Two of the olefinic protons showed signals at δ 5.77 (1H, d, *J* = 11.4 Hz) and 6.37 (1H, t, *J* = 11.4 Hz) assigned to protons H-2 and H-3, respectively, corroborating the α,β-unsaturated carbonyl system. Signals at δ 7.30 (dd, *J* = 11.4 Hz) and 5.94 (dd, *J* = 11.4, 7.0 Hz) were assigned to the protons of the second double bond conjugated to the first one. Most of the amides isolated from *Piper* species possess the (*E,E*) configuration in the double bonds of the side chain; however, this compound differed from the normal skeleton judging by the coupling constant observed for protons H-2, H-3, H-4, and H-5. In compounds with (*E*) configuration, the coupling constants are normally *J*<sub>2,3</sub> = 15.0 Hz and *J*<sub>4,5</sub> = 15.0 Hz,<sup>7,8</sup> while in the case of amide **1**, these values were observed at *J*<sub>2,3</sub> = 11.4 Hz and *J*<sub>4,5</sub> = 11.4 Hz, indicating that the two double bonds possess the (*Z,Z*) configuration. The assignments of these protons were established by analysis of the <sup>1</sup>H–<sup>1</sup>H COSY spectrum (Figure 1) and also by irradiation of the H-2, which caused the signal of H-3 to collapse to a doublet. On the other hand, the irradiation at the frequency of H-3 transformed the signal of H-2 into a singlet. The proposed stereochemistry was also corroborated by the signal of H-4 (δ 7.30, γ position), which showed significant deshielding when compared to the (*E*) configuration<sup>7</sup> caused by its proximity to the carbonyl group (Figure 1). The presence of a benzylic carbon in **1** was supported by the appearance of a fragment ion at *m/z* 135 in its MS. The assignments of methylenic protons H-7 (δ 2.59, 2H) and H-6 (δ 2.42, 2H) were confirmed by DQ–COSY spectrum, which showed correlation between such protons to each other. Additional correlation between proton H-6 and methine proton at H-5 (δ 5.94, 1H) was also observed. The <sup>13</sup>C NMR data

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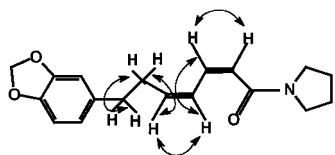
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**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Spectral Data for Compounds **1** and **2** ( $\delta$  in ppm,  $\text{CDCl}_3$ )

position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$ [m, $J$ (Hz)] <sup>a</sup>	$\delta_{\text{C}}$ <sup>b,c</sup>	$\delta_{\text{H}}$ [m, $J$ (Hz)] <sup>a</sup>	$\delta_{\text{C}}$ <sup>b,c</sup>
1		165.6 (s)		164.7 (s)
2	5.77 (1H, d, $J_{2,3} = 11.4$ )	118.1 (d)	6.03 (d, $J_{2,3} = 14.8$ )	122.3 (d)
3	6.37 (1H, t, $J_{3,2}$ and $3,4 = 11.4$ )	140.4 (d)	6.85 (dt, $J_{3,2} = 15.0$ , $J_{3,4} = 7.0$ )	144.2 (d)
4	7.30 (1H, dd, $J_{4,3} = 11.4$ )	127.9 (d)	2.38 (m)	34.4 (t)
5	5.94 (1H, dt, $J_{4,5} = 11.4$ , $J_{5,6} = 7.0$ )	141.6 (d)	2.56 (t, $J_{5,4} = 7.0$ )	34.5 (t)
6	2.42 (m)	34.9 (t)		
7	2.59 (t, $J_{7,6} = 7.0$ )	35.0 (t)		
1'		135.4 (s)		135.0 (s)
2'	6.54 (1H, d, $J_{2',6'} = 1.6$ )	108.1 (d)	6.58 (d, $J_{2',6'} = 1.6$ )	108.1 (d)
3'		145.6 (s)		145.4 (s)
4'		147.5 (s)		147.5 (s)
5'	6.65 (1H, d, $J_{5',6'} = 7.8$ )	108.8 (d)	6.62 (d, $J_{5',6'} = 7.8$ )	108.8 (d)
6'	6.59 (1H, dd, $J_{6',5'} = 7.8$ and $J_{6',2'} = 1.6$ )	121.1 (d)	6.59 (dd, $J_{6',5'} = 7.8$ , $J_{6',2'} = 1.6$ )	121.2 (d)
1''	3.47 (2H, t, $J_{1'',2''} = 7.0$ )	45.5 (t)	3.44 (t, $J_{1'',2''} = 6.5$ )	45.8 (t)
2''	1.84 (2H, dt, $J_{2'',1''} = 7.0$ and $J_{2'',3''} = 7.3$ )	26.2 (t)	1.84 (m)	26.1 (t)
3''	1.84 (2H, dt, $J_{2'',3''} = 7.3$ and $J_{3'',4''} = 7.0$ )	24.3 (t)	1.84 (m)	24.3 (t)
4''	3.39 (2H, t, $J_{4'',3''} = 7.0$ )	46.8 (t)	3.37 (t, $J_{4'',3''} = 6.5$ )	46.5 (t)
O-CH <sub>2</sub> -O	5.86 (s)	100.7 (t)	5.88 (s)	100.8 (t)

<sup>a</sup> Measured at 200 MHz. <sup>b</sup> Measured at 50 MHz. <sup>c</sup> Multiplicities of carbons (in parentheses) determined by a DEPT experiment.

**Figure 1.** Selected  $^1\text{H}$ – $^1\text{H}$  COSY correlations for **1**.

(Table 1) of **1** is in accordance with the proposed structure. The lowfield region of the spectrum exhibited an amide carbonyl at  $\delta$  165.6, 10 olefinic and/or aromatic carbons and a methylenedioxyphenyl group at  $\delta$  100.7. The assignments of the signals for the aromatic carbons based on the comparison with data described for piperdardine<sup>7</sup> indicated that a 3',4'-methylenedioxyphenyl moiety is present in **1**. Such amides with cis geometry are quite rare in nature. To date, only two cinnamoyl pyrrolidine amides showing a cis double bond at C-2 and C-3 have been reported by Sehgal et al.<sup>9</sup> and Kiuchi et al.<sup>10</sup>

*N*-[5-(3',4'-Methylenedioxyphenyl)-2(*E*)-pentadienyl]-pyrrolidine (**2**) has the molecular formula  $\text{C}_{16}\text{H}_{19}\text{NO}_3$  by analysis of the MS data. Its  $^1\text{H}$  NMR spectrum was identical to that published for the same compound previously isolated from *Piper nigrum*.<sup>10</sup> Its  $^{13}\text{C}$  NMR data (Table 1) is being published for the first time. *N*-[2-(3',4'-Methylenedioxy, 6'-methoxyphenyl)-2(*Z*)-propenyl]-pyrrolidine, a cinnamoyl pyrrolidine amide, was previously isolated from *P. peepuloides* and was identified by comparison of its spectral data.<sup>11</sup>

The antifungal activity of amide **1** was evaluated by means of direct bioautography in a TLC bioassay<sup>12</sup> and the centerpoint-inoculation-disk assay.<sup>13</sup> The MIC for **1** was found to be 8.0  $\mu\text{g}$ .

## Experimental Section

**General Experimental Procedures.** Si gel (Merck 230–400 mesh) was used for all column chromatography unless otherwise stated, and solvents were redistilled prior to use.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 200 and 50 MHz, respectively, using  $\text{CDCl}_3$

as a solvent and TMS as reference. IR spectra were obtained on a Nicolet spectrometer. ES–MS were recorded on a VG Platform II spectrometer. HPLC separations were performed on a Shimadzu LC-10AS using a reversed-phase column (Waters Nova Pack,  $\text{C}_{18}$ ;  $3.9 \times 150$  mm) eluted with MeOH– $\text{H}_2\text{O}$  (6:4), flow rate of 0.5 mL/min and detection at 254 nm.

**Plant Material.** *P. hispidum* H. B. K. (Piperaceae) leaves were collected in Parque Estadual do Morro do Diabo, Teodoro Sampaio, SP, and classified by Dr. Waldir Mantovani. The voucher specimen is deposited at Departamento de Ecologia, Instituto de Biociências-USP.

**Bioassays.** The experimental screening methods utilizing mutant yeast strains of *S. cerevisiae* have been described elsewhere.<sup>4,5</sup> The  $\text{IC}_{12}$  values refer to the concentration in  $\mu\text{g}/\text{mL}$  required to produce an inhibition zone 12 mm in diameter around a 100- $\mu\text{L}$  well during a 48-h incubation period at 37 °C. The microorganism used in the antifungal assay, *C. sphaerospermum* (Pemzig) SPC 491, has been maintained at Instituto de Botânica.

**Antifungal Assay.** Dilutions containing 750–1500  $\mu\text{g}$  of crude extract and 100, 50, 10, 1, 0.1  $\mu\text{g}$  of pure compound were applied on precoated TLC plates. TLC plates were developed with hexane–EtOAc (7:3). The chromatogram was sprayed with a spore suspension of *C. sphaerospermum* in glucose and salt solution<sup>12</sup> and incubated for 72 h in darkness in a moistened chamber at 25 °C. Clear inhibition zones appeared against a dark background, indicating the minimal amount of **1** (10  $\mu\text{g}$ ) required for inhibition of fungal growth on TLC plate. Nystatin (5  $\mu\text{g}$ ) and miconazole (1  $\mu\text{g}$ ) were used as positive controls.

**Extraction and Isolation.** The dried, powdered leaves of *P. hispidum* (3.1 g) were extracted three times at room temperature with  $\text{CH}_2\text{Cl}_2$  (150 mL each) in the course of 2 days. The resulting  $\text{CH}_2\text{Cl}_2$  extract was filtered and concentrated *in vacuo* to afford 1.84 g of a green gum. Part of this extract (1.69 g) was applied to a Si gel column (300 g) and eluted with hexane contain-

ing increasing volumes of EtOAc (up to 100%) to give 38 fractions. Fraction 25 (125 mg) was applied to a Si gel column (2.0 g) 230–400 mesh, eluted with hexane containing increasing concentrations of EtOAc (up to 70%) to give **1** (12.3 mg). The fraction 28 (198 mg) was chromatographed via Si gel column chromatography, eluted with hexane containing increasing volumes of EtOAc (up to 70%) to give *N*-[5-(3',4'-methylenedioxyphenyl)-2(*E*)-pentadienoyl] pyrrolidine (3.7 mg). Fractions containing *N*-[2-(3',4'-methylenedioxy, 6-methoxyphenyl)-2(*Z*)-propenoyl]pyrrolidine were combined and concentrated *in vacuo* to give a green gum (21.0 mg), which was further purified by reversed-phase HPLC, as described in the general experimental procedures.

***N*-[7-(3',4'-Methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine (1):** amorphous solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 262 nm (1.9); IR  $\nu_{\max}$  (KBr) 3010, 2923, 2874, 1640, 1620, 1488, 1443, 1035, 925  $\text{cm}^{-1}$ ; ES-MS  $m/z$  (rel int) 322 [ $M + Na$ ] (100); NMR see Table 1.

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