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Antifungal amides from *Piper hispidum* and *Piper tuberculatum*

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In honour of Professor Otto R. Gottlieb's 80th birthday

Abstract

Piper hispidum and *Piper tuberculatum* accumulate amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties. The isolation and characterization of several representatives including two hitherto unreported amides were performed by chromatographic techniques and by analysis of spectroscopic data. The antifungal activity of each amide was determined by direct bioautography against *Cladosporium sphaerospermum*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Piper hispidum*; *Piper tuberculatum*; Piperaceae; Amides; Antifungal activity

1. Introduction

Various amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties have been isolated from Piperaceae species (Parmar, 1997). These amides have generated interest as a result of their potent insecticidal properties (Miyako et al., 1989). In a previous paper, we described the structure of the antifungal amide *N*-[7-(3′,4′-methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine isolated from the leaves of *Piper hispidum* H.B.K. (Alécio et al., 1998).

In this paper, we describe the isolation, structure elucidation and evaluation of the antifungal activity of two new amides, (3*Z*,5*Z*)-*N*-isobutyl-8-(3′,4′-methylenedioxyphenyl)-heptadienamide (**1**), 8(*Z*)-*N*-(12,13,14-trimethoxycinnamoyl)-Δ^3-pyridin-2-one (**2**) and also of eight known antifungal amides *N*-[3-(6′-methoxy-3′,4′-methylenedioxyphenyl)-2(Z)-propenoyl]pyrrolidine (**3**), piperamine (**4**), *N*-(12,13,14-trimethoxydihydrocinnamoyl)-Δ^3-pyridin-2-one (**5**), piplartine (**6**), piperine (**7**), $\Delta^{\alpha,\beta}$-dihydropiperine (**8**), 5,6-dihydropiperlonguminine (**9**) and pellitorine (**10**). The amides isolated from stems of *Piper hispidum* (**1**, **3** and **4**) and from seeds of *Piper tuberculatum* (**2**, **5**–**10**) were active against the fungus *Cladosporium sphaerospermun* as evaluated by direct bioauto graphy (Homans and Fuchs, 1970).

2. Results and discussion

A CH_2Cl_2:MeOH (2:1) extract of the stems of *P. hispidum* was subjected to silica gel column chromatography, followed by HPLC purification to afford the compounds **1, 3** and **4**.

Compound **1**, has a molecular formula $C_{18}H_{23}NO_3$ as determined by analysis of electrospray mass spectrum (ES–MS) and of the ^{13}C NMR spectral data. The IR spectrum exhibited bands at 1640 (conjugated carbonyl group), 1620 (conjugated double bond), and 925 (methylenedioxyphenyl group) cm^{-1}. Its ^1H NMR spectrum (Table 1) revealed the presence of a 3′,4′-methylenedioxyphenyl group by the signal at δ 5.91 (2H, *s*), and four olefinic protons which showed signals at δ 5.45 (1H, *d*, $J_{3,4}=12.0$ Hz), 6.38 (1H, *dd*, $J_{4,3 \text{ and } 4,5}=12.0$ Hz), 7.24 (1H, *dd*, $J_{5,4 \text{ and } 5,6}=12.0$ Hz) and 5.94 (1H, *dt*, $J_{6,5 \text{ and } 6,7}=12.0$ and 7.0 Hz) indicative of an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl system. The coupling constant values indicated that both double bonds

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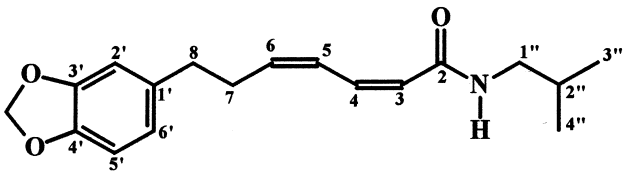

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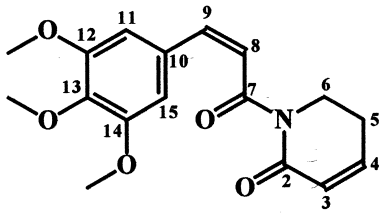

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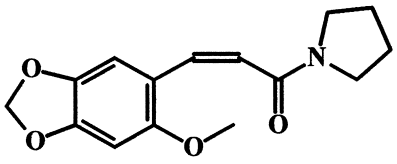

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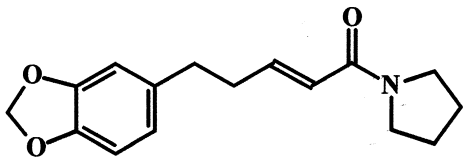

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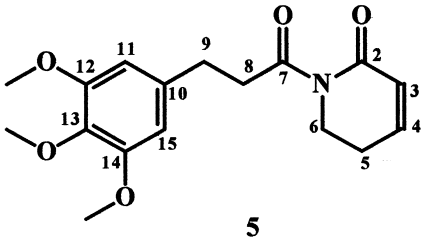

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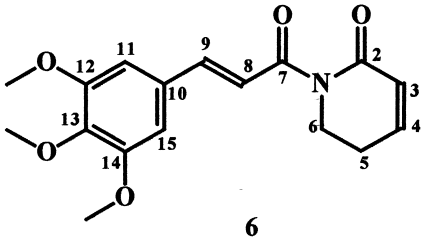

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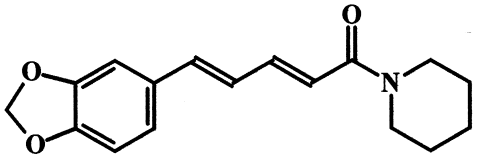

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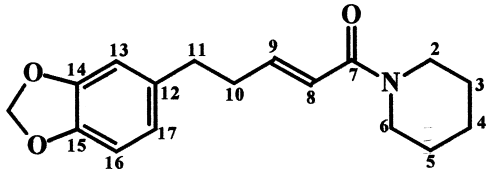

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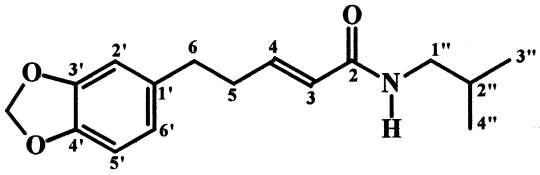

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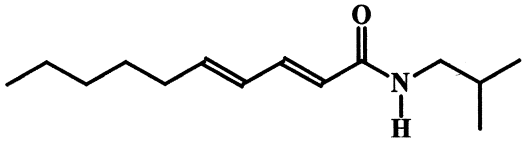

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possess the Z configuration (Alécio et al., 1998; Shah et al. 1986). The assignments of these olefinic protons were confirmed by analysis of the ^{1}H–^{1}H COSY spectrum and by comparison with the literature data (Shah et al., 1986). The irradiation at the frequencies of the H-3 and H-4 collapsed the signals of H-4 and of H-3 to a doublet and to a singlet, respectively. The assignments of methylenic protons H-8 at δ 2.62 (2H, *t*, $J_{8,7}=7.0$ Hz) and H-7 at δ 2.46 (2H, m) were confirmed by the DQ-COSY spectrum which showed mutual correlation between such protons. Additional correlation between signal assigned to the proton H-7 and to that of methine proton H-6 (δ 5.94, 1H) could be also observed.

The presence of a isobutyl moiety was confirmed by the signals at δ 3.13 (2H, *dd*, $J_{1'',2''}=7.0$ Hz), δ 1.80 (1H, *m*) and δ 0.92 (6H, *d*, $J_{3'',2''\text{ and }4'',2''}=7.0$ Hz) attributed to H-1″, H-2″, H-3″ and H-4″, respectively. The coupling between H-1″ and the NH hydrogen was confirmed by

Table 1
^{1}H and ^{13}C NMR spectral data of amides **1** and **2** (200 and 50 MHz, ppm, $CDCl_3$)

	1		**2**	
Position	^{1}H δ [m, *J* (Hz)]	^{13}C δ[a]	^{1}H δ [*m*, *J* (Hz)]	^{13}C δ[a]
2	–	166.5 (s)	–	165.7 (s)
3	5.45 (1H, *d*, $J_{3,4}$ = 12.0)	118.9 (d)	5.98 (1H, *dt*, $J_{3,4 \text{ and } 3,5}$ = 9.7, 1.9)	125.5 (d)
4	6.38 (1H, *dd*, $J_{4,3 \text{ and } 4,5}$ = 12.0)	141.3 (d)	6.89 (1H, *dt*, $J_{4,3 \text{ and } 4,5}$ = 9.7, 4.3)	145.4 (d)
5	7.24 (1H, *dd*, $J_{5,4 \text{ and } 5,6}$ = 12.0)	127.5 (d)	2.39–2.42 (2H, *m*)	24.8 (t)
6	5.94 (1H, *dt*, $J_{6,5 \text{ and } 6,7}$ = 12.0,7.0)	141.3 (d)	3.97 (2H, *t*, $J_{6,5}$ = 7.0)	41.1 (t)
7	2.46 (2H, *m*)	34.9 (t)*[b]	–	168.1 (s)
8	2.62 (2H, *t*, $J_{8,7}$ = 7.0)	35.1 (t)*	6.48 (1H, *d*, $J_{8,9}$ = 12.4)	124.5 (d)
9	–	–	6.71 (1H, *d*, $J_{9,8}$ = 12.4)	136.7 (d)
10	–	–	–	131.1 (s)
11	–	–	6.76 (1H, *s*)	106.6 (d)
12	–	–	–	152.8 (s)
13	–	–	–	145.5 (s)
14	–	–	–	152.8 (s)
15	–	–	6.76 (1H, *s*)	106.6 (d)
1′	–	135.4 (s)	–	–
2′	6.55 (1H, *d*, $J_{2',6'}$ = 1.6)	108.1 (d)	–	–
3′	–	145.6 (s)	–	–
4′	–	147.5 (s)	–	–
5′	6.70 (1H, *d*, $J_{5',6'}$ = 7.8)	108.8 (d)	–	–
6′	6.64 (1H, *dd*, $J_{6',5' \text{ and } 6',2'}$ = 7.8, 1.6)	121.1 (d)		
1″	3.13 (2H, *dd*, $J_{1'',2''}$ = 7.0)	46.7 (t)	–	–
2″	1.80 (1H, *m*)	28.6 (d)	–	–
3″	0.92 (3H, *d*, $J_{3'',2''}$ = 7.0)	20.1 (q)	–	–
4″	0.92 (3H, *d*, $J_{4'',2''}$ = 7.0)	20.1 (q)	–	–
OCH_2O	5.91 (2H, *s*)	100.7 (t)	–	–
OCH_3	–	–	3.84 (6H, *s*)	56.1 (q)
OCH_3	–	–	3.85 (3H, *s*)	60.7 (q)

[a] Multiplicities of carbons (in parentheses) determined by a DEPT experiment.
[b] *These signals can be interchanged.

exchange with D_2O in the ^{1}H NMR experiment. The ^{13}C NMR data (Table 1) of **1** are in accordance with the proposed structure. The low-field region of the spectrum exhibited signals assigned to an amide carbonyl at δ 166.5, ten olefinic and/or aromatic carbons, six saturated carbons [δ_C 46.7, 35.1, 34.9, 28.6 and 20.1(2x)], and a methylenedioxyphenyl group at δ 100.7. The assignments of the signals for the aromatic carbons are based on comparison with data described in the literature (Alécio et al., 1998; Araújo-Junior et al. 1997) and indicated that a 3′,4′-methylenedioxyphenyl moiety is present. The structure of **1** was determined as (3*Z*,5*Z*)-*N*-isobutyl-8-(3′,4′-methylenedioxyphenyl)-heptadienamide.

A CH_2Cl_2:MeOH (2:1) extract from seeds of *P. tuberculatum* was fractionated by column chromatography on silica gel followed by preparative TLC to afford compounds **2** and **5–10**.

Compound **2**, was shown to have a molecular formula $C_{17}H_{19}NO_5$ by analysis of electrospray mass spectrum (ES–MS) and of ^{13}C NMR data. Its IR spectrum revealed absorption bands at 1757 (conjugated carbonyl group), at 1615 (conjugated double bond) and at 1216 cm^{-1} (asymmetric C–O–C stretching). The ^{1}H NMR spectrum (Table 1) of **2** revealed the presence of methoxyphenyl groups by the signals at δ 3.84 (6H, *s*), δ 3.85 (3H, *s*), one aromatic proton signal at δ 6.76 (2H, *s*) which suggested a 12,13,14-trimethoxyphenyl group. Assignments of signals related to four olefinic protons were determined based on analysis of DQCOSY spectrum. The spin systems derived from H-8, H-9 and H-3, H-4 were readily recognized by starting with the 1H doublet at δ 6.48 assigned to H-8 ($J_{8,9}$ = 12.4 Hz) which showed a cross-peak with the 1H doublet at δ 6.71 assigned to H-9 ($J_{9,8}$ = 12.4 Hz). The coupling constant values indicated that this double bond possesses the *Z* configuration. The attribution of this configuration was corroborated by the shielded signals of H-8 and H-9 in the *Z* isomer when compared with the *E* isomer (Duh et al., 1990). The doublet of triplet (1H) at δ 5.98 ($J_{3,4 \text{ and } 3,5}$ = 9.7 and 1.9 Hz) assigned to H-3 showed correlation with H-4. The doublet of triplet (1H) at δ 6.89 ($J_{4,3 \text{ and } 4,5}$ = 9.7 and 4.3 Hz) assigned to H-4 showed a cross peak with the signal at δ 5.98 ($J_{3,4 \text{ and } 3,5}$ = 9.7 and 1.9 Hz) attributed to H-3 and with the multiplet centered at δ 2.40 attributed to H-5. This last proton also showed a correlation with the triplet at δ 3.97 ($J_{6,5}$ = 7.0 Hz) assigned to H-6. The low-field region of the ^{13}C NMR spectrum (Table 1) of **2** exhibited signals assigned to six aromatic carbons, four olefinic carbons, besides two carbonyl groups at δ 165.7 and 168.1. The assignments

of the carbonyl groups were based on analysis of the HMBC spectrum. The signal at δ 168.1 was assigned to the carbonyl at C-7 based on its correlation with the proton signals at δ 6.48 (H-8) and δ 6.71 (H-9). The signal of a carbonyl group at δ 165.7 was assigned to C-2 based on its cross-peak with the proton signal at δ 5.98 (H-3) and 6.89 (H-4). The high-field region exhibited signals assigned to two saturated carbons [δ_c 24.8 (C-5) and 41.1 (C-6)] and to methoxyphenyl groups at δ 56.1 and 60.7. HETCOR analysis were used to assign the signals for all proton-bearing carbons, and thus the structure of amide **2** was defined as 8(*Z*)-*N*-(12,13,14-trimethoxycinnamoyl)-Δ^3-pyridin-2-one.

N- (12, 13, 14- Trimethoxydihydrocinnamoyl)-Δ^3 -pyridin -2- one (**5**), $\Delta^{\alpha,\beta}$- dihydropiperine (**8**) and 5,6-dihydropiperlonguminine (**9**) have the molecular formula $C_{17}H_{21}NO_5$, $C_{17}H_{21}NO_3$ and $C_{16}H_{21}NO_3$, respectively, by analysis of the MS data. Their ^{1}H NMR spectra were identical to that published for the same compounds previously isolated from *Piper rugosum* (Maxwell and Rampersad, 1991), *Piper guineense* (Parmar et al., 1997) and *P. tuberculatum* (Bernard et al., 1995), respectively. Their ^{13}C NMR spectral data (Table 2) are published here for the first time.

Table 2
^{13}C NMR data of amides **5**, **8** and **9** (50 MHz, δ, ppm, $CDCl_3$)[a]

Carbons	**5**	**8**	**9**
2	165.4 (s)	43.0 (t)	166.0 (s)
3	125.8 (d)	25.5 (t)	120.8 (d)
4	145.2 (d)	24.6 (t)	142.6 (d)
5	24.6 (t)	26.7 (t)	33.8 (t)
6	41.0 (t)	46.8 (t)	34.1 (t)
7	175.5 (s)	165.5 (s)	–
8	41.0 (t)	121.1 (d)	–
9	31.5 (t)	144.0 (d)	–
10	136.9 (s)	34.4 (t)*[b]	–
11	105.4 (d)	34.5 (t)*	–
12	153.1 (s)	134.9 (s)	–
13	142.1 (s)	108.8 (d)	–
14	153.1 (s)	147.5 (s)	–
15	105.4 (d)	145.7 (s)	–
16	–	108.1 (d)	–
17	–	121.3 (d)	–
1′	–	–	134.6 (s)
2′	–	–	107.9 (d)
3′	–	–	145.5 (s)
4′	–	–	147.5 (s)
5′	–	–	108.4 (s)
6′	–	–	124.2 (d)
1″	–	–	46.7 (s)
2″	–	–	28.3 (d)
3″	–	–	19.9 (q)
4″	–	–	19.9 (q)
OCH_2O	–	100.7 (t)	100.5 (t)
OCH_3	56.0 (q)/60.8 (q)	–	–

[a] Multiplicities of carbons (in parentheses) determined by a DEPT experiment.
[b] *These signals can be interchanged.

N-[3-(6′-methoxy-3′,4′-methylenedioxyphenyl)-2(*Z*)-propenoyl]pyrrolidine (**3**) was previously isolated from *Piper peepuloides* (Shah et al., 1986) and *P. hispidum* (Alécio et al., 1998). Piperamine (**4**) was previously obtained from *Piper nigrum* (Kiuchi et al., 1988) and *P. hispidum* (Alécio et al., 1998). Piplartine (**6**) was previously described from *Piper aborescens* (Duh et al., 1990) and *P. tuberculatum* (Filho et al. 1981). Piperine (**7**) was previously described from *P. aborescens* and *P. tuberculatum* (Araújo-Júnior et al., 1997) and pellitorine (**10**) had been reported from *Cissampelos glaberrina* (Rosario et al., 1996).

The antifungal activity of amides **1–10** was evaluated by means of direct bioautography in a TLC bioassay (Homans and Fuchs, 1970). The detection limits of compounds **1–10** are shown in Table 3 and were performed according to Rahalison et al. (1994). The highest sample amount tested was 10 μg; amides producing no inhibition of *C. sphaerospermum* growth at that level were considered inactive.

The minimum quantity of compounds **1**–**4**, **6**, **8**–**10** necessary to inhibit growth of the fungus on the TLC plates was determined as 5.0 μg, while for **7** and **5** the values were 1.0 and 0.1 μg, respectively. The values for **7** and **5** were comparable to those observed for the reference compounds miconazole (0.5 μg) and nystatin (0.5 μg).

P. hispidum and *P. tuberculatum* have accumulated amides with *cis* geometry in their side chain, which is a structural feature quite rare in nature (Alécio et al., 1998; Shah et al., 1986).

3. Experimental

3.1. Instrumentation and chromatography materials

Silica gel (Merck 230–400 mesh) was used for all column chromatography unless otherwise stated and sol-

Table 3
Antifungal activity of amides **1–10** from *Piper hispidum* and *Piper tuberculatum* against *Cladosporium sphaerospermum*[a]

Compound	Antifungal activity[b] (μg)
1	5.0
2	5.0
3	5.0
4	5.0
5	0.1
6	5.0
7	1.0
8	5.0
9	5.0
10	5.0

[a] Positive controls: nystatin (0.5 μg) and miconazole (0.5 μg).
[b] Minimum amount required for inhibition of fungal growth on TLC plates.

vents were redistilled prior to use. ^1H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, using $CDCl_3$ 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 reverse phase column (Waters Nova Pack, C_{18}; 3.9 × 150 mm) eluted with MeOH:H_2O (3:2), flow rate of 0.5 ml/min and detection at 260 nm. Elemental analysis were performed on a Elemental Analyser 2400 CHN Perkin-Elmer.

3.2. Plant material

Piper hispidum H.B.K. stems were collected in Parque Estadual do Morro do Diabo, Teodoro Sampaio, SP and identified by Dr. Waldir Mantovani (Instituto de Biociências — USP). *P. tuberculatum* Jacq. seeds were collected in the Campus do INPA (Manaus) and identified by Dr. Guillermo E. D. Paredes (Universidad Nacional Pedro Ruiz Gallo, Peru). The voucher specimens are deposited at Herbarium of Instituto de Biociências — USP, São Paulo.

3.3. Antifungal assay

The microorganism used in the antifungal assay *C. sphaerospermum* (Penzig) SPC 491 has been maintained at the Instituto de Botânica, São Paulo—SP, Brazil. For the antifungal assay — 10 μl of solutions corresponding to 100 μg of crude extract and 10.0, 5.0, 1.0, 0.5, 0.1 μg of pure compound were applied to pre-coated TLC plates. TLC plates were developed with hexane:EtOAc (7:3) and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of *C. sphaerospermun* in glucose and salt solution (Rahalison et al., 1994) and incubated for 72 h in darkness in a moistened chamber at 25°C. A clear inhibition zone appeared against a dark background indicated the minimal amount of **1–10** required (Table 3). Nystatin (0.5 μg) and miconazole (0.5 μg) were used as positive controls.

3.4. Extraction and isolation of constituents

The dried and powdered stems of *P. hispidum* (28.0 g) were extracted with CH_2Cl_2 (3×150 ml), for two days at room temperature. The resulting CH_2Cl_2 extract was filtered and concentrated in vacuo to afford a green gum (1.60 g). Part of this extract (1.49 g) was applied to a silica gel column (300 g), and eluted with hexane containing increasing amounts of EtOAc (up to 100%) to give 24 fractions. Fraction 3 (0.203 g) was applied to a silica gel column (2.00 g) eluted with hexane containing increasing amounts of EtOAc (up to 70%) to give **1** (0.012 g). Fraction 28 (0.198 g) was subjected to a silica gel column chromatography, eluted with hexane containing increasing amounts of EtOAc (up to 70%) to give **3** (0.004 g). Frs containing **4** were combined and concentrated in vacuo to give a green gum (0.021 g), which was further purified by reversed-phase HPLC as described above. The dried and powdered seeds of *P. tuberculatum* (24.3 g) were extracted CH_2Cl_2:MeOH (2:1) (2 × 600 ml) for two days at room temperature. The resulting extract was filtered and concentrated in vacuo to afford a green gummy resin (2.87 g). Part of this extract (2.00 g) was fractionated by chromatographic column chromatography [eluted with Hex:-EtOAc (4:1) with gradient] to give 36 fractions (15 ml). Fraction 21 (0.043 g) was submitted to preparative TLC [Hex:EtOAc (3:2), three elutions] to yield **2** (0.024 g) and **6** (0.003 g). Fraction 20 (0.146 g) was submitted to preparative TLC [Hex:EtOAc (3:2), three elutions] to yield **5** (0.005 g). Fraction 19 (0.083 g) was submitted to preparative TLC [Hex:EtOAc (75:25), three elutions] to yield **7** (0.012 g) and **8** (0.007 g). Frs. 8 and 13 yielded **10** (0.079 g) and **9** (0.018 g), respectively.

3.5. (3Z,5Z)-N-isobutyl-8-(3′,4′-methylenedioxyphenyl)-heptadienamide (*1*)

Found: C, 71.54; H, 7.56; N, 4.45; O, 16.43%. $C_{18}H_{23}NO_3$ requires C, 71.74; H, 7.69; N, 4.65; O, 15.93%.

Amorphous solid. UV λ_{max}^{MeOH} nm: 260; IR ν_{max} (KBr): 3010, 2923, 2874, 1640, 1620, 1488, 1443, 1035, and 925 cm^{-1}; ES–MS m/z (rel. int.): 324 [M + Na] (100); ^1H and ^{13}C NMR spectra (see Table 1).

3.6. 8Z-N-(12,13,14-Trimethoxycinnamoyl)-Δ^3-pyridin-2-one (*2*)

Found: C, 63.95; H, 6.08; N, 4.28; O, 25.68%. $C_{17}H_{19}NO_5$ requires C, 64.34; H, 6.03; N, 4.41; O, 25.21%.

Amorphous solid; UV $\lambda_{max}^{CHCl_3}$ nm: 332; IR ν_{max} (KBr): 3018, 2926, 2853, 1757, 1615, 1459, 1215 cm^{-1}; ES–MS *m/z* (rel. int.): 318 [M + H] (6), 342 [M + Na + H] (13), 181 (100); ^1H and ^{13}C NMR spectra (see Table 1).

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