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ARTICLE

Synthesis and Quantitation of Six Phenolic Amides in *Amaranthus* spp.

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Cinnamoylphenethylamines are phenolic amides in which cinnamic acid provides the acid moiety and phenethylamine the amine moiety. Single ion monitoring (SIM) in LC−MS was performed on amaranth leaf extracts. Masses corresponding to sets of regioisomers, including previously reported compounds, were examined. Six peaks were detected and their corresponding standards synthesized for a quantitative LC−MS/MS investigation of cinnamoylphenethylamines in amaranth. Four cinnamoylphenethylamines (caffeoyltyramine, feruloyldopamine, sinapoyltyramine, and *p*-coumaroyltyramine) are reported in the Amaranthaceae for the first time; also, one rare compound, feruloyl-4-*O*-methyldopamine, appeared to be quite common in the genus *Amaranthus*. Feruloyldopamine showed moderate antifungal activity toward an isolate of *Fusarium culmorum*. Our LC−MS approach, in conjunction with the straightforward synthesis, provides a simple, reliable way of quantitatively investigating cinnamoylphenethylamines in plants. Concentrations of cinnamoylphenethylamines vary widely: feruloyltyramine was present in quantities of 5.26 to 114.31 μg/g and feruloyldopamine in quantities of 0.16 to 10.27 μg/g, depending on the plant sample.

KEYWORDS: LC−MS; phenolic amides; quantitation; amaranth; cinnamoylphenethylamines

INTRODUCTION

Cinnamoylphenethylamines result from the condensation of cinnamic acid and phenethylamine derivatives and have been associated with various biological activities such as the potentiation of antibiotics (*1*), inhibition of prostaglandin biosynthesis (*2*) and antioxidant effects (*3*, *4*) among others. It seems likely, therefore, that cinnamoylphenethylamines will have an impact on health if present in the diet. In fact, amaranth is currently being investigated as a highly important food crop since it is consumed both as grain and as vegetable, and in some cases both, as a dual-use crop (*5*). Several reviews have detailed the benefits and hazards of secondary metabolites in commercial crops such as potatoes (*6*) and tomatoes (*7*); these investigations can be advantageously extended both to new crops, such as amaranth, and to new secondary metabolites, especially those of known bioactivity. Further investigations of phenolics in amaranth would require both toxicological and metabolomic studies given that phenolic compounds have been linked to poor growth rates of animals fed with amaranth leaf protein extracts (*8*).

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Cinnamoylphenethylamine derivatives are found in over 30 plant families; the most ubiquitous are *p*-coumaroyltyramine and feruloyltyramine. The first reported cinnamoylphenethylamine was characterized over 40 years ago (*9*), but several have been discovered since. They are commonly reported through fortuitous discovery during phytochemical analyses. In other words, no more than three cinnamoylphenethylamines have been typically reported for a given plant species on the basis of prolonged isolation processes. Nevertheless, the cinnamic acid and phenethylamine derivatives are of great interest due to their plethora of associated biological activities.

The pupose of this study was to identify and quantify the cinnamoylphenethylamine family of compounds in plant samples of the genus *Amaranthus* using a simple synthetic procedure coupled with negative-mode LC−ESI-MS/MS.

MATERIALS AND METHODS

Preliminary Single Ion Monitoring LC−MS Analysis. Amaranth extracts were analyzed by LC−MS using selected ion monitoring (SIM) of masses corresponding to cinnamoylphenethylamine derivatives. Sets of regioisomers including compounds known from literature surveys, and representing combinations between derivatives of cinnamic acid (such as

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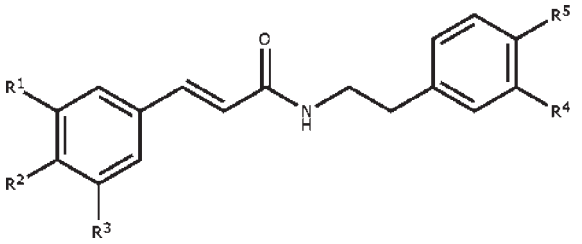
Table 1. List of Compounds



compd	no.	R^1	R^2	R^3	R^4	R^5
cinnamoylphenethylamine[a]	**1**	H	H	H	H	H
caffeoyltyramine	**2**	OH	OH	H	H	OH
feruloyldopamine	**3**	OMe	OH	H	OH	OH
sinapoyltyramine	**4**	OMe	OH	OMe	H	OH
p-coumaroyltyramine	**5**	H	OH	H	H	OH
feruloyltyramine	**6**	OMe	OH	H	H	OH
feruloyl-4-*O*-methyldopamine	**7**	OMe	OH	H	OH	OMe

[a] Common basic structure for compounds **2**−**7**.

caffeic, ferulic, sinapic, and coumaric acid) and 2-phenethylamine (such as tyramine, and dopamine, including its *O*-methyl derivatives), were selected. Six peaks corresponding to compounds **2**−**7** in **Table 1** were identifed as possible cinnamoylphenethylamines. They were chosen for synthesis and subsequent quantitation.

Chemicals. Six cinnamoylphenethylamines used for analytical experiments were synthesized in our laboratory. The acids and amines used were as follows: *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, tyramine hydrochloride, dopamine hydrochloride, purchased from Sigma-Aldrich (Munich, Germany), and 4-*O*-methyldopamine hydrochloride, purchased from Fluorochem (Glossop, England). *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide were also purchased from Sigma-Aldrich. All solvents were of analytical grade. Those used for synthesis were purchased from Labscan (Dublin, Ireland), and those used for analytical work were purchased from Rathburn (Walkerburn, Scotland).

Plant Samples. Four field-cultivated plant samples were examined analytically: two samples of *Amaranthus hypochondriacus* var. Nutrisol from Mexico (**A**, **B**), one sample of *Amaranthus mantegazzianus* var. Don Juan (**C**) and one sample of *Amaranthus hypochondriacus* var. San Antonio (**D**); the latter two originated from Argentina.

Synthesis of Cinnamoylphenethylamines. The six standards (listed as **2**−**7** previously) were synthesized in accordance with the literature (*10*). Briefly, 1.1 mmol of the corresponding acid was dissolved in 25 mL of tetrahydrofuran together with 1.2 mmol of *N*-hydroxysuccinimide and 1.3 mmol of *N*,*N*′-dicyclohexylcarbodiimide while cooling on an ice bath. The mixture was allowed to warm to room temperature and was stirred for 24 h under a slow stream of nitrogen before being filtered to remove dicyclohexylurea, evaporated to dryness and then redissolved in 15 mL of acetone. Next, 1.1 mmol of the amine was dissolved in 15 mL of saturated aqueous sodium hydrogencarbonate and mixed with the redissolved product in acetone solution. The mixture was stirred in the dark for 24 h under a slow stream of nitrogen. The acetone was evaporated and the aqueous phase acidified to pH 2 by the dropwise addition of 85% phosphoric acid. The aqueous phase was then extracted twice with 50 mL of ethyl acetate. The resulting organic phase was removed and washed first with 75 mL of saturated aqueous sodium hydrogencarbonate and then with 75 mL of saturated sodium chloride. The sample was evaporated once more and purified by UV-monitored preparative HPLC using a water−methanol gradient which started at 20% methanol on an RP-18 column (Waters Xbridge (Milford, MA) 150 × 19 mm i.d., 5 μm particle size, OBD). After evaporation of the methanol, the product was collected and lyophilized, leaving behind the amide as a white amorphous powder. Purity at the 95% level was checked by ^{1}H NMR (Pedersen et al., 2010; submitted). The percentage yields for compounds **2**−**7** were 14, 46, 63, 53, 54, and 38% respectively.

LC−MS Detection and Analysis. Analyses of plant samples were performed on an Applied Biosystems (Foster City, CA) 3200 Q Trap LC−MS set to multiple reaction monitoring (MRM). Plant samples were extracted using a Dionex (Sunnyvale, CA) ASE 350 accelerated solvent extractor using four cycles of 12.5 mL of methanol at 120 °C for a total of 50 mL for extraction of each 0.2 g sample of dry plant material, which provided the highest response among the temperature−solvent combinations tested. Before analysis, each plant sample was diluted with an equal volume of water and filtered through a Phenomenex (Torrance, CA) "Phenex" PTFE disk filter (pore size 0.45 μm). The mass couples listed in **Table 2** were monitored using the chromatographic method listed in **Table 3** at a flow rate of 0.2 mL min^{-1}. Solvent A was composed of 10% aqueous acetonitrile with 20 mM of acetic acid, and solvent B was acetonitrile with 20 mM of acetic acid. A Phenomenex Fusion 80A RP-18 column (250 × 2 mm i.d.) was used; the five compound-dependent ESI-MS/MS parameters listed in **Table 4** (declustering potential, DP; entrance potential, EP; cell entrance potential, CEP; collision energy, CE; and cell exit potential, CXP, all in volts) were applied.

Instrument parameters were as follows: curtain gas, 12; temperature, 700 °C; ion source gas 1, 40; ion source gas 2, 50; interface heater, on; collision gas, medium; and ion spray voltage, −4500 V. All measurements were in arbitrary units unless specified otherwise. Nitrogen was used as the collision and source gas. All parameters were optimized individually using flow injection analysis (FIA) of standard solutions to screen a large number of parameters.

Chromatogram peaks from plant extracts were assigned by comparison with standard chromatograms. Analyte concentration was determined using quadratic standard curves prepared by LC−MS analysis on sequential dilutions of the synthetic standards. Peak areas were measured using Analyst version 1.5 from Applied Biosystems. Solutions for standard curves were obtained by diluting the stock solution of standards 1:1 with methanolic amaranth extract (matrix) devoid of the analytes (except **6**) in question, and then diluting it further with a 1:1 mixture of water and methanolic matrix. This ensured standard solutions contained the same amount of matrix as the examined samples, so that any matrix effect in the standards would be consistent with that in the samples. In the case of analyte **6**, the unspiked sample response was subtracted from the spiked sample. The solutions were filtered through Phenomenex "Phenex" PTFE disk filters (pore size 0.45 μm) before analysis.

Recovery and Variability. Before extraction, the dry, homogenized plant samples were spiked by slowly adding 100 μL of methanolic solution

Table 2. Mass Couples and Retention Times for LC−MS/MS Analysis of Six Cinnamoylphenethylamines

compd	Q1 (*m*/*z*)	Q3 (*m*/*z*)	t_R (min)
2	298.0	135.2	5.6
3	328.2	148.1	5.8
4	341.9	220.0	7.2
5	282.1	119.0	7.3
6	312.1	148.0	7.6
7	342.2	190.1	8.4

Table 3. Chromatographic Method for LC−MS/MS of Six Cinnamoylphenethylamines

time (min)	solvent A (%)	solvent B (%)
0	70	30
10	70	30
21	10	90
23	10	90
25	70	30
35	70	30

Table 4. Mass Spectrometry Compound-Dependent Parameters

compd	DP	EP	CEP	CE	CXP
2	−35	−10	−22.7	−40	−2.0
3	−35	−9	−23.8	−40	−1.0
4	−25	−12	−24.3	−30	−1.0
5	−35	−9	−22.1	−30	−1.5
6	−35	−9	−23.2	−40	−1.0
7	−45	−11	−24.3	−30	−1.0

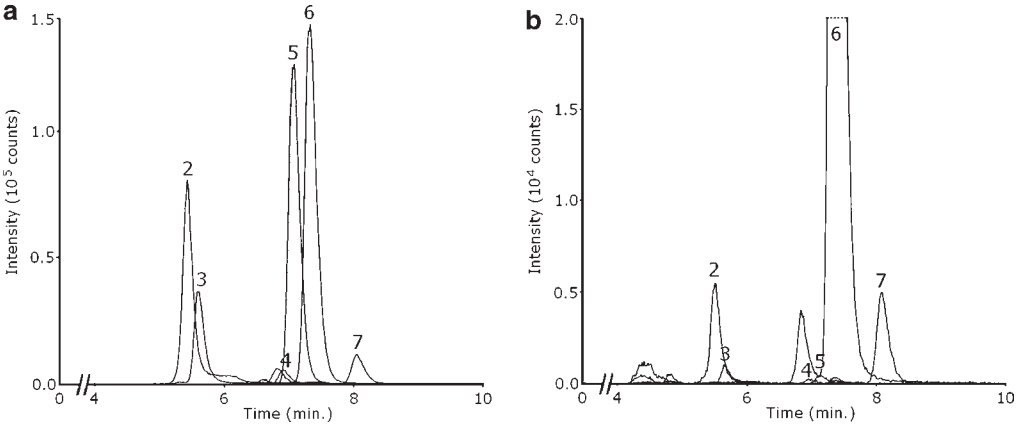


Figure 1. (**a**) MRM chromatogram of standards **2**−**7**. (**b**) MRM chromatogram of an extract from *Amaranthus hypochondriacus* var. San Antonio (**D**), from Argentina.

of the analyte to the plant matrix in the extraction vessel, and allowing the solvent to evaporate. The final low and high concentration spikes varied as follows: approximately 5 and 25 ppm for compounds **2**−**4**, approximately 0.2 and 25 ppm for **5** and **7** and approximately 0.5 and 50 ppm for **6**. The samples were then extracted and quantified as described above. The recovery percentage was calculated as a linear function of concentration (not shown) because validation experiments were carried out at two concentrations per analyte. The following experimental design was used for each concentration of each analyte in order to assess variability due to extraction, apparatus performance and day-to-day variation: spiking and extraction was repeated six times; analysis of one extract was repeated six times; and the six analyses were performed three days in a row. Student's t distribution was used to determine the 90% confidence intervals.

Antifungal Activity. The antifungal activity of the six phenethylamines was assessed by preparing analyte-laced potato dextrose agar growth medium in 5 cm Petri dishes in concentrations of 1000, 100, 10, 1, 0.1, and 0.01 ppm for each analyte. A 20 μL droplet of spore solution (1000 spores mL^{-1}) was pipetted onto the center of each plate before incubating for five days at 22 °C under black light. Fungal growth inhibition was assessed by comparing the diameter of the fungal colony with an uninhibited control. Each assay was performed in triplicate.

RESULTS AND DISCUSSION

Validation of the Quantitative Analytical Method. Compounds **2**−**7** were selected through preliminary SIM runs on plant sample extracts and confirmed by comparing retention times with standards. The method matched those compounds to plant extracts by both retention time and mass couple, giving positive identification. **Figure 1** shows the standard chromatogram (**Figure 1a**) compared to a plant sample chromatogram (**Figure 1b**).

When baseline separation was not achieved, the analytes were separated by mass and fragmentation in the mass spectrometer. Only compounds **4** and **7** could not be resolved in Q1 or Q3 due to their shared mass couple at m/z = 342 (Q1) and m/z = 190 (Q3), but they were separated chromatographically; analyte **4** was monitored using a unique Q3 fragment at m/z = 220. **Table 5** shows the quadratic functions that relate analyte concentration to LC−MS response for a range of 0 to 250 ppb. The correlation coefficient (R^2) was greater than 0.99 in all cases.

The validation experiments demonstrated the reliability of analysis down to a concentration of approximately 5 ppm for compounds **2**−**4**, 0.2 ppm for compound **5**, and 1 ppm for compounds **6** and **7**. Below these concentrations, the variability became very large, as exemplified by compounds **6** and **7** at 0.5 and 0.2 ppm, respectively (**Table 6**). According to the additivity of variances, the total standard deviation is related to those of the individual experimental procedures by the equation in **Figure 2**, where s_e, s_a, and s_{dd} are the extraction, analysis, and day-to-day

Table 5. Standard Curve Coefficients (Area = $a + b$[analyte] + c[analyte]^2) for Quantitative Analysis of Six Cinnamoylphenethylamines in LC−MS/MS

compd	a	b	c
2	109.57	3865.53	−10.78
3	267.40	2161.74	−4.94
4	63.36	675.12	−0.65
5	50.81	22493.65	−198.80
6	2113.26	10020.97	−3.85
7	159.95	2035.44	−1.55

Table 6. Recovery Values and Relative Standard Deviations of the Analytical LC−MS/MS Process[a]

compd	C_{obs}	$C_{analyte}$	recovery (%)	s_{1-6}	s_d	s_e	s_{dd}	s_a	s_{tot}	CI_{90}
2	1.8	4.8	37.87	9.5	5.4	7.2	4.8	6.3	12.4	±0.48
	9.7	24.0	40.23	3.3	2.5	2.5	2.3	2.2	4.6	±0.91
3	1.4	5.2	26.48	14.3	6.6	13.4	6.3	5.1	16.5	±0.70
	6.8	25.8	26.52	5.7	3.1	4.0	2.6	4.0	7.4	±1.55
4	3.2	5.6	57.96	8.5	4.6	1.3	3.0	8.4	12.4	±0.56
	15.2	27.8	54.80	3.5	1.7	1.5	1.0	3.2	4.8	±1.10
5	0.1	0.2	47.38	8.2	3.9	5.0	2.9	6.4	10.8	±0.02
	18.1	23.3	77.83	6.9	4.0	6.6	4.0	1.8	8.1	±1.56
6	0.3	0.5	61.90	21.4	12.7	13.3	10.7	16.8	29.2	±0.13
	35.8	52.1	68.79	2.4	1.9	2.2	1.9	1.0	3.2	±1.39
7	0.1	0.2	61.14	37.3	14.1	33.0	12.2	17.4	42.9	±0.08
	14.8	23.5	63.09	4.3	2.6	3.8	2.5	2.1	5.4	±1.04

[a] C_{obs} and $C_{analyte}$ are the observed and actual analyte concentrations in μg/g of dry plant weight. s^2_{1-6} was measured and converted to s^2_e by subtracting s^2_a; s^2_d was measured and converted to s^2_{dd} by subtracting $s^2_a/6$. s^2_{tot} is the sum of s^2_e, s^2_a, and s^2_{dd}. See the text and **Figure 2** for an explanation of the relative standard deviations given.

$$s_{tot} = \sqrt{s_e^2 + s_a^2 + s_{dd}^2} = \sqrt{s_{1-6}^2 + s_d^2 - \frac{s_a^2}{6}}$$

Figure 2. Equation relating experimentally observed variances to the total.

standard deviations expressed in terms of the experimentally observed standard deviations; s_{1-6} between six extractions analyzed, each of which was analyzed once, s_a as the pooled standard deviation of six analyses on each of three days, and s_d as the standard deviation of the means of six analyses over three days. Therefore, s_a was the only directly observable standard deviation; s_e and s_{dd} had to be calculated using the additivity of variances for a total of six standard deviations, of which three were measured and three were calculated, with one of the latter representing the

Table 7. Limits of Detection and Quantitation (μg/g) for the Quantitative Analytical LC−MS/MS Method for Six Cinnamoylphenethylamines

	2	3	4	5	6	7
L_D	1.75	2.55	2.06	0.08	0.46	0.30
L_Q	5.83	8.50	6.86	0.25	1.52	1.01

total. These experimental standard deviations are summarized in **Table 6**.

While the reproducibility was good, the recovery was quite low for compounds **2** and **3**. We believe this was related to the ease of oxidation of these compounds. However, only further investigations will determine whether such a hypothesis holds true. Interestingly, compounds **4**−**7** all possess two free hydroxyl groups, while **2** and **3** possess three. Phenolic amides are known antioxidants, and the correlation between ease of oxidation and number of free hydroxyl groups has been previously noted (*3*).

Concentration Levels in Amaranth Samples. Cinnamoylphenethylamines were quantified in four *Amaranthus* samples, two of which, *A. hypochondriacus* var. Nutrisol (**A**, **B**), were from Mexico, and the remaining two, *A. mantegazzianus* var. Don Juan (**C**) and *A. hypochondriacus* var. San Antonio (**D**), were from Argentina. The results are shown in **Table 8**. Not all of the examined cinnamoylphenethylamines were present over the limit of quantitation (L_Q) or even the limit of detection (L_D), but they are included nonetheless. Both the L_D and L_Q were calculated following EURACHEM guidelines and are defined by convention as 3 and 10 times the baseline standard deviation, respectively. Quantitation is possible below these thresholds, although the size of the standard deviation is comparable to the size of the signal at the L_D. Because the baseline is too weak in multiple reaction mode, the standard deviations of the lowest spike concentrations were used. These signals have a higher absolute standard deviation than the baseline, and therefore quantification was possible below the corresponding L_D and L_Q. Hence, the concentrations for all detected compounds were included. The limits of detection and quantitation for the entire method are given as $3s_{tot}$ and $10s_{tot}$ respectively, as listed in **Table 7**.

It is possible that recovery of phenolic amides from inside cells in the ground plant samples is less efficient than recovery of the added amide which was deposited on the surface of the plant particles. There is no definite way of assessing this error. The extraction of spiked samples for assessing recovery was employed with the intention of following the EURACHEM guidelines.

Compound **7**, specifically, showed a rare 4-*O*-methylation of the dopamine moiety. It was first isolated from the roots of *Chenopodium album* in 1993 (*11*) (whose family, Chenopodiaceae, has since been incorporated into the Amaranthaceae according to some reclassifications). Feruloyl-4-*O*-methyldopamine has since been reported several times in the Amaranthaceae (*12*−*14*), but only once outside that family (*1*), and may thus be of taxonomic significance. Our findings indicate that it is particularly common in the genus *Amaranthus*, as we here report it in two varieties of *A. hypochondriacus* as well as in *A. mantegazzianus*, species which are from distinct locations. In the samples from Mexico, the concentration of **7** exceeds even that of **6**. Compounds **2**, **4**, and **5** are well-known, but **3** is rare and none of compounds **2**−**5** have previously been reported in the Amaranthaceae. **Table 8** shows the concentration of cinnamoylphenethylamines in the investigated plant samples. Detecting four cinnamoylphenethylamines novel to the genus *Amaranthus* implies that the same approach may be advantageously applied to other genera.

Compounds **2**−**7** represent only a fraction of the naturally occurring cinnamoylphenethylamines, but one or more of them have been reported in the following families: Alliaceae (*15*), Amaranthaceae (*12*−*14*), Anacardiaceae (*16*), Annonaceae (*17*), Aristolochiaceae (*18*), Cannabidaceae (*19*), Chenopodiaceae (*11*), Convolvulaceae (*2*), Euphobiaceae (*20*), Flacourtiaceae (*21*), Fumariaceae (*22*), Hernandiaceae (*23*), Lauraceae (*24*), Leguminosae (*25*), Liliaceae (*26*), Magnoliaceae (*27*), Malvaceae (*28*), Menispermaceae (*29*), Monimiaceae (*30*), Nyctagenaceae (*1*), Papaveraceae (*31*), Piperaceae (*32*), Plumbaginaceae (*33*), Polygonaceae (*34*), Portulacaceae (*35*), Ruscaceae (*36*), Rutaceae (*9*), Solanaceae (*10*, *37*, *38*), Ulmaceae (*39*), Vitaceae (*40*), and Zygophyllaceae (*41*).

Table 8. Cinnamoylphenethylamine Occurrence in Plant Samples

	concentration in dry plant material (μg/g)			
compd	*A. hypochondriacus* var. Nutrisol, Mexico[a] (**A**)	*A. hypochondriacus* var. Nutrisol, Mexico[a] (**B**)	*A. mantegazzianus* var. Don Juan, Argentina[b] (**C**)	*A. hypochondriacus*, var. San Antonio, Argentina[b] (**D**)
2	0.71	0.48	4.47	22.31
3	0.72	0.16	0.53	10.27
4	n.d.	n.d.	0.60	5.67
5	n.d.	n.d.	0.65	0.35
6	5.26	5.26	114.31	113.99
7	10.87	7.38	9.49	31.64

[a] Mean of duplicate analysis. [b] Mean of triplicate analysis.

The fortuitous nature of cinnamoylphenethylamine discovery from natural sources, as well as the variety of synthetic work carried out to date, together with the plethora of associated biological activities of these compounds and their synthetic analogues, makes it difficult to provide a systematic overview of research carried out thus far. Tyramine derivatives predominate in the literature (*38*), but dopamine derivatives are also found (*37*) in addition to rarer, substituted, dopamine derivatives, such as **7** (feruloyl-4-*O*-methyldopamine). It has been established that cinnamate derivatives of the phenylpropanoid pathway provide the acid moieties (*42*).

Several other related compounds, such as caffedymine (*43*), rubemamin (*44*), and feruloyl-3-*O*-methyldopamine (*18*), have been documented. Outside of cinnamoylphenethylamines, cinnamoyltryptamines (*45*) have been reported as well.

Antifungal Effects. Compound **3** was found to inhibit the growth of a strain of the fungus *Fusarium culmorum* partially at a concentration of 100 ppm and completely at 1000 ppm. The remaining compounds were inactive. Certain cinnamoylphenethylamines are known, however, to be induced by a fungal challenge (*46*), suggesting that the mechanism of their function is more elaborate. Compound **7** from the roots of *Amaranthus gangeticus* has been reported as a zoospore attractant, providing antifungal defense together with zoospore motility-inhibiting nicotinamide (*14*).

Combinatorial Synthesis and Quantitative LC−MS/MS as an Alternative to Preparative Isolation. Our analysis provides reliable detection with quantitative data and presents a favorable alternative to investigation by preparative isolation. Extraction of kilogram quantities of plant sample frequently yields only a few milligrams of cinnamoylphenethylamines. Our approach is both easier and more telling. Using simple syntheses and small quantities of plant material, the occurrence of cinnamoylphenethylamines may be investigated easily by a procedure lending itself to large-scale screening experiments. The chromatographic method is only 35 min long; all compounds elute with adequate separation within the first 10 min. This method may be expanded to include other known cinnamoylphenethylamines while adjusting the chromatographic method to provide adequate separation of overlapping mass couples, and may even prove suitable for other

families of compounds that can be produced by combinatorial synthesis.

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