JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Structure−Antifungal Activity Relationship of Cinnamic Acid Derivatives

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A structure−antifungal activity relationship (SAR) study of 22 related cinnamic acid derivatives was carried out. Attention was focused on the antifungal activities exhibited against *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger*. (*E*)-3-(4-Methoxy-3-(3-methylbut-2-enyl)phenyl)acrylic acid (**16**) exhibited antifungal activity against *A. niger*, comparable to that of miconazole and a significant antifungal effect against *A. flavus* and *A. terreus* as well. A structure–activity relationship (SAR) study of related cinnamic acid derivatives has allowed a model to be proposed for the recognition of the minimal structural requirements for the antifungal effect in this series.

KEYWORDS: Antifungal; aspergillosis; cinnamic acid derivatives; structure–activity relationship

INTRODUCTION

The *Aspergillus* genus is composed of more than 180 different species. Fortunately, only a few species are able to produce infections. Three species, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus*, cause about 95% of the pathologic cases. Unfortunately, the available fungicides have not been effective in the control of these fungi, and therefore there is a real need for novel compounds against aspergillosis. *Aspergillus niger* is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis, can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections) which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympani membrane.

A major problem in corn and other cereal crops concerns contamination with aflatoxins that are produced by the fungi *A. flavus* and *Aspergillus parasiticus*. These compounds constitute a number of structurally related secondary metabolites, which differ considerably in their biological effect. Aflatoxin B_1 is by far the most potent teratogen, mutagen, and hepatocarcinogen of all aflatoxins (*1*). The carcinogenic potential of aflatoxin B_1 following oral administration has been shown in several animal species, including rodents, nonhuman primates, and fishes (*2*). The toxicological data and extensive investigations on association between aflatoxin exposure and hepatocellular carcinoma led to the classification of aflatoxin B_1 as a category 1 human carcinogen by the International Agency for Research of Cancer (*3–6*).

The study of natural products as sources for lead structures has enjoyed a resurgence of interest over the last 20 years. The reasons for this are varied, but the success and the uniqueness of natural products are probably the most important. Virtually all plants share the problems of recognition and reaction in defense mechanisms, and different processes have evolved for combating pathological challenges (*7*). Natural compounds and their derivatives could potentially serve as effective alternatives to conventional antifungal agents. Conventional fungicides are frequently perceived to present hazard to human health and environment (*8, 9*). Several phenolic compounds and derivatives have been reported possessing antifungal activity. Gallic acid has been reported to prevent aflatoxin biosynthesis by *A. flavus* (*10*). Two new natural products [1-(3′-methoxypropanoyl)-2,4,5-trimethoxybenzene and 2-(2*Z*)-(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol] isolated from the root bark of *Cordellia alliodora* have been reported as antifungal and larvicidal compounds (*11*). More recently, Kim et al. (*12*) reported antifungal effects of several phenolic compounds including cinnamic acid (**1**), *m*-coumaric acid (**12**), *p*-coumaric acid (**3**), and caffeic acid (**6**).

The exudate of the aerial parts of the perennial shrub *Baccharis grisebachii* has been shown to display activity toward dermatophytic fungi and some bacteria (*13*). Gianello and Giordano (*14*) reported the isolation of 3-(γ,γ-dimethylallyl)-*p*-coumaric acid (**14**) from the aerial parts of *B. grisebachii*

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10.1021/jf0729098 CCC: $37.00
Published on Web 11/27/2007

collected in the Province of Mendoza, Argentina. Antimicotic effects toward the dermatophytes *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton mentagraphytes*, and *Trichophyton rubrum* were reported for this compound. However, compound **14** did not show antifungal activity against *A. fumigatus*, *A. flavus*, and *A. niger* (*13*).

Our principal goal is to obtain new compounds possessing antifungal effects. In particular, we are interested in structures with antifungal activity against fungi, such as *A. flavus*, *A. terreus*, and *A. niger*. In the present study we chose 3-(γ,γ-dimethylallyl)-*p*-coumaric acid (**14**), known as drupanin, and cinnamic acid (**1**) as the starting structures. To perform a structure–activity relationship study, several derivatives of compounds **1** and **14** were prepared and tested. Thus, we report here the preparation of a series of cinnamic acid derivatives, their antifungal effects, acute toxicity, and a structure–activity relationship (SAR) study performed in this series.

MATERIALS AND METHODS

General Methods. Solvents were distilled and dried prior to use. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AC-200 and AC-400 (200 and 400 MHz and 50.2 and 100 MHz, respectively) spectrometers in $CDCl_3$; chemical shifts (δ) in parts per million are relative to TMS; coupling constants (*J*) are in Hertz. Analytical TLC was performed on Merck precoated silica gel 60 F_{254} plates. Solvents for TLC were *n*-hexane−EtOAc mixtures. *A. niger* ATCC 11394 was purchased from the American Type Culture Collection, while *A. flavus* UBA 294 and *A. terreus* INM 031783 were obtained from the Micoteca Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and Instituto "Carlos Malbran", Argentina, respectively. Fungi were maintained on PDA−slants. Spore suspensions were obtained according to reported procedures (*15*) and adjusted to 10^7 spores with colony forming ability per milliliter in PBS with added Tween 20 (5 μL/mL).

Compounds. Cinnamic acid (**1**), methyl cinnamate (**2**), (*E*)-3-(4-hydroxyphenyl)acrylic acid (*p*-coumaric acid) (**3**), (*E*)-3-(3,4-dihydroxyphenyl)acrylic acid (caffeic acid) (**6**), (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylic acid (ferulic acid) (**8**), and (*E*)-3-(3-hydroxyphenyl)acrylic acid (*m*-coumaric acid) (**12**) were purchased from Sigma-Aldrich Chemical Co., Buenos Aires, Argentina.

*Isolation of Drupanin (**14**).* (*E*)-3-(4-Hydroxy-3-(3-methylbut-2-enyl)phenyl)acrylic acid was isolated as colorless crystals (mp 147−148 °C) from aerial parts of *B. grisebachii* Hieron. Its chemical identity was confirmed by spectroscopic data, compared with previous reports (*14*).

*Preparation of (E)-Methyl 3-(4-Hydroxyphenyl)acrylate (**4**).* Compound **3** (100 mg, 0.61 mmol) was methylated with an Et_2O solution of CH_2N_2 at 0 °C for 2 h. Purification by column chromatography on Si gel using *n*-hexane−EtOAc (5:5) as solvent yielded 80.4 mg (74%) of **4**. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*16, 17*).

*Preparation of (E)-Methyl 3-(4-Methoxyphenyl)acrylate (**5**).* Compound **3** (100 mg, 0.61 mmol) was dissolved in 20 mL of acetone, 294 mg (2.14 mmol) of K_2CO_3 and 122 μL (1.29 mmol) of Me_2SO_4 were added, and and the solution was heated to reflux. After reaction completion (3 h) the reaction mixture was filtered and rinsed with EtOAc. Purification was carried out by column chromatography in Si gel with *n*-hexane−EtOAc (7:3) as solvent, yielding 79.5 mg (68.%) of **5**. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*16*).

*Preparation of (E)-Methyl 3-(3,4-Dihydroxyphenyl)acrylate (**7**).* Compound **6** (100 mg, 0.52 mmol) was dissolved in MeOH, and two drops of HCl (35%) was added. The mixture was stirred and heated to reflux overnight; after reaction completion it was neutralized with solid Na_2CO_3 and partitioned with EtOAc. Purification was carried out by column chromatography on Si gel with *n*-hexane−EtOAc (4:6) as solvent, yielding 86.4 mg (80%) of **7**: white crystals; mp 161–163 °C (*18*).

*Preparation of (E)-Methyl 3-(3,4-Dimethoxyphenyl)acrylate (**9**).* Compound **8** (100 mg, 0.51 mmol) was dissolved in 20 mL of acetone, 255 mg (1.78 mmol) of K_2CO_3 and 122 μL (1.29 mmol) of Me_2SO_4 were added, and the solution was heated to reflux. After reaction completion (3 h) the reaction mixture was filtered and rinsed with EtOAc. Purification was carried out by column chromatography on Si gel with *n*-hexane−EtOAc (7:3) as solvent, yielding 103 mg (90%) of **9** as pale yellow crystals; mp 63–66 °C. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*16*).

*Preparation of (E)-3-(3,4-Dimethoxyphenyl)acrylic Acid (**10**).* Compound **9** (50 mg, 0.22 mmol) was dissolved in 5 mL of THF, and 100 μL of H_2O was added. Then, 50 mg of KOH was added; the mixture was stirred and heated to reflux for 1 h. After reaction completion the mixture was brought to pH 4 by the addition of HCl and the solvent evaporated in vacuo. The aqueous phase was extracted three times with Et_2O and the organic layer washed with H_2O and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. Purification was carried out by column chromatography on Si gel with *n*-hexane−EtOAc (7:3) as eluent, yielding 37 mg (78%) of **10** as white crystals: mp 183–184 °C). The NMR spectra were in agreement with reported data (*19*).

*Preparation of (E)-Methyl 3-(4-Hydroxy-3-methoxyphenyl)acrylate (**11**).* Compound **8** (100 mg, 0.51 mmol) was methylated with an Et_2O solution of CH_2N_2 at 0 °C for 2 h. Purification by column chromatography on Si gel using *n*-hexane−EtOAc (5:5) as solvent yielded 74 mg (69%) of **11** as white crystals: mp 49–51 °C. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*17*).

*Preparation of (E)-Methyl 3-(3-Hydroxyphenyl)acrylate (**13**).* Compound **12** (100 mg, 0.61 mmol) was dissolved in 20 mL of MeOH, and two drops of HCl (35%) was added. The mixture was stirred and heated to reflux overnight. After reaction completion it was neutralized with solid Na_2CO_3 and partitioned with EtOAc. Purification was carried out by column chromatography on Si gel with *n*-hexane−EtOAc (5:5) as solvent, yielding 92 mg (85%) of **13**. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*17*).

*Preparation of (E)-Methyl 3-(4-Methoxy-3-(3-methylbut-2-enyl)-phenyl)acrylate (**15**).* Compound **14** (100 mg, 0.43 mmol) was dissolved in 20 mL of acetone, 208 mg (1.5 mmol) of K_2CO_3 and 122 μL (1.29 mmol) of Me_2SO_4 were added, and the solution was heated to reflux. After reaction completion (3 h) the reaction mixture was filtered, and 20 mL of EtOAc was added. Then, the solvent was evaporated under reduced pressure, and the crude residue was subjected to column chromatography on Si gel with *n*-hexane−EtOAc (7:3) as eluent, yielding 68 mg (61%) of **15** as a colorless oil. The NMR spectrum was in agreement with reported data (*20*).

*Preparation of (E)-3-(4-Methoxy-3-(3-methylbut-2-enyl)phenyl)acrylic Acid (**16**).* Compound **15** (100 mg, 0.38 mmol) was dissolved in 5 mL of THF, and 100 μL of H_2O was added. Then, 50 mg of KOH was dissolved, and the mixture was stirred and heated to reflux for 1 h. After reaction completion the mixture was brought to pH 4 by the addition of HCl and the solvent evaporated in vacuo. The aqueous phase was extracted three times with Et_2O and the organic layer washed with H_2O and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. Purification was carried out by column chromatography on Si gel with *n*-hexane−EtOAc (5:5) as eluent, yielding 72 mg (76%) of **16**. ^{1}H NMR and ^{13}C NMR data were in agreement with literature data (*21*).

*Preparation of (E)-Methyl 3-(4-Hydroxy-3-(3-methylbut-2-enyl)phenyl)acrylate (**17**).* Compound **14** (100 mg, 0.43 mmol) was methylated with an Et_2O solution of CH_2N_2 at 0 °C for 2 h. Purification by column chromatography on Si gel using *n*-hexane−EtOAc (6:4) as eluent yielded 76 mg (72%) of **17** as pale yellow crystals: mp 81–84 °C (*22, 23*). ^{1}H NMR and ^{13}C NMR data were in agreement with those previously reported (*20*).

*Preparation of (E)-3-(4-Acetoxy-3-(3-methylbut-2-enyl)phenyl)acrylic Acid (**18**).* Compound **14** (100 mg, 0.43 mmol) was dissolved in 2 mL of dry pyridine, and 2 mL of acetic anhydride was added. The mixture was kept at room temperature overnight. After all of the starting material was consumed (TLC control), the mixture was poured into a beaker containing ice and 30 mL of Et_2O. The organic layer was washed with an aqueous solution of 5% $CuSO_4$ to remove pyridine, followed by a

5% solution of $NaHCO_3$ and water. After drying over anhydrous Na_2SO_4, the organic solvent was removed in vacuum, and the residue was subjected to Si gel column chromatography using *n*-hexane−EtOAc (7:3) to yield 95.5 mg (81%) of 4-acetyl-3-prenyl-*p*-coumaric acid (**18**): colorless crystals; mp 141 °C. Spectroscopic and spectrometric data were in agreement with those previously reported (*14*).

*Preparation of Methyl 3-(4-Hydroxy-3-isopentylphenyl)propanoate (**19**).* To **17** (100 mg, 0.40 mmol) dissolved in EtOAc (50 mL) was added a catalytic amount of 5% Pd/C. The suspension was stirred vigorously under hydrogen (1 atm). When the consumption of H_2 subsided, the mixture was filtered through a thin layer of Si gel over Celite and concentrated under reduced pressure. The residue was subjected to column chromatography on Si gel with *n*-hexane−EtOAc (7:3) as eluent to yield 84.7 mg (87%) of **19** as pale yellow crystals: mp 172–175 °C. Spectroscopic data were in agreement with those reported by Carrizo et al. (*24*).

*Preparation of 3-(4-Hydroxy-3-methoxyphenyl)propanoic Acid (**20**).* To **8** (100 mg, 0.51 mmol) dissolved in EtOAc (50 mL) was added a catalytic amount of 5% Pd/C. The suspension was stirred vigorously under hydrogen (1 atm). When the consumption of H_2 subsided, the mixture was filtered through a thin layer of Si gel over Celite and concentrated under reduced pressure. The residue was subjected to column chromatography on Si gel with mixtures of *n*-hexane−EtOAc (7:3) as eluent to yield 85 mg (84%) of **20**. ^{1}H NMR and ^{13}C NMR data were in accordance with literature (*25*).

*Preparation of Methyl 3-(4-Hydroxyphenyl)propanoate (**21**).* To **4** (100 mg, 0.51 mmol) dissolved in EtOAc (50 mL) was added a catalytic amount of 5% Pd/C. The suspension was stirred vigorously under hydrogen (1 atm). When the consumption of H_2 subsided, the mixture was filtered through a thin layer of Si gel over Celite and concentrated under reduced pressure. The residue was subjected to column chromatography on Si gel with mixtures of *n*-hexane−EtOAc (7:3) as eluent to yield 83 mg (82%) of **21** as a colorless oil. ^{1}H NMR and ^{13}C NMR data were in agreement with those previously reported (*20*).

*Preparation of 3-(3-Hydroxyphenyl)propanoic Acid (**22**).* Compound **12** (100 mg, 0.61mmol) was dissolved in EtOAc and treated as described in the preparation of compound **8** to yield 89 mg (88%) of **22**. Spectroscopic data were in agreement with literature values (*22*).

Antifungal Assays. The agar dilution method was used for the antifungal evaluations, according to reported procedures (*13, 23*). Sabouraud dextrose media were used, and stock solutions of pure compounds in H_2O/DMSO, 24:1 (1000 μg/mL), were added to give final dilutions ranged from 250 to 1.95 μg/mL. The final DMSO concentration in the media did not exceed 2%. An inoculum of 10^5 spores/mL from each agar well was used. The antifungal agent myconazole (Laboratorio Saporiti, Argentina) was included as positive control. The wells were incubated for 48 h at 28 °C. MIC was defined as the lowest compound concentration showing no visible fungal growth after incubation time. MICs $\geq$250 μg/mL were considered as inactive.

Biotransformation Experiments. A two-step procedure adapted from Orden et al. (*26*) was used. Spore suspensions from *A. terreus* were incubated in liquid soybean meal−glucose medium (glucose, 20 g; yeast extract, 5 g; soybean meal, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled water to 1000 mL, pH 7) at 28 °C on a rotatory shaker at 180 rpm. Forty-eight-hour-old cultures (5 mL) were subcultured in 125 mL Erlenmeyer flasks containing 30 mL of fresh culture medium each. After 24 h, substrates (5 mg/batch), dissolved in ethanol (50 μL), were added to the fungal cultures. Biotransformation progress was monitored daily by TLC. After 8 days of incubation, the fermentation broth was filtered, and the filtrate was extracted with EtOAc (4 × 50 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Blank assays without substrates and without fungi were carried out in parallel. Each experiment was performed three times with three replicates each.

Biotransformation of **14.** Organic extracts were pooled and purified by preparative TLC using EtOAc as eluent, yielding 2-(2-hydroxypropan-2-yl)-2,3-dihydrobenzofuran-5-carboxylic acid (**23**) as pale yellow crystals: mp 156–158 °C; ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 3, CH_3), 1.37 (s, 3, CH_3), 3.20 (d, 2, J = 8.5 Hz, H-2), 3.60 (br s, 1, OH), 4.70 (t, 1, J = 8.5 Hz, H-1), 6.82 (d, 1, J = 7.4 Hz, H-7), 7.90 (d, 1, J = 1.5 Hz, H-4), and 7.92 (dd, 1, J = 7.4 Hz, J = 1.7 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 23.79 (CH_3), 25.83 (CH_3), 29.74 (C-3), 71.56 (**C**OH(CH_3)$_2$), 90.31 (C-2), 108.77 (C-7), 123.72 (C-9), 127.11 (C-4), 127.56 (C-5), 131.66 (C-6), 165.56 (C-8), and 166.65 (**C**OOH); MS [*m*/*z* (%)] 222 (47, M), 204 (37), 189 (37), 164 (100), 119 (60), and 91 (36). These data were in agreement with those reported for anodendroic acid (*27*).

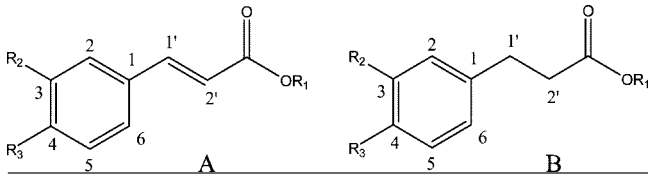
Acute Toxicity Test. The toxic effect of compounds **1**−**22** was evaluated using a toxicity test on fish and amphibians. The static technique recommended by the U.S. Fish and Wildlife Service Columbia National Fisheries Research Laboratory (*28*) was modified in order to use a lower amount of test compounds (*29*). Fish of the species *Poecilia reticulata* were born and grown in our laboratory until they reached a size of 0.7−1 cm (20 days old). Amphibians of embryo−larval stage XII (*30*) of *Bufo arenarum* were also used. In the toxicity test, at least 10 specimens were exposed to each of the three concentrations tested per drug (ranged from 10 to 2.5 μg/mL) in 2000 mL wide-mouthed jars containing 1000 mL of test solutions. The test began upon initial exposure to the potentially toxic agent and continued for 96 h. The number of dead organisms in each test chamber was recorded, and the dead organisms were removed every 24 h. General observations on the conditions of the test organism were also recorded at this time. However, the percentage of mortality was recorded at 96 h. Each experiment was performed two times with three replicates each.

RESULTS AND DISCUSSION

Kim et al. (*12*) reported that coumaric acids (**3** and **12**) were less inhibitory to fungal growth than cinnamic acid (**1**) whereas caffeic acid (**6**) showed no antifungal activity at any concentrations. Our MIC values (**Table 1**) are in agreement with those results; however, it should be noted that, in our bioassays, compound **1** displays only a moderate antifungal activity. Our results indicate that compound **14** showed no antifungal activity against *A. terreus* and *A. flavus* and only a moderate effect against *A. niger* (125 μg/mL). These results are in agreement with those previously reported by Feresin et al. (*13*). To study the structure–activity relationships, different types of structures and the effects of structural changes in different regions of the molecules were considered: elimination of the double bond of the side chain, giving the general structure type B, and changes of the R_1, R_2, and R_3 substituents in the general structure types A and B according to **Table 1**, which gives the MIC values obtained for the different compounds of this series. From these results, it is clear that compounds without the double bond in the side chain (compounds possessing the general structure type B) were inactive. This result is not unexpected; the presence of such double bond confers particular conformational and electronic characteristics to these compounds. The most active compounds in this series were compounds **8** and **16**, which possess a COOH group in the side chain. However, it is clear that the presence of this group seems to be necessary but not by itself sufficient to produce the antifungal effect. The lack of activity of compounds **3**, **6**, **10**, **12**, and **14** illustrates this situation very well. In general, it seems that a COOH group is preferred to a $COOCH_3$ group (compare MICs of compound **1** with **2**, **8** with **11**, and **16** with **15**). However, this situation is not clear when the substituents at R_2 and/or R_3 are OH groups (compare MICs of **3** with **4**, **6** with **7**, and **12** with **13**). Nevertheless, these compounds displayed only moderate or marginal antifungal effects.

Compound **16** was the most active compound in this series, displaying a MIC of 1.95 against *A. niger*, which is comparable to that of miconazole, and also possessing very interesting activities against *A. terreus* and *A. flavus* as well. The lack of activity obtained for compounds **14**, **15**, and **17** is noteworthy even though they possess a closely related structure to that of

Table 1. Structural Features and Minimal Inhibitory Concentrations (MICs) of Compounds **1–22**



					MIC (μg/mL)		
compd	type	R_1	R_2	R_3	*A. terreus*	*A. niger*	*A. flavus*
1	A	H	H	H	250	125	250
2	A	CH_3	H	H	>250	>250	>250
3	A	H	H	OH	>250	125	>250
4	A	CH_3	H	OH	125	125	125
5	A	CH_3	H	OCH_3	>250	>250	>250
6	A	H	OH	OH	>250	>250	>250
7	A	CH_3	OH	OH	125	125	>250
8	A	H	OCH_3	OH	>250	62.5	31.25
9	A	CH_3	OCH_3	OCH_3	>250	>250	>250
10	A	H	OCH_3	OCH_3	>250	>250	>250
11	A	CH_3	OCH_3	OH	>250	>250	>250
12	A	H	OH	H	>250	>250	>250
13	A	CH_3	OH	H	125	125	125
14	A	H	γ,γ-dimethylallyl	OH	>250	125	>250
15	A	CH_3	γ,γ-dimethylallyl	OCH_3	125	>250	>250
16	A	H	γ,γ-dimethylallyl	OCH_3	31.25	1.95	62.5
17	A	CH_3	γ,γ-dimethylallyl	OH	125	>250	250
18	A	H	γ,γ-dimethylallyl	OAc	>250	62.5	>250
19	B	CH_3	isopentyl	OH	>250	>250	>250
20	B	H	OCH_3	OH	>250	>250	>250
21	B	CH_3	H	OH	>250	>250	>250
22	B	H	OH	H	>250	>250	>250
miconazole (control +)					3.9	15.62	15.62

16. The replacement of COOH by $COOCH_3$ in the side chain of **15** and **17** could explain, at least in part, the lack of activity (or the only marginal effect on some fungi) obtained for these compounds. However, this is not the case for compound **14**. It is interesting to note that the only structural difference between compounds **14** and **16** is the replacement of OH by OCH_3 in R_3. Whereas compound **16** was the most active molecule in this series, compound **14** was inactive. There are several explanations for the lack of activity obtained for compound **14**. One possibility is that compounds **14** and **16** could interact chemically with the fungi in a different way. Thus, in an attempt to explain the difference in bioactivity of compounds **14** and **16** toward *A. terreus*, both compounds were used as substrates in whole cell biotransformation procedures. The inactive natural product **14** was metabolized into a more polar metabolite, **23** (**Figure 1**), after 8 days of incubation in a two-step biotransformation procedure. Compound **23** was recovered from the culture media as the sole biotransformation product; meanwhile, the substrate was completely consumed.

Its ^{1}H NMR spectrum shows two singlets at high fields (1.25 and 1.37 ppm) assigned to the methyl groups on the side chain at C-1. In the HSQC spectrum both singlets show a strong cross-peak with two carbons at 23.79 and 25.83 ppm, respectively. The HMBC shows the long-range interactions of both methyl groups with two oxygenated carbons, a singlet at 71.56 ppm and a doublet at 90.31 ppm. The doublet at 3.20 ppm in the ^{1}H NMR, which integrates for two hydrogens, is coupled with a triplet of a proton at 4.70 ppm located on an oxygenated carbon (90.31 ppm). These data are supported with the correlation observed between both signals in the COSY spectrum. In the HSQC they show correlations with C-2 at 29.73 ppm. On the other hand, the HMBC spectrum shows the long-range correlations between the methyl and the methylene signals with the oxygenated C-2 at 90.31 ppm. In the same spectra the methylene group shows a cross-peak with aromatic carbon C-4 at 127.56 ppm, oxygenated carbon C-2 at 90.31 ppm, and a cross-peak with the singlet at 71.56 ppm attributable to a quaternary alcoholic carbon. At low fields, in the ^{1}H NMR spectrum, three signals integrating for one proton each, corresponding to the aromatic hydrogens, are evident. The doublet (J = 7.4 Hz) at 6.82 ppm is coupled with a double doublet at 7.92 ppm (J = 7.4 and 1.5 Hz), indicating that the hydrogens present an ortho coupling. Meanwhile, the former signal is coupled with the other doublet (J = 1.5 Hz), indicative of meta coupling. These data are in complete agreement with the strong correlations observed in the COSY spectra. In addition, the HSQC relates the doublet at 6.82 ppm with carbon C-7 at 108.77 ppm and the other aromatic signals with carbon C-6 at 131.66 ppm and C-4 at 127.11 ppm, respectively. The described data indicate that the aromatic ring is substituted at C-5 with a carboxylic acid group (164.35 ppm). These data are consistent with the structure of the bicyclic metabolite 2-(2-hydroxypropan-2-yl)-2,3-dihydrobenzofuran-5-carboxylic acid which was previously reported as anodendroic acid from the species *Anodendron affine* (Apocynaceae) and *Eriodictyon sessilifolium* (Hydrophyllaceae) (*30*).

A probable route for the biotransformation is depicted in **Figure 1** and could be rationalized, as cleavage of acetate from

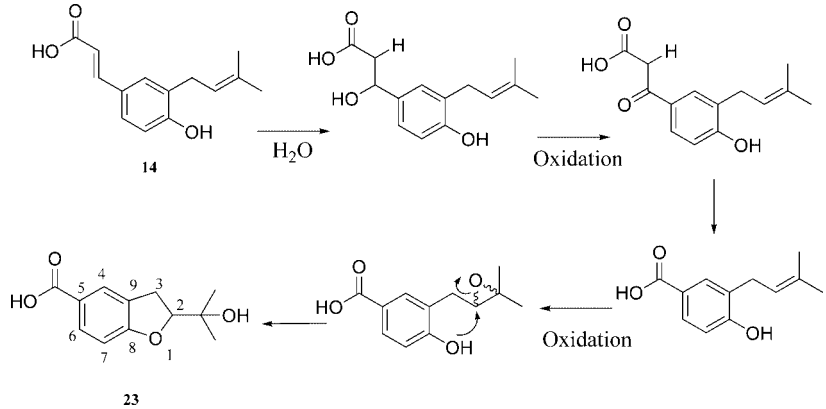


Figure 1. Drupanin biotransformation pathway.

the unsaturated side chains is one of the most common metabolic pathways of cinnamates in fungi. The possible mechanism was depicted by Rosazza et al. (*31*), who proposed water addition to the double bond, a subsequent oxidation, and a further removal of an acetate unit to yield a benzylic acid moiety. The requirements of cofactors by this process suggest the involvement of a classical β-oxidation process like fatty acid degradation. In addition, the dihydrofuran ring formation could be rationalized through the enzymatic oxidation of the side chain double bond catalyzed by a monooxygenase and the subsequent nucleophilic attack of the phenolic hydroxyl group on the less substituted carbon of the oxirane moiety. The pathway described is in agreement with the existence of several pulvinone derivatives in *A. terreus* cultures reported by Ojima et al. (*32*), since it supposes the expression of the necessary enzymes to catalyze the proposed reactions in this fungal species. This experiment does not offer enough evidence to demonstrate which of the two side chain degradation routes is occurring first. However, these data are irrelevant for the purpose of the present study, which was to find out if *A. terreus* has the ability of metabolizing the compound that was inactive in the antifungal assay.

When the most active derivative of this series, **16**, was added as substrate in a parallel experiment, no biotransformation products were recovered from the culture media, but the substrate was completely incorporated into the fungal development. From our results it is reasonable to think that compound **17** could undergo the same biotransformation process as that of compound **14**, considering that both compounds possess the same substituents at R_2 and R_3.

On the other hand, it is also possible to understand the inactivity of the derivative **18** toward *A. flavus* and *A. terreus*. It should be noted that this compound has the phenolic hydroxyl group derivatized by an acetyl moiety, which could be hydrolyzed by the fungus. The slight activity of this compound toward *A. niger* could be due to a different possibility of this strain of metabolizing the ester moiety. This result is an additional support for our assumption.

In general, the toxicity of the antifungal agents is a critical aspect for their usefulness and limitations. Thus, in addition to the antifungal evaluation, the toxic effect of compounds **1**−**22** was evaluated using a toxicity test on fish and amphibians. Only compound **17** displayed a significant acute toxicity in fish even at low concentrations (5 μg/mL). The acute toxicity in fish of this compound is comparable to those of the reference compounds (miconazole and griseofulvin). The rest of the compounds do not display any toxic effects at 2.5, 5, and 10 μg/mL. The results obtained in amphibians are in complete agreement with the percentage of mortality in fish. Only compound **17** showed toxicity (100%) at 10 μg/mL; the rest of the compounds tested did not show any toxic effect. It is interesting to note that compound **17** has been previously reported as a potent antimutagenic agent (*23*), indicating that our results are in agreement with those data previously reported. From the comparison of activities, it is clear that the antifungal activity of compounds **16** and **8** appears not to be due to their toxic properties. Only compound **17** possesses acute toxicity at low concentrations. The compounds that displayed good antifungal activities showed very low acute toxicity.

In summary, we report here a group of 22 related cinnamic acid derivatives acting as antifungal agents. Among them, compounds **16** and **8** and some of their congeners exhibited remarkable antifungal activity against *A. terreus*, *A. niger*, and *A. flavus*. The antifungal activity of these compounds along with the low toxicity gives a hope for the future development of nontoxic new antifungal agents. A SAR study on these compounds has allowed a model to be proposed for the recognition of the minimal structural requirements for the antifungal effect.

ACKNOWLEDGMENT

R.E. and M.K.-S. are members of the CONICET staff.

LITERATURE CITED

(1) Bubsy, W.; Wogan,G. Aflatoxins. In *ACS Monographs*; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1984; Vol. 2, pp 954−1136.

(2) Roebuck, B. D.; Maxuitenko, Y. Y. In *The toxicology of aflatoxins: Human health, veterinary and agricultural significance*; Eaton, D. L., Groopman, J. D., Eds.; Academic Press: San Diego, CA, 1994; pp 27–44.

(3) Bruce, R. D. Risk assessment for aflatoxin: II. Implications of human epidemiology data. *Risk Anal.* **1990**, *10*, 561–569.

(4) Gorelic, N. J. Risk assessment for aflatoxin: I. Metabolism of aflatoxin B1 by different species. *Risk Anal.* **1990**, *10*, 539–559.

(5) Kuiper-Goodman, T. Risk assessment to humans of mycotoxins in animal-derived food products. *Vet. Hum. Toxicol.* **1991**, *33*, 325–332.

(6) Smith, J. E.; Lewis, C. W.; Anderson, J. G.; Solomons, G. I. Mycotoxins in human nutrition and health. European Commission Directorate General XII, Brussels, 1994, Report No. EUR 16048 EN.

(7) Albersheim, P.; Anderson-Prouty, A. J. Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. *Annu. Rev. Plant Physiol.* **1975**, *26*, 31–52.

(8) Cuppen, J. G.; Van den Brink, P. J.; Camps, E.; Uil, K. F.; Brock, T. C. Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. *Aquat. Toxicol.* **2000**, *48*, 233–250.

(9) Staub, R. E.; Quistad, G. B.; Casida, J. E. Mechanism for benomyl action as a mitochondrial aldehyde dehydrogenase inhibitor in mice. *Chem. Res. Toxicol.* **1998**, *11*, 535–543.

(10) Mahoney, N.; Molyneux, R. J. Phytochemical inhibition of aflatoxigenicity in *Aspergillus flavus* by constituents of walnut (*Juglans regia*). *J. Agric. Food Chem.* **2004**, *52*, 1882–1889.

(11) Ioset, J. R.; Marston, A.; Gupta, M. P.; Hostettmann, K. Antifungal and larvicidal compounds from the root bark of *Cordia alliodora*. *J. Nat. Prod.* **2000**, *63*, 424–426.

(12) Kim, J. H.; Campbell, B. C.; Mahomey, N. E.; Chan, K. L.; Molyneux, R. J. Identification of phenolics for control of *Aspergillus flavus* using *Saccharomyces cerevisiae* in a model target-gene bioassay. *J. Agric. Food Chem.* **2004**, *52*, 7814–7821.

(13) Feresin, G. E.; Tapia, A.; Gimenez, A.; Gutierrez Ravelo, A.; Zacchino, S.; Sortino, M.; Schmeda-Hirschman, G. Constituents of the Argentineal medicinal plant *Baccharis grisebachii* and their antimicrobial activity. *J. Ethnopharmacol.* **2003**, *89*, 73–80.

(14) Gianello, J. C.; Giordano, O. S. Constituents from *Bacccharis grisebachii*. *An. Asoc. Quím. Argent.* **1987**, *75*, 1–3.

(15) Wright, L.; Scott, E.; Gorman, S. The sensitivity of mycelium, arthrospores and microconidia of *Trichophyton mentagrophytes* to imidazoles determined by in vitro tests. *J. Antimicrob. Chemother.* **1983**, *12*, 317–323.

(16) Dambacher, J.; Zhao, W.; El-Batta, A.; Anness, R.; Jiang, C.; Bergdahl, M. Water is an efficient medium for Wittig reactions employing stabilized ylides and aldehydes. *Tetrahedron Lett.* **2005**, *46*, 4473–4477.

(17) Dinkova-Kostova, A. T.; Abeygunawardana, C.; Talalay, P. Chemoprotective properties of phenylpropenoids, bis(benzylidene)cycloalkanones, and related Michael reaction acceptors: Correlation of potencies as phase 2 enzyme inducers and radical scavengers. *J. Med. Chem.* **1998**, *41*, 5287–5296.

(18) Etzenhouser, B.; Hansch, C.; Kapur, S.; Dias Selassie, C. Mechanism of toxicity of esters of caffeic and dihydrocaffeic acids. *Bioorg. Med. Chem.* **2001**, *9*, 199–209.

(19) Mogilaiah, K.; Randheer Reddy, G. Microwave-assisted solvent-free synthesis of trans-cinnamic acids using lithium chloride as catalyst. *Synth. Commun.* **2005**, *34*, 205–210.

(20) Menon, S. R.; Patel, V. K.; Mitscher, L. A.; Shih, P.; Pillai, S. P.; Shankel, D. M. Structure-antimutagenic activity relationship study of plicatin B. *J. Nat. Prod.* **1999**, *62*, 102–106.

(21) Warning, U.; Bohlmann, F.; Sanchez, V. H.; Del Rio, S. E.; Dominguez, X. A. New constituents of *Baccharis salicifolia*. *Rev. Latinoam. Quim.* **1986**, *17* (3–4), 199–200.

(22) Zhao, H.; Neamati, N.; Mazumder, A.; Sunder, S.; Pommier, Y.; Burke, T. R., Jr. Arylamide Inhibitors of HIV-1 integrase. *J. Med. Chem.* **1997**, *40*, 1186–1194.

(23) Zacchino, S.; Santecchia, C.; López, S.; Gattuso, S.; Muñoz, J.; Cruañez, A.; Salinas, M.; Ruiz, R.; Ruiz, S. In vitro antifungal evaluation and studies of mode of action of eight selected species from the Argentine flora. *Phytomedicine* **1988**, *5*, 389–395.

(24) Carrizo, F. R.; Sosa, M. E.; Favier, L. S.; Penna, F.; Guerreiro, E.; Giordano, O. S.; Tonn, C. E. Growth-inhibitory activities of benzofuran and chromene derivatives toward *Tenebrio molitor*. *J. Nat. Prod.* **1998**, *61*, 1209–1211.

(25) Zhang, Y.; Jiao, J.; Flowers, R. A. Mild Conversion of δ-diketones and δ-ketoesters to carboxylic acids. *J. Org. Chem.* **2006**, *71*, 4516–4520.

(26) Orden, A. A.; Cifuente, D. A.; Borkowski, E. J.; Tonn, C. E.; Kurina-Sanz, M. K. Stereo- and regioselective hydroxylation of grindelic acid derivatives by *Aspergillus niger*. *Nat. Prod. Res.* **2005**, *19*, 625–631.

(27) Arriaga-Giner, F. J.; Wollenweber, E.; Schober, I.; Yatskievych, G. Three new benzoic acid derivatives from the glandular excretion of *Eriodictyon sessilifolium* (Hydrophyllaceae). *Z. Naturforsch.* **1988**, *43c*, 337–340.

(28) Johnson, W. W.; Finley, M. T. *Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates*; U.S. Department of the Interior Fish and Wildlife Service: Washington, DC, 1980; Vol. 1, pp 1−8.

(29) Freile, M. L.; Giannini, F.; Sortino, M.; Zamora, M. A.; Juarez, A.; Zacchino, S.; Enriz, R. D. Antifungal activity and toxicity of berberine isolated from *Berberis heteropyilla*. *Acta Farm. Bonaerense* **2006**, *25*, 83–88.

(30) De Martín, M. C.; Nuñez, A. M.; Tomates, M. E. Metamorfosis en anfibios. I. Desarrollo metamórfico en larvas de *Bufo arenarum* Hensel (Amphibia: Anura). *Historia Natural.* **1985**, *5*, 289–302.

(31) Rosazza, J. P. N.; Huang, Z.; Dostal, L; Volm, T.; Rousseau, B. Review: Biocatalytic biotransformation of ferulic acid: An abundant aromatic natural product. *J. Ind. Microbiol. Biot.* **1995**, *15*, 457–471.

(32) Ojima, N.; Takenaka, S.; Seto, S. Structure of pulvinone derivatives from *Aspergillus terreus*. *Phytochemistry* **1975**, *14*, 573–576.

Received for review October 1, 2007. Revised manuscript received October 5, 2007. Accepted October 10, 2007. This work was supported by grants from CONICET (PIP 6228) and Agencia Nacional de Promociones Cientificas y Tecnológicas de la Argentina (PICTR-260). F.B. thanks CONICET for a doctoral fellowship, and F.M.G. thanks ANPCyT for a fellowship (PICTR-260).

JF0729098