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In Vitro Evaluation of Antifungal Properties of 8.O.4′-Neolignans

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Eighteen racemic 8.O.4′-neolignans with six different substitution patterns in rings A and B, in their ketone and in their *erythro* and *threo* alcoholic forms, were evaluated for antifungal activity by the agar dilution method. Only the alcohols exhibited a broad spectrum of activities against *Microsporum canis*, *Microsporum gypseum*, *Tricophyton mentagrophytes*, *Tricophyton rubrum*, *and Epidermophyton floccosum*. (±)-*erythro*-3,4-(methylenedioxy)-7-hydroxy-1′-allyl-3′,5′-dimethoxy-8.O.4′-neolignan (**11**) was the most active compound in the series, and *E. floccosum* was the most susceptible species.

Drugs from Myristicaceae species have been used in the Amazonian region as hallucinogens and arrow poisons as well as for the healing of infected wounds.[1] Chemical investigations of Amazonian Myristicaceae led to the hypothesis that the alleged usefulness of plasters made from leaves or bark resin in the treatment of skin infections may be due to the fungistatic or fungitoxic activity of neolignans.[2] Within the great structural variety of neolignans, the 8.O.4′-type represents a small group, whose members have been isolated from plants of the Myristicaceae.[3] They were reported in *Myristica fragrans*,[4−7] *Virola surinamensis*,[8] *Virola carinata*,[9] and *Virola pavonis*.[10]

Regarding their reported biological activity, 8.O.4′-neolignans showed strong activity against the penetration of cercaria of *Schistosoma mansoni*,[3] inhibition of the growth of silkworm larvae,[11] and antileukemic activity in rats.[12] Studies on the antifungal activity of 8.O.4′-neolignans have not been reported to date.

In the course of our screening program for biological activities of this type of compound, we chose to carry out systematic research into their fungistatic activity and to establish whether the antifungal activity observed in the Myristicaceae family can be ascribed to 8.O.4′-neolignans. To gain insight into structure−activity relationships, we tested neutral racemic structures with six different substitution patterns on rings A and B in their ketone (compounds **1**−**6**) and in their *erythro* and *threo* alcoholic forms (compounds **7**−**18**).[13] Amongst them, alcohols (but not ketones) have been isolated from natural sources. Alcohols with two methoxyls on ring B were isolated from *M. fragrans*[3] only in their *erythro* form (compounds **7**, **9**, and **11**); those with

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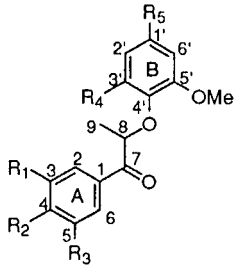
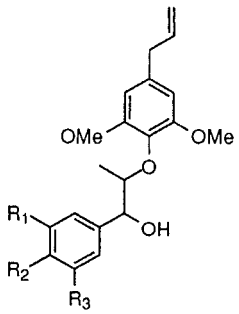
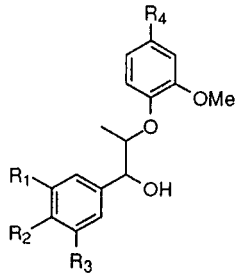
only one methoxyl on ring B were found in *M. fragrans* and *Virola* species having one, or both configurations: *erythro* **13,** in *M. fragrans*[7] and *threo* alcohols **16** and **18** in *V. surinamensis*.[8]



	R_1	R_2	R_3	R_4	R_5
1	OMe	OMe	H	OMe	allyl
2	OMe	OMe	OMe	OMe	allyl
3	-O-CH_2-O-		H	OMe	allyl
4	OMe	OMe	H	H	allyl
5	OMe	OMe	H	H	trans-propenyl
6	OMe	OMe	OMe	H	trans-propenyl



	R_1	R2	R3	conf.
7	OMe	OMe	H	erythro
8	OMe	OMe	H	threo
9	OMe	OMe	OMe	erythro
10	OMe	OMe	OMe	threo
11	-O-CH_2-O-		H	erythro
12	-O-CH_2-O-		H	threo



	R_1	R_2	R_3	R_4	conf.
13	OMe	OMe	H	allyl	erythro
14	OMe	OMe	H	allyl	threo
15	OMe	OMe	H	trans-propenyl	erythro
16	OMe	OMe	H	trans-propenyl	threo
17	OMe	OMe	OMe	trans-propenyl	erythro
18	OMe	OMe	OMe	trans-propenyl	threo

Additionally, we evaluated the minimal requirements to produce the biological response by studying the phenylpropanoid molecules 1-[3,4-(methylenedioxy)-phenyl]-1-hydroxypropane and the commercially available 4-allyl-2,6-dimethoxyphenol, moieties of molecule **11**. All diphenylpropanoids and 1-[3,4-(methylenedioxy)phenyl]-1-hydroxypropane were synthesized following methods described previously.[4,8,14−17] To the best of our knowledge, *threo* compounds **10** and **12,** *erythro* **15** and **17**, and 1-[3,4-(methylenedioxy)phenyl]-1-hydroxypropane have not been reported previously in the literature (see Experimental Section). In this study, agar dilution assays were used to determine the minimum inhibitory concentration (MIC) of compounds, using a panel of human pathogenic fungi, yeasts as well as dermatophytes.

Results and Discussion

To carry out the antifungal evaluation, concentrations of 8.O.4′-neolignans up to 250 μg/mL were incorporated into growth media according to reported procedures.[18] The agar dilution method showed that none of the compounds tested was active against the yeasts *Candida albicans*, *Saccharomyces cerevisiae*, or *Cryptococcus neoformans* nor against the filamentous fungi *Aspergillus niger*, *Aspergillus fumigatus*, or *Aspergillus flavus* (results not shown). In contrast, different results were obtained for the compounds of the series against dermatophytes. These results are shown in Table 1.

All the tested dermatophytes were inhibited at 250 μg/mL and, most often at lower concentrations. The most sensitive species was *E. floccosum*. Ketones were drastically less active than alcohols. Only structures **10**, **14**, and **17** showed significant activity against all the dermatophytes tested (MICs < 100 μg/mL). Alcohol *erythro*-**11**, with a (methylenedioxy)phenyl as ring A, showed the strongest antifungal activity with MIC = 5 μg/mL against *E. floccosum* comparable to MICs of 0.3 and 15 μg/mL found for the control drugs amphotericin B and ketoconazole.

Within the activity shown by alcohols against the most sensitive fungus, the following conclusions can be extracted: *erythro*-compounds were up to three times more active than their *threo*-isomers (see structures **11** and **12,** MICs = 5 and 15 μg/mL, respectively). The replacement of two OMe groups in structure **7** for a methylenedioxy substituent (compound **11**) enhanced the activity eight times (MICs 40 vs. 5 μg/mL). The addition of an extra OMe group in ring A (compounds **7**−**9**), decreased activity 1.25 times (MICs 40 vs. 50 μg/mL), and the suppression of a OMe in ring B (structures **7**−**13**) increased fungistatic properties 2.67 times (MICs 40 vs. 15 μg/mL). In compounds with only one OMe in ring B, the change of an allyl group for a *trans*-propenyl substituent (compounds **13**−**15**) decreased the activity 4.67 times (MICs 15 vs. 70 μg/mL), and an extra OMe in ring A (compounds **15**−**17**) increased the fungistatic action 2.8 times (MICs 70 vs. 25 μg/mL).

Because dermatophytes are a group of fungi that characteristically infect the superficial keratinized areas of the body and because dermatophytoses are of particular concern in the tropics,[19] it is interesting to note that only dermatophytes (and not other types of fungi) were affected by these alcohols, which were described in the Myristicaceae family.

Concerning the reported antifungal properties of the Myristicaceae family, it is very difficult at this point to attribute activity only to the presence of 8.O.4′-neolignans. Their moderate activity coupled with the fact that

Table 1. MIC Values (μg/mL) for Antifungal Activities of 8.O.4′-Neolignans against Dermatophytes

	dermatophytes				
compd	a	b	c	d	e
1	250	>250	>250	>250	>250
2	250	>250	>250	>250	>250
3	>250	250	>250	>250	>250
4	250	250	>250	250	250
5	>250	250	>250	>250	>250
6	>250	>250	>250	>250	>250
7	150	50	150	80	40
8	40	150	150	40	60
9	50	150	50	25	50
10	60	80	50	25	60
11	20	50	100	>250	5
12	80	40	200	20	15
13	250	>250	>250	250	15
14	20	60	40	20	25
15	50	50	150	20	70
16	50	>250	>250	>250	100
17	60	60	80	50	25
18	>250	>250	>250	125	50

[a] *M. canis.* [b] *M. gypseum.* [c] *T. mentagrophytes.* [d] *T. rubrum.* [e] *E. floccosum.*

they are minor components of the Myristicaceae species, suggest them to be contributors but not the main agents responsible for the alleged antifungal properties of the family. On the other hand, it is not possible to attribute the activity observed in Amazonian Myristicaceae to compound **11**, inasmuch as its presence was described only in *Myristica fragrans* and not in native Amazonian trees.

To gain insight into structural requirements for activity of compound **11**, we tested the phenylpropanoids synthetic (±)-1-[3,4-(methylenedioxy)phenyl]-1-hydroxypropane and commercially available 4-allyl-2,6-dimethoxyphenol (Sigma Chemical Co.), moieties corresponding to rings A and B, respectively, against *E. floccosum*. Because neither of them showed antifungal activity up to 50 μg/mL, both rings A and B appear to be a necessary structural requirement to produce the biological response, strongly suggesting a synergistic action between them. We believe that our results may be helpful in identifying and understanding the minimal structural requirements for antifungal action of 8.O.4′-neolignans and in providing guidance in the design of new compounds that exhibit strong activity against dermatophytes.

Experimental Section

General Experimental Procedures. The ^{1}H-NMR spectra were recorded at either 80.13 or 200.15 MHz; ^{13}C-NMR spectra, at 20.15 or 50.3 MHz in the Fourier transform mode and in $CDCl_3$ solutions. Carbon chemical shifts are expressed in the δ scale using $CDCl_3$ as a reference signal at 76.9 ppm; *J* values are given in Hertz. Preparative TLC was done on Si gel F_{254} with visualization under UV (254 and 365 nm). IR spectra were measured with a Bruker IFS 25 spectrophotometer. MS were measured at 70 eV for electron impact (EIMS).

Microorganisms and Media. The following microorganisms used for the fungistatic evaluation were purchased from American Type Culture Collection (Rockville, MD): *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATCC 26934, and *A. niger* ATCC 9029. Strains were grown on Saboureaud chloramphenicol agar slants for 48 h at 30 °C. Cell suspensions in sterile distilled H_2O were adjusted to give a final concentration of 10^6 viable yeast cells/mL. Dermatophytes: *M. canis* C 112, *T. rubrum* C 113, *E. floccosum* C 114, and *M. gypseum* C 115 are clinical isolates and were kindly provided by CEREMIC, Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina. *T. mentagrophytes* was ATCC 9972. Organisms were maintained on slopes of Saboureaud dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to reported procedures[20] and adjusted to 10^6 spores with colony forming ability per milliliter.

Antifungal Assays. The fungistatic activity of 8.O.4′-neolignans was evaluated with the agar dilution method by using Saboureaud−chloramphenicol agar for both yeast and dermatophyte species. The assays were carried out in 96-well microtiter plates. Stock solutions of 8.O.4′-neolignans in DMSO were diluted to give serial twofold dilutions that were added to each medium, resulting in concentrations ranging from 0.10 to 250 μg/mL. The final concentration of DMSO in the assay did not exceed 2%. Using a micropipette, an inoculum of 5 μL of the yeast cell or spore suspensions was added to each Saboureaud−chloramphenicol agar well. The antifungal agents ketoconazole (Janssen Pharmaceutica) and amphotericin B (Sigma Chemical Co.) were included in the assay as positive controls. Drug-free solution was also used as blank control. The plates were incubated 24, 48, or 72 h at 30 °C (according to the control fungus growth) up to 15 days for dermatophyte strains. MIC was defined as the lowest 8.O.4′-neolignan concentration showing no visible fungal growth after incubation time.

Test Compounds. Ketones **1**−**6** and alcohols **7**−**9**, **11**, **13**, **14**, **16**, and **18** were synthesized in accordance with our previous work.[3,16,21,22] Their ^{1}H- and ^{13}C-NMR, IR, and MS spectra were identical with the reported data.

***threo*-3,4,5-Trimethoxy-7-hydroxy-1′-allyl-3′,5′-dimethoxy-8.O.4′-neolignan (10).** A solution of $NaBH_4$ (114 mg; 3 mmol) in dry 2-propanol (10 mL) was added to a stirred solution of 15-crown−5 ether (720 mg; 3,6 mmol) in dry 2-propanol (5 mL). After 6 h, a solution of ketone **2** (416 mg, 1 mmol) in dry MeOH (5 mL) was added, and the mixture was stirred for 4 h at room temperature.Then H_2O and a few drops of HOAc were added, and the mixture was extracted with Et_2O (4 × 10 mL). The combined Et_2O extracts were washed with a saturated aqueous solution of $NaHCO_3$ and H_2O, dried (Na_2SO_4), decanted, and evaporated, affording a (9:1) mixture of *threo* (**10**) and *erythro* (**9**). Pure colorless oil **10** (292 mg, 78% yield) was obtained by preparative TLC on Si gel G F_{254} using hexane−EtOAc (80:20); IR ν_{max} (film) 3479, 2938, 1591, 1460, 1234, 1126, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.21 (3H, d, J = 6.0 Hz, H-9), 3.35 (2H, br d, H-7′), 3.81, 3.84, 3.87 (15H, s, 5 × OMe), 4.00 (1H, dq, H-8), 4.6 (1H, d, J = 8.0 Hz, H-7), 5.07 (1H, m, H-9′), 5.95 (1H, m, H-8′), 6.44, 6.57 (4H, s, ArH); ^{13}C NMR ($CDCl_3$) δ 17.55 (q, C-9), 40.35 (t, C-7′), 55.82, 55.95 (q, C-3, C-5, C-3′, and C-5′, OMe), 60.64 (q, C-4, OMe), 79.23 (d, C-7), 86.21 (d, C-8), 104.12 (d, C-2 and C-6), 105.28 (d, C-2′ and C-6′), 118.04 (t, C-9′), 135.04

(s, C-4′), 135.79 (s, C-4), 136.22 (s, C-1′), 136.87 (d, C-8′), 137.34 (s, C-1), 152.47 (s, C-3′ and C-5′), 152.91 (s, C-3 and C-5); EIMS *m/z* $[M]^+$ (19), 225 (7), 224 (28), 221-(6), 197 (5), 195 (46), 194 (100), 193 (43).

***threo*-3,4-(Methylenedioxy)-7-hydroxy-1′-allyl-3′,5′-dimethoxy-8.O.4′-neolignan (12).** The same procedure as described for alcohol **10** was followed starting with ketone **3** (368 mg, 1 mmol), $NaBH_4$ (114 mg, 3 mmol), and 15-crown−5 ether (0.69 mL, 3.6 mmol). A 8:2 mixture of crude *threo*-**12** and *erythro*-**11** was obtained. Preparative TLC on Si gel afforded pure **12** (187 mg, 73% yield), as a colorless oil; IR ν_{max} (film) 3480, 2936, 1589, 1502, 1247, 1126, 1038 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.17 (3H, d, J = 6.4 Hz, H-9), 3.35 (2H, br d, J = 6.8 Hz, H-8′), 3.87 (6H, s, 2 × OMe), 3.94 (1H, m, H-8), 4.60 (1H, d, J = 8.4 Hz, H-7), 4.99 (1H, m, H-9′), 5.15 (1H, m, H-9′), 5.91 (2H, s, OCH_2O), 6.00 (1H, m, H-8′), 6.44 (2H, s, H-2′ and H-6′), 6.73−6.87 (3H, m, H-2, H-5, and H-6); ^{13}C NMR ($CDCl_3$) δ 13.30 (q, C-9), 40.34 (t, C-7′), 55.77 (q, C-3′ and C-5′, OMe), 78.77 (d, C-7), 86.21 (d, C-8), 100.34 (t, OCH_2O) 105.26 (d, C-2′ and C-6′), 107.37 (d, C-2), 107.79 (d, C-5), 115.98 (d, C-9′), 120.86 (d, C-6), 134,60 (s, C-4′), 134,90 (s, C-1), 135,74 (s, C-1′), 136.92 (d, C-8′), 146.94 (s, C-3), 147.42 (s, C-4), 152.50 (s, C-3′ and C-5′); EIMS *m/z* $[M]^+$ (11, 221 (9), 194 (100), 193 (36), 179 (9), 163 (9), 91 (13), 65 (12).

***erythro*-3,4-Dimethoxy-7-hydroxy-1′(*E*)-propenyl-3′-methoxy-8.O.4′-neolignan (15).** An Et_2O solution of ketone **5** (428 mg, 1.2 mmol) was gradually added to a stirred suspension of $LiAlH_4$ (456 mg, 12 mmol) in dry Et_2O (36 mL). After addition was complete, the mixture was refluxed for 24 h. Excess $LiAlH_4$ was carefully destroyed by addition of EtOAc−ice and was finally diluted with H_2O (60 mL), acidified, (10% HCl), and extracted with Et_2O (3 × 60 mL). The combined Et_2O extracts were washed with 1 N aqueous NaOH and H_2O, dried (Na_2SO_4), followed by concentration affording a crude oil, which on purification by preparative TLC (hexane−EtOAc 80:20), yielded pure **15** (385 mg, 1.08 mmol); IR ν_{max} (film) 3500, 2970, 1610, 1525, 1390, 1270, 1150, 1050 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.17 (3H, d, J = 6.0 Hz, H-9), 1.88 (3H, d, J = 5.2 Hz, H-9′), 3.89, 3.91, 3.92 (9 H, s, 3 × OMe), 4.37 (1H, m, H-8), 4.86 (1H, d, J = 3.2 Hz, H-7), 5.70−6.23 (1H, m, H-8′), 6.39 (1H, d, J = 18.0 Hz, H-7′), 6.84−7.0 (6H, m, ArH); ^{13}C NMR ($CDCl_3$) δ 13.29 (q, C-9), 16.29 (q, C-9′), 55.67, 55.74 (q, C-3 and C-4, OMe), 73.31 (d, C-7), 82.40 (d, C-8), 109.10 (t, C-2′), 109.23 (t, C-5), 110.60 (t, C-2), 118.27 (t, C-5′), 118.86 (t, C-6′), 119.81 (t, C-6), 124.92 (d, C-8′), 130.30 (d, C-7′), 132.29 (s, C-1′), 133.58 (s, C-1), 145.44 (s, C-4), 147.99 (s, C-3), 148.87 (s, C-4′), 151.37 (s, C-3′); EIMS *m/z* $[M]^+$ (2) 195 (10), 194 (15), 167 (78), 165 (73), 164 (100), 149 (10), 139 (40), 121 (28), 91 (19), 77 (30).

***erythro*-3,4,5-Trimethoxy-7-hydroxy-1′(*E*)-propenyl-3′-methoxy-8.O.4′-neolignan (17).** The same procedure as for reduction of ketone **5** was used with ketone **6** (106 mg, 0.3 mmol), $LiAlH_4$ (114 mg, 3 mmol), and dry Et_2O (10 mL). After purification through preparative TLC (hexane−EtOAc 80:20), pure **17** (101 mg, 0.26 mmol) was obtained as a colorless oil; IR ν_{max} (film) 3500, 2920, 1601, 1470, 1420, 1330, 1265, 1140, 1040 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.17 (3H, d, J = 6.0 Hz, H-9), 1.88 (3H, d, J = 5.5 Hz, H-9′), 3.82, 3.85, 3.90 (12H, 4 × OMe), 4.35 (1H, m, H-8), 4.85 (1H, d, J = 3.0 Hz, H-7), 6.12−6.25 (1H, m, H-8′), 6.39 (1H, d, J = 16.0 Hz, H-7′), 6.62 (2H, s, H-2 and H-6), 6.89 (3H, m, H-3′, H-5′, and H-6′); ^{13}C NMR ($CDCl_3$) δ 13.3 (q, C-9), 16.3 (q, C-9′), 55.5 (q, C-3′, OMe), 55.9 (q, C-3 and C-5, OMe), 60.5 (q, C-4, OMe), 73.3 (d, C-7), 82.4 (d, C-8), 104.2 (d, C-2 and C-6), 109.1 (d, C-2′), 118.5 (d, C-5′), 118.7 (d, C-6′), 124.6 (d, C-8′), 130.2 (d, C-7′), 133.3 (s, C-1′), 135.5 (s, C-4), 146.4 (s, C-4′), 150.5 (s, C-3′), 152.9 (d, C-3 and C-5); EIMS *m/z* $[M]^+$ (4.39), 225 (14.65), 224 (53.64), 197 (100), 164 (99), 135 (37).

1-[3,4-(Methylenedioxy)phenyl]-1-hydroxypropane. The same procedure as for reduction of ketone **5** was used with 1-[3,4-(methylendioxy)phenyl]-1-oxopropane (103 mg, 0.58 mmol), synthesized through Grignard reaction from piperonylnitrile (Aldrich Chemical Co.),[23] $LiAlH_4$ (228 mg, 6 mmol), and dry Et_2O (10 mL). After purification through preparative TLC (hexane−EtOAc 80:20), pure **19** (90 mg, 0.50 mmol) was obtained as a colorless oil; IR ν_{max} 3520, 2940, 1630, 1540, 1500, 1470, 1450, 1055, 950 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 8.0 Hz, H-9), 1.77 (2 H, m, H-8), 4.50 (1H, t, J = 6.0 Hz, H-7), 5.95 (2H, O-CH_2-O), 6.77−6.86 (2H, ArH); ^{13}C NMR ($CDCl_3$) δ 10.05 (q, C-9), 31.71 (t, C-8), 75.80 (d, C-7), 100.84 (t, O-CH_2-O), 106.29 (d, C-2), 107.86 (d, C-5), 119.30 (d, C-6), 138.54 (s, C-1), 146.75 (s, C-3), 147.63 (s, C-4); EIMS *m/z* $[M]^+$ (51), 151 (100), 93 (95), 65 (83), 57 (14), 41(12).

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