# Antifungal Activity of Benzoic Acid Derivatives from *Piper lanceaefolium*

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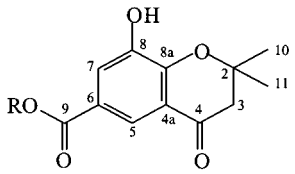
Bioactivity-guided fractionation of a methanol extract from the leaves of *Piper lanceaefolium* resulted in the isolation of four new benzoic acid derivatives (**1**−**4**), together with taboganic acid, pinocembrin, and pinocembrin chalcone. Lanceaefolic acid methyl ester (**3**) and pinocembrin chalcone displayed activity against *Candida albicans* with a minimal inhibitory concentration value of 100 $\mu$g/mL in both cases.

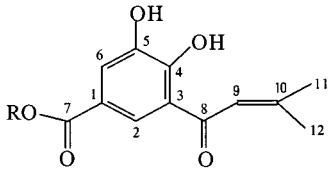
The genus *Piper* (Piperaceae) has over 700 species distributed in both hemispheres. Species belonging to this genus are included in the Indian Ayurvedic system of medicine and in folklore medicine of Latin America.[1,2] The phytochemistry of the genus has been the subject of an extensive review.[3]

Locally named in the Sibundoy Valley (southern Colombia) as "cueche", a decoction of the leaves of *Piper lanceaefolium* Kunth (Piperaceae) is taken as a bath to treat skin infections. Specifically, these refer to malignant cutaneous manifestations whose occurrence is correlated with the drizzle that usually accompanies the presence of a rainbow and is believed by the Kamsa and Inga to cause infections.[4] This species has not been the subject of phytochemical analysis, and a methanolic extract of the leaves showed sufficient activity against the pathogenic yeast *Candida albicans* to warrant bioassay-guided fractionation.[4] This led to the isolation of four hitherto undescribed prenylated benzoic derivatives (**1**−**4**) as well as the known compounds taboganic acid,[5] pinocembrin,[6] and its corresponding chalcone.[7]




1 R = $CH_3$

2 R = H




3 R = $CH_3$

4 R = H

Extraction with acetone followed by liquid−liquid partition resulted in the localization of the antifungal activity in the chloroform and ethyl ether fractions. Column chromatography, followed by preparative TLC (see Experimental Section) of the chloroform and ethyl ether fractions, resulted in the isolation of four new prenylated benzoic acid derivatives (**1**−**4**). Also, the known compounds taboganic acid,[5] pinocembrin,[6] and pinocembrin chalcone[7] were isolated for the first time from this plant and identified by comparison of their physical and spectral data with those previously reported.

Compound **1** was obtained as a white amorphous powder and had a molecular ion peak at *m*/*z* 250.08334 in HRE-IMS, suggesting a molecular formula of $C_{13}H_{14}O_5$. The IR spectrum indicated the presence of two carbonyl groups (1713, 1664 $cm^{-1}$) and an aromatic ring (1608, 1587 $cm^{-1}$). The assignments of protons and carbons were obtained by analysis of its 2D NMR (HMQC and HMBC). The $^1$HNMR spectrum (Table 1) exhibited two aromatic proton resonances at $\delta$ 7.62 (1H, d, $J$ = 2.1 Hz) and 7.96 (1H, d, $J$ = 2.1 Hz). On the basis of chemical shifts and coupling patterns, a 1,2,3,5-tetrasubstituted phenyl ring was suggested to be present in the molecule. In addition, the $^1$H NMR spectrum displayed signals for one methylene group at $\delta$ 2.83 and three methyl groups at $\delta$ 1.48 (6H, s) and 3.86 (3H, s). The $^{13}$C NMR spectrum (Table 1) and DEPT experiments of **1** showed 13 signals: three methyls, one methylene, two methines, and seven quaternary carbons, of which the signals at $\delta$ 152.7, 123.4, 121.1, 119.3, 121.2, 147.9, 166.5, and 52.3, together with a sharp singlet (3H) at $\delta$ 3.86 in the $^1$H NMR spectrum, implied the presence of a methyl ester of a 3,4,5-trisubstituted benzoic acid. The signals at $\delta$ 191.8, 48.9, 81.8, and 26.5 (×2) together with the methylene resonance (singlet) and two aliphatic methyl groups of magnetic equivalence in the $^1$H NMR spectrum, as well as the HMBC (Figure 1) correlations between C-2, C-4 and methylene (H-3), revealed the connectivity CO (4)−$CH_2$(3)−C(2)−$(CH_3)_2$. The HMBC spectrum of **1** also showed correlations from the carbonyl group at $\delta$ 191.8 to H-5, from the carboxyl group to H-5 and H-7, and from C-8a to H-7 and H-5, which indicated that the ketone carbonyl and carboxylic groups were attached at C-4a and C-6, respectively. Thus, the structure of **1** was determined to be cyclolanceaefolic acid methyl ester (the methyl ester of 8-hydroxy-2,2′-dimethyl-6-carboxychroman-4-one).

Compound **2** was isolated as white crystals. The HRE-IMS of this compound gave a molecular ion at *m*/*z* 236.06836 corresponding to a molecular formula of $C_{12}H_{12}O_5$. IR absorptions at 3233 and 1683 $cm^{-1}$ suggested the presence of hydroxyl and carbonyl groups. The $^1$H and $^{13}$C NMR data (Table 1) and DEPT experiments revealed 12 signals: two methyls, one methylene, two methines, and seven quaternary carbons. Compounds **1** and **2** were found to have

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**Table 1.** $^1$H and $^{13}$C Data for Compounds **1**−**4**[a]

| | **1** | | **2** | | | **3** | | **4** | |
|---|---|---|---|---|---|---|---|---|---|
| position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| | | | | | 1 | | 121.5 (s) | | 121.8 (s) |
| 2 | | 81.8 (s) | | 81.7 (s) | 2 | 8.12 (d, 1.9) | 123.4 (d) | 8.14 (d, 1.9) | 123.7 (d) |
| 3 | 2.83 (s) | 48.9 (t) | 2.82 (s) | 48.9 (t) | 3 | | 120.9 (s) | | 120.9 (s) |
| 4 | | 191.8 (s) | | 191.8 (s) | 4 | | 156.2 (s) | | 156.4 (s) |
| 4a | | 123.4 (s) | | 123.7 (s) | 5 | | 147.3(s) | | 147.2 (s) |
| 5 | 7.96 (d, 2.1) | 119.3 (d) | 7.96 (d, 2.1) | 119.6 (d) | 6 | 7.62 (d, 1.9) | 121.4 (d) | 7.66 (d, 1.9) | 121.3 (d) |
| 6 | | 121.2 (s) | | 121.1 (s) | 7 | | 166.5(s) | | 166.9 (s) |
| 7 | 7.62 (d, 2.1) | 121.1 (d) | 7.62 (d, 2.1) | 121.4 (d) | 8 | | 196.9(s) | | 197.0 (s) |
| 8 | | 147.9 (s) | | 147.8 (s) | 9 | 7.06 (br s) | 120.2 (d) | 7.08 (br s) | 120.3 (d) |
| 8a | | 152.7 (s) | | 152.6 (s) | 10 | | 161.8 (s) | | 161.6 (s) |
| 9 | | 166.5 (s) | | 169.9 (s) | 11 | 2.10 (s) | 28.2 (q) | 2.12 (s) | 28.2 (q) |
| 10, 11 | 1.48 (s) | 26.5 (q) | 1.48 (s) | 26.5 (q) | 12 | 2.24 (s) | 21.7 (q) | 2.24 (s) | 21.7 (q) |
| $CH_3O$ | 3.86 (s) | 52.3 (q) | | | $CH_3O$ | 3.85 (s) | 52.3 (q) | | |

[a] Spectra were recorded in $CD_3OCD_3$; chemical shifts are reported as $\delta$ values (ppm) from TMS at 400 MHz for $^1$H and 100 MHz for $^{13}$C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

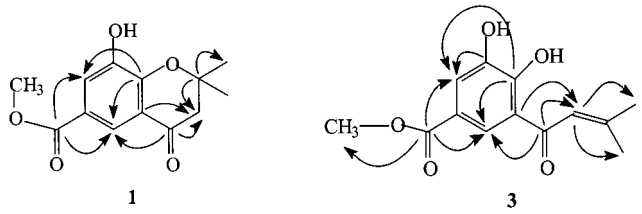


**Figure 1.** Selected HMBC correlations for **1** and **3**. (Arrows denote HMBC correlations from C to H.)

similar structures by comparison of their NMR spectra. The observed difference was the loss of the methoxy signals ($^1$H NMR at $\delta$ 3.86 and $^{13}$C NMR at $\delta$ 52.3) in the NMR spectra of **1**, which indicated that the methyl ester carbonyl was replaced in **2** by a carboxyl group. Thus, the structure of **2** was assigned as cyclolanceaefolic acid (8-hydroxy-2,2′-dimethyl-6-carboxychroman-4-one).

Compound **3** was obtained as yellowish needles. The HREIMS indicated a molecular formula of $C_{13}H_{14}O_5$. The IR spectrum indicated two carbonyl groups at 1730 and 1724 cm$^{-1}$. The $^1$H NMR spectrum contained two aromatic protons, with the chemical shift and splitting pattern typical of H-2 and H-6 of a 1,3,4,5-tetrasubstituted benzene ring. Furthermore, the signals at $\delta$ 196.9, 120.2, and 161.8 in the $^{13}$C NMR spectrum and a signal at $\delta$ 7.06 in the $^1$H NMR spectrum implied a conjugated ketone system. The signals in the $^1$H NMR spectrum at $\delta$ 2.10 (3H, s) and 2.24 (3H, s) were assigned to vinyl methyl groups positioned on a trisubstituted double bond. A sharp 3H singlet at $\delta$ 3.85, together with the $^{13}$C NMR resonances at $\delta$ 166.5 and 52.3, suggested the presence of a methyl ester. Correlations in the HMBC spectrum from C-7 to H-2 and H-6, from C-8 to H-2, and from C-3 to H-9 suggested that the ester carbonyl and the ketone carbonyl were attached to C-1 and C-3 of the benzene ring, respectively. COSY, HMQC, and HMBC NMR spectra permitted the complete assignment of all the protons and carbons as shown in Table 1. The above evidence led to the elucidation of structure **3** as lanceaefolic acid methyl ester [the methyl ester of 4,5-dihydroxy-3-(3-methyl-2-butenoyl)benzoic acid], which was confirmed by comparing the NMR data with the known compounds taboganic acid methyl ester[8] and piperoic acid.[9]

Compound **4** was obtained as orange-yellow needles. The HREIMS showed a molecular ion at *m/z* 236.06832, suggesting a molecular formula ion of $C_{12}H_{12}O_5$. The presence of a 1,3,4,5-tetrasubstituted aromatic ring in the structure became clear from the consideration of resonances attributed to the aromatic rings in the $^1$H and $^{13}$C NMR spectra. Compounds **3** and **4** showed very similar NMR spectra except that the signals of the methyl group at $\delta_H$ 3.85 and $\delta_C$ 52.3 were only present in the spectra of **3**. Thus, the structure of lanceaefolic acid was assigned as **4** [4,5-dihydroxy-3-(3-methyl-2-butenoyl)benzoic acid].

Growth inhibitory effects against *C. albicans* were detected in compound **3** and pinocembrin chalcone using disk diffusion assay and bioautographic assay.[5] Determination of the minimal inhibitory concentration of the compounds confirmed that lanceaefolic acid methyl ester (**3**) and pinocembrin chalcone are at least in part responsible for the antifungal activity of the *Piper lanceaefolium* leaf extract, with values for both compounds of 100 $\mu$g/mL. The rest of the compounds did not exhibit activity against *C. albicans* within the range of concentrations tested in the broth dilution assay.

A synthetic preparation of pinocembrin chalcone has been shown to display activity against *Candida glabrata* but not *C. albicans*.[10]

Although no activity was shown against *C. albicans*, taboganic acid exhibited activity against the plant pathogenic fungus *Cladosporium cucumerinum* in a bioautographic test on TLC (5 $\mu$g); however, it did not exhibit activity in a dilution assay.[5]

The antifungal activity of lanceaefolic acid methyl ester (**3**) and pinocembrin chalcone might be correlated with *P. lanceaefolium* traditional use in the Sibundoy Valley; however, the authors believe that the role of secondary metabolites from traditional medicinal plants and the morbid state of the host might be far more complex than merely the result of a direct antibiotic activity exerted by some of the single chemical entities present in the plant.

## Experimental Section

**General Experimental Procedures.** Melting points (uncorrected) were recorded on a Gallenkamp melting point apparatus; optical rotations were measured on a JASCO P-1010 polarimeter; IR spectra were obtained using KBr disks on a BOMEM MB-100 spectrophotometer; UV spectra were obtained on a Shimadzu UV 160U spectrophotometer; the NMR spectra were recorded on a Bruker AV-400 at 400 MHz ($^1$H) and 100 MHz ($^{13}$C); multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were run using a Bruker AV-400 NMR spectrometer; high-resolution MS spectra were obtained on a Kratos MS 50 mass spectrometer; and TLC analysis was carried out on silica gel $F_{254}$ plates (Merck). Preparative TLC was performed using silica gel 60 $F_{254}$ (Merck) (250 $\mu$m thickness). The isolated compounds were visualized by observing under UV at 254 nm, followed by development with ferric chloride spray reagent.

**Plant Material.** Leaves of *P. lanceaefolium* were collected in Sibundoy (01°11′00″ N, 076°55′00″ W) (Department of

Putumayo, Colombia) in July 1999 and identified by Dr. R. Callejas, Antioquia University Herbarium (Medellin, Colombia). A voucher specimen (Lopez 56) is kept in the herbarium of the Sinchi Institute (Santa Fe de Bogotá, Colombia).

**Extraction and Bioassay.** Fresh leaves were air-dried in the shade and ground. The resulting powder (544 g) was extracted with 300 mL of acetone on a shaker for 25 min. The resulting extract was concentrated to dryness to give 37.8 g of dried material. This extract was dissolved in methanol and passed through a glass column containing Celite 545. The resulting extract was dissolved in a mixture of methanol−water (3:1) and fractioned by liquid−liquid partition with hexane, chloroform, ethyl ether, ethyl acetate, butanol, and water. The different fractions were tested against *C. albicans* using a disk test assay.[11]

**Isolation of Compounds.** The chloroform and ethyl ether (22.3 g) were subjected to column chromatography over silica gel (346 g, silica gel 230−400 mesh, Merck) using a gradient of ethyl acetate in dichloromethane. A total of 68 fractions (230 mL each) were collected and combined into seven pools (I−VII) on the basis of similar TLC profiles. Activity against *C. albicans* was found in pools I and IV, which were eluted with $CH_2Cl_2$ and $CH_2Cl_2$/EtOAc (9:1), respectively.

Crystallization of the different pools (except pool IV) afforded compounds **1** (1.93 g), **2** (196 mg), **3** (3680 mL, 120 mg), **4** (126 mg), taboganic acid (3.06 g), and pinocembrin (6 mg). Fractions in pool IV were combined and applied to a column of silica gel (70−230 mesh) and eluted using gradients of ethyl acetate in hexane (0−100%). The fraction that displayed activity against *C. albicans* was subjected to preparative TLC to yield pinocembrin chalcone (53 mg).

**Cyclolanceaefolic acid methyl ester (1):** white amorphous powder, mp 146−147 °C; $[\alpha]^{24}_D$ −5.7° (*c* 0.5, MeOH); IR (KBr) $\nu_{max}$ 3253, 2984, 1713, 1664, 1608, 1587, 1456, 1432, 1394, 1373, 1286, 1229, 1176, 1097, 1008, 947, 929, 903, 771, 751 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 240 (4.32), 334 (3.34), 203 (3.82); $^1H$ and $^{13}C$ NMR data, see Table 1; EIMS *m*/*z* 250 $[M]^+$ (30), 235 (80), 219 (12), 194 (100), 163 (60), 135 (40), 108 (15), 91 (5), 79 (60), 77 (10), 51 (35); HREIMS *m*/*z* 250.08334 (calcd for $C_{13}H_{14}O_5$, 250.08412).

**Cyclolanceaefolic acid (2):** white crystals, mp 244−246 °C; $[\alpha]^{24}_D$ −40° (*c* 0.6, MeOH); IR (KBr) $\nu_{max}$ 3233, 1683, 1675, 1607, 1587, 1488, 1373, 1292, 1175, 1092, 996, 927, 905, 880, 771, $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 238 (4.30), 338 (3.43), 203 (3.86); $^1H$ and $^{13}C$ NMR data, see Table 1; EIMS *m*/*z* 236 $[M]^+$ (40), 221 (75), 219 (12), 180 (100), 152 (20), 135 (15), 79 (8), 51 (5); HREIMS *m*/*z* 236.06836 (calcd for $C_{12}H_{12}O_5$, 236.06847).

**Lanceaefolic acid methyl ester (3):** yellowish needles, mp 109−110 °C, $[\alpha]^{24}_D$ −8.6° (*c* 0.1 MeOH); IR (KBr) $\nu_{max}$ 3459, 1723, 1713, 1638, 1588, 1482, 1438, 1317, 1231, 1186, 1142, 1093, 1003, 910, 857, 771, 751 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 240 (4.32), 338 (3.40), 203 (3.67); $^1H$ and $^{13}C$ NMR data, see Table 2; EIMS *m*/*z* 250 $[M]^+$ (25), 235 (100), 219 (10), 194 (40), 173 (60), 135 (10), 108 (10), 83 (20), 55 (25); HREIMS *m*/*z* 250.08436 (calcd for $C_{13}H_{14}O_5$, 250.08412).

**Lanceaefolic acid (4):** orange-yellow needles (acetone); mp 253−255 °C; $[\alpha]^{24}_D$ −14.3° (*c* 0.1, MeOH); IR (KBr) $\nu_{max}$ 3477, 1713, 1688, 1638, 1584, 1482, 1422, 1313, 1283, 1134, 1096, 1057, 970, 884, 770, 695 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 236 (4.29), 338 (3.46), 205 (3.92); $^1H$ and $^{13}C$ NMR data, see Table 2; EIMS *m*/*z* 236 $[M]^+$ (30), 221 (100), 180 (55), 152 (10), 125 (10), 83 (15), 55 (15); HREIMS *m*/*z* 236.06832 (calcd for $C_{12}H_{12}O_5$, 236.06847).

**Direct Bioautographic Assay.** A bioautographic agar overlay assay was used for detection of the active fractions obtained during the chemical separation. Fractions of 700 $\mu$g each were spotted on chromatographic silica gel 60 TLC plates (Merck) using $CH_2Cl_2$/EtOAc (50:50) as solvent system. Nystatin (100 $\mu$g) was used as a positive control. After thorough drying for complete removal of solvent, an inoculum of *C. albicans* (ca. $10^7$ cells/mL) in molten agar (Saboraud broth medium agar, phenol red) was distributed over the chromatograms. The medium solidified as a thin layer (ca. 1 mm layer thickness), and the TLC plates were incubated overnight at 37 °C in a moist environment. The inhibition zones were visualized by spraying with an aqueous solution of methylthiazolytetrazolium chloride (MTT) (2.5 mg/mL). Active compounds appeared as clear yellow spots against a purple background. Two additional bioautography TLC plates were developed and sprayed with ferric chloride and vanillin/sulfuric acid in order to visualize the chromatographic profile of compounds.[12,13]

**Minimum Inhibitory Concentration (MIC) Determination.** MIC values were determined by the broth dilution method using a concentration of $6.0 \times 10^4$ colony forming units/mL of *C. albicans* grown in Sabouraud broth media. The MIC was defined as the lowest concentration of substance that prevented growth, which was determined by the appearance of turbidity after 24 h. Solutions of the test compounds were prepared in methanol and diluted with Saboraud medium to give final dilutions ranging from 400 to 0.4 $\mu$g/mL. The final concentration of methanol in the assay did not exceed 2%. The assay was carried out in 96-well microtiter plates. Incubation was at 37 °C for 24 h. Amphotericin B was used as a positive control with a MIC value of 0.055 $\mu$g/mL.[12]

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