# A Novel Antimicrobial Indolizinium Alkaloid from *Aniba panurensis*

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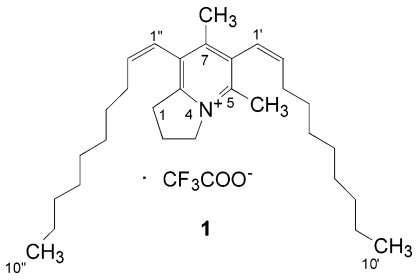
Activity-guided fractionation of an *Aniba panurensis* organic solvent extract has led to the isolation of the novel alkaloid 6,8-didec-(1*Z*)-enyl-5,7-dimethyl-2,3-dihydro-1*H*-indolizinium, as the trifluoroacetic acid salt (**1**). Its structure was determined by NMR and mass spectrometry. Bioassays performed in vitro demonstrated toxicity of compound **1** to a drug-resistant strain of *Candida albicans*.

High-throughput screening of 140 000 crude plant, marine, and fungal extracts from the Developmental Therapeutic Program (DTP) Natural Products Library has been carried out in an antimicrobial assay designed to detect novel chemotherapeutics for the treatment of drug-resistant microorganisms responsible for opportunistic infections in immunocompromised patients. Several extracts from tropical trees of the genus *Aniba* were determined to be toxic to an azole-resistant strain of *Candida albicans*.

A review of the literature revealed that the steam distillate from wood of several *Aniba* spp. that grow in Brazilian Amazonia is a commercial product known as rosewood oil. Fungal and bacterial toxicities have been ascribed to essential oils.[1,2] Other compounds known from the genus *Aniba* include alkaloids,[3−5] styrylpyrones and neolignans,[6] and flavonoids,[7] none of which have reported antimicrobial activity. To determine the identity of the substance responsible for toxicity to azole-resistant *Candida albicans*, activity-guided fractionation of an organic solvent extract of *Aniba panurensis* (Meissn) Mez (Lauraceae) was undertaken. In this publication, we report the isolation and structural elucidation of a novel 6,8-didec-(1*Z*)-enyl-5,7-dimethyl-2,3-dihydro-1*H*-indolizinium alkaloid, obtained as the trifluoroacetic acid salt (**1**).[8]




**1**

Compound **1** was isolated from an organic solvent extract of *A. panurensis* wood by activity-guided flash and HPLC chromatography. The final C8 HPLC produced 60 mg of **1** at >95% purity, an isolated yield of 0.006% from dried plant material.

LC-ESIMS analysis of compound **1** revealed a positive ion peak of *m*/*z* 424.4 [M$^+$], but no signal in the negative ion mode, suggesting a nitrogen-containing molecule. A species having a fixed positive charge was indicated because the addition of cesium iodide or potassium chloride to a methanolic solution of **1** did not result in the formation of the expected pseudomolecular ion peaks. A monoisotopic exact mass of 424.390 was established by positive ion FABMS. The initial $^1$H NMR spectrum possessed relatively few signals, most of them upfield, and was uninformative, except that this paucity of signals for a compound of MW 424 suggested an element of symmetry in the molecule. From NMR, hydrogen and carbon counts of 50 and 30, respectively, led to an elemental composition of $C_{30}H_{50}N$. The $^{13}$C NMR data indicated the presence of nine sp$^2$ carbons, which accounted for five of the seven sites of unsaturation. The two remaining sites of unsaturation were presumed to be ring structures. The remaining 21 carbons consisted of one nitrogen-bound methylene carbon, 16 carbon-bound methylene carbons, and four carbon-bound methyl carbons. The following substructures were confirmed by their $^1$H and $^{13}$C chemical shifts and number of hydrogens attached to each carbon: two identical propenyl groups, two identical ethyl groups, two equivalent 10-carbon aliphatic side chains containing both the propenyl and ethyl groups, two methyls attached to aromatic carbons, and three contiguous methylenes connected at one end to nitrogen. The five aromatic carbons were shown to be quaternary by HSQC data.

Examination of the COSY spectrum revealed the presence of two pairs of olefinic hydrogens ($\delta_H$ 6.23, 6.23, and 6.10, 6.07) which had vinylic *J* couplings of 11.4 and 11.3 Hz, indicative of a *Z*-geometry. The *Z*-configuration was supported by Advanced Chemistry Development (ACD) software calculations, which gave $J_Z$ = 11.5 Hz and $J_E$ = 16.1 Hz for the two possible partial structures. Two 1-propenyl substructures were confirmed by the couplings of the olefinic hydrogen signals at $\delta_H$ 6.10 and 6.07, which were further split into triplets with $J$ = 7.4 Hz. An extended aliphatic chain was indicated by the COSY cross-peak between the 3′ and 3″ allylic methylene hydrogens of the 1-propenyl substructure and the methylene envelope at ∼1.2 ppm. The presence of two identical octanyl groups was supported by positive ion ESIMS experiments performed at an elevated voltage setting to induce molecular fragmentation. Interestingly, the side chains appear to fragment by the incremental loss of methylene groups rather than by cleavage at the 6−1′ or 8−1″ positions. A

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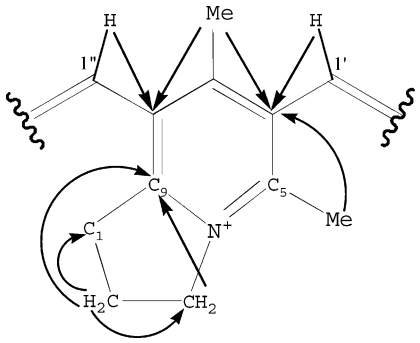


**Figure 1.** Structurally critical HMBC (8 Hz) data for **1**.

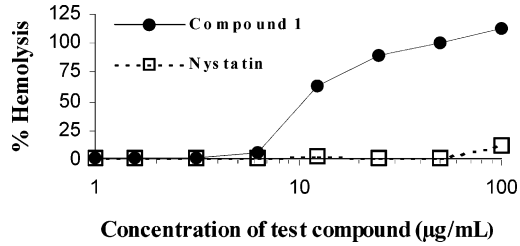


**Figure 2.** Hemolytic activity of **1** and nystatin.

high-intensity peak (*m*/*z* 310) reflected the loss of an octanyl group from the parent molecule. A second octanyl group was seen as a loss of methyl from *m*/*z* 326 followed by a cascade of methylene losses terminating with an intense *m*/*z* 212. Connectivity between the octanyl substructures and the adjoining 1-propenyl group was established by COSY, yielding identical 1-decenyl substructures.

The presence of a propyl fragment was indicated by a COSY spectrum, which demonstrated connectivity among the 1-, 2-, and 3-methylene groups. The downfield position of the 3-methylene protons in the $^1$H NMR suggested deshielding by an adjacent nitrogen. Connectivity of this propyl group at the 1-methylene ($\delta_C$ 32.6) with C-9 ($\delta_C$ 154.3) was suggested by 1,1-ADEQUATE experimental data. Further, the 1,1-ADEQUATE experiments were critical in assigning connectivities of the remaining quaternary carbons within the indolizinium ring system: C-7 ($\delta_C$ 156.0) connected to 7-methyl ($\delta_C$ 19.0), C-5 ($\delta_C$ 148.1) connected to 5-methyl ($\delta_C$ 18.1), C-6 ($\delta_C$ 136.0) and C-8 ($\delta_C$ 132.2) connected to $\delta_C$ 121.8 and $\delta_C$ 120.9, respectively. The last two carbons were assigned by three-bond HMBCs with C-6 showing cross-peaks to both 5-methyl and 7-methyl hydrogens, while C-8 shows only one HMBC to the 7-methyl hydrogens. The HMBC data supporting the assignments in the indolizinium ring are shown in Figure 1. The ring closure of C-1 to C-9 was confirmed by the HMBC connectivities from the hydrogens on C-1, C-2, and C-3 and the C-1″ vinyl groups in addition to the 1,1-ADEQUATE data given above.

The proposed structure for **1** was further supported by a number of NOE cross-peaks. The 3-methylene hydrogens exhibited NOEs with the 1- and 2-methylene and the 5-methyl groups. The 1-methylene shows a very small NOE with the 2-methylene hydrogens, 3-methylene, and 3″-methylene in the side chain in addition to the signal at 6.23 ppm, probably the 1″-methine group. The 7-methyl signal shows an NOE with H-1′ and H-2′. The 2-methylene shows NOEs only with the 1- and 3-methylene groups.

A search of the literature failed to uncover previous reports of naturally occurring tetrasubstituted indolizinium alkaloids. A compound with an identical formula but with *E*,*E*-configuration indicated has been presented.[5] Disubstituted indolizinium alkaloids found in searching the literature are juliprosine from *Prosopis juliflora*[9] and louludinium from the blue-green alga *Lyngbya gracilis*.[10]

**Table 1.** Activity of **1** against Screening Panel

| test organism | $MIC_{50}$ ($\mu$g/mL) |
|---|---|
| *Candida albicans* | 0.25 |
| *Cryptococcus neoformans* | 0.25 |
| *Enterococcus faecium* | 0.25 |
| *Staphylococcus aureus* | >25 |

**Table 2.** Spectrum of Antifungal Activity for **1**

| test organism/number of strains tested | $MIC_{90}$ range ($\mu$g/mL) |
|---|---|
| *Candida albicans*/8 | 0.5−1 |
| *Candida lusitaniae*/2 | 1−4 |
| *Candida glabrata*/2 | 2 |
| *Candida krusei*/3 | 0.5−1 |
| *Candida parapsilosis*/1 | 0.5 |
| *Cryptococcus neoformans*/4 | 0.25−0.5 |

To further evaluate the potential of compound **1** as an antimicrobial chemotherapeutic, testing against additional microorganisms was performed. Initial testing demonstrated that **1** was a potent inhibitor of the growth of an azole-resistant strain of *Candida albicans*, with an $MIC_{50}$ of ~0.25 $\mu$g/mL, but with limited activity against bacteria (Table 1). Additional testing was conducted to determine the breadth of antifungal activity to clinically relevant strains. Compound **1** proved highly toxic, with minimal inhibitory concentrations in the low to sub-microgram range to 20 recent antibiotic resistant isolates (Table 2). Further, compound **1** demonstrated a broad spectrum of activity and potent inhibition of growth against six different genera of clinically significant fungi. In side by side testing against *Candida krusei*, the potency of **1** equaled that of amphotericin B and exceeded the potency of 5-fluorocytosine and fluconazole by factors of 16 and 32, respectively. In the DTP 60 human tumor cell panel, compound **1** had an $LC_{50}$ of $10^{-5}$ M with no selectivity toward any tumor type. Additionally, this compound possessed potent hemolytic activity in the same molarity range as antifungal activity (Figure 2). The calculated log $K_{ow}$ for **1** is 9.1, an indication of lipophilicity that exceeds the value set forth by "Lipinski's rule of 5" for compounds that have favorable, drug-like properties.[11] Thus, lacking favorable chemical properties and possessing hemolytic activity, compound **1** would not be selected as a developmental candidate for antifungal or anticancer use.

## Experimental Section

**General Experimental Procedures.** IR spectra were acquired using a Perkin-Elmer Paragon 500 FT-IR spectrometer. NMR data were recorded at 27 °C in $CD_2Cl_2$ or $CD_3CN$ with TMS as internal standard on a Varian 500 MHz INOVA with spectrometer frequency set at 500 and 125 MHz for $^1$H and $^{13}$C experiments, respectively. Positive ion FAB was conducted using a VG7070-EHF high-resolution mass spectrometer. Analytical and preparative chromatography was done using a Waters 600 pump controlled by MassLynx software. Postcolumn detection was accomplished by a parallel arrangement of a Micromass ZMD electrospray ionization (ESI) mass spectrometer (cone voltage = 30), a Waters 996 photodiode array (PDA), a Sedex 75 evaporative laser light scattering detector (ELSD), and a Kratos Spectroflow 980 spectrofluorimeter.

**Plant Material.** *Aniba panurensis* (Meissn) Mez and other *Aniba* spp. were collected near Bartica, Guyana, by Dr. S. Tawari of the New York Botanical Garden on Sept 11, 1992. Identification was done by the New York Botanical Garden, which maintains herbarium vouchers. Additional vouchers of

each plant part extracted, for example, wood, bark, and leaf, are maintained at the DTP Repository in Frederick, MD.

**Extraction and Isolation.** The dried plant specimens were finely ground in a hammermill. A collection of wood of *A. panurensis* (480 g) was placed into a 3 L borosilicate glass percolator and steeped in $CH_2Cl_2$−MeOH (1:1) for 16 h at room temperature. After draining, the marc was covered with MeOH, which was drained and combined with the $CH_2Cl_2$−MeOH. Solvent was removed by rotary evaporation at <40 °C, followed by high-vacuum drying, resulting in a yield of 22.6 g.

The initial isolation by silica and reversed-phase HPLC resulted in <4 mg of **1**, which was sufficient for molecular weight determination by MS and initial $^1$H NMR analysis to be performed. To obtain an adequate amount of **1** for further biological testing and spectrometric analysis, extracts from several other plant parts of *Aniba* spp., which had been prepared in the same way and which likewise showed toxicity toward azole-resistant *Candida albicans*, were analyzed by HPLC/ELSD/diode array/MS for the presence of a mass ion of 424 amu. Several extracts contained this ion, and one extract was found that had near-identical composition and biological activity with the extract from which the original isolation had been done. The remainder of the original plus this second extract were combined, giving 35 g, which was adsorbed to silica. Nitrogen-assisted flash chromatography was performed in a 3 in. diameter glass column packed to a bed height of 12 in. with water-deactivated Davison grade 22 silica. Elution was by step gradient with $CH_2Cl_2$−MeOH−$H_2O$ mixtures (75:25:0.5, 60:40:5, 30:70:5, 0:50:50). Bioassay showed greatest toxicity to *Candida* in the material eluted with 60:40:5, 13.6 g. This was further fractionated by silica flash chromatography with the same solvent mixture but a more gradual gradient (95:5:0, 85:20:20, 75:25:2, 65:35:3, 55:45:5, 40:60:5, 20:80:10), yielding a fraction of 1.2 g, eluting at 75:25:2, which possessed increased potency against *Candida*. Analytical C8 HPLC-ESIMS of the 1.2 g fraction from above revealed a major component having a strong *m/z* 424 [M$^+$]. Two further preparative chromatographies using a Dynamax C8 HPLC column (250 × 21 mm, 8 $\mu$m, 60A, Varian), eluted with a gradient of MeOH−$H_2O$−trifluoroacetic acid (TFA) (85:15:0.02 to 100:0:0.02), yielded 60 mg of the toxic component as the TFA salt at >95% purity, which was suitable for instrumental analysis and biological evaluation.

**Compound 1:** yellow oil; IR (NaCl) $\nu_{max}$ 2928, 2856, 1779, 1734, 1688, 1602, 1467, 1199, 1143, 1033, 801, 739 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 284 (3.6) nm; $^1$H NMR ($CD_2Cl_2$, 500 MHz) $\delta$ 6.23 (1H, br ab, $J$ = 11.3 Hz, H-1′), 6.23 (1H, br ab, $J$ = 11.4 Hz, H-1″), 6.10 (1H, ab, $J$ = 11.3, 7.4 Hz, H-2′), 6.07 (1H, ab, $J$ = 11.4, 7.4 Hz, H-2″), 4.73 (2H, t, $J$ = 7.7 Hz, H-3), 3.31 (2H, t, H-1), 2.63 (3H, s, 5-C$H_3$), 2.49 (2H, p, $J$ = 7.7 Hz, H-2), 2.31 (3H, s, 7-C$H_3$), 1.80 (2H, dq, $J$ = 7.4, 1.6 Hz, H-3″), 1.74 (2H, br q, $J$ = 7.4 Hz, H-3′), 1.36 (2H, m, H-4′ and H-4″), 1.27 (2H, sextplt, H-9′ and H-9″), 1.24−1.18 (2H, m, H-5′, H-5″, H-6′, H-6″, H-7′, H-7″, H-8′, and H-8″), 0.86 (3H, t, $J$ = 7.0 Hz, H-10′ and H-10″); $^{13}$C NMR ($CD_2Cl_2$, 125 MHz) $\delta$ 156.0 (C, C-7), 154.3 (C, C-9), 148.1 (C, C-5), 139.6 (CH, C-2″), 139.4 (CH, C-2′), 136.0 (C, C-6), 132.2 (C, C-8), 121.8 (CH, C-1′), 120.9 (CH, C-1″), 58.2 ($CH_2$, C-3), 32.6 ($CH_2$, C-1), 32.21 ($CH_2$, C-8′ and C-8″), 29.75, 29.73 ($CH_2$, C-5″ and C-5′), 29.66 ($CH_2$, C-6′ and C-6″), 29.61 ($CH_2$, C-7′ and C-7″), 29.55, 29.44 ($CH_2$, C-3″ and C-3′), 29.03, 28.90 ($CH_2$, C-4′ and C-4″), 23.00 ($CH_2$, C-9′ and C-9″), 20.9 ($CH_2$, C-2), 19.0 ($CH_3$, 7-$CH_3$), 18.1 ($CH_3$, 5-$CH_3$), 14.20 ($CH_3$, C-10′ and C-10″); ESIMS *m/z* 424 [M]$^+$ (81), 408 (0.2), 396 (0.1), 394 (0.5), 380 (0.5), 366 (0.5), 352 (1), 338 (4), 326 (3.6), 324 (30), 310 (60), 296 (4), 282 (3), 268 (3.2), 254 (8), 240 (46), 226 (13), 212 (100), 198 (6), 184 (5); HRFABMS *m/z* 424.390 [M]$^+$ (calcd for $C_{30}H_{50}N$, 424.394).

**Antimicrobial and Hemolytic Activities.** Initial high-throughput biological screening was performed using four clinical isolates in a broth microdilution assay on ∼140 000 crude extracts at a single dose of 250 $\mu$g/mL. *Candida albicans* 99-788 and *Cryptococcus neoformans* 96-1034 were obtained from Michael Rinaldi, while *Staphylococcus aureus* 385 and *Enterococcus faecium* 1438 were obtained from James Jorgensen, both at the University of Texas Health Science Center in San Antonio, TX. All cultures were grown at 37 °C with shaking at 100 rpm. *S. aureus* 385 or *E. faecium* 1438 was grown in Todd Hewitt broth supplemented with 0.5% yeast extract and 2% sodium chloride. *C. albicans* 99-788 or *C. neoformans* 96-1034 was grown in YM broth. Cultures were grown to exponential growth phase, then diluted to an $A_{600}$ = 0.3 using the appropriate medium. Subsequently, *S. aureus* was diluted 1:100, *E. faecium* was diluted 1:1000, *C. albicans* was diluted 1:150, and *C. neoformans* was diluted 1:128. Extracts were prepared in polystyrene tissue culture treated 96-well microtiter plates (88 different extracts at 50 $\mu$g/well), and high-vacuum dried. Two hundred microliters of the final dilution of a culture was added to each well. Extracts demonstrating >90% microbial growth inhibition were tested in serial dilution format at a top dose of 250 $\mu$g/mL and five 2-fold dilutions by the same protocol to estimate potency. Inhibition of growth of the cultures was confirmed using ciprofloxacin at 100 $\mu$g/mL for *S. aureus*, chloramphenicol at 100 $\mu$g/mL for *E. faecium*, and nystatin at 100 $\mu$g/mL for both *C. albicans* and *C. neoformans*. *E. faecium* was incubated for 18 h, *S. aureus* and *C. albicans* were incubated for 24 h, and *C. neoformans* was incubated for 72 h. Following incubation, the density of the cultures was determined by reading the $A_{650}$. The $MIC_{50}$ was estimated as the concentration of test sample that reduced the $A_{650}$ by 50% or more relative to cultures in medium alone.

Supporting antimicrobial analysis was performed by Richard J. Hollis at the Molecular Epidemiology and Fungus Lab, University of Iowa College of Medicine, Pathology Department, 273 MRC, Iowa City, IA 52242, using 18 recent drug-resistant clinical isolates, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019. Analysis of biological activity was performed in a broth microdilution assay using RPMI 1640 buffered with MOPS and following the NCCLS guidelines.[12] Results given in Table 2 are reported as the $MIC_{90}$.

Hemolysis was determined as previously described,[13] except the highest dose of the test material contained 5% DMSO. The samples were serially diluted in phosphate-buffered saline and mixed with a final concentration of 4% sheep red blood cells in 0.2 mL. After an incubation of 2 h at 37 °C, 0.1 mL was used to determine the hemolysis.

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**Note Added in Proof:** After acceptance of the manuscript, a revised structure of the compound reported in ref 7 was published in this journal, Vol. 67, pp 1036−1038. This structure is identical to the alkaloid reported here.

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