ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1990, p. 529–533
0066-4804/90/040529-05$02.00/0

Vol. 34, No. 4

3-Methoxysampangine, a Novel Antifungal Copyrine Alkaloid from *Cleistopholis patens*

SHIHCHIH LIU,[1] BABAJIDE OGUNTIMEIN,[2] CHARLES D. HUFFORD,[1] AND ALICE M. CLARK[1,3]*

Department of Pharmacognosy[1] and Research Institute of Pharmaceutical Sciences,[3] School of Pharmacy, The University of Mississippi, University, Mississippi 38677, and Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria[2]

Received 4 October 1989/Accepted 15 January 1990

Further examination of the active ethanolic extract of the root bark of *Cleistopholis patens* by using bioassay-directed fractionation resulted in the isolation of a new alkaloid, 3-methoxysampangine (compound I), together with three known alkaloids, eupolauridine (compound II), liriodenine (compound III), and eupolauridine *N*-oxide (compound IV). The proposed structure of compound I was based on its physicochemical properties and spectral data. 3-Methoxysampangine exhibited significant antifungal activity against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. This is the first report of the isolation of liriodenine (compound III) from the root bark of *C. patens*.

The need for new, more effective, and less toxic antifungal antibiotics for the treatment of disseminated mycotic infections is obvious in light of the significant toxicities and failure rates of the currently available systemic antifungal agents. This problem has become particularly relevant in view of the fact that opportunistic disseminated mycoses are a common complication of acquired immunodeficiency syndrome. Discovery of new antibiotics has relied primarily upon isolation of such agents from natural sources. The major advantage of this approach over chemical synthesis or modification of existing agents is the probability of identifying new prototype drugs with quite different chemical structures and, hence, dissimilar toxicities and cross-resistances with present drug therapies. Although microorganisms have traditionally served as the primary source for new antibiotics, it has recently been shown that higher plants also serve as sources for a number of diverse antimicrobial agents (2–8).

We previously reported the isolation of an antifungal alkaloid, eupolauridine (compound II), from the ethanolic extract of the root bark of *Cleistopholis patens* (Benth) Engl. and Diels (*Annonaceae*) collected in Nigeria (6). Reexamination of the ethanolic extract of the same plant has resulted in the isolation of a new alkaloid, 3-methoxysampangine (compound I), in addition to the known alkaloids eupolauridine (compound II), liriodenine (compound III), and eupolauridine *N*-oxide (compound IV) (Fig. 1). Bioassay-directed isolation and characterization of the new alkaloid and evaluation of its in vitro antifungal activity are reported herein.

MATERIALS AND METHODS

Plant material. The root bark of *C. patens* was collected in the summer of 1987 on the University of Ife campus in Ife, Nigeria. The plant material was identified by Z. O. Gbile of the Forestry Research Institute of Nigeria. Herbarium specimens were deposited in the herbarium of the Forestry Research Institute of Nigeria and the Botany Department, University of Ife, Ife, Nigeria.

General experimental procedures. Melting points were determined on a Kofler hot-stage instrument and were uncorrected. The UV spectrum was recorded on a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer, and the infrared spectra were obtained with a Perkin-Elmer 281B spectrometer. Low-resolution electron impact mass spectra (MS) were determined with a Finnigan 3200 GC/MS mass spectrometer operating at 70 eV. High-resolution MS were measured at the Mass Spectrometry Laboratory, Department of Chemistry, University of Kansas, Lawrence. ^{1}H- and ^{13}C nuclear magnetic resonance (NMR) spectral data were recorded in deuteriochloroform on a Varian VXR-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Two-dimensional NMR experiments were conducted with the standard Varian software for ^{1}H homonuclear correlated, ^{1}H-^{13}C heteronuclear correlated, and long-range ^{1}H-^{13}C heteronuclear correlated spectra. Long-range ^{1}H-^{13}C heteronuclear correlated spectrum experiments were run twice, once with J = 10 Hz and once with J = 5 Hz. Thin-layer chromatography (TLC) was performed on silica gel with ethyl acetate (EtOAc)-methanol (MeOH) (4:1) as the developing solvent, and detection was achieved with a long-wavelength UV lamp and Dragendorff spray reagent.

Extraction and fractionation. Dried, ground root bark (3.85 kg) was percolated with *n*-hexane (20 liters) and then with 95% ethanol (EtOH) (60 liters) and then extracted with hot EtOH (30 liters). Evaporation of the solvent of each extract in vacuo gave residues of 27.0, 210.0, and 100.0 g, respectively. The active cold- and hot-EtOH extracts were combined (310.0 g) and partitioned between $CHCl_3$ (4 liters) and H_2O (1 liter), and the H_2O layer was further extracted with EtOAc (1 liter). The $CHCl_3$ (96 g) and EtOAc (16 g) extracts showed in vitro antifungal activity and were combined (112 g total) for further separation.

Chromatography of the active organic extract. The active fraction (112.0 g) was adsorbed onto 70 g of Celite 545 and subjected to chromatography over silica gel (1.2 kg; Macherey-Nagel; 70/240 mesh). Elution with $CHCl_3$ was followed by a stepwise gradient by increasing the percentage (5, 10, 20, and 50%) of MeOH in $CHCl_3$. Fractions (250 ml) were collected and pooled on the basis of similar TLC results. A pooled active fraction (22.0 g) eluted with 5% MeOH–$CHCl_3$ was further chromatographed on alumina (400 g of Macherey-Nagel neutral, grade III). Elution was initiated with 20% MeOH–EtOAc and then continued stepwise with increasing percentages (20, 40, 60, and 80%) of MeOH in EtOAc. Fractions (300 ml) were collected and pooled on the

* Corresponding author.

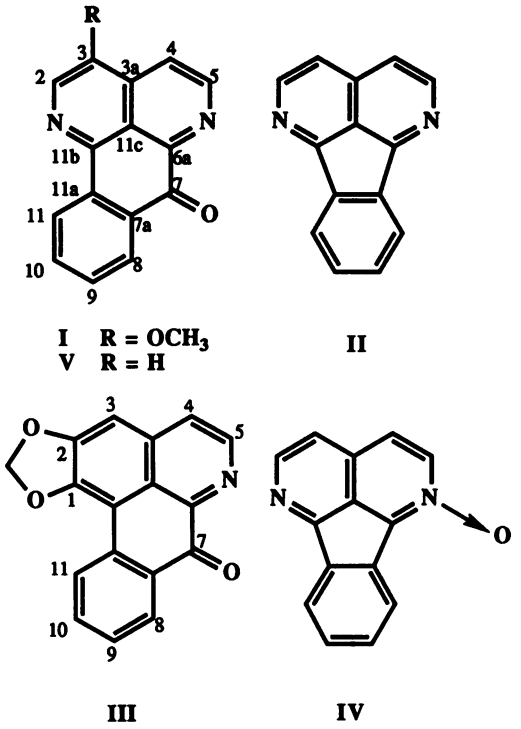

FIG. 1. Structures of antifungal alkaloids.

basis of TLC analyses to afford the following four pooled fractions: fraction 2 to 3 (20% EtOAc–MeOH, 1.27 g), fraction 7 to 9 (40% EtOAc–MeOH, 1.05 g), fraction 11 to 13 (60% EtOAc–MeOH, 2.16 g), and fraction 15 (100% EtOAc–MeOH, 6.14 g). Each fraction was separately purified over silica gel by using EtOAc–MeOH (4:1) as the eluting solvent to yield eupolauridine (compound II) (75 mg, 0.00195%), 3-methoxysampangine (compound I) (6 mg, 0.000156%), eupolauridine *N*-oxide (compound IV) (18 mg, 0.000468%), and liriodenine (compound III) (34 mg, 0.00088%), respectively.

3-Methoxysampangine (compound I) was crystallized from *n*-hexane–EtOAc as yellow needles; its characteristics were as follows: melting point, 213 to 215°C; high-resolution MS observed, 262.0742; high-resolution MS calculated for $C_{16}H_{10}N_2O_2$, 262.0742; UV λ_{max} (MeOH), 219, 253, 309, 332, and 429 nm; infrared ν_{max} (potassium bromide), 1,673, 1,598, 1,570, 1,380, 1,300, 1,238, 1,021, 954, 750, 720, and 631 cm^{-1}; electron impact MS *m/z* (relative abundance), 262 (M^+, 31), 247 (14.7), 220 (61), 219 (100), 191 (21.4), 165 (21.9), 164 (51.7), 137 (15.7). For ^1H and ^{13}C NMR spectral data, see Table 1.

Qualitative antifungal evaluation. In vitro evaluation of activity versus *Candida albicans* NIH B311, *Cryptococcus neoformans* ATCC 32264, and *Aspergillus fumigatus* ATCC 26934 was accomplished by using the agar-well difusion assay previously described (2), with the following modifications. *C. albicans* NIH B311, which is used to induce experimental disseminated candidiasis, was used for the initial qualitative evaluation of anticandidal activity. The organism was grown in Sabouraud dextrose broth for 24 h at 37°C, after which the cells were harvested by centrifugation (4°C, 2,000 rpm, 3 min). After centrifugation, the cells were washed and suspended in sterile 0.9% saline to give a final concentration of 10^6 CFU/ml (adjusted with a hemacytometer). Inocula of *C. neoformans* and *A. fumigatus* were prepared by suspension of the surface growth of stock agar slants in sterile H_2O as previously described (2, 5). Culture plates (15 by 100 mm) for the qualitative assay were prepared from 25 ml of Sabouraud dextrose agar. With sterile cotton swabs, the plates were streaked with the suspension of the appropriate test organism. Cylindrical plugs were removed from the agar plates with a sterile cork borer to produce wells with diameters of approximately 11 mm. To the wells was added 100 μl of a solution or suspension of an extract, fraction, or pure compound. Crude extracts and fractions were tested at a concentration of 20 mg/ml, whereas pure compounds were tested at 1 mg/ml. When solvents other than H_2O, EtOH, MeOH, dimethyl sulfoxide, dimethyl formamide, or acetone were required to dissolve extracts or compounds, solvent blanks were included. Antifungal activity was recorded as the width (in millimeters) of the zone of inhibition, measured from the edge of the agar well to the edge of the zone, following incubation of the plates for 24 h (37°C for *C. albicans*, 30°C for *A. flavus* and 26°C for *C. neoformans*). The antifungal agents amphotericin B and ketoconazole were included as positive controls in each assay.

Quantitative antifungal evaluation. The method used to determine the MIC was the twofold serial broth dilution assay (2, 5, 6) in yeast nitrogen broth (Difco Laboratories) for *C. albicans* and *C. neoformans* and Sabouraud dextrose broth for *A. fumigatus*. The inoculum for the MIC determination was prepared as described for the qualitative evaluation. With a calibrated sterile wire loop, each tube was inoculated with 10 μl of the suspension. The MIC was taken as the lowest concentration of a compound that inhibited the growth of the test organisms after an appropriate incubation period (37°C for 24 and 48 h for *C. albicans*; 30°C for 48 and 72 h for *A. fumigatus*; 26°C for 48 and 72 h for *C. neoformans*). The antifungal agent amphotericin B was included as a positive control in each assay.

RESULTS AND DISCUSSION

As part of our continuing search for new anticandidal drugs from natural sources, we recently reported the isolation of the antifungal alkaloid eupolauridine (compound II) from the root bark of the West African tree *C. patens*. Further examination of the active ethanolic extract of the

TABLE 1. ^1H (300 MHz) and ^{13}C (75 MHz) NMR assignments for 3-methoxysampangine (compound I)

Hydrogen no. or group (multiplicities, coupling constants)	Carbon no. or group[a]
H-2, 8.36 (1 H, s)	C-2, 126.8 (1)
	C-3, 149.9 (0)
	C-3a, 131.8 (0)
H-4, 8.21 (1 H, d, *J* = 5.4 Hz)	C-4, 118.8 (1)
H-5, 9.13 (1 H, d, *J* = 5.4 Hz)	C-5, 148.0 (1)
	C-6a, 147.2 (0)
	C-7, 182.0 (0)
	C-7a, 131.5 (0)
H-8, 8.43 (1 H, dd, *J* = 7.8, 1.2 Hz)	C-8, 128.5 (1)
H-9, 7.61 (1 H, ddd, *J* = 7.8, 7.8, 1.2 Hz)	C-9, 130.2 (1)
H-10, 7.78 (1 H, ddd, *J* = 7.8, 7.8, 1.2 Hz)	C-10, 134.6 (1)
H-11, 8.65 (1 H, dd, *J* = 7.8, 1.2 Hz)	C-11, 124.6 (1)
	C-11a, 135.7 (0)
	C-11b, 143.2 (0)
	C-11c, 119.7 (0)
OCH_3, 4.18 (3H, s)	OCH_3, 56.6 (3)

[a] The numbers in parentheses refer to numbers of attached protons as determined by the attached-proton test.

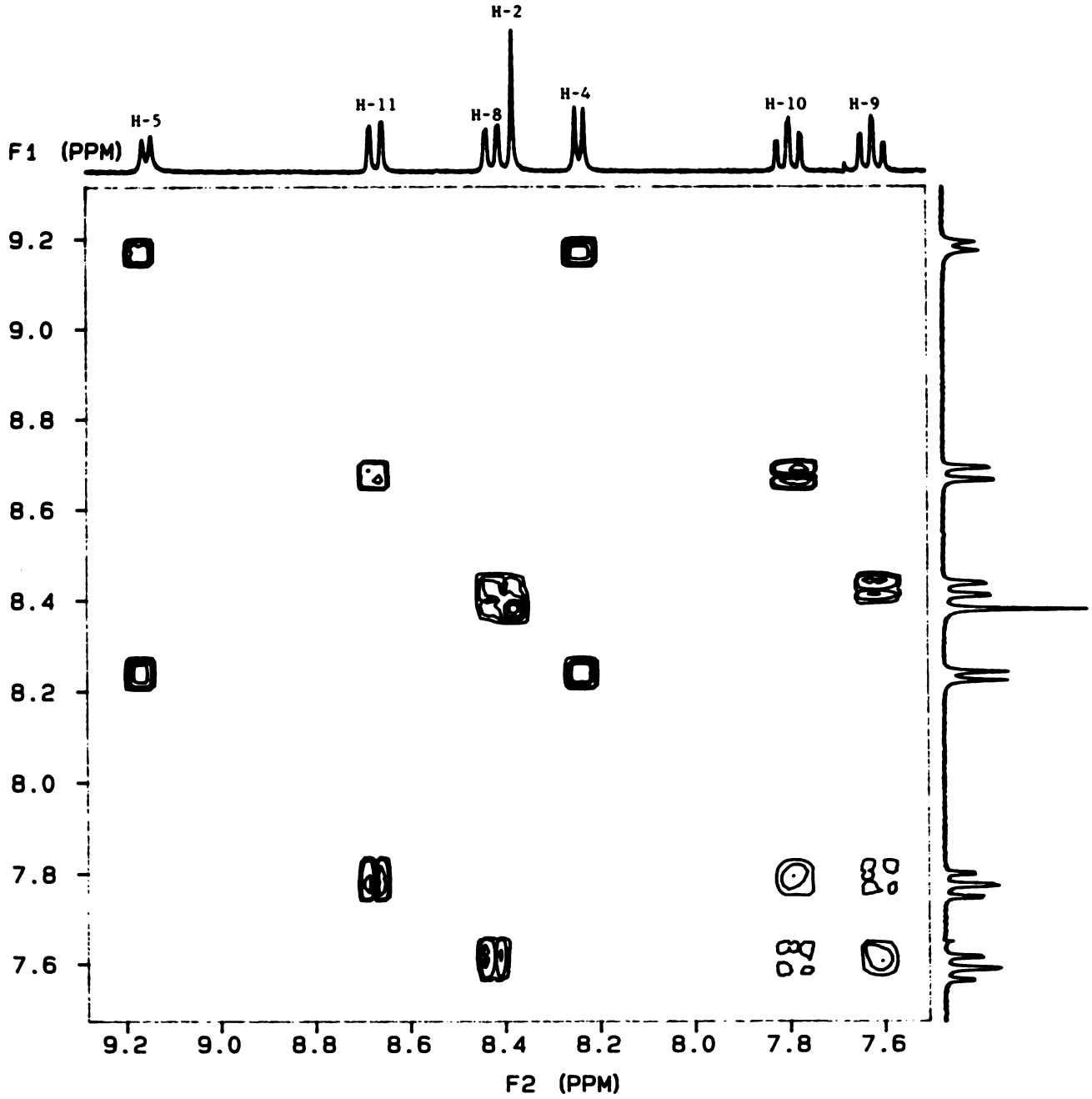

FIG. 2. ^{1}H homonuclear correlated spectrum of 3-methoxysampangine (compound I).

root bark by a bioassay-directed fractionation approach resulted in the isolation and identification of a novel antifungal alkaloid, 3-methoxysampangine (compound I).

The dried, ground root bark of *C. patens* was percolated with *n*-hexane, followed by 95% EtOH and then hot EtOH. The antifungal activity was concentrated in the ethanolic extracts, which were combined and subjected to bioassay-directed fractionation by partitioning between $CHCl_3$ and H_2O and then EtOAc and H_2O. The active $CHCl_3$- and EtOAc-soluble fractions were combined, and the combined organic fraction was chromatographed over silica gel by using $CHCl_3$ and gradually increasing percentages of MeOH in $CHCl_3$ as eluents. Fractions were pooled on the basis of similar TLC patterns, and activity was found to be concentrated in fractions eluted with 5% MeOH–$CHCl_3$, which were pooled and further purified by chromatography over neutral alumina by using mixtures of EtOAc–n-hexane as eluting solvents. Four pooled fractions were each further purified by chromatography over silica gel by using EtOAc-MeOH as the eluting solvent to yield eupolauridine (compound II), 3-methoxysampangine (compound I), eupolauridine *N*-oxide (compound IV), and liriodenine (compound III), respectively.

Eupolauridine (compound II) and liriodenine (compound III) were identified by direct comparisons (melting point, mixture melting point, TLC, and MS) with authentic samples of eupolauridine and liriodenine obtained previously in our laboratory (4, 6). The identification of eupolauridine *N*-oxide

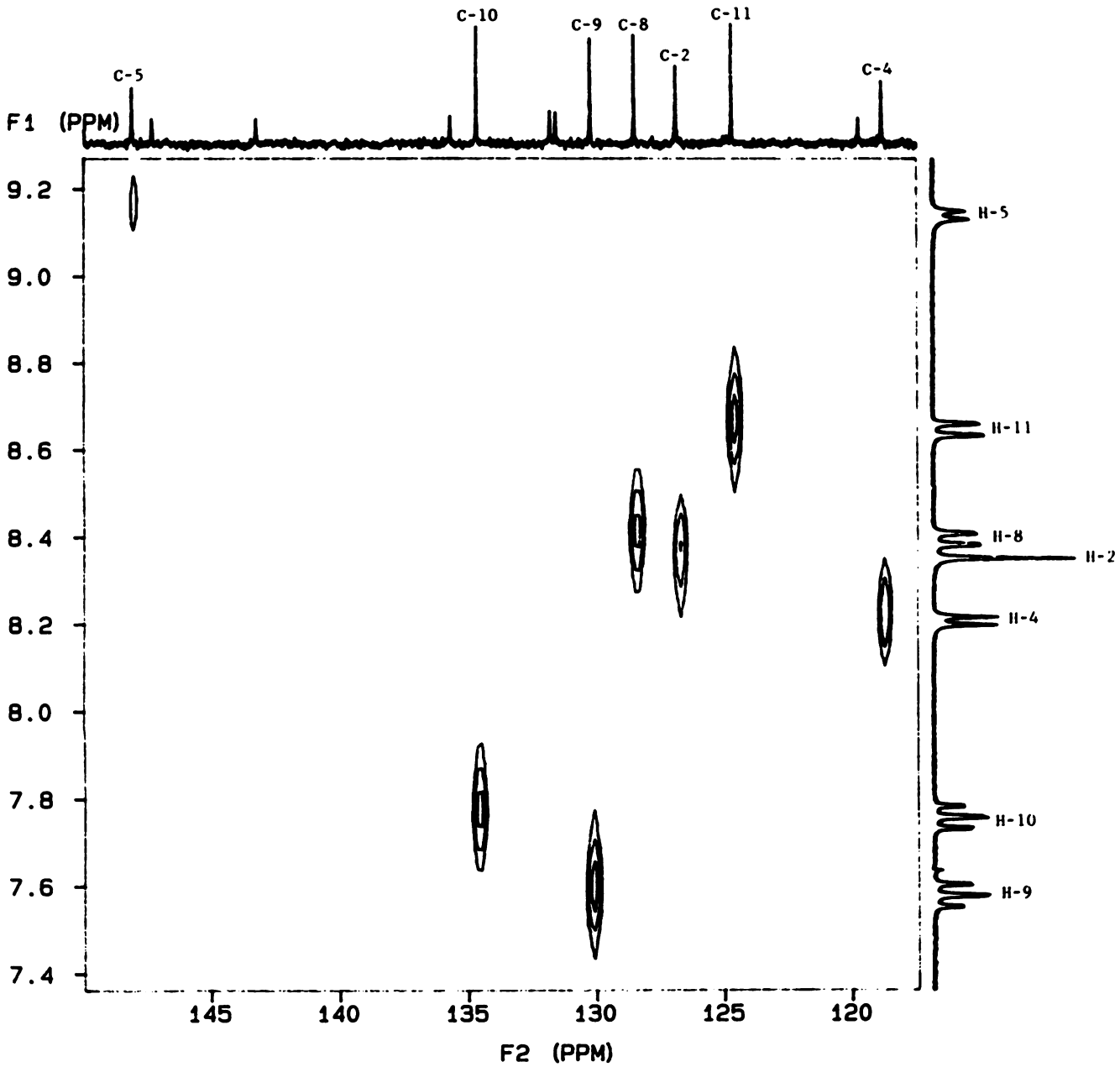

FIG. 3. ^{1}H-^{13}C heteronuclear correlated spectrum of 3-methoxysampangine (compound I).

was based on a comparison of its physicochemical and spectral properties (^{1}H and ^{13}C NMR and MS) with the data reported for compound IV (10).

The new compound, 3-methoxysampangine (compound I) was isolated in a 0.00016% yield from the root bark as yellow needles (melting point, 213 to 215°C). Electron-impact MS showed a molecular ion peak at *m/z* 262, corresponding to the molecular formula $C_{16}H_{10}N_2O_2$. This formula was confirmed by high-resolution MS and indicated the presence of a condensed-ring system (4). This was verified by the UV spectrum, which showed bands at λ_{max} 309, 332, and 429 nm, characteristic of a highly conjugated oxoalkaloid, and the infrared spectrum, which showed a carbonyl absorption band at 1,673 cm^{-1}. The 300-MHz ^{1}H NMR spectrum (Table 1) revealed a total of seven aromatic protons, including four protons at δ 8.65 (1H, dd), 8.43 (1H, dd) 7.78 (1H, ddd), and 7.61 (1H, ddd), which were determined to be an ABMX system characteristic of a 1,2-disubstituted benzene nucleus. Two pairs of aromatic doublets coupled to each other (δ 9.13 and 8.21, J = 5.4 Hz) could be assigned to H-2 and H-3 of a pyridine ring. The only remaining signals in the ^{1}H NMR spectrum were for one aromatic proton, which resonated as a singlet (δ 8.36), and a three-proton singlet for an aromatic methoxyl at δ 4.18. The ^{13}C NMR spectral data revealed 16 signals as one methyl, seven methines, and eight quaternary carbons. A search of the literature revealed that the combined spectroscopic data were similar to those of sampangine (compound V), an alkaloid isolated from *Cananga odorata* (9), whose structure was confirmed by synthesis (1). On the basis of ^{1}H and ^{13}C NMR spectral data, the methoxyl group could be located at carbon 2, 3, 4, or 5. The location of the methoxyl group at C-3 was established by unambigu-

ous assignment of all of the carbon signals of compound I by the use of two-dimensional NMR techniques.

The key experiment used in assigning the carbon signals measured the ^{1}H-^{13}C long-range heteronuclear correlated spectrum. The carbonyl carbon (δ 182.0) showed a three-bond correlation to the signal at a δ_H of 8.43, which could then be assigned to H-8. The ^{1}H homonuclear correlated and ^{1}H-^{13}C heteronuclear correlated spectra (Fig. 2 and 3) then established the ^{1}H and ^{13}C NMR assignments for 8, 9, 10, and 11. Further interpretation of the ^{1}H-^{13}C long-range heteronuclear correlated spectra allowed assignments of C-7a, 11a, and 11b, which showed appropriate three-bond correlations to these four protons (H-8, 9, 10, and 11). The signal assigned to C-11b (δ_C, 143.2) also showed a three-bond correlation to the only proton singlet (H-2, [δ, 8.36]) which then defines C-3 for the position of the methoxyl group. Carbons C-11c and 6a showed appropriate three-bond correlations to H-4 and H-5, which are also uniquely assigned. The complete ^{1}H and ^{13}C NMR assignments are listed in Table 1.

3-Methoxysampangine demonstrated significant in vitro activity against the yeasts *C. albicans* and *C. neoformans* and the filamentous fungus *A. fumigatus*. The MIC of compound I were 3.12 μg/ml for *C. albicans* and *A. fumigatus* and 0.2 μg/ml for *C. neoformans*. By comparison, the MICs of amphotericin B range from 0.78 μg/ml for *C. albicans* to 3.12 μg/ml for *C. neoformans* and 1.56 μg/ml for *A. fumigatus*. Additional studies on antifungal activity are in progress. The antifungal activities of liriodenine (compound III) and the eupolauridine (compound II) have been described previously (4, 6); however, eupolauridine *N*-oxide showed no antifungal activity.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service contracts NO1-AI-42549 and NO1-AI-72638 from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

LITERATURE CITED

1. **Bracher, F.** 1989. Synthese von Cleistopholin and Sampangin. Liebigs Ann. Chem. **1989**:87–88.
2. **Clark, A. M., F. S. El-Feraly, and W.-S. Li.** 1981. Antimicrobial activity of phenolic constituents of *Magnolia grandiflora* L. J. Pharm. Sci. **70**:951–952.
3. **El-Feraly, F. S., A. T. McPhail, and K. D. Onan.** 1978. X-ray crystal structure of canellal, a novel sesquiterpene from *Canella winterana*. J. Chem. Soc. Chem. Comm. **1978**:75–76.
4. **Hufford, C. D., M. J. Funderburk, J. M. Morgan, and L. W. Robertson.** 1975. Two antimicrobial alkaloids from heartwood of *Liriodendron tulipifera* L. J. Pharm. Sci. **64**:789–792.
5. **Hufford, C. D., S. Liu, and A. M. Clark.** 1988. Antifungal activity of *Trillium grandiflorum* constituents. J. Nat. Prod. **51**:94–98.
6. **Hufford, C. D., S. Liu, A. M. Clark, and B. O. Oguntimein.** 1987. Anticandidal activity of eupolauridine and onychine, alkaloids from *Cleistopholis patens*. J. Nat. Prod. **50**:961–964.
7. **Mitscher, L. A.** 1980. New developments in natural products of medicinal interest. Annu. Rep. Med. Chem. **15**:255–266.
8. **Oliver-Bever, B.** 1983. Medicinal plants in tropical West Africa. III. Anti-infection therapy with higher plants. J. Ethnopharmacol. **9**:1–83.
9. **Rao, J. U. M., G. S. Giri, T. Hanamaiah, and K. V. Rao.** 1986. Sampangine, a new alkaloid from *Cananga odorata*. J. Nat. Prod. **49**:346–347.
10. **Waterman, P. G., and I. Muhammad.** 1985. Sesquiterpenes and alkaloids from *Cleistopholis patens*. Phytochemistry **24**:523–527.