Journal of Natural Products
Vol. 50, No. 5, pp. 961-964, Sep-Oct 1987

ANTICANDIDAL ACTIVITY OF EUPOLAURIDINE AND ONYCHINE, ALKALOIDS FROM *CLEISTOPHOLIS PATENS*

CHARLES D. HUFFORD, SHIHCHIH LIU, ALICE M. CLARK,*

Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, Mississippi 38677

and BABAJIDE O. OGUNTIMEIN

Department of Pharmacognosy, Faculty of Pharmacy, University of Ife, Ile-Ife, Nigeria

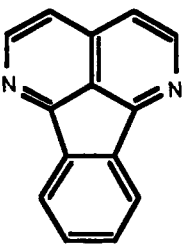
Cleistopholis patens (Benth.) Engl. and Diels (Annonaceae) is a large tree found throughout West Africa. Two previous studies on the stem and root bark have resulted in the isolation of sesquiterpenes and alkaloids (1,2). Alcoholic extracts of the root bark of *C. patens* showed significant anticandidal activity. As a part of our continuing search for new anticandidal drugs from natural sources, we report herein our results on the bioassay directed isolation of the active anticandidal component of *C. patens*.

The dried, ground root bark was extracted by percolation with *n*-hexane (inactive) and then with 95% EtOH. The active alcoholic extract was partitioned between $CHCl_3$ and H_2O. The active $CHCl_3$ extract was chromatographed over silicic acid using $CHCl_3$ and a stepwise gradient of increasing percentage of MeOH/$CHCl_3$ as eluent. Fractions were pooled on the basis of tlc analysis. The anticandidal activity was concentrated in a fraction (500 mg) that was eluted with 5% MeOH in $CHCl_3$. Further purification of this fraction over neutral alumina using EtOAc/*n*-hexane as eluting solvent resulted in the isolation of the active component as a yellow crystalline material having mp 153-155°. This substance was identified as the previously reported alkaloid, eupolauridine [**1**] (2-4), by comparison with an authentic reference sample.

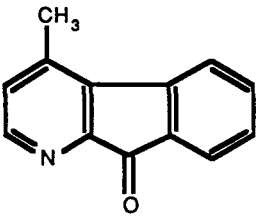
Eupolauridine [**1**] exhibited a significant zone of inhibition against three test strains of *C. albicans*. The minimum inhibitory concentration (MIC) of eupolauridine was found to be 1.56 μg/ml for each of the three strains in yeast-nitrogen broth. By comparison, the MIC value of amphotericin B, the current drug of choice for disseminated candidiasis, is 0.39 μg/ml. Consequently, eupolauridine was considered a promising potential new antifungal drug.

Because only small amounts were isolated and much larger quantities are necessary for in vivo evaluation, eupolauridine [**1**] was prepared by synthesis as previously reported (3). All of the synthetic intermediates were also evaluated for anticandidal activity. One of the intermediates [**2**] was also shown to be active against *Candida*. This intermediate is also the known alkaloid onychine, which has been reported from *C. patens* (2) and originally reported from *Onychopetalum amazonicum* (5). The original structure reported for onychine [**3**] (5) and reported in *C. patens* (2) and elsewhere (6) has been shown to be incorrect (7,8); the correct structure is represented by **2**. While the structure reported for onychine [**2**] (7,8) appears to be based on solid chemical evidence, a COSY spectrum of dihydroonychine [**4**] (2,5) clearly showed long-range couplings between H-9 and the protons of the methyl group independently confirming structure **2** for onychine.

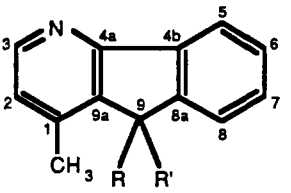
The physical and spectroscopic data reported for **1** and **2** (2,6,7) agree well with the data obtained here except for the ^{13}C-nmr assignments for onychine [**2**]. The ^{1}H-nmr assignments (2,6) were confirmed by examining the COSY spectrum of **2**. The ^{13}C-nmr assignments reported here (see Experimental section) differ significantly with those reported previously (2) and were estab-



1



3



2 R,R′=O
4 R=H, R′=OH

lished by utilizing ^{1}H-^{13}C heteronuclear correlations and by examing the proton-coupled data. The quaternary center assignments (1, 4a, 4b, 8a, 9a) were confirmed by utilizing selective low power decoupling techniques (irradiation at H for H-2, H-3, H-5, H-6, H-7, H-8, and CH_3).

Onychine is comparable to eupolauridine in its anticandidal activity with an MIC value of 3.12 μg/ml against *C. albicans* B311 in yeast-nitrogen broth. Therefore, onychine is also considered a potential candidate for further development. Studies are currently in progress to evaluate the in vivo efficacy and toxicity of eupolauridine and onychine, and those results will be reported elsewhere.

EXPERIMENTAL

PLANT MATERIAL.—The root bark of *C. patens* was collected in May 1985, on the University of Ife campus, Nigeria. The plant material was identified by Dr. Z.O. Gbile of the Forestry Research Institute of Nigeria. Herbarium specimens representing this collection are deposited in the Herbaria of the Forestry Research Institute of Nigeria, Ibadan, and Botany Department, University of Ife, Nigeria.

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot stage instrument and are uncorrected. The ir spectra were determined on a Perkin-Elmer 281B spectrometer. ^{1}H- and ^{13}C-nmr spectra were all recorded in $CDCl_3$ on a Varian VXR-300 operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The multiplicities were determined by the APT experiment. The proton coupled ^{13}C-nmr spectrum was determined by gating the decoupler off during data acquisition. The selective low power decoupling experiments centered the decoupler on the proton resonances and used $\gamma H_2/2\pi = 200$ Hz (9,10). The 2D-nmr experiments were conducted using the standard Varian software for COSY and HETCOR.

Tlc was performed on Si gel using EtOAc-*n*-hexane (1:1) or alumina using EtOAc-*n*-hexane (9:1) (2) as developing solvents and Dragendorff's reagent as the detecting spray.

EXTRACTION AND FRACTIONATION.—The dried, ground root bark was percolated with *n*-hexane (9 liters) to give 15 g of an oily residue after removal of the solvent (inactive). The air-dried marc was then percolated with 95% EtOH (15 liters). Evaporation of the EtOH left 149.0 g of residue which showed only marginal anticandidal activity. The alcoholic extract (149.0 g) was partitioned between $CHCl_3$ and H_2O (300 ml each, extracted 3 times with 300 ml $CHCl_3$). The $CHCl_3$ residue (26.7 g) showed significant anticandidal activity.

CHROMATOGRAPHY OF THE ACTIVE $CHCl_3$ EXTRACT.—The $CHCl_3$ soluble fraction (26.7 g) was adsorbed onto diatomaceous earth and chromatographed over silicic acid (500 g, 100 mesh). Elution with $CHCl_3$ was followed by a stepwise gradient of increasing MeOH in $CHCl_3$. Fractions (250 ml) were collected and pooled on the basis of tlc analyses. The activity was concentrated in pooled fraction B (500 mg, eluted with 5% MeOH/$CHCl_3$). Further purification of fraction B using dry column chromatography (80 g,

alumina, N, III) and 20% EtOAc in *n*-hexane as eluting solvent yielded an active fraction from which 5 mg of eupolauridine [**1**] was obtained by crystallization from EtOAc/*n*-hexane, mp 153-155°. The identity was confirmed by a direct comparison with an authentic sample (mmp, tlc, ir, ^{1}H and ^{13}C nmr). The physical and spectral properties have been reported for **1** (2, 4, 7, 9). There appeared to be small amounts of onychine [**2**] present in the mother liquors (tlc using reference sample).

SYNTHESIS OF ONYCHINE [**2**] AND EUPOLAURIDINE [**1**].—The preparation of **1** and **2** followed the procedure essentially as previously published (3,7). The synthetic samples were characterized by comparison of their physical and spectral properties with those previously reported (2, 3, 6). The ^{13}C-nmr spectral assignments are listed here for onychine [**2**], inasmuch as they differ considerably with those reported previously (2); δ_C (ppm) 147.1 s (C-1), 125.6 d (C-2), 152.7 d (C-3), 165.1 s (C-4a), 143.0 s (C-8a), 123.5 d (C-5), 130.6 d (C-6), 134.7 d (C-7), 120.6 d (C-8), 134.9 s (C-4b), 192.7 s (C-9), 125.8 s (C-9a), 17.2 q (CH_3). The proton-coupled data showed the 165.1 ppm signal as dd (J=2.8, 12.6 Hz), the 147.1 ppm signal as dq (J=5.9, 5.9 Hz), the 143.0 ppm as dd (J=7.3, 7.3 Hz), the 134.9 ppm signal as dd (J=8.0, 8.0 Hz), the 125.8 ppm signal as m, and all protonated carbons as dd with one-bond and three-bond couplings (C-3 shows two-bond coupling) except for C-2, which is a complex signal. Selective decoupling at δ H=2.53 (CH_3) collapsed the following signals: 147.1 and 125.8 each to d, 125.6 to dd; at δ H=8.33 (H-3) collapsed the signals 147.1 to a q, 125.8 was simplified; at δ H=6.89 (H-2) simplified the signal at 125.8; at δ H=7.33 (H-6) or 7.71 (H-8) each collapsed the 134.9 signal to a d; at δ H=7.58 (H-5) or 7.46 (H-7) each collapsed the 143.0 signal to a d.

DIHYDROONYCHINE [**4**].—Compound **4** was prepared by $NaBH_4$ reduction of **2** as described previously (2). It had mp 154-155° and other spectroscopic properties as reported (2,5). The ^{13}C-nmr spectral assignments are listed here since they have not been reported previously. The assignments were made by comparison with onychine, heterocorrelation, from proton-coupled data and from selective proton decouplings at low decoupling power levels; δ_C (ppm) 145.2 s (C-1), 123.7 d (C-2), 149.1 d (C-3), 158.3 s (C-4a), 138.9 s (C-4b), 120.8 d (C-5 or C-8), 129.4 d (C-6 or C-7), 129.0 (C-7 or C-6), 125.0 d (C-8 or C-5), 146.4 s (C-8a), 72.5 d (C-9), 137.7 s (C-9a), 17.9 q (CH_3).

QUALITATIVE ANTICANDIDAL EVALUATION.—In vitro evaluation of anticandidal activity was accomplished using the agar-well diffusion assay previously described (12,13) with the following modifications. *Candida albicans* NIH B311 used to induce experimental disseminated candidiasis was used for the initial qualitative evaluation of anticandidal activity. The organism was grown in Sabouraud-dextrose broth (SDB) for 24 h at 37°, at which time the cells were harvested by centrifugation (4°, 2000 rpm, 3 min). After centrifugation, the cells were washed and suspended in sterile 0.9% saline to give a final concentration of 10^6 (colony forming units per ml (adjusted using a hemocytometer). Culture plates (15×100 mm) for the qualitative assay were prepared from 25 ml of Sabouraud-dextrose agar. Using sterile cotton swabs, the plates were streaked with the suspension (10^6 cfu/ml) of *C. albicans* B311. Cylindrical plugs were removed from the agar plates by means of a sterile cork borer to produce wells with a diameter of approximately 11 mm. To the well was added 100 µl of 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, DMSO, DMF, or Me_2CO were required to dissolve extracts or compounds, solvent blanks were included. Anticandidal activity was recorded as the width (in mm) following incubation of the plates at 37° for 24 h. The antifungal agents amphotericin B and ketoconazole were included as standards in each assay.

QUANTITATIVE ANTICANDIDAL EVALUATION.—The method used to determine the MIC was the twofold serial broth dilution assay (12,13) in yeast nitrogen broth (Difco). In addition to *C. albicans* B311, the MIC values for eupolauridine and onychine were also determined for two additional strains of *C. albicans*: ATCC 10231 and a clinical isolate designated WH. Both compounds were initially tested using a concentration of 100 µg/ml in the first tube. The test compound was added to sterile Sabouraud-Dextrose broth as a solution in DMSO. The inoculum for the MIC determination was prepared as described for qualitative evaluation. Using a calibrated sterile wire loop, 10 µl of the 10^6 cfu/ml suspension of *C. albicans* was used as inoculum for each tube. The MIC value was taken as the lowest concentration of compound that inhibited the growth of the test organisms after 24 h and 48 h of incubation at 37°. The antifungal agents amphotericin B and ketoconazole were included as standards in each screen.

ACKNOWLEDGMENTS

This investigation was supported by contract NO1-AI-42549 from the National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland. We are grateful to Dr. W.C. Taylor, University of Syd-

ney, for kindly providing an authentic sample of eupolauridine.

LITERATURE CITED

1. S.A. Atti, A. Ammar, C.H. Phoebe, Jr., P.L. Schiff, Jr., and D.J. Slatkin, *J. Nat. Prod.*, **45**, 476 (1982).
2. P.G. Waterman and I. Muhammed, *Phytochemistry*, **24**, 523 (1985).
3. B.F. Bowden, K. Picker, E. Ritchie, and W.C. Taylor, *Aust. J. Chem.*, **28**, 2681 (1975).
4. M. Leboeuf and A. Cavé, *Lloydia*, **39**, 459 (1976).
5. M.E.L. De Almeida, R. Braz, M.V. Von Bulow, O.R. Gottlieb, and J.G.S. Maia, *Phytochemistry*, **15**, 1186 (1976).
6. M.O.F. Goulart, A.E.G Santana, A.B. De Oliveira, G.G. De Oliveira, and J.G.S. Maia, *Phytochemistry*, **25**, 1691 (1986).
7. J. Koyama, T. Sugita, Y. Suguta, and H. Irie, *Heterocycles*, **12**, 1017 (1979).
8. D. Tadic, B.K. Cassels, M. Leboeuf, and A. Cavé, *Phytochemistry*, **26**, 537 (1987).
9. W.C. Taylor, in: "The Alkaloids." Ed. by A. Brossi, Vol. XXIV, Chap. 1, Academic Press, New York, 1985, pp. 1-23.
10. C.D. Hufford, C.C. Collins, and A.M. Clark, *J. Pharm. Sci.*, **68**, 1239 (1979).
11. F.W. Wehrli and T. Wirthlin, "Interpretation of Carbon-13 NMR Spectra," Heyden & Son, Ltd., Philadelphia, PA, 1980, pp. 66-72.
12. A.M. Clark, F.S. El-Feraly, and W.S. Li, *J. Pharm. Sci.*, **70**, 951 (1981).
13. C.D. Hufford, M.J. Funderburk, J.M. Morgan, and L.W. Robertson, *J. Pharm. Sci.*, **64**, 789 (1975).

Received 23 February 1987