Isolation and *In Vitro* Antifungal Activity of 6,6′-Dihydroxythiobinupharidine

W. P. CULLEN*, R. T. LaLONDE*▲, C. J. WANG†, and C. F. WONG*

Abstract □ The isolation procedure for obtaining a new nuphar alkaloid, 6,6′-dihydroxythiobinupharidine (formerly named 6,6′-dihydroxythionuphlutine-A), and its *in vitro* activity against eight human pathogenic fungi are described.

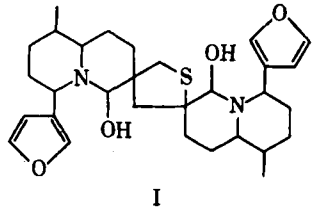
Keyphrases □ 6,6′-Dihydroxythiobinupharidine—isolation from *Nuphar luteum* subsp. *macrophyllum*, tested for antifungal activity □ *Nuphar luteum* subsp. *macrophyllum* (Beal)—isolation of 6,6′-dihydroxythiobinupharidine, tested for antifungal activity □ Antifungal agents, potential—isolation and testing of 6,6′-dihydroxythiobinupharidine from *Nuphar luteum* subsp. *macrophyllum*

The usefulness of various preparations of nuphar rhizomes in treating enteritis, gingivitis, and skin diseases was reported in 1945 (1). Subsequently, other accounts have dealt with the isolation of alkaloid mixtures, specific compounds of unknown structure, and the activity of these materials against specific organisms (2–5). This report describes the isolation of 6,6′-dihydroxythiobinupharidine[1] (I) and the results of preliminary studies dealing with the activity of this substance against eight human pathogenic fungi. The study leading to the structure of 6,6′-dihydroxythiobinupharidine and to that of a companion alkaloid, 6,6′-dihydroxythionuphlutine-B, was reported previously (6).

EXPERIMENTAL[3]

Plant Material[2]—*Nuphar luteum* subsp. *macrophyllum* (Beal) (9) rhizomes were harvested from the flats along the lower Hudson River near Columbianville, N. Y., during August 1968. The rhizomes were sliced into 0.63-cm. (0.25-in.) sections and dried under electric fans at room temperature with frequent turning. The dried rhizome sections were ground to a fine powder in a Wiley mill immediately before extraction.

Isolation of 6,6′-Dihydroxythiobinupharidine—A 500-g. quantity of powdered plant material was soaked with 1 l. of 10% aqueous ammonia for 24 hr. The resulting mixture was extracted with



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ethylene chloride (3 × 5 l.). Each separate extract was washed sequentially with 1000-, 750-, and 625-ml. portions of 10% sulfuric acid. The combined acid wash was made alkaline with aqueous ammonia in ice. The resulting turbid mixture was extracted with ~3 l. of chloroform. The deep amber-colored extract was dried over sodium sulfate. Removal of the solvent at the rotary evaporator (<40°) yielded <6 g. of dark-brown viscous oil, which was dissolved in 70 ml. of ethanol and treated with 15% ethanolic methylene-bis(salicylic) acid. The brown precipitate which resulted was filtered off and shaken with 60 ml. of 10% aqueous ammonia, and the mixture that formed was extracted with chloroform. The chloroform extract was dried over sodium sulfate. Removal of the solvent on the rotary evaporator yielded 4.2 g. of oil, 4 g. of which was eluted from a column of neutral alumina (activity grade III, 170 g.) with 13 50-ml. portions (fractions A1–A13) of *n*-hexane–methylene chloride (99:1). The column was eluted further with 50-ml. portions (fractions A14–A62) of *n*-hexane–methylene chloride containing gradually increasing amounts of the latter. After the fraction of methylene chloride had reached 50%, the column was eluted with 50-ml. portions of pure methylene chloride (fractions A63–A86) and finally with 50-ml. portions of methylene chloride–10% methanol. Fractions A72–A86 amounted to 402 mg. of a glass-like solid, 6,6′-dihydroxythiobinupharidine; $[\alpha]_D^{25}$ +44.5° (methylene chloride, c 1.2); TLC (0.25 mm. alumina GF_{254} on a 20-cm. plate); R_f (methylene chloride–0.6% methanol) 0.3; R_f (methylene chloride–1% methanol) 0.67.

Anal.—Calc. for $C_{30}H_{42}N_2O_4S$: C, 68.41; H, 8.04; N, 5.33; S, 6.09. Found: C, 68.64; H, 8.10; N, 5.26; S, 5.95.

6,6′-Dihydroxythiobinupharidine was dissolved in methylene chloride and was treated with 2 equivalents of dilute perchloric acid in the sonic oscillator. The solvents were removed at reduced pressure and the crystalline residue was recrystallized from methanol to give the bisiminium diperchlorate, m.p. (powder) 226–228° and (large crystals) 232–234°; $[\alpha]_D^{25}$ +184° [c, 0.5 methanol–acetone (16.5:3.5)].

Anal.—Calc. for $C_{30}H_{42}Cl_2N_2O_{10}S$: C, 52.09; H, 5.84; Cl, 10.25; N, 4.05; S, 4.63. Found: C, 52.19; H, 5.60; Cl, 10.28; N, 4.01; S, 4.84.

Other physical properties of 6,6′-dihydroxythiobinupharidine and its bisiminium diperchlorate were reported previously (6).

Combined fractions A47–A62 (356 mg.) were rechromatographed on 25 g. of neutral alumina (activity grade III), which was eluted sequentially with four 50-ml. portions of benzene (fractions B1–B4), three 50-ml. portions of methylene chloride (fractions B5–B7), and finally with 25 ml. of methanol. A 156-mg. portion of fraction B5 (175 mg.) was rechromatographed on 10 g. of neutral alumina (activity grade IV), which was eluted with 50 ml. of *n*-hexane (fraction C1) and three 25-ml. portions of benzene (fractions C2–C4). Fraction C3 consisted of 85 mg. of a pure, glass-like solid, 6,6′-dihydroxythionuphlutine-B; $[\alpha]_D^{25}$ −69° (c, 10 mg./ml., methylene chloride); TLC (0.25 mm. alumina GF_{254} on a 20-cm. plate); R_f (methylene chloride–1% methanol) 0.75.

Anal.—Calc. for $C_{30}H_{42}N_2O_4S$: C, 68.41; H, 8.04; N, 5.33; S, 6.09. Found: C, 68.38; H, 8.21; N, 5.34; S, 5.79.

Other physical properties of 6,6′-dihydroxythionuphlutine-B were reported previously (6).

Determination of Antifungal Properties—Twenty-five milliliters of a 0.01 *M* aqueous acetic acid solution containing the alkaloid at a concentration of 490 mcg./ml. was sterilized by filtering through a Seitz filter. The sterile alkaloid was added to four portions of sterile, melted (50°) Sabouraud dextrose agar to make the final concentrations of the alkaloid in the Sabouraud dextrose agar 100, 10, 1, and 0.1 mcg./ml. They were distributed into sterile "perfume" bottles and made into slants.

The eight human pathogenic fungi tested in this study were: *Histoplasma capsulatum* Darling (No. 1098), *Blastomyces dermati-*

[1] The alkaloid was originally named 6,6′-dihydroxythionuphlutine-A (6). While elucidating the structure of this alkaloid, it was reduced to thionuphlutine-A (6). Recently, the latter alkaloid was observed to be identical with thiobinupharidine (7), an alkaloid whose structure had remained unknown until the discovery of its identification with thionuphlutine-A. Since the name thiobinupharidine has precedence (8), the name thionuphlutine-A should be deleted from the literature.

[2] Verification of the plant material was made by Mr. S. Smith of the New York State Botanists Office, Albany, N. Y. A voucher specimen has been deposited at the New York State Botanists Office, Albany, N. Y.

[3] Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and the analytical laboratories of Stanford Research Institute, Menlo Park, Calif.

Table I—Sensitivities of Histoplasma and Blastomyces to 6,6′-Dihydroxythiobinupharidine as Expressed by Weekly Increments of the Diameter of Colony (in Millimeters) on Sabouraud Dextrose Agar at 25° (Mycelial Phase)

Concentration of Alkaloid, mcg./ml.	Week	*Histoplasma capsulatum* — Isolate No. 108						*Histoplasma capsulatum* — Isolate No. 1106						*Blastomyces dermatitidis* — Isolate No. 1099						*Blastomyces dermatitidis* — Isolate No. 1107					
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
100		0	0	0	T[a]	4[b]	7	0	0	0	0	T	T	0	0	0	T	T	T	0	0	0	2	5	5
80		0	0	2	4	5	8	0	0	0	0	0	0	0	0	T	T	T	T	0	0	2	3	6	7
60		0	3	4	7	13	17	0	0	0	0	0	T	0	0	T	2	2	2	0	0	2	3	7	7
40		0	3	8	12	14	16	0	0	0	T	2	5	0	2	3	3	4	4	0	0	8	11	13	21
20		0	5	11	16	19	24	0	6	12	17	20	23	0	6	9	11	15	18	0	0	7	12	15	25
10		2	9	14	20	23	26	2	4	7	7	13	18	2	7	9	14	19	20	4	10	16	25	28	30
Acetic acid control		4	14	25	29	31	31	0	6	14	19	28	31	8	19	26	28	29	29	6	26	31	32[c]	32	32
Control		7	12	22	26	27	27	2	10	15	27	31	31	8	18	26	28	30	30	8	25	31	32	32	32

[a] Trace of growth. [b] Average diameter of two cultures. [c] Maximal growth; surface area of this bottle is 32 × 32 mm.

tidis Gilchrist and Stokes (No. 1099), *Trichophyton mentagrophytes* (Robin) Blanchard (No. 1100), *T. tonsurans* Malmsten (No. 1101), *Microsporum gypseum* (Bodin) Guiart and Grogorakis (No. 1102), *M. canis* Bodin (No. 1103), *Cryptococcus neoformans* (Sanfelice) Vuillemin (No. 1104), and *Candida albicans* (Robin) Berkhour (No. 1105).

Each bottle was center-inoculated with a 2 × 2-mm. portion of a 2-week-old mycelial culture or a loopful of yeast-like candida or cryptococcus. All cultures were made in duplicate and incubated at 25°. Linear expansion of each colony was measured weekly up to 5 weeks and at the end of 8 weeks.

Subsequent tests were performed with two isolates each of *H. capsulatum* (Nos. 1098 and 1106) and *B. dermatitidis* (Nos. 1099 and 1107) and Sabouraud dextrose agar slants containing 100, 80, 60, 40, 20, or 10 mcg. of the alkaloid per milliliter of Sabouraud dextrose agar.

The toxicity of 6,6′-dihydroxythiobinupharidine in mice was ascertained from results (NSC 141542) obtained by the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

RESULTS AND DISCUSSION

The effect of the alkaloid 6,6′-dihydroxythiobinupharidine on the growth of histoplasma and blastomyces was measurable. At 100 mcg./ml., the alkaloid inhibited the growth of *H. capsulatum* up to 3 weeks, whereas it completely suppressed the growth of *B. dermatitidis*. At the same concentration the alkaloid suppressed the growth rate of *M. gypseum* and *M. canis* up to 3 weeks; the growth of *T. mentagrophyte* and *T. tonsurans* was suppressed up to 5 weeks. However, the alkaloid at a concentration of 100 mcg./ml. exerted no inhibitory effect against yeast-like candida or cryptococcus.

Table I reveals that isolate No. 1106 of *H. capulatum* was more sensitive to the alkaloid than isolate No. 1098. Likewise, *B. dermatitidis* isolate No. 1099 was more sensitive to the alkaloid than was No. 1107. Clearly, the alkaloid at a concentration of 40 mcg./ml. may inhibit the mycelial growth of some strains of *H. capsulatum* and *B. dermatitidis*.

These results show that 6,6′-dihydroxythiobinupharidine possesses antifungal properties *in vitro*. The effect of this alkaloid in treating animal mycoses will be investigated in the near future. In preliminary tests on mice, no deaths occurred upon intraperitoneal administration of 100 and 200 mg. of alkaloid per kilogram of mouse once daily for 30 days. At a level of 400 mg. of alkaloid per kilogram of mouse, four of six mice died within 30 days.

REFERENCES

(1) A. P. Tatarov, *Farmatsiya*, **8**, 29(1945); through *Chem. Abstr.*, **41**, 2210i(1947).

(2) V. G. Drobot'ko, E. Y. Rashba, B. E. Aizenman, S. I. Zelepukha, S. I. Novikova, and M. B. Kayanskaya, *Antibiotiki*, **1958**, 22.

(3) S. I. Novikova, *Mikrobiol. Zh., Akad. Nauk Ukr. R.S.R.*, **23**, 51(1961).

(4) K. C. Bel'tyukova and L. T. Pastushenko, *ibid.*, **25**, 36(1963).

(5) Y. A. Aleshkina, T. N. Il'inskaya, M. A. Rubinchik, and S. A. Vichkanova, British pat. 968,042 (Aug. 26, 1964); through *Chem. Abstr.*, **61**, 15939b(1964).

(6) R. T. LaLonde, C. F. Wong, and W. P. Cullen, *Tetrahedron Lett.*, **1970**, 4477.

(7) R. T. LaLonde and C. F. Wong, *Phytochemistry*, **11**, 3305 (1972).

(8) O. Achmatowicz and Z. Bellen, *Tetrahedron Lett.*, **1962**, 1121.

(9) E. O. Beal, *J. Elisha Mitchell Sci. Soc.*, **72**, 317(1956).

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▲ To whom inquiries should be directed.