Production of Psilocybin in *Psilocybe baeocystis* Saprophytic Culture

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The carpophores of six species of mushrooms were analyzed by TLC for indole derivatives. Three species, *Psilocybe baeocystis, P. caerulipes,* and *P. strictipes,* were found to contain psilocybin, and traces of psilocin were found to occur in the first two species. Neither compound could be detected in *P. atrobrunnea, Stropharia aeruginosa,* or *S. semiglobata.* Psilocybin has been isolated from *P. baeocystis* grown in submerged culture and identified by TLC, melting point, and ultraviolet and infrared spectra. Thin-layer chromatographic data for 36 indole derivatives in five solvent systems are included.

THE PSYCHOTOMIMETIC 4-substituted tryptamine derivatives, psilocybin and psilocin, were first isolated from a hallucinogenic mushroom, *Psilocybe mexicana* Heim, by Hofmann *et al.* (1). Since then, both have been reported to occur in a number of species of *Psilocybe* belonging to the section *Caerulescentes.* In addition, psilocybin has been identified as occurring in *Conocybe cyanopus* (Atk.) Kühner (2) and in *Panaeolus sphinctrinus* (Fr.) Quél. (3), though the latter has not been confirmed (4, 5). Benedict *et al.* (6) reported the occurrence of psilocin, but not psilocybin, in carpophores of *P. baeocystis* Singer and Smith, and in a subsequent investigation (2) faint traces of psilocybin were detected.

Surface cultures of various *Psilocybe* species have been investigated (7–9), and the carpophores, sclerotia, or mycelia formed were found to contain psilocybin and, in certain instances, psilocin (7, 8). The production of psilocybin in submerged culture was first investigated by Catalfomo and Tyler (10). Of the three species of *Psilocybe* studied, only one, *P. cubensis* (Earle) Singer, produced psilocybin. No psilocin was detected.

Preliminary experiments in our laboratory indicated that several species of the family *Strophariaceae* contained indole derivatives in carpophores and/or mycelial culture. Certain of these were selected for more detailed investigation.

EXPERIMENTAL

Organisms.—The sources of the specimens investigated are listed in Table I.

Cultures of *P. baeocystis* were obtained from basidiospores. Gills of a 1-week-old dried specimen were triturated with a small quantity of sterile distilled water, and the aqueous suspension was streaked on Petri dishes of Sabouraud's dextrose agar and potato dextrose agar. The plates were incubated at 15° and examined periodically with a microscope at 100× for evidence of spore germination. When the germ tube emerged, a block of agar containing the spore was removed with a fine scalpel and transferred to a second Petri dish of the respective agar. These plates were incubated at 25°. When colonial growth developed, the mycelium was examined for clamp connections and then transferred to tubes of media. After incubation at 25°, the growth was covered with sterile mineral oil, and the culture was stored at 4°.

The pileus of fresh specimens of *P. atrobrunnea* (Lasch) Gillet and *Stropharia semiglobata* (Fries) Quélet were swabbed with 0.2% $HgCl_2$ solution, the outer tissue was removed aseptically, and small portions of inner tissue were transferred to tubes containing Sabouraud's dextrose agar and potato dextrose agar. After incubation at 25°, the mycelium was examined for clamp connections and then subcultured into fresh tubes of the respective media. After growth, the tube was filled with sterile mineral oil, and the cultures were stored at 4°. Culture of the remaining species was not attempted.

Thin-Layer Chromatography.—This technique has been applied to the separation and identification of indole derivatives by various authors (11–13). However, none of the techniques available effectively separate psilocybin and psilocin from related compounds.

The apparatus used and the methods employed in the preparation of plates and developing chambers, the application of test solutions, and the development of chromatograms were essentially as described by McLaughlin *et al.* (14).

Silica Gel G[1] (SGG) and a mixture of Silica Gel G and Kieselguhr G[2] (SGG–KGG), 2:1, were used as adsorbents.

Standard solutions were prepared by weighing the compounds with a Cahn model M-10 electrobalance,[3] dissolving the compound in a suitable solvent, and diluting to a standard volume in a volumetric flask. The amount of each compound spotted and the commercial source are listed in Table II.

Ehrlich's reagent (5% *p*-dimethylaminobenzaldehyde in concentrated HCl) was used as a spray for color visualization of the compounds on developed chromatograms.

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[1] Research Specialties Co., Richmond, Calif.
[2] Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.
[3] Cahn Instrument Co., Paramount, Calif.

TABLE I.—CHROMATOGRAPHIC IDENTIFICATION OF COMPOUNDS OCCURRING IN CARPOPHORES

Organism	Source	Equivalent Wt. of Tissues Spotted, mg.	Compd. Present: Tryptophane	Psilocybin	Psilocin
P. atrobrunnea (Lasch) Gillet	Ann Arbor, Mich., 1961	12	Trace	—	—
P. baeocystis Singer and Smith	Eugene, Oreg., 1958	20	Trace	+	—
P. caerulipes Peck	Burt Lake, Mich., 1959	9	Trace	+	Trace
P. strictipes Smith	Welches, Oreg., 1944	18	—	+	—
S. aeruginosa (Curt. ex Fr.) Quélet	Mackinaw City, Mich., 1953	15	Trace	—	—
S. semiglobata (Fr.) Quélet	Douglas Lake, Mich., 1959	15	Trace	—	—

Of the 98 solvent systems tested, the following proved to be useful in these investigations: 1-propanol–5% ammonium hydroxide (5:2) on SGG–KGG (A), 1-butanol–glacial acetic acid–water (2:1:1) on SGG–KGG (B), cyclohexane–chloroform (1:1) on SGG (C), chloroform–cyclohexane–concentrated ammonium hydroxide (20:10:1) (lower phase) on SGG (D), benzene–methanol–5% ammonium hydroxide (10:15:2) on SGG (E). The R_f values for 36 compounds listed in Table II represent the average of 3 successive determinations. The standard deviation is reported for each value.

Employing the two-dimensional technique, plates were first developed with solvent system A, air-dried at room temperature for approximately 30 min., and then developed with solvent system B in the second direction. Similarly, plates were first developed with solvent system C and then with solvent system D. Compounds *1–8* are effectively separated by the C–D system and compounds *10–22*, with the exception of *11* and *12* which co-chromatograph, are effectively separated by the A–B system.

Examination of Carpophores.—Since the species examined were herbarium specimens, the methods employed in drying were undefined. The carpophores (200–350 mg.) were ground to a fine powder using a mortar and pestle. The tissue was quantitatively transferred to a 25-ml. conical flask, and 10 ml. of methanol was added. The flask was agitated mechanically on a shaker for 4–5 hr., the suspension filtered, the residue washed with small portions of methanol, and the filtrates combined.

The methanol extract was transferred to an evaporating dish and the solvent removed at room temperature with the aid of a stream of compressed air. The viscous residue was stirred with a small volume of methanol, filtered into a volumetric flask, and the residue washed with small portions of methanol to give a final volume of 1.0 ml.

Volumes of the extracts (25–75 μl.) and standard solutions of psilocybin, psilocin, and tryptophane

TABLE II.—R_f VALUES OF VARIOUS INDOLE DERIVATIVES

	Compd.	Amt. Spotted, mcg.	Source[a]	Solvent System: A	B	C	D	E
1	Indole	4	2	0.85 ± 0.01	0.82 ± 0.01	0.26 ± 0.01	0.45 ± 0.04	0.88 ± 0.02
2	2-Methylindole	2	1	0.85 ± 0.01	0.82 ± 0.01	0.26 ± 0.01	0.54 ± 0.05	0.92 ± 0.02
3	3-Methylindole	4	1	0.85 ± 0.01	0.82 ± 0.00	0.29 ± 0.01	0.53 ± 0.03	0.92 ± 0.02
4	5-Methylindole	4	6	0.85 ± 0.02	0.81 ± 0.01	0.25 ± 0.01	0.46 ± 0.02	0.92 ± 0.02
5	5-Methoxyindole	4	6	0.84 ± 0.01	0.80 ± 0.01	0.09 ± 0.01	0.34 ± 0.02	0.90 ± 0.01
6	5-Hydroxyindole	4	6	0.82 ± 0.02	0.78 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.84 ± 0.02
7	3-Indoleacetonitrile	2	1	0.85 ± 0.01	0.82 ± 0.01	0.04 ± 0.01	0.15 ± 0.01	0.89 ± 0.02
8	3-Hydroxymethyl indole	2	3	0.87 ± 0.00	0.82 ± 0.01	0.03 ± 0.01	0.21 ± 0.02	0.90 ± 0.02
9	3-Indoleacetic acid ethyl ester	2	1	0.85 ± 0.01	0.79 ± 0.01	0.03 ± 0.00	0.23 ± 0.04	0.91 ± 0.02
10	3-Indoleacetamide	2	4	0.79 ± 0.01	0.73 ± 0.01	0	0	0.80 ± 0.03
11	*N*-Methyltryptophane	2	9	0.63 ± 0.03	0.58 ± 0.02	0	0	0.37 ± 0.01
12	L-Tryptophane	0.8	1	0.61 ± 0.03	0.61 ± 0.01	0	0	0.26 ± 0.03
13	5-Hydroxytryptophane	2	5	0.56 ± 0.03	0.57 ± 0.01	0	0	0.20 ± 0.02
14	Tryptamine hydrochloride	4	1	0.80 ± 0.02	0.64 ± 0.00	0	0	0.35 ± 0.01
15	Serotonin creatinine sulfate	4	5	0.75 ± 0.02	0.61 ± 0.01	0	0	0.21 ± 0.01
16	*N,N*-Dimethyltryptamine hydrogen-oxalate	4	1	0.79 ± 0.02	0.51 ± 0.00	0	0.02 ± 0.00	0.61 ± 0.01
17	Bufotenine monooxalate	4	1	0.74 ± 0.01	0.46 ± 0.01	0	0	0.43 ± 0.02
18	Psilocin	0.8	7	0.76 ± 0.01	0.55 ± 0.01	0	0.06 ± 0.01	0.57 ± 0.02
19	Psilocybin	0.8	7	0.15 ± 0.01	0.15 ± 0.01	0	0	0.01 ± 0.00
20	3-Indolelactic acid	2	8	0.63 ± 0.03	0.70 ± 0.01	0	0	0.45 ± 0.04
21	5-Hydroxy-3-indole acetic acid	2	1	0.59 ± 0.03	0.77 ± 0.01	0	0	0.60 ± 0.03
22	3-Indole acetic acid	2	2	0.65 ± 0.02	0.81 ± 0.01	0	0	0.63 ± 0.01
23	γ-(Indole)-*N*-butyric acid	4	1	0.70 ± 0.01	0.81 ± 0.01	0	0	0.66 ± 0.03
24	β-(Indole-3)-propionic acid	4	1	0.69 ± 0.01	0.81 ± 0.01	0	0	0.67 ± 0.01
25	3-Indolecarboxylic acid	4	4	0.65 ± 0.02	0.82 ± 0.01	0	0	0.62 ± 0.02
26	β-Indole-3-acrylic acid	2	8	0.69 ± 0.03	0.81 ± 0.01	0	0	0.63 ± 0.01
27	3-Indolealdehyde	40	1	0.88 ± 0.04	0.81 ± 0.01	0	0	0.95 ± 0.01
28	3-Indoleacetaldehyde	2	3	0.88 ± 0.01	0.81 ± 0.01	0.03 ± 0.01	0	0.87 ± 0.03
29	3-Hydroxyethyl indole	2	4	0.84 ± 0.01	0.78 ± 0.00	0	0.03 ± 0.01	0.85 ± 0.03
30	Indoxylacetate	2	1	0.86 ± 0.01	0.79 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.91 ± 0.02
31	Indoxylbutyrate	2	1	0.86 ± 0.01	0.80 ± 0.01	0.06 ± 0.00	0.12 ± 0.01	0.93 ± 0.02
32	5-Methyltryptophane	2	8	0.63 ± 0.03	0.63 ± 0.01	0	0	0.31 ± 0.01
33	5-Benzyloxy-3-indole acetic acid	2	6	0.70 ± 0.03	0.81 ± 0.01	0	0	0.69 ± 0.01
34	5-Methoxy-2-carboxyindole	2	6	0.68 ± 0.03	0.78 ± 0.01	0	0	0.66 ± 0.01
35	Isatin	2	8	0.80 ± 0.02	0.74 ± 0.01	0	0	0.84 ± 0.02
36	Gramine	40	1	0.76 ± 0.01	0.56 ± 0.01	0	0	0.42 ± 0.01

[a] 1, Calbiochem, Los Angeles, Calif.; 2, Eastman Organic Chemicals, Rochester, N. Y.; 3, K & K Laboratories, Inc., Plainview, N. Y.; 4, Koch-Light & Co., Ltd., Colnbrook, England; 5, Mann Research Laboratories, Inc., New York, N. Y.; 6, Regis Chemical Co., Chicago, Ill.; 7, Sandoz Pharmaceuticals, Hanover, N. J.; 8, Sigma Chemical Co., St. Louis, Mo.; 9, Isolated from *Abrus precatorius* L.

were spotted singly and in admixture on appropriate thin-layer chromatography plates and developed with solvent systems A, B, and E. The weight of tissue represented by the volume of each extract spotted and the compounds identified as occurring in the extracts are given in Table I. Tryptophane, which barely separates from 5-methyltryptophane, can be distinguished on the basis of color; tryptophane reacts with Ehrlich's reagent to give a violet color, gradually fading to green, while 5-methyltryptophane reacts to give a blue color which persists, though fading in intensity.

Tissue Cultures.—Mycelial tissue from stock cultures of *P. baeocystis*, *P. atrobrunnea*, and *S. semiglobata* was transferred to slants of potato dextrose agar[4] and incubated at 25° for 3 weeks.

Four liquid media were chosen for preliminary investigations: Sabouraud's liquid medium[4] (SLM), malt extract broth[4] (MEB), medium No. 1 of Catalfomo and Tyler (10), modified by omitting the yeast extract and increasing the glucose concentration to 0.75% (SM-1), and the nutrient solution of Stoll *et al.* (15), modified by replacing the mannitol with 4.75% galactose and 0.25% glucose (SM-2).

Transfers were made from the potato dextrose agar slants to 500-ml. wide-mouth conical flasks containing 100 ml. of a particular medium. The flasks were incubated at 21 ± 0.5° on a model G-52 Gyrotory shaker[5] operating at approximately 280 r.p.m.

Cultures in SM-2 gave scant or no growth after 4 weeks. The mycelial pellets in the remaining media were harvested after 2, 3, and 4 weeks of growth, separated by filtration, washed with several portions of distilled water, and the filtrates combined. The tissues and the media were freeze-dried and treated in the manner described for the extraction of carpophores. The extracts were evaluated by two-dimensional TLC.

As evidenced by co-chromatography, tissues of *P. baeocystis* contained psilocybin and traces of psilocin when grown in SM-1 for 2 weeks. While growth increased in this medium in 3 and 4 weeks (approximately 80 mg./flask in 2 weeks as opposed to approximately 200 mg./flask in 3 and 4 weeks), psilocin was not detectable, and visual comparisons of the intensity and area of the spots indicated that the psilocybin concentration had decreased. Tryptophane (confirmed using solvent system E) was detected in the tissues of all three species grown in SLM and in SM-1. None of these compounds could be detected in tissues grown in MEB.

None of the compounds could be detected in the media after growth with the exception of tryptophane in SLM. Extracts of fresh SLM indicated that free tryptophane is a constituent of this medium. No attempt was made to determine whether a quantitative increase had occurred.

In order to confirm the presence of psilocybin in tissue cultures of *P. baeocystis*, growth of larger quantities of the organism was undertaken. Transfers of the organism were made from potato dextrose agar slants to 500-ml. conical flasks containing 100 ml. of SM-1. The flasks were incubated on the shaker as previously described. When the mycelial pellets first began to acquire a blue tint (10–20 days), they were removed from the shaker. Pellets were removed from the flasks, transferred to a sterile semimicro Waring blender, and homogenized for 30 sec. with 150 ml. of sterile distilled water. The resulting suspension was diluted with sterile distilled water to give an arbitrary transmittance of 65% using a Bausch & Lomb Spectronic 20 colorimeter[6] at a wavelength of 550 mμ.

Using serological pipets, 3 ml. of the diluted suspension was transferred to each of one hundred and three 500-ml. conical flasks containing 100 ml. of SM-1. The flasks were incubated on the shaker for 15 days.

The mycelial pellets were collected on a Büchner funnel, washed with several portions of distilled water, and freeze-dried. The dried tissue weighed 11.1 Gm.

Using a mortar and pestle, the tissue was ground to a fine powder and transferred to a 500-ml. flask. The tissue was extracted on a reciprocal shaker for 30-min. periods with 12 successive 100-ml. portions of methanol. The combined extracts were concentrated under vacuum using a Rinco rotating evaporator,[7] model VE-1000-A, at 40°. The brown, viscous residue was extracted with 3 successive 80-ml. portions of petroleum ether (30–60°) by stirring and decantation. The residue was mixed with 30 ml. of methanol and the suspension in turn mixed with 6 Gm. of cellulose powder.[8] This mixture was dried under vacuum at 38–40° for 8 hr. and then transferred, with the aid of an additional 1 Gm. of powdered cellulose, to the top of a 2-cm. O.D. column packed to a height of 29.5 cm. with powdered cellulose. The column was developed with 1-butanol saturated with 0.1 *N* HCl (16) at a rate of approximately 12 ml./hr. The eluate was collected in 4.5-ml. fractions using a model 230 automatic fraction collector.[9] The fractions, analyzed by TLC, indicated that traces of psilocin occurred in fractions 25–30 and that psilocybin occurred in fractions 43–120.

The fractions containing psilocybin were combined and the solvent evaporated at 40°, under vacuum, using the rotating evaporator. The dry residue (0.8 Gm.) was dissolved in 10 ml. of distilled water and the solution shaken with 0.5 Gm. Ag_2CO_3 for 20 min. The suspension was filtered on a Büchner funnel through talc which had been prewashed with distilled water. Hydrogen sulfide was passed through the dark green filtrate for 10 min., and the resulting suspension was again filtered on a Büchner funnel through prewashed talc to remove Ag_2S. Water was removed from the filtrate at 40°, under vacuum, using the rotary evaporator. The greenish-black residue was extracted with two successive 5-ml. portions, followed by three successive 2-ml. portions of methanol. The combined methanol extracts were concentrated at room temperature with the aid of a stream of nitrogen to approximately 10 ml., filtered, and placed in a freezer at −23°. Crystals, which formed overnight, melted with decomposition over a wide range. No psilocybin could be detected when a concentrated methanol solution of these crystals was analyzed by TLC. Further concentration of the filtrate to 2

[4] Difco Laboratories, Detroit, Mich.
[5] New Brunswick Scientific Co., Inc., New Brunswick, N. J.
[6] Bausch & Lomb Optical Co., Rochester, N. Y.
[7] Rinco Instrument Co., Inc., Greenville, Ill.
[8] Whatman standard grade.
[9] Packard Instrument Co., LaGrange, Ill.

ml. gave additional crystalline materials which could not be identified. TLC of the filtrate indicated that psilocybin was present in solution.

Cellulose powder was packed in a 3-cm. O.D. column to a height of 38 cm. The column was washed over a 10-hr. period with 500 ml. of 1-butanol saturated with water. Excess solvent was permitted to drain from the column.

The methanol filtrate containing psilocybin was mixed with 5 Gm. of powdered cellulose which had been washed in a similar manner, and this mixture was air-dried at room temperature for 3 hr. The mixture was packed on top of the washed cellulose column and the column developed with 1-butanol saturated with water (16), at a rate of approximately 30 ml./hr. The eluate was collected in 10-ml. fractions using the automatic fraction collector. TLC of the fractions indicated that fractions 32–35 contained psilocybin, 36–65 contained psilocybin plus slight quantities of impurities, and fractions 66–135 contained psilocybin grossly contaminated with other materials. Fractions were combined in the following manner: 32–35 (I), 36–65 (II), and 66–135 (III). The combined fractions were treated separately but in like manner. The butanol was removed under vacuum using the rotary evaporator at 40°. The residue of I was taken up in 0.5 ml. of methanol and II and III each in 2 ml. of methanol. The solutions were placed in the freezer, and in 10 days rod-shaped crystals appeared in I and II. A total of 4 mg. (1 mg. from I and 3 mg. from II) was recovered. III gave no crystals after 30 days.

Using the Fisher-Johns melting point apparatus,[10] the compound melted at 204–210°, showed no depression when mixed with reference psilocybin (m.p. 205–210°), and showed infrared[11] and ultraviolet[12] absorption spectra identical to reference psilocybin. It co-chromatographed with known psilocybin in solvent systems A and B.

DISCUSSION AND CONCLUSIONS

Of the six species of the family *Strophariaceae* which were analyzed by TLC for indole derivatives, three are members of the section *Caerulescentes*. The carpophores of three species, *P. baeocystis*, *P. caerulipes*, and *P. strictipes*, contain psilocybin while psilocin could be detected only in *P. caerulipes*. Since results of the analysis of *P. baeocystis* were not in agreement with the reports of Benedict *et al.* (2, 6), a sample of their specimen was evaluated, and indeed the results confirmed that psilocin is present and that psilocybin and tryptophane occur only in trace quantities. A recent re-examination of the two remaining carpophores of the specimen we had evaluated in January of 1959 showed psilocin to be present, but psilocybin could not be detected. The possibility that prolonged storage could account for these differences in psilocybin and psilocin content could not be substantiated by evaluating a 28-year-old specimen of *P. caerulipes*. Psilocybin and psilocin were found to occur in this specimen in quantities of the same order of magnitude as were found in the specimen collected in 1959.

From these data it would appear that differences in the amounts of psilocybin and psilocin vary among specimens of *P. baeocystis;* hence, the suggestion that this "would appear to be a chemical characteristic of some taxonomic utility for this species" (2) is invalid. It is possible that biochemical variations among individuals or differences in the biological age of individuals of this species may account for these differences.

From the limited data, there appears to be no relationship between the occurrences of tryptophane and of psilocybin and psilocin in the species examined.

Preliminary experiments with four culture media indicated that both SLM and SM-1 supported reasonable growth of *P. baeocystis*, *P. atrobrunnea*, and *S. semiglobata*. Psilocybin and traces of psilocin were detected in tissues of *P. baeocystis* grown on SM-1 for 2 weeks; but in 3 and 4 weeks, as the mycelial pellets assumed a blue color, psilocin could not be detected, and the amount of psilocybin apparently decreased.

Using these preliminary data as a guide, *P. baeocystis* was grown for 15 days on SM-1. From the 11.1 Gm. of mycelial tissue produced in this manner, 4 mg. of crystalline psilocybin was isolated and identified by TLC, melting point, ultraviolet, and infrared spectra. The isolation of psilocybin from tissue cultures indicates that the organism is a potential source of this hallucinogen.

SUMMARY

1. Thin-layer chromatographic data are presented for 36 indole derivatives in 5 solvent systems.

2. TLC evaluation of the carpophores of six species of the family *Strophariaceae* indicated that psilocybin occurs in *P. baeocystis*, *P. caerulipes*, and *P. strictipes* and that psilocin occurs in the first two species. Neither of these compounds could be detected in *P. atrobrunnea*, *S. aeruginosa*, or *S. semiglobata*.

3. *P. baeocystis*, grown in shake culture on a synthetic medium (SM-1), produced psilocybin and traces of psilocin. When grown in Sabouraud's liquid medium and in malt extract broth, neither of these compounds could be detected.

4. Crystalline psilocybin was isolated from mycelial tissue grown in SM-1 and characterized by melting point, chromatography, and ultraviolet and infrared spectra.

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[10] Fisher Scientific Co., St. Louis, Mo.
[11] Perkin-Elmer 337 grating spectrophotometer.
[12] Beckman model DB spectrophotometer.