ISOLATION OF PSILOCYBIN FROM *PSILOCYBE ARGENTIPES* AND ITS DETERMINATION IN SPECIMENS OF SOME MUSHROOMS

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Yokoyama previously reported hallucinogenic intoxication of volunteers who ingested fruiting bodies of *Psilocybe argentipes* K. Yokoyama sp. nov. This mushroom was first identified as *P. subcaerulipes* Hongo (1) but was described as a new species after more careful investigation (2). Afterwards, the same species was found in Kyoto, Osaka, Shiga, Saitama, Niigata, and Miyagi in Japan (2). In the course of this distribution survey, a case of accidental poisoning with the mushroom was reported in Niigata; three individuals had ingested an undetermined quantity of the mushroom, which they mistook for an edible mushroom, *Armillariella mellea* (Vahl in Fl. Dan ex Fr.) Karst. (Japanese name: Naratake) (2). Since the isolation of psilocybin and psilocin as the psychoactive principles from *Psilocybe mexicana* Heim. by A. Hofmann *et al.* (3), these compounds have been isolated or detected from many species of this genus and other genera (4, 5). Therefore, psilocybin or its related compounds were expected to be contained in this new species, *P. argentipes*. In July and September, 1979, and in June, July, and September, 1980, colonies of dozens of fruiting bodies of this species were found growing in the botanical garden of medicinal plants of this Institute.

Psilocybin (**1**), mp 218–228°, $C_{12}H_{17}O_4N_2P$, was obtained from the methanolic extract of the mushrooms collected in July, 1979, after preparative tlc and cellulose column chromatography. Microanalysis suggested the molecular formula for **1**, while the mass spectrum showed fragment ions at *m/e* 204 (f_1, M^+-HPO_3), 160 (f_1-$N(CH_3)_2$), 159 (f_1-$NH(CH_3)_2$), 146 (f_1-$CH_2N(CH_3)_2$). The ultraviolet (uv) maxma at 221 nm (log ϵ, 4.27), 268 (3.84), 280 (3.74) and 290 (3.64) were similar to reported data (3) and were attributed to a 4-substituted tryptamine derivative (6). The nuclear magnetic resonance (nmr) spectrum displayed the presence of two N-methyl groups (δ 2.95 ppm, 6H, s), an ethyl amino group (3.4 ppm, 4H, m), an α-hydrogen on an indole ring (7.20 ppm, 1H, s), and three adjacent hydrogens on an aromatic ring (7.3 ppm, 3H, m). On treatment with 1% HCl, **1** provided a product (**2**), which showed R_f 0.21 (silica gel, n-PrOH – 5% NH_4OH = 5:2), and t_R 5.4 min. in hplc, while I showed R_f 0.19 and t_R 6.0 min. These results are an indication that the isolated compound (**1**) was psilocybin and the hydrolysis product, psilocin (**2**).

The methanolic extract of the same mushroom collected in September, 1979, was fractionated to an etheral solution and an aqueous solution. The etheral extract was chromatographed on silica gel, and ergosterol and ergosterol peroxide were isolated. The aqueous fraction was chromatographed on a cellulose column, and psilocybin (**1**) was isolated along with

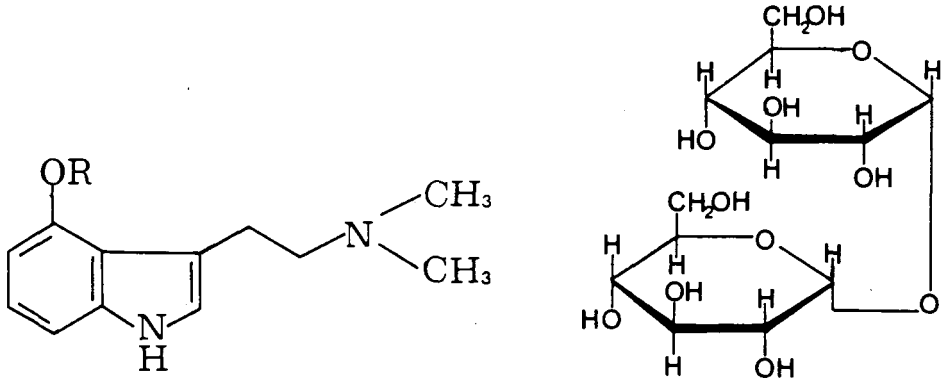

FIG. 1

1 R = H_2PO_3
2 R = H

3

α,α-trehalose (**3**), mp 95–97°, $C_{12}H_{22}O_{11}$, $[\alpha]_D$ +169°.

Quantitative determination of psilocybin was examined, and a combined method of Dowex-1 chromatography and hplc was found to be useful. The method was applied to the determination of psilocybin in specimens of *P. argentipes*, other species, and cultured fungi; the results are summarized in table 1. The values obtained for specimens of *P. argentipes* are similar to those reported in other Psilocybe species (7).

Psilocybin was found to be contained in the cultured mycelium of *P. subaeranginascens* Höhnel, although it was not detected in the cultured fluid after filtration of the mycelium. This compound was not detected in the mycelium and the fluid of cultured *P. subcaerulipes* Hongo (Japanese name: *Aizomeshi-bafutake*. It is interesting that psilocybin was not detected in specimens of *Gymnopilus spectabilis* (Fr.) A. H. Smith (4), which has been said to be psychoactive in this country. It was detected in a specimen of a species of the same genus, *G. liquiritiae* (Fr.) Karst, which has not been said to be toxic. A small amount of psilocybin was detected in specimens of *Psathyrella candolleana* (Fr.) A. H. Smith and *Agrocybe farinacea* Hongo. Numerous species of *Psilocybe*, *Panaeolina*, *Panaeolus*, *Copelandia*, *Conocybe*, *Pholiotina* and *Gymnopilus* have so far been reported as psilocybian species (4, 5). Now three new mushrooms, *Gymnopilus liquiritiae*, *Agrocybe farinacea*, and *Psathyrella candolleana*, can be added as psilocybian species.

EXPERIMENTAL[1]

ISOLATION OF PSILOCYBIN.—Dried fruiting bodies (16 g) of *Psilocybe argentipes* collected in July, 1979, at the botanical garden of medicinal plants in the Institutute of Pharmaceutical Sciences, Tohoku University, were extracted with methanol (100 ml) at room temperature for one day. The solution was decanted, and the residual mushroom was extracted again with MeOH. This procedure was repeated three times. The solution was concentrated *in vacuo* and streaked on ten preparative, 0.5–1 mm, silica gel, tlc plates (20 x 20 cm). The tlc plates were developed with n-propanol-5% NH_4OH (5:2). A band of silica gel of R_f *ca.* 0.1 was collected. The adsorbant was

[1]Melting points were determined on a Yanagimoto hot stage and are uncorrected. Microanalysis was performed in the analysis center of this institute. Ir spectra were obtained with Shimazu IR-27G photometer. ^{1}H-nmr and ^{13}C-nmr spectra were determined on a JNM FX-100 spectrometer with TMS as internal standard.

TABLE 1. Occurrence of psilocybin.

Species analyzed[c]	Origin of collection	Concentration[a] of psilocybin
Strophariaceae		
Psilocybe argentipes K. Yokoyama sp. nov.	Aobayama, Sendai (July, 1979)	0.14–0.16%[b] (2.97–3.43%)
	Aobayama, Sendai (September, 1979)	0.53–0.55% (3.59–3.65%)
	Izumi-ga-Take, Miyagi (1979)	0.018–0.027%[b] (0.38–0.55%)
	Kyoto (1979)	0.003–0.004% (2.32–2.53%)
Psilocybe subaeranginascens Höhnel (cultured)	Mycelium	0.017–0.018%
	Fluid	ND
Psilocybe subcaerulipes Hongo (cultured)	Mycelium	ND
	Fluid	ND
Naematoloma fasciculare (Fr.) Karst.	Aobayama, Sendai (October 7, 1979)	ND
Pholiota squarrosa (Fr.) Quel.	Yasóen, Sendai (October 9, 1979)	ND
Stropharia aurantiaca (Cooke) Imai	Yonezawa (October 14, 1979)	ND
Cortinariaceae		
Gymnopilus spectabilis (Fr.) A. H. Smith	Oguni, Yamagata (September, 1979)	ND
	Miyagi (Sept. 9, 1979)	ND
Gymnopilus aeruginosus (Peck) Sing.	Yonezawa, Yamagata (October 14, 1979)	ND
Gymnopilus liquiritiae (Fr.) Karst.	Yonezawa, Yamagata (October 14, 1979)	(0.012–0.029%)
Coprinaceae		
Coprinus comatus (Fr.) S. F. Gray	Aobayama, Sendai (July 25, 1979)	ND
Psathyrella candolleana (Fr.) A. H. Smith	Aobayama, Sendai (July 20, 1979)	(0.08–0.15%)
Psathyrella velutina	Miyagi (1979)	ND
Bolbitiaceae		
Conocybe antipoda (Lasch) Kühn	Aobayama, Sendai (1979)	ND
Agrocybe farinacea Hongo	Aobayama, Sendai (July 18, 1979)	(0.2–0.4%)
	Aobayama, Sendai (July 24, 1979)	ND
Agrocybe semiorbicularis (St Amans) Fayod	Aobayama, Sendai (July 18, 9)	N

[a]Based on dry weight of specimens and the figures in parentheses were obtained on the MeOH extracts. The values were determined by the combined method of an anion exchange resin chromatography and hplc except with b.

[b]MeOH extract was analyzed on hplc directly.

[c]Samples of all collections utilized have been deposited in the Institute of Pharmaceutical Sciences, Tohoku University.

extracted with methanol. The solvent was evaporated, and the residue was dissolved in *n*-butanol saturated with water. This solution was chromatographed on a cellulose column (10 g) with *n*-butanol saturated with water as the elution solvent. Fractions of five ml each were collected. Psilocybin (**1**) was obtained from fractions 32–42 as colorless needles (4 mg) after recrystallization from *n*-butanol saturated with water. It had the following properties: mp 218–228–, *Anal.* Calcd. for $C_{12}H_{17}O_4N_2P$: C, 50.75; H, 6.03; N, 9.86; P, 10.90. Found: C, 50.26; H, 6.07; N, 9.58; P, 10.55. Mass spectrum *m/e*: 204 (f_1, M^+-HPO_3), 160 ($f_1-N(CH_3)_2$, 159 ($f_1-NH(CH_3)_2$), 146 ($f_1-CH_2N(CH_3)_2$), 130, 58. Uv λ max (MeOH) nm (log ε): 221 (4.27), 268 (3.84), 280 (3.74), 290 (3.64); tlc: R_f, 0.19 (n-propanol–5% NH_4OH=5:2). Hplc: t_R, 6 min. (column: 4 mmø x 250 mm, column temp.: room temp., packing material:

Lichrosorb NH_2, eluant: 0.5 M KH_2PO_4 (pH 5.5), flow rate: 1.0 ml/min.); ^{1}H-nmr (pyr-d_5) δ: 2.95 ppm (6H, s), 3.4 (4H, m), 7.20 (1H, s), 7.3 (3H, m).

Psilocybin (**1**, 10 mg) was dissolved in 1% HCl (2 ml) and heated on a boiling water bath for one hr. After the solution was cooled to room temperature, $NaHCO_3$ was added, and the alkaline solution was extracted with ether. The ether layer was washed with water and dried over Na_2SO_4. The residue, after removal of the drying agent and the solvent, was examined by tlc and hplc to detect psilocin (R_f 0.21; t_R 5.4 min.).

ISOLATION OF PSILOCYBIN (**1**), ERGOSTEROL, ERGOSTEROL PEROXIDE AND α,α-TREHALOSE (**3**).—Dried fruiting bodies (30 g) of *P. argentipes* collected at the same place on September 9, 1979, was extracted three times with MeOH at room temperature. Methanol was evaporated *in vacuo* and the residue (4.5 g) was extracted with ether (100 ml, three times) after addition of water. The ether layer was washed with water and dried over Na_2SO_4. After removal of the inorganic reagent, the solvent was evaporated; the residue was chromatographed on silica gel (3.5 cm ϕ x 25 cm, 50 g). n-Hexane-ethyl acetate (5:1) eluted ergosterol, which was identified by tlc, mass spectrum and comparison of the ^{1}H-nmr spectrum with that of an authentic specimen. n-Hexane-ethyl acetate (5:1) eluted ergosterol peroxide, which was identified by comparison with an authentic specimen.

The aqueous fraction (2 g) was chromatographed on a 2.5 cm ϕ x 35 cm column of cellulose (50 g). After elution with n-butanol saturated with water (2 liters), psilocybin was eluted with the same solvent (600 ml), and was obtained as colorless needles (103 mg) on recrystallization from the elution solvent. After psilocybin, α,α-trehalose (**3**) was then eluted. It crystallized as colorless prisms (680 mg) from methanol, mp 95–97°, mixture mp 95–97°, $[\alpha]_D$+169°, mass *m/e*: 235, 163, 145, 127, 73; ir max (KBr) cm^{-1}: 3400–3250, 905, 840, 800; ^{1}H-nmr (CD_3OD) δ: 4.90 (2H, d, J=3 Hz, anomeric H). α,α-Trehalose acetate: mp 74–75°; ^{13}C-nmr ($CDCl_3$) ppm: 61.8 (2C, t, CH_2OH x 2), 68.2 (2C, d, C_4, $C_{4'}$), 68.6 (2C, d, C_2, $C_{2'}$), 69.9 (4C, d, C_3, $C_{3'}$ and C_5, $C_{5'}$), 92.1 (2C, d, C_1, $C_{1'}$).

SCREENING PROCEDURE FOR PSILOCYBIN.—The fresh mushroom salmpe was extracted with methanol by leaving at room temperature for one day. After removal of the marc by filtration, the filtrate was reduced to dryness *in vacuo*. The dried residue was left in a desiccator for one day; 0.1–0.5 g of the extract was weighed exactly, 1 ml of water was added, and the solution was adjusted to pH 9.5 with 5% NH_4OH. Any undissolved material was filtered, and the filtrate was applied to a column containing 10 ml of Dowex 1 anion exchange resin and eluted with 60 ml of 0.01 M NH_4HCO_3, pH 9 buffer, followed by 60 ml of 0.1 M NH_4HCO_3 buffer. The 0.1 M buffer elution fraction was reduced to dryness *in vacuo* and submitted to hplc analysis.

One ml of water was added to the dried residue, and 5 μl of the solution was submitted to the analysis. The analysis conditions were: column, Lichrosorb NH_2, 4 mm ϕ x 250 mm; room temperature; eluant: 0.5 M KH_2PO_4 (pH 5.5); detector: uv (220 nm; 250 nm). Adenosine was used as an internal standard for the correction of the determination values using a calibration curve. The limit of the detection was proved to be 1 ng by this combined method of the ion exchange resin chromatography and hlpc. A peak, which was expected to be attributed to baeocystin, was found next to the peak of psilocybin for the extract of *P. argentipes* collected in July, 1979, but work for identification is still progressing.

CULTURE OF PSILOCYBE SPP.—Mother fungi of *Psilocybe fasciata* (IFO No. 30191) and *P. subaeruginascens* (No. 30219), provided from the Institute of Fermentation at Osaka, were grown in steady culture on a pH 6.5 medium of peptone (0.5 g), malt extract (0.2 g), yeast extract (0.2 g), glucose (60 g), and dist. water (100 ml) at 25° for two months. Cultured materials were divided into mycelium and fluid. Mycelium was extracted with MeOH, and the extracts were submitted directly to hplc analysis. The fluid was submitted to ion exchange resin chromatography, and the acidic amino acid fraction was submitted to hplc.

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LITERATURE CITED

1. K. Yokoyama, *Trans. Mycol. Soc. Japan*, **14**, 317 (1973).
2. K. Yokoyama, *Trans. Mycol. Soc. Japan*, **17**, 349 (1976).
3. A. Hofmann, R. Heim, A. Brak, H. Kobel, A. Frey, H. Ott, Th. Petrzilka and F. Troxler, *Helv. Chim. Acta.*, **42**, 1557 (1959).
4. G. M. Hatfield, L. J. Valdes and A. H. Smith, *Lloydia*, **41**, 140 (1978) and the references therein.
5. R. A. Weeks, R. Singer and W. L. Hearn, *Lloydia*, **42**, 469 (1979).
6. T. R. Govindachari, N. Viswanathan, B. R. Pai and T. S. Sauitri, *Tetrahedron Letters*, 901 (1964).
7. D. B. Repke and D. T. Leslie, *J. Pharm. Sci.*, **66**, 113 (1977).