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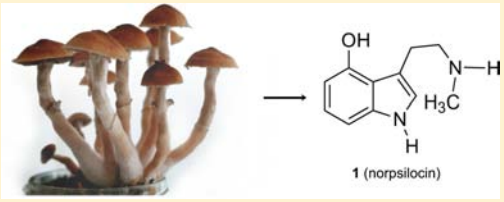
Identification of ω-*N*-Methyl-4-hydroxytryptamine (Norpsilocin) as a *Psilocybe* Natural Product

Claudius Lenz,[†] Jonas Wick,[†] and Dirk Hoffmeister*

Department of Pharmaceutical Microbiology at the Hans-Knöll-Institute, Friedrich-Schiller-Universität Jena, Winzerlaer Strasse 2, 07745 Jena, Germany

Supporting Information

ABSTRACT: We report the identification of ω-*N*-methyl-4-hydroxytryptamine (norpsilocin, **1**) from the carpophores of the hallucinogenic mushroom *Psilocybe cubensis*. The structure was elucidated by 1D and 2D NMR spectroscopy and high-resolution mass spectrometry. Norpsilocin has not previously been reported as a natural product. It likely represents the actual psychotropic agent liberated from its 4-phosphate ester derivative, the known natural product baeocystin. We further present a simple and artifact-free extraction method that prevents dephosphorylation and therefore helps reflect the naturally occurring metabolic profile of *Psilocybe* mushrooms in subsequent analyses.



Numerous fungi of the basidiomycete genus *Psilocybe* and other genera, colloquially called magic mushrooms, elicit profound changes in mood and perception following ingestion. The psychotropic effects are due to the tryptamine derivatives psilocin and its 4-*O*-phosphate ester, psilocybin, which are synthesized by these fungi.[1] These metabolites have been extracted side-by-side from these mushrooms.[2,3] Psilocybin represents the terminal pathway product and a prodrug, as it becomes rapidly dephosphorylated following oral ingestion to yield the actual psychotropically active psilocin.[4,5] This compound acts primarily as a partial agonist on $5HT_{1A}$, $5HT_{2A}$, and $5HT_{2C}$ receptors.[6] Psilocin and psilocybin were first described by Hofmann and co-workers.[1] Over the following decades, synthetic psilocybin derivatives and naturally occurring congeners have been documented, including baeocystin, norbaeocystin, and aeruginascin.[7−9] They are biosynthesized from L-tryptophan by successive decarboxylation, mono-, di-, and trimethylation, respectively, of the amino nitrogen, 4-hydroxylation, and formation of the phosphate ester.[10] Following this biosynthetic cascade, ω-*N*-methyl-4-hydroxytryptamine (norpsilocin, **1**) can be postulated as the immediate precursor of baeocystin. A very recent *in vitro* study[11] suggests that **1** directly derives from 4-hydroxytryptamine as the product of a minor shunt pathway and is then phosphorylated into baeocystin. However, compound **1**, described as a synthetic product and by artificially dephosphorylated *Psilocybe* extracts,[9,12,13] has never been identified as a natural product. Here, we describe **1** as a natural product of *Psilocybe cubensis*.

During chemical analysis of *P. cubensis* carpophore extracts, psilocin and psilocybin, along with minor amounts of baeocystin and tryptophan, were identified by LC/ESIMS (Figure 1A). Surprisingly, in the chromatogram, recorded at λ = 280 nm, an additional minor signal at t_R = 10.6 min was detected that featured the typical psilocybin signature UV−vis spectrum indicating a 4-hydroxyindole moiety. Further, the signal corresponded to a mass of *m*/*z* 191.1178 $[M + H]^+$ (calcd for $C_{11}H_{15}N_2O$ 191.1179), which is inconsistent with all known *Psilocybe* alkaloids, but matches that of psilocin (*m*/*z* 205.1 $[M + H]^+$) less a methyl group. As such a natural product has not been described yet, we sought to follow up on this observation. The compound was purified from 200 g (fresh weight) of *P. cubensis* carpophores by preparative and semipreparative RP-HPLC, which yielded 3 mg of pure compound. Subsequently, 1D and 2D NMR spectra were recorded (Table 1, Figures S1−S5). The chemical shifts and coupling constants of the ^1H NMR data indicated a typical indole ring system, except that only three aromatic signals were observed (δ 6.40, H-5, δ 6.88 H-7, and δ 6.93, H-6), which suggested a substituted aromatic ring. The coupling pattern showed three double doublets, suggesting three adjacent protons and thus a 4- or 7-hydroxylation. Subsequent HMBC spectroscopy revealed J_3 coupling between the H-7 proton and C-3a and C-5, which excludes an alcohol functionality at this position. Evidence for a monomethylated, i.e., secondary, amine came from the integral of the methyl proton signal at δ 2.71 ppm, suggesting only three magnetically equivalent protons, as opposed to six protons with psilocin. Therefore, we unequivocally demonstrated that the isolated compound is identical to ω-*N*-methyl-4-hydroxytryptamine (norpsilocin, **1**, 3-[2-(methylamino)ethyl]-1*H*-indol-4-ol, Figure 1).

We could not discount a scenario where **1** represents an artifact that may have occurred through hydrolysis of the phosphate ester of baeocystin. Previous research showed that different carpophore extraction methods heavily impact the metabolic profile, as dephosphorylation of psilocybin can occur enzymatically as well as thermally.[2,14] Therefore, we investigated if the alkaloid profile of the fungus is impacted by how

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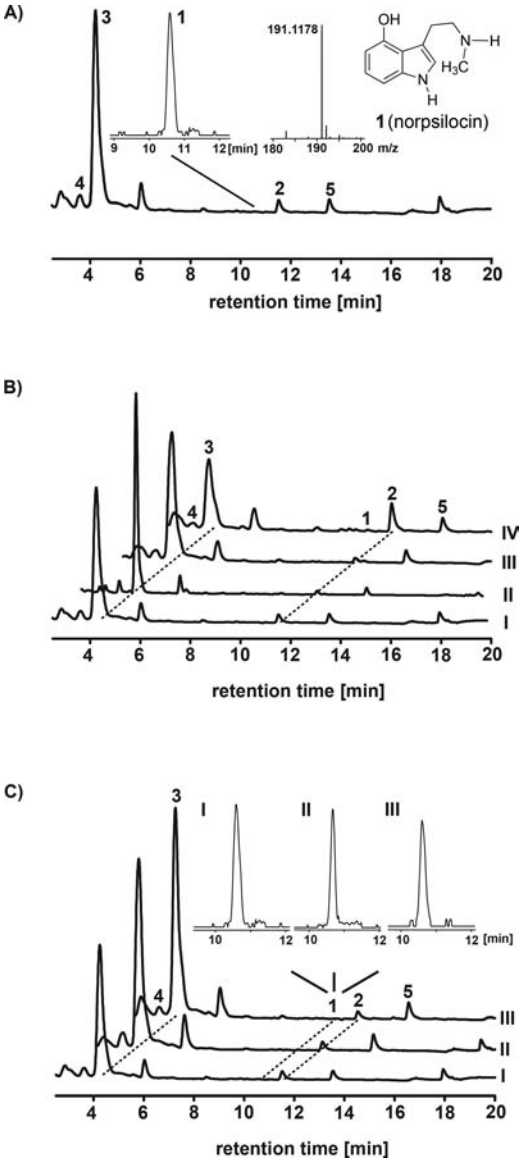

Figure 1. Chromatographic analysis of *Psilocybe* natural products. Compounds with indole UV−vis spectra are indicated by numbers: norpsilocin (**1**, t_R = 10.6 min), psilocin (**2**, t_R = 11.5 min), psilocybin (**3**, t_R = 4.1 min), baeocystin (**4**, t_R = 3.8 min), and tryptophan (**5**, t_R = 13.6 min). (A) HPL chromatographic detection of **1** after artifact-free extraction. The insets show the extracted ion chromatogram of **1** and the mass spectrum at t_R = 10.6 min. (B) Comparison of metabolic profiles after processing the carpophores by quick freezing and lyophilization (trace I), lyophilized carpophores extracted under aqueous conditions (trace II), drying at T = 37 °C prior to extraction (trace III), or using unprocessed carpophores (trace IV). (C) Comparison of metabolic profiles of *P. cubensis* isolates FSU 12407 (trace I), FSU12410 (trace II), and DSM-2530 (trace III). The insets show the extracted ion chromatograms for m/z 190.7−191.7 that prove the presence of **1** (t_R = 10.6 min) in all extracts.

the carpophore is processed prior to chromatographic analysis. Carpophores of *P. cubensis* isolate FSU12407 were harvested and either shock-frozen in liquid nitrogen and lyophilized or gradually dried at 37 °C over 2 days (protocols 1 and 3, respectively, in the Experimental Section). Lyophilized carpophores were alternatively extracted with a methanol−water mixture (4:1, v/v) to simulate the effect of water during extraction of dried biomass (protocol 2). In a fourth approach, the material was not dried at all (protocol 4). Inherent to these latter two methods, the natural products are exposed to water, either added or released by the disintegrating fungal cells during workup. In contrast, lyophilization of carpophores prior to extraction aimed at providing very mild conditions through minimized exposure to water. Subsequently, the carpophores were extracted with anhydrous methanol by shaking at ambient temperature. The respective extracts were qualitatively and quantitatively analyzed by LC/ESIMS (Figure 1B), which revealed that psilocybin, psilocin, baeocystin, and **1** were all present, regardless of the chosen method. However, quantitative differences of the indole alkaloid profile were observed which appeared in agreement with prior literature that reports dephosphorylation under aqueous conditions.[2,14] The relative amounts of norpsilocin and psilocin increased when the undried fungus was subjected to aqueous conditions and maintained close to room temperature for extended periods of time. When comparing areas under the curve, lyophilized basidiocarps showed a ratio of psilocybin:psilocin of 15:1, while basidiocarps that had not been dried showed a ratio of 5:1. Intriguingly, lyophilized carpophores extracted with a methanol−water mixture showed no increased dephosphorylation of psilocybin, and psilocin contents were also low (Figure 1B, trace II).

For *P. cubensis*, two studies investigated the amount of psilocybin and psilocin in dry biomass. Contents of 0.63% psilocybin and 0.11% psilocin are reported by one of the studies[2] and 1.07% psilocybin and 0.18% psilocin by the other.[15] Both studies therefore consistently report a 6-fold higher concentration of psilocybin over psilocin. By extracting fresh and/or poorly dried carpophores, the previous reports may have overestimated the natural psilocin concentrations in the fungus. Our results indicate that the drying process itself determines dephosphorylation and that the presence of water after drying is of minor relevance. This, in turn, supports the view that dephosphorylation is an enzymatically catalyzed, rather than spontaneous, process. To exclude strain-to-strain variation, we repeated the experiment by extracting carpophores of two other *P. cubensis* isolates (FSU12410 and DSM-2530), again applying mild conditions to prevent 4-*O*-phosphate ester cleavage. LC/ESIMS analysis revealed a virtually interchangeable metabolite profile, which confirmed

Table 1. NMR Spectroscopic Data for Norpsilocin (1)[a]

no.	δ_C, mult	δ_H, M (J in Hz)	HMBC
1			
2	123.9, CH	7.02, s	2, 3, 3a, 7a
3	111.0, C		
3a[b]	118.6, C		
4	153.5, C		
5	104.9, CH	6.40, dd (7.4, 1.0)	3a, 7
6	124.7, CH	6.93, dd (8.2, 7.4)	4, 7a
7	105.3, CH	6.88, dd (8.2, 1.0)	3a, 5
7a	141.7, C		
1′	53.2, CH_2	3.42, t (6.9)	2′, 3, N-CH_3
2′	25.9, CH_2	3.26, t (6.9)	1′, 2, 3, 3a
N-CH_3	34.5	2.71, s	1′
N-H		n.o.[c]	
4-OH		n.o.	

[a]MeOH-d_4, 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR. [b]Chemical shift of C-3a obtained from HMBC data. [c]Not observed.

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the presence of **1** in all isolates and comparably low psilocin contents (Figure 1C).

Considering the reported psychotropic effects of baeocystin,[16] **1** very likely represents its actual psychoactive form after 4-*O*-dephosphorylation *in vivo*. This assumption is made by analogy to the psilocybin/psilocin pair of alkaloids. For psilocybin, 10 mg is considered a low oral dose.[17] A self-experiment that included ingestion of 4 mg of baeocystin suggested that it is not more potent than psilocybin.[16] Given the very low concentration in carpophores, naturally occurring **1** likely contributes little to the hallucinogenic effects of ingested magic mushrooms. *Psilocybe* mushrooms have been used as illicit hallucinogenic drugs. However, the value of their alkaloid natural products becomes evident when their pharmaceutical potential is also taken into account. Recent studies indicate a positive effect of psilocybin for treatment of, for example, addictions, chronic depressions, and anxiety disorders.[17−19] Our work may add **1** to the candidates for further pharmaceutical development.

EXPERIMENTAL SECTION

General Experimental Procedures. The UV−vis spectrum of **1** was recorded by the diode-array detector during HPLC-MS experiments. Samples were dissolved in MeOH. The IR spectrum was recorded on a Jasco FT/IR 4100 instrument. To record 1D and 2D NMR spectra, the compound was dissolved in MeOH-d_4. ^{1}H and ^{13}C chemical shifts were referenced relative to residual nondeuterated solvent traces at δ_H 3.34 ppm and δ_C 49.8 ppm, respectively. NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer, at 300 K. Preparative HPLC was performed using an Agilent 1260 chromatograph equipped with a Zorbax Eclipse XDB-C_8 column (250 × 21.2 mm, 7 μm particle size). Semipreparative HPLC was done on an Agilent 1200 instrument equipped with a Zorbax Eclipse XDB-C_8 column (250 × 9.4 mm, 5 μm particle size). Analytical HPLC and mass spectrometry was performed on an Agilent 1260 chromatograph with a Zorbax Eclipse XDB C_{18} column (150 × 4.6 mm, 3.5 μm particle size), interfaced with an Agilent 6130 mass detector, and HRESIMS data were recorded on an Exactive Orbitrap instrument (Thermo Scientific). Chromatograms were recorded at λ = 280 nm; the respective diode array detectors covered the wavelength range of λ = 200−450 nm. Chemicals, solvents, and media components were purchased from Deutero, Sigma-Aldrich, Roth, and VWR.

Microbiological Methods. *Psilocybe cubensis* strains FSU12407 and FSU12410 were obtained from the Jena Microbial Resource Collection JMRC, and strain DSM-2530 was from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All strains were maintained on malt extract/peptone (MEP) solid medium (30 g/L malt extract, 3 g/L soy peptone, 18 g/L agar, pH = 5.6) at T = 23 ± 1 °C in the dark. For carpophore production of strains FSU12407, FSU12410, and DSM-2530, 200 g of rye (dry weight) was inoculated with 10 mL of a homogenized liquid MEP culture. After 4−6 d, the rye culture was used to inoculate a bark humus/perlite mixture, filled in a plastic wrap-covered flower box, which was maintained at 23 ± 1 °C in the dark for 3 days. Subsequent exposure to light induced formation of carpophores, which were harvested before the velum was ruptured.

Comparative Extraction of *Psilocybe* Natural Products. To compare extraction methods, freshly harvested carpophores similar in size and equally mature were weighed, and equal masses extracted following three different procedures. Protocol 1: Carpophores were weighed, frozen in liquid nitrogen, lyophilized for 2 days, ground with mortar and pestle to a fine powder, and then extracted in 20 mL of anhydrous MeOH per gram fresh weight. Protocol 2: Carpophores were dried as in protocol 1 but extracted with a mixture of 20 mL of methanol−water (4:1, v/v) per gram fresh weight. Protocol 3: Carpophores were weighed and subsequently dried at T = 37 °C in an oven, for 2 days, then ground to a fine powder and extracted, as above, in 20 mL of anhydrous MeOH per gram fresh weight. Protocol 4 was similar to protocol 1, but did not include lyophilization. Thus, water was not removed from the biomass.

Regardless of the protocol, the methanolic extracts were shaken at 140 rpm at RT for 18 h. A 100 μL amount of each extract was filtered, and the solvent removed under reduced pressure. The residue was taken up in 100 μL of water−acetonitrile (ACN) (9:1, v/v) and analyzed by LC/MS. To compare metabolite profiles, carpophores of strains FSU12407, FSU12410, and DSM-2530 were harvested, processed following protocol 1, and chromatographically analyzed.

Isolation of 1. Mature *P. cubensis* carpophores FSU12407 (200 g) were frozen in liquid nitrogen, ground to a fine powder, which was extracted with 50 mL of MeOH, and supplemented with sodium dithionite (0.1 g/10 g biomass) to prevent extract oxidation. The extract was transferred to Erlenmeyer flasks, shaken at 140 rpm and 23 °C, for 18 h, and subsequently filtered. The solvent was removed under reduced pressure in a rotary evaporator. The dry mycelial extract was dissolved in a 1:1 (v/v) mixture of 0.1% trifluoroacetic acid (TFA) in water−cyclohexanol (20 mL of per 100 g biomass). The solution was centrifuged at 8800*g* for 10 min. Then, the aqueous phase was filtered and used for chromatographic separation. Preparative HPLC was carried out with solvents A (ACN) and B (0.1% (v/v) TFA in water) under isocratic conditions with 5% A and 95% B, at a flow rate of 20 mL/min, for 10 min. Fractions were dried under reduced pressure. The residue was dissolved in 0.1% TFA in water (1 mL per 10 mL initial volume of the fraction). Further purification was carried out by semipreparative HPLC that was performed with the above solvents. The column was eluted with a linear gradient of 5−15% A within 2 min, followed by an isocratic hold at 15% A for 13 min. The fractions containing **1** were combined, dried under reduced pressure, and stored at −20 °C. For NMR spectroscopy, the stored fractions were dissolved in 0.1% TFA in water and repurified by semipreparative HPLC as described above. ACN and H_2O were removed by brief rotary evaporation, followed by lyophilization. As **1** is air-sensitive, the compound was cooled, whenever possible, and stored under inert gas. Open handling was minimized. To record NMR spectra, the dry sample was immediately dissolved in 160 μL of MeOH-d_4, transferred to an NMR tube, covered with nitrogen, and sealed.

Analytical HPLC and Mass Spectrometry. Solvent A was 0.1% TFA in ACN; solvent B was 0.1% TFA in water. The flow was 1 mL/min. A linear gradient from 5% to 30% A within 20 min was applied. Electrospray ionization was run in positive and negative mode, and the mass range of m/z 100−500 was scanned. For LC/HRESIMS, ionization was also in positive and negative mode, scanning the mass range of m/z 110−1000. Solvent A was ACN; solvent B was 0.1% (v/v) formic acid in water. The flow rate was 0.2 mL/min. A linear gradient from 5% to 98% A within 15 min was used.

Norpsilocin (1): amorphous, colorless solid; UV (H_2O−ACN, 4:1) λ_{max} (log ε) 3.00 (280 nm); IR (neat) 3370, 2477, 2244, 2072, 1674, 1204, 1119, 971 cm^{-1}; ^{1}H and ^{13}C NMR data (MeOH-d_4, 500 and 125 MHz, respectively), see Table 1; HRESIMS m/z 191.1178 $[M + H]^+$, calcd for $C_{11}H_{15}N_2O$ 191.1179.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00407.

1D and 2D NMR spectra of norpsilocin (**1**) (PDF)

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 3641 949850. Fax: +49 3641 949852. E-mail: dirk.hoffmeister@hki-jena.de.

ORCID

Dirk Hoffmeister: 0000-0002-5302-6461

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Author Contributions

†C. Lenz and J. Wick contributed equally.

Notes

The authors declare no competing financial interest.

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