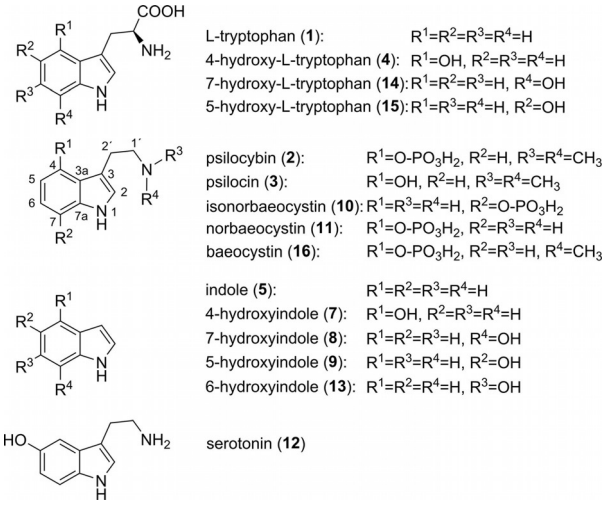
Biocatalytic Production of Psilocybin and Derivatives in Tryptophan Synthase-Enhanced Reactions

Felix Blei, Florian Baldeweg, Janis Fricke, and Dirk Hoffmeister*[a]

Abstract: Psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine) is the main alkaloid of the fungal genus *Psilocybe*, the so-called "magic mushrooms." The pharmaceutical interest in this psychotropic natural product as a future medication to treat depression and anxiety is strongly re-emerging. Here, we present an enhanced enzymatic route of psilocybin production by adding TrpB, the tryptophan synthase of the mushroom *Psilocybe cubensis*, to the reaction. We capitalized on its substrate flexibility and show psilocybin formation from 4-hydroxyindole and L-serine, which are less cost-intensive substrates, compared to the previous method. Furthermore, we show enzymatic production of 7-phosphoryloxytryptamine (isonorbaeocystin), a non-natural congener of the *Psilocybe* alkaloid norbaeocystin (4-phosphoryloxytryptamine), and of serotonin (5-hydroxytryptamine) by means of the same in vitro approach.



Scheme 1. Structures of *Psilocybe* tryptophan synthase substrates and products, and products of the in vitro reconstituted indole alkaloid synthesis pathway.

Among the most prominent natural products is the L-tryptophan (**1**)-derived alkaloid psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine **2**, Scheme 1).[1] This major metabolite of *Psilocybe* carpophores—colloquially dubbed "magic mushrooms"—rapidly dephosphorylates upon ingestion to yield psilocin (**3**), which acts as the actual hallucinogen by agonistically binding primarily to the human $5HT_{2A}$-receptor.[2] Importantly, recent clinical studies plausibly underscore the potential pharmaceutical value of **2** in the treatment of nicotine addiction, anxiety with terminal-stage cancer patients, and depression.[3]

For access to **2** and **3**, various synthetic approaches have been established.[4] Recently, a biotechnological three-enzyme route beginning from 4-hydroxy-L-tryptophan **4** to **2** has been reported. This route utilizes the *Psilocybe cubensis* enzymes PsiD, PsiK, and PsiM, which provide decarboxylase, kinase, and methyltransferase activity, respectively.[5] We sought to enhance this enzymatic procedure by enzymatically producing **4** in situ via tryptophan synthase (E.C. 4.2.1.20), thus feeding lower-priced precursors to the process. The α-subunits of $\alpha_2\beta_2$-heterotetrameric bacterial tryptophan synthases catalyze the first half reaction (Figure 1), that is, the retro aldol-type cleavage of 1-(indole-3-yl)glycerol phosphate that releases D-glyceraldehyde 3-phosphate and provides indole **5** (Scheme 1) as substrate for the subsequent second half reaction. It is catalyzed by the β-subunit and includes a pyridoxal phosphate (PLP)-dependent condensation of **5** and L-serine **6** (Figure 1) into **1**.[6] Wild type or engineered prokaryotic tryptophan synthases of *Salmonella enterica* or *Pyrococcus furiosus* proved valuable in procuring β-methyl- or L-halotryptophans, respectively, via enzymatic conversion of substituted **5** and **6** (or L-threonine) by the β-subunit.[7] A recent study showed production of

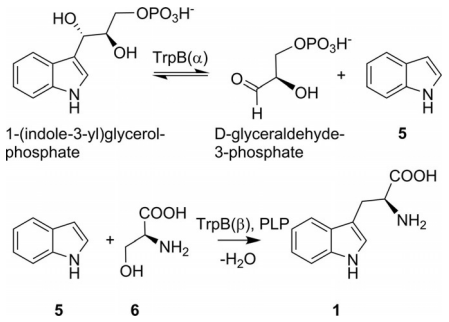


Figure 1. The tryptophan synthase reaction. Indole (**5**) formation is catalyzed by the α-subunit (TrpB(α)), L-tryptophan (**1**) production is catalyzed by the β-subunit (TrpB(β)).

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4-halogenated or 4-nitro tryptophan by engineered TrpB variants.[8] Otherwise, tryptophan synthases generally showed low tolerance for 4-substituted indoles. Furthermore, hydroxy-substituted indoles, which are relevant for our purposes, had not been included in prior studies.

We recombinantly produced *P. cubensis* L-tryptophan synthase (TrpB). Here, we report on integrating it into a one-pot procedure to synthesize **2** from **6** and 4-hydroxyindole (**7**), that is, two inexpensive compounds, in four enzymatic steps, using enzymes of the same fungus. Choosing 7-hydroxyindole (**8**) and 5-hydroxyindole (**9**), we further describe an enzymatic synthesis for isonorbaeocystin (7-phosphoryloxytryptamine **10**), that is, a non-natural isomer of the psilocybin-family alkaloid norbaeocystin **11** (Scheme 1). We also show TrpB/PsiD-catalyzed synthesis of serotonin (**12**) in two enzymatic steps.

Typically, fungal tryptophan synthases are homodimers. Each monomer is bifunctional, includes an α- and a β-domain, and shows a mass of about 75 kDa.[9] Using known fungal TrpB sequences as a reference,[10] we identified a 2648 bp candidate gene in the genome of *P. cubensis*[5] that was disrupted by ten introns, according to an in silico analysis with Augustus software.[11]

The 2103 bp coding sequence encodes a 700 aa TrpB monomer (calculated molecular mass 75.5 kDa, calculated pI 5.9). The protein was most similar (85% identical aa) to predicted proteins of the mushrooms *Hebeloma cylindrosporum* (accession # KIM48772.1) and of *Galerina marginata* (KDR73575.1). The *trpB* cDNA was inserted into expression vector pET28a to create plasmid pFB14. It was used to transform *E. coli* KRX to produce TrpB as N-terminally tagged polyhistidine fusion protein (Figure S1 in the Supporting Information).

Optimum turnover took place at pH 8.0 and at 30 °C. TrpB followed Michaelis–Menten kinetics and showed K_m values of 40 μM and 16 μM for **5** and **7**, respectively, and 2.9 mM for **6** (Figure S2, Supporting Information). The values for **5** and **6** are comparable with prior data.[7e,9] Gel permeation chromatography confirmed the dimeric state of active TrpB (Figure S3). The TrpB substrate specificity was assessed using **7**, **8**, **9**, or 6-hydroxyindole **13** (Figure 2). Indoles **7**, **8**, and **9** were converted to 4-, 7-, and 5-hydroxy-L-tryptophan (compounds **4**, **14**, and **15**, respectively, at t_R = 10.2, 9.8, and 7.9 min), as determined by LC-MS (Figure 2, found masses *m/z* 221.0920, 221.0930, and 221.0927 $[M+H]^+$, respectively, calculated for $C_{11}H_{13}N_2O_3$: 221.0926). Product formation was not detected in the reaction with **13**, a signal with the expected mass and UV/Vis spectrum was not found.

Next, we extended the described PsiD/PsiK/PsiM-dependent **2** in vitro synthesis by adding TrpB and PLP to this multienzyme assay. Substrates **6** and **7** were initially present at 3 mM, and the reaction proceeded for 4 h. LC-MS analysis unequivocally proved **2** production, along with its immediate natural precursors baeocystin (**16**) and **11** (Figure 3, Figure 4).

The substrate tolerance of the Psi enzymes outside the **2** biosynthesis pathway has not been investigated in greater detail. Considering that **15** was a TrpB product, we re-ran the four-enzyme reaction, this time adding 3 mM **6** and **9** as respective substrates. LC-MS-analysis demonstrated that a

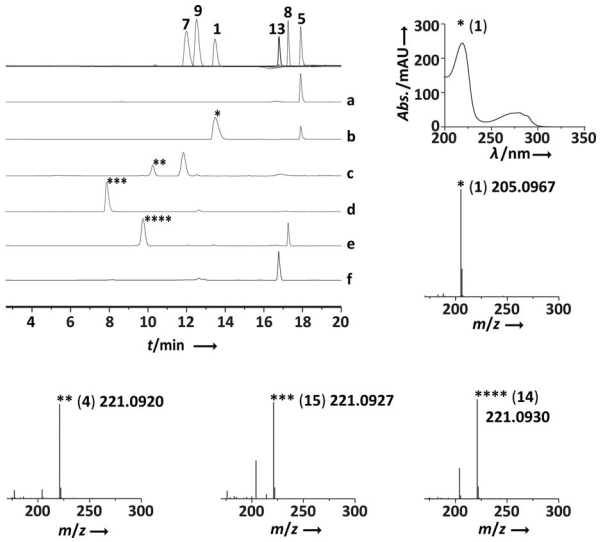

Figure 2. Product formation by *Psilocybe cubensis* TrpB. Chromatograms were extracted at $\lambda = 280$ nm, HR-ESIMS spectra of the compounds given in parentheses were recorded in positive mode, experimentally determined masses are indicated in the spectra. The calculated mass of **4**, **14**, and **15** is *m/z* 221.0926 ($[M+H]^+$), the calculated mass of **1** is *m/z* 205.0972. Top trace: overlayed separate chromatograms of authentic standards. Trace a: negative control with heat-treated TrpB and **5**. Trace b: reaction with **6** and **5**. Trace c: reaction with **6** and **7**. Trace d: reaction with **6** and **9**. Trace e: reaction with **6** and **8**. Trace f: reaction with **6** and **13**.

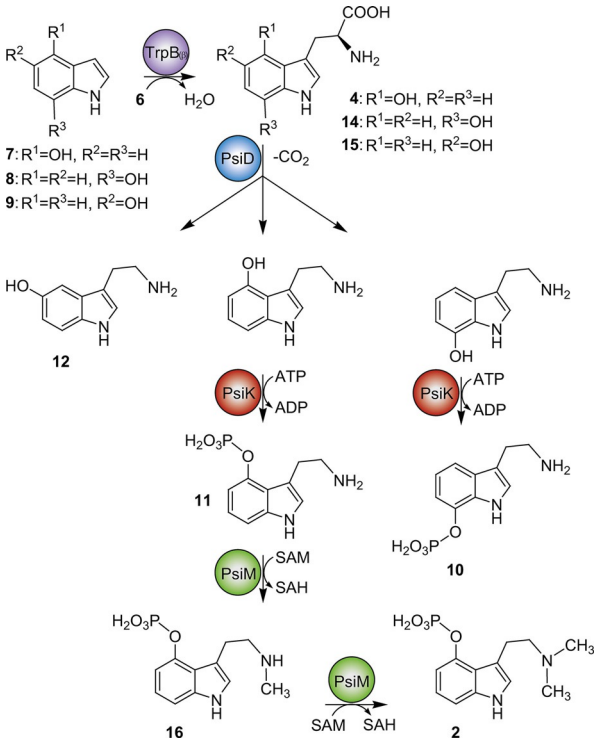

Figure 3. In vitro syntheses leading to serotonin (**12**), psilocybin (**2**), or isonorbaeocystin (**10**). The reactions included *Psilocybe cubensis* enzymes TrpB, PsiD, PsiK, and PsiM.

product had formed (t_R = 7.4 min) whose mass suggested the loss of CO_2 (*m/z* 177.1022 $[M+H]^+$) hence implying **15** forma-

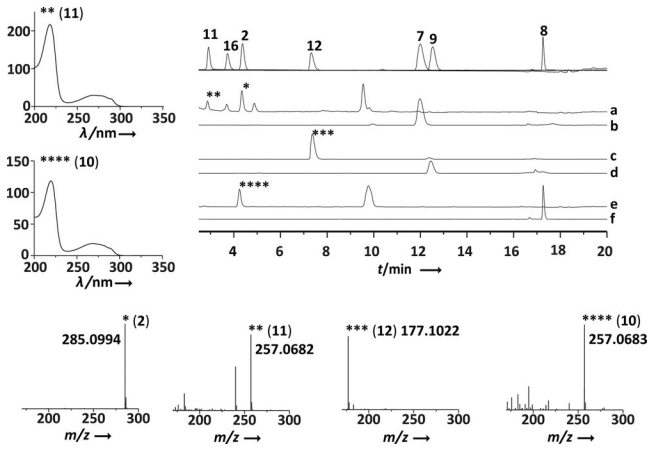

Figure 4. LC-MS analysis of product formation in multi-enzyme assays. Chromatograms were extracted at $\lambda = 280$ nm, HR-ESIMS spectra were recorded in positive mode, experimentally determined masses are indicated in the spectra. The calculated mass ($[M+H]^+$) of **2** is *m/z* 285.0999, of **10** and **11**: *m/z* 257.0686, of **12**: *m/z* 177.1022. Top trace: overlayed separate chromatograms of standards. Trace a: Reaction with TrpB, PsiD, PsiK, and PsiM and substrates **6** and **7**. The signal at $t_R = 4.8$ min is due to *S*-adenosyl-L-homocysteine. Trace b: negative control. Trace c: Reaction with TrpB and PsiD with substrates **6** and **9** (trace d: negative control). Trace e: Reaction with TrpB, PsiD, PsiK, and PsiM with substrates **6** and **8** (trace f: negative control). Signals at $t_R = 9.7$ and 9.6 min in traces a and e, respectively, represent intermediates 4- and 7-hydroxytryptamine, as identified by their UV/Vis spectra and by mass spectrometry (*m/z* 177.1 $[M+H]^+$).

tion and subsequent quantitative PsiD-catalyzed decarboxylation into **12**. This was confirmed by comparison with an authentic **12** standard. This finding further suggested that PsiD tolerates well the 5-hydroxy substitution, which confirmed a previous report.[12] The results also showed that 5-hydroxylated indoles do not serve as substrates for the kinase PsiK, as no second, that is, phosphorylated product was detectable. Assays run only with TrpB and PsiD resulted in identical chromatograms (Figure 4).

We repeated the four-enzyme reaction with **6** and **8**. LC-MS analysis indicated formation of a product whose UV/Vis spectrum and mass (*m/z* 257.0682 $[M+H]^+$) were equal to that of **11** (Figure 4, $t_R = 2.9$ min), but which differed in its retention time ($t_R = 4.2$ min), thus pointing to an isomeric product. Assuming that PsiD tolerates the 7-hydroxy group and that PsiK accepts it as phosphate acceptor substrate, we hypothesized that this product was identical to 7-phosphoryloxytryptamine **10** (Scheme 1). We chromatographically purified this compound from an upscaled assay, which yielded 2.36 mg of pure substance.

1D and 2D NMR spectra (Table 1, Figures S4–S9 in the Supporting Information) were recorded and compared to NMR data of **2** (Table 2, Figures S4 and S10–S14). In **10**, the bridgehead carbon C-3a was identified by HMBC correlations with H-1 and H-2, while C-7a was correlated to H-2. The HMBC correlation between carbons C-3a and C-7 with the H-5 triplet established the position of the phosphoryloxy group at C-7 (Figure S9). The signal at $\delta = 7.31$ ppm (H-4) showed HMBC correlations with C-3, C-6, and C-7a, thus providing final evidence for the structure of compound **10**, for which we propose the name isonorbaeocystin. Methylation of **10** by PsiM in detectable amounts was not observed.

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for **10** in [D$_6$]DMSO.

Pos.	δ_H [ppm] (mult. *J* [Hz])	δ_C [ppm]	HMBC	COSY
1	10.97 (s, 1H)	–	3, 3a	2
2	7.20 (d, 2.0, 1H)	123.9	3, 3a, 7a	1
3	–	109.9	–	–
3a	–	129.5	–	–
4	7.31 (d, 7.86, 1H)	113.8	3, 6, 7a	5
5	6.95 (t, 7.77, 1H)	118.7	3a, 7	4, 6
6	7.02 (d, 7.68, 1H)	111.3	4, 7a, 7	5
7	–	137.8	–	–
7a	–	128.2	–	–
1′	3.07 (m, 2H)	39.3[a]	3	2′, NH_2
2′	2.97 (t, 7.62, 2H)	23.2	1′, 2, 3, 3a	1′
NH_2	7.75 (s, 2H)	–	–	1′

[a] value derived from 2D ^{1}H-^{13}C correlation spectrum (HMBC).

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for **2** in [D$_6$]DMSO.

Pos.	δ_H [ppm] (mult. *J* [Hz])	δ_C [ppm]	HMBC	COSY
1	11.09 (s, 1H)	–	3, 3a	2
2	7.18 (d, 1.98, 1H)	123.7	3, 3a, 7a	1
3	–	108.2	–	–
3a	–	118.7	–	–
4	–	145.2	–	–
5	7.12 (d, 7.98, 1H)	107.6	3a, 7	6
6	6.99 (t, 7.74, 1H)	121.5	7a, 4	5, 7
7	6.93 (d, 7.89, 1H)	108.7	3a, 4, 5	6
7a	–	138.6	–	–
1′	3.32 (m, 2H)	58.1	2′, 3, $N(CH_3)_2$	2′
2′	3.18 (m, 2H)	21.2	1′, 2, 3, 3a	1′
$N(CH_3)_2$	2.82 (s, 6H)	42.3	1′, 2′	–

Therefore, we conclude that PsiM, the methyltransferase of the **2** pathway, cannot tolerate a 7-phosphoryloxy-substituted compound as acceptor substrate. This finding underscores the previously noted specificity of PsiM, which prevents *N*,*N*-dimethyltryptamine and **3** formation as intermediates in the **2** pathway.[5]

Contrasting the use of the *trpB* gene in fungal genetics as a standard selection marker in **1**-auxotrophic hosts, very few fungal tryptophan synthases were investigated biochemically.[9a,13] *P. cubensis* TrpB is the first biochemically characterized tryptophan synthase of the basidiomycetes, which represent a phylum of more than 30 000 species. The intrinsic tolerance of TrpB to substituted indoles allowed for its integration into a biocatalytic process that produces **2** congeners, including **10** and **12**, and that translates into a facile and more cost-effective enzymatic synthesis of **2**. The value of the substrate **4**, used in the previous procedure, is approximately US$ 180 mmol^{-1}, whereas the combined sales price for 1 mmol (each) of **6** and **7** as starting material in our refined process is about US$ 2.

Directed in vitro evolution and structure-based engineering of genes encoding biosynthesis enzymes proved instrumental to optimize catalytic activity, introduce new activities, and relax

specificities, to create libraries of natural product derivatives.[14] Thus, future work on enzyme engineering, in particular on the strictly specific methyltransferase PsiM, is warranted to access a larger structural diversity of **2** derivatives using only a small set of enzymes.

Experimental Section

Enzymatic reactions: All enzymatic in vitro reactions were carried out in triplicate. The enzyme concentration was 200 nM. In the case of multi-enzyme assays, each enzyme was present at this concentration. The reactions were stopped after the indicated incubation times by freezing and lyophilization. Subsequently, the residue was dissolved in methanol (MeOH), centrifuged for 10 min at 20000×g, and the supernatant was collected. The solvent was removed under reduced pressure, and the residue dissolved in H_2O:acetonitrile (9:1, v/v), filtered, and used for chromatography (see below). The in vitro characterization of TrpB was performed in a volume of 100 μL and 50 mM Tris-HCl buffer, pH 8.0 for 5 min at 30 °C (varied between 16–42 °C to determine the temperature optimum, and between pH 6.0 through 9.5 for the pH optimum). The substrates (**6**, and the respective indole substrate **5**, **7**, **8**, **9**, or **13**) were added at (each) 3 mM, the PLP concentration was 1 μM. Multi-enzyme reactions including TrpB, PsiD, PsiK, and PsiM were set up in a volume of 500 μL in 50 mM Tris-HCl buffer, pH 8.0, and proceeded for 4 h at 25 °C. The reactions yielding **2**, **10**, or **12** included **6** and indole substrates **7**, **8**, or **9** (3 mM final concentration each) as well as SAM, ATP, and $MgCl_2$ (6 mM each), the PLP concentration was 1 μM. Initially, all four enzymes were added. For subsequent assays yielding **12**, PsiK, PsiM, ATP, and SAM was omitted. The yield of **2** (20.7%) was determined by calculating the area under the curve of its chromatographic signal and compared to a standard curve, recorded with authentic compound.

Chemical analysis: LC-MS experiments were performed on an Agilent 1260 HPLC system equipped with a C_{18} column (Zorbax Eclipse XDB, 150×4.6 mm, 5 μm) and coupled to a 6130 Single Quadrupole mass detector, high-resolution mass spectrometry was performed on a Thermo Accela liquid chromatograph, following described parameters.[5] The 1D and 2D NMR spectra of **10** and **2** were recorded at 300 K on a Bruker Avance III spectrometer at 600 MHz for ^1H and at 150 MHz for ^{13}C spectra. [D_6]DMSO was used as solvent and internal standard. The solvent signals were referenced to $\delta_H = 2.50$ ppm and $\delta_C = 39.5$ ppm. Chemicals and solvents were purchased from Deutero, Key Organics, Sigma–Aldrich, Roth, TCI, and VWR. Alkaloids **2**, **11**, and **16** were purified from *P. cubensis* carpophores.

Detailed experimental procedures for microbiological and genetic methods, as well as for purification of enzymes and compound **10**, are described in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: alkaloid · biosynthesis · enzymes · psilocybin · tryptophan synthase

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