doi.org/10.1002/cplu.202000581

Taking Different Roads: L-Tryptophan as the Origin of *Psilocybe* Natural Products

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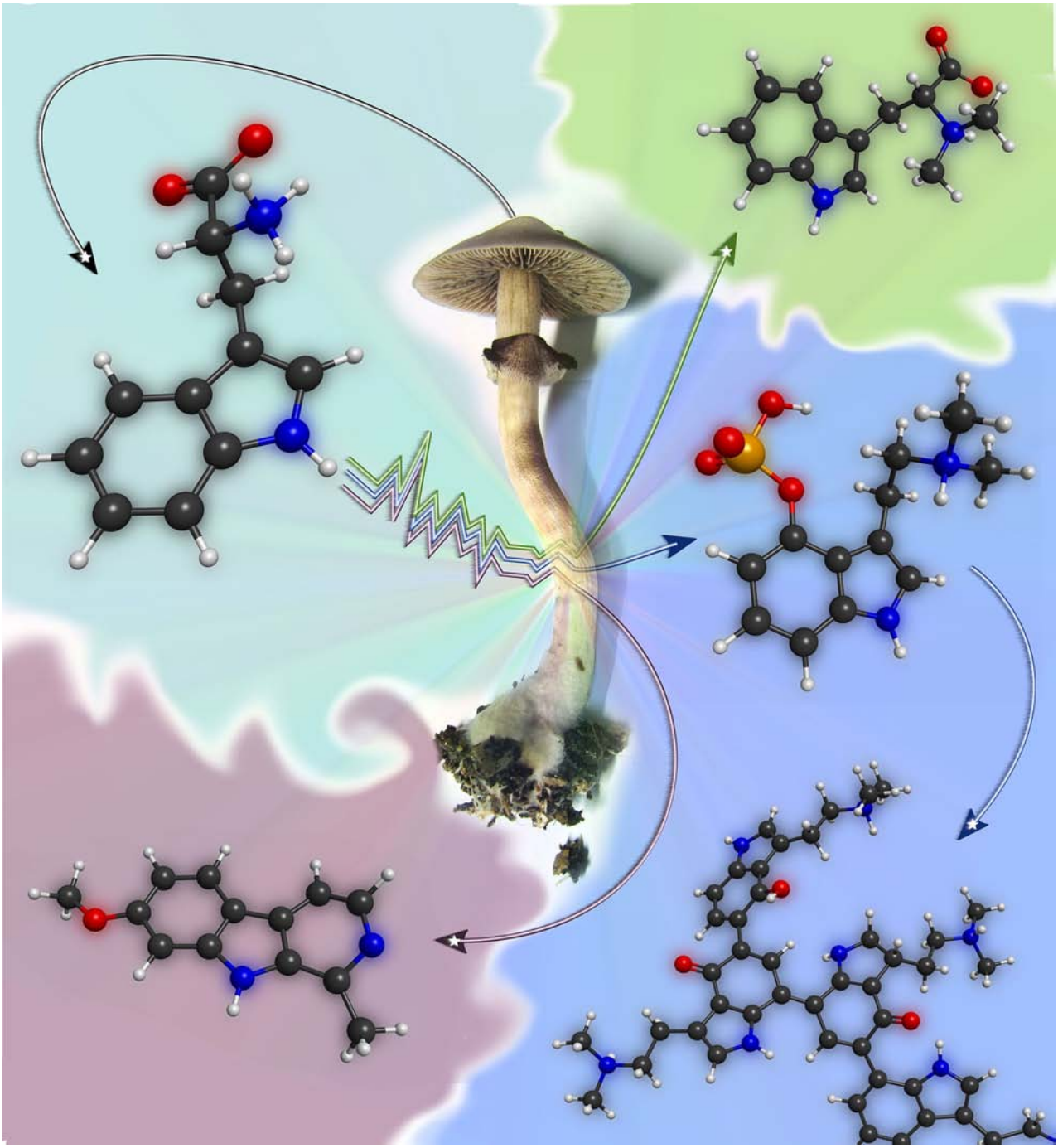

Psychotropic fungi of the genus *Psilocybe*, colloquially referred to as „magic mushrooms", are best known for their L-tryptophan-derived major natural product, psilocybin. Yet, recent research has revealed a more diverse secondary metabolism that originates from this amino acid. In this minireview, the focus is laid on L-tryptophan and the various *Psilocybe* natural products and their metabolic routes are highlighted. Psilocybin and its congeners, the heterogeneous blue-colored psilocyl oligomers, alongside β-carbolines and *N*,*N*-dimethyl-L-tryptophan, are presented as well as current knowledge on their biosynthesis is provided. The multidisciplinary character of natural product research is demonstrated, and pharmacological, medicinal, ecological, biochemical, and evolutionary aspects are included.

1. Introduction

L-Tryptophan (Figure 1) is an intriguing biomolecule in its own right. Among the 20 canonical proteinogenic amino acids, it is the only bicyclic structure, and has as the highest number of C-atoms.[1] Its *de novo* biosynthesis from D-erythrose-4-phosphate and phosphoenol pyruvate requires 13 steps and 12 enzymes, more than for any other proteinogenic amino acid, and with an average of 1.1 %, its abundance in proteins is usually the lowest of all amino acids.[1] The biogenesis of neurotransmitters and hormones such as serotonin (5-hydroxytryptamine, 5-HT, Figure 1) or melatonin begins with L-tryptophan.[2] From the perspective of natural product chemistry, a virtually limitless arsenal of bioactive plant and microbial metabolites originates from this particular amino acid, among them ergotamine, quinine, and ajmaline.[3]

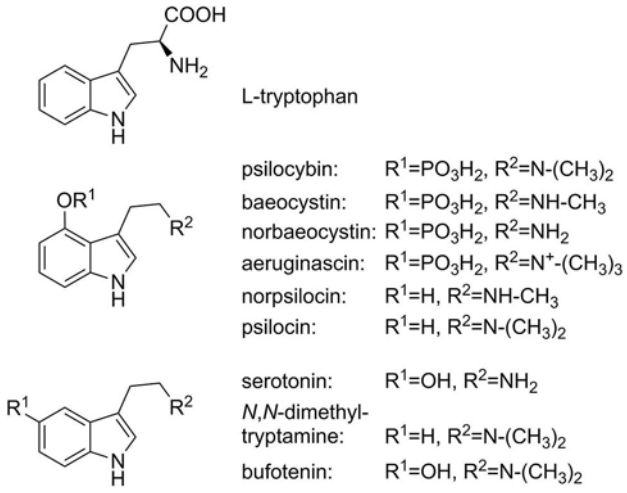

Figure 1. Structures of L-tryptophan, *Psilocybe* indole alkaloids, and other bioactive indoleethylamines.

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In this minireview, we focus on the biosynthetic routes of L-tryptophan-derived natural products in *Psilocybe* mushrooms. After looking at their biosynthesis we move on to brief forays into research areas alongside the chemistry and into the evolutionary aspects that encompass these compounds.

Usually, these fungi (e.g., *Psilocybe cyanescens*, Figure 2) are synonymous with their psychedelic effects due their capacity to produce psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine, Scheme 1).[4] This metabolite is the direct precursor of the psychotropic psilocin (Figure 1) that earned these fungi a cult status and is the reason why they have been dubbed „magic mushrooms". However, recent research has identified a more diverse set of indole compounds that were not previously recognized. These variable yet relatively simple compounds illustrate how natural product biosynthesis has evolved to create structural diversity by transforming one generic precursor L-tryptophan into bioactive molecules.

2. L-Tryptophan-derived natural products in *Psilocybe* species

2.1. Monomeric psilocybin and its congeners

2.1.1. The route from L-tryptophan to psilocybin

A hallmark feature of *Psilocybe* mushrooms and other genera is the biosynthesis of their signature natural product psilocybin.[4] Psilocin, the dephosphorylated metabolite formed immediately upon ingestion, subsequently acts as a ligand and partial agonist of the 5-HT_{2A} receptor in humans, thereby profoundly impacting human perception and consciousness.[5] For millennia, humans have made use of this phenomenal mushroom pharmacology, which is deeply rooted in Central American cultures for spiritual and divinatory purposes. The historical, pharmacological, and medicinal aspects have been reviewed.[5,6]

The isolation and structure elucidation of psilocybin was first described in the late 1950's by Albert Hofmann and colleagues at Sandoz Laboratories.[4] They used [β-^{14}C]-L-tryptophan for investigative experiments that proved psilocybin's origin from this building block.[7] Less than a decade later, Agurell and Nilsson utilized the same concept, used various radiolabeled precursors and presented a sequence of biosynthetic events which metabolize L-tryptophan to psilocybin. These authors proposed a five-step biosynthesis via decarboxylation to tryptamine as the initial step, followed by two methyltransfers, then 4-hydroxylation, and 4-*O*-phosphorylation

Figure 2. *Psilocybe cyanescens*, a psilocybin and β-carboline producer that occurs in Europe and North America. This species grows on lignin-rich substrates, such as wood chips, used to mulch plant beds or park areas. The mushrooms typically grow in clusters and appear in late fall when the temperature has dropped below ca. 10 °C (50 °F). The mature specimen shows the characteristic wavy and dark-edged cap.

as the terminal step.[8] Recent work identified the genes encoding psilocybin biosynthesis enzymes in *Psilocybe* and other genera.[9] The metabolic pathway was shown by the activity of four *Psilocybe cubensis* enzymes (PsiD, PsiH, PsiK, and PsiM), which provide L-tryptophan decarboxylase, indole-4-monooxygenase, kinase, and *N*-methyltransferase activity, respectively.[9a,10,11] Characterization of these enzymes confirmed L-tryptophan as the first substrate in the pathway. This research led to a revised biosynthetic sequence in which methylation, rather than phosphorylation, concludes the biosynthesis (Scheme 1). In the case of aeruginascin (Figure 1), the quaternary ammonium derivative of psilocybin found mainly in *Inocybe* mushrooms,[12] a third *N*-methyltransfer takes place. Collectively, these results identified norbaeocystin and baeocystin[13] as biosynthetic intermediates, and norpsilocin[14] (Figure 1) as a shunt product. Contrasting the previously assumed role as psilocybin's direct precursor, psilocin is not a pathway intermediate at all. Rather, the combined enzyme specificities cooperatively prevent psilocin formation.[9a,11] Most intriguingly, the kinase PsiK plays a dual biosynthetic and protective role: during biosynthesis, it phosphorylates 4-hydroxytryptamine to norbaeocystin. However, PsiK phosphorylates psilocin to psilocybin with a three times higher catalytic efficiency. Therefore, the cell is protected from psilocin, as it is readily oxidized and oligomerizes (see section 2.2).[11]

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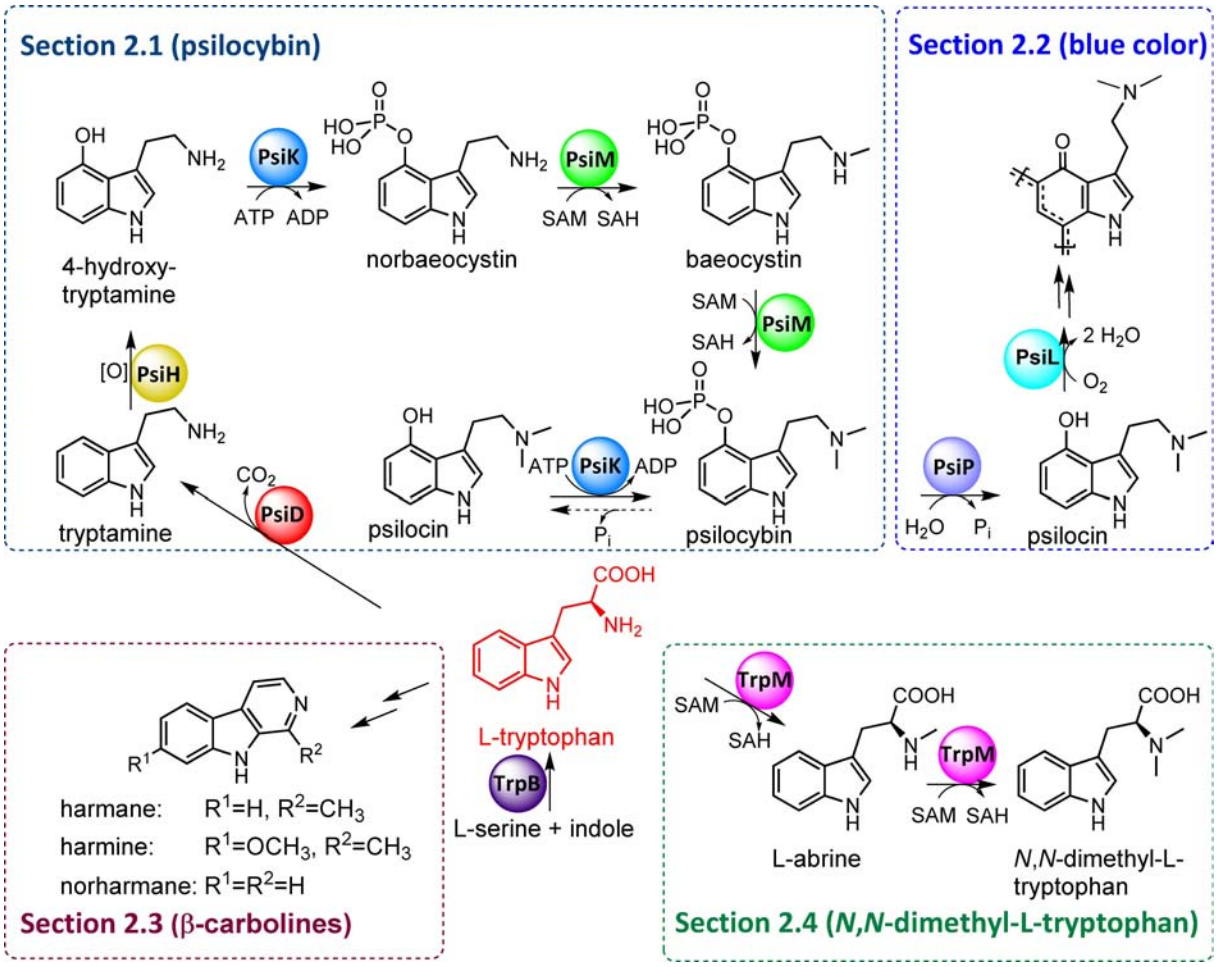
2.1.2. Psilocybin's clinical promise

Archaeological evidence has suggested that psilocybin in mushrooms has likely been consumed by humans for thousands of years.[15] Given this familiarity and its demonstrated suitable pharmacological properties, such as wide therapeutic index, oral bioavailability, and acceptable duration of action, psilocybin as a prodrug to the actual active compound psilocin is considered an ideal material to explore in controlled clinical trials.[16] With more than ten completed clinical trials over the past decade, the therapeutic potential of psychedelics has drawn considerable attention, particularly in the field of psychiatry.[17] For example, participants treated with psilocybin for alcohol use disorder (AUD)[18] or tobacco addiction[19] have both demonstrated impressive improvements in cessation post-treatment. Psilocybin-assisted therapy has also demonstrated efficacy in the treatment of depression and anxiety in cancer patients[20] as well as in an open-label study on treatment resistant depression (TRD).[21] In response to the promising early clinical data, psilocybin has been granted breakthrough designation by the United States Food and Drug Administration (FDA) for both TRD and major depressive disorder (MDD) in 2018 and 2019, respectively. Both disorders represent conditions with an unmet need where patients have not improved using conventional antidepressant treatments.

Currently available treatment options for adversely affected mental health fall short on several metrics and the need for new approaches is urgent and highly desirable. Psilocybin-assisted treatment is attractive because it requires only a small dose, has been shown to be non-addictive, and has demonstrated potential efficacy with possibly a once in a lifetime treatment.[17] Collectively, psilocybin does not fit well into a traditional model of profit-driven pharmaceutical development. Furthermore, because psilocybin is naturally-occurring and well-described, patentability possibilities are minimal. For these reasons, early developmental efforts and gaps in funding have been met philanthropically by non-profit organizations such as the Multidisciplinary Association for Psychedelic Studies (MAPS), Usona Institute, and The Heffter Research Institute.[22] A unifying goal of these organizations has been to further the understanding of the therapeutic effects of psilocybin and other consciousness-expanding medicines while influencing policy to ultimately increase their accessibility.

2.1.3. Pharmacology and medicinal chemistry

Psilocin interferes with serotonergic neurotransmission by acting as a partial agonist at mammalian serotonin (5-HT) receptors, including the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} subtypes. While psilocin's agonist activity at the 5-HT_{2A} receptor (K_i = 6 nM) is generally considered to be necessary for psychedelic activity, the role of the other subtypes is less understood.[24]



Scheme 1. Overview on L-tryptophan-fed natural product pathways in *Psilocybe* sp. and, if characterized, the participating enzymes. TrpB (violet) represents the *Psilocybe* tryptophan synthase,[23] a bifunctional protein, for which the synthase function is shown. Section 2.1 presents psilocybin and its biosynthesis. Psilocin rephosphorylation by the kinase PsiK is shown, while the minor shunt pathway to norpsilocin is not. Section 2.2 is dedicated to the formation and function of the blue color of injured *Psilocybe* fruiting bodies. Section 2.3 highlights β-carbolines. Biosynthesis enzymes for these compound are not known from *Psilocybe* sp. Section 2.4 covers the TrpM-catalyzed *N*,*N*-dimethyl-L-tryptophan biosynthesis in *P. serbica*.

Very recently, the dephosphorylated analog of aeruginascin, 4-hydroxy-*N*,*N*,*N*-trimethyltryptamine, as well as norpsilocin, were shown *in vitro* to bind to the 5-HT_{2A} receptor as well.[25] Aside from imparting psychedelic activity, psilocybin, which readily dissociates to psilocin *in vivo*, possesses several advantageous pharmacological properties in humans, including oral bioavailability, blood-brain-barrier penetrability, and a relatively long duration of action. Critically, the action of the monooxygenase PsiH on L-tryptophan-derived tryptamine in *Psilocybe* results in metabolites bearing hydroxylation at the C4-position on the indole core (Scheme 1).[9a]

Counterintuitively, this seemingly simple structural modification is directly responsible for the unique pharmacokinetic properties of psilocin in humans. The discovery of this insight would have otherwise required a great deal of serendipity, if not for the *Psilocybe* mushrooms, as the 4-hydroxyindole motif provides little impetus for casual exploration by chemists given that the electron-rich indoles are prone to oxidation and are relatively challenging to synthesize and store. It is the history of consumption of *Psilocybe* mushrooms for their psychoactive effects that provided direct correlation between the structures of psilocin and psilocybin and their pharmacokinetic properties in humans.

The production of psilocybin in fungi is one of many examples where nature has provided unique and potentially therapeutic substances with pharmacology that may never have been discovered. Both the presence and position of the C4 hydroxy group on the *N*,*N*-dimethyltryptamine (DMT, Figure 1) scaffold are essential to the unique psychedelic pharmacology of psilocin. This nuance is best understood by comparing the structure of psilocin to two structurally related naturally occurring tryptamines DMT and bufotenin (Figure 1) and contrasting the known activity of each in humans. DMT is structurally analogous to psilocin except that it lacks the C4-hydroxylation.

While DMT is known to elicit a psychedelic effect in humans, the compound is not orally active in the absence of monoamine oxidase inhibitors (see also section 2.3.) and is rapidly metabolized when administered parenterally.[26] Bufotenin is best known as a toad poison, but has also been isolated from

Anandenanthera trees (legumes), and from the mushroom *Amanita citrina* (false death cap). Chemically, it represents the positional isomer of psilocin with hydroxylation at the C5-position instead of the C4 position. Despite its structural similarity, bufotenin has a contrasting pharmacological effect to that of psilocin, having a duration of action of about 15–30 minutes when administered intravenously. Additionally, its effects are exerted predominantly on the peripheral nervous system,[27] often resulting in variable and unpleasant toxicological responses. Evidence suggested that bufotenin does not readily cross the blood brain barrier, unlike psilocin.[28] In contrast to DMT and bufotenin, a relatively small dose of psilocybin (<20 mg) as a prodrug to psilocin is both orally bioavailable and produces profound psychedelic alteration of consciousness in humans that typically lasts for 4–6 hours without significant toxicological side effects, which highlights the importance of the C4-hydroxylation. These pharmacological differences are not only important for ongoing clinical trials but would have significant implications for scaling when more psychedelic-assisted therapies reach diverse population groups.

2.1.4. Psilocybin's potential ecological role

The ecological impetus for psilocybin production has remained obscure. However, there must be a strong ecological benefit for the producer to justify the energetic burden to synthesize and accumulate a secondary metabolite at typically 0.5–2% of total dry weight.[29] Inspired by the pharmacology in humans as a ligand to 5-HT receptors, the "monomer hypothesis" assumes that monomeric psilocin represents the ecologically relevant compound, which is presented in this section. The following section (2.2.) then introduces an alternative hypothesis that instead describes oligomers as the ecologically functional species.

Methanolic *Psilocybe* crude extracts (2 to 23 $\mu g\,ml^{-1}$) have shown a lethal effect on brine shrimp (Arthropoda), along with ambiguous effects on *Caenorhabditis elegans* (Nematoda).[30] These data, along with alkaloid production that is triggered in the fungus upon formation of the ephemeral fruiting bodies,[31] suggest that nematodes are not psilocybin's primary target. In addition, toxicity of psilocybin in mammals is near negligible in terms of acute physiological damage, with a LD_{50}/ED_{50} ratio >1000 in mice.[32] Regarding any potential antiinsect activity, a preprint article describes that only a single individual of *Sciaridae* sp. (dark winged fungus gnat) completed the entire developmental cycle (i.e., egg to adult) feeding solely on *P. cyanescens*.[33] Furthermore, psilocybin seems to attenuate the web building behavior of the spider *Araneus diadematus*.[34]

Structurally, psilocin is closely related to 5-HT. As 5-HT receptors are also present in invertebrates (e.g., nematodes, arthropods and molluscs) potentially feeding on mushrooms,[35] the monomer hypothesis looks appealing at first glance. However, a comparison of 5-HT receptors across species in terms of ligand specificity and ligand-activity relationships must be treated with caution as an agonistic action of psilocin on human 5-HT receptor subtypes may not be consistent with activity in arthropods. Notably, with larvae of *Drosophila melanogaster*, 5-HT receptor activation would potentially lead to increased feeding without increasing locomotion,[36] which would be an undesirable outcome for the mushroom. A study on parasitic fungi (*Massospora* spp.) that effectively alter the behavior of cicadas has shown that these fungi contain psilocybin or cathinone while infecting their host.[37] However, metagenomic data did not support the presence of known psilocybin biosynthesis genes, which led the authors to speculate that these fungi may have independently evolved the respective biosynthesis. Despite this unresolved aspect, the study may point to an interesting direction as it suggests that psilocybin-producing mushrooms may protect themselves against, and interfere with, small animals, by neurochemically induced behavioral manipulation.

Taken together, little evidence exists yet to support monomeric psilocybin as a defense agent impactful to animals. Of those, spiders, brine shrimp, and humans can be excluded as an evolutionary force to develop and maintain psilocybin biosynthesis since they interact with *Psilocybe* mushrooms occasionally or not at all. Furthermore, mammals evolved at a time where wood-decaying fungi had already resisted invertebrates for millions of years.[9b]

2.2. Psilocyl Oligomers

2.2.1. The blueing reaction of Psilocybe *mushrooms*

Psilocybin-containing mushrooms instantly develop a blue hue when the mycelium is injured (Figure 3), or as they age. For decades, this iconic phenomenon has intrigued chemists and amateur mycologists alike. Oligomerization converts the L-tryptophan-derived indole nucleus into a blue chromophore.[38]

From a chemical perspective, psilocybin may be viewed as the stabilized form of psilocin with a phosphate ester as a protecting group, which is readily removed when mycelium is injured and psilocybin is exposed to intrinsic or animal phosphatases, followed by subsequent oligomerization

Figure 3. The blueing reaction of magic mushrooms. Left: intact mature carpohore of *Psilocybe cubensis*; right, carpophore about one minute after injury with a blade. Reproduced from reference [38a] with permission from Wiley-VCH.

(Scheme 2).[39] The sequence involves psilocybin dephosphorylation to psilocin, catalyzed by the phosphatase PsiP and followed by immediate oxidative coupling by the laccase PsiL. This reaction sequence results in formation of a heterogeneous mixture of psilocyl 3- to 13-mers, coupled preferentially via carbons 5 and 7 of the indole nucleus.[38a]

2.2.2. A possible ecological role for the blue hue

Based on the above reaction, psilocybin may fulfill its true ecological function as a nascent or complete oligo-/polymer. The complex and dynamically developing coupling products share a polyphenolic character and aryl coupling with tannins and melanins. These are substance families of outstanding ecological importance that make tissue resistant to microbial attack and damage, e.g., by light.[40] Like tannins, the blue oligomers precipitate proteins.[38a] In addition, various tannins are assumed to exert defensive purposes as they generate reactive oxygen species.[41]

Reminiscent of conditions favoring psilocin oxidation, tannin-based oxidations are also facilitated by a basic environment, such as the intestines of arthropods feeding on live or decomposing plant matter. For example, the saprophagous larvae of *Pentheria holosericea* (sharing an ecological niche with wood/manure-inhabiting fungi like *Psilocybe*) were shown to maintain extraordinarily high pH values (10–12) and strongly oxidizing conditions within their midguts,[42] which seamlessly integrates into the concept of the „polymer hypothesis". In this scenario, psilocybin would represent the deactivated precursor of a „defense-on-demand" oligomer whose production can be triggered instantly. Hypothetically, the highly reactive psilocyl radicals may directly react with proteins and may have an immediate deleterious effect on feeding insects as well. Future work is warranted to resolve whether psilocybin/psilocin fulfills its role as a monomer, or in an oligo-/polymeric state, or both.

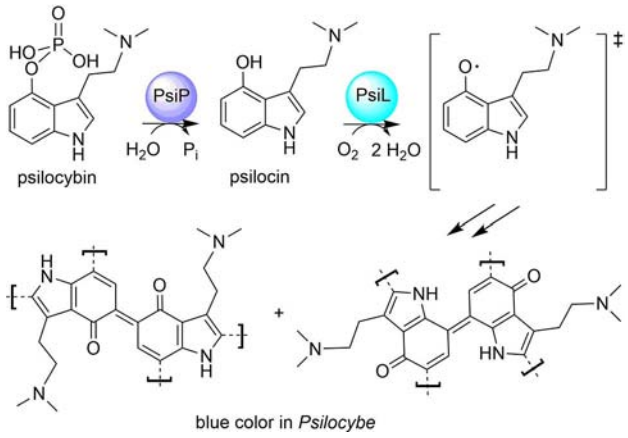
2.3. β-Carbolines

2.3.1. Another L-tryptophan-fed pathway in Psilocybe

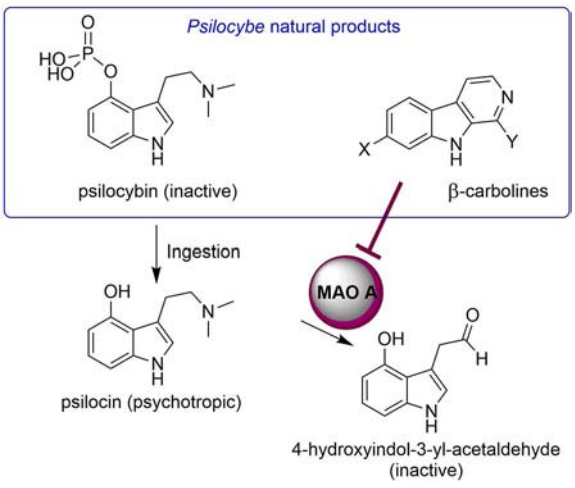
Despite decades of analytical work on the genus *Psilocybe* and although known from other sources,[43] several β-carbolines, including harmane, norharmane, and harmine (Scheme 1), were discovered as natural products of various *Psilocybe* species only very recently.[44] The 1,2,3,4-tetrahydrocarboline core represents the primary cyclization product formed via a Schiff base intermediate, when tryptamine is condensed by Pictet-Spengler-type chemistry with aldehydes or an α-keto acid. Therefore, the β-carbolines contrast psilocybin biosynthesis (as well as that of *N,N*-dimethyl-L-tryptophan, presented in the following section). None of the latter includes the biosynthetic addition of a moiety to expand the core scaffold structure, except that SAM-dependent methylation of the primary amine takes place. Although the enzymes participating in β-carboline biosynthesis in *Psilocybe* species have not been identified yet, stable-isotope labeling with $^{13}C_{11}$-L-tryptophan, leading to a mass increase of *m/z* 10, proved the mushroom origin of the found β-carbolines and ruled out a carry-over from medium or other external sources.

2.3.2. A synergistic pharmacology

β-carbolines, primarily harmane and harmine, have long been established as indirect neuroactive compounds as they strongly and reversibly inhibit monoamine oxidase isoenzyme (MAO)-A.[45] This flavin-dependent enzyme is involved in the breakdown of serotonin and other neuroactive amines by oxidative deamination. Consequently, MAO A inhibition enhances their effect and prevents the bioactivity from being lost. MAO-A's substrate spectrum also includes psilocin, which aside from renal elimination, is eliminated by formation of 4-hydroxyindol-3-yl-acetaldehyde (Scheme 3). β-carboline-mediated MAO-A



Scheme 2. Enzymatic cascade initiating the blueing reaction in *Psilocybe cubensis*, catalyzed by the phosphatase PsiP and by the laccase PsiL. The bottom structures symbolize the mixture of heterogeneous oligomers that cause the blue color.



Scheme 3. Hypothetical synergistic effects of mushroom-produced psilocybin and β-carbolines through inhibition of monoaminoxidase A and hence, of psilocin degradation.

inhibition consequently would intensify the effects of psilocin. We therefore encounter an intriguing, and in the fungal arena, unique scenario: one precursor, L-tryptophan, feeds into two different biosynthetic routes which result in metabolites that act on dissimilar targets (receptor versus catabolic enzyme), yet act synergistically by contributing to the same pharmacology. Mechanistically, this scenario is somewhat reminiscent of the natural products of the soil bacterium *Streptomyces clavuligerus*. It produces cephamycin, a β-lactam antibiotic, and simultaneously, clavulanic acid, i.e., an inhibitor of β-lactamase that could otherwise inactivate cephamycin.[46] However, these biosyntheses do not involve L-tryptophan.

The detected β-carboline concentrations in the fruiting bodies are very low (around 0.2 $\mu g\,g^{-1}$, and tenfold higher in sclerotia). Therefore, from an anthropocentric perspective, it is currently unknown if the co-occurrence of β-carbolines and psilocin (released from its prodrug psilocybin) in *Psilocybe* mushrooms is of pharmacological relevance. Furthermore, numerous clinical studies with synthetic psilocybin have demonstrated that the pharmacological effects can be attributed to the pure substance.

2.4. *N*,*N*-Dimethyl-L-tryptophan

2.4.1. Perfectly tuned substrate specificities prevent crosstalk between pathways

P. serbica has long been known as a prolific psilocybin producer. Surprisingly, chromatographic analysis identified another set of L-tryptophan-derived natural products in this species, L-abrine and *N*,*N*-dimethyl-L-tryptophan (Scheme 1).[47] This somewhat surprising finding raised the question: Which enzyme would catalyze this L-tryptophan modification and how does it relate to psilocybin biosynthesis?

The search for the relevant methyltransferase gene in *P. serbica* was guided by the striking similarity to the reaction catalyzed by EgtD, a thoroughly studied methyltransferase of mycobacteria[48] which occurs as a two-domain bifunctional enzyme EgtDB in fungi. EgtD catalyzes trimethylation of the amino group of L-histidine as a gateway step of the ergothioneine biosynthesis pathway. Two *egtD*-like genes were identified in the *P. serbica* genome. One gene was fused to an *egtB* homolog, to encode the bifunctional sulfoxide synthase/methyltransferase typical for ergothioneine assembly in fungi. However, the second gene, *trpM*, encoded a discrete EgtD-type methyltransferase that is only infrequently found in basidiomycetes. Heterologously produced TrpM catalyzed the formation of L-abrine and *N*,*N*-dimethyl-L-tryptophan. We need to keep in mind that the specificity profiles of the psilocybin enzymes prevent psilocin formation by avoiding dimethyltryptamine, which could be hydroxylated to psilocin (section 2.1.1.). TrpM's substrate specificity supports this biosynthetic feature: it is strictly specific for L-tryptophan and did not accept tryptamine or 4-hydroxytryptamine, the very intermediates of psilocybin biosynthesis that could be potentially converted to psilocin.[47] Hence, TrpM works completely separately from the psilocybin pathway, and the two routes mutually avoid interfering with each other (Scheme 1).

2.4.2. TrpM implies a mechanism for natural product gene evolution

An unexpected, yet intriguing aspect around this pathway was revealed upon reconstructing TrpM's evolutionary history. As a member of methyltransferase class 33, it is phylogenetically unrelated to PsiM, which falls into class 10, even though the catalyzed reactions are highly similar. Instead, TrpM shows a close relationship to the above-mentioned EgtD. The *trpM* gene originates from a very ancient partial duplication of the *egtDB* gene.[47] In the course of basidiomycete evolution, most fungi lost *trpM*, while *P. serbica* retained the gene. Even more surprisingly, *trpM* independently re-evolved multiple times from *egtDB* through gene duplication in various basidiomycetes. This finding implies that weakly selected genes are preserved in the genetic material of more strongly conserved pathways. Therefore, the *Psilocybe* tryptophan metabolism helped discover a mechanism of how natural product pathway genes are evolved.

3. Summary and Perspective

In this minireview, we have summarized what is currently known about L-tryptophan-derived compounds in *Psilocybe* mushrooms and have highlighted recent developments.

From the medicinal and applied perspective, the large-scale production of pharmaceutical-grade psilocybin under Good Manufacturing Practice (GMP) guidelines has become a high priority because of psilocybin's status as the prototype molecule for modern clinical research into psychedelic medicine. Current efforts are directed toward psilocybin's approval as a therapeutic in the not so distant future. Efforts for sustainable psilocybin production are supported by the elucidated biosynthetic pathways and characterized enzymes that allow for chemoenzymatic strategies. From the perspective of pure research, the chemical mechanism of the blueing reaction is now understood, and β-carbolines were added to the natural product repertoire of *Psilocybe*.

Still, the fundamental question remains to be answered: why do *Psilocybe* mushrooms make psilocybin? Is the ecologically relevant compound mono- or oligomeric? And why do these fungi make β-carbolines? Do these two classes of compounds exert their ecological functions independently or synergistically? And do the β-carbolines contribute to the psychedelic pharmacology in humans? These questions show that *Psilocybe* compounds exemplify natural product research as an interfacing endeavour and a hub that connects multiple disciplines.

Acknowledgements

Psilocybin-related work in D.H.s laboratory was funded by the Deutsche Forschungsgemeinschaft (DFG, grant HO2515/7-1) and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, SFB 1127/2 ChemBioSys 239748522). The authors also acknowledge the Usona Institute, Madison, WI for support. Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: biosynthesis · fungi · natural products · psilocybin · tryptophan

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Manuscript received: August 14, 2020
Revised manuscript received: September 30, 2020
Accepted manuscript online: October 1, 2020