Immobilized Enzyme Approach to Alkylating Tryptamines for the Facile Production of Psilocybin Analogues

Patent Disclosure

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1. Title of Invention

Immobilized Enzyme Approach to Alkylating Tryptamines for the Facile Production of Psilocybin Analogues (tentative title)

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3. Overview of the Invention

The following invention outlines a means by which psilocybin analogues can be enzymatically synthesized using an immobilized enzyme approach.

Problem

Psilocybin, the psychoactive compound found in various members of the *Psilocybe* genus colloquially known as 'magic mushrooms', has regained a resurgence in the medical research community in the last decade as a potential treatment for major depressive disorder (MDD)[1] and treatment-resistant depression (TRD)[2]. Research into the compound's potential therapeutic benefits was greatly stifled by legislative restrictions that set psilocybin and other psychedelic compounds as Schedule I substances during the 1960s.[3] However, pioneering work and outcomes from the Usona Institute and institutional partners have led to the United States Food and Drug Administration granting two "Breakthrough Therapy" designations to psilocybin.[4] Clinical trials for the use of psilocybin for substance use disorders and existential distress from cancer are underway as well.[5]

With these new developments, the demand for psilocybin, that conforms to Current Good Manufacturing Practices (cGMP) for pharmaceutical purposes, is on the rise. The majority of pure psilocybin used in clinical trials have been sourced from methodologies relying on multi-gram[6] to kilogram-scale organic syntheses[7]. Despite these impressive improvements over older

syntheses[8] from the research literature, authors report challenges in sustaining increased scale in the protocols, namely a.) the need for strong kinetic control over reaction conditions to limit side reactions, b.) difficulties in workup conditions due to instability of intermediates, c.) the use of toxic reagents and their associated byproducts, and d.) limited yield.

Synthetic biology has opened the opportunity to produce psilocybin through biosynthetic, enzymatic, or chemoenzymatic means.[8] A notable advancement in psilocybin production was the work of Schäfer et al. in 2025.[9] The researchers demonstrated the production of psilocybin using a set of immobilized enzymes both found endogenously in various species of *Psilocybe* as well as exogenously from other microorganisms, elegantly coupling the biosynthetic pathway to form the desired psychoactive without the previously outlined issues associated with chemical synthesis or the challenges of in vivo biosynthesis in workhorse microbial species (i.e. *Escherichia coli*, *Aspergillus niger, Saccharomyces cerevisiae*), such as extracting the psilocybin from pyrogen or endotoxin-containing media.

While much work has been done on expanding the scale of psilocybin production, other groups have focused their attention on expanding the structural-chemical analogues of psilocybin available for basic and clinical research. One goal has been to better study the structure-activity relationship between these psilocybin analogues and the various 5-hydroxytryptamine (5-HT) receptors which they act upon. Another goal has been to screen libraries of analogues in the search for more potent or psychiatrically appropriate variants suitable for specific indications. A notable example of synthetic biology in the production of psilocybin analogues has been the generation of halogenated or methoxylated variations at the indole core in positions 4 to 7 within a gene-edited biosynthetic system using *Escherichia coli*.[10] The project highlighted the promiscuity of the enzymes in the psilocybin biosynthetic cascade to incorporate tryptophan entities with bulkier groups.

That said, some notable designs and syntheses of psilocybin analogues, in which the dimethylamine moiety has been modified, remain in the purview of chemical synthesis with its accompanying technical challenges.[11,12] Arguably, it is the dimethylamine structural analogues that give rise to differential binding to the 5-HT receptors and modifies the perceptual activity of the substance in patients.[11]

To fill in this missing technological gap, the following invention provides an immobilized enzyme pathway for constructing structural analogues of psilocybin at the dimethylamine moiety, to construct a large chemical library for the purpose of screening new drug candidates in the psilocybin family with more desirable treatment properties.

Description

Psilocybin is produced by various species of the *Psilocybe* genus, with special focus on high-yielding species *Psilocybe cubensis* and *Psilocybe cyanescens*, through a multi-step cascade involving a four-enzyme pathway.[13] Namely, L-tryptophan first undergoes a decarboxylation reaction via putative decarboxylase PsiD to form tryptamine. This intermediate undergoes hydroxylation via putative hydrolase PsiH to generate 4-hydroxytryptamine, followed by phosphorylation by putative kinase PsiK to norbaeocystin. Two methylation steps achieved by methyltransferase PsiM yields psilocybin.

Methyltransferase PsiM relies on the presence of endogenous co-factor S-adenosyl-L-methionine (SAM) to provide the needed methyl groups to undergo the final methylation step. Modification of SAM, either biochemically or synthetically, can yield analogues that transfer alkyl groups beyond the methyl group. For example, researchers have found that the rare amino acid, ethionine, which contains an ethyl group in lieu of the methyl group found in methionine, can be coupled to adenosine to form S-adenosyl-L-ethionine (SAE). This SAE is capable of biological ethylation reactions analogous to the methylation properties of SAM.[14]

Similarly, synthetic chemists have discovered several approaches to chemically coupling adenosine to a variety of methionine analogues to form SAM analogues to achieve alkylations in biological settings.[15] However, no known native biological analogue has been found beyond ethionine, while synthetic analogues of SAM possess limitations in that they have a limited half-life of several hours to several days and are not easily taken up by biosynthetic workhorses such as *Saccharomyces cerevisiae* and *Escherichia coli* due to their poor stability and high polarity.[16] As such, their potential as alkyl donors in relevant living systems is limited in scope.

In 2023, Mohr et al. discovered the enzyme class of *O*-acetyl-L-homoserine sulfhydrolases (OAHS) derived from *Saccharomyces cerevisiae* is capable of catalyzing the coupling of L-homocysteine, the precursor to L-methionine, with a thiol-bearing alkyl group to form

analogues of L-methionine. The promiscuity of the enzyme to accept various alkyl mercaptans ranged from ethyl mercaptan to allyl and benzyl mercaptan with yields between 60 to 75 percent. Following the enzymatic displacement reaction, the L-methionine analogues must undergo an additional conversion in the presence of a L-methionine adenosyltranferase (MAT) to form the corresponding SAM analogues. In nature, SAM is synthesized by MATs from L-methionine and ATP. Mutations of the active site of MATs have been shown to support the accommodation of larger alkyl chains of L-methionine analogues. These SAM analogues were used to successfully conduct an alkyl transfer onto hydroxyl-bearing substrates such as anthranilate and vanillin using the corresponding downstream *O*-methyltransferase.

The main embodiment of the invention involves using the known co-immobilized enzyme cascade system outlined by Schäfer et al.[9], in which aromatic aldehyde synthase IasA from *Psilocybin mexicana*, gene edited at Phe329 into tyrosine, is co-immobilized with kinase PsiK and methyltransferase PsiM. Additionally, *S*-adenosyl-L-homocysteine nucleosidase (MtnN) and adenine deaminase (Ade) are also immobilized onto the column to remove *S*-adenosyl-L-homocysteine, a byproduct of SAM methylation that inhibits PsiM activity.

Methionine-coupling enzyme, *O*-acetyl-L-homoserine sulfhydrolase (OAHS), and L-methionine adenosyltransferase (MAT) would additionally be immobilized onto this column to generate the needed L-methionine analogue and *S*-adenosyl-L-methionine analogue, respectively. A solution of precursors and cofactors of the desired alkyl thiol analogue, 4-hydroxy-L-tryptophan, L-methionine, adenosine triphosphate (ATP), and pyroxidal phosphate (PLP) would flow through the immobilized column and yield the desired psilocybin analogue. The immobilized enzyme system provides a clean, simple means in which to separate the desired product from the milieu, using magnetic beads as the core of the immobilization substrate that can be removed using a magnet and later washed for future reactions. Point mutation or directed evolution of the enzymes may be required to accommodate the increased steric bulk of the new analogues side chains.

Other embodiments of the invention could include the separate enzymatic production and isolation of the L-methionine analogue via L-methionine, the alkyl thiol analogue, and the OAHS to avoid co-reaction of L-methionine in the enzyme cascade to form psilocybin as a side product. The L-methionine analogue would then be used as a precursor for the above immobilized enzyme column reaction, sans OAHS as one of the immobilized enzymes.

Additionally, the SAM analogue could be constructed in a separate immobilized enzyme cascade containing the two enzymes OAHS and MAT and isolated, purified, and then reacted in the above immobilized enzyme embodiment, sans OAHS and MAT. The desired SAM analogue could also be chemically synthesized according to known literature as the precursor for the enzymatic cascade.

Furthermore, a set of gene cassettes or a polycistronic gene embedded in a single mRNA strand could be inserted into a workhorse microorganism (*Escherichia coli, Saccharomyces cerevisiae*, *Aspergillus nidulans*, etc.) to express the above enzyme systems with the accompanied production of the psilocybin analogue. In this embodiment, the *Psilocybe* cytochrome P450 oxidase *psiH* gene for tryptamine 4-hydroxylation would need to be included in the gene cassettes or polycistronic gene system to endogenously form 4-hydroxy-L-trytophan.

This enzyme is excluded from the above immobilized enzyme cascades due to the inherent insolubility and poor activity of PsiH. Additionally, the desired alkyl thiol analogue would need to be added to the culture media such that the workhorse species uptakes the thiol and incorporates it into the formation of the L-methionine analogues and subsequent psilocybin biosynthesis. This system would not be ideal as multiple competing biosynthetic pathways would likely interfere with the incorporating of the alkyl thiol, or some alkyl thiols are likely to inhibit the growth of the gene-edited microbial species.

An additional embodiment involves incorporating tryptophan synthetase (TrpB) into the immobilized enzyme cascade, and use L-serine and substituted indoles in lieu of 4-hydroxy-tryptophan to expand the chemical library further, as suggested by Shäfer et al.[9] Following the biosynthesis[10] of psilocybin derivatives substituted at the more facile 6 or 7 positions with the halogen series (fluorine, chlorine, bromine, iodine) or the methoxy substituent with point mutation or directed evolution of TrpB to accommodate the additional bulk of these substituents, this gives us 5 to 10 times as many potential analogues for each dimethylamine dialkyl variant driven by the alkyltransferase OAHS/MAT system. Again, these enzymes can either be expressed, purified, and immobilized into a single biosynthetic system or inserted into a workhorse microorganism for bioreactor scale up and production.

Conclusion

The above invention expands the potential for the rapid synthesis of psilocybin analogues that may be too challenging, costly, or time-consuming to synthesize via conventional chemical means, opening up the door for molecular entities with new activities and some with greater potential to treat psychiatric disorders. The immobilized enzyme system further provides an avenue for rapid scale-up of the specific psilocybin analogues that prove viable post-screening, for future animal studies and clinical trials, in a cGMP-compliant manner.

4. Prior Art

1959 - Psilocybin and psilocin and processes for their preparation (GB911946A)

2017 - Engineered synthase for production of tryptophan derivatives and intransigent substrates (US20180057806A1)

2018 - Large scale method for the preparation of Psilocybin and formulations of Psilocybin so produced (GB2571696B)

2018 – Preparation of psilocybin, different polymorphic forms, intermediates, formulations and their uses (JP2024069473A)

2019 – Heterologous production of psilocybin (US11952580B2)

2020 – Genetic engineering of fungi to modulate tryptamine expression (IL291839A)

2020 - Processes for the production of tryptamines (US20210108238A1)

2020 – Scalable synthetic routes to psilocybin and psilocin (JP7719164B2)

2020 – Biosynthetic production of psilocybin and related intermediates in recombinant organisms (US11441164B2)

2020 - Deuterated derivatives of psilocybin and uses thereof (US11000534B1)

2021 - Aza-substituted psilocin analogs and methods of synthesizing the same (US11939292B1)

2021 - Tryptamine derivatives and their therapeutic uses (US20230406824A1)

2021 – Novel psilocin derivatives having prodrug properties (US20230295086A1)

2021 – Deuterated n,n-dimethyltryptamine compounds (US20210403426A1)

2021 - Modified indole alkaloids for therapeutic uses (WO2022133314A1)

2021 - Glycosylated psilocybin derivatives and methods of using (US11891360B2)

2021 - Halogenated psilocybin derivatives and methods of using (US11998557B2)

2021 - Hydroxylated psilocybin derivatives and methods of using (US20250100967A1)

2021 - Nitrilated psilocybin derivatives and methods of using (US11931338B2)

2021 – Aldehyde and ketone psilocybin derivatives and methods of using (US11845727B2)

2021 – Carboxylated psilocybin derivatives and methods of using (US11752130B2)

2021 – Nitrated psilocybin derivatives and methods of using (US20230040398A1)

2022 – Multi-substituent psilocybin derivatives and methods of using (US12128058B2)

2022 – Prenylated psilocybin derivatives and methods of using (US11891359B2)

2022 - Processes of preparing psilocin and psilocybin (CA3231520A1)

2022 – Methods for the production of tryptophans, tryptamines, intermediates, side products and derivatives (US20240417764A1)

2023 – Improved Synthesis of Psilocybin Derivatives (CN119948042A)

2023 – Preparation of stable psilocin salts, esters and conjugates and uses thereof (KR20250035567A)

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