

VIP Very Important Paper

A „Magic Mushroom“ Multi-Product Sesquiterpene Synthase

Eike Schäfer^{+, [a]}, Paula S. Seibold^{+, [a, b]}, Stefan Bartram,^[c] Felix Trottmann,^[d] Veit G. Haensch,^[d] Markus Gressler,^[a] Andrew R. Chadeayne,^[e] Christian Hertweck,^[b, d, f] Sarah E. O'Connor,^[c] and Dirk Hoffmeister^{*[a, b]}

Psilocybe “magic mushrooms” are chemically well understood for their psychotropic tryptamines. However, the diversity of their other specialized metabolites, in particular terpenoids, has largely remained an open question. Yet, knowledge on the natural product background is critical to understand if other compounds modulate the psychotropic pharmacological effects. CubA, the single clade II sesquiterpene synthase of *P. cubensis*, was heterologously produced in *Escherichia coli* and characterized *in vitro*, complemented by *in vivo* product formation assays in *Aspergillus niger* as a heterologous host. Extensive

GC-MS analyses proved a function as multi-product synthase and, depending on the reaction conditions, cubebol, β -copaene, δ -cadinene, and germacrene D were detected as the major products of CubA. In addition, mature *P. cubensis* carpophores were analysed chromatographically which led to the detection of β -copaene and δ -cadinene. Enzymes closely related to CubA are encoded in the genomes of various *Psilocybe* species. Therefore, our results provide insight into the metabolic capacity of the entire genus.

Introduction

Members of the fungal genus *Psilocybe* and other genera are colloquially referred to as magic mushrooms as they produce

the psychedelic, i.e., mind-altering psilocybin (Figure 1).^[1] More specifically, this distinctive 4-O-phosphorylated tryptamine derivative is the immediate precursor of the actual bioactive compound, psilocin, which mainly targets the human 5-HT_{2A}-receptor as a partial agonist and elicits profound changes of perception.^[2] Given these significant pharmacological effects, natural product chemists investigating *Psilocybe* metabolites have traditionally focused on tryptamine-derived compounds.^[1,3]

Whether pure psilocybin/psilocin alone explains all observed somatic and perceptual effects after mushroom uptake remains the subject of considerable debate. Some studies have demonstrated that extracts of *Psilocybe* mushrooms have different *in vivo* properties compared to pure psilocybin, presumably due to the presence of other compounds which contribute to a so-called “entourage effect”.^[4] This term was coined during research with *Cannabis* natural products and the endocannabinoid receptors. It describes the observation that the pure ligand elicited a different response than a complex (and potentially synergistic) mixture of compounds co-occurring in the plant tissue and extracts thereof.^[5] The possibility and importance of an entourage effect in *Psilocybe* mushrooms remains controversial. Although not systematically investigated yet, this phenomenon prompted more comprehensive chromatographic work into the natural product metabolome of *Psilocybe*. Very recently, other nitrogen-containing natural products, including various β -carbolines, lumichrome, and diketopiperazines were identified.^[6]

However, *Psilocybe* terpenoid metabolism is surprisingly poorly understood, even though other basidiomycete members of this class of natural products demonstrated remarkable structural diversity and biological effects.^[7,8] Besides their inherent bioactivities, terpenoids as usually non-nitrogen containing natural products can bind to and interfere with

[a] E. Schäfer,⁺ P. S. Seibold,⁺ Dr. M. Gressler, Prof. Dr. D. Hoffmeister
Department of Pharmaceutical Microbiology at the Hans Knöll Institute
Friedrich-Schiller-Universität Jena
Winzerlaer Str. 2, 07745 Jena (Germany)
E-mail: dirk.hoffmeister@leibniz-hki.de

[b] P. S. Seibold,⁺ Prof. Dr. C. Hertweck, Prof. Dr. D. Hoffmeister
Cluster of Excellence Balance of the Microverse
Friedrich-Schiller-Universität Jena
Neugasse 23, 07743 Jena (Germany)

[c] Dr. S. Bartram, Prof. Dr. S. E. O'Connor
Max Planck Institute for Chemical Ecology
Department Natural Product Biosynthesis
Hans-Knöll-Strasse 8, 07745 Jena (Germany)

[d] Dr. F. Trottmann, Dr. V. G. Haensch, Prof. Dr. C. Hertweck
Department Biomolecular Chemistry
Leibniz Institute for Natural Product Research and Infection Biology
Hans Knöll Institute
Beutenbergstrasse 11a, 07745 Jena (Germany)

[e] A. R. Chadeayne
CaaMTech, LLC
58 East Sunset Way, Issaquah, WA 98027 (USA)

[f] Prof. Dr. C. Hertweck
Institute of Microbiology
Friedrich-Schiller-Universität Jena
Neugasse 23, 07743 Jena (Germany)

[*] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbic.202300511>

© 2023 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

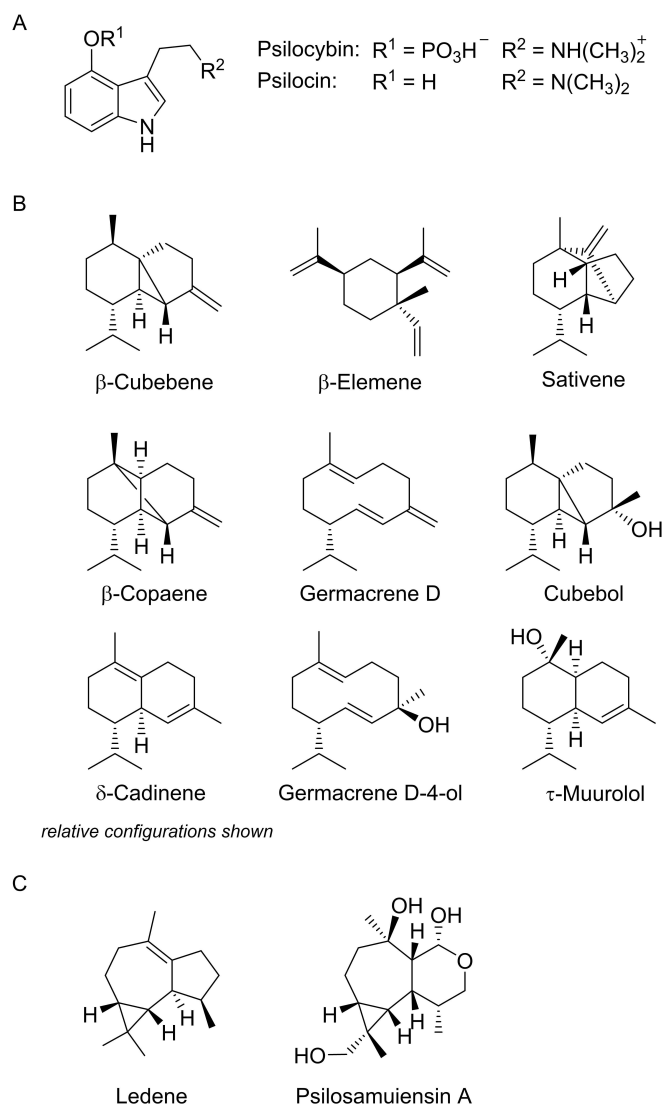


Figure 1. Structures of *Psilocybe cubensis* natural products. A) psilocybin and its psychoactive follow-up product psilocin; B) sesquiterpene/terpenoid products of CubA identified *in vivo* or *in vitro* during this study, structures represent relative stereochemistry; C) psilosamuinsin A and its likely precursor ledene.

receptors. This was shown, for example, for GABA and nicotinic acetylcholine receptors.^[9] Thus, terpenoids are candidates that may contribute to a possible entourage effect of magic mushrooms.

From the non-psychedelic fungal genus *Deconica* that is related to *Psilocybe*, terpenoids have been described.^[10] Yet, the only literature available regarding *Psilocybe* terpenoids is a single report on a *seco*-aromadendrane-type, ledene-derived sesquiterpenoid, psilosamuinsin A of *P. samuensis* (Figure 1).^[11] Its precursor ledene is likely biosynthesized by a so-called clade II synthase.

Generally, the mode by which the universal sesquiterpene building block (2*E*,6*E*)-farnesylidiphosphate (2*E*,6*E*-FPP) is cyclized, and in some cases isomerized to (3*R*)-nerolidylidiphosphate (NPP), correlates with phylogenetically distinct categories that sesquiterpene synthases fall into. These categories include

clades I–IV (Figure S1),^[12] and a fifth clade has been proposed.^[13] Clade II synthases isomerize FPP to NPP, which is subsequently cyclized via C-1 and C-10 to the *Z,E*-germacradienyl cation and then to various scaffolds.

Given that the assembly of psilosamuinsin A suggests an active clade II synthase, we considered this particular clade a promising choice to establish what terpenoids are biosynthesized by the genus *Psilocybe*. Here, we report on the functional characterization and the product range of the enzyme CubA *in vitro* and *in vivo* and assign it as a *P. cubensis* clade II multi-product synthase. We also report the first detection of β -copaene and δ -cadinene in *P. cubensis* fruiting bodies.

Results and Discussion

Genetic characterization of *cubA*

Genomic sequences of various *Psilocybe* species^[6,14] revealed an unexpected wealth of genes putatively encoding sesquiterpene synthases, ranging from 17 genes in *P. mexicana* to 24 in *P. serbica*.^[6] Intriguingly, both *P. cubensis*, *P. cyanescens* and *P. serbica* encode only a single gene for a clade II synthase, in *P. cubensis* now referred to as *cubA* (Figure S2). In a published transcriptome of this species,^[15] *cubA* was four-fold upregulated in fruiting bodies vs. vegetative mycelium, which pointed to a function of this gene in the mushrooms. CubA is encoded by a 1265 bp gene and interrupted by four introns. Notably, the *cubA* gene is not embedded in a cluster of other typical biosynthetic genes. Full splicing results in a predicted 1032 bp *cubA* reading frame. The canonical sesquiterpene synthase motifs that coordinate an active site trinuclear Mg^{2+} cluster^[16] are present in CubA as well (⁸⁵DExxD⁸⁹ and ²²⁰NDXXSXXXE²²⁸, Figure S2). The enzymes most similar to CubA include *Agrocybe aegerita* Agr4^[12e] and *Coprinopsis cinerea* Cop4^[17] (71 % and 66 % identical amino acid residues, respectively), which are both multi-product clade II sesquiterpene synthases. Like CubA, these enzymes fall into subclade IIb (Figure S1), which catalyze the biosynthesis of germacrene D (Figure 1) and other sesquiterpenes derived from the *Z,E*-germacradienyl cation,^[12a,17] whereas members of subclade IIa lead to viridiflorene, ledene, and other follow-up products of the bicyclogermacrene intermediate.^[12c] We determined the expression levels of *cubA* by qRT-PCR. Using intron 3 and flanking up- and downstream sequences, accurately spliced mRNA leads to a 113 bp amplicon. The glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) served as control. We found a 3.7-fold increased *cubA* expression in carpophores, which is consistent with previous transcriptomic data.^[15]

Characterization of CubA *in vitro*

The *cubA* cDNA was cloned to yield expression plasmid pES02. The sesquiterpene synthase CubA was produced in *E. coli* KRX \times pES02 as a 41.5 kDa 363 aa *N*-terminally tagged hexahistidine fusion protein (Figure S3) and purified by metal affinity

chromatography. Ethyl acetate extracts of *in vitro* assays with FPP as substrate were analyzed by gas chromatography and electron impact mass spectrometry (GC-EIMS). This analysis revealed product peaks and mass fragmentation patterns that were compatible with typical cyclic sesquiterpenoids (m/z 161, 207, and 222 $[M]^+$ for hydroxylated; m/z 105, 161, 204 for non-hydroxylated compounds).^[18] Optimum product formation was detected at 29 °C (Figure S4). The most prominent products were preliminarily identified as sativene, β -copaene, germacrene D, cubebol, δ -cadinene, and germacrene D-4-ol (Figure 1) by GC analysis, which is consistent with products known from other clade II synthases, including *Agrocybe aegerita* Agr4,^[12e] *Coprinopsis cinerea* Cop4,^[12f,17] *Phanerochaete chrysosporium* PcSTS-04,^[13c] *Coniophora puteana* Copu3,^[18] and *Antrodia cinnamomea* ACTPS9.^[19] Interestingly, the product spectrum was a function of pH, which confirms a finding for *C. cinerea* Cop4 which became a selective germacrene D synthetase at low and unphysiologically high pH values.^[17] Optimum turnover to the germacrene D follow-up products cubebol, sativene, and β -copaene was observed in MOPS buffer at pH 7.0 (Figure S5).

After optimum reaction conditions for CubA activity had been established, the *in vitro* product formation assays were repeated and the ethyl acetate extracts subjected to in-depth GC-EIMS measurements. Comparative chromatographic analysis with *Piper cubeba* berry and *Angelica archangelica* root essential oil, and authentic samples as references,^[20] confirmed cubebol as main product (44.6% of the total peak area, t_R = 29.03 min) and identified germacrene D (t_R = 27.67 min), β -copaene (t_R = 25.57 min), germacrene D-4-ol (t_R = 31.35 min), sativene (t_R = 24.23 min), and δ -cadinene (t_R = 29.33 min), with 16.7, 15.0, 13.2, 5.2, and 3.5% of the total peak area, respectively, as minor compounds, along with minute amounts of further sesquiterpenoids (Figure 2, Table S1, a comprehensive biosynthetic scheme is provided in Scheme S1).

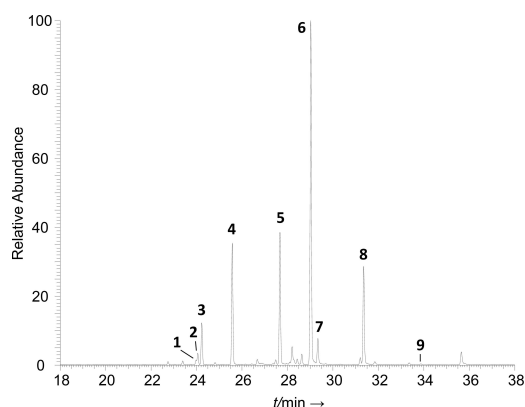


Figure 2. Gas chromatographic analysis of CubA-catalyzed sesquiterpenoid formation *in vitro*. Compound numbers are assigned as follows: β -cubebene (1), β -elemene (2), sativene (3), β -copaene (4), germacrene D (5), cubebol (6), δ -cadinene (7), germacrene D-4-ol (8), and τ -muurolol (9). Corresponding structures are shown in Figure 1.

Characterization of CubA *in vivo*

Neither cubebol nor the other sesquiterpene products found after the *in vitro* reaction have previously been described from *P. cubensis* or any other species of this genus. To confirm the activity and product spectrum of CubA under physiological conditions, the enzyme was heterologously produced in *Aspergillus niger*. The transformant tES02 carried *cubA* gDNA as transgene, controlled by the ATNT doxycycline-dependent inducible expression system.^[21] Ethyl acetate extracts of mycelium, harvested from induced and non-induced cultures, and from an untransformed control strain were analyzed by GC-MS. Contrasting the *in vitro* findings, the amount of cubebol, which remains the major product, was decreased (t_R = 29.07 min, 32.7% of the cumulated peak areas) as well as the production of germacrene D and germacrene D-4-ol (t_R = 27.69 min; 7.5% and t_R = 31.37 min; 3.8%, respectively). The production of β -copaene (t_R = 25.61 min), δ -cadinene (t_R = 29.36 min), τ -muurolol (t_R = 33.93 min), β -cubebene (t_R = 24.00 min), and β -elemene (t_R = 24.07 min) was significantly increased for which 23.9, 13.3, 6.1, 3.8 and 2.2%, respectively, of the cumulated peak areas were determined (Figure 3, Table S2). Due to a not entirely silent promoter controlling transgene expression, terpenes were produced in minor quantities in uninduced cultures as well, yet they were absent from the empty vector control.

The cumulative *in vivo* and *in vitro* product profile of CubA is qualitatively reminiscent of the compounds detected with Cop4, which produces mainly cubebol but also germacrene D under standard conditions *in vitro*.^[17] However, CubA differs from Cop4, Agr4, and *Stereum hirsutum* terpene synthases, produced in *E. coli* and *Aspergillus oryzae*, respectively,^[12c,e,17] as δ -cadinene was identified as their major *in vivo* product. Still, all of the above enzymes are established multi-product synthases. β -Elemene, although formed from 2E,6E-FPP via germacrene A,^[22] was also found as a minor product of the *Coniophora*

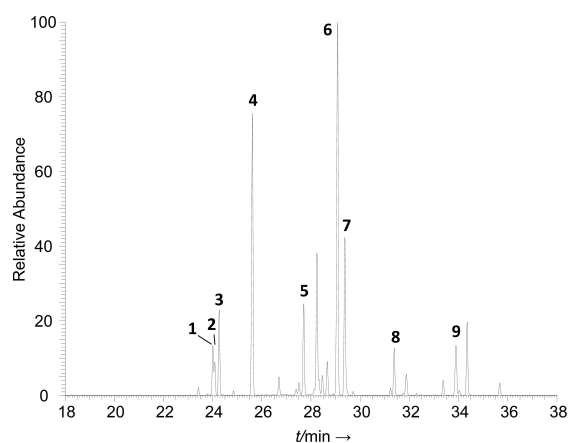


Figure 3. Gas chromatographic analysis of CubA-catalyzed sesquiterpene formation in *Aspergillus niger* as heterologous host. Shown are ethyl acetate extracts of a doxycycline-induced culture of *A. niger* tES02. Chromatograms of controls are shown in Figure S6. Compound numbers: β -cubebene (1), β -elemene (2), sativene (3), β -copaene (4), germacrene D (5), cubebol (6), δ -cadinene (7), germacrene D-4-ol (8), and τ -muurolol (9).

puteana synthase Copu3^[18] and the *Omphalotus olearius* clade II terpene synthases Omp5a and Omp5b.^[23]

Natural product analysis of *P. cubensis* mycelium and fruiting bodies

Our experiments identified *P. cubensis* as a potential producer of various mono-, bi-, and tricyclic sesquiterpenes/-terpenoids which expands the realm of the *Psilocybe* natural products repertoire to nitrogen-free compounds previously unknown from this genus. The results also prompted analyses if the compounds, produced by CubA *in vitro* and heterologously by *A. niger*, are in fact present in vegetative *P. cubensis* mycelium or fruiting bodies. Ethyl acetate extracts of mycelium, grown in submerge culture for 10 d, and of mature fruiting bodies were analyzed by GC-EIMS. While the mycelial extracts did not contain any of the previously identified sesquiterpenes in quantities that would be detectable by gas chromatography, traces of both β -copaene and δ -cadinene were found in fruiting bodies (Figure 4).

Secondary metabolite production as a function of the developmental stage of a mushroom-forming fungus has previously been found within and outside the genus *Psilocybe*.^[12d] For *P. cubensis*, psilocybin production sets in by massively upregulated transcription of biosynthetic genes when fruiting body formation is initiated and the fungus enters its reproductive phase.^[24] Conversely, the melleolides, i.e., bioactive sesquiterpene aryl esters of the honey mushroom *Armillaria mellea*, accumulate in vegetative mycelium whereas fruiting bodies contain only traces.^[25]

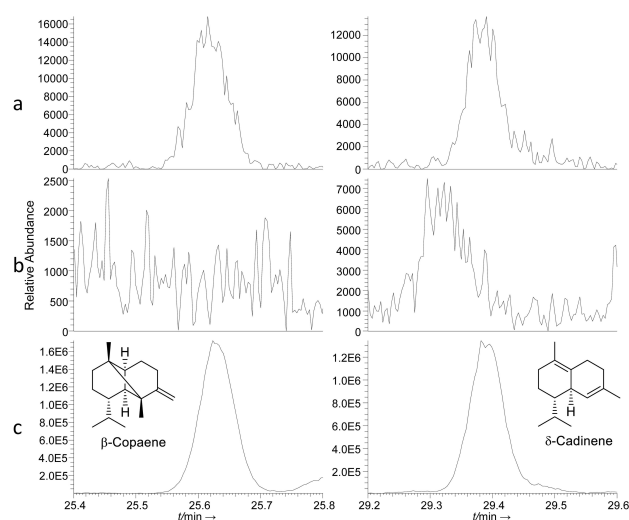


Figure 4. Analysis of sesquiterpenes (extracted ion traces of base peak; m/z 161 [M]⁺) in *P. cubensis* carpophores (a), submerge-grown mycelium (b), and reference *A. archangelica* root oil (c).

Conclusions

Previous knowledge of *Psilocybe* terpenoids was virtually non-existent. This first study into the enzymatic basis of terpenoid metabolism in the mushroom genus *Psilocybe* identified *P. cubensis* CubA as a multifunctional sesquiterpene synthase. As highly similar enzymes are encoded by other *Psilocybe* species as well, our results may be transferable to these. Furthermore, our results contribute to more profound insight into their capacity to make terpenes and help investigate the elusive phenomenon of the “entourage effect” on a rational basis.

Experimental Section

Molecular biology procedures and fungal strains. DNA restriction and ligation, and plasmid isolation were carried out following the instructions provided by the manufacturers of kits and enzymes (NEB, Promega, Thermo Fisher, Macherey–Nagel). Chemicals, media ingredients, and solvents were purchased from Oshida, Primavera, Carl Roth, Sigma-Aldrich, and VWR. Oligonucleotides were synthesized by IDT Europe. *Psilocybe cubensis* FSU12409 was maintained on MEP (15 g L^{−1} malt extract, 3 g L^{−1} peptone, 18 g L^{−1} agar) plates. For heterologous gene expression, *Aspergillus niger* ATNT16Δ-pyrGx24 was used, grown on *Aspergillus* minimal medium (AMM), supplemented with 25 mM L-glutamine.

Creation of the *cubA* expression plasmids. The cDNA of *P. cubensis cubA* (GenBank accession OQ784618) was amplified by PCR from randomly reverse-transcribed mRNA,^[24] using the oligonucleotide pair oES3/oES4 (Table S3) and Phusion polymerase (Thermo Fisher). The thermal cycling protocol was 98 °C, 2 min initial hold, followed by 35 cycles of 98 °C, 20 s, 60 °C, 20 s, 72 °C, 50 s, and a final hold at 72 °C for 5 min. The PCR product was purified on an agarose gel, restricted with *Nde*I and *Hind*III and ligated to vector pET28a, opened equally, to construct *E. coli* expression plasmid pES02. DNA sequencing confirmed correct amplification and cloning. For heterologous gene expression in *A. niger*, gDNA of *P. cubensis* was amplified with oligonucleotides oES11 and oES12 (Table S3) using the protocol described above, and cloned via the *Nsi*I and *Nco*I sites into vector pSMXpress2,^[21b] to yield plasmid pES06 which was used to transform *A. niger* protoplasts.

Heterologous production of CubA and *in vitro* assays. CubA was produced as N-terminally hexahistidine-tagged fusion protein in *E. coli* KRX × pES02, grown in LB medium and 50 μg mL^{−1} kanamycin as selection marker. The protein was purified as previously described,^[24] concentrated on an Amicon Ultra-15 centrifugal filter, and eluted with MOPS buffer (pH 7) 50 mM, 20 mM MgCl₂. Protein concentrations were determined using the Pierce BCA-Protein Assay Kit (Thermo Fisher), and the identity of the produced protein was verified by mass spectrometry-based peptide fingerprinting, as described.^[26] *In vitro* reactions were carried out in triplicates and with 1 μM CubA (2 μM to determine the pH optimum), and 300 μM farnesyl diphosphate, in a volume of 1 mL (200 μL to record the optimal temperature) at 29 °C for 10 min (15 min for the pH optimum). To determine optimum conditions, the temperature was varied between 4 and 40 °C, and the pH between 5 and 9 (5 to 7 in 50 mM citrate, 6.5 to 7.5 in 50 mM MOPS, 7 to 9 in 50 mM TRIS buffers). The reaction volumes (1 mL) were extracted with 500 μL ethyl acetate (200 μL reaction volumes with an equal volume), centrifuged, filtered, and subjected to GC-MS analysis.

Transformation of *Aspergillus niger* and transgene expression. Preparation of *Aspergillus niger* protoplasts and PEG8000-mediated transformation followed a described protocol.^[27] To select for

prototrophy, i.e., integration of plasmid pES06, transformants were grown on uracil-free medium. To genetically verify full-length integration of *cubA*, genomic DNA of transformants was isolated and subjected to a diagnostic PCR, using oligonucleotides oES12/oMG370 (Table S3) and *Taq* polymerase and following the above thermal cycling protocol. One positive transformant, *A. niger* tES02, was chosen for further work (Figure S7). This transformant was grown in 100 mL liquid YPD medium (20 g L⁻¹ peptone, 20 g L⁻¹ D-glucose, 10 g L⁻¹ yeast extract) for 24 h at 30 °C, shaking at 180 rpm. Expression of *cubA* was induced by adding 30 µg mL⁻¹ doxycycline, and cultivation was continued for 12 h. For negative control, *A. niger* tNAL000^[28] carrying the insertless vector pSMX2-URA, was included.

Extraction of fungal biomass. For chromatographic analysis, the biomass of *A. niger* tES02 was separated from the broth by filtration, rinsed with water, blotted dry, frozen in liquid nitrogen, and subsequently extracted with 5 mL ethyl acetate for 20 min. Terpenoid analysis of *Psilocybe* biomass was carried out with i) mycelium grown in MEP liquid medium, incubated for 7 d at 25 °C and shaken at 140 rpm and ii) with *P. cubensis* carpophores that were produced as described.^[3c] Mycelium and carpophores were collected and shock-frozen in liquid nitrogen prior to work-up for chromatography. To extract natural products, 5 g biomass (mycelium or carpophores) was powdered under liquid nitrogen, extracted with 15 mL ethyl acetate for 20 min, centrifuged and subjected to GC-MS.

Gas chromatography and mass spectrometry. The gas chromatographic separation of CubA products was performed with an ISQ GC-MS-System (Thermo Fisher) using a ZB-5MS-column with inactive guard column (30 + 10 m × 0.25 mm × 0.25 µm, Phenomenex). The temperature program had a heating rate of 3 °C min⁻¹ from 60–260 °C. As carrier gas helium was used at a flow of 1 mL min⁻¹ with splitless injection or in split-mode (1:10) with an injector temperature of 220 °C. The column was connected via a transfer line (280 °C) to an ISQ quadrupole-mass spectrometer (Thermo Fisher). Electron impact ionization was performed at 70 eV, the ion source was heated to 250 °C. All spectra were measured in positive ionization mode. Deconvolution and retention index calculation according to van den Dool and Kratz^[29] was accomplished with MassFinder (Hochmuth Scientific Consulting, Hamburg, Germany) and data visualization with Xcalibur software. Total ion current (TIC) values were recorded in the mass range of 33–450 amu, with a scan time of 0.2 s. and a MS delay of 5 min. Samples in *n*-hexane or ethyl acetate (1 µL) were injected. GC-MS results were analyzed using XcaliburTM v3.1.66.10 (Thermo Fisher, Waltham, MA, USA). Compounds were identified by comparing their mass spectra and the respective retention index with established databases and literature data.^[30] Additionally, retention times and mass spectra were compared with GC-MS analysis under the same conditions of pure compounds or essential oils of *Piper cubeba* berries and *Angelica archangelica* roots.^[20]

Chromatographic runs to determine temperature and pH optima were performed on a Trace 1310 GC coupled with a TSQ 9000 electron impact (EI)-triple quadrupole mass spectrometer (Thermo Fisher). A 4 mm SSL GC inlet glass liner with glass wool and a BPX5 capillary column (30 m, 0.25 mm i. d., 0.25 µm film, Trajan Scientific Europe Ltd., Milton Keynes) was used. The column was operated with helium carrier gas (1.5 mL min⁻¹) and split injection (split ratio 1:10) or splitless, depending on the sample concentration). The injector temperature was set to 200 °C, the MS transfer line to 300 °C, and the ion source temperature to 200 °C. The GC temperature profile was as follows: 40 °C for 0–1 min, heating to 100 °C over 1–3 min (30 °C min⁻¹), heating to 300 °C over 3–24 min (10 °C min⁻¹). GC-MS results were analyzed using XcaliburTM

v4.4.16.14 and ChromeleonTM v7.3 software (both Thermo Fisher, Waltham, MA, USA).

Expression analysis. *P. cubensis* mycelium and fruiting bodies were ground under liquid nitrogen to a fine powder. RNA was isolated with the SV Total RNA Isolation System (Promega) using the manufacturer's protocol. 1 µg RNA was reverse transcribed to cDNA using the RevertAid RT kit (Thermo Fisher) and an anchored oligo-(dT)₁₈ primer. Expression analysis was carried out essentially as described^[24] and based on three biological replicates and three technical replicates each, with primers with a minimum efficiency of 95 % (Table S4). Melting curves were obtained by heating from 60 to 94 °C. A gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) served as an internal housekeeping reference gene. Gene expression levels were determined as described.^[31]

Bioinformatic methods. The evolutionary relationship of basidiomycete terpene synthases and the phylogenetic placement of CubA was inferred using the Maximum Likelihood method and Le_Gascuel_2008 model.^[32] References to amino acid sequences and database entries are given in Table S5. The bootstrap consensus tree was inferred from 1000 replicates.^[33] Evolutionary analyses were conducted in MEGA11.^[34]

Supporting Information

The authors have cited additional references within the Supporting Information (Ref. ^[35]).

Acknowledgements

We thank Jonas Fiedler and Mark Ellerhorst (Leibniz Institute for Natural Product Research and Infection Biology) for peptide fingerprinting. This work was in part supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2051 – Project-ID 390713860 and by the Carl Zeiss Foundation. The laboratories of C.H. and D.H. are also funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – SFB 1127, Project-ID 239748522. Financial statement: A.R.C. reports an ownership interest in CaaMTech, LLC. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biosynthesis · enzyme · *Psilocybe* · sesquiterpene · terpene synthase

- [1] A. Hofmann, R. Heim, A. Brack, H. Kobel, A. Frey, H. Ott, T. Petrzilka, F. Troxler, *Helv. Chim. Acta* **1959**, *42*, 1557–1572.
- [2] a) F. Tylys, T. Palenicek, J. Horacek, *Eur. Neuropsychopharmacology* **2014**, *24*, 342–356; b) H. A. Geiger, M. G. Wurst, R. N. Daniels, *ACS Chem. Neurosci.* **2018**, *9*, 2438–2447.
- [3] a) A. Y. Leung, A. G. Paul, *J. Pharm. Sci.* **1968**, *57*, 1667–1671; b) N. Jensen, J. Gartz, H. Laatsch, *Planta Med.* **2006**, *72*, 665–666; c) C. Lenz, J. Wick, D. Hoffmeister, *J. Nat. Prod.* **2017**, *80*, 2835–2838.
- [4] a) J. Gartz, *Int. J. Crude Drug Res.* **1989**, *27*, 141–144; b) J. Gartz, *J. Bas. Microbiol.* **1994**, *34*, 17–22; c) Y. Matsushima, O. Shirota, R. Kikura-Hanajiri, Y. Goda, F. Eguchi, *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1866–1868; d) O. Zhuk, I. Jasicka-Misiak, A. Poliwoda, A. Kazakova, V. V. Godovan, M. Halama, P. P. Wiczorek, *Toxin Rev.* **2015**, *7*, 1018–1029.
- [5] a) S. Ben-Shabat, E. Fride, T. Sheskin, T. Tamiri, M. H. Rhee, Z. Vogel, T. Bisogno, L. De Petrocellis, V. Di Marzo, R. Mechoulam, *Eur. J. Pharmacol.* **1998**, *353*, 23–31; b) R. Mechoulam, S. Ben-Shabat, *Nat. Prod. Rep.* **1999**, *16*, 131–143; c) E. B. Russo, *Br. J. Pharmacol.* **2011**, *163*, 1344–1364; d) E. B. Russo, *Front. Plant Sci.* **2019**, *9*, 1969.
- [6] S. Dörner, K. Rogge, J. Fricke, T. Schäfer, J. M. Wurlitzer, M. Gressler, D. N. K. Pham, D. R. Manke, A. R. Chadeayne, D. Hoffmeister, *ChemBioChem* **2022**, *23*, e202200249.
- [7] J. S. Dickschat, *Nat. Prod. Rep.* **2017**, *34*, 310–328.
- [8] B. Sandargo, C. Chepkirui, T. Cheng, L. Chaverra-Muñoz, B. Thongbai, M. Stadler, H. Hüttel, *Biotechnol. Adv.* **2019**, *37*, 107344.
- [9] a) D. Janzen, B. Slavik, M. Zehe, C. Sottriffer, H. M. Loos, A. Buettner, C. Villmann, *J. Neurochem.* **2021**, *159*, 101–115; b) S. Nurulain, T. Prytkova, A. M. Sultan, O. Ievlevskiy, D. Lorke, K.-H. S. Yang, G. Petroianu, F. C. Howarth, N. Kabbani, M. Oz, *Neurosci* **2015**, *15*, 91–99.
- [10] F. Surup, B. Thongbai, E. Kuhnert, E. Sudarman, K. D. Hyde, M. Stadler, *J. Nat. Prod.* **2015**, *78*, 934–938.
- [11] S. Pornpakakul, S. Suwanchaoen, A. Petsom, S. Roengsumran, N. Muangsin, N. Chaichit, J. Piapukiew, P. Sihanonth, J. W. Allen, *J. Asian Nat. Prod. Res.* **2009**, *11*, 12–17.
- [12] a) M. B. Quin, C. M. Flynn, G. T. Wawrzyn, S. Choudhary, C. Schmidt-Dannert, *ChemBioChem* **2013**, *14*, 2480–2491; b) M. B. Quin, C. M. Flynn, C. Schmidt-Dannert, *Nat. Prod. Rep.* **2014**, *31*, 1449–1473; c) S. Nagamine, C. Liu, J. Nishishita, T. Kozaki, K. Sogahata, Y. Sato, A. Minami, T. Ozaki, C. Schmidt-Dannert, J. I. Maruyama, H. Oikawa, *Appl. Environ. Microbiol.* **2019**, *85*, e00409–19; d) M. Gressler, N. A. Löhr, T. Schäfer, S. Lawrinowitz, P. S. Seibold, D. Hoffmeister, *Nat. Prod. Rep.* **2021**, *38*, 702–722; e) C. Zhang, X. Chen, A. Orban, S. Shukal, F. Birk, H.-P. Too, M. Rühl, *ACS Chem. Biol.* **2020**, *15*, 1268–1277; f) S. Agger, F. Lopez-Gallego, C. Schmidt-Dannert, *Mol. Microbiol.* **2009**, *72*, 1181–1195; g) J. Wu, X. Yang, Y. Duan, P. Wang, J. Qi, J.-M. Gao, C. Liu, *J. Fungi* **2022**, *8*, 913.
- [13] a) N. B. Kreuzenbeck, E. Seibel, J. W. Schwitalla, J. Fricke, B. H. Conlon, S. Schmidt, A. Hammerbacher, T. G. Köllner, M. Poulsen, D. Hoffmeister, C. Beemelmans, *mSystems* **2022**, *7*:e0121421; b) I. Burkhardt, N. B. Kreuzenbeck, C. Beemelmans, J. S. Dickschat, *Org. Biomol. Chem.* **2019**, *17*, 3348–3355; c) H. Ichinose, S. Ukeba, T. Kitaoka, *Enzyme Microb. Technol.* **2022**, *158*, 110037.
- [14] a) J. Fricke, F. Blei, D. Hoffmeister, *Angew. Chem. Int. Ed.* **2017**, *56*, 12352–12355; b) H. T. Reynolds, V. Vijayakumar, E. Gluck-Thaler, H. B. Korotkin, P. B. Matheny, J. C. Slot, *Evol. Lett.* **2018**, *2*, 88–101; c) K. McKernan, L. T. Kane, S. Crawford, C.-S. Chin, A. Trippe, S. McLaughlin, *F1000Research* **2021**, *10*:281.
- [15] M. P. Torrens-Spence, C. T. Liu, T. Pluskal, Y. K. Chung, J. K. Weng, *ACS Chem. Biol.* **2018**, *13*, 3343–3353.
- [16] M. Seemann, G. Zhai, J.-W. de Kraker, C. M. Paschall, D. W. Christianson, D. E. Cane, *J. Am. Chem. Soc.* **2002**, *124*, 7681–7689.
- [17] F. Lopez-Gallego, S. A. Agger, D. Abate-Pella, M. D. Distefano, C. Schmidt-Dannert, *ChemBioChem* **2010**, *11*, 1093–1106.
- [18] W. Mischko, M. Hirte, M. Fuchs, N. Mehlmer, T. B. Brück, *Microb. Cell Fact.* **2018**, *17*, 164.
- [19] Y.-L. Lin, L.-T. Ma, Y.-R. Lee, J.-F. Shaw, S.-Y. Wang, F.-H. Chu, *J. Agric. Food Chem.* **2017**, *65*, 1874–1886.
- [20] a) R. Bos, H. J. Woerdenbag, O. Kayser, W. J. Quax, K. Ruslan, Elfami, J. Essent. Oil Res. **2007**, *19*, 14–17; b) E. Al-Sayed, H. A. Gad, D. M. El-Kersh, *ACS Omega* **2021**, *6*, 25652–25663; c) J. C. Chalchat, R. P. Garry, *J. Essent. Oil Res.* **1997**, *9*, 311–319.
- [21] a) M. Gressler, P. Hortschansky, E. Geib, M. Brock, *Front. Microbiol.* **2015**, *6*, 184; b) E. Geib, M. Brock, *Fungal Biol. Biotechnol.* **2017**, *4*, 13.
- [22] H. Xu, J. S. Dickschat, *Chem. Eur. J.* **2020**, *26*, 17318–17341.
- [23] G. T. Wawrzyn, M. B. Quin, S. Choudhary, F. López-Gallego, C. Schmidt-Dannert, *Chem. Biol.* **2012**, *19*, 772–783.
- [24] R. Demmler, J. Fricke, S. Dörner, M. Gressler, D. Hoffmeister, *ChemBioChem* **2020**, *21*, 1364–1371.
- [25] M. Dörfer, M. Gressler, D. Hoffmeister, *Mycol. Prog.* **2019**, *18*, 1027–1037.
- [26] C. Lenz, J. Wick, D. Braga, M. García-Altare, G. Lackner, C. Hertweck, M. Gressler, D. Hoffmeister, *Angew. Chem. Int. Ed.* **2020**, *59*, 1450–1454.
- [27] M. Gressler, P. Hortschansky, E. Geib, M. Brock, *Front. Microbiol.* **2015**, *6*, 184.
- [28] N. A. Löhr, F. Eisen, W. Thiele, L. Platz, J. Motter, W. Hüttel, M. Gressler, M. Müller, D. Hoffmeister, *Angew. Chem. Int. Ed.* **2022**, *61*, e202116142.
- [29] H. Van den Dool, P. D. Kratz, *J. Chromatogr.* **1963**, *11*, 463–471.
- [30] a) R. P. Adams, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, Allured Publishing Corp., Carol Stream, IL, **2007**; b) D. Joulain, W. A. König, *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*, E. B. Verlag, Hamburg, **1998**; c) National Institute for Standards and Technology, Mass Spectral & Retention Index Library, **2017**.
- [31] M. W. Pfaffl, *Nucleic Acids Res.* **2001**, *29*, e45.
- [32] S. Q. Le, O. Gascuel, *Mol. Biol. Evol.* **2008**, *25*, 1307–1320.
- [33] J. Felsenstein, *Evolution* **1985**, *39*, 783–791.
- [34] K. Tamura, G. Stecher, S. Kumar, *Mol. Biol. Evol.* **2021**, *38*, 3022–3027.
- [35] a) T. Rehka, D. Sharma, F. Lin, Y. Khai Choong, C. Lim, C. Jobichen, C. Zhang, *ACS Catal.* **2023**, *13*, 4949–4959; b) H. Koo, C. Vickery, Y. Xu, G. V. Louie, P. E. O'Maille, M. Bowman, C. M. Nartey, M. D. Burkart, J. P. Noel, *J. Antibiot.* **2016**, *69*, 524–533; c) S. Garms, T. G. Köllner, W. Boland, *J. Org. Chem.* **2010**, *75*, 5590–5600; d) C. Nakano, T. Tezuka, S. Horinouchi, Y. Ohnishi, *J. Antibiot.* **2012**, *65*, 551–558; e) Y.-Y. Li, X.-M. Tan, J. Yang, L.-P. Guo, G. Ding, *J. Agric. Food Chem.* **2020**, *68*, 9827–9838; f) D. Arigoni, *Pure Appl. Chem.* **1975**, *41*, 219–245.

Manuscript received: July 14, 2023

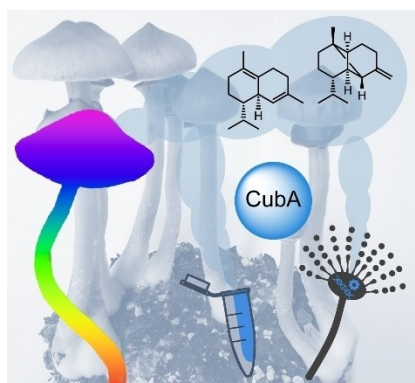
Revised manuscript received: August 22, 2023

Accepted manuscript online: August 23, 2023

Version of record online: ■■■■■

RESEARCH ARTICLE

Shrooms going terpenoid: The sesquiterpene synthase CubA of the “magic mushroom” *Psilocybe cubensis* was identified as a multi-functional enzyme producing cubebol, β -copaene, and various other mono-, bi-, and tricyclic sesquiterpenes or -terpenoids. As related enzymes are encoded in numerous *Psilocybe* species, our results provide further insight into the true metabolic diversity of this genus.



E. Schäfer, P. S. Seibold, Dr. S. Bartram, Dr. F. Trottmann, Dr. V. G. Haensch, Dr. M. Gressler, A. R. Chadeayne, Prof. Dr. C. Hertweck, Prof. Dr. S. E. O'Connor, Prof. Dr. D. Hoffmeister*

1 – 7

A „Magic Mushroom“ Multi-Product Sesquiterpene Synthase

