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Biosynthesis

Ibotenic Acid Biosynthesis in the Fly Agaric Is Initiated by Glutamate Hydroxylation**

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In memory of Professor Rolf Huisgen

Abstract: *The fly agaric, Amanita muscaria, is widely known for its content of the psychoactive metabolites ibotenic acid and muscimol. However, their biosynthetic pathway and the respective enzymes are entirely unknown. 50 years ago, the biosynthesis was hypothesized to start with 3-hydroxyglutamate. Here, we build on this hypothesis by the identification and recombinant production of a glutamate hydroxylase from A. muscaria. The hydroxylase gene is surrounded by six further biosynthetic genes, which we link to the production of ibotenic acid and muscimol using recent genomic and transcriptomic data. Our results pinpoint the genetic basis for ibotenic acid formation and thus provide new insights into a decades-old question concerning a centuries-old drug.*

Amanita muscaria, the fly agaric, is perhaps the most prominent of all mushrooms, known for its extravagant appearance with a red cap covered by white specks, and its infamous toxicity. While the mushroom is actually less deadly than generally assumed, the psychoactive effects are mediated by ibotenic acid (**1**) and its decarboxylation product muscimol (**2**).[1–3] They possess structural similarity to the neurotransmitters glutamate (**3**) and GABA (**4**), respectively, activating the corresponding receptors in the brain (Figure 1 A).[4,5] Due to their activity, **1** and **2** have been used as lead compounds for pharmacological research; the ibotenic acid derivative AMPA, for example, has given its name to the most common glutamate receptor in the human brain.[6]

Shortly after the structure of ibotenic acid was solved in 1964, there was speculation concerning its biosynthetic origin.[1] Based on co-occurring metabolites, Eugster and co-workers hypothesized that ibotenic acid is derived from 3-hydroxyglutamate (**5**).[7] However, 3-hydroxyglutamate has not been identified in the fly agaric and the biosynthesis of ibotenic acid and muscimol has remained obscure.

To identify the biosynthetic genes, we assumed that the formation of ibotenic acid is initiated with the hydroxylation of either glutamine or glutamate. Hitherto, no enzyme that catalyzes this reaction on free substrate has been experimentally verified.[8] Nevertheless, 3-hydroxyglutamine (**7**, Figure 3) occurs as a component of the non-ribosomal peptide pneumocandin B_0 from *Glarea lozoyensis*. Its biosynthetic gene cluster (BGC) includes a putative dioxygenase, GloE, which has been proposed as a candidate enzyme for the

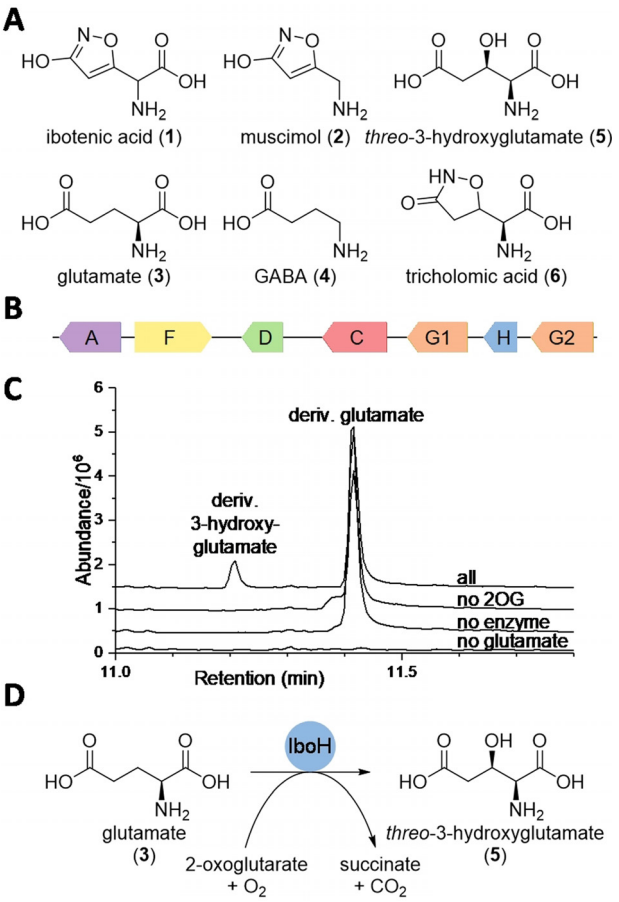

Figure 1. Discovery of the *ibo* BGC. A) Structures of *Amanita muscaria* metabolites and analogues. B) Schematic of the *ibo* BGC. C) GC-MS total ion chromatograms of IboH assays: 3-hydroxyglutamate formation was dependent on 2-oxoglutarate (2OG), enzyme, and glutamate. D) Stereoselective glutamate hydroxylation by IboH.

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hydroxylation of glutamine.[9] Therefore, we used its protein sequence to screen the *A. muscaria* genome.[10] Indeed, a homologous protein, IboH (GenBank entry KIL56739), is encoded in a genetic region that features six additional biosynthetic enzymes. This putative *ibo* BGC (Figure 1B) comprises a cytochrome P450 enzyme (IboC KIL56737), a flavin-dependent monooxygenase (FMO, IboF KIL56733), an adenylating enzyme (IboA KIL56732), two mutually similar pyridoxal phosphate (PLP)-dependent enzymes (IboG1 KIL56738 and IboG2 KIL56740), and a decarboxylase (IboD KIL56734). The genes include all functionalities putatively needed for the biosynthesis of ibotenic acid (see below).

Consequently, the assignment of the BGC was verified experimentally. The *iboH* gene was expressed in *Escherichia coli* with an N-terminal GST tag (GenBank entry MN520442). As IboH is predicted to be an Fe^{II}/2-oxoglutarate-dependent dioxygenase, the purified protein was incubated aerobically with Fe^{2+}, 2-oxoglutarate, ascorbic acid, and one of the putative substrates glutamine and glutamate. The reaction mixtures were derivatized with ethyl chloroformate/ethanol and analyzed by GC-MS. While the glutamine assay was negative, glutamate was transformed to a product that was detected as a new peak in the GC-MS chromatogram (Figure 1C, 11.2 min). Control experiments lacking either enzyme, glutamate, or 2-oxoglutarate did not yield the product, thus confirming that the enzyme is indeed 2-oxoglutarate-dependent.

As the yield with the purified enzyme was too low for product isolation, a whole-cell approach was used: incubation of live *E. coli GST-iboH* cells with L-glutamate gave sufficient amounts of the product (Supporting Information, Figure S1), which was extracted from the cell supernatant by cation-exchange chromatography as the hydrochloride. NMR analysis showed the presence of *threo*-3-hydroxyglutamate, confirming the role of IboH as being an L-glutamate 3-(*R*)-hydroxylase (Figures 1D, S2, and S3).[11] This is the first report of an enzymatic hydroxylation of free glutamate.

To determine the biological relevance of glutamate hydroxylation in *A. muscaria*, mushroom samples (collected near Feldberg, Black Forest, Germany) were analyzed by GC-MS. Ibotenic acid was detected along with low levels of 3-hydroxyglutamate (Figures S4 and S5). This hints towards IboH being active in its native organism, coinciding with the production of ibotenic acid. Furthermore, public RNA-seq data revealed that the seven genes in the *ibo* BGC are highly expressed when *A. muscaria* was artificially grown in symbiosis with *Populus*, which is close to its natural condition (Figure 2A).[12] To investigate whether the genes are functionally linked, coexpression network analysis was conducted. The data showed that *ibo* genes have a very similar expression pattern. From a total of 11915 expressed genes, all seven *ibo* genes clustered closely together, indicating tight coregulation and hence a common metabolic function (Figure 2B).[13]

To further probe the link of the *ibo* BGC to the production of ibotenic acid, transcriptomic data of another ibotenic acid producer, *Amanita pantherina*, was screened for homologous genes.[14,15] The RNA-seq reads were mapped to the *ibo* BGC. Indeed, *A. pantherina* actively expresses close homologues of each of the *ibo* genes (>80% nucleotide identity, Figure S6), confirming the link to ibotenic acid production.

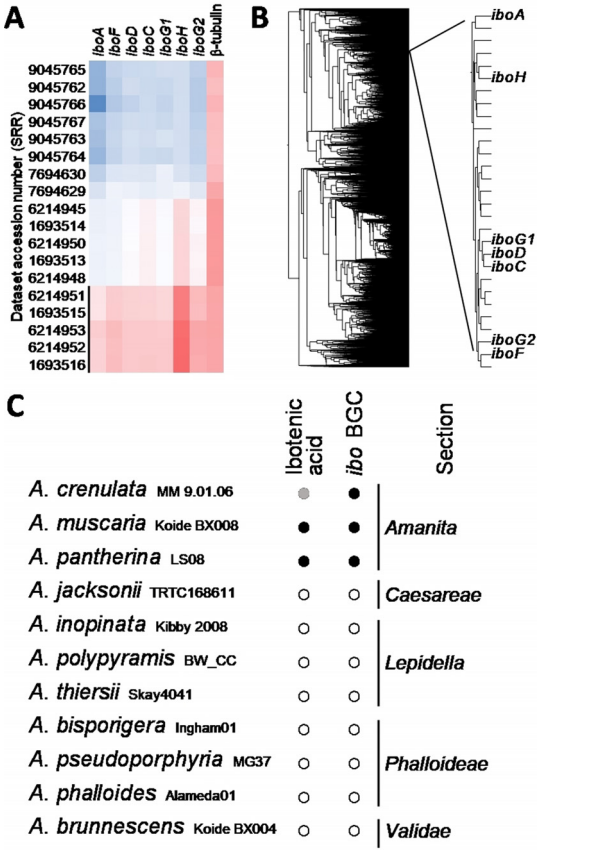

Figure 2. Genomic and transcriptomic data of the *ibo* BGC. A) Normalized expression of *ibo* genes across RNA-seq datasets from NCBI SRA: low expression (blue) to high expression (red). The black line marks cocultivation experiments of *Amanita muscaria* with *Populus tremula x tremuloides*. The β-tubulin gene is included for comparison. B) Location-independent coexpression clustering of 11915 expressed *A. muscaria* genes. The *ibo* genes cluster closely together, indicating coregulation. C) Co-occurrence of the *ibo* genes and (putative) ibotenic acid production of *Amanita* species with sequenced genomes/transcriptomes. The three species containing *ibo* genes belong to *Amanita* section *Amanita*.

Furthermore, the transcriptome of the related mushroom species *Amanita crenulata* was checked for *ibo* homologues. Again, all genes had expressed matches in *A. crenulata* (>80% nucleotide identity, Figure S7). Thus, we predict that this species is capable of producing ibotenic acid and muscimol. This correlates with previous reports of human intoxication with symptoms that resemble those after ibotenic acid and muscimol exposure.[16]

Additionally, we analyzed genomes of eight *Amanita* species from four taxonomic sections, which do not produce ibotenic acid. None of these contain the *ibo* BGC, substantiating the correlation between ibotenic acid and the *ibo* genes (Figure 2C). Apparently, the presence of the *ibo* BGC is confined to *Amanita* section *Amanita*, which is in accordance with previous studies on the taxonomic distribution of ibotenic acid production.[17,18]

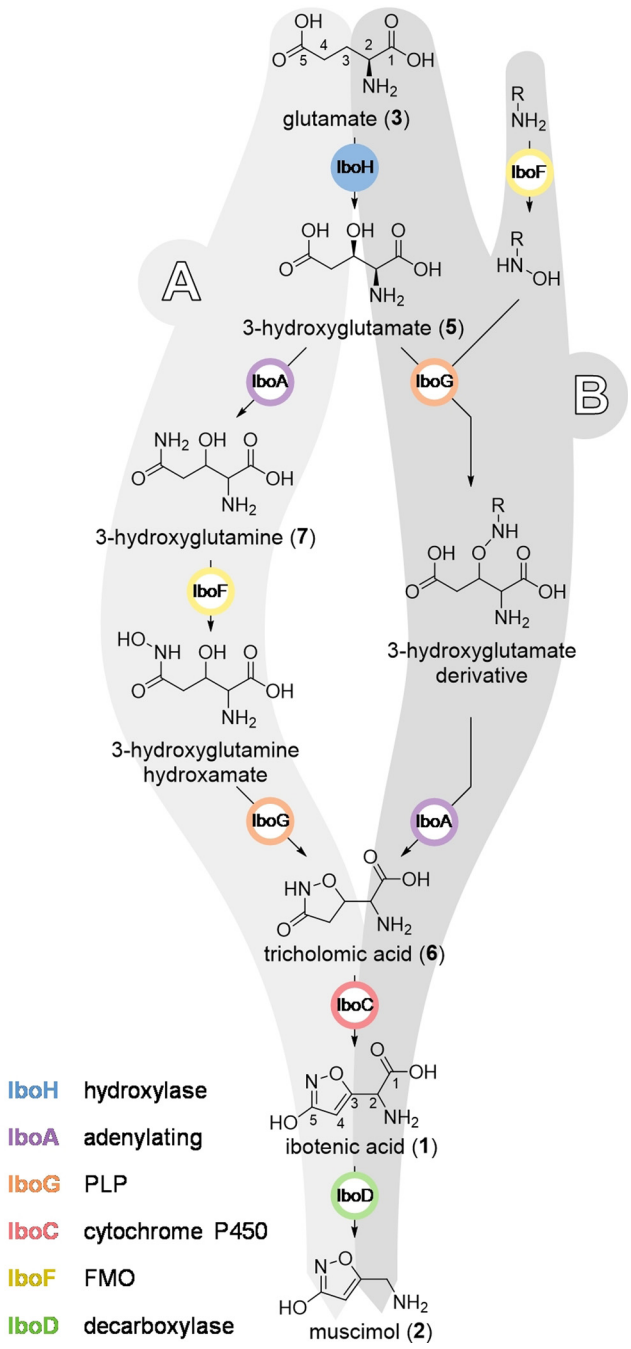

Figure 3. Proposed alternative biosynthetic pathways of ibotenic acid. Pathway A involves N-hydroxylation of the amide of **7** by IboF. Pathway B involves N-hydroxylation of a hypothetical external compound which does not end up in the final structure of ibotenic acid (**1**). Enzymes are indicated by colored circles: filled for verified function, non-filled for inferred function. PLP: pyridoxal phosphate-dependent enzyme; FMO: flavin-dependent monooxygenase.

To deduce the biosynthetic functions of the *ibo* proteins, their sequences were matched against known enzymes. From this, we propose the functions illustrated in Figure 3 and described in the following. Related proteins with known functions are listed in brackets along with amino acid sequence identity values for comparison.

The first committed step is glutamate hydroxylation by IboH, and the last step is decarboxylation[19] of ibotenic acid to muscimol by IboD (tryptophan decarboxylase P0DPA6,[20] 32 %). The order of the intermediate reactions is somewhat ambiguous. IboA (adenylation domain of F8P9P5,[21] 21 %) likely activates the carboxylic acid at position 5 to introduce an amide bond, and the flavin monooxygenase IboF (heteroatom oxygenases B8NM63, B8NM73,[22] 21–24 %) generates the N–O bond. There are several options for the latter step. One option (Figure 3 A) is that IboF directly hydroxylates the amide nitrogen formed by IboA to produce a hydroxamic acid species (cf. trichostatin biosynthesis[23]). Another option (Figure 3 B) is that IboF hydroxylates an external N-containing compound, whose resulting N–O bond is subsequently introduced into the hydroxyglutamate scaffold (cf. cycloserine biosynthesis[24]).

The paralogous PLP-dependent enzymes IboG1 and IboG2 (cystathionine γ-synthase I1RZK8,[25] 38–39 %) are likely involved in substitution of the OH group at position 3 by the O-N moiety. Similar substitution reactions are known from other PLP-dependent enzymes, such as cystathionine β-synthase.[26] An alternative pathway that could proceed without IboG1/IboG2 is given in the Supporting Information (Figure S8).

The first cyclic intermediate is most probably tricholomic acid (**6**, Figure 1 A), which is likely desaturated to ibotenic acid by the cytochrome P450 IboC (A1CFL5, A1CFL6,[27] A0A286LF02,[20] 27–30 %). Tricholomic acid (**6**) is a metabolite produced by *Tricholoma muscarium*.[28] As *Tricholoma* and *Amanita* are related (taxonomic order *Agaricales*), the biosynthesis of **6** should be similar to that of ibotenic acid, omitting the desaturation step. Further ibotenic acid- and tricholomic acid-producing fungi have been proposed, ranging diverse taxa from *Amantia* species to *Ustilago* and *Ophiocordyceps*.[17,18,29,30] The BGC reported here offers the opportunity to reassess the proposed producers on the genetic level, and thus verify or refute the hypothesis for further producers.

Taken together, our findings indicate that the *ibo* genes are responsible for ibotenic acid production in—at least—three *Amanita* species. The identified BGC contains the glutamate hydroxylase IboH, whose activity was demonstrated in a heterologous system. This discovery revives the long-dormant research on psychoactive toxin biosynthesis in the fly agaric. Full elucidation of the biosynthetic pathway will reveal the reactions that lead to the isoxazole core, and will enable the utilization for biotechnological applications.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biosynthesis · enzyme catalysis · fly agaric · hydroxylation · ibotenic acid

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