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THE BIOGENESIS OF β-*N*-(γ-GLUTAMYL)-4-HYDROXYMETHYLPHENYLHYDRAZINE (AGARITINE) IN *AGARICUS BISPORUS*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Agaricus bisporus*; Agaricaceae; synthesis; tracer study; agaritine; phenylhydrazine; γ-glutamyl derivative; 4-(2-nitrobenzylidene)hydrazinobenzyl alcohol; 4-(4-nitrobenzylidene)hydrazinobenzyl alcohol; glyphosate

Abstract—According to the biogenetic scheme of Schütte *et al.* (*An. Quim.*, 1972, **68**, 899), agaritine [AG; β-*N*-(γ-glutamyl)-4-hydroxymethylphenylhydrazine (HMPH)], a constituent of the fruit body of the common mushroom *Agaricus bisporus*, is generally held to be a shikimate-derived metabolite. A critical evaluation of the methodological basis of this tenet showed it is not supported by the experimental data presented. A major doubt concerns the conditions used for hydrolysis of the hydrazide bond; therefore, two derivatives of HMPH, 2- and 4-nitrobenzaldehyde hydrazones, were synthesized as reference compounds for the assessment of the desglutamyl product. The UV-, IR- and ^{1}H NMR spectroscopic properties of the two new substances are listed. Using (i) the established shikimate-derived metabolite γ-glutaminyl-4-hydroxybenzene, which co-occurs with AG, as the positive control, (ii) the shikimate-chorismate pathway inhibitor 'glyphosate' as a tool, and (iii) complementary results from physiological experiments, it was demonstrated that there is no *de novo* synthesis of the desglutamyl moiety of AG in the fruit body. The likely site of AG synthesis is the vegetative hyphae in contact with the wheat straw compost. A speculative scheme is presented for the assembly of AG, which postulates an exogenous origin of both the aryl and the hydrazide moieties of this, resulting, respectively, from the breakdown of lignin by the fungus and the diazotrophic activity of a bacterial commensal in the substratum.

INTRODUCTION

Fruit bodies of the common cultivated mushroom *Agaricus bisporus* and some other *Agaricus species* [1, 2] contain various unusual phenolics (Figure 1 and references [3–14]). Of these, only agaritine (**1**) and γ-glutaminyl-4-hydroxybenzene (GHB; **6**) are present in more than traces, contributing some 0.5–5% to the dry matter of the sporocarp [2, 15–22]. Compound **6** is derived from the shikimate-chorismate pathway, with 4-aminobenzoic acid (4-ABA) as an intermediate [16, 22–25]. The same has long been assumed to hold for **1** [26–30]. This generally accepted view is based on an *in vivo* tracer study of Schütte *et al.* [31]; and the recent identification of agaritinal (**2**), 4-hydrazinobenzoate (**4**) and agaritinate (**3**) is also considered a convincing confirmation of this hypothesis [2, 7–9]. Further, the occurrence in the *A. bisporus* fruit body of a special γ-glutamyltransferase (GT), termed agaritine GT (AGT; EC 2.3.2.9 [32]) has likewise been taken as indicative of a common biosynthetic route for **1** and **6**. This is because AGT has the ability to utilize aryl hydrazines and their γ-glutamyl derivatives as acceptor and donor substrates, respectively, while not effecting the cleavage and formation of typical γ-glutamyl peptides (as performed by GT EC 2.3.2.2), but to accept the desglutamyl moiety of **6**. For a number of reasons (as detailed in Results and Discussion) Schütte's scheme [31] cannot, however, be regarded as satisfactory.

Theoretically, three possibilities may be considered for the origin of agaritine (**1**) in the sporocarp of the fungus: (i) synthesis *in situ* from a simple non-aromatic compound *via* the polyketide or the shikimate pathway, of which participation of the latter is clearly

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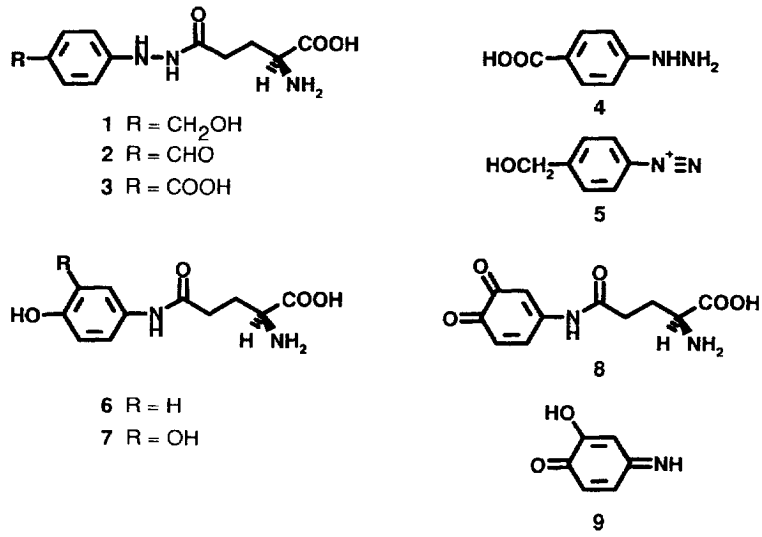


Fig. 1. Structural formulae of non-ubiquitous N-containing aromatic components (and derivatives thereof) of the common mushroom *A. bisporus* and some closely related species. **1**, *β-N*-(γ-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine [3–6]); **2**, *β-N*-(γ-glutamyl)-4-formylphenylhydrazine (agaritinal [7]); **3**, *β-N*-(γ-glutamyl)-4-carboxyphenylhydrazine (agaritinic acid [8]); **4**, 4-hydrazinobenzoic acid [9]; **5**, 4-hydroxymethylbenzenediazonium ion [10]; **6**, γ-glutaminyl-4-hydroxybenzene (GHB [11]); **7**, γ-glutaminyl-3,4-dihydroxybenzene (GDHB, agaridoxin [12]); **8**, γ-glutaminyl-3,4-benzoquinone (GBQ [13]); **9**, 2-hydroxy-4-imino-2,5-cyclohexadienone [14].

indicated by the substitution pattern of **1** [33]; (ii) *de novo* synthesis by the vegetative mycelium, followed by translocation of the product into the fruit body; and (iii) synthesis of the desglutamyl residue through transformation of an aromatic compound generated during the breakdown of lignin by exo-enzymes of the fungus [34, 35]. The present study was undertaken to re-visit the subject of the origin of the aromatic residue of agaritine (**1**) in the *A. bisporus* fruit body, using the established shikimate pathway-derived metabolite **6** as the reference, and to specify sites of synthesis of **1** and **6** within the fruit body–substrate mycelium continuum.

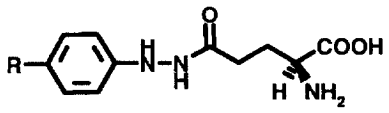
RESULTS AND DISCUSSION

The following considerations [(i)–(iv)] cast doubt on the reliability of the experimental evidence presented by Schütte *et al.* [31] for their biogenetic scheme: (i) under the conditions used in that study for column chromatography, agaritine (**1**) is quickly degraded, whereas co-occurring GHB (**6**) is not (D. Baumgartner and D. M. Rast, unpublished observations); (ii) the authors explicitly state that only one radioactive metabolite was detected following administration of labelled 4-ABA; (iii) data are lacking demonstrating the presence of radioactivity in the aryl residue of **1** following application of the tracer substance; and (iv) **1** has been reported to be unusually sensitive to destruction by acid [36], to the extent that standard procedures for the hydrolysis of γ-glutamylated compounds (as perhaps used by Schütte *et al.* [31]; details are not given) would probably have decomposed agaritine (**1**) or, in any case, would not have yielded stoichiometric amounts of the γ-glutamyl and desglutamyl moieties of **1**. Therefore, a comparative study of the hydrolytic behaviour of some γ-glutamyl hydrazides was performed.

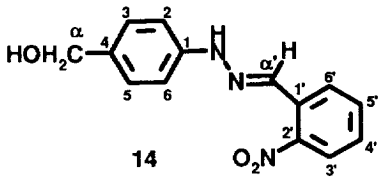
Hydrolysis of agaritine (1) and two other aromatic γ-glutamyl hydrazides

Hydrolytic cleavage, with 1–6 M HCl, of amide bonds, e.g., as in GHB (**6**, [16]), or of hydrazide linkages (see [37]), is a common method used in structural elucidation as well as in metabolic studies for the assessment of labelling patterns of such compounds. Upon hydrolytically treating **1**, however, this was found to be quickly destroyed even in dilute acid, thus confirming an earlier report [4]. In contrast to this, agaritane (**10**, *β-N*-(γ-glutamyl)-4-methylphenylhydrazine) and xanthodermine (**11**, *β-N*-(γ-glutamyl)-4-hydroxyphenylhydrazine [38]) were readily hydrolyzed with 1–6 M HCl, but stable in dilute acid (TLC-evidence). The unusual acid lability of **1** was confirmed by ^{1}H NMR spectroscopy. Thus, upon exposure of **1** to 0.1 M DCl, the aromatic signals disappeared within 24 hrs, whereas **10** showed no spectral changes under the same conditions [22]. Furthermore, there was no free glutamic acid detectable with **1**, as well as with **10** and **11** (TLC-evidence), unless the concentration of the acid and the temperature were raised to 1 M and 80°, respectively. With **10**, this procedure afforded >80% of the desglutamyl residue isolated as its 4-nitrobenzaldehyde hydrazone (**15**), with **1**, however, the yield was <10%.

The reason for the unusual behaviour of **1** is to be seen in its 4-hydroxymethyl functionality allowing the



10 R = CH_3
11 R = OH



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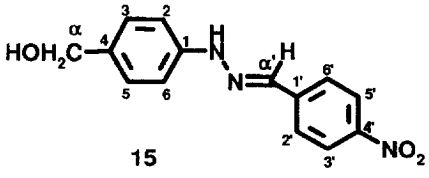

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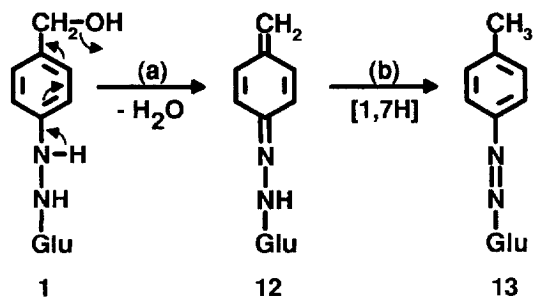
elimination of a water molecule across the aromatic ring [6]. The resulting intermediate could regain aromaticity through a 1,7-H-shift yielding the 4-(γ-glutamylazo)toluene **13** (Figure 2). Despite the mechanistically high plausibility of the reaction sequence presented in Fig. 2, there were, nevertheless, no ^{1}H NMR signals of **12** and **13** detectable, indicating that the concentrations of these are below detection limits and/or that **1** undergoes other destructive reactions as well, some of which may be due to the general reactivity of the *para* substituted hydroxymethyl group as well as the hydrazino functionality.

As chemical hydrolysis of agaritine to obtain its γ-glutamyl and aryl moieties intact from the same sample is, thus, not possible—since under mild conditions the former is not released, and under harsh conditions the latter is destroyed—the methodological basis of Schütte's biogenetic scheme for agaritine [31] is suspect. Enzymatic hydrolysis with GT [36, 39], however, resulted in the neat cleavage of the hydrazide linkage of **1** and **10**, yielding glutamic acid and the respective aryl moiety in stoichiometric amounts. It is, therefore, this procedure that served in the determination of labelling patterns in tracer experiments with **1**.



1 **12** **13**

Fig. 2. Reaction scheme for the protic transformation of agaritine (**1**) in aqueous solution. (a) Elimination of H_2O [6], and (b) re-aromatization of the intermediate, 4-glutamylhydrazono-1-methylene-cyclohexa-2,5-diene (**12**), through 1,7-H-shift, yielding 4-(γ-glutamylazo)toluene (**13**).

For the specific detection and quantitation of the desglutamyl part of **1**, two methods were considered, which are both widely used: (i) oxidation of the hydrazine with SeO_2, followed by coupling of the azo compound with 2-naphthol, and (ii) derivatization of the hydrazine to the nitrobenzaldehyde hydrazone. Since (i) yielded more than one product [22], procedure (ii) was chosen. Moreover, (ii) is a one-step-reaction, and the resulting hydrazones can easily be quantitatively extracted and analyzed by HPLC. Despite the simplicity of reaction (ii) to derivatize aromatic phenylhydrazines, compounds **14** and **15** have not been described hitherto, in contrast to their 4-carboxyphenyl and 4-methylphenyl analogues [40]. The spectroscopic properties of **14** and **15** are presented in Table 1. The UV/VIS and IR characteristics of the two new compounds are in good agreement with those of other nitrobenzaldehyde hydrazones [41, 42]. This also holds for the ^{1}H NMR spectra, which display the expected chemical shifts calculated from the increments for the substituents of **14** and **15** according to [43].

Since these hydrazones are intensely coloured, the corresponding benzaldehydes can conveniently be used as chromogenic agents. Using 4-nitrobenzaldehyde as a spray reagent for the determination of phenylhydrazines on TLC plates, the detection limit for 4-hydrazinobenzyl alcohol is 0.5–1 nmole. In addition, the reagent shows group specificity, inasmuch as it reacts only with aromatic hydrazines, producing a deep orange colour. This contrasts favourably with one of the most commonly used colorimetric methods for the determination of phenylhydrazines, namely, the one relying on 4-dimethylaminocinnamaldehyde, since this reacts also with aromatic amines, e.g., 4-ABA, aniline and 4-aminophenol as well as with the γ-glutamyl derivatives **1**, **3**, **10** and **11**.

*Synthesis of agaritine (**1**) and GHB (**6**) in the sporocarp of A. bisporus*

To test hypothesis (i) (see Introduction), fruit bodies detached from the substrate were administered various ^{14}C-labelled potential precursors through the stipe cut at the base, and the incorporation rates as well as the intramolecular distribution of the radioactivity between the two moieties of **1** was determined. The synthesis of the co-occurring established shikimate-derived metabolite GHB (**6**, [16]) was assessed simultaneously, as a positive control. As shown by the

Table 1. Spectroscopic properties of two new phenylhydrazones

Compound	UV (EtOH) λ_{max} nm (log ϵ)	IR (KBr) ν_{max} cm^{-1}	^1H NMR (200 MHz, CD_3OD, TMS as internal standard) δ-values
14	405 *sh* (4.0)	3600–3100 *m br*	8.23 (1H, *s*, H–C(α′))
	348 (4.15)	3290 *s*	8.22 (1H, *dxd*, J=7.4 and 1.4 Hz, H-3′)
	293 (3.97)	1615 *m*	7.92 (1H, *dxd*, J=8.1 and 1.0 Hz, H-6′)
	253 (4.24)	1570 *s*	7.64 (1H, *dxdxm*, J=8.1 and 7.3 Hz, H-4′)
	228 (4.25)	1540 *s*	7.41 (1H, *dxdxm*, J=8.0 and 7.5 Hz, H-5′)
		1510 *m*	7.3–7.1 (4H, AA′BB′, J_{AB}=8.7 Hz, H-(2,3,5,6)
		1495 *s*	4.51 (1H, *s*, H–C(α))
15	425 (4.39)	3600–3300 *m br*	8.3–7.7 (4H, AA′BB′, J_{AB}=8.8 Hz, H-(2′,3′,5′,6′)
	292 *sh* (4.06)	3220 *m*	7.80 (1H, *s*, H–C(α′))
	264 (4.20)	1610 *m*	7.3–7.1 (4H, AA′BB′, J_{AB}=8.7 Hz, H-(2,3,5,6)
		1595 *m*	4.52 (1H, *s*, H–C(α))
		1550 *s*	
		1540 *s*	
		1500 *s*	
		1460 *m*	
		1410 *m*	

Table 2. Incorporation of labelled precursors into agaritine (**1**) and GHB (**6**) in fruit bodies of *Agaricus bisporus*

Tracer substance	Precursor fed		Agaritine (**1**) isolated		GHB (**6**) isolated	
	Amount (μmol)	Sp. act (μCi/μmol)	Sp. act (μCi/μmol)	Dilution factor* (x)	Sp. act. (μCi/μmol)	Dilution factor* (x)
[U-^{14}C]Acetic acid	2.5	58	0.02	405	0.33	283
[U-^{14}C]Glucose	0.5	270	0.07	593	0.11	198
[U-^{14}C]Shikimic acid	0.4	21	0.002	2146	0.22	25
[U-*ring*-^{14}C]4-ABA	0.4	52	0.01	770	2.24	6
[*Carboxy*-^{14}C]4-ABA	0.2	57	0.001	2800	0.009	1326

* Corrected for different precursor amounts and the different pool sizes of **1** and **6**. Representative data are given from one out of 3 independent experiments.

Table 3. Distribution of radioactivity in ^{14}C-labelled agaritine (**1**) and GHB (**6**) isolated following administration of various precursors to *A. bisporus* fruit bodies*

Tracer substance	Agaritine (**1**)		GHB (**6**) (control)	
	Proportion (%) of label found in			
	4-Hydrazino-benzyl alcohol	Glutamic acid	4-Amino-phenol	Glutamic acid
[U-^{14}C]Acetic acid	1	99	18	82
[U-^{14}C]Glucose	1	99	43	57
[U-^{14}C]Shikimic acid	nd†	nd†	99	1
[U-*ring*-^{14}C]4-ABA	nd†	nd†	99	1
[*Carboxy*-^{14}C]4-ABA	nd†	nd†	nd†	nd†

* For incorporation rates, see Table 2. † nd, not determined: The specific activity was too low for determination of labelling pattern.

results presented in Tables 2 and 3, the mushroom fruit body synthesizes the desglutamyl moiety of agaritine (**1**) neither by the acetate-malonate nor the shikimate pathway (were there to occur synthesis at all of the aromatic residue of **1** at this location), since the specific activity (sp. act) of isolated **1** is always very low. With carboxy-labelled 4-ABA, i.e. with the compound that, on Schütte's hypothesis, would be

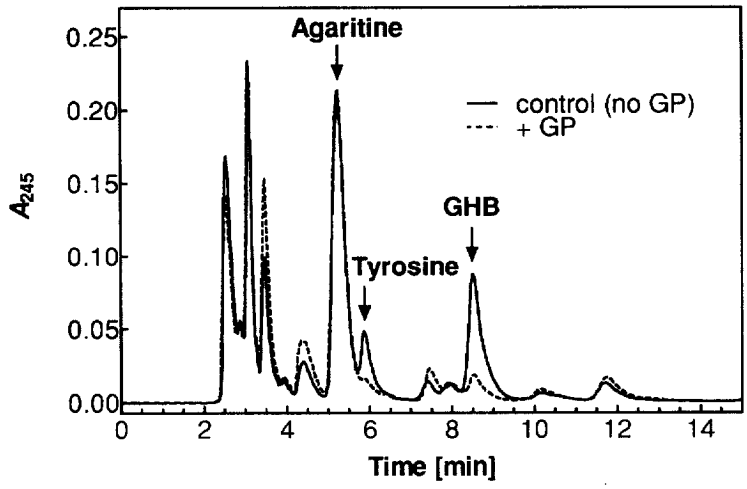


Fig. 3. Inhibition of the accumulation of GHB (**6**), but not that of agaritine (**1**), by the shikimic acid pathway inhibitor *N*-(phosphonomethyl)glycine (glyphosate®, GP) in *A. bisporus* fruit bodies growing on GP-supplemented casing soil under conditions of commercial mushroom cultivation (for methodological details, see Experimental).

expected to have the same incorporation rate as ring-labelled 4-ABA, the sp. act was zero (the tiny amount of ^{14}C incorporated into **1** from this tracer substance can easily be attributed to randomization of the label). Moreover, in the two cases yielding agaritine labelled sufficiently to locate the radioactivity within the molecule, ^{14}C was situated only in the γ-glutamyl moiety of the isolated product. This is in sharp contrast to the situation observed with GHB (**6**) in the same hyphae, which unequivocally displays the biogenetic behaviour of a metabolite derived from shikimate *via* 4-ABA, as demonstrated previously using the same experimental set-up as followed in the present study [16, 23]. Unpublished data of another group who also could not detect incorporation of labelled 4-ABA into **1** (K. Sasaoka, pers. letter communication), but in co-occurring **6** [23], further support our results. These, together with the methodological arguments presented in the above section, strongly suggest that the compound whose synthesis the biogenetic scheme of Schütte *et al.* [31] is referring to is in all probability GHB (**6**) and that there is no *de novo* synthesis of the aromatic residue of **1** in the *A. bisporus* fruit body.

This conclusion gets additional confirmation from experiments using N-(phosphonomethyl)glycine (glyphosate®, GP) as a tool, which is an inhibitor of 5-enol-pyruvoylshikimate-3-P synthase that catalyzes a step in the conversion of shikimate to chorismate [44, 45]. 4-ABA is derived from this by amination, the amido group of glutamine acting as the donor ([28, 46–48]; for *A. bisporus*, see [49]). According to the above conclusion, application of GP to growing fruit bodies should, therefore, lead to a reduction of their content of **6**, but not of **1**. This, indeed, is what was observed (Figure 3). The similar effect of GP on the pool size of chorismate-derived tyrosine as on GHB is in keeping with this result and, thus, with the hypothesis of a plainly different biosynthetic origin of the desglutamyl moieties of **1** and **6** in the sporocarp. This does, of course, not discount the possibility that the reversible γ-glutamyl transfer catalyzed by AGT *in vitro* between the two components (see Introduction) may have its *in vivo* counterpart as a 'metabolic shuttle' for γ-glutamyl units [32, 36].

The substrate mycelium as the site of synthesis of agaritine (1) in A. bisporus

The results shown in the above section clearly suggest that **1** present in the sporocarp is a translocate from the vegetative hyphae. Although alone not constituting strict proof of this conjecture, i.e. of hypothesis (ii) as stated in Introduction, the consistent increase observed of the pool sizes of agaritine in fruit bodies connected with substrate mycelium, in contrast to those of sporocarps detached from the mushroom bed (Table 4), fits the proposition. To elaborate on the quantitative differences in the pool sizes of individual sporocarps appears not meaningful, because even mushrooms growing on the same bed and having the same morphological appearance are unlikely to be physiologically identical, because the population density, the frequency of clusters, the position of individuals within these and also other factors always vary to some extent. Since leakage of **1** into the water was found to be negligible (data not shown), its decrease in post-harvest mushrooms must be due to catabolism. This most probably embodies an oxidative transformation of the desglutamyl moiety of **1**, occurring either on the intact molecule [22] or following removal of the glutamyl residue [10]. It remains to be established whether incorporation of **1** into melanin (as has been shown to occur with **6** [16, 24, 50]) and/or cleavage of the aromatic ring (as efficiently performed by the *A. bisporus* fruit body with benzoic acid as the substrate [16]), contribute to the observed decline of

Table 4. Changes in the pool sizes of agaritine (**1**) in *A. bisporus* fruit bodies during growth on compost (a) and with the cut stipes dipped in water (b)*

No. of Exp.	Pool size of agaritine (**1**) [mg (%)] per sporocarp following treatment		
	none	(a)	(b)
1	1.85 (100)	3.14 (170)	0.90 (49)
2	1.68	2.61 (155)	1.19 (71)
3	2.05	4.10 (200)	1.33 (65)
4	1.90	3.42 (180)	1.16 (61)

* Twelve stage-2-sporocarps growing on a mushroom bed were selected, four of which were collected and analyzed immediately (zero control; $t = 0$). The others were kept, respectively, under conditions (a) and (b) for 24 hrs.

agaritine in sporocarps detached from the nutrient medium.

For the localization of the site of agaritine synthesis in the vegetative mycelial system colonizing the substratum, rhizomorphs were tested for their ability to perform *de novo* synthesis of **1** (again, with **6** as the control). To this purpose, intact sporocarps with attached pseudorhiza were fed [U-^{14}C]glucose, conditions otherwise being the same as used for the tracer experiments with fruit bodies cut from the mushroom bed at the base of the stipe (see above). At the end of the metabolic time, the rhizomorphs were removed and analyzed. The sp. activities of the isolated products amounted to 0.06 and 0.5 μCi μmole^{-1} for **1** and **6**, respectively; and the dilution factors were 3546 and 303 ×. The sample size was too small to locate ^{14}C in **1**, but sufficient for that purpose for **6**, where 70% of the radioactivity resided in the desglutamyl moiety. The rhizomorph contents of **1** and **6** were 2.1 and 2.6 mg g^{-1} dry wt, respectively, values which, thus, lay well within the range given by other authors [21]. The outcome of this experiment, taken together with the data presented above (Tables 2–4), points to the thin hyphae in tight contact with the solid-state fermentation medium as the likely site of agaritine synthesis within the vegetative mycelium–fruit body plectenchyme continuum.

The compost-casing soil unit colonized by *A. bisporus* represents a complex ecosystem, the biosphere of which encompasses other fungi as well as bacteria [51]. Considering (i) the small contribution of the mushroom mycelium to the total compost biomass even at the end of the growth phase (cf. [52]), (ii) the strong adsorptive nature of humic materials (for mechanisms of binding, see [53]), (iii) the high chemical reactivity of humic acids from compost, peat and similar sources that is due to the presence of permanent organic free radicals [54, 55], which react especially well with phenols and compounds akin to them, whereby these are covalently linked to the humic polymer [56, 57], and (iv) the fact that **1** is a good substrate of the phenoloxidases produced, and (partly) exuded into the medium by *A. bisporus* [22, 34], the chances to reliably quantify agaritine in compost and casing-soil samples are low.

Attempts were made, nevertheless, to probe for the presence of **1** as well as of potential precursors of its desglutamyl moiety, for which phenylhydrazine (PH) and 4-hydrazinobenzoic acid (**4**) were chosen as model compounds. The sample series included crude press juices and the aqueous supernatants of homogenates from compost and from casing soil, prepared before and after colonization with mushroom hyphae, with or without inhibitors of phenol-oxidizing enzymes. However, in no case was there found any agaritine or free phenylhydrazine. Further, neither of the three compounds could be detected following addition to the extraction medium in amounts 10 times exceeding the detection limits of the HPLC and the TLC/specific colour reaction used in the present study. Hyphae not contaminated by compost and, yet, fully dependent on this as the nutrient source were obtained by cultivating the fungus in a 'sandwich' arrangement of the compost, the central zone of which consisted of a layer of glass beads (see Experimental for details), through which the hyphae grew upwards from the spawned bottom layer into the non-colonized top layer (in the insert, they preferentially closely clinged to the beads). The agaritine content of the 'sandwich' mycelium was in the range of 1 mg g^{-1} dry wt (U. Wäspi and D. M. Rast, unpublished data; [58]).

The inability of the *A. bisporus* sporocarp to generate **1** *de novo* (Tables 2–3; Fig. 3) and the decline of **1** in fruit body hyphae detached from the compost/casing soil unit (Table 4), together with the restricted systematic distribution of the compound even within the genus *Agaricus*, leave no doubt that the agaritine located in the fruit body arrives there by translocation, and that its assembly in the fungus reflects a specific metabolic activity of the vegetative mycelium. This is not to postulate, however, that the substrate hyphae would necessarily be self-sufficient for the synthesis of the desglutamyl moiety of **1**. The aryl partial structure could as well be considered to be derived from the wheat straw lignin component of the compost [hypothesis (iii) of Introduction], as has long been suggested to hold for some other simple aromatic secondary metabolites found in fruit bodies of lignin-degrading basidiomycetes [59]. The lignin of wheat straw, as that of any other grass litter, contains

a sizeable proportion of 4-hydroxycinnamyl alcohol residues that, upon oxidation by enzymes of lignin-degrading fungi, give rise to C_6–C_1 compounds, of which 4-hydroxybenzoic acid (4-HBA) is mentioned most often (see [60]). As any other lignin-degrading fungus, *A. bisporus* can be supposed to have a transporter for the uptake of 4-HBA; this is fairly unspecific [61].

The origin of the hydrazino functionality and the step of its introduction within the pathway leading to the assembly of **1** upon the 4-HBA foundation can only be speculated upon. Theoretically, two mechanisms may be considered whereby the N–N partial structure of agaritine could be generated, i.e. (i) oxidative coupling of amines following generation of phenolic radicals by a phenol-oxidizing enzyme [57, 62] and (ii) fixation of nitrogen through the nitrogenase system (see [63] for a minireview). However, there is no evidence that either reaction is used by *A. bisporus* for the synthesis of **1**, since (i) none of the phenoloxidases of the fungus [34] display the required high substrate specificity, such as to restrict the number of coupling products to basically only one simple low-molecular-weight metabolite—on the contrary, the end products of oxidative coupling reactions are often mixed polymers, and since (ii) the existence of a diazotrophic fungus would represent a novelty, indeed. Considering components of the consortium of microorganisms present in wheat straw compost [51, 64, 65] that would be most suitable to yield the hydrazino partial structure, the idea appears not far-fetched, that *A. bisporus* is provided with **4** by a physically tightly associated nitrogen-fixing commensal (e.g. *Pseudomonas*) that uses 4-HBA resulting from lignin degradation by the fungus as an acceptor for the N–N structure generated by incomplete reduction of dinitrogen. Following uptake by the fungus, **4** could be transformed into agaritinic acid (**3**) by derivatization with glutamate through the action of AGT, which displays the appropriate substrate specificity [32, 36]. Chemically, acylation is a general procedure to protect reactive -OH and -NH_2 substituents; γ-glutamylation represents just a particular case thereof. Biochemically, γ-glutamylation is regarded as a detoxification reaction. Since free hydrazines are highly toxic (lit. cit's in [66, 67]), conjugation of **4** with glutamate, therefore, can confer stability not only to the parent phenylhydrazine structure, but also prevent this from causing harmful effects to the hyphal cells exposed to it. It is mainly for this reason that the reduction of the carboxyl to the benzylalcohol group is proposed to occur with (**3**) and not with (**4**), because 4-hydrazinobenzyl alcohol is quite susceptible to oxidation [68]. The existence in *A. bisporus* of detectable amounts of **3** [8] and **2** [7] represents additional support for this opinion. In any case, the reduction of the phenolic carboxyl to the benzylalcohol group is a simple reaction sequence that has been shown to be performed by many fungi, including lignin-degraders [69–71].

Disregarding cellular compartmentation phenomena, the new scheme presented for the synthesis of **1** encompasses reactions/enzymes that are common to large groups of organisms—with one exception, the AGT-catalyzed transfer of a glutamyl residue to a phenylhydrazine [36]. Yet, the systematic distribution of **1** within fungi is restricted to only some *Agaricus* species [1, 2]. This even holds if the synthesis of xanthodermine (**11**), the 4-hydroxy analogue of **1** synthesized by *Agaricus xanthoderma* [38], is considered but a variation of the scheme, as AGT is not specific for the type of phenylhydrazine it binds as an acceptor [36]. The cause enabling nature to elaborate the exclusive agaritine entity must, therefore, be sought at a higher level of biological hierarchy, that of biochemical ecology. It is by a combination [(i)–(iv)] of specific metabolic activities of representatives of three biologically largely different groups of organisms that the rare natural γ-glutamylphenylhydrazine **1** is assembled: (i) the synthesis of cinnamyl units through the shikimic acid pathway by a grass (*Triticum*) and polymerization of such C_6–C_3 building blocks into recalcitrant polymeric biomass that is accessible only to microbial specialists; (ii) degradation of this by a lignin-degrading fungus through special oxidative exoenzymes (*Agaricus*); (iii) interaction of a diazotroph with a monomeric product of lignin breakdown (e.g. *Pseudomonas*) such as to generate a phenylhydrazine; and (iv) specific γ-glutamylation of the bacterial metabolite by a fungus (*Agaricus*).

EXPERIMENTAL

Fungus material

Fruit bodies of *A. bisporus* were produced under conditions of commercial mushroom growing with short-composted horse manure on wheat straw basis as the substrate and a peat/limestone/$CaCO_3$ mixt. as the casing material. Unless stated otherwise, stage-2-sporocarps [72] served for experimentation. Compost-free vegetative mycelium was obtained by inclusion of a 2 cm layer of glass beads (3 mm diameter) into the substrate contained in plexiglass trays ($50 \times 50 \times 20$ cm), the insert being sepd from the compost by tightly fitting framed stainless steel nets with mesh size 2×2 mm (modified from [73] and [34]). The thickness of the compost layers was 7 cm. Harvesting was 25 d after spawning. *N*-(phosphonomethyl)glycine (glyphosate™, GP) was supplied to these cultures with the water, in a formal concn of 4 mM.

Administration of radioactive compounds

The freshly harvested fruit bodies (cap diam. 1.2–1.7 cm, *ca* 1.5 g fr. wt) were handled as described before [16].

Isolation and quantitation of agaritine (1)

Freshly picked mushrooms were minced in a mortar containing liquid N_2 and the small pieces extracted with 95% MeOH supplemented with 20 μM salicylhydroxamic acid (an inhibitor of phenolase [74]) in a Sorvall Omni-Mixer. After centrifugation (15 000 *g*, 20 min), washing of the sediment with 95% MeOH, and a further centrifugation step, the supernatants were combined and evapd to dryness. The residue was dissolved in water and filtered (0.45 μm) prior to HPLC analysis. The recovery of added agaritine (**1**) was 96–99%.

Hydrolysis of γ-glutamyl derivatives

For chemical hydrolysis, aq. solns of the compounds were repeatedly degassed and satd with Ar. The reaction was started by adding HCl to a final concn of 0.1–6 M, and the mixt. kept at 27° or 80°. For enzymatic hydrolysis, the compounds were incubated for 12 hrs at 25° with γ-glutamyltranspeptidase [GT, EC 2.3.2.2; 16 nkat (μmole of substrate)$^{-1}$] in Tris buffer (0.1 M, pH 8.1) containing 15 mM $CaCl_2 \times 6\ H_2O$, according to [39]. For the quantitative isolation of the desglutamyl part of **1**, 4-nitrobenzaldehyde (1.1 mole eq., 0.3 M in MeOH) was added at the end of the incubation time and the mixt. kept several hrs at 4° for crystallization. The red needles were collected by filtration, washed with ice-cold EtOH and dried. Yields were calculated using spectroscopic quantitation at 430 nm or HPLC analysis.

Determination of the labelling pattern of agaritine (1) and GHB (6)

The crude extract of the fungus material was prepared as described above. **1** and **6** were separated on a Sephadex LH-20 column (1.6 × 90 cm) using water as the eluent (15 ml hr^{-1}). Further purification of **1** and **6** was achieved by chromatography on Lobar B (RP18), resulting in 98% pure preparations of **1** and **6**. The radioactivity in the glutamyl and the aryl residue was determined using HPLC analysis of the enzymatic hydrolyzate of **1**, and the chemical hydrolyzate of **6** [16].

Synthesis of 4-(2-nitrobenzylidene)hydrazinobenzyl alcohol (14) and 4-(4-nitrobenzylidene)hydrazinobenzyl alcohol (15)

To obtain **14**, equivalent amounts of 4-hydrazinobenzyl alcohol (prepared according to [68]) and 2-nitrobenzaldehyde in MeOH were mixed and heated to 60° for 10 min. The solid hydrazone **14** precipitated as the soln was cooled to 4°. Recrystallization from EtOH gave **14** (77% yield) as garnet-coloured needles with mp 174–175°. TLC (solvent B): R_f 0.54 (dark-orange spot). Found: C, 61.92; H, 4.87; N, 15.74. $C_{14}H_{13}N_3O_3$ (271.26) requires: C, 61.98; H, 4.83; N, 15.49. For spectral data, see Tab. 1. To obtain **15**, equimolar solns of 4-nitrobenzaldehyde and 4-hydrazinobenzyl alcohol (prepared according to [68]) in MeOH were heated under reflux until the hydrazine educt was completely reacted upon (TLC-evidence). The resulting soln was left for crystallization at 4° overnight. Recrystallization from EtOH yielded **15** (67%) as ruby-coloured needles with mp 155–156°. TLC (solvent B): R_f 0.48 (orange-red spot). Found: C, 61.96; H, 5.05; N, 15.31. $C_{14}H_{13}N_3O_3$ (271.26) requires: C, 61.98; H, 4.83; N, 15.49. For spectral data see Tab. 1.

Chromatography

Analytical TLC was performed on Silica gel 60 F_{254} (Merck 5554) with (A) *n*-BuOH–HOAc–H_2O (12:3:5) or (B) CH_2Cl_2–MeOH (10:1) as the mobile phase. Detection was under UV light at 254 nm. The following solns were used as group specific spray reagents: ninhydrin (0.3% in *n*-BuOH–HOAc (97:3)), 4-nitrobenzaldehyde (60 mM in MeOH–H_2O (4:1)), 4-dimethylaminocinnamaldehyde (60 mM in MeOH–H_2O (4:1), [2]). For HPLC analysis, the samples were separated on a Nucleosil 5C18 column (4 × 250 mm) with 0.9 ml min^{-1} of the eluent. For mushroom metabolites this consisted of 98% MeOH containing 50 mM KH_2PO_4, and of 80% CH_3CN for the hydrazones **14** and **15**. Identification and quantitation of peaks was effected with a diode array detector (HP 1040A) and the Hewlett–Packard Chemstation software, using authentic compounds for calibration. For **1**, **6** and **10** the detection limit was about 5 pmol μl^{-1}.

Spectroscopy

Spectra were taken on an Uvikon 810 (UV), a Perkin–Elmer 781 (IR, all bands above 1400 cm^{-1} with an intensity >10% of the background are reported) and a Varian XL 200 (^{1}H NMR, the number of protons in the assignment corresponds within ±10% to the integration).

Chemicals and biochemicals **1**, **6** and **10** were synthesized as described previously [13, 22, 68]. **11** was a gift from Prof. W. Steglich. As checked by HPLC, the purity of the products was >99%. Tracer substances were purchased from Amersham and New England Nuclear, glyphosate® from Riedel deHaën and γ-glutamyl transpeptidase (bovine kidney) from Sigma. Other chemicals were *puriss p.a.* from Fluka.

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