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Department of Chemistry, Faculty of Science, Kyoto University, Kyoto (Japan) KOICHIRO SUMIZU

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An aromatic diazonium compound in the mushroom *Agaricus bisporu*

The isolation, structure, and synthesis of agaritine (I), a unique phenylhydrazine derivative of L-glutamic acid from basidiomycetes of the family Agaricaceae, has recently been reported[1,2]. As a prelude to studies on the enzymic synthesis of this substance, it was essential to determine relative levels of agaritine within successive sections of the stipe and fruiting cap of *Agaricus bisporus*. Routine analyses were based on the observation[3] that acidic solutions of β-*N*-acyl-phenylhydrazines very slowly form typical azo chromophores with *N*-1-naphthylethylenediamine, the coupling reagent employed in the BRATTON–MARSHALL test for arylamines[4]. On examining extracts prepared from serial sections of basidiomycete tissue (Fig. 1), a coupling response was observed which, in its rapidity, was clearly more characteristic of a

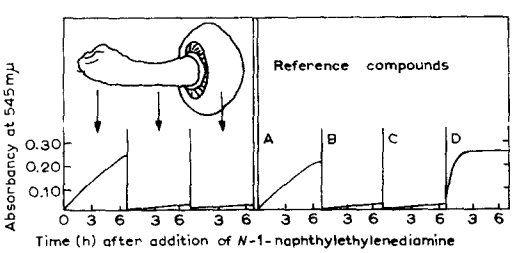


Fig. 1. Azo-coupling responses in extracts of carpophore segments and with certain arylhydrazine derivatives and diazotized arylamines. Sections of uniform volume, free of adhering soil particles, were cut from the indicated portions of the carpophore, suspended in 1.0 ml of cold 0.07 *N* HCl in a thick-walled centrifuge tube, and disrupted with a loose-fitting glass homogenizer. After centrifugation, an aliquot of the clear supernatant fluid was diluted to 0.80 ml with 0.05 *N* HCl and treated at 23° with 0.10 ml of 0.2 % coupler solution. The rate of formation of the arylazo dye in extracts from the base of the stalk was undiminished on including 1 % ammonium sulfamate in the dilute acidic medium used for tissue disintegration. Reference compounds: A, diazotized *p*-aminobenzyl alcohol; B, *p*-tolylhydrazine; C, agaritine; D, diazotized *p*-aminobenzoic acid.

fully oxidized aryldiazonium ion rather than a hydrazide or hydrazine moiety. This "fast-coupling" substance was confined chiefly to the basal portion of the stipe; it could not be detected at all in extracts of the cap, the tissue highest in agaritine content. Azo coupling with extracts from the base occurred at a rate resembling most closely that of diazotized *p*-aminobenzyl alcohol. In this report, evidence is presented in support of II as the structure of the aromatic diazo derivative present in *A. bisporus*.

Identification of arylazo product. In Table I R_F values are given in several solvent systems for arylazo compounds synthesized from the couplers *N*,*N*-dimethylaniline and *N*-1-naphthylethylenediamine. Chromophore development was allowed to proceed for 30 min at 27°, after which the derivatives were extracted into ether from strongly alkaline solution, re-extracted into dilute, aqueous HCl, and finally returned to fresh ether under basic conditions. For purposes of comparison, and to illustrate the resolving power of the solvents employed, R_F values are included for the corresponding arylazo compounds formed from diazotized *p*-toluidine. For both types of azo derivatives produced in this way from concentrates of the basal-stalk extract, R_F values and hues* were indistinguishable from those of the authentic coupled *p*-hydroxymethylbenzene diazonium ion.

TABLE I

PAPER CHROMATOGRAPHY OF ARYLAZO DERIVATIVES

Chromatographic solvents: I, ethanol – 0.05 *M* ammonium acetate (1:1); II, *n*-butanol – pyridine – water (210:35:45); III, *n*-butanol – acetic acid – water (18:2:5); IV, ethanol – 0.05 *M* potassium phosphate buffer, pH 6.5 (35:65); V, 8% aq. NaCl – acetic acid (100:1).

Aryldiazonium compound derived from:	*R_F of arylazo compound formed by coupling with:*					
	*Naphthylethylenediamine**			*Dimethylaniline**		
	Solvent					
	I	*II*	*III*	*I*	*IV*	*V*
A. bisporus, basal tissue	0.60	0.53	0.63	0.75	0.31	0.30
p-Aminobenzyl alcohol	0.61	0.53	0.62	0.75	0.31	0.29
p-Toluidine	0.32	0.69	0.80	0.62	0.12	0.07

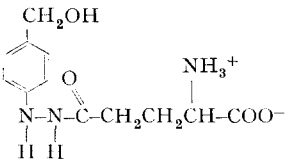
* The reaction with naphthylethylenediamine was performed in 0.05 *N* HCl; that with dimethylalanine was done at pH 7 in dil. sodium acetate buffer. Chromatography was carried out in a descending manner on Whatman No. 3 papers. Azo dye-colors were developed by spraying chromatograms with ethanol–1 *N* HCl (1:1).

Hydrolysis of mushroom aryldiazonium compound to p-hydroxybenzyl alcohol. Aromatic diazo compounds are converted to phenols when heated with mineral acid[5]. When extracts of the basal portion of the mushroom stalk were treated with 0.25 *N* HCl for 2 min at 100° almost complete disappearance of the azo-coupling response occurred. The hydrolysate was extracted with ether several times and the combined ethereal fractions were evaporated. Portions were chromatographed on paper[6] and phenols were visualized with the spray reagents of AMES AND MITCHELL[7]. Control samples, consisting of aliquots in which acid hydrolysis was not carried out, or in

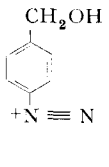
* The blue and violet hues of the arylazo compounds derived from coupling of the isomeric diazotized monomethyl or monohydroxymethyl anilines with naphthylethylenediamine are each characteristically distinguishable from one another on paper chromatograms by simple visual observation.

which extracts of the cap and upper portion of the stipe were used in place of the basal-stalk fraction, were also subjected to analysis. Additional controls, carried through the entire procedure, included solutions of *o*- and *p*-tolylhydrazine, I, *p*-aminobenzyl alcohol, and diazotized *p*-aminobenzyl alcohol. Only in the latter case and in the basal-stalk extract did a phenol spot appear. In each instance the phenolic derivative migrated with the same R_F and gave the same orange-yellow hue as an authentic sample of *p*-hydroxybenzyl alcohol. Furthermore, when the arylazobenzene-*p*-sulfonate spots were eluted and chromatographed according to CHANG *et al.*[8], their R_F values were indistinguishable from that of authentic 4-hydroxyphenylazobenzene-*p*-sulfonic acid.

Although these results provide evidence for the occurrence of *p*-hydroxymethylbenzene diazonium ion in basal-stalk sections of the carpophore, we can only speculate at present on its involvement in the metabolism of agaritine. It is equally as likely



I
Agaritine



II
p-Hydroxymethylbenzene diazonium ion

that II may arise by an enzymically catalyzed diazotization process (to consider just one of several possibilities) as that it be formed by chemical or enzymic oxidation of a related arylhydrazine*. Further investigations in progress in this laboratory on the mode of formation of the N–N bond of I should offer more direct information on this point.

The mushrooms used in this investigation (*Agaricus bisporus*, strain 303) were obtained from the Michigan Mushroom Co., Niles, Mich. (U.S.A.). *p*-Aminobenzyl alcohol was synthesized by reduction of *p*-nitrobenzyl alcohol essentially as described[9]. This study was supported by Grant E-2966 from the National Institutes of Health, U.S. Public Health Service.

Department of Biological Chemistry, BRUCE LEVENBERG**
The University of Michigan, Ann Arbor, Mich. (U.S.A.)

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* An enzyme present in extracts of this organism cleaves I to *p*-hydroxymethylphenylhydrazine and L-glutamic acid[1].

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