Agaritine: Isolation, Degradation, and Synthesis[1]

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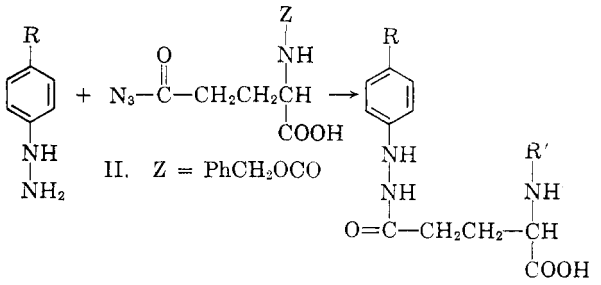
Agaritine has been isolated from *Agaricus bisporus* by an improved procedure and the proposed structure, L-glutamic acid γ-(α-hydroxy-*p*-tolylhydrazide) (IIIa), confirmed by degradation and synthesis.

Recently Levenberg[2] reported the isolation of agaritine from *Agaricus bisporus*, the commercial mushroom, for which he proposed structure IIIa. This novel derivative of L-glutamic acid occurs predominantly in the fruiting body of young mushrooms and the concentration diminishes with age.[3] Levenberg[2] also observed that glutamic acid is freed from agaritine by an enzyme present in *A. bisporus*.



II. Z = $PhCH_2OCO$

Ia. R = H
b. R = CH_3
c. R = CH_2OH
d. R = $COOCH_3$

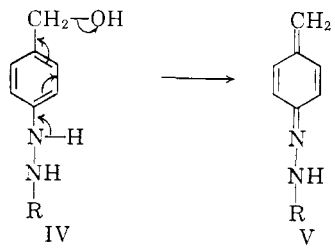
IIIa. R = CH_2OH, R′ = H
b. R = H, R′ = $PhCH_2OCO$
c. R = CH_3, R′ = $PhCH_2OCO$
d. R = R′ = H
e. R = CH_3, R′ = H

By an improved extraction procedure, followed by ion exchange chromatography, we have isolated agaritine from mushroom sporophores in *ca.* 0.04% yield (based on fresh tissue) and we have confirmed its structure as IIIa by degradation and synthesis.

The n.m.r. spectrum of agaritine indicated the presence of a *p*-disubstituted benzene ring with a CH_2OH group as one of the substituents. In accordance with the n.m.r. spectrum and the proposed structure, oxidation of agaritine with ferric chloride[4] yielded glutamic acid and benzyl alcohol with the concomitant evolution of nitrogen.

Since agaritine is stable only at pH values near neutrality, a synthesis of γ-phenylhydrazides of L-glutamic acid employing mild and essentially neutral conditions was required. For this purpose the condensation of the γ-azide of *N*-carbobenzoxy-L-glutamic acid (II) with a phenylhydrazine in cold ether was found to be appropriate. With phenylhydrazine (Ia) and *p*-tolylhydrazine (Ib), yields of up to 70% of the substituted analogs IIIb and IIIc, respectively, were obtained.

The procurement of α-hydroxy-*p*-tolylhydrazine[5] (Ic) required for the synthesis of agaritine, however, was complicated by its extreme sensitivity. This sensitivity, and that of agaritine (IV. R = γ-glutamyl), is assumed to be due to the facile elimination of water across the benzene ring (IV → V). The isolation of *p*-tolylhydrazine in 41% yield after reduction of *p*-carboxymethylphenylhydrazine (Id) with lithium aluminum hydride in boiling ether can be regarded as good evidence for the ease of operation of the elimination IV→V, especially since, with related aromatic carbonyl compounds, much more severe conditions are required for the lithium aluminum hydride reduction and hydrogenolysis of the resulting benzyl alcohol.[6] The reduction is envisaged as proceeding through the



IV V

desired alcohol (IV) which undergoes elimination of water to give the intermediate V. The latter is then reduced to *p*-tolylhydrazine.[7,8]

Some α-hydroxy-*p*-tolylhydrazine was finally obtained by reduction of *p*-carboxymethylphenylhydrazine (Id) with lithium aluminum hydride for a short time (90 min.) in boiling ether. But it was not until a technique was devised whereby the reduction mixture could be worked up under essentially neutral conditions that any success was achieved. This technique involved the decomposition of excess lithium aluminum hydride by the addition of a small quantity of saturated

(1) A preliminary account of this work has been published by the authors, *J. Am. Chem. Soc.*, **83**, 3333 (1961).
(2) B. Levenberg, *ibid.*, **83**, 503 (1961).
(3) B. Levenberg, private communication.
(4) H. B. Milne, J. E. Halvon, D. S. Ho, and M. S. Mason, *J. Am. Chem. Soc.*, **79**, 637 (1957).
(5) No account of the isolation and characterization of this compound could be found in the literature.
(6) Norman S. Gaylord, "Reduction with Complex Metal Hydrides," Interscience Publishers, Inc., New York, 1956. See especially Chap. 16 for lead references; see also ref. 7.
(7) *Cf.* L. H. Conover and D. S. Tarbell, *J. Am. Chem. Soc.*, **72**, 3586 (1950).
(8) *Cf.* the reduction of vinylogous amides with lithium aluminum hydride. See ref. 6, also J. Szmuszkovicz, *ibid.*, **82**, 1180 (1960).

sodium chloride solution and removal by filtration of inorganic salts, which, by hydration, acted as a drying agent. Since no satisfactory method was found for the isolation and purification of α-hydroxy-*p*-tolylhydrazine, the filtrate was treated directly with a cold ethereal solution of carbobenzoxy-L-glutamic γ-azide (II).

Decarbobenzoxylation was effected by hydrogenolysis under conditions whereby the benzyl alcohol group of agaritine was not hydrogenolyzed. In the case of the substituted phenyl analog (IIIb) and the *p*-tolyl analog (IIIc), the respective decarbobenzoxylated compounds, L-glutamic acid γ-phenylhydrazide (IIId) and L-glutamic acid γ-(*p*-tolylhydrazide) (IIIe), were crystallized without further purification. The crude agaritine was purified by chromatography over Dowex-50 resin. Spectral analysis of the effluent indicated a 6% yield of agaritine (based on the carbobenzoxy-L-glutamic acid γ-hydrazide from which the azide II was prepared), half of which was isolated in pure crystalline form.[9]

Agaritine is quantitatively converted to the *p*-tolyl analog (IIIe) by prolonged catalytic hydrogenation. This conversion constitutes an independent confirmation of IIIa as the structure of agaritine.

So far as we know, agaritine represents the first reported occurrence of a phenylhydrazine derivative in natural products and its discovery may have interesting biogenetic implications.

Experimental

Isolation of Agaritine.—Sporophores from 2- to 3-day-old mushrooms[10] (2.9 kg.) were homogenized in 5.6 l. of methanol at 4–10° for 1 min. in a Waring Blendor. After standing at room temperature for 30 min., 80 g. of Celite was added and the mixture was filtered. The filtrate was concentrated *in vacuo* to a volume of 2.0 l. The concentrate was applied to a column (7.5 × 45 cm.) of Dowex-2 (X-8, Ac^-) resin and the column was eluted with water at the rate of 600 ml./hr. The effluent volume between 1360 ml. and 3700 ml., having the absorption at 240 mμ > the absorption at 260 mμ, was collected and adjusted to pH 7.0 with 1 *N* sodium hydroxide solution. This was passed through a column (10 × 60 cm.) of Dowex-50 (X-4, NH_4^+) resin and the column was eluted with water at the rate of 800 ml./hr. The agaritine-containing fractions, selected by ultraviolet absorption, were concentrated *in vacuo* at 38° to 50 ml., 50 ml. of ethanol and 200 ml. of *n*-butyl alcohol were added, and the resulting solution was evaporated *in vacuo* to incipient turbidity. This solution, left at 4°, deposited 1.037 g. of crystalline agaritine. After recrystallization, m.p. 205–208° (dec.), λ_{max}^{water} 237 mμ (ε 12,000) and 280 mμ (ε 1400), $[\alpha]^{25}D$ + 7° (*c*, 0.8 in water) and infrared spectrum in agreement with IIIa.

Anal. Calcd. for $C_{12}H_{17}O_4N_3$: C, 53.92; H, 6.41; N, 15.72; NH_2, 5.2. Found: C, 53.77; H, 6.60; N, 15.47; NH_2, 5.2.

Ferric Chloride Oxidation of Agaritine.—A solution of 404 mg. of agaritine in 10 ml. of water was treated dropwise at 38° with a 20% solution of ferric chloride hexahydrate until the rapid evolution of nitrogen had ceased (3.5 ml. of ferric chloride solution). The solution was extracted with three 10-ml. portions of benzene. Vapor phase chromatography of a portion of the benzene extract showed the presence of a single component indistinguishable from benzyl alcohol.

A *p*-nitrophenylurethane derivative (33 mg.) was prepared from the remainder of the benzene extract. It had m.p. 155–157° and was identical with an authentic sample of the *p*-nitrophenylurethane derivative of benzyl alcohol (m.p., mixed m.p., and infrared spectrum).

Glutamic acid was detected in the extracted aqueous solution (above) by paper chromatography.

N-Carbobenzoxy-L-glutamic Acid γ-Phenylhydrazide (IIIb).—A solution of 54.4 g. (0.18 mole) of N-carbobenzoxy-L-glutamic acid γ-hydrazide[11] in 300 ml. of 3 *N* hydrochloric acid and 100 ml. of water was cooled on an ice bath, then covered with 1 l. of cold (−10°) ether. A cold solution of 15.2 g. (0.22 mole) of sodium nitrite in 100 ml. of water was added to the cold stirred mixture over a period of 15 min. The lower layer was separated and extracted with two 1-l. portions of cold (−10°) ether. The ether extracts were quickly washed with two 500-ml. portions of cold water, combined, and dried briefly over sodium sulfate. The dried ice-cold ether extract was added portionwise with stirring over a period of 20 min. to 43.2 g. (0.40 mole) of phenylhydrazine in 500 ml. of ether cooled in an ice bath. The resulting solution was cooled in an ice bath for 8 hr., then allowed to remain at room temperature for 8 hr. The heavy precipitate which had formed was collected, washed with ether, and dissolved in a mixture of 1 l. of ethyl acetate and 500 ml. of 1 *N* sulfuric acid. After mixing well, the layers were separated and the aqueous layer was extracted with two 1-l. portions of ethyl acetate. The ethyl acetate extracts were washed with two 500-ml. portions of 1 *N* sulfuric acid, then with two 250-ml. portions of water, dried over sodium sulfate, and evaporated *in vacuo* at 38°. The residue, on crystallization from 60% methanol, yielded 47.3 g. (70%) of product. Recrystallized from 65% ethanol, it melted at 70–80°, slowly resolidified and melted at 148–149°, λ_{max}^{EtOH} 235 mμ (ε 10,300) and 280 mμ (ε 1500) and infrared spectrum consistent with IIIb.

Anal. Calcd. for $C_{19}H_{21}O_5N_3$: C, 61.44; H, 5.70; N, 11.32. Found: C, 61.12; H, 5.86; N, 11.25.

N-Carbobenzoxy-L-Glutamic Acid γ-(*p*-Tolylhydrazide) (IIIc).—This was prepared by the procedure detailed in the preceding experiment from 21.5 g. (0.073 mole) of N-carbobenzoxy-L-glutamic acid γ-hydrazide and 18.3 g. (0.15 mole) of *p*-tolylhydrazine. The precipitate was worked up with methylene chloride and 1 *N* hydrochloric acid rather than ethyl acetate and 1 *N* sulfuric acid. The crude product, on crystallization from 75% methanol, yielded 15.0 g. of crystalline product. Recrystallized from methanol–ether, then from 80% ethanol, m.p. 153–155° λ_{max}^{EtOH} 236 mμ (ε 11,000) and 285 mμ (ε 1400), infrared spectrum consistent with IIIc.

Anal. Calcd. for $C_{20}H_{23}O_5N_3 \cdot C_2H_5OH$: C, 61.24; H, 6.77; N, 9.74. Found: C, 61.16; H, 6.64; N, 10.01.

L-Glutamic Acid γ-Phenylhydrazide (IIId).—*N*-Carbobenzoxy-L-glutamic acid γ-phenylhyrazide (IIIb; 5.0 g.) in 200 ml. of 50% methanol was hydrogenated over 500 mg. of palladium-charcoal (10%) catalyst at 2 atm. for 1 hr. The catalyst was removed by filtration and washed well with warm water. The combined washings and filtrate were evaporated *in vacuo* at 36° to a volume of 300 ml. and extracted with 200 ml. each of methylene chloride, ethyl acetate, and ether. The extracted aqueous solution was concentrated *in vacuo* at 38° to a volume of 100 ml. The product (1.99 g., 65%) crystallized upon addition of 100 ml. of ethanol. Twice recrystallized from 50% ethanol, m.p. 203°, λ_{max}^{water} 229 mμ (ε 10,000) and 275 mμ (ε 1400),

(9) The 6% yield given in our preliminary communication (ref. 1) was in error; this is the yield computed from spectral analysis and not the yield of crystalline agaritine.

(10) Procured from Michigan Mushroom Co., Niles, Michigan.

(11) S. G. Waley, *J. Chem. Soc.*, 517 (1955).

infrared spectrum consistent with IIId and $[\alpha]^{25}D$ +24° (*c*, 0.41 in 0.5 *N* HCl).

Anal. Calcd. for $C_{11}H_{15}O_3N_3$: C, 55.68; H, 6.47; N, 17.71. Found: C, 55.50; H, 6.16; N, 17.73.

L-Glutamic Acid γ-(*p*-Tolylhydrazide) (IIIe).—*N*-Carbobenzoxy-L-glutamic-acid γ-(*p*-tolylhydrazide) (IIIe; 3.85 g.) in 250 ml. of 60% ethanol was hydrogenated over 250 mg. of palladium–charcoal (10%) catalyst for 1 hr. at 2 atm. The catalyst was removed by filtration and washed with warm water. The combined filtrate and washings were evaporated *in vacuo* at 38° to a volume of 100 ml. and 150 ml. of water was added to redissolve a precipitate. The aqueous solution was extracted with two 150-ml. portions of ethyl acetate, then with 150 ml. of ether and the organic extracts were washed with two 100-ml. portions of water. The extracted aqueous solution and the washings were combined and evaporated *in vacuo* to a volume of 200 ml. and the product (1.94 g., 77%) crystallized by the addition of 200 ml. of ethanol. It had m.p. 183–184°, λ_{max}^{water} 232 mμ (ε 11,700) and 283 mμ (ε 1500), infrared spectrum consistent with IIIe and $[\alpha]^{25}D$ +25° (*c*, 0.88 in 0.5 *N* HCl).

Anal. Calcd. for $C_{12}H_{17}O_3N_3$: C, 57.29; H, 6.82; N, 16.71; O, 19.18. Found: C, 57.42; H, 6.69; N, 16.25; O, 19.34.

***p*-Carboxymethylphenylhydrazine (Id).**—A solution of 20.0 g. of *p*-hydrazinobenzoic acid in 200 ml. of absolute methanol and 20 ml. of concentrated sulfuric acid was refluxed for 1 hr., then concentrated *in vacuo* to a volume of 75 ml. Ice (400 g.) was added to the residue and the resulting solution was cooled to −10° while treated with 30% sodium hydroxide solution.[12] The product crystallized when the solution became strongly alkaline and was promptly collected and washed with ice water until neutral. The dried product weighed 14.3 g. (66%). Recrystallized from ether, with charcoal treatment, it melted at 110° (dec.), λ_{max}^{water} 218 mμ and 287 mμ. It decomposes at room temperature and is best kept as the hydrochloride.

Anal. Calcd. for $C_8H_{10}O_2N_2$: N, 16.86. Found: N, 16.78.

The **hydrochloride**, prepared by treating a cold methanolic solution of the ester with hydrogen chloride gas, after crystallization from methanol had m.p. 234–236° (dec.), λ_{max}^{EtOH} 217 mμ (ε 2,000) and 287 mμ (ε 12,500) and infrared spectrum consistent with the hydrochloride of Id.

Anal. Calcd. for $C_8H_{10}O_2N_2 \cdot HCl$: C, 47.41; H, 5.47; N, 13.83. Found: C, 47.36; H, 5.56; N, 13.96.

Synthesis of Agaritine (IIIa).—A solution of 2.08 g. (0.0125 mole) of *p*-carboxymethylphenylhydrazine (Id) in 125 ml. of ether was added to a refluxing suspension of 1.00 g. of lithium aluminum hydride in 125 ml. of ether over a period of 10 min. The reaction mixture was stirred under reflux for 90 min., then allowed to remain at room temperature for 30 min. The apparatus was flushed with nitrogen while 5 ml. of saturated sodium chloride solution was added in one portion. The inorganic salts were collected by filtration, resuspended in ether with anhydrous sodium sulfate, and again collected and washed with ether. The ether filtrates and washings were promptly cooled in an ice bath while treated over a period of 60 min. with a cold ethereal solution of N-carbobenzoxy-L-glutamic acid γ-azide prepared as above from 1.77 g. (0.006 mole) of N-carbobenzoxy-L-glutamic acid γ-hydrazide. The mixture was then allowed to remain at room temperature for 2 hr. The solvent was evaporated *in vacuo* at 30° and the residue was hydrogenated in 65 ml. of 60% methanol over 350 mg. of palladium–charcoal (10%) catalyst for 1 hr. at 2 atm. The catalyst was removed by filtration and washed with 50 ml. of warm 50% methanol. The combined filtrate and washings were evaporated *in vacuo* at 30° to a volume of 10 ml., diluted with 50 ml. of water, and extracted with two 30-ml. portions of ethyl acetate, then with two 30-ml. portions of ether. The extracted aqueous solution was applied to a column of 5 l. of Dowex 50 (NH_4^+) (10 × 60 cm.) and the column was eluted with water. The effluent volume between 9500 ml. and 11,000 ml., having λ_{max} 236 and 280 mμ, was combined and evaporated *in vacuo* at 40° to a volume of 7 ml. *n*-Butyl alcohol (25 ml.) was added and the mixture was evaporated *in vacuo* until a single phase resulted. This solution deposited 50 mg. (3%) of agaritine which was identical with a sample of natural agaritine (m.p., mixed m.p., ultraviolet and infrared spectra, $[\alpha]^{25}D$, and paper chromatography).

Anal. Calcd. for $C_{12}H_{17}O_4N_3$: C, 53.92; H, 6.14; N, 15.72. Found: C, 53.92; H, 6.57; N, 15.45.

Conversion of Agaritine to L-Glutamic Acid γ-(*p*-Tolylhydrazide) (IIIe).—Agaritine (100 mg.) in 15 ml. of water was reduced for 7 hr. at 2 atm. over 50 mg. of palladium–barium sulfate (10%) catalyst. The catalyst was removed by filtration and the filtrate applied to paper strips along with agaritine and IIIe. When developed in *n*-butyl alcohol, acetic acid, water (4:1:1), and sprayed with either ninhydrin solution or potassium ferricyanide–ferric sulfate solution, only one spot was detected. This differed from agaritine and had the same R_f value as L-glutamic acid γ-(*p*-tolylhydrazide) (IIIe).

Reduction of *p*-Carboxymethylphenylhydrazine with Lithium Aluminum Hydride.—The ester (1.66 g., 0.01 mole) was added to a boiling suspension of 2.0 g. of lithium aluminum hydride in 180 ml. of ether from a Soxhlet apparatus over a period of 40 min. After refluxing 19 hr., the apparatus was flushed with nitrogen while 5 ml. of saturated sodium chloride solution was added in one portion. The inorganic salts were removed by filtration and washed well with dry ether. The filtrate and washings were evaporated *in vacuo* and the residue, in 50 ml. of methanol, was treated with 0.8 ml. of concentrated nitric acid. The resulting precipitate (1.37 g.), on crystallization from methanol–chloroform, gave 760 mg. (41%) of *p*-tolylhydrazine nitrate with m.p. 149–152°. It was identical to a sample of *p*-tolylhydrazine nitrate prepared from authentic *p*-tolylhydrazine (m.p., mixed m.p., ultraviolet and infrared spectra).

Acknowledgment.—We thank Wm. A. Struck and associates for analytical data, Dr. George Slomp for n.m.r. work, Dr. G. R. Umbreit for vapor phase chromatography, and John Woltersom for technical assistance.

(12) Rapid manipulation is necessary from this point until the product is thoroughly washed with cold water.