for another 20 min. Evaporation of acetone was followed by partitioning the residue between chloroform and water followed by the usual work-up. Filtration of the residue on a silica gel column (1:1 ether–hexane) gave the compound (62 mg, 40%): mp 152–153 °C; $[\alpha]^{25}D$ +120.47° (*c* 1.07, $CHCl_3$); NMR δ 0.92 (3 H, s), 3.73 (3 H, s), 6.52–7.1 (3 H, m).

(+)-17α-Ethynyl-9(10→19)*abeo*-estradiol 3-Methyl Ether (12). The procedure employed here is essentially the same as that described for the preparation of **7**. Thus the ketone **11** (570 mg, 1.9 mmol) was treated with lithium acetylide–ethylenediamine complex (3.0 g) in anhydrous dioxane (15 ml). The product was obtained from methanol–water (315 mg, 51%): mp 154–156 °C $[\alpha]^{25}D$ +5.52° (*c* 1.31, $CHCl_3$); NMR δ 0.93 (3 H, s), 2.5 (1 H, s), 3.8 (3 H, s), 6.55–7.1 (3 H, m).

(+)-9(10→19)*abeo*-Estradiol (13). Although this compound was prepared from the alcohol **9**, direct hydrolysis of compound **10** gave better yields. Thus, under similar reaction conditions to those reported for the preparation of **8**, compound **10** (48 mg, 0.13 mmol) was treated with boron tribromide (0.1 ml) in methylene chloride (5 ml). The work-up furnished a residue (29 mg, 78%) which gave the analytical sample upon crystallization from methanol: mp 234–238 °C; ir (KBr) 3550, 3250 cm^{-1}; mass spectrum *m/e* 286 (M^+).

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Agaridoxin, a Mushroom Metabolite. Isolation, Structure, and Synthesis

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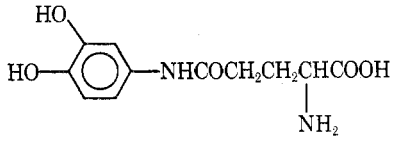
Agaridoxin, a strongly autoxidizable substance, was isolated from the common mushroom *Agaricus campestris*. Its structure was established, largely by NMR, mass spectroscopy, uv spectroscopy, and polarography, to be 3,4-dihydroxy-(γ-L-glutamyl)anilide (**1**). This metabolite was synthesized starting with the reaction between 3,4-(isopropylidenedioxy)aniline (**5**) and *N*-phthaloylglutamic anhydride (**6**). The resulting substituted phthalimide (**7**) was converted into (γ-L-glutamyl)-3,4-(isopropylidenedioxy)anilide (**9**) by treatment with hydrazine in ethanol. Removal of the isopropylidene protecting group by use of boron trichloride gave agaridoxin in good yield.

The isolation of 4-hydroxy(γ-L-glutamyl)anilide from the gill tissue of *Agaricus bisporus*,[1] its enzymatic conversion to the sulfhydryl enzyme inhibitor, *N*-(γ-L-glutamyl)amino-3,4-benzoquinone,[2] and the direct isolation of the quinone from this mushroom have recently been described. We wish to report the isolation, identification, and confirmatory synthesis of the putative intermediate, 3,4-dihydroxy(γ-L-glutamyl)anilide (**1**), obtained from the very closely related species *Agaricus campestris*. Our interest in **1** resulted from a long and continual search by one of us (A.S.-G.) for compounds with low electron affinity[3] which would autoxidize readily and would be expected to suppress cell division. Since we observed that aqueous extracts of *Agaricus campestris* (var. *bifidis*) autoxidized rapidly, the isolation of the substance responsible for this reaction was undertaken. The autoxidation was markedly promoted by manganese salts with the development of a red color, a reaction used to follow the isolation of the mushroom factor. We now wish to report on the isolation, identification and synthesis of this substance which we named agaridoxin.

Agaridoxin was isolated from a methanol extract of the powdered mushroom by treating with lead acetate, separating from insolubles, concentrating to dryness, and subjecting the residue in aqueous solutions to Sephadex G10 chromatography.

Primary identification studies led us to believe that agaridoxin was a dihydroxy(γ-glutamyl)anilide represented by **1** or **2**. This conclusion was based on NMR, gas–liquid chro-



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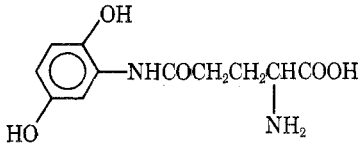

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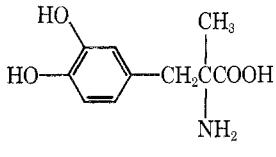
matography–mass spectrographic analysis, ir, and elemental analyses. The NMR spectral parameters of agaridoxin in D_2O (Varian HA-100 spectrometer) are listed in Table I. Signals at δ 3.82, 2.57, and 2.24 indicated the presence of a $>CHCH_2CH_2-$moiety. The suspicion that this grouping represented a glutamyl residue was virtually confirmed when it was observed that the methine signal exhibited a pH dependency typical of the α proton in amino acids. The ir spectrum showed a sharp absorption at 3380 cm^{-1} indicating a peptide linkage. The aromatic resonances between δ 6.81 and 7.02 indicated a 1,2,4-trisubstituted system having one or more electron-donating substituents. Two of these were tentatively considered to be hydroxyls on the basis of the chemical shifts of the aromatic protons and the ease of autoxidation shown by the compound. Since the disposition of the hydroxyl groups would not be definitively assigned from the NMR spectrum, we resorted to comparisons of the polarographic and uv spectrographic behavior of agaridoxin with those of the model compounds L-3,4-dihydroxy-α-methylphenylalanine (**3**) and 3,4-dihydroxyacetanilide (**4**) and *p*-hydroquinone. Polaro-



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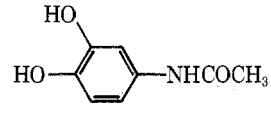

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grams of agaridoxin and the model compounds in pH 5 acetate buffer are shown in Table II. Clearly, agaridoxin resembles 3,4-dihydroxyacetanilide in its oxidation properties. The uv comparison of agaridoxin and **4** in Table III confirms assignment of the hydroxyl groups in the 3,4 positions.

The combined gas–liquid chromatography and mass spectrometry was carried out on the tetratrimethylsilyl derivative of agaridoxin which was prepared by heating a mixture of **1** and bis(trimethylsilyl)trifluoroacetamide in acetonitrile at 105 °C for 15 min. Only one component was observed on the gas chromatogram. This component gave a mass spectrum with strong signals at *m/e* 614 [M^+, corresponding to the molecular weight of the tetratrimethylsilyl (TMSi) derivative of **1** with fragmentation peaks at *m/e* 497 (CO_2TMSi), 396 [$CH(CO_2TMSi)NHTMS$)] and the base peak *m/e* 383 [M − 231, loss of $CH_2{=}C(CO_2TMSi)NHTMSi$], and 571 ($M^+ - CH_3CO$), fragments expected to be lost from TMSi derivatives of amino acids.

Confirmation of the glutamyl moiety was obtained from the acid hydrolysate of agaridoxin (6 N HCl, 111 °C, 20 h) by Spinco analysis. Thin layer chromatography in two systems showed glutamic acid to be the sole ninhydrin-positive amino acid in the molecule. The acid hydrolysate, passed through a column of Pittsburg OL Carbon to remove aromatics, was compared by optical rotatory dispersion with a sample of L-glutamine that had been similarly hydrolyzed and treated. The ORD curves were essentially identical. Thus, agaridoxin

Table I. NMR Parameters and Assignments for Agaridoxin (in D_2O)

δ^a	Multiplicity[b]	Assignment
7.02	(d) (J = 2.0)	Aromatic
6.89	(d) (J = 8.5)	Aromatic
6.81	(d,d) (J = 8.5, 2.0)	Aromatic
3.82	(t) (J = 6.0)	CH
2.57	(t) (J = 7.5)	$CH_2C{=}O$
2.24	(q) (J = 6.0, 7.5)	CH_2C

[a] Relative to internal DSS. [b] d = doublet, t = triplet, q = quartet.

Table II. Polarographic Data for 1, 3, 4, and Hydroquinone

Compd	$E_{1/2}$, V vs. SCE
3	0.24
Agaridoxin	0.18
4	0.18
p-Hydroquinone	0.13

Table III. Uv Data for 1 and 4

	λ_{max}, nm	ϵ	λ_{max}', nm	ϵ'	ϵ'/ϵ
1	285	4240	248	9670	2.28
4	285	3460	246	8100	2.34

contains a glutamine moiety with the natural L configuration and is 3,4-dihydroxy(γ-L-glutamyl)anilide (structure **1**).

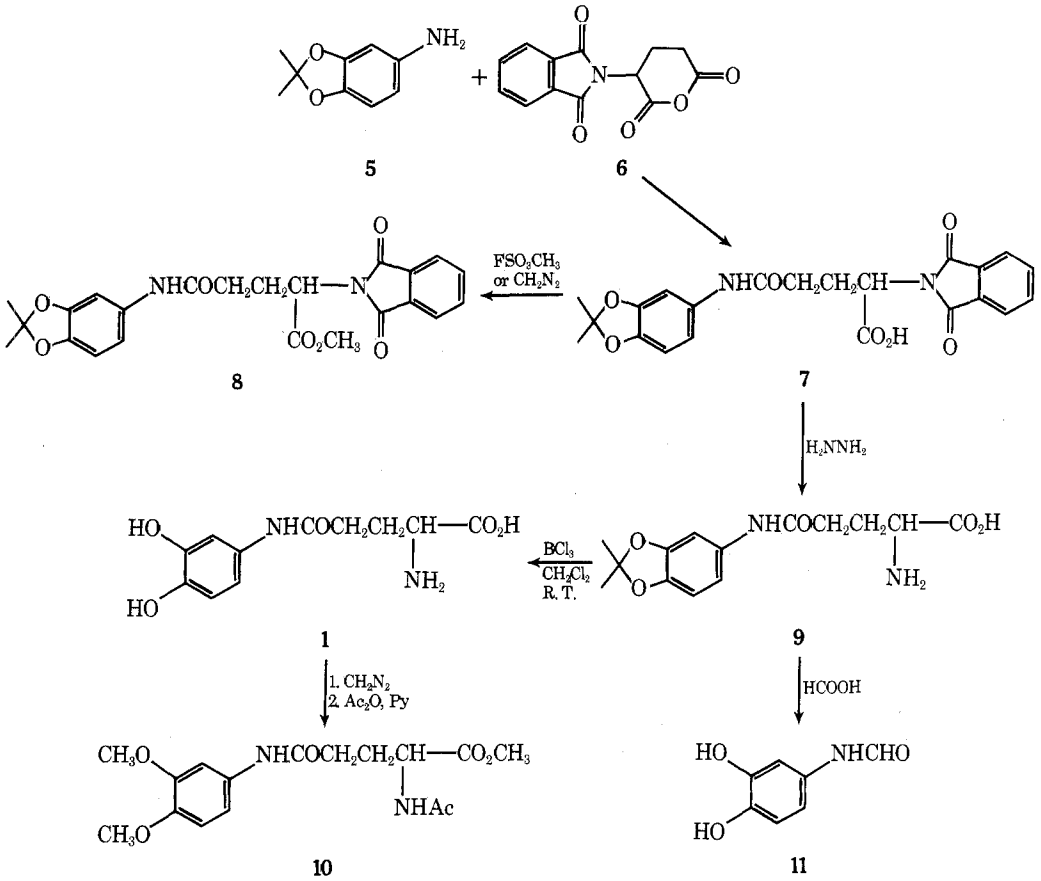
The synthesis of agaridoxin involves the formation of a peptide bond between the γ-carboxyl group of glutamic acid and the amino group of 4-aminopyrocatechol. This scheme entails the usual problems of peptide synthesis: protection of some functional groups, peptide formation, and removal of the protective groups. The starting materials of our successful synthesis were isopropylidene-3,4-dioxyaniline (**5**) and the anhydride of *N*-phthaloyl-L-glutamic acid (**6**) and the scheme that we employed is outlined in Scheme I. The anhydride **6** was prepared by acetic anhydride[4] treatment of *N*-phthaloyl-L-glutamic acid which in turn was prepared from *N*-carboethoxyphthalimide and L-glutamic acid by the elegant method of Nefkens, Tesser, and Nivard,[5] a sequence shown by them to take place without significant racemization.

The reaction of *N*-phthaloyl-DL-glutamic anhydride with various nucleophilic reagents has been extensively investigated,[4-7] and the results indicate that cleavage of the anhydride ring to form γ derivatives is a general occurrence. Thus, the condensation of the aniline **5** and the anhydride **6** in ether at room temperature provided **7** in 90% yield. Treatment of this acid **7** with methyl fluorosulfonate or diazomethane yielded a methyl ester **8**, which gave a three-proton singlet at δ 3.70 in the NMR spectrum. In addition, the character of the compound was further verified by the appearance of the corrected molecular ion (*m/e* 438) in the mass spectrum as well as by its elemental analysis.

Removal of the phthaloyl group was accomplished by boiling an ethanol solution of hydrazine hydrate[6] and the phthaloyl acid **7** for 2 h. The amino acid **9** was readily separated from the reaction mixture in 71% yield.

Removal of the isopropylidene protecting group by treatment with acidic, aqueous, or alcoholic medium is generally a facile process. However, this process could not be applied in our case, owing to the sensitive amide group. Ordinary conditions for hydrolysis of the acetal group also brought about cleavage of the amide portion of the molecule. It is worthy of note that *N*-formylaminopyrocatechol was obtained

Scheme I



when the amino acid **9** was refluxed with 97% formic acid for 0.5 h, and that even at room temperature the amide group was cleaved. Success was finally achieved by use of boron trichloride, a reagent proving to be a useful Lewis acid,[8,9] and superior in some instances to boron trifluoride and boron tribromide. The latter two reagents gave intractable mixtures in our case.

Selective cleavage of aromatic methyl ethers with boron trichloride has been utilized by several workers.[10-12] Likewise, the use of boron trichloride as a reagent for the cleavage of cyclic acetals of hexitols has been studied.[13] However, removal of an isopropylidene group with this reagent has not been reported.

Amino acid **9** on treatment with boron trichloride in methylene chloride at room temperature followed by workup with minimum air exposure yielded agaridoxin in 80% yield. The synthetic product was identical in physical properties, including spectral behavior, with the natural substance. In addition, the *N*-acetyl dimethoxy methyl ester **10** obtained by treating the synthetic agaridoxin with diazomethane followed by reaction with acetic anhydride and pyridine was identical with the compound obtained from the natural agaridoxin by the same sequence of reactions.

It is interesting to note that while agaridoxin showed no antitumor activity in our animal tests γ-L-glutaminyl-4-hydroxybenzene and γ-L-glutaminyl-3,4-benzoquinone were recently found to have interesting biological activities.[14] They appear to be involved in the initiation and maintenance of dormancy of the mushroom spores and to possess modest bactericidal action against a variety of microorganisms.

Experimental Section

Melting points were determined with a Mel-Temp apparatus and are uncorrected. Infrared (ir) spectra were recorded on a Perkin-Elmer 137 spectrophotometer; only bands characteristic of the functional groups present are reported. The nuclear magnetic resonance (NMR) spectra were obtained with a Varian Model A-60 and HA-100 spectrometer. Tetramethylsilane was employed as the internal reference, except where noted. Ultraviolet (uv) spectra were taken on a Perkin-Elmer 202 spectrophotometer. GLC–mass spectra were carried out on a LKB 9000 instrument and the polarography was done with a Melabs Pulse Polarographic Analyzer, Model CPA3.

Except where noted solvents were reagent grade and were used as received. In all work-up procedures, the drying process involved swirling over anhydrous magnesium sulfate and filtering prior to evaporation.

Isolation of Agaridoxin (1). Fresh mushrooms, *Agaricus campestris*, were frozen by storage in a deep freeze refrigerator, pulverized using a hammer–mincer, and dropped directly into methanol (90 l. for 45-kg lots) containing 0.1% formic acid. The brei was homogenized by agitation and the solid was then separated on a drum centrifuge lined with a wire net and cheese cloth. To the somewhat turbid filtrate, 2 l. of 50% lead acetate was added and the precipitate was allowed to settle overnight.

The supernatant was clarified on a Sharples supercentrifuge, and the sediment filtered on a large Büchner funnel. The combined clear filtrates were concentrated at reduced pressure to 4.5 l. on a thin-film evaporator and further concentrated on a flash evaporator to 1 l. Three volumes of methanol were added to the aqueous concentrate and the resulting solution stored overnight at 0 °C. The supernatant was decanted, and the sediment filtered and washed with methanol. To the combined filtrates and washings was added 500 ml of 50% lead acetate and enough sodium hydroxide to bring the pH of the liquid to 8.5. The precipitate was quickly separated on a laboratory model Sharples and the pH of the liquid adjusted to 3–4 with sulfuric acid. After storage at 0 °C overnight, the clear liquid was concentrated to a sticky gum. To eliminate excess acid (acetic and formic), the residue was stored in a vacuum desiccator containing NaOH pellets. Eventually the dried material was dissolved in a small volume of water and passed twice through a Sephadex G10 column. The active material was contained in the fourth void volume. The elution was followed by adding 1 drop of 0.1 M manganese acetate solution to 0.5-ml samples and then neutralizing the solution with sodium bicarbonate. The developing red color indicated the presence of agaridoxin. The eluate was concentrated and the solid residue was recrystallized by dissolving in a minimum of water and adding several volumes of

methanol, mp 218–221 °C. About 3 g was obtained from 1 ton of mushrooms.

Agaridoxin was obtained as a grayish-white powder which darkened on storage in air. It gives a beautiful blue color with ferric chloride. NMR data are summarized in Table I; ir, sharp absorption at 3380 cm^{-1} (CONH–), broad band 3200–2400 cm^{-1}, multiple absorption 1650–1500 cm^{-1}. The hydrochloride showed absorption at 1720 cm^{-1} (COOH) and bands at 1640–1660 and 1510 cm^{-1} (CONH–); polarographic and uv data are summarized in Tables II and III. For mass spectral studies a sample was silylated (0.5 mg heated in a mixture of 50 μl of bis(trimethylsilyl)trifluoroacetamide and 50 μl of acetonitrile at 105 °C for 15 min) and examined by combined gas–liquid chromatography and mass spectrometry with the LKB Model 9000 instrument; GLC operating conditions, 6 ft × 3.5 mm i.d. glass spiral column, 2% SE-30 stationary phase on 100–120 mesh acid-washed and silanized Gas Chrom T, 180 °C, 25 ml/min flow rate; MS operating conditions, 70 eV ionizing potential, 270 °C ion source temperature, 50-μA filament current, 3.5-kV accelerating potential; MS data in discussion section.

Anal. Calcd for $C_{11}H_{14}N_2O_5$: C, 51.97; H, 5.52; N, 11.03. Found: C, 51.59; H, 5.58; N, 10.92.

3,4-Dihydroxyacetanilide.[15] A solution of 4-nitropyrocatechol (4.49 g, 32.2 mmol) in 50 ml of absolute ethanol was hydrogenated at 40 psig over 0.25 g of 5% Pd/C. Hydrogenation was complete in 1 h. The mixture was filtered from the catalyst and evaporated in vacuo to an oily residue. Acetic anhydride (3.64 ml) was added to the residue and allowed to stand for 1 h at room temperature. The reaction mixture was quenched by addition of 3 ml of water, evaporated to an oil in vacuo, and dissolved in 30 ml of water containing 0.2 g of Na_2SO_3 to yield 4.3 g (80%) of dark but well-formed crystals. Recrystallization from 25 ml of water containing 0.4 g of Na_2SO_3 and using 0.6 g of Darco G-60 for decolorization yielded 2.768 g (51.5% overall yield) of 3,4-dihydroxyacetanilide (mp 171–173 °C with softening at 167 °C) with satisfactory elemental analysis. The NMR spectrum confirmed the absence of *O*-acetate and the aromatic portion of the spectrum was superimposable on that of agaridoxin.

***N*-Phthaloyl(γ-L-glutamyl)-3,4-(isopropylidenedioxy)anilide (7).** To a partially dissolved solution of 26 g (0.1 mol) of *N*-phthaloyl-L-glutamic anhydride[4,5,16] (**6**) in 500 ml of absolute ether was added dropwise a solution of 17 g (0.1 mol) of 3,4-(isopropylidenedioxy)aniline[17] (**5**) in dry ether at room temperature. The mixture was stirred at room temperature for 40 min. The yellowish solid was filtered and recrystallized from ethyl acetate and dimethoxyethane to give 32 g (76%) of pale yellow solid: mp 132–134 °C; NMR ($CDCl_3$–NMe_2SO-d_6, 1:1) δ 1.6 (6 H, s), 2.45 (4 H, m), 4.95 (1 H, m), 6.75 and 7.1 (3 H, m), and 7.87 (4 H, s).

Anal. Calcd for $C_{22}H_{20}N_2O_7$: C, 62.26; H, 4.75; N, 6.60. Found: C, 62.27; H, 15.15; N, 6.61.

***N*-Phthaloyl(γ-L-glutamyl)-3,4-(isopropylidenedioxy)anilide Methyl Ester (8).** Equimolar amounts of the acid **7** and methyl fluorosulfonate were refluxed for 1 h in ethanol. Following the work-up, a quantitative yield of ester **8** was obtained. Recrystallization from benzene gave a yellow solid: mp 148–149 °C; NMR ($CDCl_3$) δ 1.6 (6 H, s), 2.5 (4 H, m), 3.75 (3 H, s), 6.9 (1 H, s), 6.4 and 7.1 (3 H, m), and 7.75 (4 H, narrow multiplet); mass spectrum (70 eV) *m/e* 438 (parent).

(γ-L-Glutamyl)-3,4-(isopropylidenedioxy)anilide (9). To a solution of 7.58 g (0.179 mol) of purified phthaloyl acid **7** in 300 ml of absolute ethanol was added 1 g (0.2 mol) of hydrazine hydrate at room temperature. The reaction mixture was refluxed for 2 h, and ethanol was removed on a rotary evaporator. The white residue was taken up in 60 ml of 5% HCl at ice bath temperature and after standing for 30 min, the solid *N,N'*-phthaloyl hydrazide was separated by filtration. The filtrate was made alkaline with ammonium hydroxide solution at 0 °C to give 3.75 g (71%) of amino acid **9**, mp 185–191 °C. An analytical sample was recrystallized from water–methanol: mp 194–195 °C; mass spectrum (based on trisilylated compound of amino acid **5**) *m/e* (rel intensity) 510 (7), 495 (13.4), 393 (42.5), 292 (39), 279 (100), 164 (17), 116 (22.8), 73 (74).

Anal. Calcd for $C_{14}H_{18}N_2O_5$: C, 57.14; H, 6.16; N, 9.52. Found: C, 57.52; H, 6.03; N, 10.07.

3,4-Dihydroxy-*N*-formylaniline (11). A mixture of 400 mg (1.36 mmol) of amino acid acetal **9** and 7 ml of 97% formic acid was refluxed for 0.5 h. The mixture was allowed to cool and was then poured over crushed ice. Thorough extraction with ethyl acetate and concentration in vacuo yielded 116 mg (55%) of *N*-formyl product as a grayish solid which became crystalline on triturating with ethyl acetate: mp 182–183 °C; NMR (Me_2SO-d_6) δ 6.7 (2 H, m), 7.24 (1 H, s), 8.3 (2 H, m). 9.5 (1 H, s); mass spectrum (70 eV) *m/e* (rel intensity) 153 (100), 125 (50), 79 (35); uv λ_{max} (H_2O) 285 nm (ε 3650) and 247 (8960).

Agaridoxin (3,4-Dihydroxy-(β-L-glutamyl)anilide, 1). Boron trichloride (tenfold excess) was bubbled into methylene chloride (50 ml) at 0°C. To this solution was added in portions 1 g (0.0034 mol) of acetal **9**. The suspension was stirred for 4 h at room temperature. Most of the excess boron trichloride was then removed by blowing nitrogen gas into the reaction flask. Finally, solvent was removed on a rotary evaporator until a completely dry white residue remained in the flask. Methanol was added at 0 °C and was removed in vacuo at room temperature. The darkish residue was taken up in 95% ethanol and basified with triethylamine or ammonium hydroxide solution. The solvent was then removed under reduced pressure at room temperature and the residue was triturated with 95% ethanol to yield 690 mg (80%) of agaridoxin as a grayish solid (mp 215–219 °C). This was recrystallized by dissolving in a minimum amount of water containing 100 mg of sodium bisulfite, adding an equal volume of methanol, and chilling in a refrigerator giving a grayish solid: mp 220–221 °C, mixture melting point with natural unchanged; NMR (D_2O, DSS as internal reference) δ 2.24 (2 H, q), 2.57 (2 H, t), 3.82 (1 H, t), 6.81 (1 H, dd), 6.89 (1 H, dd), 7.02 (1 H, d); uv λ_{max} (H_2O) 285 nm (ε 4240) and 248 (9670).

Anal. Calcd for $C_{11}H_{14}N_2O_5$: C, 51.97; H, 5.55; N, 11.02. Found: C, 51.87, H, 5.49; N, 10.79.

The NMR of the synthetic preparation was identical with that of the natural product. The NMR spectrum of a 1:1 mixture of the two solutions was indistinguishable from the individual curves.

***N*-(4-Acetylamino-4-carbomethoxybutyryl)-3,4-dimethoxyaniline (10).** To 200 mg of agaridoxin solid was added methanolic hydrogen chloride at 0 °C. Solvent was then completely removed in vacuo at room temperature. The residue was taken up in methanol and treated with excess diazomethane at 0 °C. The solvent was evaporated and the residue stirred with acetic anhydride and pyridine at room temperature for 10 h. After removal of the excess acetic anhydride and pyridine, the product was dissolved in ethyl acetate and washed first with 5% HCl and then saturated $NaHCO_3$ solution. Drying and evaporation of ethyl acetate provided a thick oil. The white solid product was obtained by crystallization from a minimum amount of ethyl acetate at freezer temperature: mp 169–171 °C; NMR ($CDCl_3$) δ 2.05 (3 H, s), 2.25 (4 H, m), 3.7 (3 H, s), 3.83 (6 H, s), 6.8 (3 H, m). A sample prepared from natural agaridoxin was identical in all respects with the synthetic product.

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