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A NEW AMINO ACID ISOLATED FROM *MORCHELLA ESCULENTA* AND RELATED SPECIES*

SHIN-ICHI HATANAKA

Biological Institute, College of General Education, University of Tokyo, Meguro, Tokyo

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Abstract—A new amino acid was isolated from mushrooms, *Morchella esculenta*, and related species. From the results of elementary analysis, deamination, and NMR spectrum, its structure was shown to be *cis*-3-amino-L-proline. It is present in a free state in fruit bodies of *M. esculenta*, *M. conica*, and *M. crassipes*, as well as in their cultured mycelia and its occurrence seems to be confined to the genus *Morchella*.

INTRODUCTION

DURING a paper chromatographic survey of non-protein amino acids of fungi,[1] fruit bodies of *Morchella esculenta* were found to contain a considerable amount of a substance giving a specific greenish blue coloration with ninhydrin, which apparently differed from known natural and synthetic amino acids. This paper reports its isolation and identification.

RESULTS AND DISCUSSION

Further paper chromatographic survey revealed that two other related species *Morchella conica* and *M. crassipes* also contained this substance. Therefore, fruit bodies of these three species were used separately for the isolation. Since the new amino acid occurred both in pilei and stipes, the whole fruit bodies were used as material, which had been collected from natural habitats. It was isolated by ion-exchange chromatography[2] and isolated as the hydrochloride. The yield of crude material indicated that it was the principal constituent of the soluble nitrogen fraction. In the case of *M. conica* its content amounted to at least 0·5% of dry weight of the fruit bodies. Semiquantitative chromatographic estimation showed considerable differences in content among different collections of *Morchella*, but its relation to the species or to the maturity is not yet clear.

Elementary analysis agreed with the formula $C_5H_{10}N_2O_2 \cdot HCl$, indicating the presence of one carboxy and two amino or imino groups, and the molecular weight, determined by mass spectrometry of the *N*-acetyl ethyl ester, of 242 agreed with this assumption. Van Slyke determination showed 1·4 N atoms per molecule, indicating that the compound contained at least one primary amino group. It showed no appreciable absorption in the u.v. The free compound and its hydrochloride were both readily soluble in water. On heating to 110° in 6 N HCl in a sealed tube for 24 hr, it was recovered unchanged, but oxidation with $KMnO_4$ afforded aspartic acid and β-alanine, together with other minor decomposition

* Part II in the series Biochemical Studies on Nitrogen Compounds of Fungi; for Part I, see *Botan. Mag., Tokyo* **81**, 259 (1968).

[1] S. HATANAKA and H. TERAKAWA, *Botan. Mag. Tokyo* **81**, 259 (1968).
[2] P. B. HAMILTON and R. A. ANDERSON, *J. Biol. Chem.* **211**, 95 (1954).

products. It was not hydrogenated in the presence of Adams catalyst. It formed a copper-complex on a paper chromatogram, which had been dusted lightly with basic copper carbonate,[3] indicating that it was an α-amino or α-imino acid; from its optical rotation, it seems to belong to the L-series. Pauli and Ehrlich reactions proved also to be negative.

On deamination, it gave *cis*- and *trans*-3-hydroxyprolines,[4] which were detected on an amino acid analyser. Although the yields were very low (a few per cent), the ratios of ninhydrin colorations 440/570 nm were similar. These results strongly suggest that the new amino acid is *cis*-3-aminoproline. The formation of a small amount of its *trans*-isomer is seemingly due to the Walden inversion during the deamination procedure.

The proposed structure, *cis*-3-aminoproline, was further examined with the NMR spectrum in D_2O (Fig. 1). Decoupling experiments at appropriate frequencies confirmed the assignment of signals to individual protons Among others, the C-2 proton peak

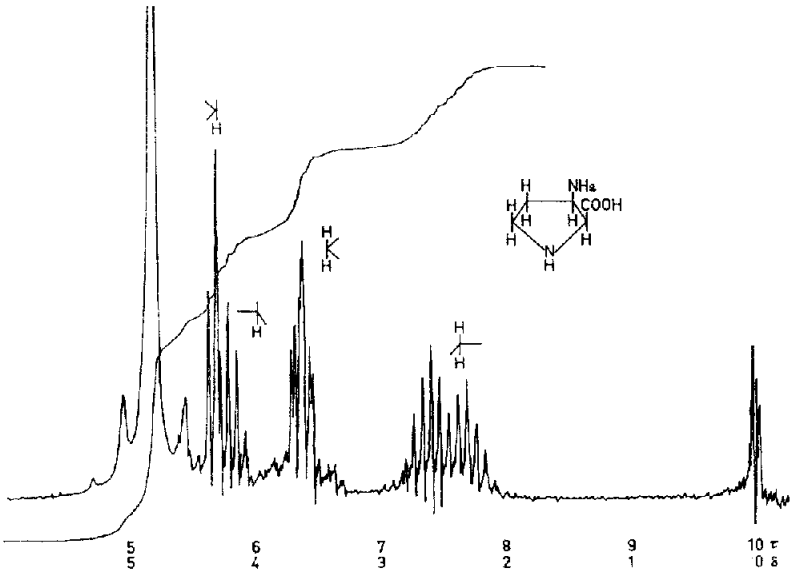

FIG. 1. NMR SPECTRUM OF A NEW AMINO ACID FROM *Morchella*.

appeared as a doublet showing $J_{2,3}=6$ cps. It was previously reported[4] that C-2 proton of *cis*-3-hydroxyproline was a doublet indicating $J_{2,3}=4$ cps and that of *trans*-isomer was an unresolved but broader peak, indicating $J_{2,3}<1$ cps. The higher value of $J_{2,3}$ of C-2 proton of the *Morchella* acid is probably due to the nitrogen atom attached to C-3 instead of oxygen in the case of *cis*-3-hydroxyproline. All the above experimental results show that the newly isolated amino acid is *cis*-3-amino-L-proline.

This amino acid was also produced by the cultured mycelia of *M. esculenta* and *M. conica*, obtained by culturing spores of these organisms. After 7 days' culture, the filtered culture medium also proved to contain a considerable amount of *cis*-3-amino-L-proline together with other usual amino acids. It is, however, not yet certain whether this and the other amino acids were exuded from living mycelia or they were due to the decomposition of the mycelia during the culture.

[3] H. R. CRUMPLER and C. E. DENT, *Nature* **164**, 441 (1949).
[4] F. IRREVERRE, K. MORITA, A. V. ROVERTSON and B. WITKOP, *J. Am. Chem. Soc.* **85**, 2824 (1963).

The new acid does not seem to be present in a bound form within proteins. The fruit bodies of *M. esculenta* were extracted exhaustively with 80% ethanol and the hydrolysate of the residue remaining was examined paper chromatographically. They did not contain *cis*-3-amino-L-proline in any detectable concentration, whilst under the same conditions the usual thirteen protein amino acids appeared.

Morel mushroom, one of the common edible fungi, especially *M. esculenta*, have long been known and cultured. Several works have appeared on some nutritional and environmental conditions affecting the growth of these cultured mycelia.[5,6] Recently Litchfield and his co-workers[7] reported the proximate analyses and quantitative amino acid composition of mycelial proteins of *M. esculenta*, *M. hortensis*, and *M. crassipes*, and also pointed out the occurrence of some unusual amino acids, such as α-aminoisobutyric acid and 2,4-diaminobutyric acid. No reference, however, was made to our new amino acid.

EXPERIMENTAL

Isolation of 3-*Amino-*L-*Proline*

Fruit bodies of *Morchella esculenta* (900 g) collected from natural habitats were washed with tap water and extracted five times with 80% ethanol (10 l.). The combined extract was filtered and passed through a column of 150 ml Amberlite IR-120 (H^+) and the amino acid fraction was obtained by eluting with 1·5 l. of 2 N NH_4OH. The eluate was then evaporated to dryness under reduced pressure, the residue dissolved in 0·2 M citrate buffer, pH 5·0 (18 ml), and applied to a column of Dowex-50W (200–400 mesh, ×8, Na^+) (26 × 540 mm), equilibrated previously with 0·1 M citrate buffer of pH 3·4. The fractionation was carried out with 0·2 M citrate buffer, pH 5·0.[2] After the acidic and neutral amino acids were displaced from the column, the new acid was eluted without any contamination by ninhydrin-positive substances. About 2 l. buffer sufficed for the elution. The combined fractions (500 ml) were desalted with a column of 300 ml Dowex-50W (H^+), followed by the elution again with 3 l. of 2 N NH_4OH. The ammonia was removed under reduced pressure, the remaining residue taken with a small amount of water and decolorized with active charcoal. The filtrate was neutralized with 2 N HCl and ethanol added. Crystals separated immediately; after standing for 2 days in a refrigerator, the crystals were washed with chilled ethanol and dried in a desiccator. Yield was 270 mg crude crystals from 900 g starting material. It was purified by repeated recrystallization from methanol–water and had m.p. 215° (decomp.), $[\alpha]_D^{20} = +5{\cdot}8°$ ($c=2$, H_2O), $+23{\cdot}0°$ ($c=2$, 5 N HCl). Found: C, 36·30; H, 6·75; N, 16·59; O, 19·79; Cl, 21·21. $C_5H_{10}N_2O_2 \cdot HCl$ required: C, 36·04; H, 6·66; N, 16·82; O, 19·20; Cl, 21·24. It was also isolated from two other related species. The yields of crude crystals were as follows: 490 mg from 980 g *M. conica* and 400 mg from 1500 g *M. crassipes*.

Paper Chromatography

The following solvents were used: *n*-butanol–acetic acid–water (63:10:27, v/v) (1) and phenol–water (100:36, w/w, in NH_3 vapour) (2). In the two-dimensional method, the solvent (1) was used for the first descending and the solvent (2) for the second ascending development. Paper used was Toyo No. 50. For R_fs, see Table 1. For distinguishing α- and the other amino acids, the method of two-dimensional paper chromatography by Crumpler and Dent[3] was adapted. After applying the sample to the paper in duplicate (Whatman No. 4, 18 × 18 cm), a sheet of paper was dusted lightly with finely powdered $CuCO_3 \cdot Cu(OH)_2$ along the path of first development of the amino acids. Two chromatograms were then run in the usual way in two directions, successively, both with the solvent (2). α-Amino acids showed the different behaviour as in the control without basic copper carbonate.

Deamination[4]

3-Aminoproline hydrochloride (50 mg) was dissolved in 1 N HCl (3 ml), immersed in boiling water and treated with $NaNO_2$ (130 mg). After 5 min it was cooled and the nitrosamino acids were extracted with ethyl acetate (9 ml × 3). The combined extract was evaporated to dryness *in vacuo*, dissolved in 6 N HCl (2 ml), followed by the addition of ammonium sulphamate (8 mg). It was then heated at 121–123° for 90 min in a sealed tube. After the excess of HCl was removed by evaporation *in vacuo*, the residue was taken with a small amount of water and passed through a small amount of Amberlite IR-120 (H^+). The free imino acids were eluted with 2 N NH_4OH and the eluate was evaporated to dryness. The residue was dissolved again in an appropriate amount of water and examined with a Beckman-Spinco amino acid analyser.

[5] T. D. Brock, *Mycologia* **43**, 402 (1951).
[6] J. H. Litchfield, R. C. Overleck and R. S. Davidson, *J. Agri. Food Chem.* **11**, 158 (1963).
[7] J. H. Litchfield, V. G. Vely and R. C. Overbeck, *J. Food Sci.* **28**, 741 (1963).

TABLE 1. R_fS OF *cis*-3-AMINO-L-PROLINE AND SOME COMMON AMINO ACIDS

(Temperature: 26–28°, paper: Toyo No. 50)

Amino acids	R_fs in	
	n-Butanol–acetic acid–water, descending	Phenol–water (NH_3), ascending
Alanine	0·34	0·63
Arginine	0·22	0·90
Aspartic acid	0·24	0·18
Glutamic acid	0·31	0·28
Histidine	0·20	0·74
Lysine	0·18	0·84
3-Aminoproline	0·20	0·86
Valine	0·52	0·82

Oxidation with Potassium Permanganate

The hydrochloride (1·7 mg, 10 μM) was dissolved in water (1 ml), followed by the dropwise addition of 1% $KMnO_4$ (0·6 ml). The precipitate was filtered with a small amount of charcoal. After the evaporation, the residue was subjected to an amino acid analyser. Aspartic acid (0·46 μM) and β-alanine (0·34 μM) were identified.

Culture of the Mycelia and Their Analyses

The mycelial cultures of *M. esculenta* and *M. conica* were originally isolated by Dr. H. Terakawa in the spring of 1967 by culturing spores from stipe tissues of the fruit bodies collected from natural habitats. Stock cultures were maintained since then as slants on potato-extract agar medium (pealed and chopped potato was extracted with an equal weight of boiling water for 20 min and the filtrate was diluted with 5 vol. of water, followed by adding agar to 1·5%) at 24–26° under diffuse light. Fresh transfers were made at least every month.

A portion of the growth on an agar slant was inoculated to a Petri dish containing 150 ml of the potato-extract medium (without agar) and incubated at 24–26° under diffuse light. The 7-day-old cultures with thirty Petri dishes gave 80 g (wet weight) mycelia of *M. esculenta*.

Cultured mycelia were separated with a glass filter and extracted with boiling 80% ethanol and the extract was treated with Amberlite IR-120 to obtain amino acid fraction as usual. The presence of 3-aminoproline was shown on a two-dimensional paper chromatogram. Amino acids in the filtered culture medium were also examined in the same way and the new acid was therein demonstrated.

Examination with Ethanol-Insoluble Fraction

Ethanol (80%)-insoluble residue (100 mg), obtained after repeated extraction of fruit bodies of *M. esculenta*, was hydrolysed with 6 N HCl (8 ml) at 110° for 24 hr. After it was filtered, diluted with water (to 70 ml) and treated with Amberlite IR-120 (5 ml), 2 N NH_4OH (50 ml) eluted the amino acids. Ammonia was removed by evaporation under reduced pressure and the residue dissolved in water (1 ml). A small portion (40 μl) of this solution was examined by two-dimensional paper chromatography. No detectable amount of 3-aminoproline was found to be present, while the thirteen usual protein amino acids appeared.

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