[Chem. Pharm. Bull. 36(4)1366—1370(1988)]

A New Acidic Amino Acid from a Basidiomycetes, *Lactarius piperatus*

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(Received October 7, 1987)

A new acidic amino acid was isolated from a Basidiomycetes, *Lactarius piperatus* (Fr.) S. F. GRAY. The structure of this compound has been determined as 2(*S*),3′(*S*)-1-(3-amino-3-carboxypropyl)-5-oxo-2-pyrrolidinecarboxylic acid (**1**) on the basis of nuclear magnetic resonance analysis and chemical synthesis.

Keywords——Basidiomycetes; *Lactarius piperatus*; isolation; synthesis; acidic amino acid; COSY NMR; reductive amination

Lactarius piperatus (Fr.) S. F. GRAY (Japanese name: tsuchikaburi) is a large, ivory-white colored mushroom that is widely distributed in Japan, Europe and North America, but is usually not eaten due to its bitter taste. The investigations on the constituents of this mushroom have been focused mainly on the bitter constituents, which have been betermined to be sesquiterpene aldehydes such as piperadial and velleral.[1,2] Little is known about amino acid components of this mushroom and other mushrooms of the *Lactarius* family.[3] In the course of our study to isolate biologically active amino acids from Basidiomycetes,[4,5] the amino acid fraction of *Lactarius piperatus* (Fr.) S. F. GRAY has been examined. Pipecolic acid derivatives which have some biological activities have been isolated from *Russula subnigricans* HONGO (Japanese name: nisekurohatsu), an anatomically analogous mushroom to *L. piperatus*.[4] Herein, we describe the isolation of a new acidic amino acid named Lp-2, as well as its structure determination and synthesis.

In the water extract of *Lactarius piperatus*, an unusual band was noticed on the paper chromatogram (PC); when tested with ninhydrin, it gave a violet coloration, indicating a new amino acid. This amino acid (*Rf*, 0.15 on PC; t_R, 13.0 min on high-performance liquid chromatography (HPLC)) was found to be an acidic amino acid from the elution behavior on ion exchange resins and a yellow coloration with bromocresol green. The isolation and purification of this amino acid were achieved by means of the following procedures from the water extract of the mushroom. The acidic amino acid portion obtained from the extract in the usual manner was applied to a Dowex 50W × 4 column which was buffered with an ammonia–formate buffer (pH 2.70). The separation of each fraction was monitored by HPLC. The fractions containing the amino acid were separated by gel filtration using Cellulofine GCL-25-m. The final purification was carried out on a cellulose column to give the acidic amino acid as a colorless amorphous mass.

The amino acid, Lp-2 (**1**), $[\alpha]_D$ −17.5° (c=0.939, H_2O), had the molecular formula $C_9H_{14}N_2O_5$; field desorption mass spectrum (FDMS), m/z 231 $(M+1)^+$ and showed infrared (IR) absorptions at 3000—3400 cm^{-1} (NH) and 1590—1700 cm^{-1} (CONH and COO^-). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** exhibited signals due to ten protons (δ 2.18—2.36, 3H, m; 1.75—1.85, 2H, m; 1.995, 1H, m; 2.952, 1H, ddd, J=4.49, 7.52,

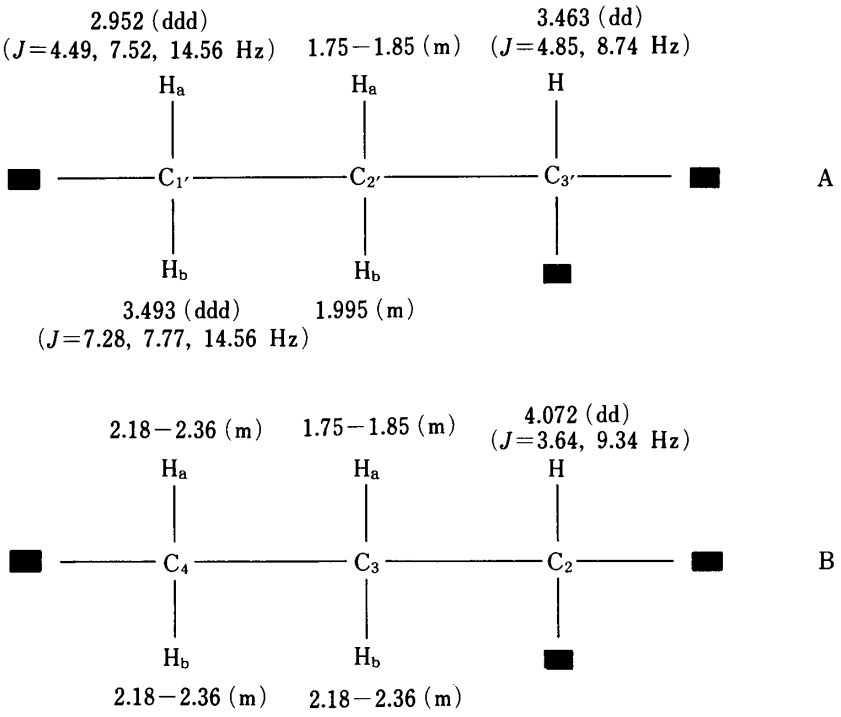


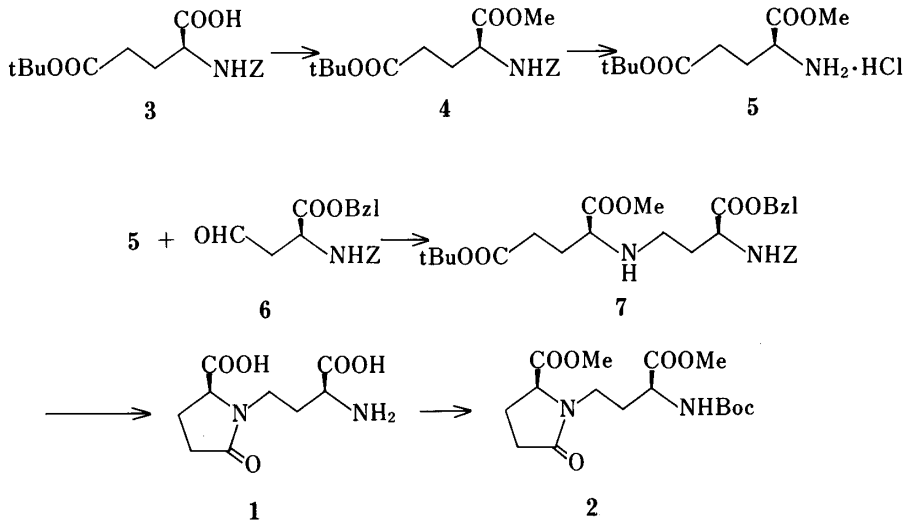
Fig. 1

14.56 Hz; 3.463, 1H, dd, $J=4.85$, 8.74 Hz; 3.493, 1H, ddd, $J=7.28$, 7.77, 14.56 Hz; 4.072, 1H, dd, $J=3.64$, 9.34 Hz). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** showed three signals due to carbonyl carbons at δ 174.3 (s), 179.4 (s) and 179.7 (s) and six signals at δ 23.3 (t), 28.2 (t), 29.9 (t), 38.6 (t), 52.7 (d) and 63.0 (d). Analysis of the ^{1}H-NMR spectrum of Lp-2 with the aid of 2D correlated spectroscopy (COSY) experiments demonstrated the presence of the partial structures A and B, where ■ denotes a hetero atom or a carbonyl group (Fig. 1).

Treatment of Lp-2 with di-*tert*-butyl dicarbonate and triethylamine yielded the *tert*-butoxycarbonyl (Boc) derivative, which on methylation with diazomethane gave the Boc-dimethyl ester **2** (δ 3.630, 3H, s; 3.698, 3H, s; 1.350, 9H, s). This indicates that two carboxyl groups and one amino group exist in the molecule, and the latter is assumed to be primary since Lp-2 showed a violet coloration with ninhydrin. The downfield shift of the C-3′ methine proton (−0.50 ppm) and the C-2 methine proton (−0.21 ppm) signals in the ^{1}H-NMR spectrum of the Boc-dimethyl ester **2** indicate the *tert*-butoxycarbonylamino group to be located at C-3′ and two methoxy-carbonyl groups at C-3′ and C-2, respectively. The ^{1}H-NMR arising from partial structure B agreed closely with the corresponding signals of 2-pyrrolidine-5-carboxylic acid (pyroglutamic acid) suggesting Lp-2 to be a derivative of pyroglutamic acid. Therefore, the three carbonyl groups in Lp-2 have been assigned. The situation of the C-1′ methylene group (partial structure A) adjacent to nitrogen is implied by the chemical shifts of the protons (δ 2.952 and 3.493) in the ^{1}H-NMR spectrum.

From the accumulated data, it was thought to be most probable that Lp-2 has structure **1**. Further, the stereochemistry of the chiral centers can be presumed as 2(*S*) and 3′(*S*) from the circumstantial evidence that most of the amino acids isolated from the Basidiomycetes have the stereochemistry corresponding to the L-series. This assumption was confirmed by the following synthesis.

Benzyloxycarbonyl (*Z*)-L-glutamic acid γ-*tert*-butyl ester **3**, prepared from L-glutamic acid according to Itoh's method,[6] was methylated with diazomethane to give the methyl ester **4**. The amino protecting group of **4** was removed by hydrogenolysis to yield the amine **5**,



tBu=*tert*-butyl

Chart 1

which was reductively coupled with the aspartic-β-semialdehyde **6**[7,8] by the action of sodium cyanoborohydride ($NaBH_3CN$)[8,9] giving rise to a product **7**, $[\alpha]_D$ $-13.5°$ in 38% yield (from **4**). An initial attempt to prepare the pyrrolidine ring by selective removal of the *tert*-butyl group of **7** was unsuccessful.[10] Therefore, benzyl-type protecting groups were removed at this stage. Hydrogenolysis of **7** in 1 N hydrochloric acid and subsequent alkaline hydrolysis proceeded smoothly accompanied with pyrrolidine ring formation to give the desired Lp-2 in 43% yield. The synthetic **1** was shown to be identical with the natural substance (including the optical rotation: $[\alpha]_D - 17.8°$). Consequently, the structure of Lp-2 was elucidated as 2(*S*), 3′(*S*)-1-(3-amino-3-carboxypropyl)-5-oxo-2-pyrrolidinecarboxylic acid.

There are two groups of amino acid derivatives in which amino acid moieties are connected by an *N*-alkyl bond. One group is the phytosiderophores, such as mugineic acids[11,12] and nicotianamine,[13] which play a role in iron uptake and transport in the plant. The other group is the opines which are synthesized and catabolized by *Agrobacterium tumefaciens* and play a central role in the crown gall biology.[14,15] Lp-2 is analogous to the glutamyl opines as represented by succharopine,[16,17] which has also been identified in the mushroom *Lentinus edodes*[18] and *Flammulina velutipes*.[19] The glutamyl moiety of succharopine is known to lactamize to give the pyroglutamyl opine, whose contents are dependent on the age and storage conditions of the crown gall and the method of extraction.[15] While this indicates the non-enzymatic formation of the 5-oxopyrrolidine ring of Lp-2, enzymatic formation of the ring can not be excluded because it is well known that pyroglutamic acid is synthesized from glutamic acid enzymatically in the γ-glutamyl cycle.[20] Although the pathway of production of Lp-2 is not certain, taking into account the biological significance of the related compounds, it is expected to have some biological activities and therefore further study is under way.

Experimental

General Procedure——Optical rotations were determined on a JASCO DIP-340 spectrometer. Low-resolution electron impact mass spectra (LRMS) were recorded on a Hitachi M-52G spectrometer. FDMS were recorded on a JEOL JMS-01 SG-2 spectrometer. IR spectra were recorded on a JASCO A-100S spectrometer. ^1H- and ^{13}C-NMR spectra were taken on a JEOL FX-100 and JNM-GX 500 spectrometer. Chemical shifts are reported in δ units downfield from internal tetramethylsilane. HPLC was carried out on an instrument (JASCO TRIROTAR) equipped with a refractive index detector (Shodex RI SE-31). A stainless steel column packed with Hitachi gel #2618 (cation

exchange resin) was used and eluted with ammonia–formate buffer (pH 2.70) at a rate of 0.5 ml/min. PC was performed using *n*-butanol–acetic acid–water (4 : 1 : 5 upper layer) by the ascending technique on Toyo No. 50 paper.

Isolation of 2(*S*),3′(*S*)-1-(3-Amino-3-carboxypropyl)-5-oxo-2-pyrrolidinecarboxylic Acid (Lp-2) (1)——*Lactarius piperatus* (Fr.) S. F. GRAY (7 kg) collected near Sendai was extracted with water (20 l). After filtration of the extract, the filtrate was applied to Amberlite IR-120B resin (H^+, 6.0 × 40 cm). After being washed with water, the column was eluted with 1 N pyridine (2 l), affording the acidic and neutral amino acids. The column was subsequently eluted with 1N ammonia (2 l) to give the basic amino acids. The acidic and neutral amino acid fraction was concentrated, and one-third of the concentrate was applied to Dowex 1 × 8 resin (CH_3COO^-, 100—200 mesh, 4.0 × 40 cm). The column was washed with water (1 l) to give the neutral amino acids and subsequent elution with 1 N acetic acid (1 l) yielded the acidic amino acids (2.3 g). The acidic amino acid fraction was concentrated and subjected to chromatography on Dowex 50W × 4 resin [100—200 mesh, 4.5 × 35 cm, buffered with a pH 2.70 ammonia–formate buffer and eluted with the same buffer (60 ml fractions)]. Each fraction was monitored by HPLC. The fractions containing Lp-2 were collected and desalted using Dowex 50W × 4 (100—200 mesh) to yield crude Lp-2 (252 mg). The crude Lp-2 (195 mg) was subjected to gel chromatography (Cellulofine GCL-25-m, 1.5 × 95 cm, eluted with water). The fractions which showed a single peak on HPLC were collected. The final purification was carried out on a cellulose column (2.2 × 25 cm) with ethanol–water (2 : 1) to give Lp-2 (**1**) as a colorless amorphous mass. $[\alpha]_D$ −17.5° (c = 0.939, H_2O). FDMS m/z: 231 $(M+H)^+$. IR ν_{max}^{KBr} cm^{-1}: 3400, 3100, 2040, 1660, 1590, 1510, 1400, 1230. ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 1.75—1.85 (2H, m, C_3-H_a and C_2-H_a), 1.995 (1H, m, C_2-H_b), 2.18—2.36 (3H, m, C_3-H_b, C_4-H_a and C_4-H_b), 2.952 (1H, ddd, J = 4.49, 7.52, 14.56 Hz, C_1-H_a), 3.463 (1H, dd, J = 4.85, 8.74 Hz, C_3-H), 3.493 (1H, ddd, J = 7.28, 7.77, 14.56 Hz, C_1-H_b), 4.072 (1H, dd, J = 3.64, 9.34 Hz, C_2-H). ^{13}C-NMR (25 MHz, D_2O, TMS) δ: 23.3 (t), 28.2 (t), 29.9 (t), 38.6 (t), 52.7 (d), 63.0 (d) 174.3 (s), 179.4 (s), 179.7 (s).

Methyl 2(*S*),3′(*S*)-1-(3-*tert*-Butoxycarbonylamino-3-methoxycarbonylpropyl)-5-oxo-2-pyrrolidinecarboxylate (2)——Di-*tert*-butyldicarbonate (20 mg) and triethylamine (20 μl) in dioxane (0.5 ml) was added to a solution of Lp-2 (**1**) (19.4 mg) in water (0.5 ml). The reaction mixture was stirred at room temperature for 24 h, then 10% citric acid was added and the whole was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to yield a colorless oil. An ethereal solution of diazomethane was added to the methanolic solution (1 ml) of the residual oil at 0°C till it became slightly yellow. The solvent was evaporated off, and the residue was subjected to silica gel chromatography with hexane–ethyl acetate (5 : 1) to give **2** (5.6 mg, 6%). EIMS m/z: 302 $(M-tBu)^+$, 258 $(M-CO_2tBu+H)^+$. ^{1}H-NMR (500 MHz, CD_3OD, TMS) δ: 1.350 (9H, s, tBu), 1.773 (1H, m, C_2-H_a), 1.910 (1H, m, C_3-H_a), 2.000 (1H, m, C_2-H_b), 2.20—2.38 (3H, m, C_4-H_a, C_4-H_b and C_3-H_b), 2.935 (1H, m, C_1-H_a), 3.605 (1H, m, C_1-H_b), 3.630 (3H, s, CO_2Me), 3.698 (3H, s, CO_2Me), 3.964 (1H, dd, J = 4.20, 8.75 Hz, C_3-H), 4.284 (1H, dd, J = 3.00, 8.51 Hz, C_2-H).

Methyl 2(*S*)-2-Benzyloxycarbonylamino-4-*tert*-butoxycarbonylbutanoate (4)——An ethereal solution of diazomethane was added to the methanolic solution of **3** (517 mg, 10 ml) synthesized from L-glutamic acid according to Itoh's procedure till it became slightly yellow. The solvent was evaporated off and the residue was chromatographed on silica gel with hexane–ethyl acetate (10 : 1) to give **4** (331 mg, 64%) as a colorless oil. $[\alpha]_D$ +4.9° (c = 0.35, $CHCl_3$). FDMS m/z: 352 $(M+H)^+$. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3425, 2975, 2950, 1720. ^{1}H-NMR (60 MHz, $CDCl_3$, TMS) δ: 1.42 (9H, s, tBu), 1.8—2.0 (4H, m, C_3-H_2 and C_4-H_2), 3.76 (3H, s, CO_2Me), 4.35 (1H, m, C_2-H), 5.08 (2H, s, $CH_2C_6H_5$), 5.49 (1H, d, J = 4.6 Hz, NH), 7.30 (5H, s, C_6H_5).

Methyl 2(*S*),3′(*S*)-2-*N*-(3-Benzyloxycarbonylamino-3-benzyloxycarbonylpropyl)amino-4-*tert*-butoxycarbonylbutanoate (7)——A 5%Pd–C catalyst (50 mg) and 1 N hydrochloric acid (0.95 ml) were added to a methanolic solution of **4** (331 mg). The mixture was stirred under a hydrogen atmosphere at room temperature for 2 h, then the catalyst was filtered off and the filtrate was concentrated to dryness to yield the hydrochloride **5**. The amine hydrochloride **5** and aspartic-β-semialdehyde derivative **6** (273 mg) were dissolved in methanol (10 ml) and the solution was adjusted to pH 6 with phosphate buffer. Sodium cyanoborohydride (50.4 mg) was added to the solution and the reaction mixture was stirred at room temperature for 24 h. Methanol was removed by evaporation, and the residue was taken up in water (3 ml). The aqueous solution was extracted with ethyl acetate and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was chromatographed on silica gel with hexane–ethyl acetate (2 : 1) to give **7** (195 mg, 38% from **4**) as a colorless oil. $[\alpha]_D$ −13.5° (c = 0.619, $CHCl_3$). FDMS m/z: 245 (M^+). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3440, 2980, 1740, 1730. ^{1}H-NMR (60 MHz, $CDCl_3$, TMS) δ: 1.42 (9H, s, tBu), 1.73—2.00 (4H, m, C_3-H_2 and C_2-H_2), 2.13—2.73 (4H, m, C_4-H_2 and C_1-H_2), 3.13 (1H, m, C_2-H), 3.65 (3H, s, CO_2Me), 4.50 (1H, m, C_3-H), 5.07 (2H, s, $CH_2C_6H_5$), 5.10 (2H, s, $CH_2C_6H_5$), 6.00 (1H, brd, NH), 7.30 (10H, s, C_6H_5 × 2).

2(*S*),3′(*S*)-1-(3-Amino-3-carboxypropyl)-5-oxo-2-pyrrolidinecarboxylic Acid (1)——A 5% Pd–C catalyst (50 mg) was added to a solution of **7** (178 mg) in 1 N methanolic hydrochloric acid (20 ml), and the mixture was stirred under a hydrogen atmosphere at room temperature for 2 h. The catalyst was filtered off and the filtrate was evaporated to dryness under reduced pressure. Benzene (1 ml) was added to the residue and the mixture was evaporated *in vacuo*. This operation was repeated three times. Then 1 N sodium hydroxide (2.5 ml) was added to the residue and stirred at 0 °C for 1 h. The reaction mixture was applied to Amberlite IRC-50 resin (H^+) and eluted with water. The eluate was concentrated *in vacuo* and the residue was applied to a column of Dowex 50W × 4 resin (100—200 mesh, buffered with

pH 2.70 ammonia–formate buffer) and eluted with the same buffer. The fractions containing Lp-2 were collected and desalted using Dowex 50W × 4 (H^+) to give Lp-2 (**1**) (32.5 mg, 43%) as a colorless amorphous mass. $[\alpha]_D -17.8°$ ($c = 1.05$, H_2O). Synthetic **1** was identical with the natural product (PC, HPLC, IR, ^1H- and ^{13}C-NMR spectra).

Acknowledgement This work was supported by a Grant-in-Aid for General Scientific Research (No. 61470145) from the Ministry of Education, Science and Culture, Japan.

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