(2*R*), (1′*R*) and (2*R*), (1′*S*)-2-Amino-3-(1,2-dicarboxyethylthio)propanoic Acids from *Amanita pantherina*. Antagonists of *N*-Methyl-D-aspartic Acid (NMDA) Receptors

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An antagonist of NMDA-sensitive glutamate receptors in rat brain membrane and rat spinal motoneurones was isolated from *Amanita pantherina* and identified as a diastereoisomeric mixture of 2-amino-3-(1,2-dicarboxyethylthio)propanoic acids. The mixture was separated and the absolute configurations of the components were determined as (2*R*), (1′*R*) and (2*R*), (1′*S*), by analysis of the optical properties.

Keywords *Amanita pantherina*; glutamate receptor; antagonist; diastereoisomer; configuration

Agonists and antagonists of excitatory amino acid (EAA) receptors are of great importance not only as experimental tools for investigating fundamental mechanisms of the mammalian central nervous system (CNS) but also as potential drugs for treating CNS disorders.[1-3] Since important glutamate agonists such as ibotenic acid and acromelic acids are known to be distributed in Basidiomycetes,[4,5] we hoped to isolate neurologically active compounds from mushrooms.[6,7] In the course of the studies, we isolated a diastereoisomeric mixture of 2-amino-3-(1,2-dicarboxyethylthio)propanoic acids from a toadstool, *Amanita pantherina* (DC.: Fr.) KROMBH., which showed an antagonistic action to *N*-methyl-D-aspartic acid (NMDA)-sensitive glutamate receptors in rat brain and spinal motoneurons. Although this diastereoisomeric mixture had been found in human urine, guinea-pig kidney,[8] calf lens[9] and *Asparagus officinalis*,[10] separation of the mixture and details of its biological activities have not been reported. Herein, we report the separation of the mixture by the chromatographic methods, stereochemical assignments of the components, and their biological activities.

The neutral and acidic amino acid fraction, obtained in a usual manner from the 65% aqueous ethanol extract of fresh fruiting bodies (6.6 kg) of *Amanita pantherina*, was applied to a Dowex 50W × 1 (AcO^-) column. After elution of neutral amino acids by washing the column with water, the column was eluted with 1 N AcOH to give the acidic amino acid fraction I (A-I) and further elution with 4 N AcOH yielded the acidic amino acid fraction II (A-II). In A-II, an unusual peak was noticed on HPLC (t_R 19.0 min). This amino acid, KI-II (*Rf* 0.24 on TLC) was found to be acidic from its elution behavior on ion exchange resins and its yellow coloration with bromocresol green. The isolation of KI-II was achieved by means of the following procedures. A-II was applied to a Dowex 50W × 4 column which was buffered with an ammonia–formate buffer (pH 2.50). The column was eluted with the same buffer system changing the pH from 2.50 to 2.70 and then to 3.00. Elution was monitored by HPLC. The fractions containing KI-II were collected and desalted to give KI-II as a colorless amorphous mass.

The amino acid, KI-II (**1**), $[\alpha]_D$ −23.8° (c = 0.168, H_2O), showed a blue to purple coloration with ninhydrin. The molecular formula, $C_7H_{11}NO_6S$, of KI-II was determined by FABMS; m/z 238 ($M^+ + H$) and by HRMS of the carbobenzoxy (Cbz)-trimethyl ester **2**; m/z 413.1125 (M^+) Calcd for $C_{18}H_{23}NO_8S$ 413.1144. The IR spectrum of KI-II exhibited characteristic amino acid absorptions at 3200—2800, 1720, 1620—1580 and 1400 cm^{-1}. In order to characterize the functional groups in KI-II, KI-II was treated with Cbz chloride and then with diazomethane to give the Cbz-trimethyl ester **2**. This indicates that three carboxyl groups and one amino group exist in the molecule, and further, α-amino acid nature is indicated by the coloration with ninhydrin and the chemical shifts of the methine signals (4.02 ppm) in the ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum of KI-II exhibited signals due to four methylene groups at 2.7—2.9 (4H) and 3.1—3.3 (4H) ppm and four methine groups at 3.73 (2H) and 4.02 (2H) ppm. These signals were attributed to two sets of partial structures A and B (Fig. 1) by analyzing the coupling constants and the chemical shifts. The ^{13}C-NMR spectrum also exhibited two sets of signals at 31.8 (t); 32.7 (t), 36.8 (t); 37.3 (t), 43.4 (d); 44.6 (d), 53.7 (d); 53.9 (d) and 176.6 (s); 176.8 (s) ppm besides two independent signals at 172.3 (s) and 175.4 (s) ppm. These NMR data suggest that KI-II is a mixture of similar compounds, most likely a diastereoisomeric mixture. Taking into account the molecular formula, partial structure A should be linked to partial structure B through a sulfur atom, giving the structure **1**. KI-II was eventually deduced to be a diastereoisomeric mixture of 2-amino-3-(1,2-dicarboxyethylthio)propanoic acids (Chart 1).

In order to determine the absolute configuration of each isomer and to examine individual biological activities, we attempted to separate KI-II. Thus, KI-II was applied to a Dowex 50W column equilibrated with pH 2.70 ammonia–

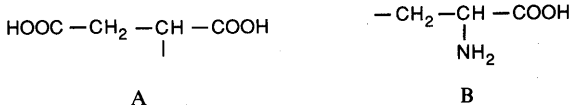

Fig. 1

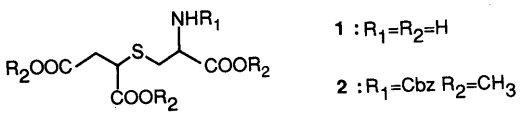

Chart 1

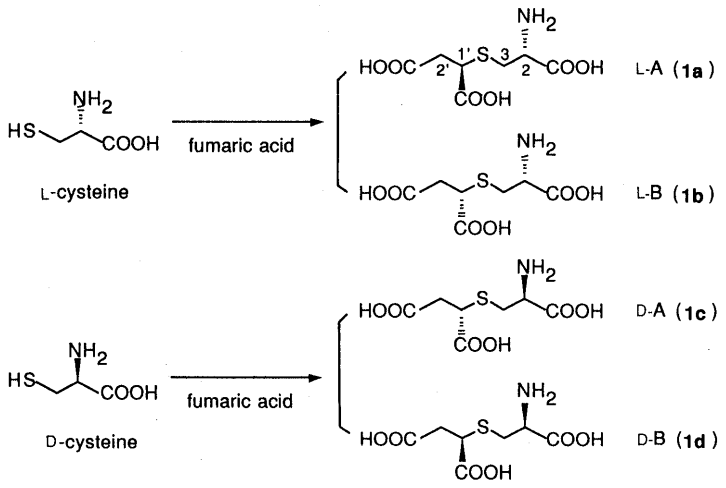

Chart 2

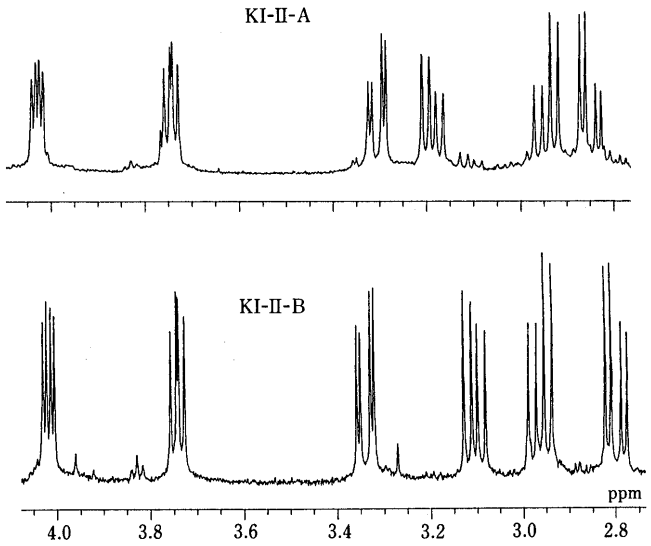

Fig. 2. ^{1}H-NMR Spectra of KI-II-A and KI-II-B

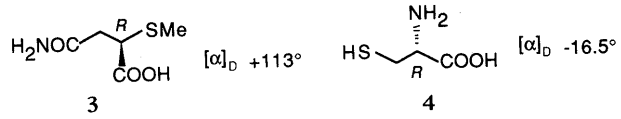

Chart 3

TABLE I. $[\alpha]_D$ Data

Synthetic				Natural	
L-A	+30.0°	D-A	−34.1°	KI-II-A	+23.1°
L-B	−96.0°	D-B	+84.0°	KI-II-B	−80.0°

formate buffer and the column was eluted with the same buffer to give two isomers, KI-II-A (**1a**) and KI-II-B (**1b**), whose ^{1}H-NMR spectra are clearly distinguishable (Fig. 2). The former has a shorter retention time on HPLC. The fractions still containing the mixture were again subjected to the same procedure.

In order to compare the optical properties, four stereoisomers of KI-II, L-A (**1a**), L-B (**1b**), D-A (**1c**) and D-B (**1d**), were prepared by reaction of L- or D-cysteine with fumaric acid,[8] respectively, followed by separation on a Dowex 50W column (Chart 2). Here, L- or D- means the isomer derived from L- or D-cysteine, respectively, and series A has a shorter retention time on HPLC, so L-A is the enantiomer of D-A and L-B is that of D-B. KI-II-A and KI-II-B were identical with L-A (or D-A) and L-B (or D-B), respectively, in all respects except the optical properties. The agreement of the $[\alpha]_D$ values between KI-II-A and L-A and between KI-II-B and L-B (Table I) indicates that KI-II-A and KI-II-B both have 2*R* configuration (L-isomers).

The configurations at C-1′ (α-thiocarboxylic acid moiety) were deduced as follows. Since the $[\alpha]_D$ value of *R*-α-thiomethylcarboxylic acid (**3**) (+113.0°)[11] is much larger than that of *R*-cysteine (L-cysteine) (**4**) (−16.5°) (Chart 3), the $[\alpha]_D$ value of the 1′*R* isomer is presumed to be positive and that of the 1′*S* isomer is anticipated to be negative, irrespective of the configuration at C-2. From the $[\alpha]_D$ data of the isomers listed in Table I, the configurations were confirmed as 2*R*, 1′*R* for KI-II-A (**1a**) and 2*R*, 1′*S* for KI-II-B (**1b**).

The activity of KI-II (**1**) on glutamate receptors was demonstrated by a receptor binding assay. KI-II (**1**) inhibited the binding of ^{3}H-glutamate to the glutamate receptors in bovine cerebral cortical membranes with an IC_{50} value of 2×10^{-5} M. Representative antagonists, D-2-amino-5-phosphonopentanoic acid (D-AP5) and (±)-3-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), inhibited the binding with IC_{50} values of 2×10^{-6} and 1×10^{-6} M, respectively, and the agonist, NMDA inhibited the binding with an IC_{50} value of 4×10^{-6} M. KI-II (**1**) also inhibited the binding of ^{3}H-MK-801 to the NMDA sensitive glutamate receptors. This indicates that KI-II (**1**) is an NMDA antagonist, because MK-801 can bind to the binding sites when the cationic ion channels, coupled to the NMDA-sensitive glutamate receptors, are opened by an NMDA agonist, but can not bind in the presence of an NMDA antagonist.[12] The activities of the four synthetic isomers were also evaluated electrophysiologically. They inhibited the depolarizing action of NMDA on spinal motoneurones in newborn rats. The activities of the four isomers are almost the same and are weaker by about one

order of magnitude than that of D-AP5. The details of the biological activities will be reported elsewhere.

Considering its wide distribution, KI-II (**1**) may have an important biological role and therefore further studies on its stereochemistry and the biological activities are under way.

Experimental

General Procedure Optical rotations were determined on a JASCO DIP-340 spectrometer. Low-resolution electron impact mass spectra (LRMS) and FABMS were recorded on a JMS-DX-303 and JMS-AX-500 spectrometr. High-resolution electron impact mass spectra (HRMS) were recorded on a JMS-OISG-2 spectrometer. IR spectra were recorded on a JASCO A-100S spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL FX-100 and JEOL JNM-GX 500 spectrometer. Chemical shifts are reported in δ units downfield from internal tetramethylsilane. HPLC was carried out on a JASCO Trirotar equipped with a refractive index detector (GL Science RI Model 504). A stainless steel column packed with Hitachi gel #2618 (cation exchange resin) was used and eluted with an ammonia–formate buffer at a rate of 0.5 ml/min. TLC was performed using *n*-butanol–acetic acid–water (4 : 1 : 2) and isopropanol–ammonia–water (7 : 1 : 2) by the ascending technique on cellulose (Merck 5728).

Isolation of 2-Amino-3-(1,2-dicarboxyethylthio)propanoic Acids (KI-II) (1) *Amanita pantherina* (DC.: Fr.) KROMDH. (6.6 kg) collected near Sendai was extracted with 65% aqueous ethanol. After filtration of the extract, the filtrate was concentrated *in vacuo* to remove ethanol. The concentrate 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 (4 l), affording the acidic and neutral amino acids. The column was subsequently eluted with 1 N ammonia (3 l) and 4 N ammonia to give the basic amino acid fractions I and II, respectively. The acidic and neutral amino acid fraction was concentrated, and applied to Dowex 1 × 8 resin (AcO^-, 100—200 mesh, 4.0 × 31 cm). The column was washed with water (3 l) to give the neutral amino acids (11.0 g) and subsequent elution with 1 N (2 l) and 4 N acetic acid (2.2 l) yielded the acidic amino acid fractions I (A-I) (6.7 g) and II (A-II) (1.8 g), respectively. A-II was concentrated, subjected to chromatography on Dowex 50W × 4 resin (100—200 mesh, 3.5 × 18 cm) buffered with pH 2.50 ammonia–formate buffer, eluted with the same buffer and fractionated. The column was subsequently eluted with a pH 2.70 buffer (0.9 l) and then with a pH 3.00 buffer (0.3 l). Each fraction was monitored by HPLC. The fractions containing KI-II were collected and desalted using Dowex 50W × 4 (100—200 mesh) to yield KI-II (**1**) (70 mg) as a colorless amorphous mass, $[\alpha]_D$ −23.8° (c = 0.168, H_2O). FABMS *m/z*: 238 $(M+H)^+$. IR ν_{max}^{KBr} cm^{-1}: 3200—2800, 1720, 1620—1580, 1400. ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.02 (1H, dd, J = 4.4, 8.3 Hz), 4.01 (1H, dd, J = 4.5, 8.7 Hz), 3.74 (1H, dd, J = 6.4, 8.3 Hz), 3.73 (1H, dd, J = 6.4, 8.3 Hz), 3.33 (1H, dd, J = 3.4, 15.1 Hz), 3.30 (1H, dd, J = 4.3, 15.6 Hz), 3.18 (1H, dd, J = 8.8, 14.7 Hz), 3.10 (1H, dd, J = 7.8, 16.6 Hz), 2.95 (1H, dd, J = 7.8, 16.6 Hz), 2.94 (1H, dd, J = 8.3, 16.6 Hz), 2.84 (1H, dd, J = 5.9, 16.6 Hz), 2.79 (1H, dd, J = 5.9, 16.6 Hz). ^{13}C-NMR (100 MHz, D_2O, TMS) δ: 31.8 (t), 32.7 (t), 36.8 (t), 37.3 (t) 43.4 (d), 44.6 (d), 53.7 (d), 53.9 (d), 172.3 (s), 175.4 (s), 176.6 (s), 176.8 (s).

Separation of KI-II to KI-II-A (1a) and KI-II-B (1b) KI-II (70 mg) was subjected to chromatography on Dowex 50W resin [200—400 mesh, 1.5 × 90 cm, buffered with a pH 2.50 ammonia–formate buffer and eluted with a pH 2.70 buffer (5 ml fractions)]. Fractions 61—66 and 68—70, which showed peaks at t_R 18.5 and 19.5 min on HPLC were respectively combined and desalted using Dowex 50W to give KI-II-A (**1a**) (10 mg) and KI-II-B (**1b**) (5 mg). **1a**: mp 125—126 °C, $[\alpha]_D$ +23.11° (c = 0.130, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3200—2800, 1720, 1620—1580, 1400. ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.03 (1H, dd, J = 4.3, 8.0 Hz), 3.74 (1H, dd, J = 6.0, 8.6 Hz), 3.31 (1H, dd, J = 4.3, 15.0 Hz), 3.19 (1H, dd, J = 8.0, 15.0 Hz), 2.94 (1H, dd, J = 8.6, 17.0 Hz), 2.85 (1H, dd, J = 6.0, 17.0 Hz). *Rf* on cellulose TLC: 0.24 (*n*-butanol–acetic acid–water (4 : 1 : 2)), 0.05 (isopropanol–ammonia–water (7 : 1 : 2)). **1b**: mp 125—126 °C, $[\alpha]_D$ −80.55° (c = 0.102, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3200—2800, 1720, 1620—1580, 1400. ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.02 (1H, dd, J = 4.3, 8.6 Hz), 3.73 (1H, dd, J = 6.4, 8.5 Hz), 3.34 (1H, dd, J = 4.3, 14.5 Hz), 3.12 (1H, dd, J = 8.6, 14.5 Hz), 2.96 (1H, dd, J = 8.5, 17.1 Hz), 2.80 (1H, dd, J = 6.4, 17.1). *Rf* on cellulose TLC: 0.24 (*n*-butanol–acetic acid–water (4 : 1 : 2)), 0.05 (isopropanol–ammonia–water (7 : 1 : 2)).

Preparation of the Four Stereoisomers (L-A (1a), L-B (1b), D-A (1c) and D-B (1d)) Fumaric acid (213.8 mg) was added to a solution of L-cysteine (220.2 mg) in water (3 ml). The reaction mixture was stirred at room temperature for 24 h, then acetone (15 ml) was added. The precipitate was collected by filtration and recrystallized from water–acetone to give the diastereoisomeric mixture (310.0 mg, 70% yield). The separation was carried out in the same manner as for KI-II to give L-A (**1a**) and L-B (**1b**). D-A (**1c**) and D-B (**1d**) were prepared by the reaction of D-cysteine and fumaric acid in the same way. **1a**: mp 125—126 °C, $[\alpha]_D$ +30.0° (c = 0.447, H_2O). ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.02 (1H, dd, J = 4.3, 8.1 Hz), 3.74 (1H, dd, J = 6.0, 8.5 Hz), 3.30 (1H, dd, J = 4.3, 14.5 Hz), 3.18 (1H, dd, J = 8.1, 14.5 Hz), 2.94 (1H, dd, J = 8.5, 17.1 Hz), 2.85 (1H, dd, J = 6.0, 17.1). **1b**: mp 125—127 °C, $[\alpha]_D$ −96.4° (c = 0.671, H_2O). ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.01 (1H, dd, J = 3.9, 8.6 Hz), 3.72 (1H, dd, J = 6.4, 8.5 Hz), 3.33 (1H, dd, J = 3.9, 14.5 Hz), 3.10 (1H, dd, J = 8.6, 14.5 Hz), 2.95 (1H, dd, J = 8.5, 17.1 Hz), 2.79 (1H, dd, J = 6.4, 17.1 Hz). **1c**: mp 115—117 °C, $[\alpha]_D$ −34.1° (c = 0.270, H_2O). ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.02 (1H, dd, J = 4.3, 8.1 Hz), 3.74 (1H, dd, J = 6.0, 8.6 Hz), 3.31 (1H, dd, J = 4.3, 14.5 Hz), 3.18 (1H, dd, J = 8.1, 14.5 Hz), 2.94 (1H, dd, J = 8.6, 17.1 Hz), 2.85 (1H, dd, J = 6.0, 17.1 Hz). **1d**: mp 115—116 °C, $[\alpha]_D$ +84.0° (c = 0.617, H_2O). ^{1}H-NMR (500 MHz, D_2O, TMS) δ: 4.01 (1H, dd, J = 3.9, 8.6 Hz), 3.74 (1H, dd, J = 6.4, 8.5 Hz), 3.34 (1H, dd, J = 3.9, 14.5 Hz), 3.10 (1H, dd, J = 8.6, 14.5 Hz), 2.96 (1H, dd, J = 8.5, 17.1 Hz), 2.80 (1H, dd, J = 6.4, 17.1 Hz).

Carbobenzoxy-KI-II Trimethyl Ester (2) Carbobenzoxy chloride (7 mg) and 1 N NaOH (0.5 ml) was added to a solution of KI-II (**1**) (8.0 mg) in water (0.5 ml). The reaction mixture was stirred at 0 °C for 3 h, then acidified with 1 N HCl and 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 a methanolic solution (0.5 ml) of the residual oil at 0 °C. The solvent was evaporated off, and the residue was subjected to silica gel TLC (hexane–ethyl acetate (2 : 1)) to give **2** (9.8 mg, 70%). LRMS *m/z*: 413 (M^+), 381 $(M-MeOH)^+$, 354 $(M-CO_2Me)^+$, 278 $(M-Cbz)^+$. HRMS *m/z*: 413.1125; Calcd for $C_{18}H_{23}NO_8S$: 413.1144.

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