Pergamon

*Tetrahedron Letters*, Vol. 35, No. 44, pp. 8201-8204, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)01746-8

# Three New Amino Acids from a Poisonous Mushroom, *Clitocybe Acromelalga*

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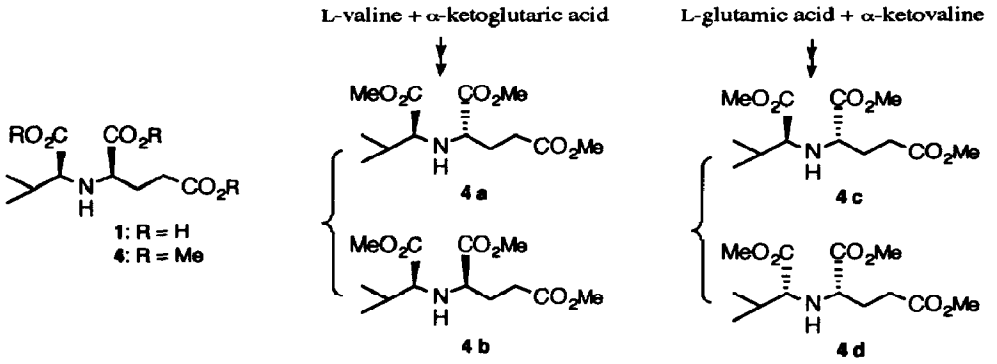
**Abstract**: Three new amino acids, valinopine, *epi*-leucinopine and isoleucinopine, were isolated from a poisonous mushroom, *Clitocybe acromelalga*. The structures were determined to be **1**, **2** and **3**, respectively, by spectroscopic analysis and chemical synthesis.

*Clitocybe acromelalga* (Tricholomataceae) is one of the most poisonous mushrooms in Japan. Ingestion of this mushroom causes violent pain and red coloration in the fingers and toes, similar to acromelalgia or erythromelalgia, and the pain continues for two to four weeks. The uniqueness of the symptoms has attracted considerable attention to the active principles, and many neurologically active substances have been isolated so far. These compounds are acromelic acids A and B,[1] potent neuroexcitatory and neurotoxic amino acids,[2] and the congeners, acromelic acids C,[3] D and E,[4] stizolobic acid and stizolobinic acid,[5] competitive antagonists of the quisqualic acid receptor subtype,[6] β-cyano-L-alanine and its γ-glutamyl peptide,[7] the so-called neurolathyrogens,[8] and *N*-(γ-aminobutyryl)-L-glutamic acid, a convulsant.[9] The varieties of structural features and neurological activities of the isolated compounds are characteristic of *C. acromelalga*. Continuation of our investigation has led to the isolation of three new amino acids belonging to the opine family for which the names of valinopine (V-P), *epi*-leucinopine (E-P) and isoleucinopine (I-P) are proposed. Herein, we describe the isolation and structures of these amino acids.

The neutral and acidic amino acid fraction obtained by the usual manner from the 70 % aqueous ethanol extract of the fresh fruiting bodies (6.2 kg) of *C. acromelalga* was subjected to Dowex 1 ($AcO^-$) column. The column was successively eluted with $H_2O$, 1N AcOH, 4N AcOH and 1N HCl to give neutral, acidic I, acidic II and acidic III amino acid fractions, respectively. The acidic III fraction was fractionated into four parts by a charcoal column eluting successively with $H_2O$, 50 % aq. MeOH, MeOH and acetone. The 50 % aq. MeOH fraction was applied to a Dowex 50 W column equilibrated with an ammonia-formate buffer (pH 2.50), and the column was eluted with the same buffer system whose pH was stepwise changed from 2.50 to 2.70 and 3.00 to afford V-P (6.0 mg) and the mixture of E-P and I-P (15.0 mg).

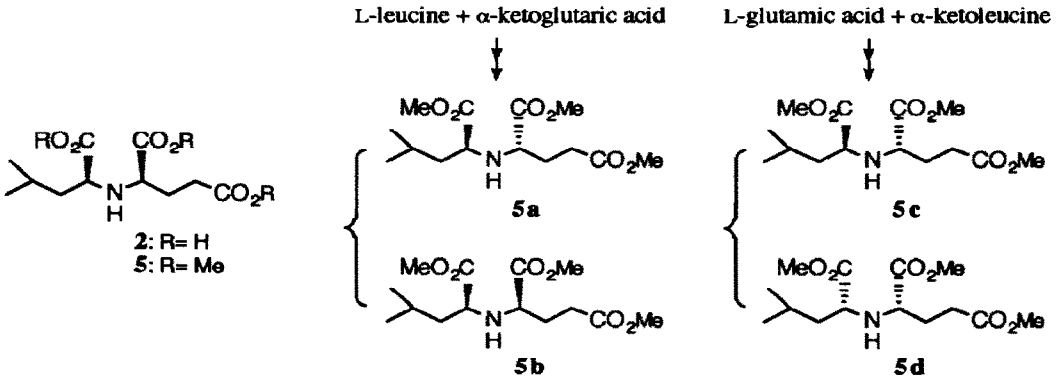
V-P, $[\alpha]_D^{21}$ +6.3°(*c* 0.32, $H_2O$), *Rf* 0.78 on cellulose TLC (*n*BuOH/AcOH/$H_2O$= 4/1/2), showed a purple coloration with ninhydrin. The molecular formula $C_{10}H_{17}NO_6$ of V-P was determined by FAB-MS;*m/z* 248 $(M+H)^+$ and 270 $(M+Na)^+$ and $^{13}C$-NMR spectrum[10], and by HREI-MS of trimethyl-V-P (**4**); *m/z* 289.1543 ($[M]^+$, $C_{13}H_{23}NO_6$) obtained by treating V-P with $CH_2N_2$. Analysis of the $^1H$-NMR spectrum ($D_2O$, 500 MHz) of V-P with the aid of decoupling experiments indicated the presence of 1, 1-disubstituted-2- methylpropane (δ 0.93, 3H, d, *J*=7.0 Hz; 0.98, 3H, d, *J*=7.0 ; 2.18, 1H, m; 3.55, 1H, d, *J*=4.0) and 1, 1, 3- trisubstituted-propane

(δ 2.52, 2H, t, $J$=7.0 Hz; 2.07, 2H, m; 3.62, 1H, t, $J$=6.0) structures in-V-P. And, they were presumed to be valine and glutamic acid units, respectively, from the chemical shifts of these signals and the tricarboxylic acid nature of V-P. The situation of two units connected through the same nitrogen atom is suggested by the fact that V-P has only one nitrogen atom. V-P was thus thought to be *N*-(1-carboxy-2-methylpropyl)glutamic acid (**1**). In order to confirm this assumption and to determine the absolute configurations, the stereoisomers of trimethyl-V-P (**4**) were synthesized as follows. Reductive coupling of L-valine and α-ketoglutaric acid by use of $NaBH_3CN$ followed by methylation with $CH_2N_2$ gave a diastereoisomeric mixture of trimethylesters, **4a** and **4b**, which were separated by HPLC.[11] L-Glutamic acid and α-ketovaline (3-methyl-2-oxobutyric acid) were also coupled and methylated, and the products were separated into **4c** and **4d**. In these stereoisomers, **4a** was proved to be the same as **4c** by comparing the $^1$H-NMR spectra,[11] optical rotations (**4a**: $[\alpha]_D^{21}$-33.0°($c$ 0.91, $CHCl_3$), **4c**: $[\alpha]_D^{21}$-38.2°($c$ 0.25, $CHCl_3$)) and *Rt* s on HPLC (**4a**: 18.0 min, **4c**: 18.0 min). The $^1$H-NMR spectrum and *Rt* on HPLC (24.0 min) of **4b** are identical with those of **4d** (24.0 min), whereas the optical rotation of **4b** ($[\alpha]_D^{21}$+3.1°($c$ 0.26, $CHCl_3$)) is the opposite of **4d** ($[\alpha]_D^{21}$-5.4°($c$ 0.11, $CHCl_3$)), indicating **4b** to be the enantiomer of **4d**. Taking into account the starting compounds of the synthesis, it is concluded that **4a** (=**4c**), **4b** and **4d** are trimethyl- $L^{glu}$, $L^{val}$, trimethyl-$D^{glu}$, $L^{val}$ and trimethyl-$L^{glu}$, $D^{val}$, respectively. The $^1$H-NMR spectrum, *Rt* on HPLC (24.0 min) and optical rotation ($[\alpha]_D^{21}$+3.9°($c$ 0.10, $CHCl_3$)) of trimethyl-V-P (**4**) were identical with those of **4b**, establishing the structure (2*R*, 1'*S*)-*N*-(1-carboxy-2-methylpropyl)glutamic acid ($D^{glu}$, $L^{val}$) (**1**) for V-P.




E-P and I-P were proved to be isomers, having the molecular formulas $C_{11}H_{19}NO_6$, by FAB-MS and $^{13}$C-NMR spectrum of the mixture. The mixture (5 mg) could be separated by HPLC after methylation with $CH_2N_2$, into trimethyl-E-P (**5**) (1.5 mg), $[\alpha]_D^{21}$-7.1°($c$ 0.57, $CHCl_3$), HR-EIMS; *m/z* 303.1677 $[M]^+$, $C_{14}H_{25}NO_6$, and trimethyl-I-P (**6**) (2.7 mg), $[\alpha]_D^{21}$+5.1°($c$ 0.59, $CHCl_3$), HR-EIMS; *m/z* 303.1677 $[M]^+$, $C_{14}H_{25}NO_6$. The $^1$H-NMR spectrum ($CDCl_3$, 500 MHz) of trimethyl-E-P (**5**) showed signals due to 1, 1-disubstituted-3-methylbutane (δ 0.90, 3H, d, $J$=6.5 Hz; 0.92, 3H, d, $J$=6.5; 2.00, 1H, m; 1.47, 2H, t, $J$=7.0; 3.25, 1H, t, $J$=7.0) and 1, 1, 3-trisubstituted-propane (δ 2.42, 1H, ddd, $J$=6.5, 8.0, 16.5 Hz; 2.50, ddd, $J$=7.0, 8.0, 16.5; 1.80, 1H, m; 1.91, 1H, m; 3.26, 1H, dd, $J$=5.0, 8.0) as well as three carboxymethyl signals (δ 3.69, 3H, s; 3.70, 3H, s; 3.72, 3H, s). These data suggested that the valine moiety of V-P (**1**) should be replaced by leucine in E-P (**2**). In order to confirm this assumption and to determine the absolute configurations, the stereoisomers were

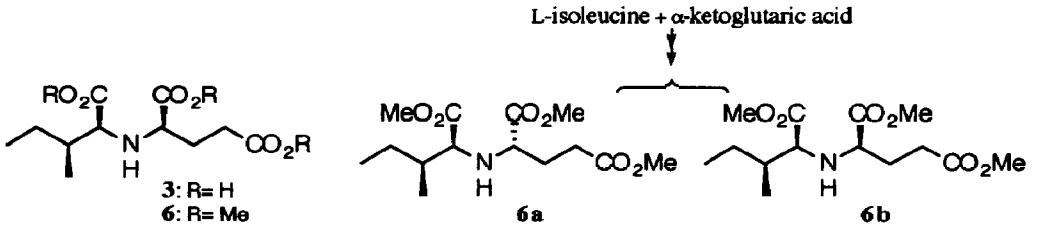
synthesized from L-leucine and α-ketoglutaric acid (**5a** and **5b**) and from L-glutamic acid and 2-ketoleucine (4-methyl-2-oxopentanoic acid) (**5c** and **5d**) by the same method as mentioned above. **5a** was demonstrated to be identical with **5c** by comparing the $^1$H-NMR spectra,[12] optical rotations (**5a**:$[\alpha]_D^{21}$-36.6°(*c* 0.57, $CHCl_3$), **5c**: $[\alpha]_D^{21}$-34.7°(*c* 0.81, $CHCl^3$)) and *Rt*s on HPLC (**5a**: 25.0 min, **5c**: 25.0 min). The enantiomeric relationship between **5b** and **5d** was also verified by the same $^1$H-NMR spectra and *Rt*s on HPLC (**5b**: 34.0 min, **5d**: 34.0 min) and opposite optical rotations (**5b**:$[\alpha]_D^{21}$-6.3°(*c* 0.57, $CHCl_3$), **5d**:$[\alpha]_D^{21}$+5.7°(*c* 0.56, $CHCl_3$)). Thus, **5a** (= **5c**), **5b** and **5d** are inevitably assigned as trimethyl-$L^{glu}$, $L^{leu}$, trimethyl-$D^{glu}$, $L^{leu}$ and trimethyl-$L^{glu}$, $D^{leu}$, respectively. Trimethyl-E-P (**5**) was proved to be identical with **5b** in all respects: $^1$H-NMR spectrum, *Rt* on HPLC (34.0 min) and optical rotation ($[\alpha]_D^{21}$-7.1°(*c* 0.57, $CHCl_3$)). E-P is thus concluded to be (2*R*, 1'*S*) -*N*-(1-carboxy-2-methylbutyl)glutamic acid ($D^{glu}$, $L^{leu}$) (**2**).




Leucinopine has been isolated as a characteristic compound of some crown-gall tumors incited by *Agrobacterium tumefaciens* strain Bo 542.[13] The absolute stereochemistry of leucinopine was determined as $L^{glu}$, $L^{leu}$ on the basis of specificity of catabolism of four synthetic diastereoisomers by *A. tumefaciens* strains carrying the tumor-inducing plasmid pTi Bo 542.[14] E-P is thus the epimer of leucinopine in regard to C-2.

The $^1$H-NMR spectrum (pyridine-$d_5$, 500 MHz) of trimethyl-I-P (**6**) showed signals due to 1, 1-disubstituted-2-methylbutane (δ 0.83, 3H, t, *J*=7.5 Hz; 0.93, 3H, d, *J*=7.0; 1.23, 1H, m; 1.62, 1H, m, 1.78, 1H, m; 3.23, 1H, d, *J*=6.5) and 1, 1, 3-trisubstituted-propane (δ 2.08, 1H, m; 2.17, 1H, m; 263, 2H, m; 3.43, 1H, dd, *J*=5.0, 8.0) as well as three carboxymethyl signals (δ 3.62, 3H, s; 3.64, 3H, s; 3.68, 3H, s). These data suggested that the leucine moiety of E-P (**2**) should be replaced by isoleucine in I-P (**3**). In order to confirm this assumption and to determine the absolute configurations, two stereoisomers (**6a** and **6b**) were synthesized from L-isoleucine and α-ketoglutaric by the same method mentioned above. By comparing the behavior patterns on HPLC and optical rotations of **6a** (*Rt* 42.0 min, $[\alpha]_D^{21}$ +6.0°(*c* 0.59, $CHCl_3$)) and **6b** (*Rt* 29.0 min, $[\alpha]_D^{21}$-14.3°(*c* 0.57, $CHCl_3$)) with those of the stereoisomers of trimethyl-V-P (**4a** and **4b**) and trimethyl-E-P (**5a** and **5b**), **6a** and **6b** were deduced to be trimethyl-$D^{glu}$, $L^{ile}$ and trimethyl-$L^{glu}$, $L^{ile}$, respectively. Trimethyl-I-P (**6**) was proved to be identical with **6b** in all respects: $^1$H-NMR spectrum, *Rt* on HPLC (42.5 min) and optical rotation ($[\alpha]_D^{21}$ +5.1°(*c* 0.59, $CHCl_3$)), which led to the conclusion that I-P is (2*R*, 1'*S*, 2'*S*)- *N*-(1-carboxy-2-methylbutyl)glutamic acid ($D^{glu}$, $L^{ile}$) (**3**).

Crown gall tumors are induced by virulent *Agrobacterium tumefaciens* in many higher plants, and a segment of bacterial Ti plasmid DNA is transferred to the plant cells to direct the host plant to synthesize some unusual amino acid derivatives called opines whose structures are specifically determined by the inciting strain. The inciting bacteria can catabolite them and use as nutritional substrates.[14] V-P (**1**), E-P (**2**) and I-P (**3**) structurally belong to the opine family. Saccaropine and its lactam have also been isolated from *Lentinus edodes*, *Flammulina veltipes* and *Pleurotous ostreatus*.[15] The biological significance of these mushroom opines is not certain, but considering their structural features, they seem to have some activities toward the glutamic acid receptors, and the study on this respect is under way.




**Acknowledgments**
This work was supported by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture, Japan.

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10) $^{13}$C-NMR of V-P ($D_2O$, 75 MHz) δ 17.2, 18.2, 23.6, 29.1, 30.5, 48.9, 61.0, 66.7, 172.3, 172.6, 177.3.
11) HPLC was carried out by using a reverse phase column (Cosmosil 5C18-MS, 10 mmφ x 250 mm) and 60% aq. MeOH was eluted at a rate of 1.8 ml/min.
12) $^1$H-NMR data. **4a** (pyridine-$d_5$, 500 MHz) δ 0.83 (6H, d, *J*=7.0 Hz), 1.90 (2H, m), 2.06 (1H, m), 2.50 (2H, m), 3.22 (1H, d, *J*=6.0), 3.44 (3H, s), 3.45 (1H, dd, *J*=6.0, 8.0), 3.49 (3H, s), 3.52 (3H, S). **5a** ($CDCl_3$, 500 MHz) δ 0.91 (3H, d, *J*=7.0 Hz), 0.93 (3H, d, J=7.0), 1.49 (2H, m), 1.72 (1H, m), 1.86 (1H, m), 2.03 (1H, m), 2.48 (2H, m), 3.29 (1H, dd, *J*=5.5, 8.0), 3.33 (1H, dd, *J*=6.0, 8.0), 3.68 (3H, s), 3.70 (3H, s), 3.72 (3H, s). **6a** (pyridine-$d_5$, 500 MHz) δ 0.83 (3H, t, *J*=7.5 Hz), 0.94 (3H, d, *J*=7.0), 1.23 (1H, m), 1.62 (1H, m), 1.78 (1H, m), 2.03 (1H, m), 2.20 (1H, m), 2.63 (2H, m), 3.45 (1H, d, *J*=6.0), 3.59 (3H, s), 3.61 (1H, dd, *J*=5.0, 8.0), 3.65 (3H, s), 3.67 (3H, s).
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(*Received in Japan* 2 *June* 1994; *accepted* 22 *August* 1994)