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New Opine Type Amino Acids from a Poisonous Mushroom, *Clitocybe Acromelalga*

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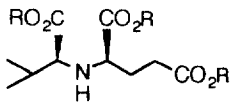
Abstract: Four new opine type amino acids, valinopine, epileucinopine, isoleucinopine and phenylalaninopine, were isolated from a poisonous mushroom, *Clitocybe acromelalga*. The structures were determined to be **1**, **2**, **3** and **4**, respectively, by spectroscopic analysis and chemical synthesis.

Clitocybe acromelalga (Tricholomataceae) is a famous poisonous mushroom in Japan, its ingestion causes unique symptoms similar to acromelalgia or erythromelalgia.[1] Efforts directed towards the isolation of the toxic principles resulted in the isolation of many kinds of neurologically active amino acid derivatives. 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] and 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 these compounds are characteristic of *C. acromelalga*. A further survey has revealed the occurrence of other amino acids which show different behavior patterns on TLC and HPLC from those of above mentioned amino acids. An attempt to separate these amino acids has resulted in the isolation of four new amino acids. Since these amino acids were shown to have structures analogous to the opines, the names of valinopine, epileucinopine, isoleucinopine and phenylalaninopine were given. The isolation and structure of valinopine, epileucinopine and isoleucinopine has been outlined in the preceding communication.[10] In the present paper, we describe in detail evidence leading to structures **1**, **2**, and **3** for valinopine, epileucinopine and isoleucinopine, respectively, as well as the structure of the fourth compound, phenylalaninopine (**4**).



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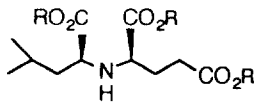
5: R= Me



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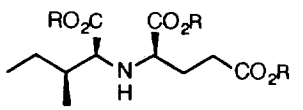

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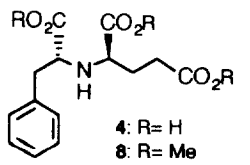
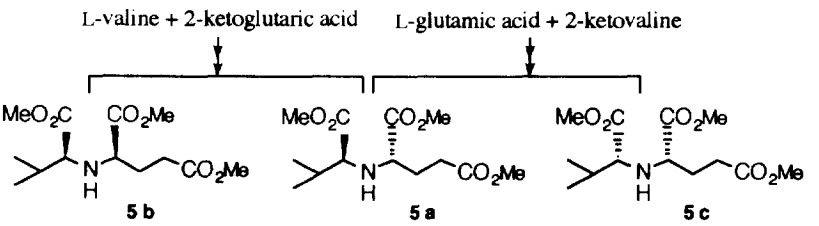


4: R= H
8: R= Me

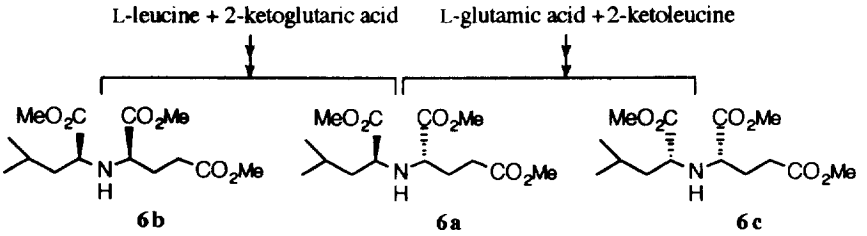
The FAB-mass spectrum of valinopine showed the peaks at *m/z* 248 and 270 attributable to $(M+H)^+$ and $(M+Na)^+$, respectively. The ^{13}C-NMR spectrum of valinopine showed ten carbon signals. Treatment of valinopine with CH_2N_2 gave valinopine trimethyl ester (**5**), whose HREI-mass spectrum showed the molecular ion peak at *m/z* 289.1543 corresponding to the formula $C_{13}H_{23}NO_6$. These data revealed that valinopine is a monoamino (imino) tricarboxylic acid having the molecular formula $C_{10}H_{17}NO_6$. Analysis of the ^1H-NMR spectrum of valinopine with the aid of decoupling experiments demonstrated the presence of a 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 a 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) in valinopine. The chemical shifts of these signals and the tricarboxylic acid nature of valinopine indicated that these partial structures consist in valine and glutamic acid units, respectively. Since valinopine has only one nitrogen atom, the two units should be connected through the nitrogen atom. Valinopine was thus concluded 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 valinopine trimethyl ester (**5**) were synthesized as follows.[11] Reductive coupling of L-valine and 2-ketoglutaric acid by use of $NaBH_3CN$ followed by methylation with CH_2N_2 gave a diastereoisomeric mixture of trimethyl esters, **5a** and **5b**, which was separated by HPLC. L-Glutamic acid and 2-ketovaline (3-methyl-2-oxobutyric acid) were also coupled and methylated, and the products were separated into **5a** and **5c**. The ^1H-NMR spectrum and *Rt* on HPLC of **5b** are identical with those of **5c** (24.0 min), whereas the optical rotation of **5b** (+3.1°) is the opposite of **5c** (-5.4°), indicating **5b** to be the enantiomer of **5c**. Taking into account the starting compounds of the synthesis, it is concluded that **5a**, **5b** and **5c** are trimethyl- L^{glu}, L^{val}, trimethyl-D^{glu}, L^{val} and trimethyl-L^{glu}, D^{val}, respectively. The ^1H-NMR spectrum, *Rt* on HPLC (24.0 min) and optical rotation (+3.9°) of valinopine trimethyl ester (**5**) are identical with those of **5b**, establishing the structure of valinopine as (2*R*, 1'*S*)-*N*-(1-carboxy-2-methylpropyl)glutamic acid (D^{glu}, L^{val}) (**1**).



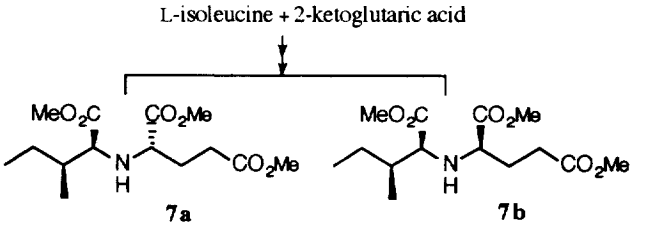
Epileucinopine and isoleucinopine were proved to be isomers, having the same molecular formulas $C_{11}H_{19}NO_6$, by FAB-mass and ^{13}C-NMR spectra of the mixture. The mixture could be separated by HPLC after methylation with CH_2N_2, into epileucinopine trimethyl ester (**6**) and isoleucinopine trimethyl ester (**7**). The HREI-mass of **6** showed the molecular ion peak at *m/z* 303.1677 corresponding to the formula $C_{14}H_{25}NO_6$. The ^1H-NMR spectrum of **6** showed signals due to a 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 a 1, 1, 3-trisubstituted-propane (δ 2.42, 1H, ddd, *J*=6.5, 8.0, 16.5 Hz; 2.50, 1H, ddd, *J*=7.0, 8.0, 16.5; 1.80, 1H, m; 1.91, 1H, m; 3.26, 1H, dd, *J*=8.0, 5.0) as well as three carboxymethyl signals (δ 3.69, 3H, s; 3.70, 3H, s; 3.72, 3H, s). These data suggest that the valine moiety of valinopine (**1**) is replaced by leucine in epileucinopine (**2**). In order to confirm this assumption and to determine the absolute configurations, the stereoisomers were synthesized from L-leucine

and 2-ketoglutaric acid (**6a** and **6b**) and from L-glutamic acid and 2-ketoleucine (4-methyl-2-oxopentanoic acid) (**6a** and **6c**) by the same method as mentioned above. The enantiomeric relationship between **6b** and **6c** was verified by the same ^{1}H-NMR spectra and *Rt*s on HPLC (34.0 min) and opposite optical rotations (**6b**: -6.3°, **6c**: +5.7°). Thus, **6a**, **6b** and **6c** were inevitably assigned as trimethyl-L^{glu}, L^{leu}, trimethyl-D^{glu}, L^{leu} and trimethyl-L^{glu}, D^{leu}, respectively. Epileucinopine was proved to be identical with **6b** in all respects: ^{1}H-NMR spectrum, *Rt* on HPLC (34.0 min) and optical rotation (-7.1°). Epileucinopine is thus concluded to be (2*R*, 1'*S*) -*N*-(1-carboxy-3-methylbutyl)glutamic acid (D^{glu}, L^{leu}) (**2**).

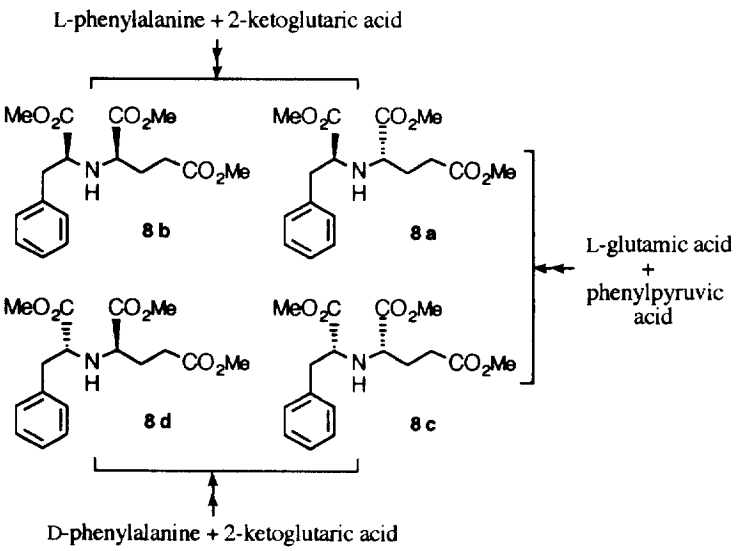
Leucinopine has been isolated as a characteristic compound of some crown-gall tumors incited by ***Agrobacterium tumefaciens*** strain Bo 542.[12] The absolute stereochemistry of leucinopine was determined as L^{glu}, L^{leu} on the basis of specificity of catabolism of four synthetic diastereoisomers by *A. tumefaciens* strains carrying the tumor-inducing plasmid pTi Bo 542.[13] Epileucinopine is thus the epimer of leucinopine in regard to C-2.



The HREI-mass of isoleucinopine trimethyl ester (**7**) showed the molecular ion peak at *m/z* 303.1677 corresponding to the formula $C_{14}H_{25}NO_6$ The ^{1}H-NMR spectrum of **7** showed signals due to a 1, 1-di-substituted-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 a 1, 1, 3-trisubstituted-propane (δ 2.08, 1H, m; 2.17, 1H, m; 2.63, 2H, m; 3.43, 1H, dd, *J*=8.0, 5.0) 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 epileucinopine (**2**) is replaced by isoleucine in isoleucinopine. In order to confirm this assumption and to determine the absolute configurations, the stereoisomers **7a** and **7b** were synthesized from L-isoleucine and 2-ketoglutaric acid by the same method mentioned above. By comparing the behavior patterns on HPLC and optical rotations of **7a** (*Rt* 29.0 min, -11.1°) and **7b** (*Rt* 42.5 min, +6.0°) with those of the stereoisomers of valinopine trimethyl esters (**5a** and **5b**) and epileucinopine trimethyl esters (**6a** and **6b**), **7a** and **7b** were deduced to be trimethyl-L^{glu}, L^{ile} and trimethyl-D^{glu}, L^{ile}, respectively. The ^{1}H-NMR spectrum, *Rt* on HPLC (42.5 min) and optical rotation (+5.1°) of **7** were identical with those of **7b**, which led to the conclusion that isoleucinopine is (2*R*, 1'*S* , 2'*S*)-*N*-(1-carboxy-2-methylbutyl)glutamic acid (D^{glu}, L^{ile}) (**3**).



FAB-mass spectrum of phenylalaninopine showed the ion peak at *m/z* 296 corresponding to $[M+H]^+$. The ^{13}C-NMR spectrum of phenylalaninopine exhibited 14 carbon signals. Treatment of phenylalaninopine with CH_2N_2 gave phenylalaninopine trimethyl ester (**8**), whose HREI-mass spectrum showed the molecular ion peak at *m/z* 337.1541 corresponding to the formula $C_{17}H_{23}NO_6$. These data revealed that phenylalaninopine is a monoamino (imino) tricarboxylic acid having the molecular formula $C_{14}H_{17}NO_6$. The presence of a phenyl group in phenylalaninopine was demonstrated by the UV spectrum (ν_{max} 256 nm) and the ^1H-NMR spectrum (δ 6.94 - 7.02, 5H) of phenylalaninopine. Analysis of the ^1H-NMR spectrum of phenylalaninopine with the aid of decoupling experiments demonstrated the presence of a 1, 1, 2-trisubstituted-ethane (δ 2.87, 1H, dd, *J*=7.5, 15.0 Hz; 2.98, 1H, dd, *J*=6.5, 15.0 ; 3.60, 1H, dd, *J*=7.5, 6.5) and a 1, 1, 3- trisubstituted-propane (δ 1.81, 1H, m; 1.88, 1H, m; 2.15, 1H, dt, *J*=16.0, 7.0; 2.20, 1H, dt, *J*=16.0, 5.0; 3.37, 1H, dd, *J*=5.0, 7.0) in phenylalaninopine. These data suggested that phenylalaninopine consists of phenylalanine and glutamic acid units, and the structure **4** is proposed for phenylalaninopine. To confirm this assumption and to determine the absolute configurations, L-phenylalanine and 2-ketoglutaric acid, and L-glutamic acid and phenylpyruvic acid were reductively coupled and methylated, respectively, to give the stereoisomers **8a**, **8b** and **8c**, which were assigned as trimethyl-L^{glu}, L^{phe}, trimethyl-D^{glu}, L^{phe} and trimethyl-L^{glu}, D^{phe}, respectively, in a similar manner as above. In this case, the ^1H-NMR spectrum and *Rt* on HPLC (27.5 min) of phenylalaninopine trimethyl ester (**8**) were proved to be different from those of **8b** (**8C**), but identical with those of **8a**. Whereas, the optical rotation of phenylalaninopine trimethyl ester (**8**) (+25.8°) is the opposite of **8a** (-19.8°). These findings indicate that phenylalaninopine trimethy ester (**8**) is the enantiomer of **8a**, which was proved by direct comparison with **8d** derived from D-phenylalanine and 2-ketoglutaric acid. Phenylalaninopine is thus concluded to be (2*R*, 1'*R*)-*N*-(1-carboxy-2-phenylethyl)glutamic acid (D^{glu}, D^{phe}) (**4**).



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.[13] Although saccaropine and its lactam have been isolated from mushrooms such as *Lentinus edodes*, *Flammulina veltipes* and *Pleurotous ostreatus*[14], their biological significance have not clarified yet. Valinopine (**1**), epileucinopine (**2**), isoleucinopine (**3**) and phenylalaninopine (**4**) structurally belong to the opine family. Since these compounds contain the skeleton of glutamic acid, it would be expected to have excitatory action on the mammalian central neuron. However, when the action of these compounds was electrophysiologically examined in the isolated spinal cord of the newborn rat,[15] they did not show any excitatory or inhibitory action at the concentration of 1 mM.

Experimental

General procedures The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on JOEL JNM-GSX 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) and Hitachi R-3000 spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C), with TMS as int. standard (in the case of D_2O soln., DOH peak at 4.68 ppm was used as a standard). High resolution electron impact (HREI) mass spectra were recorded on a JEOL JMSDX303 spectrometer. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMSDX303 spectrometer using glycerol as a matrix. Optical rotations were recorded on a JASCO DPI-370 spectrometer. Cellulose TLC was performed using *n*-BuOH-HOAc-H_2O (4:1:2 v/v). HPLC was carried out on an instrument equipped with a refractive index detector and a photodiodearray detector. For analysis of free amino acids, a stainless steel column (0.26 ϕ x 50 cm) packed with Hitachi gel 2618 (cation exchange resin) was used and eluted with NH_3-HCO_2H buffer (pHs being shown in parentheses) at a rate of 0.5 ml/min. For analysis of methylesters, a reverse phase column (Cosmosil 5C18-MS, 1.0 ϕ x 25 cm) was used and eluted with 60% aq.MeOH at a rate of 1.8 ml/min.

***Isolation of valinopine* (1), *epileucinopine* (2), *isoleucinopine* (3) *and phenylalaninopine* (4)** Fruiting bodies of *Clitocybe acromelalga* (6.2 kg) collected in Miyagi prefecture on October 1992, were cut into small pieces and soaked in 70% aqueous EtOH (39.5 l) at room temperature for 1 day. After filtration of the extract, the filtrate was concentrated under reduced pressure. The residual aqueous suspension was extracted successively with AcOEt and *n*-BuOH and the aq. layer was applied to Ambelite IR 120B resin (H^+, 20-50 mesh, 6.0 x 50 cm). After washing with H_2O, the column was eluted with 1M pyridine (4 l), affording the acidic and neutral amino acids. The column was subsequently eluted with 1M NH_3 (4 l) and 4M NH_3 (4 l) to give the basic amino acid fractions. The neutral and acidic amino acid fraction was subjected to Dowex 1x8 resin (AcO^-, 50-100 mesh, 5.0 x 35 cm). The column was eluted successively with H_2O (3 l), 1M HOAc (2 l), 4M HOAc (2 l) and 1M HCl (2 l) to give neutral, acidic I, acidic II and acidic III amino acid fractions, respectively. The acidic III fraction (1 g) was applied to an active charcoal column (Wako Pure Chemical Industries Ltd., 8 g) and the column was eluted successively with H_2O, 50% aq. MeOH, MeOH and acetone (each 200 ml). The 50% aq. MeOH fraction (150 mg) was applied to a Dowex 50 Wx4 column (200-400 mesh, 2.0 x 45 cm) equilibrated with an ammonia-formate buffer (pH 2.50), and the column was eluted with the same buffer system whose pH was changed stepwise from 2.50 to 2.70 and 3.00 (each 250 ml, 50 ml fractions). Each fraction was desalted using Dowex 50Wx4 (100-200 mesh) and monitored by cellulose TLC to yield crude valinopine (pH 2.50- V) and the mixture of epileucinopine and isoleucinopine (pH 2.70- I and II), which were purified by HPLC (pH 2.45) to give valinopine (**1**) (6.0 mg, *Rt* 18) and the mixture of epileucinopine (**2**) and isoleucinopine (**3**) (15.0 mg, *Rt* 26-28).

Fruiting bodies of *C. acromelalga* (2.2 kg) collected in the same place on October 1994, were treated similarly, and the extract was separated into the amino acid fractions. Without being subjected to active charcoal column, the acidic III fraction (550 mg) was applied to a Dowex 50 Wx4 column (100-200 mesh, 3.5 x 30 cm) equilibrated with an ammonia-formate buffer (pH 2.50), and the column was eluted with the same buffer system whose pH was changed stepwise from 2.50 to 2.70 and 3.00 (each 250 ml, 60 ml fractions). In the fraction (pH 2.70- 4, 64 mg), an unusual spot was noticed on the cellulose TLC (*Rf* 0.28), which gave a pale yellow coloration when tested with ninhydrin and showed an absorption under UV light. This fraction was applied to the Dowex 50Wx4 column again and fractionated. The fraction showing the spot on TLC was desalted using Dowex 50Wx4 (100-200 mesh) to give phenylalaninopine (**4**) (3.2 mg).

***Valinopine* (1)** Colorless amorphous mass. $[\alpha]_D^{21}$ +6.3°(*c* 0.32, H_2O). FAB-MS: *m/z* 248 $(M+H)^+$, 270 $(M+Na)^+$. ^{1}H-NMR (D_2O, 500 MHz): δ 0.93 (3H, d, *J*=7.0 Hz), 0.98 (3H, d, *J*=7.0 Hz), 2.07 (2H, m), 2.18 (1H, m), 2.52 (2H, t, *J*=7.0 Hz), 3.55 (1H, d, *J*=4.0 Hz), 3.62 (1H, t, *J* =6.0 Hz). ^{13}C-NMR (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.

***Phenylalaninopine* (4)** White powder. UV λ_{max} (H_2O): 256 nm (log ε =1.27). FAB-MS: *m/z* 296 $(M+H)^+$. ^{1}H-NMR [D_2O-pyridine-d_5 (4/1), 500 MHz]: δ 1.81 (1H, m), 1.88 (1H, m), 2,15 (1H, dt, *J*=16.0, 7.0 Hz), 2.20 (1H, dt, *J*=16.0, 7.0 Hz), 2.87 (1H, dd, *J*=15.0, 7.5 Hz), 2.98 (1H, dd, *J*=15.0, 6.5 Hz), 3.37 (1H, dd, *J*=7.0, 5.0 Hz), 3.60 (1H, dd, *J*=7.5, 6.5 Hz), 6.94 (2H, m), 7.02 (3H, m). ^{13}C-NMR [D_2O-pyridine-d_5 (4/1), 75 MHz]: δ 28.8, 35.9, 38.7, 64.8, 65.7, 129.6, 131.0 (2C), 131.4 (2C), 136.9, 174.8, 175.2, 183.2.

***Valinopine trimethyl ester* (5)** An ethereal solution of CH_2N_2 was added to a solution of valinopine (**1**) (2.00 mg) in MeOH (1 ml) till it became pale yellow. After removal of the solvent, the crude product was purified by silica gel chromatography [hexane-AcOEt (4:1)] to afford valinopine trimethyl ester (**5**) (1.8 mg) as a colorless gum. $[\alpha]_D^{21}$ +3.9°(*c* 0.10, $CHCl_3$). EI-MS: *m/z* 289 $(M)^+$, 230 (100 %). HREI-MS: *m/z* 289.1543 $(M)^+$; calcd. for $C_{13}H_{23}NO_6$, 289.1526. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.78 (3H, d, *J*=7.0 Hz), 0.79 (3H, d, *J*=7.0 Hz), 1.80 (1H, dq, *J*=16.0, 8.0 Hz), 1.92 (1H, ddt, *J*=16.8, 8.0, 5.0 Hz), 1.98 (1H, dq, *J*=7.0, 6.0 Hz), 2.43 (1H, dt, *J*=16.0, 8.0 Hz), 2.49 (1H, dt, *J*=16.0, 8.0 Hz), 3.01 (1H, d, *J*=6.0 Hz), 3.31 (1H, dd, *J*=8.0, 5.0 Hz), 3.46 (3H, s), 3.47 (3H, s), 3.51 (3H, s).

***Epileucinopine trimethyl ester* (6) *and isoleucinopine trimethyl ester* (7)** An ethereal solution of CH_2N_2 was added to the solution of the mixture of epileucinopine (**2**) and isoleucinopine (**3**) (5 mg) till it became pale yellow. After removal of the solvent, the crude product was separated by HPLC (Cosmosil 5C18-MS, 1.0 x 25 cm, 60% MeOH) into epileucinopine trimethyl ester (**6**) (2.7 mg, *Rt* 34.0) and isoleucinopine trimethyl ester (**7**) (1.5 mg, *Rt* 42.5).

***Epileucinopine trimethyl ester* (6)** Colorless gum. $[\alpha]_D^{21}$ -7.1° (*c* 0.39, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 244 (100 %). HREI-MS: *m/z* 303.1677 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.90 (3H, d, *J*=6.5 Hz), 0.92 (3H, d, *J*=6.5 Hz), 1.47 (2H, t, *J*=7.0 Hz), 1.80 (1H, m), 1.91 (1H, m), 2.00 (1H, m), 2.42 (1H, ddd, *J*=16.5, 8.0, 6.5 Hz), 2.50 (1H, ddd, *J*=16.5, 8.0, 7.0 Hz), 3.25 (1H, t, *J*=7.0 Hz), 3.26 (1H, dd, *J*=8.0, 5.0 Hz), 3.69 (3H, s), 3.70 (3H, s), 3.72 (3H, s).

***Isoleucinopine trimethyl ester* (7)** Colorless gum. $[\alpha]_D^{21}$ +5.1° (*c* 0.59, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 288 $(M-CH_3)^+$, 244 (100 %). HREI-MS: *m/z* 303.1677 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.83 (3H, t, *J*=7.5 Hz), 0.93 (3H, d, *J*=7.0 Hz), 1.23 (1H, m), 1.62 (1H, m), 1.78 (1H, m), 2.08 (1H, m), 2.17 (1H, m), 2.63 (2H, m), 3.23 (1H, d, *J*=6.5 Hz), 3.43 (1H, dd, *J*=8.0, 5.0 Hz), 3.62 (3H, s), 3.64

(3H, s), 3.68 (3H, s). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.5, 15.8, 25.2, 28.1, 30.2, 38.8, 51.6, 51.7, 52.0, 60.2, 65.3, 173.7, 174.6, 175.0.

***Phenylalaninopine trimethyl ester* (8)** According to the procedure to prepare trimethylvalinopine (**5**), phenylalaninopine (**4**) (2.3 mg) was converted to phenylalaninopine trimethyl ester (**8**) (1.2 mg). Colorless gum. $[\alpha]_D^{29}$ +25.8° (*c* 0.12, $CHCl_3$). EI-MS: *m/z* 337 $(M)^+$. HREI-MS: *m/z* 337.1541 $(M)^+$, calcd. for $C_{17}H_{23}NO_6$; 337.1525. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.90 (1H, m), 2.02 (1H, m), 2.45 (2H, q, *J*=7.0), 2.97 (1H, dd, *J*=12.0, 7.0 Hz), 3.12 (1H, dd, *J*=12.0, 5.0 Hz), 3.41 (1H, m), 3.64 (1H, m), 3.645 (3H, s), 3.65 (3H, s), 3.67 (3H, s), 7.21-7.25 (3H, m), 7.31 (2H, m).

***Synthesis of trimethyl-L^{glu}, L^{val}* (5a) *and trimethyl-D^{glu}, L^{val}* (5b)** $NaBH_3CN$ (315 mg, 3.0 mmol) was added by portions to a solution of L-valine (118 mg, 1.0 mmol) and 2-ketoglutaric acid (146 mg, 1.0 mmol) in water (5 ml). The mixture was stirred for 48 hrs at room temperature, whose pH was kept at 6 - 7 during the reaction by adding $NaHCO_3$. Dowex 50Wx4 (100-200 mesh, 50 ml) was added to the reaction mixture. The suspension was stirred for 4 hrs and subjected to a glass filter. After washing with water until the filtrate became neutral, the resin was washed with 1M pyridine (200 ml). The pyridine eluent was evaporated to dryness *in vacuo*. The residue was dissolved in water (5 ml) and subjected to a Dowex 1x8 column (2 x 9 cm), which was eluted successively with water (100 ml) and 1M HCl (100 ml). 1M HCl eluent was evaporated to dryness and MeOH (5 ml) was added to the residue. An ethereal solution of CH_2N_2 was added to the MeOH solution till it became pale yellow. After removal of the solvent, the crude product was separated by HPLC (Cosmosil 5C18-MS, 1.0 x 25 cm, 60% MeOH) into trimethyl-L^{glu}, L^{val} (**5a**) (47 mg, *Rt* 18.0) and trimetyl-D^{glu}, L^{val} (**5b**) (17 mg, *Rt* 24.0).

***Trimethyl-L^{glu}, L^{val}* (5a)** Colorless gum. $[\alpha]_D^{21}$ -33.0°(*c* 0.91, $CHCl_3$). EI-MS: *m/z* 289 $(M)^+$, 230 (100 %). HREI-MS: 289.1535 $(M)^+$, calcd. for $C_{13}H_{23}NO_6$; 289.1526. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.83 (6H, d, *J*=7.0 Hz), 1.88 (1H, q, *J*=7.0 Hz), 1.92 (1H, q, *J*=7.0 Hz), 2.06 (2H, dq, *J*=7.0, 6.0 Hz), 2.48 (1H, dt, *J*=16.0, 8.0 Hz), 2.52 (1H, dt, *J*=16.0, 8.0 Hz), 3.22 (1H, d, *J*=6.0 Hz), 3.44 (3H, s), 3.45 (1H, dd, *J*=8.0, 5.0 Hz), 3.49 (3H, s), 3.52 (3H, s).

***Trimethyl-D^{glu}, L^{val}* (5b)** Colorless gum. $[\alpha]_D^{21}$ +3.1°(*c* 0.26, $CHCl_3$). EI-MS: *m/z* 289 $(M)^+$, 230 (100 %). HREI-MS: 289.1543 $(M)^+$, calcd. for $C_{13}H_{23}NO_6$; 289.1526. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.78 (3H, d, *J*=7.0 Hz), 0.79 (3H, d, *J*=7.0 Hz), 1.80 (1H, dq, *J*=16.0, 8.0 Hz), 1.91 (1H, ddt, *J*=16.0, 8.0, 5.0 Hz), 1.99 (1H dq, *J*=7.0, 6.0 Hz), 2.43 (1H, dt, *J*=16.0, 8.0 Hz), 2.49 (1H, dt, *J*=16.0, 8.0 Hz), 3.01(1H, d, *J*=6.0 Hz), 3.31 (1H, dd, *J*=8.0, 5.0 Hz), 3.46 (3H, s), 3.47 (3H, s), 3.51 (3H, s).

***Synthesis of trimethyl-L^{glu}, L^{val}* (5a) *and trimethyl-L^{glu}, D^{val}* (5c)** According to the preceding procedure, trimethyl-L^{glu}, L^{val} (**5a**) (3 mg, *Rt* 18.0) and trimethyl-L^{glu}, D^{val} (**5c**) (1.5 mg, *Rt* 24.0) were obtained from L-glutamic acid (147 mg, 1.0 mmol) and 2-ketovaline (116 mg, 1.0 mmol).

***Trimethyl-L^{glu}, L^{val}* (5a)** Colorless gum. $[\alpha]_D^{21}$ -38.2°(*c* 0.25, $CHCl_3$). EI-MS: *m/z* 289 $(M)^+$, 230 (100 %). HREI-MS: 289.1535 $(M)^+$, calcd. for $C_{13}H_{23}NO_6$; 289.1526. ^{1}H-NMR (pyridine-d_5, 300 MHz): δ 0.83 (6H, d, *J*=7.0 Hz), 1.90 (2H, m), 2.06 (2H, dq, *J*=7.0, 6.0 Hz), 2.48 (1H, dt, *J*=16.0, 8.0 Hz), 2.52 (1H, dt, *J*=16.0, 8.0 Hz), 3.22 (1H, d, *J*=6.0 Hz), 3.44 (3H, s), 3.45 (1H, dd, *J*=8.0, 5.0 Hz), 3.49 (3H, s), 3.52 (3H, s).

***Trimethyl-L^{glu}, D^{val}* (5c)** Colorless gum. $[\alpha]_D^{21}$ -5.4°(*c* 0.11, $CHCl_3$). EI-MS: m/z 289 $(M)^+$, 230 (100 %).

HREI-MS : 289.1515 $(M)^+$, calcd. for $C_{13}H_{23}NO_6$; 289.1526. ^{1}H-NMR (pyridine-d_5, 300 MHz): δ 0.78 (3H, d, *J*=7.0 Hz), 0.79 (3H, d, *J*=7.0 Hz), 1.80 (1H, dq, *J*=16.0, 8.0 Hz), 1.92 (1H, ddt, *J*=16.0, 8.0, 5.0 Hz), 1.98 (1H, dq, *J*=7.0, 6.0 Hz), 2.43 (1H, dt, *J*=16.0, 8.0 Hz), 2.49 (1H, dt, *J*=16.0, 8.0 Hz), 3.01 (1H, d, *J*=6.0 Hz), 3.31 (1H, dd, *J*=8.0, 5.0 Hz), 3.46 (3H, s), 3.47 (3H, s), 3.51 (3H, s).

***Synthesis of trimethyl-L^{glu}, L^{leu}* (6a) *and trimethyl-D^{glu}, L^{leu}* (6b)** According to the preceding procedure, trimethyl-L^{glu}, L^{leu} (**6a**) (26.4 mg, *Rt* 25.0) and trimethyl-D^{glu}, L^{leu} (**6b**) (39.8 mg, *Rt* 34.0) were obtained from L-leucine (396 mg, 3.0 mmol) and 2-ketoglutaric acid (438 mg, 3.0 mmol).

***Trimethyl-L^{glu}, L^{leu}* (6a)** Colorless gum. $[\alpha]_D^{21}$ -36.6°(*c* 0.54, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 244 (100 %). HREI-MS: *m/z* 303.1677 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.91 (3H, d, *J*=6.5 Hz), 0.93 (3H, d, *J*=6.5 Hz), 1.44 (2H, dd, *J*=7.0, 5.0 Hz), 1.73 (1H, tt, *J*=14.0, 7.0 Hz), 1.82 (1H, dq, *J*=14.0, 7.0 Hz), 2.03 (1H, dq, *J*=12.0, 8.0 Hz), 2.43 (2H, dt, *J*=15.0, 7.5 Hz), 3.32 (2H, dq, *J*=14.0, 7.0 Hz), 3.68 (3H, s), 3.70 (3H, s), 3.72 (3H, s).

***Trimethyl-D^{glu}, L^{leu}* (6b)** Colorless gum. $[\alpha]_D^{21}$ -6.3° (*c* 0.70, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 244 (100 %). HREI-MS: *m/z* 303.1680 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.91 (3H, d, *J*=6.5 Hz), 0.92 (3H, d, *J*=6.5 Hz), 1.47 (2H, dd, *J*=7.0, 5.0 Hz), 1.80 (1H, tt, *J*=15.0, 7.5 Hz), 1.91 (1H, ddt, *J*=15.0, 6.5, 5.0 Hz), 2.00 (1H, m), 2.42 (1H, dt, *J*=15.0, 7.5 Hz), 2.49 (1H, dt, *J*=15.0, 7.5 Hz), 3.26 (2H, m), 3.69 (3H, s), 3.70 (3H, s), 3.72 (3H, s).

***Synthesis of trimethyl-L^{glu}, L^{leu}* (6a) *and trimethyl-L^{glu}, D^{leu}* (6c)** According to the preceding procedure, trimethyl-L^{glu}, L^{leu} (**6a**) (24.0 mg, *Rt* 25.0) and trimethyl-L^{glu}, D^{leu} (**6c**) (16.2 mg, *Rt* 34.0) were obtained from L-glutamic acid (147 mg, 1.0 mmol) and 2-ketoleucine (130 mg, 1.0 mmol).

***Trimethyl-L^{glu}, L^{leu}* (6a)** Colorless gum. $[\alpha]_D^{21}$ -34.7°(*c* 0.81, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 244 (100 %). HREI-MS: *m/z* 303.1673 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.91 (3H, d, *J*=6.5 Hz), 0.93 (3H, d, *J*=6.5 Hz), 1.44 (2H, dt, *J*=7.0, 5.0 Hz), 1.73 (1H, tt, *J*=14.0, 7.0 Hz), 1.82 (1H, dq, *J*=14.0, 7.0 Hz), 2.03 (1H, dq, *J*=12.0, 8.0 Hz), 2.43 (2H, t, *J*=10.0 Hz), 3.32 (2H, dq, *J*=14.0, 7.0 Hz), 3.68 (3H, s), 3.70 (3H, s), 3.72 (3H, s).

***Trimethyl-L^{glu}, D^{leu}* (6c)** Colorless gum. $[\alpha]_D^{21}$ +5.7°(*c* 0.56, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 244 (100 %). HREI-MS: *m/z* 303.1680 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.91 (3H, d, *J*=6.5 Hz), 0.92 (3H, d, *J*=6.5 Hz), 1.47 (2H, dd, *J*=7.0, 5.0 Hz), 1.80 (1H, tt, *J*=15.0, 7.5 Hz), 1.91 (1H, ddt, *J*=15.0, 6.5, 5.0 Hz), 2.00 (1H, m), 2.42 (1H, dt, *J*=15.0, 7.5 Hz), 2.49 (1H, dt, *J*=15.0, 7.5 Hz), 3.26 (2H, m), 3.69 (3H, s), 3.70 (3H, s), 3.72 (3H, s).

***Synthesis of trimethyl-L^{glu}, L^{ile}* (7a) *and trimethyl-D^{glu}, L^{ile}* (7b)** According to the preceding procedure, trimethyl-L^{glu}, L^{ile} (**7a**) (19.0 mg, *Rt* 29.0) and trimethyl-D^{glu}, L^{ile} (**7b**) (34.3 mg, *Rt* 42.5) were obtained from L-isoleucine (396 mg, 3.0 mmol) and 2-ketoglutaric acid (438 mg, 3.0 mmol).

***Trimethyl-L^{glu}, L^{ile}* (7a)** Colorless gum. $[\alpha]_D^{21}$ -11.1°(*c* 0.57, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 288 $(M\text{-}CH_3)^+$, 244 (100 %). HREI-MS: *m/z* 303.1659 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.81 (3H, t, *J*=7.5 Hz), 0.94 (3H, d, *J*=6.5 Hz), 1.23 (1H, ddt, *J*=16.0, 15.0, 7.5 Hz), 1.62 (1H, ddt, *J*=15.0, 12.0, 7.5 Hz), 1.80 (1H, m), 2.05 (1H, dq, *J*=15.0, 8.0 Hz), 2.20 (1H, ddt, *J*=15.0, 8.5, 6.0 Hz), 2.65 (2H, dt, *J*=14.0, 7.0 Hz), 3.45 (1H, d, *J*=6.0 Hz), 3.57 (3H, s), 3.63 (1H, dd, *J*=8.0, 5.0 Hz), 3.65 (3H, s),

3.67 (3H, s).

***Trimethyl-D^{glu}, L^{lle}* (7b)** Colorless gum. $[\alpha]_D^{21}$ +6.0°(*c* 0.59, $CHCl_3$). EI-MS: *m/z* 303 $(M)^+$, 288 $(M\text{-}CH_3)^+$, 244 (100 %). HREI-MS: *m/z* 303.1700 $(M)^+$, calcd. for $C_{14}H_{25}NO_6$; 303.1682. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 0.81 (3H, t, *J*=7.5 Hz), 0.93 (3H, d, *J*=7.5 Hz), 1.23 (1H, m), 1.62 (1H, m), 1.78 (1H, m), 2.08 (1H, m), 2.17 (1H, m), 2.63 (2H, m), 3.23 (1H, d, *J*=7.5 Hz), 3.43 (1H, dd, *J*=8.0, 5.0 Hz), 3.61 (3H, s), 3.62 (3H, s), 3.65 (3H, s).

***Synthesis of trimethyl-L^{glu}, L^{phe}* (8a) *and trimethyl-D^{glu}, L^{phe}* (8b)** According to the preceding procedure, trimethyl-L^{glu}, L^{phe} (**8a**) (21.6 mg, *Rt* 27.5) and trimethyl-D^{glu}, L^{phe} (**8b**) (36.8 mg, *Rt* 34.5) were obtained from L-phenylalanine (495 mg, 3.0 mmol) and 2-ketoglutaric acid (438 mg, 3.0 mmol).

***Trimethyl-L^{glu}, L^{phe}* (8a)** Colorless gum. $[\alpha]_D^{21}$ -19.9°(*c* 2.12, $CHCl_3$). EI-MS: *m/z* 338 $(M+H)^+$, 278 $(M\text{-}CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 338.1609 $(M+H)^+$, calcd. for $C_{17}H_{24}NO_6$; 338.1604. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.78 (1H, m), 1.95 (1H, m), 2.41 (2H, t, *J*=7.3 Hz), 2.85 (1H, dd, *J*=13.0, 8.0 Hz), 3.04 (1H, dd, *J*=13.0, 6.0 Hz), 3.30 (1H, dd, *J*=8.0, 5.5 Hz), 3.57 (1H, dd, *J*=8.0, 6.0 Hz), 3.61 (3H, s), 3.65 (6H, s), 7.16-7.33 (5H, m).

***Trimethyl-D^{glu}, L^{phe}* (8b)** Colorless gum. $[\alpha]_D^{27}$+14.2°(*c* 3.50, $CHCl_3$). EI-MS: *m/z* 338 $(M+H)^+$, 278 $(M\text{-}CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 338.1578 $(M+H)^+$, calcd. for $C_{17}H_{24}NO_6$; 338.1604. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.80 (1H, m), 1.90 (1H, m), 2.26 (2H, t, *J*=7.3 Hz), 2.85 (1H, dd, *J*=13.0, 7.0 Hz), 2.96 (1H, dd, *J*=13.0, 7.0 Hz), 3.18 (1H, dd, *J*=7.7, 5.0 Hz), 3.45 (1H, t, *J*=7.0 Hz), 3.64 (6H, s), 3.66 (3H, s), 7.16-7.30 (5H, m).

***Synthesis of trimethyl-L^{glu}, L^{phe}* (8a) *and trimethyl-L^{glu}, D^{phe}* (8c)** According to the preceding procedure, trimethyl-L^{glu}, L^{phe} (**8a**) (46.0 mg, *Rt* 27.5) and trimethyl-L^{glu}, D^{phe} (**8c**) (66.5 mg, *Rt* 34.5) were obtained from phenylpyruvic acid (492 mg, 3.0 mmol) and L- glutamic acid (441 mg, 3.0 mmol).

***Trimethyl-L^{glu}, L^{phe}* (8a)** Colorless gum. $[\alpha]_D^{31}$ -19.8°(*c* 1.68, $CHCl_3$). EI-MS: *m/z* 337 $(M)^+$, 278 $(M\text{-}CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 337.1496 $(M)^+$, calcd. for $C_{17}H_{23}NO_6$; 337.1525. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.78 (1H, m), 1.95 (1H, m), 2.41 (2H, t, *J*=7.3 Hz), 2.85 (1H, dd, *J*=13.5, 8.0 Hz), 3.04 (1H, dd, *J*=13.5, 6.0 Hz), 3.30 (1H, dd, *J*=8.0, 5.5 Hz), 3.57 (1H, dd, *J*=8.0, 6.0 Hz), 3.61 (3H, s), 3.65 (6H, s), 7.16-7.33 (5H, m).

***Trimethyl-L^{glu}, D^{phe}* (8c)** Colorless gum. $[\alpha]_D^{32}$ -16.6°(*c* 2.21, $CHCl_3$). EI-MS: *m/z* 337 $(M)^+$, 278 $(M\text{-}CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 337.1489 $(M)^+$, calcd. for $C_{17}H_{23}NO_6$; 337.1525. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.80 (1H, m), 1.90 (1H, m), 2.26 (2H, t, *J*=7.3 Hz), 2.85 (1H, dd, *J*=13.5, 7.5 Hz), 2.96 (1H, dd, *J*=13.5, 7.0 Hz), 3.18 (1H, dd, *J*=7.7, 5.2 Hz), 3.45 (1H, t, *J* =7.2 Hz), 3.64 (6H, s), 3.66 (3H, s), 7.16-7.30 (5H, m).

***Synthesis of trimethyl-D^{glu}, D^{phe}* (8d) *and trimethyl-L^{glu}, D^{phe}* (8c)** According to the preceding procedure, trimethyl-D^{glu}, D^{phe} (**8d**) (8.4 mg, *Rt* 27.5) and trimethyl-L^{glu}, D^{phe} (**8c**) (13.6 mg, *Rt* 34.5) were obtained from D-phenylalanine (495 mg, 3.0 mmol) and 2-ketoglutaric acid (438 mg, 3.0 mmol).

***Trimethyl-D^{glu}, D^{phe}* (8d)** Colorless gum. $[\alpha]_D^{28}$ +18.5°(*c* 0.84, $CHCl_3$). EI-MS: *m/z* 337 $(M)^+$, 278 $(M\text{-}CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 337.1498 $(M)^+$, calcd. for $C_{17}H_{23}NO_6$; 337.1525. ^{1}H-NMR ($CDCl_3$,

300 MHz): δ 1.78 (1H, m), 1.95 (1H, m), 2.41 (2H, t, *J*=7.6 Hz), 2.85 (1H, dd, *J*=13.5, 8.0 Hz), 3.04 (1H, dd, *J*=13.5, 6.0 Hz), 3.30 (1H, dd, *J*=8.0, 5.5 Hz), 3.57 (1H, dd, *J*=8.0, 6.0 Hz), 3.61 (3H, s), 3.65 (6H, s), 7.17-7.33 (5H, m).

Trimethyl-L^{glu}, D^{phe} **(8c)** Colorless gum. $[\alpha]_D^{29}$ -15.0°(*c* 1.36, $CHCl_3$). EI-MS: *m/z* 337 $(M)^+$, 278 $(M-CO_2Me)^+$, 246 (100 %). HREI-MS: *m/z* 337.1494 $(M)^+$, calcd. for $C_{17}H_{23}NO_6$; 337.1525. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.82 (1H, m), 1.90 (1H, m), 2.26 (2H, t, *J*=7.3 Hz), 2.85 (1H, dd, *J*=13.5, 7.5 Hz), 2.96 (1H, dd, *J*=13.5, 6.2 Hz), 3.18 (1H, dd, *J*=7.7, 5.1 Hz), 3.45 (1H, t, *J*=7.3 Hz), 3.64 (6H, s), 3.66 (3H, s), 7.16-7.30 (5H, m).

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