STRUCTURAL ELUCIDATION OF AIBELLIN, A NEW PEPTIDE ANTIBIOTIC WITH EFFICIENCY ENHANCING ACTIVITY ON RUMEN FERMENTATION

SHIGENORI KUMAZAWA, MINA KANDA, HIDEYUKI AOYAMA, MASAMI UTAGAWA, JUN KONDO, SEIHO SAKAMOTO, HIROYUKI OHTANI, TAKASHI MIKAWA, ISAO CHIGA and TETSUO HAYASE

Yokohama Research Center, Mitsubishi Chemical Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227, Japan

TSUNEO HINO

Department of Agriculture, Meiji University, Higashimita, Tama-ku, Kawasaki 214, Japan

TOSHIFUMI TAKAO and YASUTSUGU SHIMONISHI

Institute for Protein Research, Osaka University, Yamada, Suita 560, Japan

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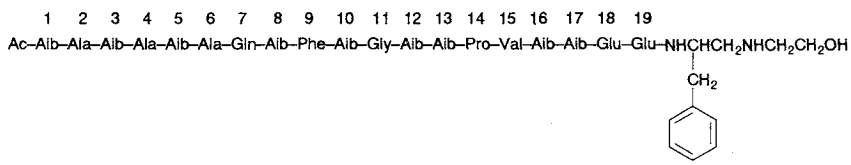
A new peptide antibiotic, aibellin, that had the efficiency enhancing activity on rumen fermentation, was isolated from the culture broth of the fungus, *Verticimonosporium ellipticum* D1528, and its primary structure was elucidated from spectrometric analysis and chemical degradation. Aibellin is a 20-residue peptaibol, and it has a unique structural feature in the novel *C*-terminal amino alcohol. Moreover, aibellin is the first peptaibol that possesses two acidic amino acids in the *C*-terminal region and a Phe residue in the middle of the sequence.

In ruminants, the positive correlation between an increase in feed conversion and enhanced propionate production in the rumen after the treatment of ionophore antibiotics such as monensin and lasalocid has been reported[1~3]. These antibiotics enhance propionate production and reduce methanogenesis, thus leading to greater efficiency of energy metabolism in ruminant animals[3,4].

During our search for novel ruminant growth performance enhancers from microbial sources using an *in vitro* rumen fermentation system, a new linear peptide antibiotic, aibellin, was found in the culture broth of the fungus *Verticimonosporium ellipticum* D1528. Aibellin was found to enhance propionate production and to reduce methanogenesis by rumen microorganisms[5].

In this paper, we report the isolation and structural elucidation of aibellin. Aibellin had structural features common to peptaibol[6~24] [Aib (α-aminoisobutyric acid)-containing linear peptides with an

Fig. 1. Primary structure of aibellin.



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acylated *N*-terminus and a *C*-terminal amino alcohol]. However, it had unique features in its structure. The *C*-terminal amino alcohol component of aibellin, 2-(2-amino-3-phenylpropylamino)ethanol, is reported here for the first time as a component of natural products. Moreover, aibellin is the first peptaibol that possesses two acidic amino acids in the *C*-terminal region (two Glu residues at positions 18 and 19) and a Phe residue in the middle of the sequence (at position 9).

Results

Fermentation

The producing fungus, strain D1528, was isolated from a dead leaf sample collected at Noto Peninsula, Japan and identified as *Verticimonosporium ellipticum*[25]. One loopful of a culture from an agar slant of *V. ellipticum* D1528 was inoculated into an Erlenmeyer flask (500-ml) containing 80 ml of a medium consisting of starch 2%, glucose 0.5%, soybean oil 5%, Pharmamedia (Tranders Oil Mill) 2%, soybean protein 2%, Staminol (Sapporo Brewery) 0.2% and $CaCO_3$ 1% (pH 6.0 before sterilization). A total of 100 flasks were shaken on a rotary shaker (210 rpm) for 7 days at 27°C.

Isolation of Aibellin

The culture broth of *V. ellipticum* D1528 (8 liters) was extracted with an equal volume of methanol and centrifuged. The supernatant was concentrated to 8 liters and distributed with 4 liters of ethyl acetate twice. The aqueous phase was then extracted with 4 liters of *n*-butanol twice and concentrated to yield 3 g of residue. The fraction was subjected to reversed-phase column chromatography of ODS (1 liter) with 50% methanol in 0.1% trifluoroacetic acid. Fractions having an efficiency enhancement activity on rumen fermentation[5] were collected. Aibellin was the major component of the fraction and purified by preparative HPLC using an ODS column (2 × 25 cm) with a mobile phase of 50% acetonitrile in 0.1% trifluoroacetic acid to give a pure compound (1.3 g).

Characterization of Aibellin

Aibellin was observed to have the following physico-chemical properties: MP 192~194°C; $[\alpha]_D^{25}$ −20.4° (*c* 0.5, MeOH); IR ν_{max} (KBr) cm^{-1} 3300 (NH), 1660 and 1540 (amide); UV λ_{max}^{MeOH} nm (ε) 220 (12,000), 252 (340), 258 (330) and 264 (280); ^1H NMR (500 MHz, CD_3OH) δ 2.02 (COMe), 7.0~8.5 (amide and phenyl protons); FAB-MS *m/z* 2,001 $(M+H)^+$ and 2,023 $(M+Na)^+$. The IR and NMR data suggested aibellin to be a peptide. Its negative reactivity for ninhydrin and the ^1H NMR signal of an acetyl group indicated the *N*-terminus to be acetylated.

Amino acid analysis of the acid hydrolysate (6 N HCl, 110°C, 24 hours) of aibellin showed its composition to be as follows: Aib (8~10 residues), Ala (3), Glu (3), Gly (1), Phe (1), Val (1) and Pro (1). Since the sensitivity of the amino group of Aib toward the ninhydrin reagent was much less than that of other amino acids, the exact number of Aib residues in aibellin could not be determined by amino acid analysis. Nine quarternary carbon signals were observed at δ 57~58 in the DEPT NMR spectrum, and thus the number of Aib residues was determined to be nine.

Structure of Aibellin

Sequence Determination of Aibellin

The positive FAB mass spectrum of aibellin showed the pseudomolecular ion peaks, $(M+H)^+$ at *m/z* 2,001 and $(M+Na)^+$ at *m/z* 2,023 (Fig. 2), indicating a molecular weight of 2000. The mass spectrum

Fig. 2. Positive FAB mass spectrum of aibellin.

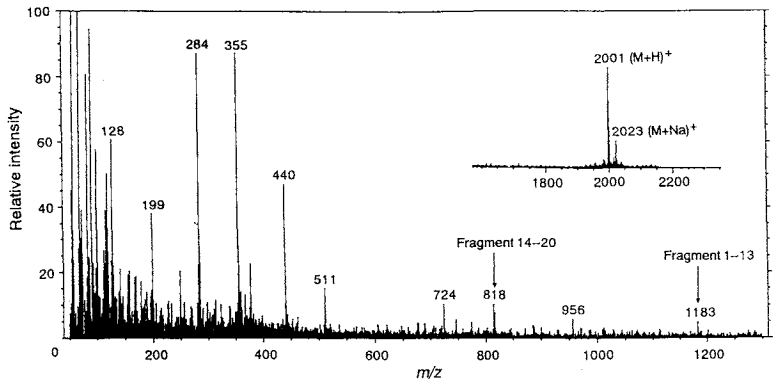
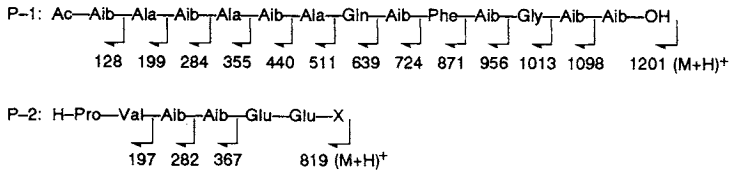


Fig. 3. Sequence-specific ions in the FAB mass spectra of two fragment peptides of aibellin.

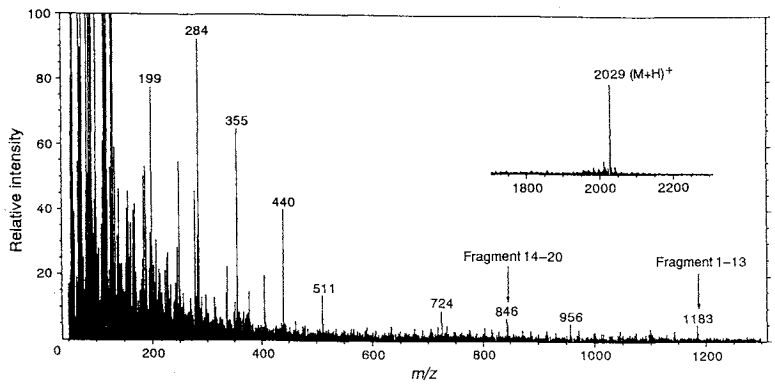


showed two characteristic fragment peaks at *m/z* 1,183 and *m/z* 818. The sum of the mass numbers of these fragment peaks was approximately equal to the molecular weight of aibellin. The previous studies showed that the Aib-Pro linkage in peptaibols was very labile and tended to be preferentially cleaved when samples were subjected to FAB mass measurement[16~24]. Thus, these two fragment peaks were considered to be obtained from the cleavage at the Aib-Pro linkage. Other than those peaks, some sequence-specific peaks, such as *m/z* 956, 724, 511, 440, 355, 284, 199 and 128 were observed in Fig. 2. These fragments, however, did not present enough information to determine the whole sequence, thus analysis of the partial degradation products was performed as follows.

Aibellin was subjected to partial acid hydrolysis (6 N, 37°C, 12 hours) yielding two peptides, P-1 and P-2. The FAB mass spectra of these peptides indicated a molecular weight of 1200 for P-1 and of 818 for P-2, the sum of which agreed with the molecular weight of aibellin, 2000. Sequence-specific fragment ions assignable to the sequence of Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Phe-Aib-Gly-Aib-Aib for P-1 and (Pro, Val)-Aib-Aib- for P-2 were observed (Fig. 3). Gas phase sequencing of P-2 indicated the sequence to be Pro-Val-Aib-Aib-Glu-Glu-X, where X was an unknown component. The previously described Aib-Pro linkage in peptaibols is very labile. Thus, acid hydrolysis of the bond in aibellin is most likely to result in the formation of the two fragment peptides and P-1 may be the *N*-terminal half and P-2 may be the other half. The reactivity toward ninhydrin (negative for P-1 and positive for P-2) supports this consideration. Therefore, the characteristic acylium fragment ion of aibellin at *m/z* 1,183 in Fig. 2 corresponds to the *N*-terminal half and the other fragment ion at *m/z* 818 to the *C*-terminal half. It follows from the above considerations that the entire sequence of aibellin is Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Phe-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Glu-Glu-X.

It should be noted that two Glu residues in the sequence of P-2 may possibly have been formed

Fig. 4. Positive FAB mass spectrum of Me-aibellin.



through deamination of Gln residues during the partial acid hydrolysis. The three Glx (Gln or Glu) of aibellin were identified by the following procedure. Methylesterification of the carboxylic acids of aibellin was quantitatively carried out using trimethylsilyldiazomethane[26] to yield the dimethyl ester derivative, Me-aibellin, the molecular weight of which was determined to be 2028 by FAB mass spectrometry (Fig. 4). The *C*-terminus of aibellin was an amino alcohol (described below), thus indicating the two Glu residues to be methylated. A comparison of the FAB mass spectrum of Me-aibellin with that of aibellin provided the following clarification. The fragment peak of aibellin at *m/z* 818 corresponding to P-2 shifted to *m/z* 846 in the spectrum of Me-aibellin (Fig. 4). Another characteristic fragment ion at *m/z* 1,183 corresponding to P-1 was not affected by methylation leading to the assignment of Gln at position 7, and Glu at positions 18 and 19. The amino acid sequence of aibellin was thus determined as **1** (Fig. 1).

Structural Elucidation of the *C*-Terminal Amino Alcohol

The structure of the unknown *C*-terminal component, X, was determined as follows. The ^1H NMR spectrum of aibellin indicated X to be a phenylalaninol-like amino alcohol. The ^1H-^1H COSY and HOHAHA spectra of aibellin demonstrated the presence of a 2-amino-3-phenylpropyl group and 2-aminoethanol moiety. In the HMBC spectrum, ^1H-^{13}C long range couplings between the two methylene groups (2- and 4-positions in Fig. 5) through the nitrogen atom were observed, indicating the amino group of aminoethanol to be substituted by the 2-amino-3-phenylpropyl group. The structure of X was thus determined to be **6** in Fig. 6; 2-(2-amino-3-phenylpropylamino)ethanol, $C_{11}H_{18}N_2O$ (MW 194). Compound **6** was synthesized for confirmation of the structure. Carbobenzoxy (*Z*)-L-phenylalanine **2** and ethanolamine **3** were coupled by dicyclohexylcarbodiimide to give *Z*-L-Phe-ethanolamine **4**. *Z* group of **4** was then removed (H_2-Pd) to give L-Phe-ethanolamine **5** and followed by reduction (BH_3) to give **6** (Fig. 6). The authentic compound **6** thus synthesized was co-chromatographed on reversed-phase HPLC with the amino alcohol purified from the acid hydrolysate of aibellin.

Absolute Configuration of the Amino Acids and Amino Alcohol

The absolute configurations of the optically active amino acids and the *C*-terminal amino alcohol were determined as follows. The chiral amino acids in the acid hydrolysate of aibellin were analyzed by HPLC using a MCI GEL CRS-10W chiral column[27]. A comparison with authentic D (or L)-amino acids

Fig. 5. Structure of the *C*-terminal component of aibellin and a part of the HMBC spectrum in CD_3OH. The ^1H-^{13}C long range couplings are indicated by broken lines.

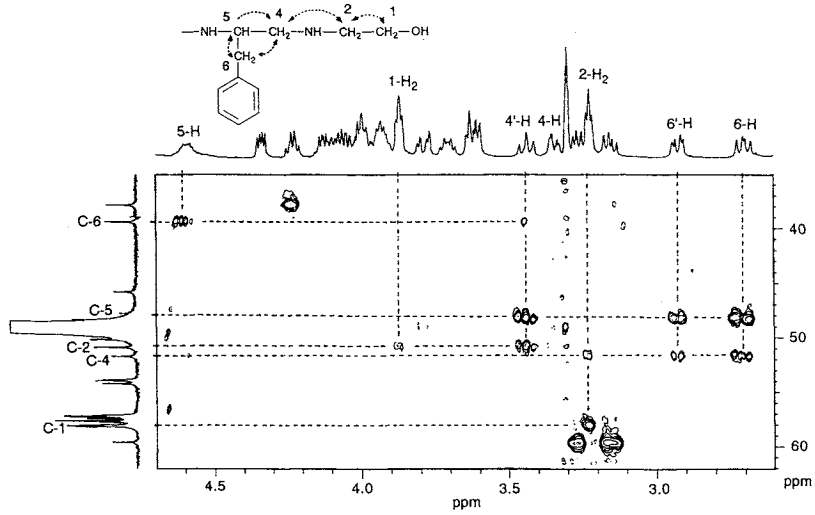
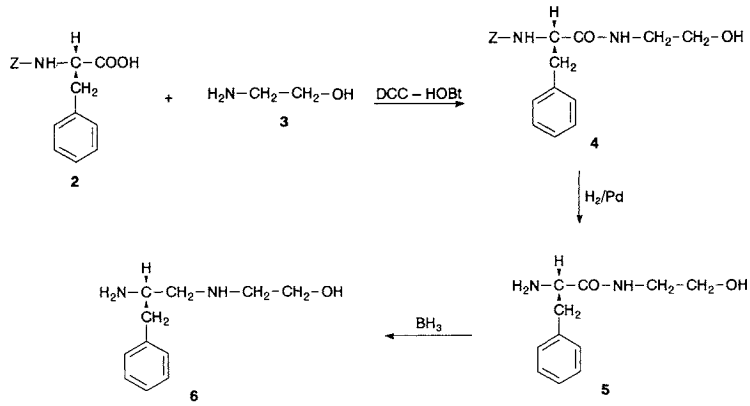


Fig. 6. Synthesis scheme for the *C*-terminal component of aibellin.



indicated all the chiral amino acids (Ala, Gln, Glu, Phe, Pro and Val) in aibellin to have the *S*-configuration (L-amino acids). HPLC analysis with a SUMICHIRAL OA-5000 chiral column of the authentic compound **6** (or its stereoisomer) synthesized from L (or D)-phenylalanine (described above) showed the *C*-terminal amino alcohol of aibellin to possess the *S*-configuration corresponding to L-phenylalanine.

Discussion

The structure of a new peptide antibiotic, aibellin, was elucidated from spectrometric analysis and chemical degradation. It was revealed that aibellin contained an acetylated *N*-terminal residue, a *C*-terminal amino alcohol and high content of hydrophobic amino acids including α-aminoisobutyric acid (Aib). Thus, aibellin belongs to the peptaibol class. However, aibellin has the following three unique features in its structure. (i) Aibellin has a novel *C*-terminal amino alcohol, 2-(2-amino-3-phenylpropylamino)ethanol. Although this amino alcohol has been shown to be an intermediate of peptide synthesis[28], it is reported here for the first time to be a component of natural products. (ii) Aibellin has two Glu residues in the *C*-terminal region (at positions 18 and 19). Alamethicins[8], trichotoxins A-40[11] and trichorzianines B[19]

are known as Glu-containing peptaibols, and they have one Glu residue. Aibellin is shown for the first time to be a peptaibol possessing two acidic amino acids in the *C*-terminal region. (iii) Aibellin contains a Phe residue at position 9. Antiamoebins[6,9], emerimicins[7] and tricholongins B[21] have been reported to contain one Phe residue in the *N*-terminal region (at position 1 or 3). Aibellin is the first peptaibol that possesses a Phe residue in the middle of the sequence.

Peptaibols have a broad range of biological activities, many of which are related to the interaction with biological membranes[29~31]. The interaction with membranes is considered to reflect the preference of the helical structure of these peptides[19,21]. In the case of aibellin, its circular dichroism spectrum clearly indicated a helical conformation (data not shown). Thus, a possibility exists that aibellin also has an interaction with biological membranes.

Aibellin enhanced propionate production and reduced methanogenesis in mixed rumen microorganisms without significant effects on protozoal growth or cellulose digestion[5]. Alamethicins, which are the membrane-modifying peptaibols, have also been reported to affect the production of volatile fatty acids by rumen bacteria[32]. Thus, the activity on rumen fermentation efficiency of alamethicins and aibellin may be related to the interaction with biological membranes. The relationship between the structure and activity of aibellin is presently being studied.

Experimental

General Procedures

NMR spectra were measured on a Bruker AM-500 spectrometer at room temperature with TMS as an internal standard. FAB mass spectra were obtained on a JEOL HX-100 spectrometer with glycerol as matrix. IR spectra were recorded on a Jasco FT/IR-8000 spectrometer. UV spectra were obtained on a Shimadzu UV-3100S spectrometer. Optical rotations were measured with a Jasco DIP-370 polarimeter.

In Vitro Assay of the Efficiency Enhancing Activity on Rumen Fermentation

The detail of this assay was reported previously[5]. Briefly, this assay is as follows. The rumen bacteria were separated from rumen fluid taken from a goat, and fermented under anaerobic conditions after adding the test sample. The culture continued 2 days. An aliquot of the culture broth was taken every 12 hours, and methane and volatile fatty acids including propionate were determined. The efficiency enhancing activity on rumen fermentation was measured by the reduction of methane and the increase of propionate in the culture broth.

Identification of the Amino Acids and Determination of Their Absolute Configuration

Aibellin (0.1 mg) was hydrolyzed in 6 N HCl at 110°C for 24 hours. The hydrolysate was analyzed by a Hitachi amino acid analyzer (L-8500) for amino acid composition analysis. For the chiral separation of Pro, Val, Glu and Phe, the hydrolysate was subjected to chiral HPLC analysis with a MCI GEL CRS-10W column (0.46 × 15 cm) [eluent 2 mM $CuSO_4$ MeOH - H_2O (15 : 85, v/v); flow rate 1.0 ml/minute; UV detector (254 nm)]. Ala was also analyzed by HPLC with the same column [eluent 0.1 mM $CuSO_4$; flow rate 0.8 ml/minute; UV detector (254 nm)].

Identification of the *C*-Terminal Amino Alcohol and Determination of Its Absolute Configuration

Aibellin (30 mg) was hydrolysed in 6 N HCl at 110°C for 24 hours. The hydrolysate was fractionated by HPLC with an ODS column (1 × 30 cm) [eluent 10% acetonitrile in 0.1% trifluoroacetic acid; flow rate 2.0 ml/minute; UV detector (215 nm)] to give the *C*-terminal component of aibellin (1.2 mg), $[\alpha]_D^{25}$ −5.3° (*c* 0.1, MeOH); IR ν_{max} (KBr) cm^{-1} 3300 (NH), 3000, 1670 (NH), 1190 and 1120; ^1H NMR (500 MHz, CD_3OD) δ 3.02 (2H, m, 6-CH_2), 3.14 (2H, m, 2-CH_2), 3.32 (2H, m, 4-CH_2), 3.77 (2H, m, 1-CH_2), 3.86 (1H, m, 5-CH) and 7.36 (5H, m, Ph); HRFAB-MS $C_{11}H_{19}N_2O$ (obsd *m/z* 195.1472, calcd 195.1497 $(M+H)^+$). The absolute configuration was determined by HPLC analysis with a SUMICHIRAL OA-5000 column (0.46 × 15 cm) [eluent 2 mM $CuSO_4$; flow rate 1.0 ml/minute; UV detector (254 nm)].

Partial Acid Hydrolysis of Aibellin

Aibellin (1 mg) was hydrolyzed in 6 N HCl at 37°C for 12 hours. The hydrolysate was subjected to

HPLC with a C8 column (0.46×25 cm) [eluent 25% acetonitrile in 0.1% trifluoroacetic acid; flow rate 1.0 ml/minute; UV detector (215 nm)]. Two major fragment peptides were collected, P-1 (0.2 mg): FAB-MS *m/z* 1201 $(M+H)^+$], and P-2 (0.4 mg): FAB-MS *m/z* 819 $(M+H)^+$. The amino acid sequence of P-2 was determined by direct sequencing on a gas-phase sequencer (Applied Biosystems 470A).

Z-L-Phe-Ethanolamine **4**

Z-L-Phe-OH **2** (300 mg, 1 mmol), *N*,*N*-dicyclohexylcarbodiimide (DCC) (206 mg, 1 mmol) and 1-hydroxybenzotriazole (HOBt) (135 mg, 1 mmol) were added successively to a stirred solution of ethanolamine **3** (61 mg, 1 mmol) in DMF (10 ml). After 24 hours, the product was extracted with ethyl acetate. The extract was washed with 1 N HCl, 5% $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized from $CHCl_3$ - *n*-hexane to give *Z*-L-Phe-ethanolamine **4** (250 mg, 73%), MP 125~127°C; $[\alpha]_D^{25}$ −3.66° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1} 3300 (OH), 1680 (NCO), 1640 (NCO), 1530 and 1250; ^1H NMR (500 MHz, $CDCl_3$) δ 2.45 (1H, br s, OH), 3.03 (2H, m, βCH_2), 3.22 (2H, m, NHCH_2), 3.50 (2H, m, CH_2OH), 4.46 (1H, m, αCH), 5.00 (2H, m, PhCH_2 in Z), 5.92 (1H, br d, *J*=8 Hz, N*H*CH), 6.85 (1H, br m, N*H*CH_2) and 7.22 (10H, m, Ph); FAB-MS *m/z* 343 $(M+H)^+$.

Anal Calcd for $C_{19}H_{22}N_2O_4$: C 66.70, H 6.43, N 8.19.
Found: C 66.82, H 6.40, N 8.12.

Z-D-Phe-Ethanolamine (Enantiomer of **4**)

This compound was prepared in the same way and on the same scale as described for *Z*-L-Phe-ethanolamine, starting with *Z*-D-Phe-OH (300 mg, 1 mmol). *Z*-D-Phe-ethanolamine (280 mg, 82%), MP 127~129°C; $[\alpha]_D^{25}$ +4.21° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1} 3300 (OH), 1680 (NCO), 1640 (NCO), 1530 and 1250; ^1H NMR (500 MHz, $CDCl_3$) δ 2.45 (1H, br s, OH), 3.03 (2H, m, βCH_2), 3.26 (2H, m, NHCH_2), 3.52 (2H, m, CH_2OH), 4.41 (1H, m, αCH), 5.03 (2H, m, PhCH_2 in Z), 5.50 (1H, br d, *J*=8 Hz, N*H*CH), 6.25 (1H, br m, N*H*CH2) and 7.22 (10H, m, Ph); FAB-MS *m/z* 343 $(M+H)^+$.

Anal Calcd for $C_{19}H_{22}N_2O_4$: C 66.70, H 6.43, N 8.19.
Found: C 66.88, H 6.67, N 8.10.

L-Phe-Ethanolamine **5**

Z-L-Phe-ethanolamine **4** (200 mg, 0.56 mmol) in MeOH (10 ml) was hydrogenated by H_2 gas over 10% palladium-on-charcoal with stirring. After removal of the catalyst by filtration, the filtrate was concentrated. The residue was recrystallized from MeOH - $CHCl_3$ to give L-Phe-ethanolamine **5** (105 mg, 91%), MP 182~184°C; $[\alpha]_D^{25}$ +48.7° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1} 3250 (NH), 2950, 1660 (NCO), 1550, 1490 and 1050; ^1H NMR (500 MHz, CD_3OD) δ 3.11 (2H, m, βCH_2), 3.29 (2H, m, NHCH_2), 3.52 (2H, m, CH_2OH), 4.03 (1H, dd, *J*=7 and 12 Hz, αCH) and 7.33 (5H, m, Ph); FAB-MS *m/z* 209 $(M+H)^+$.

Anal Calcd for $C_{11}H_{16}N_2O_2$: C 63.50, H 7.69, N 13.50.
Found: C 63.57, H 7.53, N 13.72.

D-Phe-Ethanolamine (Enantiomer of **5**)

This compound was prepared in the same way and on the same scale as described for L-Phe-ethanolamine, starting with *Z*-D-Phe-ethanolamine (200 mg, 0.56 mmol). D-Phe-ethanolamine (102 mg, 88%), MP 183~185°C; $[\alpha]_D^{25}$ +46.8° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1} 3250 (NH), 2950, 1660 (NCO), 1550, 1490 and 1050; ^1H NMR (500 MHz, CD_3OD) δ 3.11 (2H, m, βCH_2), 3.28 (2H, m, NHCH_2), 3.52 (2H, m, CH_2OH), 4.03 (1H, dd, *J*=7 and 12 Hz, αCH) and 7.33 (5H, m, Ph); FAB-MS *m/z* 209 $(M+H)^+$.

Anal Calcd for $C_{11}H_{16}N_2O_2$: C 63.50, H 7.69, N 13.50.
Found: C 63.72, H 7.51, N 13.72.

S-2-(2-Amino-3-phenylpropylamino)ethanol **6**

L-Phe-ethanolamine **5** (80 mg, 0.41 mmol) was dissolved in a mixture of 20 ml of dry tetrahydrofuran and 40 ml of dry 1,2-dimethoxyethane (DGM). The solution was cooled on ice and flushed with N_2, and 20 mmol of BH_3 (as 1 M solution in tetrahydrofuran) was added slowly (in 45 minute) with stirring. The reaction mixture was refluxed for 5 hours under anhydrous conditions. 15 ml of 1 M HCl/MeOH was added slowly to the reaction mixture after cooling on ice. The solution was then refluxed for 1 hour, and evaporated

to dryness under reduced pressure. The product was purified by HPLC using an ODS column (2×25 cm) with 10% acetonitrile in 0.1% trifluoroacetic acid to give a pasty substance, *S*-2-(2-amino-3-phenylpropylamino)ethanol **6** (52 mg, 65%), $[\alpha]_D^{25}$ −7.75° (*c* 0.1, MeOH); IR ν_{max} (KBr) cm^{-1} 3350 (NH), 3000, 1670 (NH), 1190 and 1120; ^1H NMR (500 MHz, CD_3OD) δ 3.02 (2H, m, 6-CH_2), 3.15 (2H, m, 2-CH_2), 3.32 (2H, m, 4-CH_2), 3.77 (2H, m, 1-CH_2), 3.86 (1H, m, 5-CH) and 7.36 (5H, m, Ph); HRFAB-MS $C_{11}H_{19}N_2O$ (obsd *m/z* 195.1500, calcd 195.1497 $(M+H)^+$).

R-2-(2-Amino-3-phenylpropylamino)ethanol (Enantiomer of **6**)

This compound was prepared in the same way and on the same scale as described for *S*-2-(2-amino-3-phenylpropylamino)ethanol, starting with D-Phe-ethanolamine (80 mg, 0.41 mmol). *R*-2-(2-amino-3-phenylpropylamino)ethanol (44 mg, 55%), $[\alpha]_D^{25}$ +6.94° (*c* 0.1, MeOH); IR ν_{max} (KBr) cm^{-1} 3350 (NH), 3000, 1670 (NH), 1190 and 1120; ^1H NMR (500 MHz, CD_3OD) δ 3.03 (2H, m, 6-CH_2), 3.15 (2H, m, 2-CH_2), 3.32 (2H, m, 4-CH_2), 3.77 (2H, m, 1-CH_2), 3.86 (1H, m, 5-CH) and 7.36 (5H, m, Ph); HRFAB-MS $C_{11}H_{19}N_2O$ (obsd *m/z* 195.1514, calcd 195.1497 $(M+H)^+$).

Acknowledgments

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