ENDOTHELIN-BINDING INHIBITORS, BE-18257A AND BE-18257B

II. STRUCTURE DETERMINATION

SHIGERU NAKAJIMA, KENJI NIIYAMA, MASAKI IHARA,
KATSUHISA KOJIRI† and HIROYUKI SUDA†

Central Research,
† Exploratory Research Laboratories,
Banyu Pharmaceutical Co., Ltd.,
2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

(Received for publication May 31, 1991)

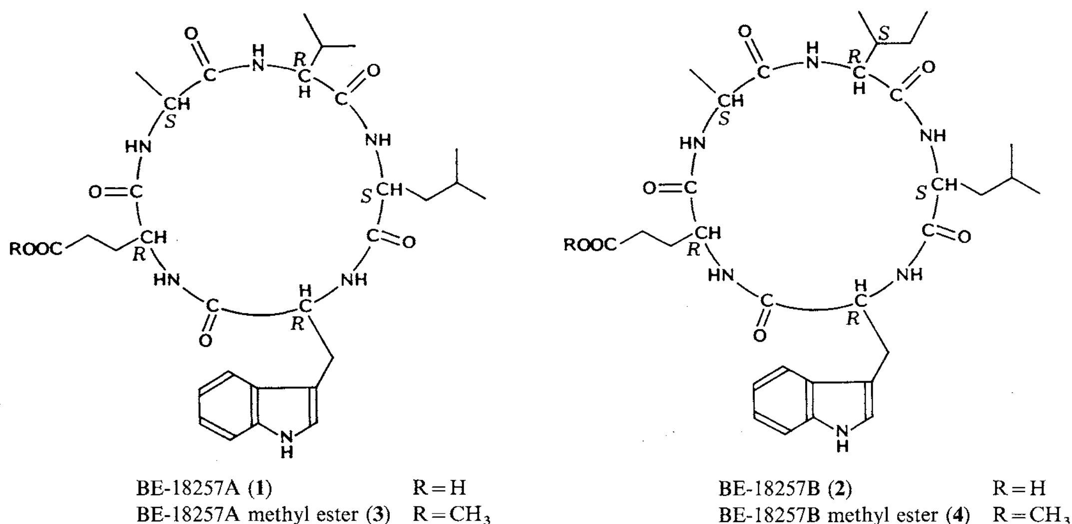
The structures of novel endothelin-binding inhibitors, BE-18257A and BE-18257B, were elucidated by spectral analyses and chemical studies. Both inhibitors were found to have a cyclic pentapeptide structure containing three D-form amino acid residues, namely, the structures of BE-18257A and BE-18257B were elucidated to be cyclo(-D-Glu-L-Ala-D-Val-L-Leu-D-Trp-) and cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-), respectively.

Endothelin binding inhibitors, BE-18257A (**1**) and B (**2**) were isolated from the culture broth of *Streptomyces misakiensis* BA18257 as described in a previous paper[1]. The structure determination studies of these compounds are described in this paper. The structures of **1** and **2** are shown in Fig. 1.

The Structure of BE-18257A

The physico-chemical properties of **1** and **2** were described in a previous paper[1]. The molecular formula of **1** was determined as $C_{30}H_{42}N_6O_7$ from HRFAB-MS (Calcd: *m/z* 599.3193, Found: *m/z* 599.3249 $(M+H)^+$) and ^{13}C NMR spectral data. Hydrolysis of **1** was performed with 6 N HCl at 105°C for 18 hours. The amino acid analysis of the hydrolysate indicated that **1** consists of one mole each of Ala, Glu, Leu, Trp and Val. No *N*-terminal amino acid was detected by Edman degradation. The absolute

Fig. 1. Structures of BE-18257A (**1**) and B (**2**).



configurations of amino acid residues of **1** were examined by a direct HPLC method, using a chiral column. Retention times of amino acids in the hydrolysate were compared with those of reference amino acids (Table 1). These data indicate that **1** consists of L-Ala, D-Glu, L-Leu, D-Trp and D-Val.

Table 1. The HPLC data of amino acids in BE-18257A (**1**) and BE-18257B (**2**) on Crownpac CR (+).

Condition[a]	Amino acid	Rt (minutes)		
		Authentic amino acid	**1**	**2**
1	D-Ala	5.18	—	—
	L-Ala	8.77	8.72	8.74
	D-Glu	5.71	5.69	5.66
	L-Glu	19.49	—	—
	D-Ile[b]	10.56	—	10.25
	L-Ile[b]	14.57	—	—
	D-Leu	13.13	—	—
	L-Leu	31.15	*ca.* 31[c]	*ca.* 31[c]
	D-Val	6.27	6.25	—
	L-Val	7.58	—	—
2	D-Trp	31.15	31.06	31.13
	L-Trp	36.11	—	—

[a] See experiment.
[b] Ile and allo-Ile could not be separated.
[c] Shoulder peaks interrupted by the impurities.
—: Not detected.

The ^{1}H and ^{13}C NMR data are shown in Tables 2 and 3, respectively. The assignments of proton and carbon signals were done using ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY spectra and an heteronuclear multiple-bond connectivity (HMBC) experiment[2,3]. The data obtained from these studies supported the presence of the above five amino acids in **1**. In the ^{1}H NMR spectrum of **1**, the γ-methylene signal of Glu was shifted from δ 2.16 to δ 1.95 by addition of 1 N NaOD, while the other signals remained essentially identical, indicating that the free carboxyl group observed in the spectrum is the γ-carboxyl group of the Glu residue. Furthermore, no chemical shift change was observed after addition of 1 N DCl. These results and the existence of five doublet amide protons in the ^{1}H NMR spectrum of **1** together with its molecular formula and also the above-mentioned result of Edman degradation strongly supported the idea that **1** has a cyclic peptide structure. However, amino acid sequence could not be determined through the analysis of its HMBC spectrum only, because the carbonyl carbons of Ala and Leu residue were indistinguishable in this spectrum. The amino acid sequence was finally determined through rotating frame NOESY (rotating-frame Overhauser enhancement spectroscopy: ROESY) spectral analyses[4,5] (Fig. 2) as follows. Strong ROE cross peaks were observed between the amide proton of Trp and the α-proton of Leu, the amide proton of Leu and the α-proton of Val, the amide proton of Val and the α-proton of Ala, the amide proton of Ala and the α-proton of Glu and also between the amide proton of Glu and the amide proton of Trp. On the basis of these results, the structure of **1** was assigned to be cyclo(-D-Glu-L-Ala-D-Val-L-Leu-D-Trp-) as shown in Fig. 1. Treatment of **1** with excess trimethylsilyl diazomethane in MeOH - benzene at room temperature gave the corresponding methyl ester derivative (**3**). The fragment peak data of FAB-MS spectrum of **3** also supported the structure of **1** (Table 4).

Furthermore, this novel, unique structure of **1** was confirmed by means of an alternate chemical synthesis of **1** (Scheme 1). The synthesized compound showed identical FAB-MS, IR and ^{1}H NMR spectra and optical rotation with those of the natural product. The endothelin-binding inhibitory activity (IC_{50}) of the synthetic compound was the same as the natural product (3.0 μM).

The Structure of BE-18257B

The molecular formula of **2** was determined as $C_{31}H_{44}N_{6}O_{7}$ from HRFAB-MS (Calcd: m/z 613.3550, Found: m/z 613.3377 $(M+H)^{+}$) and ^{13}C NMR spectral data. Hydrolysis of **2** was performed with 6 N HCl at 105°C for 18 hours. The amino acid analysis of the hydrolysate indicates that **2** consists of one mole each of Ala, Glu, Ile, Leu and Trp. The absolute configurations of amino acids in the hydrolysate

Table 2. ^{1}H NMR data for BE-18257A (**1**) and B (**2**).

1[a]	Assignment	**2**	Assignment
12.09 (1H, br s)	COOH	12.02 (1H, br s)	COOH
10.79 (1H, br s)	Trp-NH	10.78 (1H, br s)	Trp-NH
8.75 (1H, d, J=8.4[b])	Trp-amide	8.78 (1H, d, J=7.0)	Trp-amide
8.72 (1H, d, J=8.0)	Ala-amide	8.75 (1H, d, J=7.0)	Ala-amide
8.55 (1H, d, J=6.5)	Leu-amide	8.60 (1H, d, J=6.6)	Leu-amide
7.53 (1H, d, J=7.6)	Trp-H4	7.52 (1H, d, J=7.6)	Trp-H4
7.51 (1H, d, J=9.2)	Val-amide	7.49 (1H, d, J=7.8)	Glu-amide
7.39 (1H, d, J=7.3)	Glu-amide	7.45 (1H, d, J=9.4)	Ile-amide
7.31 (1H, d, J=7.6)	Trp-H7	7.31 (1H, d, J=7.6)	Trp-H7
7.12 (1H, d, J=1.2)	Trp-H2	7.12 (1H, d, J=1.8)	Trp-H2
7.04 (1H, t, J=7.6)	Trp-H6	7.04 (1H, t, J=7.6)	Trp-H6
6.96 (1H, t, J=7.6)	Trp-H5	6.96 (1H, t, J=7.6)	Trp-H5
4.40 (1H, dq, J=7.1)	Ala-αCH	4.45 (1H, dq, J=7.1)	Ala-αCH
4.28 (1H, m)	Trp-αCH	4.34~4.21 (3H, m)	Ile-αCH,
4.26 (1H, m)	Glu-αCH		Trp-αCH,
4.14 (1H, m)	Val-αCH		Glu-αCH
4.10 (1H, m)	Leu-αCH	4.06 (1H, m)	Leu-αCH
3.26 (1H, br d)	Trp-βCH_2	3.28 (1H, dd, J=2.3, 14.6)	Trp-βCH_2
2.91 (1H, dd, J=11.8, 14.4)	Trp-βCH_2	2.90 (1H, dd, J=11.8, 14.6)	Trp-βCH_2
2.16 (2H, m)	Glu-γCH_2	2.16 (2H, m)	Glu-γCH_2
1.88 (2H, m)	Glu-βCH_2	1.89 (2H, m)	Glu-βCH_2
1.78 (1H, m)	Val-βCH	1.58 (1H, m)	Ile-βCH
1.20 (2H, t, J=7.6)	Leu-βCH_2	1.29 (1H, m)	Ile-γCH_2
1.14 (3H, d, J=6.9)	Ala-βCH_3	1.20 (2H, m)	Leu-βCH_2
1.01 (1H, m)	Leu-γCH	1.13 (3H, d, J=7.1)	Ala-βCH_3
0.83 (3H, d, J=6.6)	Val-γCH_3	1.07 (1H, m)	Ile-γCH_2
0.82 (3H, d, J=6.6)	Val-γCH_3	0.95 (1H, m)	Leu-γCH
0.74 (3H, d, J=6.4)	Leu-δCH_3	0.87 (3H, t, J=7.4)	Ile-γCH_3
0.64 (3H, d, J=6.4)	Leu-δCH_3	0.78 (3H, d, J=6.7)	Ile-δCH_3
—	—	0.73 (3H, d, J=6.4)	Leu-δCH_3
—	—	0.63 (3H, d, J=6.4)	Leu-δCH_3

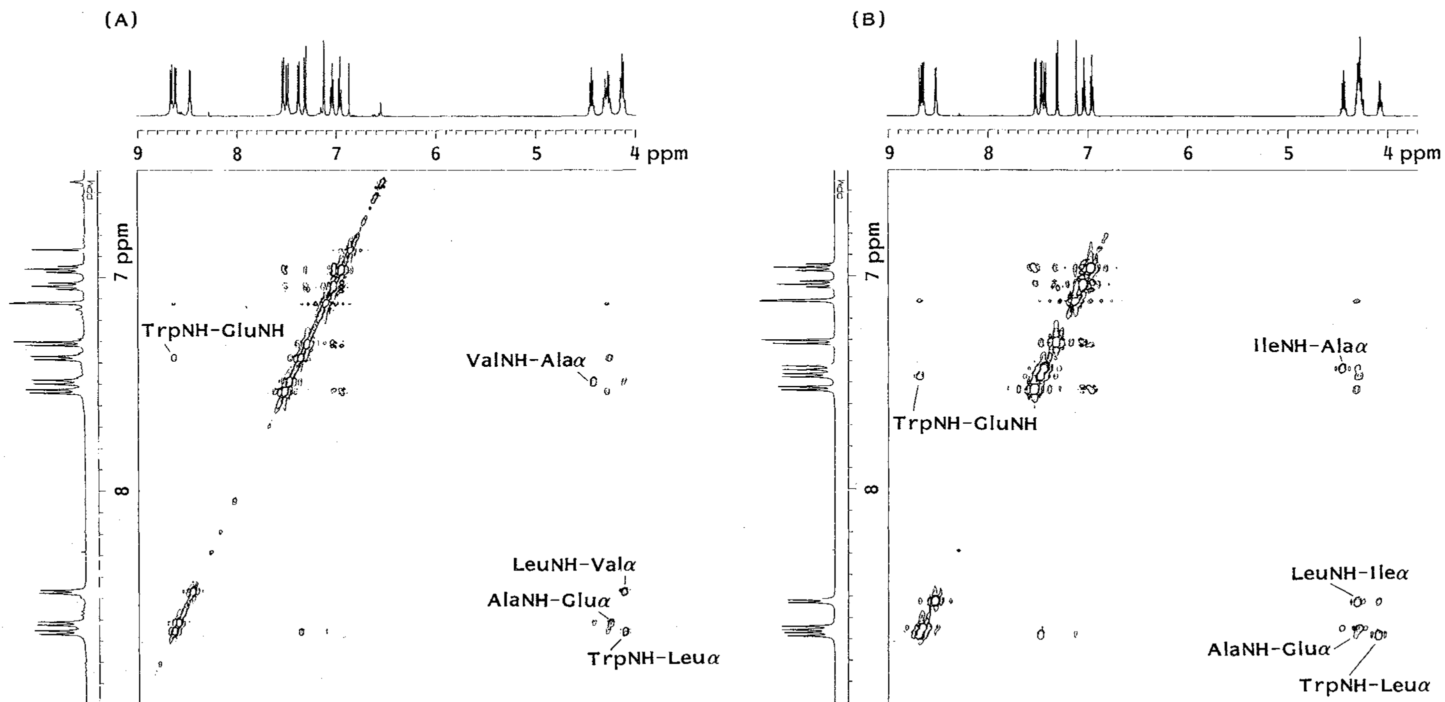
[a] Chemical shifts from TMS (0 ppm) in DMSO-d_6.
[b] Coupling constants in Hz.

Table 3. ^{13}C NMR data for BE-18257A (**1**) and B (**2**).

1[a]	Assignment	**2**	Assignment	**1**[a]	Assignment	**2**	Assignment
173.5 (s)	Glu-COOH	173.4 (s)	Glu-COOH	52.1 (d)	Glu-αCH	52.2 (d)	Leu-αCH
171.5 (s)	Val-CO	171.8 (s)	Ile-CO	51.9 (d)	Leu-αCH	52.1 (d)	Glu-αCH
171.4 (s)	Leu-CO	171.4 (s)	Leu-CO	47.1 (d)	Ala-αCH	47.0 (d)	Ala-αCH
171.4 (s)	Ala-CO	171.4 (s)	Ala-CO	38.5 (t)	Leu-βCH_2	38.4 (t)	Leu-βCH_2
171.2 (s)	Trp-CO	171.1 (s)	Trp-CO	30.6 (d)	Val-βCH	37.1 (d)	Ile-βCH
169.9 (s)	Glu-CO	169.7 (s)	Glu-CO	30.0 (t)	Glu-γCH_2	30.0 (t)	Glu-γCH_2
135.9 (s)	Trp-C7a	135.8 (s)	Trp-C7a	27.1 (t)	Trp-βCH_2	27.1 (t)	Trp-βCH_2
126.6 (s)	Trp-C3a	126.5 (s)	Trp-C3a	26.7 (t)	Glu-βCH_2	26.7 (t)	Glu-βCH_2
123.4 (d)	Trp-C2	123.3 (d)	Trp-C2	23.7 (d)	Leu-γCH	25.7 (t)	Ile-γCH_2
120.5 (d)	Trp-C6	120.4 (d)	Trp-C6	22.2 (q)	Leu-δCH_3	23.6 (d)	Leu-γCH
118.0 (d)	Trp-C5	117.9 (d)	Trp-C5	21.9 (q)	Leu-δCH_3	22.3 (q)	Leu-δCH_3
117.8 (d)	Trp-C4	117.7 (d)	Trp-C4	19.0 (q)	Val-γCH_3	21.8 (q)	Leu-δCH_3
111.0 (d)	Trp-C7	111.0 (d)	Trp-C7	18.0 (q)	Val-γCH_3	14.4 (q)	Ile-γCH_3
110.3 (s)	Trp-C3	110.3 (s)	Trp-C3	14.2 (q)	Ala-βCH_3	14.1 (q)	Ala-βCH_3
57.1 (d)	Val-αCH	55.2 (d)	Ile-αCH	—	—	11.3 (q)	Ile-δCH_3
54.9 (d)	Trp-αCH	54.8 (d)	Trp-αCH				

[a] Chemical shifts from TMS (0 ppm) in DMSO-d_6.

Fig. 2. Portions of 500 MHz ROESY spectra of BE-18257A (A) and B (B).



Scheme 1. Synthesis of BE-18257A.

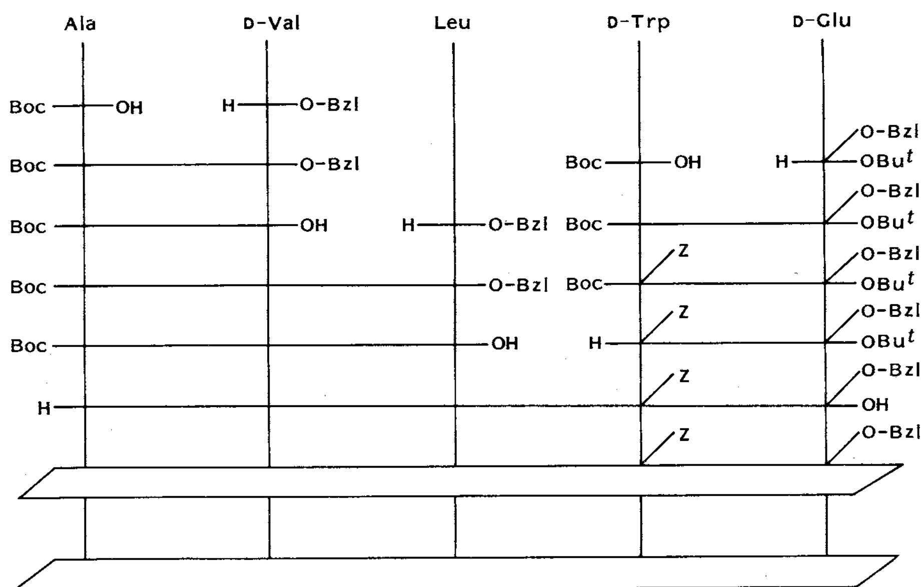


Table 4. FAB-MS data of **3** and **4**[a].

3		4	
m/z	Fragmentation	m/z	Fragmentation
613	$(M+H)^+$	627	$(M+H)^+$
427	$(M-Trp+H)^+$	441	$(M-Trp+H)^+$
314	$(M-Trp\text{-}Leu+H)^+$	328	$(M-Trp\text{-}Leu+H)^+$
215	$(M-Trp\text{-}Leu\text{-}Val+H)^+$	215	$(M-Trp\text{-}Leu\text{-}Ile+H)^+$
144	$(M-Trp\text{-}Leu\text{-}Val\text{-}Ala)^+$		

[a] Abbreviation means amino acid residue (NH-CH(R)-CO).

Table 5. The HPLC data of GITC-amino acid derivatives in **1** and **2** on Nucleosil.

GITC-amino acids	Rt (minutes)		
	Authentic derivatives	**1**	**2**
D-Ala	12.15		
L-Ala	10.65	10.79	10.81
D-Glu	9.75	9.91	9.93
L-Glu	9.30		
allo-D-Ile	40.32		40.64
allo-L-Ile	28.48		
D-Ile, D-Leu	41.73		
L-Ile, L-Leu[a]	29.75	29.84	29.93
D-Trp	56.88	57.04	57.41
L-Trp	41.95		
D-Val	24.77	25.03	
L-Val	18.35		

[a] L-Ile and L-Leu could not be separated.

of **2** were determined by the direct chiral column HPLC method. The configurations of Ala, Glu, Leu and Trp were found to be L, D, L and D, respectively. The configuration of α-carbon of Ile was determined to be D-form (Table 1), however, the configuration of β-carbon of Ile could not be resolved by this direct chiral column HPLC method. Therefore, the absolute configuration of Ile in the hydrolysate was determined by reverse-phase HPLC analyses of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[6] derivatives of amino acids contained in the hydrolysate of **2**. Five peaks of GITC-amino acids were observed in the HPLC spectrum and their retention times were compared with those of authentic GITC-amino acids. As shown in Table 5, the absolute configurations of Ala, Glu, Ile, Leu and Trp in **2** were determined to be L, D, allo-D, L and D, respectively.

The ^{1}H and ^{13}C NMR data are shown in Tables 2 and 3, respectively. The chemical shift assignments

for **2** were done by ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC spectral analyses. In the ^{1}H and ^{13}C NMR spectra of **2**, signals for an isoleucine moiety were observed instead of a valine moiety, which was observed in **1**, and the other signals were quite similar to those of **1**. The upfield shift of the γ-methylene signal of Glu was also observed from δ 2.16 to δ 1.90 by addition of 1 N NaOD, while the other signals remained essentially identical. These results suggest that **2** is also a cyclic compound. ROESY spectral analyses of the five amide- and α-protons of the amino acids of **2** were performed (Fig. 2). From these results, the structure of **2** was elucidated to be cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-) as shown in Fig. 1. The FAB-MS data of the methyl ester derivative of **2** (**4**) also supported the structure of **2** (Table 4).

Experimental

MS was carried out on a Jeol JMS-DX 300 spectrometer. MP was taken with a Yanaco MP-S3 melting point apparatus and was uncorrected. Optical rotation was determined with a Horiba Sepa-200 high sensitive polarimeter. IR spectra were recorded on a Hitachi 270-30 spectrometer. Generally NMR spectra were recorded on a Varian VXR 300 NMR spectrometer with ^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz. In all the measurements, TMS was used as an internal standard. The ROESY spectra were recorded on a Jeol JNM-GSX 500 spectrometer. The HMBC spectra were recorded on a Jeol JNM-EX 400 spectrometer. Packed column of the Inertsil ODS was purchased from Gasukuro Kogyo Inc. The Crownpak CR (+) was obtained from Daicel Chemical Industries, Ltd. The Nucleosil C_{18} was obtained from Macherey-Nagel.

Hydrolysis of BE-18257A (1) and B (2)

About 1.5 mg of **1** or **2** was hydrolyzed with 6 N HCl (1.5 ml) at 105°C for 18 hours in a sealed tube. The resulting solution was concentrated to dryness. A procedure of dissolution of the residue in H_2O and evaporation of the solvent was repeated several times to remove HCl.

Detection of Amino Acids

Amino acids were detected after conversion to their DNP-derivatives. Two hundred μl of a 5% ethanol solution of 2,4-dinitrofluorobenzene was added to a mixture (0.5 ml) containing the hydrolysate of **1** or **2** and 2% aq solution of $NaHCO_3$. The solution was allowed to stand at room temperature for 6 hours. The reaction mixture was extracted with diethyl ether three times. Then the aqueous layer was extracted with BuOH under acidic conditions (pH 2.0). The BuOH layer was concentrated to dryness and the residue was dissolved in 200 μl of MeOH. The MeOH solution was then subjected to HPLC analysis (Jasco Tri Rotar SR 2). The HPLC system was operated by using a reversed phase column of a Inertsil ODS (0.46 × 25 cm) at a flow rate of 1.0 ml/minute with a linear gradient of solvent A (100 ~ 0%) and solvent B (0 ~ 100%) for 15 minutes and then with solvent B for 25 minutes isocratically (solvent A: MeOH - 50 mM K-phosphate buffer (pH 6.0) - H_2O, 30 : 30 : 40, solvent B: MeOH - 50 mM K-phosphate buffer (pH 6.0), 70 : 30). The detection was carried out with a UV spectrometer at 254 nm. Rt of each DNP-amino acid was as follows, (minutes); DNP-Glu: 6.57, DNP-Ala: 15.42, DNP-Trp: 19.84, DNP-Val: 20.14, DNP-Ile: 22.71, DNP-Leu: 23.00.

Determination of Absolute Configuration of Amino Acids

Direct Method: The hydrolysate of **1** or **2** was dissolved in 100 μl of aq $HClO_4$ (pH 1.5). An aliquot (10 μl) of this solution was subjected to HPLC analysis (Jasco Trirotar VI), equipped with a UV detector (Jasco Uvidec 100 VI).

Condition 1: The Crownpak CR (+) (0.4 × 15 cm) was used at a flow rate of 0.5 ml/minute with aq $HClO_4$ (pH 1.5) at 5°C. The detection was carried out with a UV spectrometer at 200 nm.

Condition 2: The Crownpak CR (+) (0.4 × 15 cm) was used at a flow rate of 1.3 ml/minute with aq $HClO_4$ (pH 2.0) at 25°C. The detection was carried out with a UV spectrometer at 200 nm.

Derivatization Method: The hydrolysate of **1** or **2** (*ca.* 1 mg) was dissolved in 1 ml of H_2O. To an aliquot of this solution (10 μl), 50 μl of 50% acetonitrile containing 0.4% triethylamine and 100 μl of 0.2%

2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) in acetonitrile were added, then an aliquot (10 μl) of the mixture was analyzed by HPLC (Hitachi 6200). The reversed phase column of ODS Nucleosil (0.46 × 25 cm) was used at a flow rate of 0.6 ml/minute with a solution of 50% methanol in 10 mM phosphate buffer (pH 2.8) as eluent. Detection was carried out with a UV spectrometer at 250 nm.

Chemical synthesis of **1** was performed as follows (Scheme 1).

Boc-L-Ala-D-Val-OBzl (**5**)

To a solution of *N-tert*-Boc-L-alanine (56.8 mg, 0.30 mmol), D-valine benzyl ester (62.2 mg, 0.30 mmol) and HOBt (50.9 mg, 0.33 mmol) in DMF (0.50 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) (63.3 mg, 0.33 mmol). The mixture was stirred at room temperature for 2 hours and concentrated. After addition of aq $NaHCO_3$ (2 ml) the mixture was extracted with CH_2Cl_2 (10 ml × 3). The organic layers were combined and dried over $MgSO_4$ and concentrated. The residual solid was chromatographed on a silica gel column (Kieselgel 60, 5 g). The $CHCl_3$ - MeOH (100 : 1) eluate was concentrated to give 113.7 mg (0.30 mmol) of **5** as colorless powder: IR (KBr) cm^{-1} 3340, 1740, 1689, 1659, 1530, 1272; ^1H NMR (300 MHz, $CDCl_3$) δ 0.85 (3H, d, J=6.8 Hz), 0.93 (3H, d, J=7.2 Hz), 1.37 (3H, d, J=7.0 Hz), 1.45 (9H, s), 2.15~2.25 (1H, m), 4.15~4.25 (1H, m), 4.57 (1H, dd, J=4.6 and 8.8 Hz), 4.85~4.95 (1H, br s), 5.12 (1H, d, J=12.2 Hz), 5.20 (1H, d, J=12.2 Hz), 6.65~6.75 (1H, br s), 7.35 (5H, s).

Boc-L-Ala-D-Val-OH (**6**)

A mixture of **5** (94.6 mg, 0.25 mmol), 10% Pd-C (10.0 mg) and THF (1.2 ml) was stirred under H_2 atmosphere (1 atm) at room temperature for 3 hours. The mixture was filtered and the filtrate was concentrated to give 70.1 mg (0.24 mmol) of **6** as colorless powder: IR (KBr) cm^{-1} 3328, 2974, 1665, 1530, 1173; ^1H NMR (300 MHz, $CDCl_3$) δ 0.98 (6H, d, J=6.8 Hz), 1.38 (3H, d, J=7.1 Hz), 1.44 (9H, s), 2.20~2.35 (1H, m), 4.40~4.65 (2H, m), 5.25~5.35 (1H, br s), 6.90~7.00 (1H, br s).

Boc-L-Ala-D-Val-L-Leu-OBzl (**7**)

To a solution of **6** (57.7 mg, 0.20 mmol), L-leucine benzyl ester tosylate (82.3 mg, 0.20 mmol), HOBt (33.7 mg, 0.22 mmol) and *N*-methylmorpholine (22.0 μl, 0.20 mmol) in DMF (0.20 ml) was added EDCI (42.2 mg, 0.22 mmol) at 0°C. The mixture was stirred at 0°C for 1 hour. After addition of 5 ml of water the mixture was extracted with CH_2Cl_2 (10 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated. The residual solid was subjected to preparative TLC (Kieselgel 60, $CHCl_3$ - MeOH (10 : 1)) to give 77.8 mg (0.16 mmol) of **7** as colorless powder: IR (KBr) cm^{-1} 3328, 1740, 1677, 1647, 1542, 1515; ^1H NMR (300 MHz, $CDCl_3$) δ 0.90~0.95 (9H, m), 0.95 (3H, d, J=6.9 Hz), 1.35 (3H, d, J=7.1 Hz), 1.44 (9H, s), 1.55~1.70 (3H, m), 2.20~2.30 (1H, m), 4.10~4.20 (1H, m), 4.31 (1H, dd, J=5.9 and 8.7 Hz), 4.55~4.65 (1H, m), 4.90~5.00 (1H, br s), 5.11 (1H, d, J=12.2 Hz), 5.17 (1H, d, J=12.2 Hz), 6.55~6.65 (1H, br s), 6.66 (1H, d, J=9.0 Hz), 7.30~7.40 (5H, m).

Boc-L-Ala-D-Val-L-Leu-OH (**8**)

A mixture of **7** (334 mg, 0.70 mmol), 10% Pd-C (34 mg) and THF (3.5 ml) was stirred under H_2 atmosphere (1 atm) at room temperature for 2 hours. The mixture was filtered and the filtrate was concentrated. After addition of aq NaCl (3 ml) the mixture was extracted with ethyl acetate (30 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated to give 281 mg (0.70 mmol) of **8** as colorless powder: IR (KBr) cm^{-1} 3316, 2968, 1720, 1695, 1650, 1542, 1167; ^1H NMR (300 MHz, $CDCl_3$) δ 0.85~1.05 (12H, m), 1.36 (3H, d, J=6.8 Hz), 1.44 (9H, s), 1.55~1.80 (3H, m), 2.10~2.20 (1H, m), 4.10~4.60 (3H, m), 5.35~5.50 (1H, br s), 7.20~7.50 (2H, br s).

Boc-D-Trp-D-Glu(OBzl)-*O-tert*-Bu (**9**)

To a solution of *N*-Boc-D-tryptophan (274 mg, 0.90 mmol), D-glutamic acid α-*tert*-butyl-γ-benzyl diester (264 mg, 0.90 mmol), HOBt (145 mg, 0.95 mmol) in DMF (1.8 ml) was added EDCI (181 mg, 0.94 mmol). The mixture was stirred at room temperature for 2 hours and then concentrated. After addition of aq $NaHCO_3$ (2 ml), the mixture was extracted with CH_2Cl_2 (20 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated. The residual solid was chromatographed on a silica gel column (Kieselgel 60, 25 g). The eluate with $CHCl_3$ - MeOH (50 : 1) was concentrated to give 492 mg

(0.85 mmol) of **9** as pale yellow powder: IR (KBr) cm^{-1} 3352, 2980, 1725, 1500, 1395, 1371, 1254, 1155, 741; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (18H, s), 1.80~1.95 (1H, m), 2.10~2.25 (3H, m), 3.15 (1H, dd, J=6.1 and 14.3 Hz), 3.30~3.40 (1H, m), 4.35~4.55 (2H, m), 5.00~5.10 (1H, br s), 5.09 (2H, s), 6.53 (1H, d, J=7.1 Hz), 7.01 (1H, d, J=2.3 Hz), 7.10~7.20 (2H, m), 7.24 (1H, d, J=7.3 Hz), 7.30~7.45 (5H, m), 7.61 (1H, d, J=7.3 Hz), 7.92 (1H, br s).

Boc-D-Trp(*Z*)-D-Glu(OBzl)-*O*-*tert*-Bu (**10**)

To a suspension of **9** (232 mg, 0.40 mmol), tetra-*n*-butylammonium hydrogen sulfate (1.4 mg, 0.004 mmol) and NaOH (26 mg, 0.60 mmol) in CH_2Cl_2 (2.0 ml) was added benzyl chloroformate (86 μl, 0.60 mmol) at 0°C. The mixture was stirred at 0°C for 1 hour. After addition of aq $NaHCO_3$ (2 ml) the mixture was extracted with CH_2Cl_2 (20 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated. The residual solid was chromatographed on a silica gel column (Kieselgel 60, 5 g). The eluate with hexane - ethyl acetate (4 : 1) was concentrated to give 258 mg (0.36 mmol) of **10** as colorless powder: IR (KBr) cm^{-1} 3350, 1740, 1665, 1458, 1401, 1371, 1251, 1161; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (18H, s), 1.80~1.95 (1H, m), 2.10~2.40 (3H, m), 3.16 (2H, d, J=5.9 Hz), 4.35~4.45 (2H, m), 5.06 (2H, s), 5.41 (2H, s), 6.51 (1H, d, J=7.3 Hz), 7.20~7.50 (14H, m), 7.59 (1H, dd, J=1.4 and 6.5 Hz), 8.15 (1H, d, J=6.8 Hz).

D-Trp(*Z*)-D-Glu(OBzl)-*O*-*tert*-Bu (**11**)

To ice-cooled TFA (1.8 ml) was added **10** (250 mg, 0.35 mmol) in small portions. The mixture was stirred at 0°C for 10 minutes and then concentrated (below 10°C). After addition of aq $NaHCO_3$ (2 ml) the mixture was extracted with CH_2Cl_2 (20 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated. The residual oil was chromatographed on a silica gel column (Kieselgel 60, 10 g). The eluate with CH_2Cl_2 - MeOH (50 : 1) was concentrated to give 193 mg (0.32 mmol) of **11** as pale yellow oil: IR (neat) cm^{-1} 3376, 2980, 1743, 1671, 1458, 1404, 1362, 1251; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (9H, s), 1.90~2.00 (1H, m), 2.10~2.40 (3H, m), 2.83 (1H, dd, J=9.3 and 14.6 Hz), 3.31 (1H, dd, J=4.0 and 14.6 Hz), 3.71 (1H, dd, J=4.0 and 9.3 Hz), 4.45~4.55 (1H, m), 5.10 (2H, s), 5.43 (2H, s), 7.20~7.50 (13H, m), 7.62 (1H, dd, J=1.4 and 7.0 Hz), 7.84 (1H, d, J=8.0 Hz), 8.10~8.20 (1H, br d).

Boc-L-Ala-D-Val-L-Leu-D-Trp(*Z*)-D-Glu(OBzl)-*O*-*tert*-Bu (**12**)

To a solution of **11** (15.3 mg, 0.025 mmol), **8** (10.0 mg, 0.025 mmol) and HOBt (4.6 mg, 0.030 mmol) in DMF (0.25 ml) was added EDCI (5.8 mg, 0.030 mmol). The mixture was stirred at room temperature for 1 hour and then concentrated. After addition of aq $NaHCO_3$ (1 ml), the mixture was extracted with CH_2Cl_2 (10 ml × 3). The organic layers were combined, dried over $MgSO_4$ and concentrated. The residual solid was subjected to preparative TLC (Kieselgel 60, $CHCl_3$ - MeOH (20 : 1)) to give 21.1 mg (0.021 mmol) of **12** as colorless powder: IR (KBr) cm^{-1} 3304, 2974, 1737, 1640, 1533, 1458, 1401, 1371, 1251, 1164; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.78 (3H, d, J=6.3 Hz), 0.81 (3H, d, J=6.6 Hz), 0.91 (6H, d, J=6.9 Hz), 1.25~1.50 (6H, m), 1.34 (9H, s), 1.43 (9H, s), 1.95~2.25 (3H, m), 2.35~2.45 (2H, m), 3.31 (2H, d, J=6.5 Hz), 3.95~4.05 (1H, m), 4.05~4.20 (2H, m), 4.40~4.50 (1H, m), 4.55~4.65 (1H, m), 5.09 (2H, s), 5.41 (2H, s), 5.60~5.70 (1H, br s), 6.75~6.85 (1H, br d), 6.95~7.10 (3H, m), 7.20~7.55 (13H, m), 7.60 (1H, dd, J=1.2 and 7.8 Hz), 8.10~8.20 (1H, br d).

L-Ala-D-Val-L-Leu-D-Trp(*Z*)-D-Glu(OBzl)-OH (**13**)

12 (15.0 mg, 0.015 mmol) was dissolved in TFA (0.15 ml) and the solution was left for 3 hours at room temperature, and then concentrated to give 12.5 mg (0.015 mmol) of **13** as colorless powder: FAB-MS *m*/*z* 840 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.59 (3H, d, J=6.1 Hz), 0.63 (3H, d, J=6.4 Hz), 0.85 (3H, d, J=6.4 Hz), 0.90 (3H, d, J=6.5 Hz), 1.15~1.30 (3H, m), 1.48 (3H, d, J=6.6 Hz), 1.90~2.35 (5H, m), 3.05~3.15 (1H, m), 3.20~3.30 (1H, m), 4.00~4.30 (4H, m), 4.50~4.60 (1H, m), 5.01 (2H, s), 5.34 (2H, s), 7.10~7.45 (16H, m), 7.52 (1H, s), 7.58 (1H, d, J=8.8 Hz), 8.00~8.10 (1H, br d).

Cyclo(-D-Glu(OBzl)-L-Ala-D-Val-L-Leu-D-Trp(*Z*)-) (**14**)

To a solution of **13** (16.8 mg, 0.020 mmol) and HOBt (4.6 mg, 0.030 mmol) in DMF (4.0 ml) was

added a solution of EDCI (5.8 mg, 0.030 mmol) in DMF (1.0 ml) over 1 hour at 0°C. The mixture was stirred at room temperature for 2 hours and then concentrated. The residual solid was washed successively with 1 N HCl, aq $NaHCO_3$, and water and then dried to give 12.3 mg (0.015 mmol) of **14** as pale yellow powder: FAB-MS *m/z* 823 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6) δ 0.51 (3H, d, J=6.6 Hz), 0.58 (3H, d, J=6.7 Hz), 0.80 (3H, d, J=6.4 Hz), 0.82 (3H, d, J=5.9 Hz), 1.12 (3H, d, J=7.1 Hz), 1.20~1.35 (3H, m), 1.70~2.05 (3H, m), 2.25~2.40 (2H, m), 2.85~2.95 (1H, m), 3.20~3.30 (1H, m), 3.95~4.05 (1H, m), 4.10~4.20 (1H, m), 4.25~4.35 (1H, m), 4.35~4.50 (2H, m), 5.09 (2H, s), 5.45 (2H, s), 7.20~7.60 (16H, m), 8.00~8.10 (1H, br s), 8.55~8.65 (1H, br s), 8.75~8.90 (2H, m).

Cyclo(-D-Glu-L-Ala-D-Val-L-Leu-D-Trp-) (1)

To a mixture of ethane dithiol (25 μl, 0.30 mmol) and a 1-M solution of both trimethylsilyl trifluoromethanesulfonate and thioanisole in TFA (1.4 ml, 1.40 mmol) was added **14** (12.3 mg, 0.015 mmol) at 0°C. The mixture was stirred at 0°C for 2.5 hours and then concentrated. The residue was triturated with 3 ml of ether and the precipitates were collected, washed with water and ether and then dried to give 6.2 mg (0.010 mmol) of **1** as colorless powder: MP >300°C; $[\alpha]_D^{20}$ $-9.8°$ (*c* 1.0, DMSO); FAB-MS *m/z* 599 $(M+H)^+$; IR (KBr) cm^{-1} 3292, 2968, 1647, 1533, 1248; ^1H NMR (300 MHz, DMSO-d_6) δ 0.64 (3H, d, J=6.4 Hz), 0.74 (3H, d, J=6.4 Hz), 0.82 (3H, d, J=6.6 Hz), 0.83 (3H, d, J=6.6 Hz), 0.95~1.10 (1H, m), 1.14 (3H, d, J=6.9 Hz), 1.20 (2H, t, J=7.6 Hz), 1.75~1.85 (1H, m), 1.85~2.00 (2H, m), 2.10~2.25 (2H, m), 2.91 (1H, dd, J=11.8 and 14.4 Hz), 3.26 (1H, br d), 4.05~4.20 (2H, m), 4.20~4.35 (2H, m), 4.40 (1H, dq, J=7.1 Hz), 6.96 (1H, t, J=7.6 Hz), 7.04 (1H, t, J=7.6 Hz), 7.12 (1H, d, J=1.2 Hz), 7.31 (1H, d, J=7.6 Hz), 7.39 (1H, d, J=7.3 Hz), 7.51 (1H, d, J=9.2 Hz), 7.53 (1H, d, J=7.6 Hz), 8.55 (1H, d, J=6.5 Hz), 8.72 (1H, d, J=8.0 Hz), 8.75 (1H, d, J=8.4 Hz), 10.79 (1H, br s), 12.09 (1H, br s).

Acknowledgments

We express our thanks to Mr. O. Kamo, Jeol Co., Ltd., for measuring the ROESY spectra.

References

1) Kojiri, K.; M. Ihara, S. Nakajima, K. Kawamura, K. Funaishi, M. Yano & H. Suda: Endothelin-binding inhibitors, BE-18257A and BE-18257B. I. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 44: 1342~1347, 1991
2) Seto, H.; K. Furihata & M. Ohuchi: Particular utility of the HMBC technique to polypropionate derived metabolites as exemplified by erythromycin A. J. Antibiotics 41: 1158~1160, 1988
3) Bax, A. & M. F. Summers: ^1H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986
4) Bax, A. & D. G. Davis: Practical aspects of two dimensional transverse NOE spectroscopy. J. Magn. Reson. 63: 207~213, 1985
5) Kessler, H.; C. Griesinger, R. Kerssebaum, K. Wagner & R. R. Ernst: Separation of cross-relaxation and *J* cross-peaks in 2D rotating-frame NMR spectroscopy. J. Am. Chem. Soc. 109: 607~609, 1987
6) Kinoshita, T.; Y. Kasahara & N. Nimura: Reversed-phase high-performance liquid chromatographic resolution of non-esterified enantiometric amino acids by derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4-tri-*O*-acetyl-α-D-arabinopyranosyl isothiocyanate. J. Chromatogr. 210: 77~81, 1981