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Isolation and Structural Determination of Phepropeptins A, B, C, and D, New Proteasome Inhibitors, Produced by *Streptomyces* sp.

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We have isolated four related compounds named phepropeptins A, B, C, and D, as inhibitors of proteasome proposed to regulate many cellular functions. From an NMR analysis, the phepropeptins appeared as cyclic hexapeptides, differing in the two residues of the constituent amino acids from one another, with four conserved amino acid moieties. Based on an amino acid analysis, we synthesized two possible cyclic peptides to phepropeptin B that differ in the configurations. A comparison of the properties between the natural and synthesized compounds revealed that the structure of phepropeptin B was *cyclo*(-L-Leu-D-Phe-L-Pro-L-Phe-D-Leu-L-Val-). The phepropeptins showed inhibition to the proteasomal chymotrypsin-like activity but not to α-chymotrypsin.

Proteasome is the multicatalytic protease complex with chymotrypsin-like (ChT-L), trypsin-like (T-L), peptidyl glutamyl peptide hydrolase (PGPH), branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP) activities[1,2] responsible for the degradation of the functional proteins used for cell cycle progression, immune responses, and inflammation depending on ubiquitin in the presence of ATP[3~5]. Because proteasome regulates many important functions in cellular events, a proteasome inhibitor is considered to be useful for therapeutic applications[6,7].

Synthetic peptides including PS-341[7] and natural products such as lactacystin[8,9] and epoxomicin[10] which suppress tumor growth and regulate antigen presentation[11], respectively, have been reported as proteasome inhibitors. A mechanistic analysis of the lactacystin and peptide aldehyde inhibitors revealed that their mechanism of inhibition is the inhibitory binding to the amino terminal threonine residue of the proteasomal β-subunit which catalyzes the ChT-L activity[9,12]. Though this research indicated that the inhibition of the ChT-L activity is efficient toward the major catalytic activities of proteasome, it has been considered that other types of inhibitors could affect the cellular responses in different ways[13].

We then screened new inhibitors of proteasome in microbial secondary metabolites and isolated four related inhibitors, named phepropeptins A, B, C, and D (Fig. 1), as products of *Streptomyces* sp. MK600-cF7. Their structures were determined as cyclic hexapeptides and we determined the stereochemistry of the phepropeptins based on an amino acids analysis and synthetic study. We described the isolation procedure, structural determination including stereochemistry, and proteasome inhibition of the phepropeptins in this report.

Materials and Methods

Proteasome Preparation

The proteasome used in this study was partially purified from mouse livers as follows. A 7.3 g sample of livers from five mice were homogenized in 20 ml of homogenizing buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 2 mM ATP, 0.25 M sucrose and ultracentrifuged at 35,000 rpm for 1 hour at 4°C. The supernatant was then further ultracentrifuged at 40,000 rpm for 5 hours at 4°C. The obtained precipitate of this centrifugal fractionation was resolved in 2 ml of standard

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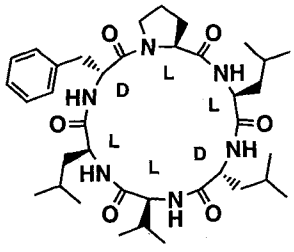
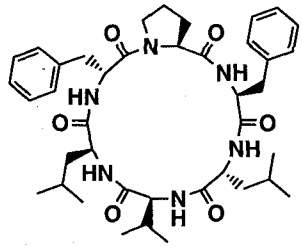
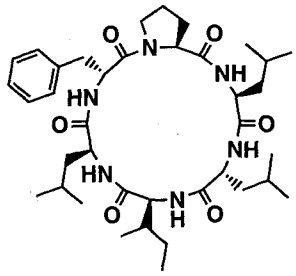
Fig. 1. Structures of phepropeptins.



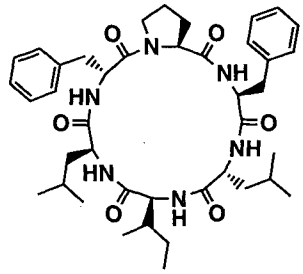
phepropeptin A (**1**)



phepropeptin B (**2**)



phepropeptin C (**3**)



phepropeptin D (**4**)

buffer consisting of 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 mM ATP, 20% glycerol and centrifuged at 20,000 *g* for 30 minutes to remove any insoluble particles. The supernatant was divided and stored at −20°C as the proteasome preparation.

Peptidase Assay

The peptidase activity of proteasome was represented as the ChT-L activity using a fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-MCA (Peptide Institute, Inc., Osaka). Briefly, 80 μl of the total reaction mixture containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 2 mM ATP, 0.05% (w/v) SDS, 20 μM fluorogenic substrate and the proteasome preparation described above was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 50 μl of 10% (w/v) SDS solution, and then the fluorescence of the released aminomethylcoumarin was measured. To measure the proteasomal T-L and PGPH activities, the fluorogenic substrates were replaced by Boc-Leu-Arg-Arg-MCA and Z-Leu-Leu-Glu-MCA (Peptide Institute, Inc., Osaka), respectively, and SDS was not added during the reaction when measuring the T-L activity.

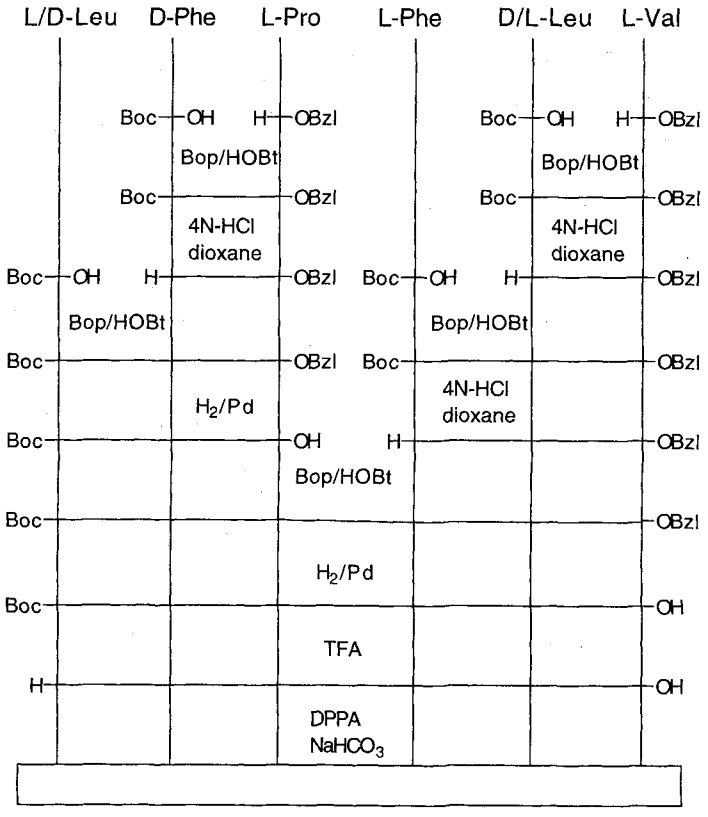
Fermentation

The MK600-cF7 strain identified as *Streptomyces* sp. was inoculated into a 500-ml Erlenmayer flask containing 110 ml of medium consisting of 2.5% soybean meal, 2% soybean oil, 1% maltose, 0.2% yeast extract, 0.05% K_2HPO_4 and 0.025% $MgSO_4$. The flask for the seed culture was shaken on a rotary shaker for 5 days at 27°C. Two ml portions were inoculated into 500-ml Sakaguchi flasks containing 125 ml of the same medium into 40 of 500-ml Sakaguchi. These flasks were reciprocated for 5 days at 27°C.

Amino Acids Analysis

A 56 mg sample of **2** was hydrolyzed in 4 ml of 6 N HCl at 105°C for 22 hours. The hydrolysate was dried, resolved in 5 ml of 0.05 M pyridine-HCOOH (pH 3.1) and charged on an ion exchanging column (Dowex 50W×8, 200~400 mesh, 21 mm i.d.×400 mm) equilibrated with the same buffer. The column was eluted with the following buffer systems, 0.1 M pyridine-HCOOH (pH 3.1) for proline, 0.2 M pyridine-HCOOH (pH 3.1) for valine, and 0.2 M pyridine-AcOH (pH 3.1) for leucine and phenylalanine (18.3 mg) separately in that order. To further purify the proline, valine

Scheme 1. Synthetic route of **2** and its stereoisomer.



and leucine, each fraction was charged on a column (Dowex 50W×4, 50~100 mesh, 20 mm i.d.×60 mm) and eluted with 1 N NH_4OH to give proline (9.2 mg), valine (7.4 mg), and leucine (6.6 mg), respectively.

1 was hydrolyzed and the constituent amino acids were similarly separated.

The configurations of the resulting amino acids were analyzed by thin layer chromatography on an HPLC-chiral plate (Art. 14101, Merck) with the solvent system of MeOH - CH_3CN - H_2O, 1 : 4 : 1 (in volume).

Synthesis of **2** and Its Stereoisomer

The syntheses of **2** and its stereoisomer were summarized in Scheme 1. Briefly, the Boc tripeptide benzyl esters are synthesized by ordinary liquid phase stepwise elongation using benzotriazole-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate/1-hydroxybenzotriazole (Bop/HOBt) in the presence of triethylamine in 90% overall yield.

The Boc-D/L-Leu-D-Phe-L-Pro benzyl ester was deprotected by hydrogenation with palladium black to yield the corresponding Boc-tripeptide acid. The Boc-L-Phe-D/L-Leu-L-Val benzyl ester was treated with 4 N HCl/dioxane to yield the corresponding tripeptide ester hydrochloride.

Fragment condensation of these tripeptide acids and amino-ester hydrochloride derivatives using Bop/HOBt in the presence of triethylamine afforded the Boc-hexapeptide benzyl esters in 90~95% yield.

After deprotection by hydrogenation with palladium black, The Boc-hexapeptide benzyl esters were treated with 4 N HCl/dioxane to yield the corresponding hexapeptide hydrochloride.

Cyclization of the hexapeptide was fulfilled using diphenylphosphoryl azide and sodium bicarbonate (DPPA/$NaHCO_3$) in DMF (15 ml per 100 mg peptide) at 5°C for 3 days in 30~40% yield.

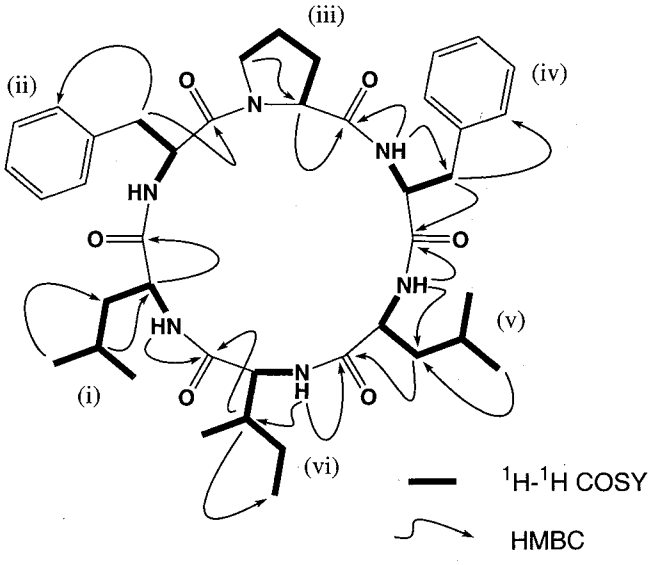
Cyclo(-D-Leu-D-Phe-L-Pro-L-Phe-L-Leu-L-Val-): MP 136°C, $[\alpha]_D^{26}$ −56.4° (*c* 0.25, MeOH), Chemical shifts of ^{13}C-NMR (ppm in $CDCl_3$) 18.7 (q), 19.5 (q), 21.1 (q), 21.8 (q), 22.8 (q), 23.1 (q), 24.3 (t), 25.0 (d), 25.4 (d), 28.9 (t), 29.1 (d), 35.3 (t), 38.3 (t), 38.6 (t), 40.7 (t), 47.7 (t), 52.0 (d), 53.0 (d), 59.8 (d), 61.2 (d), 126.7 (d), 127.1 (d), 128.5 (d), 128.6 (d), 129.1 (d), 129.4 (d), 136.2 (s), 138.0 (s), 171.4 (s), 171.7 (s), 171.9 (s), 172.0 (s), 172.5 (s), 174.5

Table 1. Physico-chemical properties of **1**, **2**, **3** and **4**.

	1	2	3	4
Appearance	white powder	white powder	white powder	white powder
Melting point (°C)	115-117	125-127	118-121	125-127
Optical rotation in MeOH	$[\alpha]_D^{26}$ −85.9° (c 1.0)	$[\alpha]_D^{23}$ −107.5° (c 0.4)	$[\alpha]_D^{26}$ −81.6° (c 1.0)	$[\alpha]_D^{25}$ −94.4° (c 1.0)
Molecular formula HRFAB-MS (m/z)	$C_{37}H_{58}O_6N_6$	$C_{40}H_{56}O_6N_6$	$C_{38}H_{60}O_6N_6$	$C_{41}H_{58}O_6N_6$
Found	683.4489 $(M+H)^+$	717.4350 $(M+H)^+$	697.4633 $(M+H)^+$	731.4485 $(M+H)^+$
Calcd.	683.4496 (as $C_{37}H_{59}O_6N_6$)	717.4340 (as $C_{40}H_{57}O_6N_6$)	697.4653 (as $C_{38}H_{61}O_6N_6$)	731.4496 (as $C_{41}H_{59}O_6N_6$)
UVλmax (nm)	End	End	End	End
in MeOH	258 (<300)	258 (300)	258 (<300)	258 (300)
IR ν max (cm^{-1})	2958, 2871, 1641,	2958, 2871, 1641,	2958, 2873, 1641,	2958, 2873, 1641,
KBr	1516, 1448, 702	1516, 1448, 702	1514, 1450, 702	1514, 1448, 702
Retention time[1] (min.)	7.8	8.1	9.8	10.3

[1] HPLC: Capcell Pak UG120 (4.6 mmΦ x 150 mm, Shiseido), 60% CH_3CN, 1 ml/min.

Fig. 2. Summary of ^1H-^1H COSY and HMBC experiments of **4**.



(s). The retention time was 5.9 minutes. (under the same conditions described in Table 1).

Cyclo(-L-Leu-D-Phe-L-Pro-L-Phe-D-Leu-L-Val-): MP 125~127°C, $[\alpha]_D^{23}$ −107.4° (*c* 1.1, MeOH), Chemical shifts of ^1H- and ^{13}C-NMR (ppm in $CDCl_3$) were identical with those of **2**. The retention time was 8.1 minutes. (under the same conditions described in Table 1).

General Procedures

Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Mass spectra were obtained using a JEOL JMS-SX102 spectrometer. All NMR spectra were recorded with a JEOL JNM-A500 spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C) in $CDCl_3$. The fluorescence of AMC was measured by a Millipore CytoFluor 2300 fluorometer with filters of 360 nm (ex) and 460 nm (em).

Results and Discussion

Isolation

The fermented broth of the producing strain described above was centrifuged to separate the mycelium. The mycelium cake was extracted with 2 liters of MeOH and filtered to remove any insoluble materials. The filtrate was concentrated *in vacuo* and combined with the broth filtrate. This material (5 liter) was extracted with EtOAc and the organic layer was evaporated to dryness to give 19.4 g of an oily residue. This residue was suspended in 200 ml of MeOH and extracted with 400 ml of hexane. The crude material (10.9 g) that resided in the methanolic phase was charged on a silica gel column (Wako Gel C-200, 40 mm i.d.×160 mm) and eluted with 400 ml each of the solvent mixture of $CHCl_3$-MeOH (100:0, 100:1, 100:2). The fractions containing the phepropeptins were collected and dried to give 2.78 g of an oily residue. This material was

Table 2. ^{1}H chemical shifts of **1**, **2**, **3** and **4** in $CDCl_3$.

Position	Carbon	1	2	3	4
i	1	--	--	--	--
	2	4.33 m	4.50 dd	4.44 dd	4.51 dd
	3	1.60 m,1.67 m	1.70 m, 1.73 m	1.56 m, 1.65 m	1.68 m, 1.75 m
	4	1.53 m	1.60 m	1.53 m	1.63 m
	5	0.89 d	0.95 d	0.89 d	0.95 d
	6	0.92 d	0.93 d	0.92 d	0.92 d
	NH	6.44 brd	6.63 brd	6.40 d	6.58 d
ii	1	--	--	--	--
	2	4.33 m	4.35 m	4.34 m	4.35 m
	3	2.96 dd, 3.07 dd	2.98 dd, 3.06 dd	2.97 dd, 3.08 dd	2.96 dd, 3.07 dd
	4	--	--	--	--
	5	7.21 d	]	7.21 d	]
	6	7.27 dd	7.10-7.30	7.29 dd	7.10-7.30
	7	7.26 dd	(5 H)	7.27 dd	(5 H)
	NH	7.21 d	7.35 brd	7.43 d	7.49 brd
iii	1	--	--	--	--
	2	4.38 dd	4.32 dd	4.38 m	4.31 m
	3	1.86 m, 2.00 m	1.64 m	1.86 m, 2.01 m	1.65 m
	4	1.61 m, 1.75 m	1.05 m, 1.35 m	1.75 m, 1.60 m	1.05 m, 1.35 m
	5	2.56 m, 3.71 m	2.43 m, 3.55 m	2.55 m, 3.72 m	2.48 m, 3.55 m
iv	1	--	--	--	--
	2	4.48 m	4.65 m	4.48 m	4.65 m
	3	1.65, 1.90	3.07 dd, 3.38 dd	1.67, 1.88	3.07 dd, 3.38 dd
	4	1.64 m	--	1.64 m	--
	5	0.90 d	]	0.89 d	]
	6	0.95 d	7.10-7.30	0.96 d	7.10-7.30
	7		(5 H)		(5 H)
	NH	7.13 d	7.25	7.14 d	7.29
v	1	--	--	--	--
	2	4.30 dd	4.29 dd	4.30 m	4.27 m
	3	1.74 m	1.64 m, 1.78 m	1.74 m	1.64 m, 1.77 m
	4	1.62 m	1.65 m	1.65 m	1.69 m
	5	0.90 d	0.90 d	0.89 d	0.89 d
	6	0.97 d	0.96 d	0.94 d	0.94 d
	NH	7.55 d	7.45 d	7.57 d	7.45 d
vi	1	--	--	--	--
	2	4.34 m	4.40 dd	4.36 m	4.42 dd
	3	2.27 m	2.34 m	1.99 m	2.03 m
	4	0.91 d	0.92 d	1.14 m, 1.43 m	1.13 m, 1.45 m
	5	0.99 d	1.00 d	0.93 t	0.93 t
	6			0.96 d	0.97 d
	NH	6.73 d	6.72 d	6.77 d	6.77 d

Chemical shifts in ppm, from TMS as an internal standard.

charged on a silica gel column (Wako Gel C-200, 40 mm i.d.×160 mm) again and eluted stepwise with each 1 liter of mixture of hexane-EtOAc (1 : 1, 1 : 2, and 1 : 3). Several fractions mainly containing the phepropeptins (71 mg) were further purified by repeated HPLC (Capcell Pak UG120, 20 mm i.d.×250 mm, Shiseido) using 60% CH_3CN as the mobile phase to give 5.4 mg of **1**, 3.4 mg of **2**, 13.4 mg of **3**, and 24.7 mg of **4**.

Structural Analysis

The physico-chemical properties of the phepropeptins are shown in Table 1. The phepropeptins as white powders were soluble in MeOH, $CHCl_3$, or EtOAc, and insoluble in hexane and H_2O. Their IR absorption bands at 1641 cm^{-1} and 1514~1516 cm^{-1} suggested that the phepropeptins were peptide substances. The UV spectra of the phepropeptins suggested that the presence of a benzyl group (slight absorption around 258 nm) originated from the Phe moiety.

The ^{1}H- and ^{13}C-NMR data of the phepropeptins are shown in Tables 2 and 3, respectively. The results of the ^{1}H-^{1}H COSY and HMBC experiments of **4** are summarized in Fig. 2. The HMBC experiments of **4** indicated that it included a Pro-Phe-Leu-Ile-Leu residue and a Phe moiety in the molecule. Although no HMBC correlation was

Table 3. ^{13}C chemical shifts of **1**, **2**, **3** and **4** in $CDCl_3$.

Position	Carbon	1	2	3	4
i	1	173.1	173.3	173.1	173.2
	2	50.8	50.9	50.9	50.9
	3	39.8	39.8	40.0	40.0
	4	24.8	24.9	25.1	24.9
	5	22.3	22.6	22.3	22.6
	6	22.7	22.7	22.7	22.7
ii	1	171.0	170.9	171.2	171.0
	2	54.3	54.5	54.6	54.6
	3	36.7	36.7	36.7	36.7
	4	135.5	135.5	135.5	135.5
	5	129.3	129.3	129.2	129.3
	6	128.7	128.7	128.7	128.6
	7	127.4	127.4	127.4	127.4
iii	1	171.8	171.9	171.9	171.9
	2	60.9	60.6	60.9	60.6
	3	29.3	29.0	29.3	29.0
	4	24.2	23.4	24.3	23.4
	5	47.0	46.8	47.0	46.9
iv	1	173.9	173.0	173.9	173.0
	2	50.9	53.7	50.9	53.7
	3	38.3	35.1	38.4	35.2
	4	25.1	137.8	24.8	137.8
	5	21.5	129.1	21.4	129.1
	6	23.1	128.4	23.1	128.4
	7		126.6		126.6
v	1	171.7	171.4	171.7	171.4
	2	51.8	52.1	51.7	51.9
	3	37.5	37.7	37.5	37.7
	4	24.5	24.5	24.5	24.5
	5	22.4	22.4	22.2	22.3
	6	22.8	22.7	22.8	22.7
vi	1	171.8	172.0	171.7	171.7
	2	59.5	59.4	59.0	59.0
	3	29.2	29.3	36.0	36.0
	4	17.3	17.3	24.8	24.8
	5	19.5	19.5	11.7	11.7
	6			16.0	16.0

observed between the Phe group positioned at (ii) and other parts of the molecule, its molecular formula indicated that this Phe was connected to Leu at (i) and Pro at (iii) through peptide bonds. Then, **4** consisted of these six amino acids as a cyclic peptide. From the NMR analysis of other phepropeptins, all the phepropeptins are closely related cyclic peptides consisting of six amino acids. Therefore, all phepropeptins have Leu-Phe-Pro and another Leu moiety as their common structures with the differences shown by the changes in the other two amino acid moieties. We then determined the tentative structures of the phepropeptins as *cyclo*(-Leu-Phe-Pro-X-Leu-Y-) which have not been reported. The constituent amino acids were properly numbered in Roman numerals, described in the Fig. 2 inset, and used in Tables 2 and 3.

Next, we analyzed the configurations of the constituent amino acids of the phepropeptins. We decomposed **1** and **2**, which differ only in the amino acid next to Pro, and the Rf values of the amino acids separated from the hydrolysates of **1** and **2** are shown in Table 4. By conparison with the reference amino acids, it was revealed that they have L-Pro, D-Phe, L-Leu, D-Leu and L-Val as the consistent amino acids in their common structural parts. Whereas Phe from **2** was mixtures in both configurations, that from **1** was

Table 4. Rf values of amino acids from **1** and **2** on a chiral TLC.

Amino acid	L-configuration	D-configuration	from **1**	from **2**
Pro	0.56	0.46	0.55	0.55
Val	0.69	0.56	0.69	0.69
Leu	0.69	0.57	0.57, 0.69	0.57, 0.69
Phe	0.69	0.53	0.53	0.53, 0.69

Rf value(s) were determined by a nynhidrin-colormetric reaction on a chiral TLC (HPLC-Fertigplatten CHIR, Art.14101, Merck) with a solvent system (MeOH-CH_3CN-H_2O, 1:4:1 in volume).

determined to be D-Phe. The common Phe moiety in the phepropeptins was then considered to have a D-configuration, and it was suggested that the amino acids next to Pro should have the L-conformations in their structures.

We then synthesized two possible stereoisomers of phepropeptin B for comparison with the natural compound. Two synthetic compounds related to **2** showed different properties (MP, optical rotations, NMR signals, Rt on the HPLC and inhibitory activities to proteasome). Actually, the IC_{50} values of the synthesized *cyclo*(-D-Leu-D-Phe-L-Pro-L-Phe-L-Leu-L-Val-) and *cyclo*(-L-Leu-D-Phe-L-Pro-L-Phe-D-Leu-L-Val-) toward the ChT-L activities were 18.0 μg/ml and 11.0 μg/ml, respectively. These observations of *cyclo*(-L-Leu-D-Phe-L-Pro-L-Phe-D-Leu-L-Val-) were identical to the properties of natural **2**. Hence, it has revealed that the absolute structure of **2** is *cyclo*(-L-Leu-D-Phe-L-Pro-L-Phe-D-Leu-L-Val-). Based on these results, we determined that the absolute structures of the phepropeptins are *cyclo*(-L-Leu-D-Phe-L-Pro-L-X-D-Leu-L-Y-), where X is Leu for **1** and **3**, Phe for **2** and **4**, whereas Y is Val for **1** and **2**, Ile for **3** and **4**.

Inhibition of Proteasome

The inhibitory activities to proteasome by the phepropeptins are shown in Table 5 using IC_{50} values. All the phepropeptins showed no inhibitions toward proteasomal T-L and α-chymotrypsin from bovine pancreas (Sigma C-4129), up to a concentration of 100 μg/ml. The proteasomal PGPH activity was slightly inhibited by the phepropeptins but the IC_{50} values were over 100 μg/ml. (data not shown)

Compared with other proteasome inhibitors like MG-132 or lactacystin, the phepropeptins have weaker inhibitory effects on the proteasomal ChT-L activity. However the phepropeptins differ from all known proteasome inhibitors which have been shown to bind to the β-subunits of proteasome, in that, as small, stable, cyclic peptides, the phepropeptins are unlikely to react with the enzymes.

Table 5. Inhibitory activities to proteasome.

Compound	IC_{50} value (μg/ml)
1	21.0
2	11.0
3	12.5
4	7.8
MG-132	0.05
Lactacystin	1.4

Given that the proteasomes are believed, *via* their multi-catalytic activities, to regulate some important, but undefined, cellular activities, the phepropeptins may contribute to the development of new therapeutic or biochemical tools.

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