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Cycloleonuripeptide D, A New Proline-Rich Cyclic Decapeptide from *Leonurus heterophyllus* [1)]

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Abstract : A new proline-rich cyclic decapeptide, cycloleonuripeptide D (**1**), *cyclo* (-Ser-Pro-Pro-Pro-Tyr-Phe-Gln-Thr-Pro-Ile-), was isolated from the fruits of *Leonurus heterophyllus* and the structure was elucidated by extensive 2D NMR, chemical and enzymatic degradation studies, and tandem MS method. The solid state conformation of cycloleonuripeptide D was clarified by X-ray diffraction study. The cyclic decapeptide backbone of **1** contained two β-turns, one type I β-turn at Pro-Ile and one type III β-turn at Pro-Tyr. A transannular 4 → 1 backbone hydrogen bond between Ser-NH and Thr-CO, and a 5 → 1 hydrogen bond between Phe-NH and Pro-CO encompassing Pro-Pro-Tyr, in which the peptide linkage between the two proline residues was shown to be in the *cis* conformation, were observed.

Recently a number of naturally occurring cyclic peptides with unique structures and interesting biological activities have been isolated from various sources, most of recently isolated ones being from microorganism and marine invertebrates. As part of our ongoing investigation of bioactive cyclic peptides from higher plants, we recently isolated three new cyclic nonapeptides, cycloleonuripeptides A - C, showing cytotoxic activities, from the fruits of *Leonurus heterophyllus* (Labiatae),[2)] which have been used as a Chinese drug for invigorating blood circulation, regulating menstrual disturbance and to dispel edema,[3)] and determined their structures and conformations.[2)] By further fractionation of the fruit extract, we obtained a new proline-rich cyclic decapeptide, having a cyclooxygenase inhibitory activity and named it cycloleonuripeptide D. In this paper, we describe the isolation and structure elucidation of this new cycloleonuripeptide D (**1**) by using extensive 2D

NMR, chemical and enzymatic degradation studies and tandem MS method. The solid state conformation of cycloleonuripeptide D with four proline residues containing three successive prolines, analyzed by X-ray diffraction study, was also described.

Results and Discussion

Structure determination of cycloleonuripeptide D (1)

The methanolic extract of the fruits of *L. heterophyllus* (Labiatae) was partitioned between *n*-BuOH and H_2O. The *n*-BuOH soluble material was subjected to Diaion HP-20 column (H_2O - MeOH) and 80% MeOH eluted fraction was chromatographed on a silica gel column, followed by MPLC and HPLC on ODS to yield a peptidic compound, named cycloleonuripeptide D (**1**: 0.002 %), together with a known cyclic peptide, cycloleonurinin.[4)]

Table 1. ^1H and ^{13}C NMR signal assignments of cycloleonuripeptide D (**1**) in DMSO-d_6

assignment		^1H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C
Ser[1]			
	α	4.33 (1H, m)	54.55
	β	3.55 (1H, m)	60.68
		3.76 (1H, m)	
	OH	5.81 (1H, br s)	
	NH	7.12 (1H, d, 8.1)	
	C=O		166.85[a)]
Pro[2]			
	α	4.39 (1H, m)	58.38
	β	1.69 (1H, m)	27.78
		2.25 (1H, m)	
	γ	1.86 (1H, m)	24.87
		2.01 (1H, m)	
	δ	3.57 (1H, m)	46.88
		3.81 (1H, m)	
	C=O		169.91[a)]
Pro[3]			
	α	4.73 (1H, br d)	56.79
	β	1.91 (1H, m)	27.42
		2.06 (1H, m)	
	γ	1.99 (1H, m)	24.15
		2.14 (1H, m)	
	δ	3.63 (1H, m)	46.57
		3.73 (1H, m)	
	C=O		170.19[a)]
Pro[4]			
	α	4.41 (1H, m)	60.04
	β	1.83 (1H, m)	30.16
		2.20 (1H, m)	
	γ	0.92 (1H, m)	21.18
		1.63 (1H, m)	
	δ	2.91 (1H, m)	45.71
		3.27 (1H, m)	
	C=O		170.23[a)]
Tyr[5]			
	α	4.24 (1H, m)	55.89[b)]
	β	3.05 (2H, m)	35.17
	γ		128.82
	δ	7.27 (2H, m)	130.04
	ε	6.65 (2H, d, 8.4)	114.66
	ζ		155.58
	OH	9.13 (1H, br s)	
	NH	8.54 (1H, d, 7.8)	
	C=O		170.30[a)]
Phe[6]			
	α	4.59 (1H, m)	53.44
	β	2.89 (1H, dd, 5.9, 13.7)	35.40
		2.99 (1H, m)	
	γ		137.28
	δ	7.25 (2H, m)	127.85
	ε	7.13 (2H, m)	129.90
	ζ	7.24 (1H, m)	126.00
	NH	7.97 (1H, d, 8.2)	
	C=O		170.39[a)]
Gln[7]			
	α	3.89 (1H, m)	55.27
	β	1.88 (1H, m)	27.19
	γ	2.18 (1H, m)	31.42
	δ		173.61
	NH_2	12.08 (2H, br s)	
	NH	7.35 (1H, d, 5.1)	
	C=O		170.46[a)]
Thr[8]			
	α	4.85 (1H, dd, 3.3, 8.3)	55.42
	β	4.31 (1H, m)	66.94
	γ	1.04 (3H, d, 5.8)	19.04
	OH	4.78 (1H, d, 7.7)	
	NH	7.66 (1H, d, 8.3)	
	C=O		170.56[a)]
Pro[9]			
	α	4.29 (1H, m)	61.34
	β	1.77 (1H, m)	29.63
		2.35 (1H, m)	
	γ	1.90 (2H, m)	24.87
	δ	3.71 (1H, m)	47.72
		3.88 (1H, m)	
	C=O		170.62[a)]
Ile[10]			
	α	4.36 (1H, m)	55.97[b)]
	β	2.04 (1H, m)	35.07
	γ	0.93 (1H, m)	23.55
		1.24 (1H, m)	
	γMe	0.79 (3H, d, 6.6)	15.72
	δ	0.78 (3H, t, 7.0)	11.82
	NH	7.03 (1H, d, 9.7)	
	C=O		171.28[a)]

a,b) Assignment may be interchanged.

Cycloleonuripeptide D (**1**), colorless needles, mp. 200 - 202 °C, $[\alpha]_D$ -99.0° (c 0.21, MeOH), showed a molecular formula, $C_{56}H_{77}N_{11}O_{14}$, which was permitted by HR FAB-MS spectrum, indicating the presence of 24 degrees of unsaturation in the molecule. Amino acid analysis of **1** showed it consisted of Pro × 4, Ser, Thr, Glu, Ile, Phe and Tyr, all of which were proved to be L-amino acids by Marfey's derivatization,[5] followed by HPLC analysis. Methylation of **1** with trimethylsilyldiazomethane[6] gave **2** with one methyl ether attached to an aromatic residue (Tyr) (^{1}H: δ 3.61, 3H, s; ^{13}C: 55.11, q). Therefore, **1** contains a Gln residue and not Glu. In the NMR spectra, eight amide proton signals corresponding to the above six amino acids except for four prolines and eleven amide carbonyl signals corresponding to the above ten amino acids were observed. The four proline-containing cyclic decapeptide structure satisfies the 24 degrees of unsaturation, implied by the molecular formula.

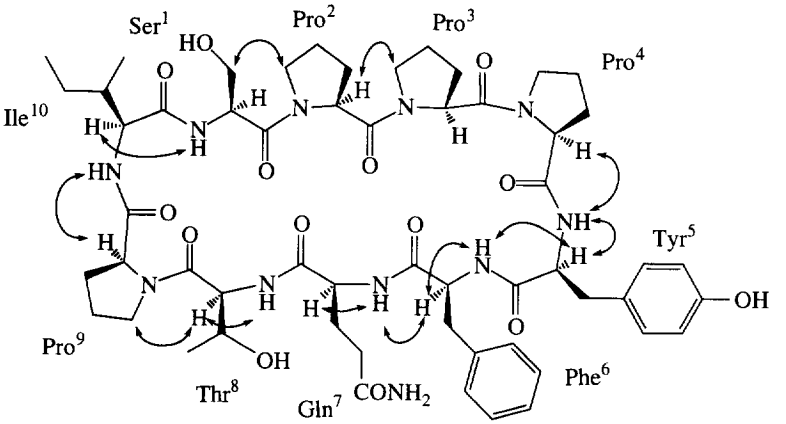

Fig. 1 Structure of cycloleonuripeptide D (**1**), The arrows show NOE relationship in a phase sensitive NOESY spectrum. Ser was provisionally numbered as the first amino acid.

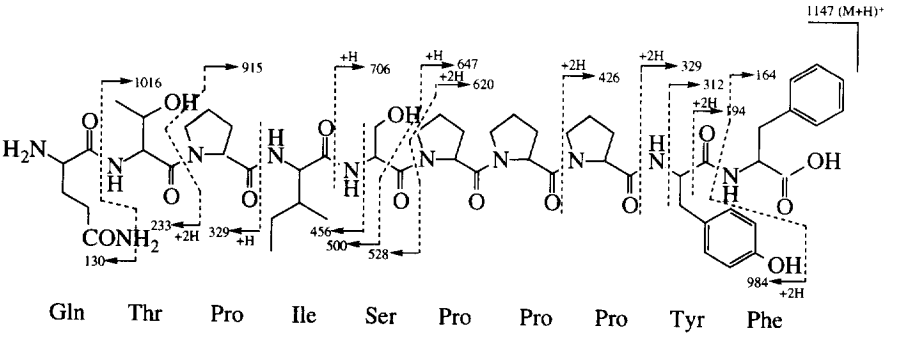

Fig. 2 ESI MS/MS fragmentations of **3**

Since considerable overlapped signals, the NMR spectra were measured both in DMSO-d_6 and pyridine-d_5. Extensive 2D NMR analysis, including DQF-COSY, HOHAHA[7] and HMQC spectra, was used to assign the ^1H and ^{13}C signals of the ten amino acids. The NMR signals in DMSO-d_6 are summarized in Table 1. As the chemical shifts of ten amide carbonyl carbon resonances were very close to each other, the sequential resonance assignment of **1** was established on the basis of the data from both NOE and tandem MS experiments as follows.

The NOE enhancements were observed between Thr^8-Hα and one of Pro^9-Hδ, and between Pro^9-Hα and Ile^{10}-NH in a phase sensitive NOESY spectrum. The NOEs were also observed between Ile^{10}-Hα and Ser^1-NH, between Ser^1-Hβ and Pro^2-Hδ, and between Pro^2-Hα and Pro^3-Hδ, implying the partial amino acid sequence of Thr-Pro-Ile-Ser-Pro-Pro. Other NOEs, suggesting the amino acid sequence of Pro-Tyr-Phe-Gln, were also observed as shown in Fig. 1. Further amino acid sequence analysis was conducted by using electrospray ionization (ESI)[8] tandem MS/MS experiments used as a tool for sequence determination of peptides.[9] The ESI MS spectrum of **1** in 50% MeOH produced rather stable ions such as $(M+Na)^+$ and $(M+K)^+$ only. A peptide fragment (**3**) generated by digestion of **1** with α-chymotrypsin produced its $(M+H)^+$ ion, which was then analyzed in a second mass spectrometer to be Gln-Thr-Pro-Ile-Ser-Pro-Pro-Pro-Tyr-Phe (Fig. 2). Therefore, the structure of **1** was unequivocally established to be a new cyclic decapeptide, representing *cyclo* (-Ser-Pro-Pro-Pro-Tyr-Phe-Gln-Thr-Pro-Ile-).

Solid state molecular structure of cycloleonuripeptide D (1)

The conformation of cycloleonuripeptide D is interesting, as it contained three successive prolines in the structure. The cyclic decapeptide backbone of **1** is considered to be quite flexible, and to find the conformation of cyclic peptides, it is important to explore the structural role of hydrogen bonding for which X-ray data are available. Therefore, the conformational analysis in the solid state was conducted by X-ray diffraction study.

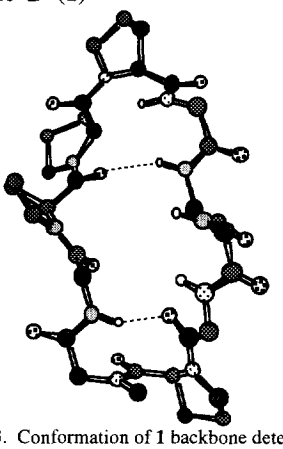

Fig. 3. Conformation of **1** backbone determined by X-ray analysis. Side chain atoms have been omitted and only amide hydrogens are included. Broken lines indicate hydrogen bonds.

Cycloleonuripeptide D was crystallized from a CH_3CN - MeOH mixture in orthorhombic crystals of space group $P2_12_12_1$. The determination of the lattice constants and intensity data collection were carried out on a Nonius CAD4 automated diffractometer. Because the crystals deteriorated rapidly upon contact with air, they were sealed in thin-walled glass capillaries in contact with the mother liquor. Figure 3 shows the backbone structure of **1** with intramolecular hydrogen bonds, as illustrated by the broken lines. The stereoscopic views (Fig. 4) show that the phenyl ring in Phe^6 is inside the peptide backbone structure. The peptide bonds of

this cyclic decapeptide, consisting entirely of L-amino acid residues, assume generally the common planar *trans* conformation except for one *cis* proline amide bond at residue 4.

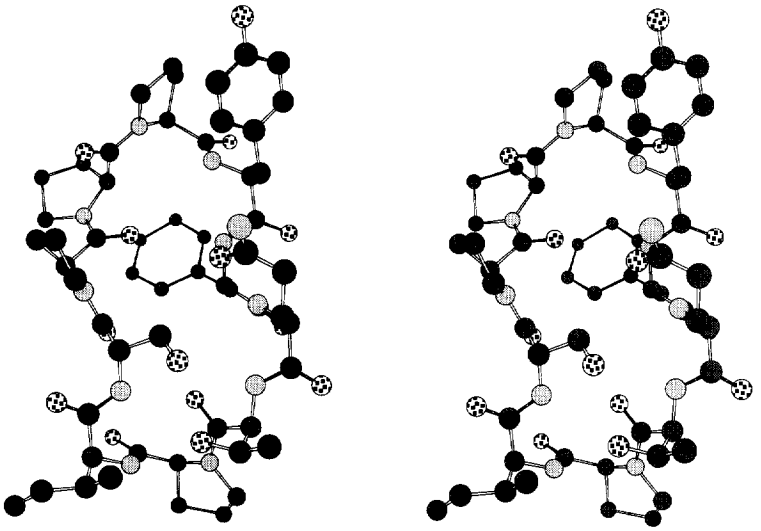

Fig. 4. Stereoscopic view of the X-ray structure of **1**; Hydrogen atoms are omitted.

Range of backbone bond distances (N_i-C_i^{α} 1.434-1.507, C_i^{α}-C_i 1.514-1.555, C_i-O_i 1.207-1.244, and C_i-N_{i+1} 1.337-1.365Å) and angles (C_{i-1}-N_i-C_i^{α} 118.1-124.3°, N_i-C_i^{α}-C_i, 105.2-114.0°, C_i^{α}-C_i-N_{i+1}, 113.8-118.8°, C_i^{α}-C_i-O_i 119.0-123.5°, and O_i-C_i-N_{i+1} 119.1-124.5°), are within the corresponding acceptable ranges reported for cyclic peptides and suggest that the crystal conformation does not have unusual strain.[10]

Table 2 shows the backbone dihedral angles in **1**, and Figure 5 shows the ϕ and ψ-angles along the main chain of **1** in the Ramachandran plot. The (ϕ, ψ)-values of all residues lie within the allowed region of the Ramachandran plot. The (ϕ, ψ)-value of Phe^6 shows that it is in the left-handed α-helix region, thus producing a small α-helix-like stretch, whereas those of the other residues fall generally in the β-sheet region in the diagram. In addition, individual ω angles of four Pro residues deviate by -17° to +11° from the ideal value and the average in angles is 180°. Of the four proline residues, according to the torsion angles (ψ), the residues Pro^2 and Pro^3 are of collagen type with large positive ψ angles, and the residues Pro^4 and Pro^9 are of the α-helix type with small negative ψ angles. The torsion angles (θ, χ^1, χ^2, χ^3 and χ^4) around the four proline rings, summarized in Table 3, indicate that the prolyl conformations at residues Pro^2, Pro^3 and Pro^4 are of C_2-C^{γ}-endo form and that at residue Pro^9, C_2-C^{γ}-exo form.[11]

Side chain conformations mostly take the favorable orientation of g^+, g^-, t (χ=60°, -60°, 180°, respectively), whereas the torsion angles, $\chi^{2,1}$ of Gln^7 (94.3°) significantly deviate from the ideal values. The conformational deviation of the side chain of Gln^7 from its more stable conformation is probably due to the hydrogen bonding between the side chain carbonyl oxygen of Gln^7 and the backbone amide hydrogen of Gln^7.

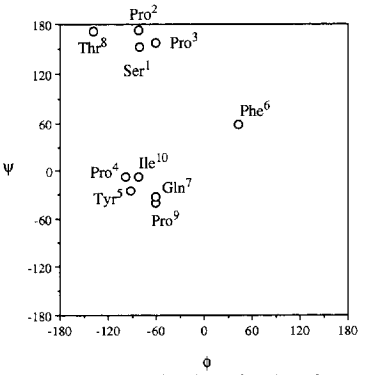

Fig. 5 Ramachandran plot (ϕ,ψ plot) of the crystal structure of **1**

Table 2 Torsion angles (°) along the peptide backbone of **1**

residue	ϕ	ψ	ω
Ser^1	-81	152	-177
Pro^2	-82	173	163
Pro^3	-61	157	-170
Pro^4	-99	-7	-169
Tyr^5	-93	-25	179
Phe^6	42	58	-179
Gln^7	-61	-39	174
Thr^8	-139	171	-179
Pro^9	-61	-32	-178
Ile^{10}	-82	-7	178

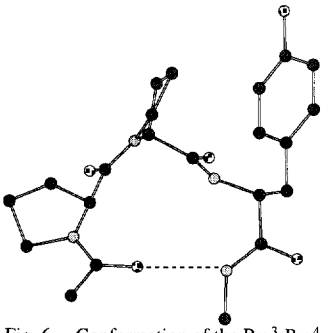

Fig. 6 Conformation of the Pro^3-Pro^4-Tyr^5 sequence constructed by a 5→ 1 type hydrogen bond, Broken line refers to the internal NH - C=O hydrogen bond (NH-O 2.326 Å).

Table 3 Conformation of the four proline rings in **1**

Residue	Conformation	Torsion angles (°)				
		θ	$\chi1$	$\chi2$	$\chi3$	$\chi4$
Pro^2	C_2-Cγ-endo	-11.3	26.7	-33.2	26.3	-8.2
Pro^3	C_2-Cγ-endo	-6.1	25.1	-35.4	30.6	-15.1
Pro^4	C_2-Cγ-endo	-20.4	33.1	-35.8	23.2	-1.9
Pro^9	C_2-Cγ-exo	14.2	-33.2	40.9	-32.3	11.5

Table 4 Hydrogen bonds and intramolecular short contacts of **1**

From	To	Distance (Å)
Ser^1-NH	Thr^8-CO	2.025
Ser^1-NH	Thr^8-O*	2.855
Ile^{10}-NH	Thr^8-O*	2.817
Thr^8-NH	Ser^1-O*	2.478
Ser^1-OH	Gln^7-CO*	3.270
Gln^7-NH	Gln^7-CO*	1.873
Phe^6-NH	Pro^2-CO	2.326
Tyr^5-NH	Pro^2-CO	3.150

* side chain of Ser^1, Gln^7, and Thr^8.

Intramolecular hydrogen bonds and β-turn structure

Cyclic peptides are constrained as they contain turns in the backbones, which have been implicated in the bioactivity of several of these naturally occurring peptides, and these turns are often stabilized by intramolecular hydrogen bonds, which are also crucial for structural determinants of the cyclic peptides and for establishing the molecular dimensions and conformational angles of such turns. In the crystal structure of **1**, the protons of the four NH groups in Ser^1, Phe^6, Gln^7 and Thr^8 are located inward of the ring and a significant intramolecular NH···O hydrogen bonding contact of the type 4 → 1 between Ser^1-NH and Thr^8-CO exists [N---O of 2.956(8) Å and N-H---O of 152.1°]. This hydrogen bond makes a 10-membered ring inside the covalent 30-membered ring. Accordingly, the end of the molecule is constrained by β-turns formed by the residues Pro^9 → Ile^{10} and by the residues Pro^4 → Tyr^5. The Pro^9 → Ile^{10} turn is denoted as type I β-turn formed with the intramolecular hydrogen bond between Ser^1-HN and Thr^8-CO, supported by the NOE data described above. While the Pro^4 → Tyr^5 turn formed the type III β-turn without transannular hydrogen bonds.

Other hydrogen bonds and short intramolecular distances in the crystal are summarized in Table 4. There is a weak intramolecular hydrogen bond between Phe^6-NH and Pro^2-CO of a type 5 → 1, which is very rare and only found in [Phe^4, Val^6]antamanide.[12] The detailed conformation around this 5 → 1 hydrogen bond is shown in Fig. 6 and the conformational angles around three related residues, Pro^3-Pro^4-Tyr^5, are almost the same as those in [Phe^4, Val^6] antamanide.

The conformation of **1** revealed by the present study appears to be an intrinsic property of the molecule. The geometry of one Pro amide bond was found to be *cis* in solid state. One of the proline amide bonds in solution (Pro^4) was also shown to be *cis* by the ^{13}C chemical shifts (δ 30.16 and 21.18) of β and γ positions in Pro^4 residue.[13]

Cycloleonuripeptide D showed potent cyclooxygenase inhibitory activity (72.6 % inhibition at 100 μM). We are also interesting in the solution conformation of cycloleonuripeptide D and the biological activities relationship, though the analysis could not be tried because of the ^1H signal overlapping in NMR spectra. Studies on the further conformational analyses and biological evaluations of cycloleonuripeptides D are currently being done in our laboratories.

Experimental

M.p.s were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer and ESI MS/MS spectrum with Finnigan MAT TSQ-700 spectrometer. IR spectrum was recorded on a JASCO A-302 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20mm i.d. ×250mm and 30mm i.d. ×250mm, GL Science Inc.) packed with 10μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent. Proton and carbon NMR

spectra were recorded on Bruker spectrometers (AM500) and processed on a Bruker data station with an Aspect 3000 computer. 15 mg of cycloleonuripeptide D in a 5mm tube (0.5ml pyridine-d_5 or DMSO-d_6, degassed) was used for the homonuclear and heteronuclear 2D NMR measurements. The spectra were recorded at 303K. A phase sensitive NOESY experiment was performed with mixing times of 0.6 sec. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Extraction and Isolation

After the fruits of *Leonurus heterophyllus* (10 kg) were defatted with n-hexane two times, they were extracted with hot 70% methanol two times to give a methanol extract (ca. 500 g) which was treated with *n*-butanol and water. The *n*-butanol soluble fraction (ca. 250 g) was subjected to Diaion HP-20 column chromatography using a water - methanol gradient system (1:0 - 0:1). The fractions (47 g) eluted by 80% methanol were further subjected to silica gel column chromatography using a methylene chloride - methanol gradient system (1:0 - 0:1). The fraction eluted by 15% methanol was subjected to ODS MPLC with 65 % MeOH solvent system, and ODS HPLC with 30 % CH_3CN solvent system to give cycloleonuripeptide D (0.002%) as colorless needles.

Cycloleonuripeptide D (1). - Colorless needles, mp. 200-202°C, $[\alpha]_D$ -99.0° (c 0.21, MeOH); *m/z* 1129 (Found: $(M+H)^+$, 1128.5684. $C_{56}H_{78}N_{11}O_{14}$ requires, 1128.5729); ν_{max} (KBr)/cm^{-1} 3400 (NH and OH), 2980, 1670 (amide C=O), 1630, 1520 and 1440; λ_{max} (MeOH) / nm 276 (ε 1730).

Acid Hydrolysis of **1**

Solution of **1** (1 mg) in 6N HCl was heated at 110°C for 24h in a sealed tube. After cooling, the solution was concentrated to dryness. The hydrolysate was dissolved in 0.02N HCl and applied to the analysis by an amino acid analyzer (Hitachi L-8500 Amino Acid Analyzer).

Absolute Configuration of Amino Acids[5)]

Solution of **1** (1 mg) in 6N HCl was heated at 110°C for 12h in a sealed tube. After being cooled, the solution was concentrated to dryness. The residue was dissolved in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35°C for 1h. After being cooled, 2M HCl was added and then concentrated to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10μm), Merck), flow rate 1 ml/min, detection 340nm, solvent : 10 - 50% CH_3CN / 50mM triethylamine phosphate buffer. The t_R values were L-Ser 20.08; L-Thr 21.37; L-Pro 27.47; L-Tyr 30.95; L-Ile 39.83; L-Phe 40.18; L-Glu 23.52 min.

Methylation of **1** by $TMSCHN_2$

Trimethylsilyldiazomethane (2.0 M hexane solution, 67 μl) was added to a stirred solution of **1** (10 mg) and N,N-diisopropylethylamine (23.5 μl) in methanol-acetonitrile (1:9, 2.0 ml) at room temperature. The mixture was stirred for 48 h at room temperature, and concentrated in vacuo. The

residue was worked up in the usual extractive manner and then subjected to ODS-HPLC to give **2** (methyl ether of **1**, 8.0 mg) as colorless powder; m/z 1098 $(M\text{-}CONH_2)^+$; ^{1}H-NMR (pyridine-d_5) 0.86 (3H, t, J=7.0 Hz), 0.87 (3H, d, J=7.0 Hz), 1.07 (3H, d, J=6.7 Hz), 3.61 (3H, s); ^{13}C-NMR (pyridine-d_5) 10.71 (q), 14.18 (q), 16.37 (q), 22.00 (t), 23.20 (t), 24.54 (t), 24.91 (t), 25.31 (t), 25.99 (t), 26.81 (t), 27.67 (t), 28.17 (t), 29.16 (t), 30.42 (t), 34.42 (t), 35.40 (d), 36.77 (t), 46.57 (t), 46.68 (t), 47.24 (t), 47.36 (t), 53.59 (d), 55.11 (q), 56.32 (d), 57.06 (d), 58.77 (d), 59.28 (d), 59.57 (d), 61.03 (d), 62.24 (t), 68.20 (d), 114.10 (d), 126.80 (d), 128.77 (d), 129.30 (d), 130.06 (d), 131.08 (d), 131.49 (d), 138.52 (s), 158.63 (s), 167.92 (s), 170.10 (s), 170.61 (s), 171.06 (s), 171.22 (s), 171.92 (s × 2), 171.97 (s), 172.09 (s), 172.75 (s).

Enzymatic Hydrolysis of **1**

α-Chymotrypsin (250 μg dissolved in 25 μl of 0.001% HCl, Wako Pure Chemical Industries) was added to **1** (1.0 mg) in NH_4HCO_3 solution (1%, 0.45 ml) and the mixture was incubated at 35 °C with the pH maintained at 8.0 by the manual addition of 0.1N HCl. After 24 h, the reaction was stopped by adjusting the solution to pH 2.2 with 1N HCl. The digestion mixture was lyophilized to dryness and hydrolysates were subjected to HPLC (Inertsil PREP-ODS column, 20mm i.d. ×250mm, GL Science Inc., packed with 10μm ODS, eluted with 20% CH_3CN / 0.05%TFA) to give compounds **3** (0.6 mg) as amorphous powder.

Crystallographic analysis of compound **1**

Crystal data: $C_{56}H_{77}N_{11}O_{14}\cdot 6H_2O$, orthorhombic, space group $P2_12_12_1$, Z=4, a=16.2815, b=24.6967, c=15.5430 Å, V=6249.8 Å^3. A colorless prismatic crystal of approximately 0.58 × 0.33 × 0.20 mm in length was sealed in a thin walled glass capillary and was mounted on a Nonius CAD4 diffractometer with graphite-monochromated CuKα radiation (μ=22.4 cm^{-1}) at 23°C. A total of 6722 reflections were observed. The structure was determined by the direct method using the SHELXS-86 program[14] and the refinement was carried out by the block-diagonal-matrix least-squared method. The final R value was 0.097. The molecular structure determined by this method is illustrated in Fig. 4.

Assay for Cyclooxygenase Inhibitors

This assay was performed by the use of cyclooxygenase + PGH_2/PGE_2 isomerase kit (Eldan Tec. Co. Ltd., Israel), briefly, 2 ml of samples in various concentrations and indometacin (1×10^{-4} M) solutions, and 10 ml of cofactors' solution (includes epinephrine, tryptophan, hydroquinone and GSH) were added to 100 ml of sheep vesicular gland microsomes solutions (0.2 mg/ml) which were dissolved in 50 mM Tris-HCl buffer solution. Their mixture solutions were pre-incubated with shaking for 3 min at 25 °C. After pre-incubation, 2 ml of arachidonic acid solution (1 mg/ml) was added to above enzymatic solutions and incubated continually for further 3 min. At the end of reaction was added 10 ml of $FeCl_3$ solution (25 mM) to the reaction mixtures. After completion of the reactions, they were centrifuged at 3000 × g at 4 °C for 10 min. The contents of PGE_2 in the

supernatant solutions were determined by using the prostaglandin E_2 enzyme immunoassay kit (Cayman chemical company, USA).

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