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Hibispeptin B, a Novel Cyclic Peptide from *Hibiscus syriacus*

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Abstract : A new cyclic peptide, designated as hibispeptin B, has been isolated from the root bark of *Hibiscus syriacus*. Hibispeptin B has a unique amino acid unit assigned as 2-amino-3-(2-hydroxy-5-aminoacetylbenzyl)pentanoic acid (Ahabpa) in cyclic core. Its structure was established as *cyclo*[-Ahabpa(-pyro-Glu)-Pro-Leu-Leu-] on the basis of various spectroscopic analyses. Configurations of all normal amino acids were determined as L by chiral-TLC analysis.

Introduction

Many cyclic peptides with unique structures have been isolated from microbial and marine origins. They also exhibit various biological activities such as antimicrobial, antitumor, immunosuppresive, enzyme inhibitory, receptor antagonistic and cardiotonic effects and some of them are used clinically. Despite of their importance, only a few compounds including lyciumins[1a], citrusins[1b], curcacycline A[1c], cleromyrine I[1d], yunnanins[1e], astins[1f], segetalins[1g], dichotomins[1h] and pseudostellarins[1i] were isolated from higher plants. We herein deal with a peptide with unusual amino acid unit in cyclic isolated from a higher plant.

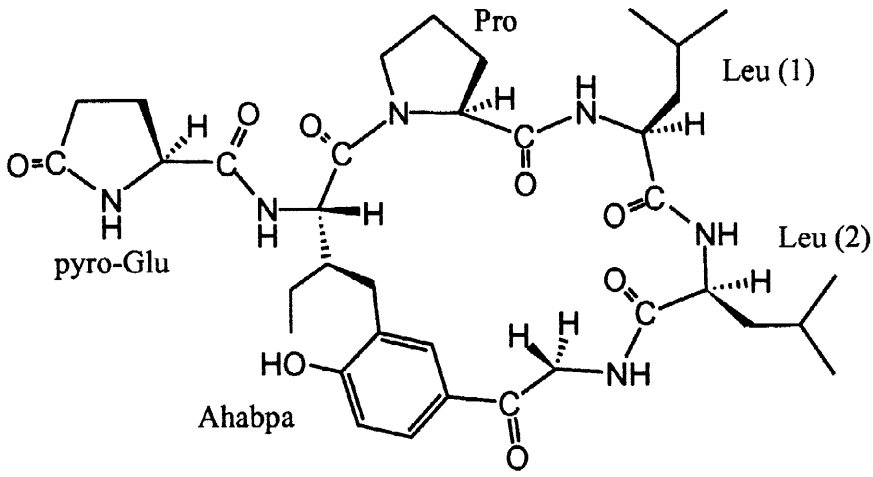

Fig. 1. The structure of hibispeptin B.

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In the course of screening for biologically active novel constituents from higher plants using as the traditional Chinese medicines[2], we have isolated a unique cyclic peptide, named hibispeptin B, from the root bark of *Hibiscus syriacus* Linne (Malvaceae), which has been used as antipyretic, anthelmintic and antifungal agents in the Orient[3]. Previously some flavonoids, fatty acids, naphethalenes, polyphenols and hibispeptin A have been isolated from *H. syriacus*[4]. In this paper, we describe the isolation and structural elucidation of hibispeptin B.

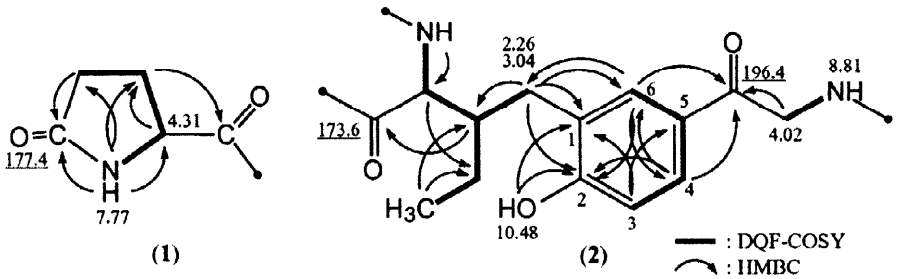
Results and discussion

A methanolic extract of the dried root bark of *H. syriacus* was washed with hexane and then partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction was chromatographed on silica gel and Sephadex LH-20 columns followed by preparative RP-TLC developed with 65% aq. MeOH to give hibispeptin B (6 mg).

Hibispeptin B was obtained as white powder and its molecular formula was established as $C_{36}H_{52}N_6O_8$ by high resolution FAB mass measurement (glycerol/PEG, *m/z* 697.3940 $(M+H)^+$ +1.6mmu, *m/z* 719.3740 $(M+Na)^+$ -0.5mmu). The IR absorptions near 3420, 1690, 1675-1640 and 1540 cm^{-1} attributed to the hydroxyl, α,β-unsaturated or aryl group attached carbonyl, amide carbonyl and amide NH groups, respectively, indicated that this compound has peptidic character. That was also supported by ninhydrin reagent showing that its hydrolysate (6N HCl, 110°C, 24 hours) was positive. The UV absorptions at 272 and 360 nm in MeOH suggested the presence of aromatic functions in hibispeptin B. The ^1H NMR spectrum in DMSO-d_6 showed seven α protons between δ 3.65 to δ 4.35, three aromatic methine protons at δ 7.41, 7.38 and 6.74 and six exchangeable protons at δ 10.48, 8.81, 8.48, 7.77, 7.69, 6.90, which were collapsed on shaking with D_2O. Also several methylene protons observed between δ 2.40 to δ 1.20 together with five methyl protons indicated that hibispeptin B was composed of nonpolar amino acids. The ^{13}C NMR spectrum suggested the presence of an α,β-unsaturated carbonyl (δ 196.4), six amide carbonyls (δ 177.4, 174.7, 173.6, 171.8, 171.6, 170.2), an oxygenated sp^2 quaternary (δ 160.0), three sp^2 methine (δ 132.8, 128.7, 114.1), two sp^2 quaternary (δ 126.9, 124.9), α carbons between δ 60 to δ 50 and sp^3 carbons between δ 10 to δ 50, as shown in Table 1.

Component amino acids. A DEPT experiment established the multiplicities of the carbon resonances while the HMQC[5] data assigned all of the proton-bearing carbons. The DQF-COSY[6] data revealed the presence of seven partial structures as shown in Fig. 2. These partial structures in combination with HMQC data assumed to be one mol each of the glutamine (Gln) or pyro-glutamic acid (pyro-Glu), glycine and proline and two mol of leucine (Leu (1) and Leu (2)), together with 1,2,5-trisubstituted benzene and an unusual amino acid. Of these partial units, pyro-glutamic acid (**1**), but not glutamine, was assigned by the long-range correlations from the amide proton at δ 7.77 of pyro-Glu to the carbonyl carbon at δ 177.4 that was long-range coupled with γ protons of pyro-Glu at δ 2.06 and 2.14, implying the presence of five membered cyclic amide in component amino acids. The structure of unusual amino acid with isoleucine moiety was also elucidated by DQF-COSY and HMBC data as shown in (**2**). The methylene protons at δ 2.26 and 3.04 of unusual amino acid showed the long-range correlations to three sp^2 carbons at δ 124.9 (C-1), 160.0 (C-2) and 132.8 (C-6) of 2-hydroxyphenyl, and H-4 and H-6 of hydroxyphenyl were correlated to the carbonyl carbon at δ 196.2, which was long-range coupled with α methylene protons at δ 4.02 for glycine

moiety. Therefore, it was determined to be 2-amino-3-(2-hydroxy-5-aminoacetylbenzyl)pentanoic acid (Ahabpa) connected phenyl and glycine through C-C bond.



^{13}C chemical shift values were underlined

Sequence of amino acids. From the above results, we found that hibispeptin B was composed of pyro-Glu, Pro, Leu (1), Leu (2) and Ahabpa. The chemical connectivities of these partial structures were further established by the HMBC experiment[7], as shown in Fig. 2. It showed long-range correlation(s) from amide and/or α proton(s) of each amino acid to carbonyl carbon of neighboring amino acid. Namely, the long-range couplings from α and β protons of Pro at δ 4.16 and 2.25, respectively, to a carbonyl carbon at δ 170.2, which was correlated with amide proton of Leu (1) at δ 7.69, revealed the connection of Pro and Leu (1). Leu (1) and Leu (2) were connected by long-range correlations from α proton of Leu (1) at δ 4.06 and amide proton of Leu (2) at δ 6.90 to a carbonyl carbon at δ 171.6. Also the correlations from methylene (δ 4.02) and NH (δ 8.81) protons of Gly moiety and α proton of Leu (2) to a carbonyl carbon at δ 171.8 connected Leu (2) to Ahabpa, and the correlations from β methylene protons (δ 2.33, 1.90) of pyro-Glu and amide proton (δ 8.48) of Ahabpa to carbonyl carbon at δ 174.7 connected Ahabpa to pyro-Glu. Thus the sequencing of the amino acids for hibispeptin B was determined as Pro-Leu (1)-Leu (2)-Ahabpa-pyro-Glu. The remaining carbonyl carbon of Ahbpa at δ 173.6 should be connected to nitrogen of Pro by the process of elimination. Therefore, the structure of hibispeptin B was assigned as a unique cyclic peptide composed of five amino acids, as shown in Fig. 1. The ^{1}H and ^{13}C NMR spectral data are summarized in Table 1.

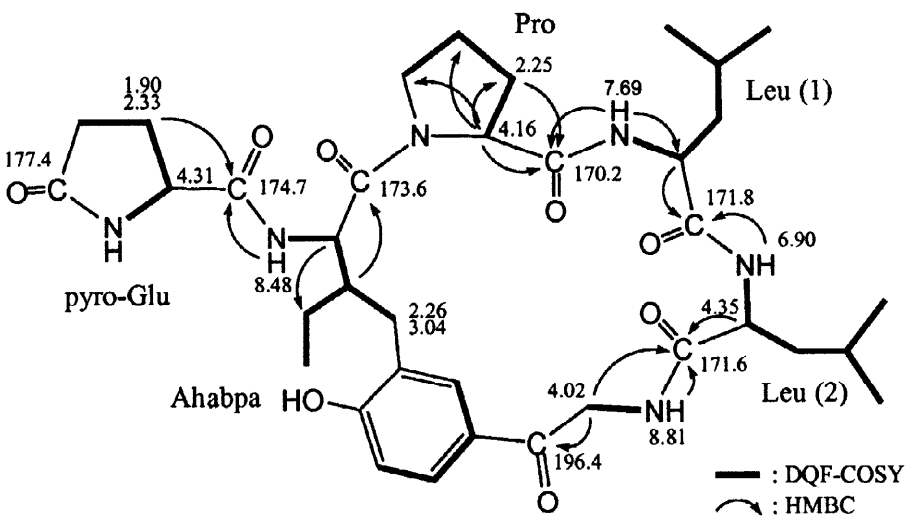


Fig. 2. The structure of hibispeptin B elucidated by the DQF-COSY and HMBC experiments.

Table 1. ^1H and ^{13}C NMR spectral data of hibispeptin B in DMSO-d_6[a].

Position		δ_H	δ_C	Position		δ_H	δ_C
pyro-Glu				Pro			
	α	4.31 (1H, br. s)[b]	54.8		α	4.16 (1H, br. d, 5.0)	59.8
	β	1.90 (1H, m)	25.3		β	1.87 (1H, m)	30.8
		2.33 (1H, m)				2.25 (1H, m)	
	γ	2.06 (1H, m)	29.3		γ	1.37 (2H, m)	21.3
		2.14 (1H, m)			δ	3.30 (2H, m)	45.8
	δ		177.4		CO		170.2
	NH	7.77 (1H, s)		Leu (1)			
	CO		174.7		α	4.06 (1H, m)	53.5
Ahabpa					β	1.29 (1H, m)	41.1
	α	3.65 (1H, br. s)	53.0			1.50 (1H, m)	
	β	2.25 (1H, m)	37.5		γ	1.34 (1H, m)	24.4
	γ	1.34 (1H, m).	22.0		δ	0.70 (3H, d, 5.5)	20.8
		1.60 (1H, m)				0.81 (3H, d)	23.1
	δ	0.94 (3H, t, 7.2)	12.4		NH	7.69 (1H, d, 9.0)	
	CH_2	2.26 (1H, d, 12.0)	33.0		CO		171.6
		3.04 (1H, br. d, 12.0)		Leu (2)			
	1		124.9		α	4.35 (1H, m)	51.2
	2		160.0		β	1.33 (1H, m)	40.9
	2-OH	10.48 (1H, s)				1.50 (1H, m)	
	3	6.74 (1H, d, 8.0)	114.1		γ	1.34 (1H, m)	24.7
	4	7.41 (1H, d, 8.0)	128.7		δ	0.81 (3H, d)	22.5
	5		126.9			0.85 (3H, d)	22.3
	5-CO		196.4		NH	6.90 (1H, d, 8.0)	
	$5\text{-}CH_2$	4.02 (2H, br. s)	47.3		CO		171.8
	5-NH	8.81 (1H, br. s)					
	6	7.38 (1H, br. s)	132.8				
	NH	8.48 (1H, br. s)					
	CO		173.6				

[a] taken in 500 MHz for ^1H and 125 MHz for ^{13}C at 298 K

[b] Proton resonance integration, multiplicity and coupling constant (J=Hz) are in parenthesis

In order to determine the absolute stereochemistry, hibispeptin B was subjected to complete hydrolysis with 6N HCl at 110°C for 24h in a sealed tube. Each amino acid from the hydrolysate was purified through silica gel TLC and then analysed by comparing the Rf-values with standard amino acids on chiral-TLC. Consequently, all normal component amino acids (Gln, Pro, Leu (1) and Leu (2)) in hibispeptin B were established to be L-configuration. As far as we know, all component amino acids of cyclic peptides isolated from higher plants have L-configuration. Thus, it would be reasonable that isoleucine moiety in Ahabpa formed by condensation of isoleucine, hydroxyphenyl and glycine is to be assumed as L-configuration. The geometry of the proline amide bond was determined to be *cis* by the ^{13}C chemical shifts at δ 30.8 and 21.3 for β and γ positions, respectively[8].

The biological activities of hibispeptin B, along with hibispeptin A previously reported[4], including antioxidative, tyrosinase inhibitory, antimicrobial and cytotoxic activities were investigated. Free radical scavenging activity was evaluated by the inhibitory activities of compounds against lipid peroxidation in rat liver microsomes according to the method of Yagi *et al*[9]. Hibispeptin A inhibited lipid peroxidation with IC_{50} value of 9.2 μg/ml (vitamin E, 0.15 μg/ml) but B did not show even at the concentration of 30 μg/ml. Morita *et al*.[1i] reported that pseudostellarins, cyclic pentapeptides isolated from a higher plant, have

tyrosinase inhibitory activity but hibispeptins did not exhibit this activity up to the concentration of 50 μg/ml. Also antimicrobial activity against Gram (+), (-) bacteria and phytopathogenic fungi and cytotoxic effect against some cancer cell lines were not detected.

Experimental

General Methods

Specific rotation was determined by using a Polartronic polarimeter. Mass spectra were measured by using a JEOL JMS-HX 110A/HX-110A spectrometer in the FAB mode using glycerol matrix with polyethylene glycol as internal standard. UV and IR spectra were recorded on a Shimadzu UV-300 and a FT-IR Equinox 55 spectrophotometer, respectively. NMR spectra were obtained on a Varian UNITY 500 NMR spectrometer with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard. All NMR experiments were performed on 6 mg of hibispeptin B dissolved in 0.8 ml of DMSO-d_6. Analytical silica gel TLC (Merck, Kiesel gel 60 F_{254}, 0.25 mm), reverse phase TLC (Merck, RP-18 F_{254S}) and chiral TLC (Merck, TLC plates CHIR, Merck Art. 12381) plates were used without activation.

Plant Material and Isolation Procedures

The root bark of *H. syriacus* was collected at Yusong, Chungnam Province, Korea, in October 1995, and identified by staff at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea. Fresh root bark was dried in a dark, well-ventilated place. The voucher specimen is deposited in the Herbarium of KRIBB. The dried root bark of *H. syriacus* (1.6 kg) was ground into powder and extracted with MeOH twice at room temperature for 2 days. The methanolic extract was filtered and the filtrate was concentrated *in vacuo*. The residue was partitioned between *n*-hexane and water and then water layer was extracted with chloroform, successively. The chloroform layer was concentrated under reduced pressure and the residue was chromatographed on a silica gel column eluted with chloroform only and chloroform-mathanol (100:1 to 1:1, stepwisely) mixture. The peptidic fractions were collected and combined by monitoring with reaction to ninhydrin reagent for hydrolysate (6N HCl, 110°C, 24 hours) in combination with analytical TLC analysis. The fraction was concentrated *in vacuo* and rechromatographed on a silica gel column eluted with chloroform-acetone (1:1), followed by Sephadex LH-20 column chromatography eluted with $CHCl_3$-MeOH (1:1). The peptidic fraction was finally purified by reverse-phase (ODS) preparative TLC developed with 65% aq. methanol to give hibispeptin B (6.0 mg). *Hibispeptin B* - White powder; $[\alpha]_D$ = -42.7° (*c* = 0.75, $CHCl_3$/MeOH (1:1)); IR (KBr) : 3420, 2925, 1690, 1675-1640, 1600, 1540, 1440, 1285, 1115 cm^{-1}; UV λ_{max} nm (ε) in MeOH : 209 (37500), 221 (sh, 24400), 272 (23300), 325 (9800), 360 (9000); FAB-MS : *m/z* 697 $(M+H)^+$; High resolution FAB-MS : *m/z* 697.3940 $(M+H)^+$, *m/z* 719.3740 $(M+Na)^+$ ($C_{36}H_{52}N_6O_8$ requires 697.3924 and 719.3745, respectively); ^{1}H-NMR and ^{13}C- NMR see Table 1.

Acid Hydrolysis of Hibispeptin B

A solution of hibispeptin B (1 mg) in 6N HCl (2 ml) was heated at 110°C for 24h in a sealed tube. After removal of traces of HCl by repeated evaporation under reduced pressure, the residual hydrolysate was purified by preparative silica gel TLC with BuOH-MeOH-H_2O (4:1:1). Each amino acid giving positive

response to ninhydrin reagent was scraped off TLC plates. The purified amino acids for their configurations were analysed by comparing the Rf-values with standard amino acids on chiral-TLC. The Rf-values of standard amino acids eluted with CH_3CN-MeOH-H_2O (4:1:1) were 0.75 (L-Glu), 0.68 (D-Glu), 0.69 (L-Leu), 0.56 (D-Leu), 0.55 (L-Pro) and 0.44 (D-Pro). Amino acids from hibispeptin B were in good agreement with L-form. Acid hydrolysis during the isolation procedure for hibispeptin B was performed under the same condition as above.

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