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Lyngbyaureidamides A and B, Two Anabaenopeptins from the Cultured Freshwater Cyanobacterium *Lyngbya* sp. (SAG 36.91)

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Abstract

Two anabaenopeptin-type peptides, lyngbyaureidamides A and B, together with two previously reported peptides lyngbyazothrins C and D, were isolated from the cultured freshwater cyanobacterium *Lyngbya* sp. (SAG 36.91). Their structures were determined by spectroscopic and chemical methods. Lyngbyazothrins C and D were also able to inhibit the 20S proteasome with IC₅₀ values of 7.1 μM and 19.2 μM, respectively, while lyngbyaureidamides A and B were not active at 50 μM.

Keywords

Cyanobacterium; *Lyngbya* sp. (SAG 36.91); Peptide; 20S proteasome

1. Introduction

Cyanobacteria are known to be a rich source of secondary metabolites with diverse chemical structures and biological activities (Wagoner *et al.*, 2007; Tan, 2007; Müller *et al.*, 2006; Luesch *et al.*, 2001; Singh *et al.*, 2005; Jaiswal *et al.*, 2008). Many of these metabolites have been found to inhibit various proteases and we have initiated a screening program to discover cyanobacterial inhibitors of the 20S proteasome, the catalytic core of the major proteolytic system in eukaryotic cells and an established anticancer target. One of the active extracts was *Lyngbya* sp. (SAG 36.91), which showed inhibitory activity against the 20S proteasome at a concentration of 50 μg/mL. Bioassay-guided fractionation led to the isolation of four cyclic peptides, including two new anabaenopeptin-type peptides lyngbyaureidamides A (1) and B (2) and the previously reported peptides lyngbyazothrins C (3) and D (4). Lyngbyazothrins C (3) and D (4) were originally reported as an inseparable mixture with antibacterial activity from this strain (Zainuddin *et al.*, 2009). Lyngbyazothrins C (3) and D (4) were recently reported individually as portoamides A and B (Leão *et al.*, 2010).

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2. Results and Discussion

Lyngbya sp. (SAG 36.91) was mass cultured in Z medium (Mian *et al.*, 2003). The organic extract was active in our 20S proteasome assay and was fractionated using a diaion HP20SS resin column. The activity was recovered in fraction 3, eluting with 40% isopropanol in water and further purified by HPLC, which led to the isolation of four peptides: lyngbyaureidamides A (**1**) and B (**2**) and the previously reported peptides lyngbyazothrins C (**3**) and D (**4**).

Lyngbyaureidamide A (**1**) was obtained as a yellowish gum. The molecular formula was determined as $C_{46}H_{61}N_7O_9$ by HRMS (m/z 878.4426 $[M + Na]^+$). The proton spectrum of **1** contained signals for six amide protons (δ_H 8.92, 8.75, 7.13, 7.03, 6.61 and 5.93), a *N*-methyl group (δ_H 2.64) and six α -protons (δ_H 4.95, 4.78, 4.16, 3.95, 3.87 and 3.77). The latter set correlated with six carbon resonances (δ_C 54.9, 49.0, 53.4, 55.2, 57.3 and 56.1) in the HSQC spectrum. Detailed 2D NMR spectroscopic analysis (Table 1 and Figure 2) indicated the presence of six amino acid residues, three of which were the common amino acids Lys, Ile and Phe. The structures of three remaining non-standard amino acid residues were determined by analysis of COSY, HSQC and HMBC data. The structure of *N*-MeAla was determined by the HMBC correlation from the *N*-methyl singlet to the *N*-MeAla C-1 (δ_C 170.6), in combination with a COSY correlation between *N*-MeAla H- α and *N*-MeAla H₃- β (δ_H 1.25). The presence of a homophenylalanine (Hph) residue was deduced by the HMBC correlation from Hph H₂- γ (δ_H 2.39) to C-2'/C-6' (δ_C 128.6) connecting the two partial structures established by COSY correlations (Hph NH to H₂- γ and a mono substituted aromatic ring C-1'–C-6'). Similarly, the presence of a homotyrosine (Hty) residue was deduced by the HMBC correlations from Hty H₂- γ (δ_H 2.48 and 2.67) to C-2'/C-6' (δ_C 129.5), connecting Hty NH to H₂- γ partial structure to the *para*-substituted aromatic ring (Hty C-1'–C-6').

The structure of **1** was established by interpretation of the HMBC spectrum (Table 1 and Figure 2). The correlations from the NH of Hph to C-1 of *N*-MeAla (δ_C 170.6), from H_{NMe} of *N*-MeAla to C-1 of Hty (δ_C 171.8), from the NH of Hty to C-1 of Ile (δ_C 173.2), from the NH of Ile to C-1 of Lys (δ_C 172.9) and from ϵ -NH of Lys to C-1 of Hph (δ_C 171.5), indicated that these five residues formed a 19-membered peptide ring, characteristic of the anabaenopeptin-type peptides (Welker and von Döhren, 2006). Finally, analysis of the correlations from H- α of Lys and H- α of Phe to the same sp^2 carbon at δ_C 157.1 suggested that the remaining Phe residue was attached to the 19-membered ring through an ureido group. This ureido group also accounted for the remaining carbonyl moiety implied by the molecular formula. The absolute configurations of amino acid residues of **1** were determined by Marfey's method (Marfey, 1984). The result of the HPLC analysis showed that *N*-MeAla, Hty, Ile and Hph all were of the L configuration, while Lys and Phe both were D.

Lyngbyaureidamide B (**2**) was obtained as a yellow gum. The HRMS quasimolecular peak at m/z 864.4255 $[M + Na]^+$ suggested a molecular formula of $C_{45}H_{59}N_7O_9$. The 1D and 2D NMR spectroscopic data of **2** were very similar to those of **1**. The only difference was the loss of the Hph moiety and the appearance of an additional Phe moiety in **2**. The placement of the second Phe moiety was determined by analysis of the ROESY spectrum of **2**. The correlations between NH of Phe⁴ (δ_H 8.75) and H- α of *N*-MeAla³ (δ_H 4.78) and ϵ -NH of Lys⁵ (δ_H 7.16) indicated that Hph had been replaced by Phe. This was confirmed by the HMBC correlations from the NH of Phe⁴ to C-1 of *N*-MeAla³ (δ_C 170.6), and from ϵ -NH of Lys⁵ to C-1 of Phe⁴ (δ_C 171.5). The amino acid residues of **2** were defined as L-Ile, L-Hty, L-*N*-MeAla, L-Phe, D-Phe and D-Lys by Marfey's method, indicating the replacement of L-Hph⁴ by L-Phe⁴. This assignment was further supported by comparison of the NMR spectroscopic data of lyngbyaureidamide B with those published for other anabaenopeptins

containing a L-Hty-L-N-MeAla-L-Phe-D-Lys sequence as part of their 19-membered peptide ring (Williams *et al.*, 1996; Zafrir-Ilan *et al.*, 2010). No significant deviation (<0.8 ppm) was found for these ^{13}C chemical shifts. In addition, the NMR chemical shifts for Phe⁶ of lyngbyaureidamide B closely matched those of Phe⁶ of **1**. Together, this indicated L-Phe to be in position 4.

Anabaenopeptins, a group of cyclic hexapeptides, are characterized by a 19-membered peptide ring that is formed by cyclization between the C-terminal amino acid and the ϵ -amine of a lysine residue. The α -amine of the lysine is further linked through an ureido group to a side-chain amino acid. Anabaenopeptin-type peptides have been obtained from several cyanobacterial species, including *Anabaena* (Harada *et al.*, 1995; Grach-Pogrebinsky *et al.*, 2008), *Aphanizomenon* (Murakami *et al.*, 2000), *Lyngbya* (Matthew *et al.*, 2008), *Microcystis* (Williams *et al.*, 1996; Beresovsky *et al.*, 2006; Gesner-Apter *et al.*, 2009; Zafrir-Ilan *et al.*, 2010), *Nodularia* (Fujii *et al.*, 1997), *Oscillatoria* (Sano *et al.*, 1995; Shin *et al.*, 1997; Itou *et al.*, 1999), *Planktothrix* (Sano *et al.*, 2001; Okumura *et al.*, 2009), *Schizothrix* (Reshef *et al.*, 2002), and *Tychonema* (Müller *et al.*, 2006), as well as from sponges (Kobayashi *et al.*, 1991a, 1991b; Schmidt *et al.*, 1997; Uemoto *et al.*, 1998; Robinson *et al.*, 2007; Plaza *et al.*, 2010). The exocyclic amino acid for all anabaenopeptin-type peptides reported to date has L configuration and lyngbyaureidamide A (**1**) and B (**2**) are the first examples of a D-configuration amino acid residue at this position (D-Phe).

All isolates were evaluated for their 20S proteasome inhibitory activity (chymotrypsin-like activity). Lyngbyazothrins C (**3**) and D (**4**) were found to be active with IC_{50} values of $7.1\text{ }\mu\text{M}$ and $19.2\text{ }\mu\text{M}$, respectively, while lyngbyaureidamide A (**1**) and B (**2**) were found to be inactive at $50\text{ }\mu\text{M}$. None of the four compounds showed cytotoxic activities against the human colon cancer cell line designated HT-29 at $50\text{ }\mu\text{M}$. Lyngbyazothrins C (**3**) and D (**4**) were originally obtained as an inseparable mixture from this cyanobacterial strain and shown to inhibit the growth of *E. coli* (Zainuddin *et al.*, 2009). The same two structures were recently also reported as pure compounds, named portoamides A and B, obtained from an *Oscillatoria* sp. (Leão *et al.*, 2010). The portoamides were found to act synergistically to inhibit the growth of the green microalga *Chlorella vulgaris*. We here were also able to separate lyngbyazothrins C (**3**) and D (**4**), the two major components of *Lyngbya* sp. (SAG 36.91) by HPLC. Comparison of the spectroscopic data confirmed that lyngbyazothrins C and D were identical to portoamides A and B, respectively.

3. Conclusions

Bioassay-guided investigation (proteasome inhibition) of the cyanobacterium *Lyngbya* sp. (SAG 36.91) led to the isolation of four peptides. The previously reported lyngbyazothrins C (**3**) and D (**4**) accounted for the 20S proteasome inhibitory activity. In addition, two new anabaenopeptin-type peptides, lyngbyaureidamide A (**1**) and B (**2**) were obtained.

4. Experimental

4.1. General experimental procedures

The optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Varian Cary 50 Bio spectrophotometer. IR spectra were obtained on a Jasco FTIR-410 Fourier transform infrared spectrometer. ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance DRX600 MHz NMR spectrometer with a 5 mm CPTXI Z-gradient probe and a Bruker Avance II900 MHz NMR spectrometer with a 5 mm ATM CPTCI Z-gradient probe, referenced to the corresponding solvent peaks. HRMS spectra were obtained on a Shimadzu IT-TOF spectrometer. HPLC separations were performed on an instrument consisting of a Waters 600 controller, a Waters 600 pump, and a Waters 2487 dual

wavelength absorbance detector, with an Alltima (250 × 10 mm i.d.) preparative column packed with C₁₈ (5 μm) or a Varian (250 × 10 mm i.d.) semipreparative column packed with C₈ (5 μm).

4.2. Biological Material

Lyngbya sp. (SAG 36.91) from Culture Collection of Algae, Göttingen, Germany) was grown in 21 L of aerated inorganic Z media (Mian *et al.*, 2003). Cultures were illuminated with fluorescent lamps at 1.93 klx with an 18/6 h light/dark cycle. The temperature of the culture room was maintained at 22 °C. After 7 weeks, the biomass of cyanobacteria was harvested by centrifugation and freeze-dried (yield 11.24g).

4.3. Extraction and isolation

Freeze-dried *Lyngbya* biomass (11.24 g) was extracted five times, each for two hrs with 200 ml of CH₂Cl₂/MeOH (1:1) at room temperature to yield a crude extract (984.5 mg). A portion of the crude extract (849.8 mg) was fractionated using a Diaion HP-20 column (10 g, 130 × 25 mm) with increasing amounts of *i*-PrOH:H₂O (0:100; 20:80; 40:60; 70:30; 80:20; 90:10; 100:0, fraction size 100 ml) to generate eight sub-fractions. Fraction 3 (eluted with *i*-PrOH 40:60, v/v) was the most active fraction and showed 78.4% inhibition against 20S proteasome at 50 μg/mL. This fraction (103.5 mg) was subjected to reversed-phase HPLC (Alltima C₁₈, 5 μm, 250 × 10 mm) with a solvent gradient of MeOH:H₂O (35–65→90:10) over 50 min to afford two subfractions (3a at 32–33 min, and 3b at 35–36 min). Subfraction 3a (8.9 mg) was further separated by reversed-phase HPLC (Varian C₈, 5 μm, 250 × 10 mm), using MeOH–H₂O (55:45) as mobile phase, to yield **1** (1.6 mg, eluting after 112 min) and **2** (1.1 mg eluting after 110 min). Subfraction 3b (67.3 mg) was subjected to reversed-phase HPLC (Varian C₈, 5 μm, 250 × 10 mm), eluted by MeOH–H₂O (65:35), to give **3** (20.5 mg, eluting after 183 min) and **4** (10.3 mg, eluting after 188 min).

4.3.1 Lyngbyaureidamide A (1)—yellowish gum; [α]_D –27.3 (c 0.09 MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.21) nm; IR (neat) ν_{max} 3295, 3250, 2932, 2863, 1643, 1548, 1503, 1452, 1250, 1007 cm^{–1}; For ¹H and ¹³C NMR spectroscopic data, see Table 1; HRMS *m/z* 878.4426 [M + Na]⁺ (calcd for C₄₆H₆₁N₇O₉Na, 878.4428).

4.3.2 Lyngbyaureidamide B (2)—yellow gum; [α]_D –41.1 (c 0.06 MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.27) nm; IR (neat) ν_{max} 3286, 2928, 1643, 1548, 1524, 1504, 1250, 1024 cm^{–1}; For ¹H and ¹³C NMR spectroscopic data see Table 1; HRMS *m/z* 864.4255 [M + Na]⁺ (calcd for C₄₅H₅₉N₇O₉Na, 864.4272).

4.3.3 Lyngbyazothrin C (3)—yellow gum; [α]_D –14.6 (c 0.1 MeOH); IR (neat) ν_{max} 3321, 2957, 1652, 1601, 1506, 1250, 1214 cm^{–1}; ¹H and ¹³C NMR data as described (Zainuddin *et al.*, 2009; Leao *et al.*, 2010); HRMS *m/z* 1554.7771 [M + Na]⁺ (calcd for C₇₄H₁₀₉N₁₃O₂₂Na, 1554.7708).

4.3.4 Lyngbyazothrin D (4)—yellow gum; [α]_D –17.2 (c 0.1 MeOH); IR (neat) ν_{max} 3307, 2971, 1664, 1605, 1470, 1207, 1128 cm^{–1}; ¹H and ¹³C NMR data as described (Zainuddin *et al.*, 2009; Leao *et al.*, 2010). HRMS *m/z* 1524.7673 [M + Na]⁺ (calcd for C₄₅H₅₉N₇O₉Na, 1524.7602).

4.4. Racemization of D-Homotyrosine

LD-Hty was obtained from D-Hty via an acetylation–deacetylation process (Sealock *et al.*, 1946). D-homotyrosine hydrobromide (100 mg, 0.00036 mol; Chem–Impex International Inc., Wood Dale, USA) and NaOH (46.6 mg, 0.00115 mol) were dissolved in H₂O (1.12

mL), and Ac₂O (600 μ L, Sigma–Aldrich Inc., Louis, USA) was added in ten portions at 3 min intervals with continuous stirring. The reaction mixture was stirred at 65 °C for 6 h, and then evaporated *in vacuo* to give a thick syrup (132 mg), which was extracted by acetone and concentrated *in vacuo* to afford crude diacetylated LD-homotyrosine (99 mg) as yellow oil, confirmed by IT–TOF MS: HRMS m/z 302.1004 [M + Na]⁺ (calcd for C₁₄H₁₇NO₅Na, 302.1004). The crude diacetylated LD-homotyrosine was dissolved in 2 N NaOH (400 μ L), and stirred at room temperature for 30 min. The resulting solution was neutralized to pH 7 by 6 N HCl and evaporated *in vacuo* to give a thick syrup which was extracted by acetone and concentrated *in vacuo* to afford crude *N*-acetylated LD-homotyrosine (78 mg) as yellow oil, confirmed by IT–TOF MS: HRMS m/z 260.0898 [M + Na]⁺ (calcd for C₁₂H₁₅NO₄Na, 260.0899). The residue was hydrolyzed with 6 N HCl (2 mL) in a sealed thick glass tube at 102 °C for 3.5 h. The solvent was removed under reduced pressure to give crude LD-homotyrosine (70.1 mg) as a red solid, [α]_D $\approx$ 0, confirmed by IT–TOF MS. LD-Homotyrosine: HRMS m/z 196.0968 [M + H]⁺ (calcd for C₁₀H₁₄NO₃, 196.0895).

4.5. Acid hydrolysis of **1** and **2**, and Marfey's analyses of the hydrolyzates

In separate reactions, compounds **1** (0.2 mg) or **2** (0.2 mg) were dissolved in 6 N HCl (0.4 ml), and heated at 110 °C in a sealed vial for 13 h. The cooled reaction mixture was evaporated to dryness under reduced pressure. The amino acid mixture was resuspended in H₂O (40 μ L). A solution of 1% (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA) in acetone (100 μ L) and 1 M NaHCO₃ (100 μ L) was added to each reaction vessel. The reaction mixture was stirred at 40 °C for 2 h and the resulting solution was neutralized using 2 M HCl. The FDAA-amino acid derivatives, from hydrolyzates, were compared with similarly derivatized standard amino acids by HPLC analysis at 340 nm. The column (Alltima RP-C₈, 5 μ m, 250 $\times$ 4.6 mm) was eluted with a linear gradient of (A) CH₃CN containing 0.65% AcOH and (B) H₂O containing 0.65% AcOH from 20% to 42% (A) over 70 min followed by another gradient elution from 42% to 45% (A) over 20 min at flow rate: 0.5 mL/min. The standards gave the following retention times in min: 48.2 for FDAA; 67.5 for L-Ile, 76.4 for D-Ile; 66.8 for L-*allo*-Ile, 75.9 for D-*allo*-Ile; 69.5 for L-Phe, 76.1 for D-Phe; 71.1 for L-Lys, 73.4 for D-Lys; 77.8 for L-Hph, 85.8 for D-Hph; 58.0 for L-Hty, 63.2 for D-Hty; 42.1 for L-*N*-MeAla, 47.2 for D-*N*-MeAla.

4.6. 20S Proteasome Assay

The proteasome assay was performed according to the protocol provided with the BIOMOL 20S Proteasome Assay Kit for Drug Discovery (BIOMOL International LP, Plymouth Meeting, PA, USA catalog number AK740-0001). The protocol was modified such that the 10 min incubation period was performed at 37 °C. Enzyme was acquired from BostonBiochem (20S proteasome, human, BostonBiochem, USA) and substrate from BIOMOL (Suc-LLVY-AMC). This substrate is specific for the chymotrypsin-like activity of the 20S proteasome. Fluorescence was measured using either a Tecan Genios Pro microplate reader or a Hewlett Packard model AF10000 fluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The positive control was bortezomib (IC₅₀ 2.5 nM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structure of lyngbyaureidamide A (1) and B (2), lyngbyazothrins C (3) and D (4)

Figure 2.
 ^1H - ^1H COSY and key HMBC correlations of **1**.

Table 1

NMR spectroscopic data (δ) of compounds **1** and **2** in DMSO- d_6

unit	1		2		COSY	HMBC	δ_C^a	δ_H^b	COSY	HMBC
	C/H no.									
Ile ¹	NH		7.03	H $_{\alpha}$		C-1 _{Lys}		7.14	H $_{\alpha}$	C-1 _{Lys}
	C-1	173.2					173.0			
	C $_{\alpha}$	57.3	3.87	NH, H $_{\beta}$			57.1	3.97	NH, H $_{\beta}$	
	C $_{\beta}$	35.9	1.54	H $_{\alpha}$, H $_{\gamma}$, H' $_{\gamma}$		C-1 _{Ile}	36.1	1.77	H $_{\alpha}$, H $_{\gamma}$, H' $_{\gamma}$	C-1 _{Ile}
	C $_{\gamma}$	25.4	1.45	H $_{\beta}$, H $_{\delta}$			25.3	1.56	H $_{\beta}$, H $_{\delta}$	
	C' $_{\gamma}$	15.5	0.89	H $_{\beta}$			15.4	1.01	H $_{\beta}$	
	C $_{\delta}$	10.8	0.73	H $_{\gamma}$			10.7	0.82	H $_{\gamma}$	
Hty ²	NH		8.92	H $_{\alpha}$		C-1 _{Ile}		8.94	H $_{\alpha}$	C-1 _{Ile}
	C-1	171.8					171.4			
	C $_{\alpha}$	49.0	4.78	NH, H $_{\beta}$			49.2	4.71	NH, H $_{\beta}$	
	C $_{\beta}$	33.8	1.93	H $_{\alpha}$, H $_{\gamma}$		C-1 _{Hty}	33.7	1.87	H $_{\alpha}$, H $_{\gamma}$	C-1 _{Hty}
			1.80	H $_{\alpha}$, H $_{\gamma}$				1.72	H $_{\alpha}$, H $_{\gamma}$	
	C $_{\gamma}$	30.9	2.67	H $_{\beta}$		C-1', 2', 6'	31.0	2.64	H $_{\beta}$	C-1', 2', 6'
			2.48	H $_{\beta}$				2.43	H $_{\beta}$	
N-MeAla ³	C-1'	131.2					131.7			
	C-2', 6'	129.5	7.04	H-3', 5'			129.5	7.01	H-3', 5'	
	C-3', 5'	115.5	6.69	H-2', 6'			115.6	6.67	H-2', 6'	
	C-4'	156.0					156.0			
	4'-OH		9.26					9.26		
	N-Me	28.8	2.64			C-1 _{NMe-Ala} , C $_{\alpha}$, C-1 _{Hty}	27.4	1.77		C-1 _{NMe-Ala} , C $_{\alpha}$, C-1 _{Hty}
	C-1	170.6					170.6			
Hph ⁴ /Phe ⁴	C $_{\alpha}$	54.9	4.95	H $_{\beta}$			54.7	4.78	H $_{\beta}$	
	C $_{\beta}$	14.5	1.25	H $_{\alpha}$		C-1 _{NMe-Ala}	14.3	1.06	H $_{\alpha}$	C-1 _{NMe-Ala}
	NH		8.75	H $_{\alpha}$		C-1 _{NMe-Ala}		8.75	H $_{\alpha}$	C-1 _{NMe-Ala}

unit	C/H no.	1					2				
		δ_C^a	δ_H^b	COSY	HMBC		δ_C^c	δ_H^d	COSY	HMBC	
Lys ⁵	C-1	171.5					171.5				
	C _{α}	53.4	4.16	NH, H _{β}			55.4	4.35	NH, H _{β}		
	C _{β}	34.5	2.08	H _{α} , H _{γ}	C-1 _{Hph}		37.9	3.29	H _{α}		C-1 _{Phe4} and C-1', 2', 6'
	C _{γ}	32.3	2.39	H _{β}	C-1', 2', 6'			2.78	H _{α}		
	C-1'	141.9					139.1				
	C-2',6'	128.6	7.07				129.3	7.05			
	C-3',5'	128.5	7.23				128.7	7.20			
	C-4'	126.2	7.15				126.4	7.15			
	NH		6.61	H _{α}				6.62	H _{α}		
	C-1	172.9					172.9				
Phe ⁶	C _{α}	55.2	3.95	NH, H _{β}	C _{Ureido}		55.4	3.96	NH, H _{β}		C _{Ureido}
	C _{β}	32.7	1.54	H _{α} , H _{γ}	C-1 _{Lys}		32.7	1.59	H _{α} , H _{γ}		C-1 _{Lys}
	C _{γ}	20.7	1.26	H _{β}			20.8	1.29	H _{β}		
	C _{δ}	28.5	1.45	H _{ϵ}			28.5	1.48	H _{ϵ}		
			1.38					1.43			
	C _{ϵ}	38.7	3.50	ϵ -NH, H _{δ}			38.8	3.56	ϵ -NH, H _{δ}		
			2.79	ϵ -NH, H _{δ}				2.81	ϵ -NH, H _{δ}		
	ϵ -NH		7.13	H _{ϵ}	C-1 _{Hph}			7.16	H _{ϵ}		C-1 _{Phe4}
	NH		5.93	H _{α}				5.96	H _{α}		
	C-1	173.0					173.3				
	C _{α}	56.1	3.77	NH, H _{β}	C _{Ureido}		55.4	3.96	NH, H _{β}		C _{Ureido}
	C _{β}	38.2	3.00	H _{α}	C-1 _{Phe} and C-1', 2', 6'		38.2	3.01	H _{α}		C-1 _{Phe6} and C-1', 2', 6'
			2.94	H _{α}				2.96	H _{α}		
	C-1'	140.1					139.8				
	C-2',6'	130.1	7.06				130.0	7.11			
	C-3',5'	127.6	7.12				127.9	7.19			

unit	C/H no.	1				2			
		δ_C^a	δ_H^b	COSY	HMBC	δ_C^c	δ_H^b	COSY	HMBC
C-4'		125.6	7.07			126.0	7.14		
Ureido	C _{Ureido}	157.1				157.3			

^a Chemical shifts were determined by HSQC and HMBC experiments recorded at 600 MHz.

^b Recorded at 600 MHz.

^c DEPTQ experiment recorded at 226 MHz.