Nostophycin, a Novel Cyclic Peptide from the Toxic Cyanobacterium *Nostoc* sp. 152

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For the elucidation of the biosynthetic relationship between nontoxic and hepatotoxic peptides produced by cyanobacteria, we investigated the secondary metabolites from the toxic cyanobacterium *Nostoc* sp. strain 152, which produces microcystins. A novel cyclic peptide, nostophycin, possessing weakly cytotoxic activity was isolated together with microcystins from this strain. Nostophycin is composed of six amino acids, D-glutamine, glycine, L-phenylalanine, D-*allo*-isoleucine, 2 mol of L-proline, and a novel β-amino acid moiety, (2*S*,3*R*,5*R*)-3-amino-2,5-dihydroxy-8-phenyloctanoic acid (Ahoa). The sequence of the constituent amino acids in nostophycin and its structure were determined by 2D-NMR techniques and MS/MS experiments, and the advanced Marfey's method has been used to simultaneously determine their absolute configurations including the β-amino acid moiety. The stereochemistries at the C-2 and C-5 positions of Ahoa were determined by the ^{1}H NMR spectral analysis of its lactone derivative with the acetylation. These results suggest that nostophycin is biosynthetically related to the microcystins, because they have a β-amino acid and two D-amino acids in common.

Introduction

Some cyanobacteria produce the hepatotoxic cyclic peptides microcystins and nodularin.[1] Microcystins are known to be produced by strains of four cyanobacterial genera, *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc*, and nodularin is produced by *Nodularia spumigena*.[1] They have caused the deaths of wild and domestic animals all over the world and led to the deaths of 60 patients from hemodialysis in Brazil in 1996.[2,3] Recently, a large number of peptides other than hepatotoxic peptides have also been isolated from several cyanobacteria.[4] We have focused on the biosynthetic relationship between these peptides and hepatotoxic peptides. Initially, we carried out the detection, isolation, and structural determination of peptides produced together with hepatotoxic peptides by toxic cyanobacteria. Aeruginopeptins, cyclic depsipeptides possessing a 3-amino-6-hydroxy-2-piperidone (Ahp) moiety, were isolated from not only the cultured cells of toxic *Microcystis aeruginosa* but also bloom samples.[5,6] Anabaenopeptins, cyclic peptides possessing an ureido linkage, were isolated from *Anabaena flos-aquae* NRC 525-17 that simultaneously produced anatoxin-a(s) and microcystins.[7] Anabaenopeptilides, whose structures are similar to those of aeruginopeptins, were also isolated along with anabaenopeptins from the toxic *Anabaena* sp. strains which coproduced microcystins.[8] On the other hand, no peptide has been detected from the neurotoxic *Anabaena* sp. strains which produced anatoxin-a.[9] Furthermore, we compared the products from the toxic and nontoxic *N. spumigena*. While we isolated two groups of peptides, cyclic peptides such as anabaenopeptins and linear peptides composed of three amino acids and a fatty acid, together with nodularin from toxic *N. spumigena*, two glycosidic compounds were isolated instead of these peptides from nontoxic *N. spumigena*.[9] These results strongly suggested that the production of these peptides is closely related to that of the hepatotoxic peptides.

For the elucidation of this biosynthetic relationship, we further investigated the products from one of the toxic cyanobacterial genera, *Nostoc*. The toxic cyanobacterium *Nostoc* sp. strain 152 isolated from a lake in Finland was the first strain of that genus found to produce microcystins.[10] Seven microcystins has been isolated and characterized from this strain (Figure 1).[11,12] Microcystins

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(1) Carmichael, W. W. *Sci. Am.* **1994**, *270*, 64−72.
(2) Jochimsen, E. M.; Carmichael W. W.; An, J. C.; Cardo, D. M.; Cookson, S. T.; Holmes, C. E. M.; Antunes, M. B. C.; Filho, D. A. M.; Lyra, T. M.; Barreto, V. S. T.; Azevedo, S. M. F. O.; Jarvis, W. R. *N. Engl. J. Med.* **1998**, *338*, 873−878.
(3) Pouria, S.; Andrade, A.; Barbosa, J.; Cavalcanti, R. L.; Barreto, V. T. S.; Ward, C. J.; Preiser, W.; Poon, G. K.; Neild, G. H.; Codd, G. A. *Lancet* **1998**, *352*, 21−26.
(4) Namikoshi, M.; Rinehart, K. L. *J. Ind. Microbiol.* **1996**, *17*, 373−384.
(5) Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett.* **1993**, *34*, 6091−6094.
(6) Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Park, H.; Watanabe, M. F. *35th Symp. Chem. Nat. Prod. Symp. Pap. Kyoto* **1993**, 377−384.
(7) Harada, K.-I.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett.* **1995**, *36*, 1511−1514.
(8) Fujii, K.; Harada, K.-I.; Suzuki, M.; Kondo, F.; Ikai, Y.; Oka, H.; Carmichael, W. W.; Sivonen, K. *Harmful and Toxic Algal Blooms*; Yasumoto, T., Oshima, Y., Fukuyo, Y., Eds.; Intergovernmental Oceanographic Commission of UNESCO: Sendai, Japan, 1996; pp 559−562.
(9) Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, Y.; Shimizu, Y.; Sano, H.; Hirayama, M.; Suzuki, M.; Harada, K.-I. *Tetrahedron Lett.* **1997**, *38*, 5525−5528, 5529−5532.
(10) Sivonen, K.; Carmichael, W. W.; Namikoshi, M.; Rinehart, K. L.; Dahlem, A. M.; Niemelä, S. I. *Appl. Environ. Microbiol.* **1990**, *56*, 2650−2657.

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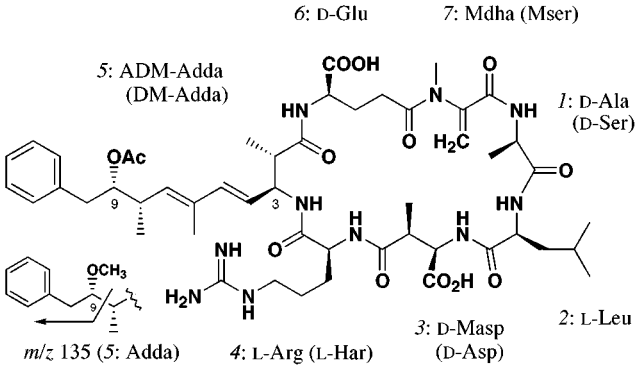


Figure 1. Structure of [ADM-Adda[5]]microcystin LR produced by *Nostoc* sp. strain 152 as a major microcystin. This strain produces seven microcystins, [ADM-Adda[5]]microcystin LR, [ADM-Adda[5]]microcystin LHar, [D-Asp[3], ADM-Adda[5]]microcystin LHar, [D-Ser[1], ADM-Adda[5]]microcystin LR, [D-Asp[3], ADM-Adda[5]]microcystin LR, [ADM-Adda,[5] Mser[7]]microcystin LR, and [DM-Adda[5]]microcystin LR. In the case of the Adda as the fifth constituent amino acid, the microcystins give the prominent fragment ion at *m*/*z* 135 under FAB ionization. Abbreviations: Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(*E*),6(*E*)-decadienoic acid; ADM-Adda, 9-*O*-acetyl-9-*O*-demethyl-Adda; DM-Adda, 9-*O*-demethyl-Adda; Har, L-homoarginine; Masp, D-*erythro*-*β*-methylaspartic acid; Mdha, *N*-methyldehydroalanine; Mser, *N*-methylserine.

are normally characteristic cyclic heptapeptides composed of D-gulutamic acid, D-alanine, D-*erythro*-*β*-methylaspartic acid, *N*-methyldehydroalanine (Mdha), a unique *β*-amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(*E*),6(*E*)-decadienoic acid (Adda), and two variable L-amino acids.[1] However, microcystins produced by the *Nostoc* sp. strain 152 possess an interesting modification in their Adda moiety, which has an acetoxy group instead of the *O*-methyl group at the C-9 position (Figure 1).[11] In addition, one of the microcystins has a *N*-methylserine variant of Mdha and the other has D-serine in place of D-alanine.[12] During the investigations of peptides other than the microcystins from this toxic cyanobacterium, we found a novel cyclic peptide possessing a *β*-amino acid moiety together with these microcystins. In this paper, we report the isolation and the structural determination of this novel cyclic peptide, nostophycin (**1**), from the toxic *Nostoc* sp. strain 152.

Results and Discussion

A method for the differentiation of microcystins from groups of peptides other than the microcystins is required. We have already established the analytical methods for microcystins using HPLC with photodiode array detection and Frit-FAB LC/MS.[13,14] These methods have the advantage that peptides other than microcystins can also be detected and characterized, and we have successfully applied these techniques to previous studies.[5−9] The HPLC analysis using photodiode array detection and

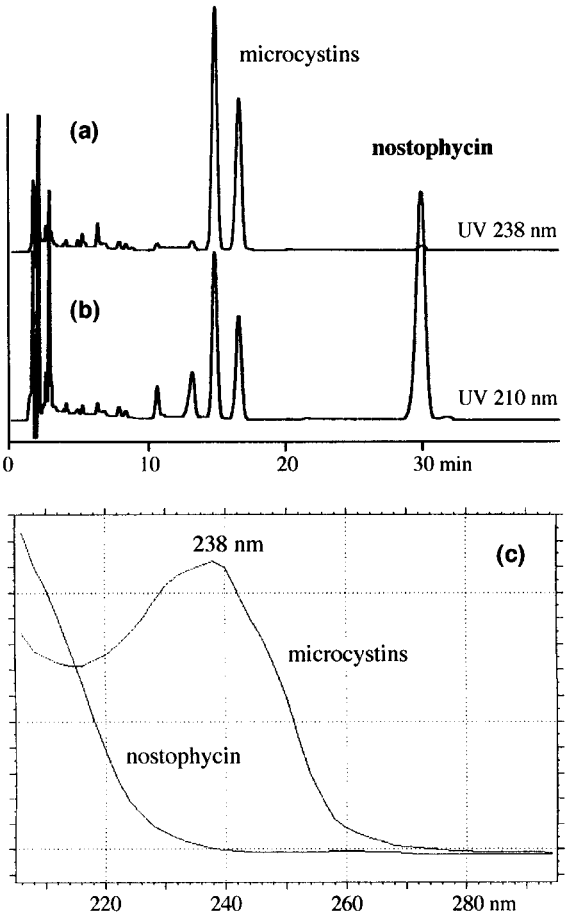


Figure 2. HPLC chromatograms at UV 238 nm (a) and 210 nm (b) of the 5% AcOH aqueous extract after cleanup with ODS silica gel and UV spectra (c) of microcystins and nostophycin using photodiode array detection.

mass chromatography monitored at *m*/*z* 135 using Frit-FAB LC/MS permit the rapid identification of microcystins, because these methods recognize the most unusual structural feature of the microcystins, Adda, which gives the characteristic UV spectra with an absorption maxima at 238 nm and the fragment ion at *m*/*z* 135 under FAB ionization (Figure 1). Unfortunately, microcystins produced by the *Nostoc* sp. strain 152 could not be detected by Frit-FAB LC/MS using mass chromatography monitored at *m*/*z* 135, because these microcystins containing 9-acetoxy-Adda give no fragment ion at *m*/*z* 135. However, an unidentified peak, whose UV spectrum was not consistent with those of the microcystins, was detected by HPLC analysis using photodiode array detection along with those of the microcystins in a 5% AcOH aqueous extract of the cultured cells of *Nostoc* sp. strain 152 as shown in Figure 2. Consequently, the compound designated as nostophycin was isolated from the extract and was purified by repeated silica gel and TOYOPEARL HW-40F chromatographies on the basis of the results of TLC analysis using iodine vapor as the detection.

Nostophycin (**1**) is a colorless amorphous powder which is negative to ninhydrin: $[\alpha]^{27}_{D}$ −27.1° (*c* 0.100, MeOH). The positive ion and negative ion fast atom bombardment (FAB) mass spectra showed $[M + H]^+$, $[M + H - H_2O]^+$, and $[M - H]^-$ peaks at *m*/*z* 889, 871, and 887, respectively, indicating a molecular weight of 888 for **1**. The molecular formula of **1** was established to be $C_{46}H_{64}N_8O_{10}$ based on the HRFABMS (*m*/*z* 889.4825, $[M + H]^+$, Δ +

(11) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Sivonen, K.; Carmichael, W. W. *J. Org. Chem.* **1990**, *55*, 6135−6139.
(12) Sivonen, K.; Namikoshi, M.; Evans, W. R.; Fürdig, M.; Carmichael, W. W.; Rinehart, K. L. *Chem. Res. Toxicol.* **1992**, *5*, 464−469.
(13) Harada, K.-I.; Suzuki, M.; Dahlem, A. M.; Beasley, V. R.; Carmichael W. W.; Rinehart, K. L., Jr. *Toxicon* **1988**, *26*, 433−439.
(14) Kondo, F.; Ikai, Y.; Oka, H.; Matsumoto, H.; Yamada, S.; Ishikawa, N.; Tsuji, K.; Harada, K.-I.; Shimada, T.; Oshikata, M.; Suzuki, M. *Nat. Toxins* **1995**, *3*, 41−49.

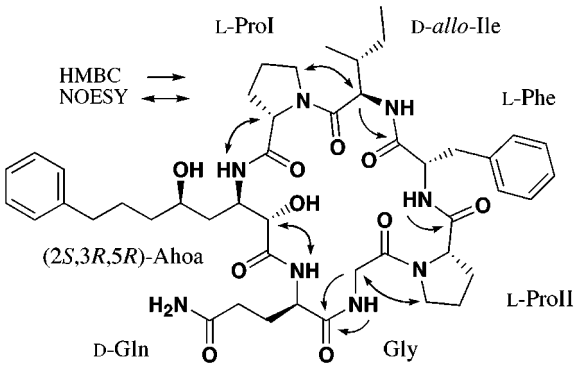
Table 1. ^{13}C and ^{1}H NMR Spectral Data for Nostophycin in DMSO-d_6[a]

amino acid unit	assgn	^{13}C	^{1}H (mult; *J*, Hz)
Pro I	1	171.0	
	2	60.0	4.27 (dd; 5.4, 7.8)
	3	29.2	1.29 (m), 1.79 (m)
	4	24.5	1.87 (m), 2.04 (m)
	5	47.6	3.62 (m), 3.72 (−[b])
allo-Ile	1	170.0	
	2	54.2	4.56 (t; 8.4)
	2-NH		7.37 (br d; 8.4)
	3	36.2	1.95 (m)
	4	25.4	1.04 (m), 1.30 (m)
	5	11.4	0.92 (t; 7.2)
	6	14.4	0.90 (d; 6.6)
Phe	1	170.6	
	2	53.9	4.43 (−[b])
	2-NH		7.69 (br d; 9.0)
	3	35.8	2.80 (dd; 12.0, 13.8), 3.25 (−[b])
	4	138.4	
	5	128.6	7.19 (m)
	6	128.0	7.26 (m)
	7	126.0	7.18 (m)
Pro II	1	170.9	
	2	60.9	4.01 (dd; 6.3, 8.7)
	3	28.8	1.29 (m), 1.97 (m)
	4	23.8	1.57 (m), 1.74 (m)
	5	46.0	3.45 (m), 3.55 (−[b])
Gly	1	168.7	
	2	41.4	3.75 (−[b]), 4.15 (−[b])
	2-NH		7.07 (br dd; 8.4, 1.8)
Gln	1	171.7	
	2	54.2	4.17 (−[b])
	2-NH		7.74 (−[b])
	3	26.4	2.05 (m)
	4	32.2	2.18 (m)
	5	173.6	
	5-NH_2		6.72 (br s), 7.29 (br s)
Ahoa	1	171.6	
	2	70.5	4.04 (−[b])
	2-OH		5.02 (−[b])
	3	49.7	3.80 (−[b])
	3-NH		7.82 (br d; 9.0)
	4	35.7	1.37 (m), 1.69 (m)
	5	67.3	3.56 (−[b])
	5-OH		4.39 (−[b])
	6	35.8	1.27 (m), 1.38 (m)
	7	26.7	1.56 (m), 1.68 (m)
	8	35.0	2.51 (m), 2.57 (m)
	9	142.2	
	10	128.1	7.17 (m)
	11	127.9	7.25 (m)
	12	125.4	7.15 (m)

[a] The chemical shifts of ^{1}H resonances, which overlapped with other ^{1}H resonances in 1D spectrum, were determined using $^{1}H-^{1}H$ COSY and HSQC experiments. [b] The multiplicity of ^{1}H resonances were not determined due to the broadening and overlapping with other ^{1}H resonances.

0.2 mmu) and NMR spectral data (Table 1). It was suggested that **1** is a peptide compound based on the ^{1}H and ^{13}C NMR spectra. The 2D-NMR spectral analysis of **1** confirmed the presence of 1 mol each of glutamine (Gln), glycine (Gly), phenylalanine (Phe), and isoleucine (Ile) and 2 mol of proline (Pro) and additionally indicated the presence of an unusual amino acid. This structure was finally determined to be a β-amino acid, 3-amino-2,5-dihydroxy-8-phenyloctanoic acid (Ahoa), by the interpretation of the ^{1}H and ^{13}C NMR, $^{1}H-^{1}H$ COSY, HSQC, and HMBC spectra, although broadening and overlapping of the ^{1}H and ^{13}C signals were partly observed in these spectra. Although several ^{1}H resonances of methylene protons for Ahoa particularly overlapped each other and were very close with those of two Pro, each proton was separately assigned by a spin network system with adjacent protons using the HSQC techniques. The coupling network of protons from H-2 to H-8 for Ahoa was observed by the COSY spectrum, and each methine proton at H-2 (4.04 ppm), H-3 (3.80 ppm), and H-5 (3.56 ppm) was correlated with the active protons (Table 1). On the basis of the chemical shifts of these methine carbons, it was suggested that an amino group was combined with the carbon at C-3 (49.7 ppm) and two hydroxy groups were bound with the carbons at C-2 (70.5 ppm) and C-5 (67.3 ppm). Furthermore, the interpretation of the HMBC spectrum revealed that another phenyl group was combined with C-8 (35.0 ppm) and supported this proposed structure. This novel β-amino acid is structurally related to 7,8-epoxy-5-hydroxy-6-methyl-8-phenyl-2-octenoic acid in cryptophycins isolated from *Nostoc* sp.[15]

The sequence of the constituent amino acids of nostophycin (**1**) has been established with the help of the HMBC and NOESY spectra as shown in **1**. The partial



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sequences of −Gln−Gly− and −ProII−Phe−Ile− were determined by the following HMBC correlations: Gln *CO*/Gly H-2 and *NH*, ProII *CO*/Phe *NH*, Phe *CO*/Ile H-2. The remaining sequences were determined by the following NOESY correlations: −Gly−ProII− from Gly H-2/ProII H-5, −Ile−ProI− from Ile H-2/ProI H-5, −ProI−Ahoa− from ProI H-2/Ahoa *NH*, and −Ahoa−Gln− from Ahoa H-2/Gln *NH*. Furthermore, the MS/MS method under FABMS conditions was applied to confirm the established structure for **1** and to especially complement the sequence of constituent amino acids, which was determined by NOESY correlations. In the product ion spectrum for the $[M + H]^+$ at *m*/*z* 889 of **1**, several ions were prominently observed, which were assigned as the ions of each constituent amino acid residue lost from the precursor ion, $[M - Gly]^+$ at *m*/*z* 832, $[M - Ile]^+$ at *m*/*z* 775, and $[M - Phe]^+$ at *m*/*z* 741, and the following two fragmentation series, indicating the partial sequences of −ProI−Ahoa−Gln−Gly−ProII−: (1) [ProI−Ahoa−Gln−Gly−(ProII − *CO*) + H]$^+$ at *m*/*z* 601, [ProI−Ahoa−Gln−Gly + H]$^+$ at *m*/*z* 532, [ProI−Ahoa−Gln + H]$^+$ at *m*/*z* 475, [ProI−Ahoa + H]$^+$ at *m*/*z* 347, and [ProI−(Ahoa − *CO*) − H_2O + H]$^+$ at *m*/*z* 301; (2) [*CO*(ProI)−Ahoa−Gln−Gly−ProII − H_2O + 2H]$^+$ at *m*/*z* 543, [*CO*(ProI)−Ahoa−Gln−Gly − H_2O + 3H]$^+$ at *m*/*z* 447, and [*CO*(ProI)−Ahoa−Gln − H_2O + 3H]$^+$ at *m*/*z* 390.[16,17]

(15) Trimurtulu, G.; Ohtani, I.; Paterson, G. M. L.; Moore, R. E.; Corbett, T. H.; Valeriote, F. A.; Demchik, L. *J. Am. Chem. Soc.* **1994**, *116*, 4729−4737.

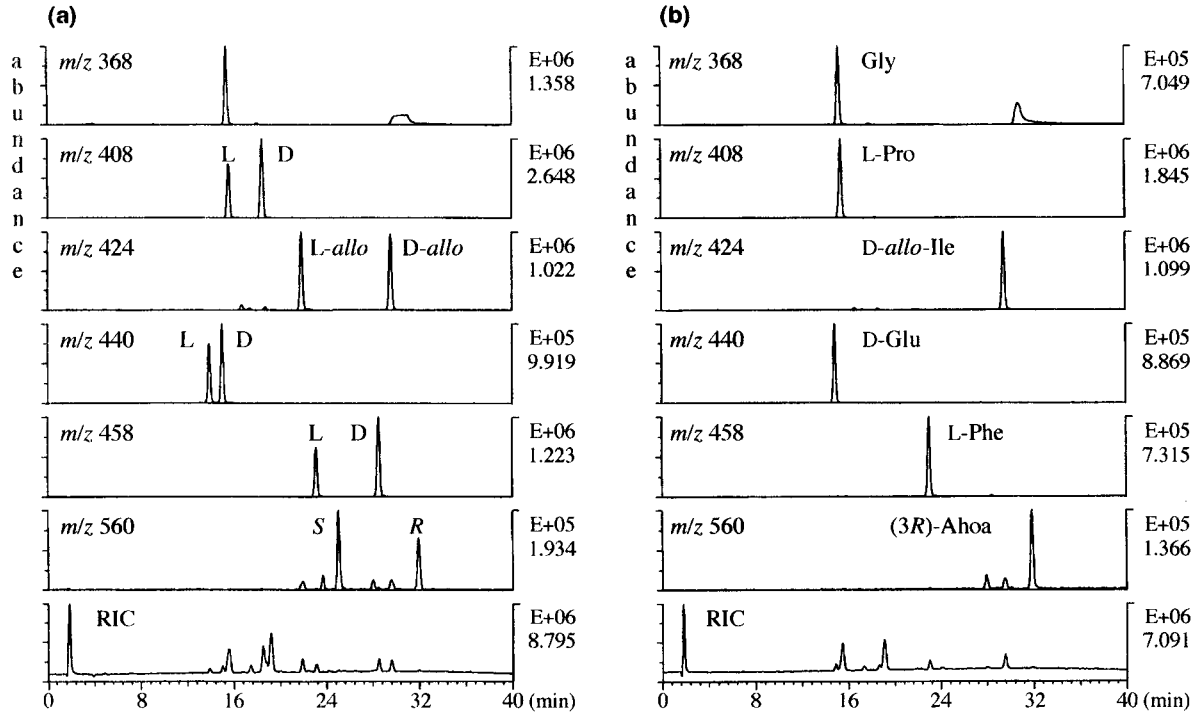


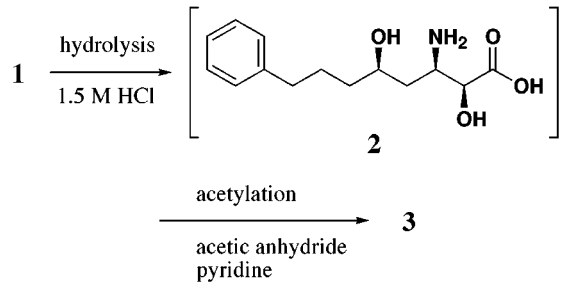
Figure 3. Mass chromatograms monitored at the *m*/*z* values of the deprotonated ions of the DL- (a) and L-FDLA derivatives (b) of each constituent amino acid in nostophycin using ESI LC/MS in the negative ion mode.

The absolute configurations of the constituent amino acids and that at C-3 of Ahoa were simultaneously determined by the advanced Marfey's method.[18,19] The 1.5 M hydrolysate of nostophycin (**1**, 100 μg) was divided into two portions, and each portion was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide or -D-leucinamide (L- or D-FDLA). The L-FDLA derivatives alone and the equal mixture of D- and L-FDLA derivatives, the DL-FDLA derivatives, were analyzed using electrospray ionization (ESI) LC/MS in the negative ion mode. Figure 3a,b shows the mass chromatograms monitored at the *m*/*z* values of the deprotonated ions of the DL- (a) and L-FDLA derivatives (b). The DL-FDLA derivatives of each constituent amino acid except for Gly were detected as two peaks, and each peak that eluted prior to another peak was assigned to the L-amino acid derivatized with L-FDLA in the cases of these amino acids on the basis of the separation mechanism for this method.[18] As a result, we can conclude that Pro and Phe have L-configurations and that Ile and Glu (from Gln) have D-configurations. The complete configuration of Ile in **1** was determined as the D-*allo*-configuration (in this method, the D-FDLA derivatives of D-Ile and D-*allo*-Ile can be separated under another condition[20]). In addition, the DL-FDLA derivatives of Ahoa were also detected as two peaks (retention time: 24.6 and 31.5 min) and its L-FDLA derivatives were detected at 31.5 min on the mass chromatograms monitored at *m*/*z* 560 ([M − H]$^-$). According to the separation mechanism for this method, the absolute configuration at the C-3 of Ahoa was determined as *R*, because the L-FDLA derivative of (3*S*)-Ahoa should be eluted prior to that of (3*R*)-Ahoa for the reason that the hydroxy-phenyl-pentyl moiety in Ahoa can be estimated to be more hydrophobic than the hydroxy-carboxylic acid moiety.

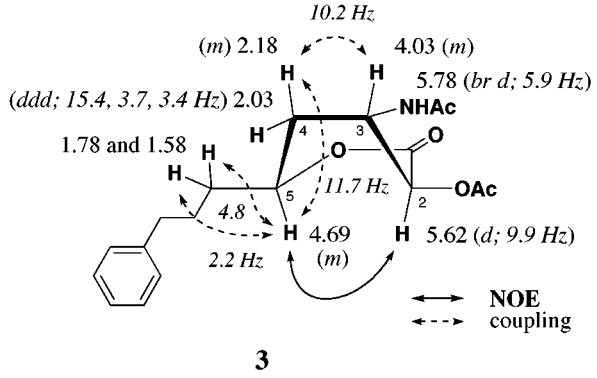
Similar β-amino acids have been found in several peptides isolated from cyanobacteria.[21−25] The β-amino acid Ahoa (**2**) from nostophycin is structurally quite similar to (2*S*,3*R*,5*S*)-3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid (Ahda) in scytonemin A isolated from the cyanobacterium *Scytonema* sp., and both have the α,δ-dihydroxy-β-amino acid moiety in common.[21] The relative stereochemistry of an α,δ-dihydroxy-β-amino acid moiety in Ahda was determined by the ^{1}H NMR spectral analysis of its lactone derivative (triAc-Ahda lactone) with acetylation, and the absolute configurations of C-2, C-3, and C-5 were finally determined by a CD study of the triAc-Ahda lactone.[21] Because the absolute configuration at the C-3 in **2** has already been determined as detailed above, the remaining absolute configurations at the C-2 and C-5 positions of **2** can be determined by the elucidation of the relative stereochemistry of **2** using the

(16) El Hajji, M.; Rebuffat, S.; Lecommandeur, D.; Bodo, B. *Int. J. Pept. Protein Res.* **1987**, *29*, 207−215.
(17) Pettit, G. R.; Clewlow, P. J.; Dufresne, C.; Doubek, D. L.; Cerny, R. L.; Rützler K. Can. *J. Chem.* **1990**, *68*, 708−711.
(18) Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K.-I. *Anal. Chem.* **1997**, *69*, 3346−3352 and 5146−5151.
(19) Fujii, K.; Shimoya, T.; Ikai, Y.; Oka, H.; Harada, K.-I. *Tetrahedron Lett.* **1998**, *39*, 2579−2562.
(20) The D-FDLA derivatives of D-Ile and D-*allo*-Ile were separated by HPLC on a TSKgel ODS-80Ts (150 × 4.6 mm i.d.) column maintained at 40 °C with UV detection at 340 nm using 0.01 M TFA(aq)/CH_3CN (65:35) as the eluent. At a flow of 1.0 mL/min, the D-FDLA derivatives of D-Ile and D-*allo*-Ile eluted at 29.8 and 29.0 min, respectively.
(21) Helms, G. L.; Moore, R. E.; Niemczura, W. P.; Patterson, G. M. L.; Tomer, K. B.; Gross, M. L. *J. Org. Chem.* **1988**, *53*, 1298−1307.
(22) Moore, R. E.; Bornemann, V.; Niemczura, W. P.; Gregson, J. M.; Chen, J.-L.; Patterson, G. M. L.; Helms, G. L. *J. Am. Chem. Soc.* **1989**, *111*, 6128−6132.
(23) Moon, S.-S.; Chen, J. L.; Moore, R. E.; Patterson, G. M. L. *J. Org. Chem.* **1992**, *57*, 1097−1103.
(24) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 501−504.
(25) Pergament, I.; Carmeli, S. *Tetrahedron Lett.* **1994**, *35*, 8473−8476.

^{1}H NMR spectral analysis of its δ-lactone derivative with acetylation. Compound **2** was smoothly isolated using an



ODS silica gel cartridge from the 1.5 M HCl hydrolysate of **1**, and the subsequent acetylation of **2** gave the desired lactone **3** after workup and HPLC preparative separation. The structure of **3** was primarily determined by the ^{1}H NMR spectral analysis including the COSY technique in $CDCl_3$. Although **3** has the same lactone moiety as the triAc-Ahda lactone, the chemical shifts of each ^{1}H signal are obviously different (Table 2), indicating that **3** possesses a different absolute configuration and/or conformation on the lactone moiety from the triAc-Ahda lactone. Additionally, it was found that **3** is labile in MeOH, because it was easily cleaved to give the linearized methyl ester. An NOESY spectrum (in $CDCl_3$) of **3** was measured to verify its conformation. As shown in **3**, an NOE was observed between H-2 (5.62 ppm) and H-5 (4.69 ppm), indicating that **3** is present in a boat conformation, and both H-2 and H-5 are axially oriented. On the basis of this result and the large coupling (9.9 Hz) observed between H-2 and H-3 (4.03 ppm), the relative stereochemistry of **3** was determined as shown in **3**. To further confirm this relative stereochemistry, the coupling constants of the ^{1}H signals on the lactone moiety of **3** were investigated by ^{1}H homodecoupling NMR experiments irradiated at one of the H-4 (2.03 ppm) signals (Table 2). As shown in **3**, H-3 and H-5 were coupled to



one of the H-4 (2.18 ppm) signals by 10.2 and 11.7 Hz, respectively. These results definitely supported the relative stereochemistry of **3** having the boat conformation. On the basis of the relative stereochemistry of **3**, the absolute stereochemistries of the C-2 and C-5 in (3*R*)-Ahoa were, therefore, determined to be 2*S* and 5*R* as shown in **2**.

Thus, these results led to the complete structure of nostophycin (**1**) as shown in **1**, and it is structurally characterized as a 22-membered cyclic peptide composed of seven amino acid residues including a β-amino acid and two D-amino acids. The structure of **1** including the stereochemistry is quite similar to the partial structure

Table 2. Comparison of ^{1}H Chemical Shifts at the Lactone Moiety between Lactone 3 from Nostophycin and TriAc-Ahda Lactone from Sytonemin A in $CDCl_3$

	^{1}H (mult; *J*, Hz)		
posn	lactone **3**	lactone **3** (irr; H-4, 2.03)[a]	triAc-Ahda lactone[b]
2	5.62 (d; 9.9)	5.62 (d; 9.9)	5.09 (d; 11.2)
3	4.03 (m)	4.03 (ddd; 10.2, 9.9, 5.9)	4.40 (m)
NH	5.78 (br d; 5.9)	5.78 (br d; 5.9)	5.58 (br d; 7.4)
4	2.18 (−[c])	2.18 (−[c])	2.35 (dt; 13.8, 4.0)
	2.03 (ddd, 15.4, 3.7, 3.4)	irradiation	1.65 (q; 12.4)
5	4.69 (m)	4.69 (ddd; 11.7, 4.8, 2.2)	4.41 (m)
6	1.78 (m)	1.78 (m)	1.31−1.60
	1.58 (m)	1.58 (m)	

[a] ^{1}H homodecoupling NMR spectral data irradiated at the H-4 signals (2.03 ppm). [b] ^{1}H NMR spectral data from ref 21. [c] Not determined due to overlapping acetyl proton signals.

of 34-membered cyclic peptides possessing α-hydroxy-β-amino acid, scytonemin A, and schizotrin A, which were isolated from cyanobacteria, *Scytonema* and *Schizotrix* sp., respectively.[21,25] Although these peptides showed activity against a wide spectrum of bacteria and fungi, **1** showed no activity against them but was weakly cytotoxic.[26]

In the present study, we investigated the secondary metabolites produced by the toxic cyanobacterium *Nostoc* sp. strain 152 in connection with a study for the elucidation of the biosynthetic relationship between hepatotoxic peptides and other types of peptides from cyanobacteria. A novel cyclic peptide, nostophycin (**1**), was isolated together with microcystins and has a characteristic structure composed of a β-amino acid, Ahoa, two D-amino acids, and the four usual amino acids. As mentioned in the Introduction, there are many peptides produced by terrestrial cyanobacteria in addition to microcystins and nodularin. Nostophycin is very similar to microcystins, because both have a β-amino acid and two D-amino acids. In consideration of the biosynthesis of these peptides, this strain is interesting and it required further study, particularly a genetic study.[27]

Experimental Section

General Aspects. Optical rotations were recorded at 27 °C at the sodium D line. FABMS and HRFABMS spectra were obtained using glycerol as the matrix. NMR spectra were measured at 600 MHz for the ^{1}H and at 150 MHz for the ^{13}C in DMSO-d_6. The ^{1}H and ^{13}C chemical shifts are referenced to the solvent peaks (^{1}H, 2.49 ppm, and ^{13}C, 39.5 ppm, in DMSO-d_6). Product ion spectra were taken using a JMS-HX110A/HX110A tandem ADS instrument: ion source, FAB; ion mode, positive; matrix, glycerol/NBA (3:1); accelerating voltage, 10 kV; gun voltage, 6 kV; emission current, 5 mA; floating voltage, 8 kV. ESI mass spectra for LC/MS were measured on a TSQ7000 mass spectrometer using a HP1050 HPLC system. All chemicals and solvents were of analytical grade.

Materials. *Nostoc* sp. strain 152 (isolation and strain history described in ref 10) was cultivated in the defined inorganic nutrient culture medium, called Z8, minus its normal

(26) Nostophycin (**1**) showed no activity (20 μg/mL) against *Aspergillus niger*, *Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. At 10 μg/mL, **1** showed 40% growth inhibition against the lymphocytic mouse leukemia L1210 cells.

(27) Dittman, E.; Neilan, B. A.; Erhard, M.; Döhren, H.; Börner, T. *Mol. Microbiol.* **1997**, *26*, 779−787.

concentration of nitrogen ingredients. Cells were harvested after 2 weeks of cultivation and freeze-dried.

Isolation. Dried cells (5.4 g) were extracted three times with 0.5% AcOH(aq) (400 mL) for 30 min while stirring. The combined extracts were centrifuged at 9000 rpm for 30 min, and the supernatant was applied to a preconditioned ODS silica gel cartridge (20 g, Chromatorex ODS) after the filtration on a glass microfiber filter (GF/C). The cartridge was rinsed with water (200 mL) and 20% MeOH(aq) (200 mL) and then eluted with MeOH (400 mL) to give a fraction containing microcystins and nostophycin (**1**). The fraction (72.7 mg) was separated to give **1** (12.0 mg) using the following chromatography: silica gel [Silica gel 60 (230−400 mesh)] using AcOEt/*i*-PrOH/H_2O (4:3:7, upper layer), $CHCl_3$/MeOH/H_2O (65:15:5, lower phase), and TOYOPEARL HW-40F (890 × 11 mm i.d.; flow rate, 0.3 mL/min; detection, UV 230 nm) using MeOH.

Nostophycin (1): amorphous powder; $[\alpha]^{27}_D$ −27.1° (*c* 0.100, MeOH); positive FABMS (glycerol) *m*/*z* 889 $[M + H]^+$, *m*/*z* 871 $[M + H - H_2O]^+$, negative FABMS (glycerol) *m*/*z* 887 $[M - H]^-$; HRFABMS *m*/*z* 889.4825 $[M + H]^+$, calcd for $C_{46}H_{65}N_8O_{10}$, 889.4823; FAB MS/MS (889) *m*/*z* 871, 853, 832, 817, 775, 769, 748, 741, 668, 601, 543, 832, 475, 447, 390, 347, 302, 301; ^1H and ^{13}C NMR (see Table 1); TLC R_f 0.63 and 0.38 [silica gel (Silica gel 60 F_{254}), AcOEt/*i*-PrOH/H_2O (4:3:7, upper layer) and $CHCl_3$/MeOH/H_2O (65:15:5, lower phase); detection, iodine vapor].

Preparation of FDLA Derivatives. A 100 μg of nostophycin was hydrolyzed at 110 °C for 14 h with 200 μL of 1.5 M HCl. This solution was divided into two portions, and each portion was derivatized with L- or D-FDLA. Each solution was then evaporated to dryness, and the residue was dissolved in 50 μL of water. To each amino acid solution was added 20 μL of 1 M sodium bicarbonate, and then 100 μL of 1% L- or D-FDLA in acetone. These solutions were vortexed and incubated at 37 °C for 60 min. These reactions were quenched by the addition of 20 μL of 1 M HCl. After dilution with 210 μL of acetonitrile, 4 μL of the L-FDLA derivative and an equal mixture of the L- and D-FDLA derivatives were analyzed by ESI LC/MS.

ESI LC/MS Conditions. The separation of the L- and DL-FDLA derivatives of nostophycin was performed on a Develosil ODS-HG-5 (150 × 2.0 mm i.d.) column maintained at 40 °C using acetonitrile−water containing 0.01 M TFA as the mobile phase under a linear gradient elution mode (acetonitrile, 30−70%, 40 min) at a flow rate of 0.2 mL/min. All mass spectra were acquired using Q1 as the scanning quadrupole. The ESI voltage was 4.5 kV with the auxiliary and sheath gas nitrogen pressure set at 5 units and 60 psi, respectively, and the capillary was heated to 250 °C. A mass range of *m*/*z* 300−1000 was covered with a scan time of 1.5 s, and data were collected in the negative ion mode using an electron multiplier voltage of 1200 V.

Preparation of 3. A solution of 3.0 mg of nostophycin in 500 μL of 1.5 M HCl was refluxed for 16 h. After the solution was cooled to room temperature, 5 mL of water was added and the new solution was evaporated to dryness under reduced pressure. The hydrolysate was applied to a preconditioned ODS silica gel cartridge (2 g, Sep-Pak Vac) after being dissolved in 5 mL of water. The cartridge was rinsed with water (20 mL) and then eluted with 50% MeOH (20 mL) to give Ahoa, **2**: FABMS (glycerol), *m*/*z* 268 $[M + H]^+$, *m*/*z* 266 $[M - H]^-$; TLC R_f 0.75 [silica gel, $CHCl_3$/MeOH/H_2O (30:20:4); detection, ninhydrin]. The obtained Ahoa was dissolved in 1 mL of pyridine, 1 mL of acetic anhydride was added to the solution, and the mixture was stirred overnight at room temperature. After the standard workup, the residue was purified by HPLC on a COSMOSIL 5C_{18}-AR-II column (150 × 4.6 mm i.d.) with UV detection at 210 nm using 0.05% TFA(aq)/CH_3CN (63:37) as the eluent. At a flow of 1.0 mL/min, the major fraction was eluted at 18.4 min which gave, after evaporation, 0.5 mg of pure lactone **3**. The treatment of **3** using MeOH readily gave the diacetyl-Ahoa methyl ester: HPLC, retention time, 10.3 min; FABMS (glycerol), *m*/*z* 366 $[M + H]^+$.

Compound **3** had the following properties: FABMS (glycerol) *m*/*z* 334 $[M + H]^+$, *m*/*z* 332 $[M - H]^-$; HRFABMS *m*/*z* 334.1646 $[M + H]^+$, calcd for $C_{18}H_{24}NO_5$, 334.1654; ^1H NMR ($CDCl_3$) δ 5.62 (d, J = 9.9 Hz, H-2), 4.03 (m, H-3), 2.18 (m, H_{ax}-4), 2.03 (ddd, J = 15.4, 3.7 and 3.3 Hz, H_{eq}-4), 4.69 (m, H-5), 1.78 (m, H-6), 1.58 (m, H-6′), 1.86 (m, H-7), 1.76 (m, H-7′), 2.64 (m, 2H, H-8), 7.1−7.3 (m, 5H, Ph), 5.78 (br d, J = 5.9 Hz), 1.96 (s, Ac), 2.18 (s, Ac); ^1H homodecoupling NMR (irradiation: δ 2.03 (H_{eq}-4), $CDCl_3$) δ 4.03 (ddd, J = 10.2, 9.9 and 5.9 Hz, H-3), 4.69 (ddd, J = 11.7, 4.8 and 2.2 Hz, H-5).

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Supporting Information Available: ^1H, ^{13}C, COSY, HSQC, HMBC, NOESY, and MS/MS spectra for **1** and ^1H, ^1H homodecoupling, COSY, and NOESY spectra for **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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