Ferintoic Acids A and B, New Cyclic Hexapeptides from the Freshwater Cyanobacterium *Microcystis aeruginosa*

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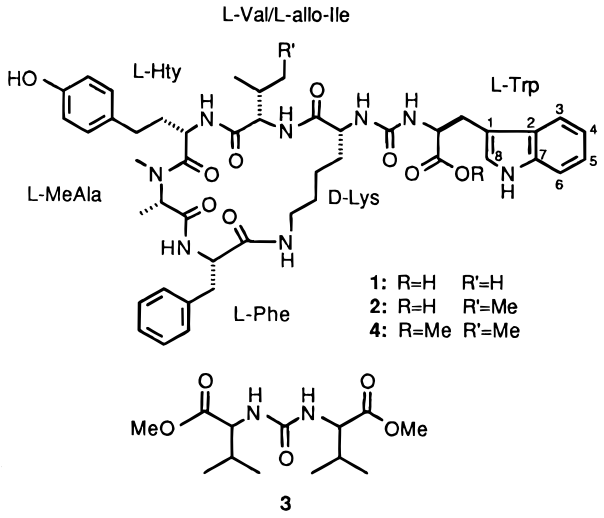
Received October 31, 1995[⊗]

The cyclic hexapeptides ferintoic acids A (**1**) and B (**2**) have been isolated from cells of *Microcystis aeruginosa* harvested during a natural bloom of the toxic cyanobacterium. The structures of **1** and **2** were determined by a combination of spectroscopic analysis and chemical degradation.

Introduction

Freshwater cyanobacteria belonging to the genus *Microcystis* are a rich source of biologically active peptides. The microcystins, a family of more than 45 closely related hepatotoxic cyclic heptapeptides, are the best known examples.[1,2] Microcystins produced by natural blooms of *Microcystis* species in freshwater lakes and drinking water reservoirs are responsible for the deaths of livestock and wild animals[1,3] and represent a potential threat to the health of humans.[4] Recently, it has been shown that the microcystins are some of the most potent *in vitro* inhibitors of protein phosphatases-1 and -2A ($IC_{50} \approx 0.1$ nM) currently known.[8,9] Microcystins are also extremely potent tumor promoters that act by the "okadaic acid pathway" involving *in vivo* inhibition of PP-1 and PP-2A.[10] Several other peptides that have enzyme inhibitory activities have also been isolated from cultures and natural blooms of *Microcystis* species. These include the following: microginin, an angiotensin-converting enzyme inhibitor;[11] micropeptins A and B, plasmin and trypsin inhibitors;[12] aeruginosin 298-A, a thrombin and trypsin inhibitor;[13] and aeruginosins 98-A and -B, which are also trypsin inhibitors.[14]

Cyanobacterial blooms are common summer events in freshwater lakes and reservoirs located in the prairie provinces of Canada. In August of 1991, a dense cyanobacterial bloom occurred in Little Beaver Lake, the drinking water reservoir for the town of Ferintosh, Alberta. Microscopic examination of cyanobacterial cells collected from the lake during the bloom showed that it was essentially an unialgal growth of *Microcystis aeruginosa* Kütz (Chroococcaceae). The density of the Little Beaver Lake bloom made it possible to collect many tens of kilograms wet weight of *M. aeruginosa* cells. As part of our ongoing interest in the distribution and environmental impact of microcystin/nodularin hepatotoxic peptides in marine[15−18] and freshwater habitats,[19] we have undertaken a detailed chemical examination of the methanol extract obtained from the *M. aeruginosa* cells collected from the Little Beaver Lake bloom. The major constituents isolated from the extracts were microcystin-LR, its $\Delta^{6,7}$ geometric isomer, and microcystin-LA.[1] A number of previously unreported microcystins, such as the very lipophilic analog microcystin-LL,[19] and the two novel cyclic hexapeptides, ferintoic acids A (**1**) and B (**2**), were isolated as very minor components of the extract. Details of the isolation and structure elucidation of ferintoic acids A (**1**) and B (**2**) are presented below.



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Results and Discussion

M. aeruginosa was harvested from Little Beaver Lake using a plankton net towed with a small boat. Freshly collected cells were lyophilized, and the dry cells were repeatedly extracted with MeOH. The MeOH extracts were combined, concentrated *in vacuo*, and then partitioned between $MeOH/H_2O$ (3:2) and $CHCl_3$. Repeated fractionation of the $MeOH/H_2O$-soluble materials via reversed-phase flash chromatography and reversed-phase HPLC gave samples of pure ferintoic acids A (**1**) and B (**2**).

Ferintoic acid A (**1**) was obtained as a pale yellow optically active glass that gave a $[M + H]^+$ ion in the HRFABMS at *m/z* 867.44172 that was appropriate for a molecular formula of $C_{46}H_{58}N_8O_9$ (ΔM 1.40 ppm). The ^{13}C/APT/HMQC NMR data obtained for **1** identified 42 carbon resonances (4 × CH_3, 8 × CH_2, 17 × CH, 13 × C), indicating that there were elements of symmetry in the molecule. Many of the carbon resonances in the ^{13}C NMR spectrum of **1** were doubled, which was attributed to a slow conformational equilibrium involving some portion of the molecule. Both the ^1H and ^{13}C NMR spectra (Table 1) of **1** had features that suggested a peptide structure. Six resonances that had chemical shifts appropriate for amide or carboxylic acid carbonyls (δ 169.7, 170.7, 170.9, 172.2, 172.5, 174.0 ppm) and six

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[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0163-3864(96)00108-5 CCC: $12.00

Table 1. 500 MHz NMR Data for Ferintoic Acids A (**1**) and B (**2**) and Methyl Ester **4** Recorded in DMSO-d_6

	ferintoic acid A (**1**)				ferintoic acid B (**2**)			ester **4**
atom	δ ^{1}H	δ ^{13}C[a]	HMBC[b]	NOE[c]	δ ^{1}H	δ ^{13}C[a]	ROESY[d]	δ ^{1}H
Trp								
*C*O		174.0				174.0, 173.9		
a	4.42 m	53.2			4.42 m	53.2		4.47 m
b	3.12 dd (14.5, 5)	27.7			3.11 dd (14.3, 4.5)	27.8		3.09 dd (14.5, 6)
b′	3.02 dd (14.5, 6.8)				3.02 dd (14.3, 6.5)			3.02 dd (14.5, 7)
1		109.5, 109.4				109.5		
2		127.4				127.4		
3	7.48 d (7.8)	118.3			7.48 d (7.8)	118.33, 118.31		7.43 d (7.3)
4	6.96 t (7.8)	118.3			6.95 t (7.8)	118.28, 118.27		6.97 t (7.3)
5	7.06−7.03[e]	120.9			7.06−7.03[e]	120.8		7.07−7.04[e]
6	7.32 d (8)	111.3, 111.2			7.32 d (8)	111.3		7.33 d (8)
7		136.0, 135.9				136.0		
8	7.08 s	123.6, 123.4			7.08 s	123.6		7.09 d (2)
COO*H*	12.57 bs				12.25 bs			
COO*Me*								3.54 s
α-N*H*	6.23 d (8)				6.26 d (8.5)		3.93, 6.54	6.38 d (8)
7-N*H*	10.86 s				10.86 s			10.88 s
Urea								
*C*O		157.0, 156.9	6.23, 6.51			157.0		
Lys								
*C*O		172.6, 172.5				172.6		
α	3.94 m	54.5, 54.4			3.93 m	54.6	6.26, 6.86	3.91 m
β	1.60 m	31.8, 31.7			1.60 m	31.7		1.62−1.54[f]
b′	1.60 m				1.60 m			1.62−1.54[f]
γ	1.28 m	20.2			1.29−1.09[f]	20.2		1.25 m
g′	1.11 m				1.29−1.09[f]			1.16 m
δ	1.42 m	28.1			1.43 m	28.0		1.42 m
d′	1.42 m				1.43 m			1.42 m
ϵ	3.57 m	38.2, 38.1			3.56 m	38.2		3.57 m
e′	2.79 m				2.78 m			2.78 m
α-N*H*	6.51 d (7)				6.54 d (7)		6.26	6.53 d (7)
ϵ-N*H*	7.12[e]				7.12[e]		4.37	7.13[e]
Phe								
*C*O		170.7, 170.6	4.36, 2.79			170.7		
α	4.36 m	54.9, 54.8			4.37 m	54.9	7.12	4.38 m
β	3.29 m	37.4			3.30 m	37.5		3.31[e]
b′	2.74 t (13.5)				2.73 t (13)			2.74 t (13.5)
1		138.2				138.3		
2,6	7.04 d (7)	128.8			7.04 d (7.3)	128.8		7.05 d (7.5)
3,5	7.18 t (7)[g]	128.3			7.18 t (7.3)[g]	128.2		7.18 t (7.5)[g]
4	7.13 t (7)[g]	126.0			7.15 t (7.3)[g]	126.1		7.15[e]
α-N*H*	8.65 d (10)			4.78	8.60 d (10)		4.78	8.60 d (10)
N-Me-Ala								
*C*O		169.8, 169.7	4.78, 1.05, 8.65			169.8		
α	4.78 q (6.5)	54.2		8.65	4.78 q (6.5)	54.2	4.71, 8.60	4.79 q (7)
Me	1.05 d (6.5)	13.8			1.05 d (6.5)	13.8		1.06 d (7)
N*Me*	1.76 s	27.0			1.77 s	27.0	4.71	1.78 s
Htyr								
*C*O		170.9				170.9		
α	4.71 m	48.7, 48.6			4.71 m	48.8	4.78, 1.77	4.72 m
β	1.85 m	33.2, 33.2			1.86 m	33.1		1.86 m
b′	1.71 m				1.68 m			1.69 m
γ	2.62 tm (12.4)	30.5			2.62 tm (10.5)	30.5		2.59 m
g′	2.42 m				2.44 m			2.43 m
1		131.0				131.0		
2,6	7.00 d (7.5)	129.0			6.99 d (8)	129.0		6.99 d (8.5)
3,5	6.66 d (7.5)	115.1; 115.0			6.66 d (8)	115.1		6.66 d (8.5)
4		155.5				155.5		
α-N*H*	8.91 d (0.5)			3.89	8.90 d (4.5)		4.06	8.90 d (4.5)
4O*H*	9.16 s				9.16 s			9.15 s
4O*Me*								
Val/Ile								
*C*O		172.2, 172.1				172.1		
α	3.89 t (7.2)	58.1−57.9 br		8.91	4.06 t (7.4)	56.4	8.90	4.09 t (7.7)
β	1.92 m	30.0, 29.9			1.76 m[e]	36.2		1.77[e]
Me/Ile *γ*	1.02 d (6.5)	19.2			1.62 m[e]	25.0		1.62−1.54[f]
Ile *γ*′					1.14 m			1.13 m
Me′/*δ*-Me	0.92 d (6.5)	18.9			0.88 t (8)	11.5		0.88 t (7)
Ile *β*-Me					0.87 d (7)	14.9		0.87 d (6.5)
α-N*H*	6.92 d (7.2)				6.86 d (7.4)		3.93	6.86 d (7.7)

[a] In **1** the majority of the carbons appeared as two resonances (1:1−1:0). In **2** many resonances were doubled. [b] Chemical shifts of proton resonances correlated to the carbon resonance listed in the δ ^{13}C column. [c] Chemical shifts of proton resonances showing positive difference NOEs when the proton resonance in the δ ^{1}H column is selectively irradiated. [d] Chemical shifts of proton resonances that show ROESY correlations to the proton resonances in the δ ^{1}H column. [e] Obscured region. [f] Overlapping resonances. [g] A distorted triplet.

resonances that had chemical shifts appropriate for amino acid α-carbons (δ 48.7, 53.2, 54.2, 54.5, 54.9, 58.1 ppm) were observed in the ^{13}C NMR spectrum, suggesting a hexapeptide. COSY, HMQC, and HMBC data showed that ferintoic acid A (**1**) contained tryptophan, phenylalanine, lysine, valine, and homotyrosine residues. COSY correlations between a methyl doublet at δ 1.05 (J = 6.5 Hz) and a methine quartet at δ 4.78 (J = 6.5 Hz) indicated the presence of an alanine residue. The absence of coupling between the alanine α-methine proton at δ 4.78 and a NH proton indicated that the alanine residue was N-substituted. An HMBC correlation between the alanine α-methine proton resonance (δ 4.78) and a methyl carbon resonance at δ 27.0 that was correlated in the HMQC spectrum to a proton resonance at δ 1.76 (s, 3H) demonstrated that the alanine residue was N-methylated.

Hydrolysis of ferintoic acid A (**1**) with 6 N HCl followed by Marfey's HPLC[20] and chiral GC[21,22] analyses of the amino acids in the hydrolysate confirmed the presence of L-phenylalanine, L-valine, D-lysine, and L-*N*-methylalanine. Tryptophan was missing, apparently not surviving the hydrolysis conditions, and an unidentified component was attributed to the homotyrosine. Hydrazinolysis of ferintoic acid A (**1**), using Akabori's method[23] for the characterization of carboxyl-terminal amino acids, liberated tryptophan that was shown to have the L configuration by both Marfey's HPLC[20] and chiral GC[21,22] analyses. The stereochemistry of the homotyrosine residue in **1** was determined by partial racemization with 4-(dimethylamino)pyridine (DMAP) according to literature procedures.[24−27] Previous work by Namikoshi *et al.*[24−27] had shown that the pentafluoropropionamide isopropyl ester derivatives of D-homotyrosine and D-tyrosine eluted faster off a chiral GC column than did the corresponding derivatives of L-homotyrosine and L-tyrosine, respectively. Since both enantiomers of tyrosine were available as standards, L-tyrosine was treated under identical racemization conditions as a control for this experiment. Thus, heating L-tyrosine at 110 °C for 22 h in a 1:1 mixture of H_2O/MeOH containing DMAP produced a 1:30 D:L ratio as determined by chiral GC analysis.[21,22] As reported by Namikoshi *et al.*,[24−27] the D-tyrosine derivative eluted first from the chiral GC column. Treatment of the ferintoic acid A (**1**) HCl hydrolysate mixture under identical racemization conditions followed by chiral GC analysis showed that homotyrosine had been converted into a 1:10 mixture of D (early-eluting GC derivative) and L (late-eluting GC derivative) enantiomers, respectively. Therefore, we concluded that the homotyrosine residue in ferintoic acid A (**1**) had the L configuration.

The six amino acid residues identified in ferintoic acid A (**1**) accounted for all eight of the nitrogen atoms and 45 of the 46 carbon atoms in the molecular formula. Akabori hydrazinolysis[23] liberated only tryptophan, indicating that it was present as a carboxyl-terminal residue and that the carbonyl functionalities of the other five amino acids were part of amide bonds. Thus, the six amino acid residues in ferintoic acid A (**1**) also accounted for seven of the eight oxygen atoms, all of the 58 hydrogen atoms, and 20 of the 22 sites of unsaturation demanded by the molecular formula. A resonance at δ 157.0 was the only resonance in the ^{13}C NMR spectrum that could not be assigned to one of the six component amino acids. This carbon resonance (δ 157.0) showed HMBC correlations to both the tryptophan α-amino proton resonance (δ 6.23) and the lysine α-amino proton resonance (δ 6.51), suggesting that there was a urea linkage between the tryptophan and lysine α-amino nitrogens as shown in **1**. A literature precedent for a urea linkage between the α-amino groups of two amino acids was provided by the two Okinawan sponge hexapeptides konbamide[28] and keramamide A.[29] The chemical shift assignments of the urea carbonyl in these two compounds was δ 157.5 and 156.8 ppm, respectively. In addition, in the course of this study we synthesized the model compound urea *N*,*N*′-divalinylmethyl ester (**3**) in which the urea carbonyl ^{13}C NMR chemical shift was assigned as δ 157.7 ppm. These three assignments were in excellent agreement with the chemical shift of the proposed urea carbonyl in ferintoic acid A (**1**). Since the urea carbonyl and six amino acid fragments accounted for only 21 of the 22 sites of unsaturation required by the molecular formula, it was apparent that **1** also contained an additional ring.

HMBC correlations (Table 1) established amide linkages between the *N*-methylalanine carbonyl and the phenylalanine amino nitrogen and between the phenylalanine carbonyl and the lysine ϵ-amino nitrogen. Difference NOEs (Table 1) demonstrated the presence of an amide bond between the homotyrosine amino nitrogen and the valine carbonyl. The final two amide bonds in ferintoic acid A (**1**), between the valine amino nitrogen and the lysine carbonyl and between the *N*-methylalanine nitrogen and the homotyrosine carbonyl, were required to complete the final ring demanded by the molecular formula of **1**. Some very weak and poorly resolved correlations observed in the HMBC spectrum of **1** were consistent with these two final amide linkages, but they did not provide unambiguous proof for the two bonds. However, analysis of the ROESY data obtained for ferintoic acid B (**2**) (Table 1) confirmed these connectivities.

Ferintoic acid B (**2**) was also obtained as a pale yellow optically active glass with a molecular formula of $C_{47}H_{60}N_8O_9$, which differed from the molecular formula of ferintoic acid A (**1**) simply by the addition of CH_2. Treatment of ferintoic acid B (**2**) with MeI in the presence of K_2CO_3 afforded the monomethyl ester **4** (OMe δ 3.54, ^1H NMR Table 1) which provided chemical evidence for a free carboxylic acid functionality. The HRFABMS of **4** showed an intense peak at m/z 863.44536 attributed to a stable hydantoin ion generated by loss of methanol between the carboxy group of tryptophan and the α-NH of lysine, which corroborated the presence of the ureido bond.[28]

Acid hydrolysis of ferintoic acid B (**2**) followed by amino acid analysis[20−22] and examination of the ^1H, ^{13}C, COSY, HMQC, and HMBC NMR data (Tables 1) of **2** showed that the valine of ferintoic acid A (**1**) had been replaced with isoleucine in ferintoic acid B (**2**). Analysis of the ROESY data (Table 1) provided the complete sequence of amide linkages shown for ferintoic acid B (**2**).

Through a combination of both Marfey's HPLC[20] and chiral GC analyses[21,22] it was possible to determine the complete configuration of the isoleucine in the hydrolysate mixture of ferintoic acid B (**2**) as L-allo (in Marfey's HPLC method the L- and L-alloisoleucine are unresolv-

able as are the D- and D-alloisoleucine and in the GC method the L-allo- and D-isoleucine are unresolvable). Hence, the complete structures of ferintoic acids A and B were established as shown in **1** and **2**.

Ferintoic acids A (**1**) and B (**2**) are related to the recently reported metabolites anabaenopeptins A and B[30] and oscillamide Y[31] in which L-tyrosine or L-arginine residues replace the L-tryptophan residue of ferintoic acids A (**1**) and B (**2**), and L-valine and L-alloisoleucine appear to be interchangeable as is the case with **1** and **2**. The NMR assignments reported for the anabaenopeptins A and B and oscillamide Y are completely consistent with the NMR assignments given in Table 1 for metabolites **1** and **2**.

Ferintoic acids A (**1**) and B (**2**) represent two additional members of a new class of cyclic peptide metabolites isolated from microcystin producing cyanobacteria. The present work represents the first example of the isolation of members of this class of hexapeptides from a natural cyanobacterial bloom. One member of this peptide family, oscillamide Y, has been reported to strongly inhibit chymotrypsin activity at 1.0×10^{-5} M.[31] Ferintoic acids A (**1**) and B (**2**) showed no inhibition of chymotrypsin activity at concentrations up to 1.5×10^{-4} M.

Experimental Section

General Experimental Procedures. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer. ^{1}H chemical shifts are referenced to the residual DMSO-d_6 signal (δ 2.49 ppm), and ^{13}C chemical shifts are referenced to the DMSO-d_6 solvent peak (δ 39.5 ppm). Low- and high-resolution FABMS were recorded on a Kratos Concept II HQ mass spectrometer with xenon as the bombarding gas and a thioglycerol sample matrix. Low- and high-resolution EIMS were recorded on Kratos AEI MS-59 and AEI MS-50 mass spectrometers, respectively. The UV spectra were recorded on a Bausch and Lomb Spectronic 2000 instrument. Optical rotations were measured using a JASCO J-710 spectrophotometer. Melting points were taken using a Fisher-Johns apparatus, and the reported values are uncorrected.

Merck Type 5554 silica gel plates and Whatman MKC18F plates were used for analytical thin layer chromatography. Merck silica gel G60 (230−400 mesh) was converted to C_{18} reversed-phase material according to Kühler and Lindsten[32] and used for reversed-phase flash chromatography.

Reversed-phase HPLC purifications were performed on a Waters 600E system controller liquid chromatograph attached to a Waters 486 tunable absorbance detector. HPLC analysis of amino acid derivatives employed a Waters 600E system controller and a Waters 994 programmable photodiode array detector. All solvents used for HPLC were Fisher HPLC grade.

Chiral capillary GC analysis of amino acid derivatives was carried out on a Hewlett-Packard 5880A gas chromatograph using a Chirasil-Val column[21] (0.25 mm × 50 m, Alltech, Deerfield, IL) and helium as carrier gas (flow rate: 1 mL/min; split ratio: 40:1). The program rate for the amino acid derivatives was 90° (4 min) to 220° (27.5 min) at 4°/min. The other conditions were as follows: injector temperature 250°; detector temperature 275°; makeup gas N_2 (30 mL/min).

Algal Material. *M. aeruginosa* was collected using a plankton net during a bloom in Little Beaver Lake, near Ferintosh, Alberta, Canada, in August 1991. Microscopic analysis showed that the bloom was an almost unialgal culture of *M. aeruginosa*. A voucher sample of the algal cells has been deposited in the North East Pacific Culture Collection housed in the Oceanography Department at UBC.

Purification of Ferintoic Acids A (1) and B (2). The freeze-dried algal cells (163 g) were extracted repeatedly with MeOH (2 × 500 mL), and the combined extracts were concentrated *in vacuo* before being partitioned between $CHCl_3$ (5 × 200 mL) and MeOH/H_2O (3:2, 1000 mL). The combined $CHCl_3$ extracts were back extracted with fresh MeOH/H_2O (3:2, 3 × 200 mL). The combined aqueous extracts were reduced to dryness *in vacuo* to yield 27.6 g of brown oil. Fractionation of the oil by C_{18} reversed-phase flash chromatography (gradient elution: H_2O to MeOH) gave a fraction (1.2 g, eluting with 3:7 H_2O/MeOH) that contained a complex mixture of microcystins as well as ferintoic acids A (**1**) and B (**2**). This complex mixture was fractionated via repetitive C_{18} reversed-phase HPLC. Preparative HPLC using a Rainin Dynamax-60A column with 3:1 MeOH/(0.05% TFA/H_2O) as eluent gave a mixture of **1**, **2**, and hydrophilic microcystins (including-LR). Recycling the fraction containing **1** and **2** (eluent: 13:7 MeOH/(0.05% TFA/H_2O)) gave one fraction containing principally **1** and the geometric isomer of microcystin-LR and a second more hydrophobic fraction that contained **2**. Subsequent semipreparative HPLC, using a Whatman Magnum-9 Partisil 10 ODS-3 column, with 11:9 MeOH/(0.05% TFA/H_2O) as eluent followed by 47:53 MeCN/(0.05% TFA/H_2O) as eluent, gave pure ferintoic acid A (**1**) (8.0 mg), and from the more hydrophobic fraction with 13:7 MeOH/(0.05% TFA/H_2O) as eluent a fraction was obtained that was further chromatographed using 1:1 MeCN/(0.05% TFA/H_2O) as eluent to give pure ferintoic acid B (**2**) (3.7 mg).

Ferintoic acid A (1): isolated as a pale yellow glass; $[\alpha]^{25}_D$ −13.8° (*c* 0.034, MeOH); UV (MeOH) λ max 222 (ϵ 19 046), 280 (ϵ 548) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; positive ion HRFABMS $[M + H]^+ m/z$ 867.441 72 ($C_{46}H_{59}N_8O_9$ ΔM 1.40 ppm).

Ferintoic acid B (2): isolated as a pale yellow glass; $[\alpha]^{25}_D$ −81.7° (*c* 0.006, MeOH); UV (MeOH) λ max 224 (ϵ 20 172), 280 (ϵ 3668) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; positive ion HRFABMS $[M + H]^+ m/z$ 881.456 70 ($C_{47}H_{61}N_8O_9$ ΔM 0.62 ppm).

Preparation of Urea *N,N′*-Divalinylmethyl Ester (3). L-Valine methyl ester hydrochloride (101 mg, 604 μmol) and 1,1-carbonyldiimidazole (49 mg, 301 μmol) were dissolved in 3 mL of DMF. The reaction mixture was stirred for 16 h, the volume of the resulting solution was reduced *in vacuo* to near dryness, and 10 mL of H_2O was added. The aqueous solution was then extracted with $(Et)_2O$ (4 × 4 mL). The combined $(Et)_2O$ extracts were dried over Na_2SO_4, filtered and concentrated in vacuo to yield 77 mg of clear glassy material. The glass was chromatographed on a Whatman Magnum-9 Partisil 10 ODS-3 reversed-phase column using 2:3 MeCN/(0.05% TFA/H_2O) as eluent to give urea *N,N′*-divalinylmethyl ester (**3**) (64 mg) as clear irregular crystals: mp 138−140 °C; $[\alpha]^{25}_D$ +9.8° (*c* 0.127, MeOH); ^{1}H NMR (500-MHz, DMSO-d_6) δ 6.40 (d, J = 8.3 Hz,

2H), 4.05 (dd, J = 8.3,5.5 Hz, 2H), 3.60 (s, 6H), 1.97 (quintet, J = 6.5 Hz, 2H), 0.85 (d, J = 6.5 Hz, 6H), 0.83 (d, J = 6.5 Hz, 6H) ppm; ^{13}C NMR (125-MHz, DMSO-d_6) δ 173.1 (2 × C), 157.7 (C), 57.8 (2 × CH), 51.5 (2 × CH_3), 30.6 (2 × CH), 19.0 (2 × CH_3), 17.7 (2 × CH_3) ppm; HREIMS $[M]^+$ m/z 288.168 67 ($C_{13}H_{24}N_2O_5$ Dev −0.5 ppm); LREIMS m/z (relative intensity) 288 (M^+, 0.2), 256 (0.2), 229 (10), 197 (10), 182 (4), 154 (6), 143 (7), 115 (31), 98 (24), 88 (24), 72 (100), 68 (29), 55 (45), 41 (34).

Preparation of Methyl Ferintoate B (4). To 0.8 mg of ferintoic acid B (**2**) (0.91 μmole) was added ~3 mL of DMF (freshly distilled) and 0.5 mg of K_2CO_3. The solution was stirred for 2 h, 100 μL of MeI (passed over dry basic alumina) was added, and the reaction was stirred for a further 15 h. The volume of the solution was reduced *in vacuo* to ~0.5 mL. The methyl ester adduct of **2** was then purified on reversed-phase HPLC, using a Whatman Magnum-9 Partisil 10 ODS-3 column with 1:1 MeCN/H_2O as eluent, to yield 0.6 mg (0.67 μmol) of pure methyl ferintoate B (**4**).

Methyl ferintoate B (4): white powder; ^{1}H NMR, see Table 1; positive ion HRFABMS $[M + H]^+$ m/z 895.467 89 ($C_{48}H_{63}N_8O_9$ ΔM −4.37 ppm), $[M + H - MeOH]^+$ m/z 863.445 369 ($C_{47}H_{59}N_8O_8$ ΔM −0.26 ppm).

Hydrolysis of Ferintoic Acids A (1) and B (2). Purified metabolites **1** (500 μg, 0.577 μmol) and **2** (500 μg, 0.568 μmol) were hydrolyzed in 0.5 mL of 6 N HCl (freshly distilled, constant boiling HCl) at 108 °C with stirring for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixtures were evaporated to dryness and traces of HCl removed from the residual hydrolysates by repeated evaporation from H_2O (3 × 0.4 mL). The resultant hydrolysate mixture obtained from **2** was split into three equal portions.

Hydrazinolysis of Ferintoic Acid A (1).[23] Purified ferintoic acid A (**1**) (480 μg, 0.554 μmol) and 0.5 mL of anhydrous hydrazine were heated at 125 °C with stirring for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. After cooling, the excess hydrazine was evaporated *in vacuo* over H_2SO_4, and traces were removed from the residue by repeated evaporation from H_2O (3 × 0.4 mL). The resultant mixture was divided into two portions. One portion was derivatized with Marfey's reagent[20] and the other derivatized for chiral GC analysis,[21,22] as described below.

Racemization of L-Tyrosine and the Homo-Tyrosine in the Acid Hydrolysate of Ferintoic Acid B (2).[24−27] L-Tyrosine (700 μg, 3.86 μmol) and 4-(dimethylamino)pyridine (DMAP, 233 μg, 1.91 μmol) in MeOH (250 μL) and H_2O (250 μL) were heated in a threaded Pyrex tube sealed with a Teflon screw cap at 110 °C for 22 h. The mixture was evaporated to dryness under N_2 and derivatized for chiral GC analysis[21,22] as described below. The GC chromatography showed about a 1:30 D:L ratio.

One third of the dried acid hydrolysate of ferintoic acid B (**2**), obtained above, was dissolved in MeOH and H_2O (each 250 μL). DMAP (177 μg, 1.45 μmol) was added, and the mixture was heated at 110 °C for 22 h, evaporated to dryness, and then derivatized for GC analysis.[21,22]

Derivatization of Amino Acids with Marfey's Reagent and HPLC Analysis.[20] To a 0.5 mL vial containing 2.0 μmol of the pure amino acid standard in 40 μL of H_2O was added 2.8 μmol of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDAA) in 80.0 μL of acetone followed by 20 μL of 1 N $NaHCO_3$. The mixture was heated for 1 h at 40 °C. After the mixture was cooled to RT, 10 μL of 2 N HCl was added, and the resulting solution was filtered through a 4.5 μm filter and stored in the dark until HPLC analysis.

The hydrolysate and half the residual hydrazinolate of ferintoic acid A (**1**) in 69 and 33 μL of H_2O were reacted with 5.66 and 2.72 μmol of FDAA in 162 and 78 μL of acetone, respectively, as described above. In addition, one third of the dried acid hydrolysate of **2** in 23 μL of H_2O was reacted with 1.86 μmol of FDAA in 53 μL of acetone. In each case, a 5 μL aliquot of the resulting mixture of FDAA derivatives was analyzed by reversed-phase HPLC. An Alltech Econosil C_{18} 5 μm column with a linear gradient of (A) 9:1 triethylammonium phosphate (50 mM, pH 3.0)/MeCN and (B) MeCN with 0% B at start to 40% B over 55 min (flow rate 1 mL/min) was used to separate the FDAA derivatives with UV detection at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time and photodiode array UV spectrum with that of the FDAA derivative of the pure amino acid standard and by coinjection. In all cases, a peak at 42.63 min was observed which was attributed to excess FDAA.

PFP−IPA Derivatization of Amino Acids and Chrial GC Analysis.[21,22] Acetyl chloride (1.25 mL) was slowly added to 2-propanol in an ice bath. The resulting isopropyl acetate solution (250 μL) was distributed among the following samples: half the dried hydrazinolate residue of ferintoic acid A (**1**), a third of the dried acid hydrolysate of ferintoic acid B (**2**), the DMAP-treated hydrolysate of **2**, the DMAP-treated L-Tyr, and 1−2 mg of the pure dry amino acid standard, each in a 5 mL screw-capped vial. The vials were heated to 110 °C for 45 min. Excess reagent was removed under a stream of N_2. After the vials were cooled, in an ice bath, CH_2Cl_2 (250 μL) and pentafluoropropyl anhydride (100 μL) were added and the vials heated to 110 °C for 15 min. Excess reagent was evaporated under dry N_2, and CH_2Cl_2 (200 μL) was added to each vial. A 2 μL aliquot of each sample was analyzed by chiral GC.

Acknowledgment. The authors would like to thank David Graham, Fisheries and Oceans Inspections and Special Services Branch, Burnaby, B.C., for testing the ferintoic acids for mouse toxicity. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada and the Medical Research Council of Canada.

References and Notes

(1) Rinehart, K. L.; Namikoshi, M.; Choi, B. W. *J. Appl. Phycol.* **1994**, *6*, 159−176.
(2) Namikoshi, M.; Sun, F.; B. Choi, W.; Rinehart, K. L.; Carmichael, W. W.; Evans, W. R.; Beasley, V. R. *J. Org. Chem.* **1995**, *60*, 3671−3679.
(3) Carmichael, W. W. In *Natural toxins: Characterization, pharmacology and therapeutics;* Ownby, C. L., Odell, G. V., Ed.; Pergamon: London, 1988; pp 3−16 and references cited therein.
(4) For circumstantial evidence of human intoxication see refs 5−7.
(5) Sargunar, H.; Sargunar, A. In *Proceedings of the 4th international IUPAC symposium on Mycotoxins and Phycotoxins*, Lausanne, Switzerland; Abstract #610.
(6) Falconer, I. R.; Beresford, A. M.; Runneger, M. T. C. *Med. J. Aust.* **1983**, *1*, 511−514.
(7) Hashimoto, Y. In *Marine toxins and other bioactive marine metabolites;* Japan Scientific Societies Press: Tokyo, 1979; pp 53−55.

(8) Yoshizawa, S.; Matsushima, R.; Watanabe, M. F.; Harada, K.-I.; Ichihara, A.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1990**, *116*, 609−614.
(9) Honkanen, R. E.; Zwiller, J.; Moore, R. E.; Daily, S. L.; Khatra, B. S.; Dukelow, M.; Boynton, A. L. *J. Biol. Chem.* **1990**, *265*, 19401−19404.
(10) Nishiwaki-Matsushima, R.; Ohta, T.; Nishiwaki, S.; Suganuma, M.; Kohyama, K.; Ishiwaka, T.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 420−424.
(11) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 501−504.
(12) Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 8131−8134.
(13) Murakami, M.; Okita, Y.; Matsuda, H.; Okino T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, *35*, 3129−3132.
(14) Murakami, M.; Ishida, K.; Okino,T.; Okita,Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 2785−2788.
(15) de Silva, E. D.; Williams, D. E.; Andersen, R. J.; Holmes, C. F. B.; Allen, T. M. *Tetrahedron Lett.* **1992**, *33*, 1561−1564.
(16) Chen, D. Z. X.; Boland, M. P.; Smillie, M. A.; Klix, H.; Ptak, C.; Andersen, R. J.; Holmes, C. F. B. *Toxicon* **1993**, *31*, 1407−1414.
(17) Andersen, R. J.; Luu, H. A.; Chen, D. Z. X.; Holmes, C. F. B.; Kent, M. L.; LeBlanc, M.; Taylor, F. J. R.; Williams, D. E. *Toxicon* **1993**, *31*, 1315−1323.
(18) Boland, M. P.; Smillie, M. A.; Chen, D. Z. X.; Holmes, C. F. B. *Toxicon* **1993**, *31*, 1393−1405.
(19) Craig, M.; McCready, T. L.; Luu, H. A.; Smillie, M. A.; Dubord, P.; Holmes, C. F. B. *Toxicon* **1993**, *31*, 1541−1549.
(20) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(21) Frank, H.; Nicholson, G. J.; Bayer, E. *J. Chromatogr. Sci.* **1977**, *15*, 174−176.
(22) Alltech amino acid derivatization and GC absolute configuration kit, cat. no. 18093 and 13637.
(23) Akabori, S.; Ohno, K.; Narita, K. *Bull. Chem. Soc. Jpn.* **1952**, *25*, 214−218.
(24) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Sivonen, K.; Carmichael, W. W. *J. Org. Chem.* **1990**, *55*, 6135−6139.
(25) Dhaon, M. K.; Olsen, R. K.; Ramasamy, K. *J. Org. Chem.* **1982**, *47*, 1962−1965.
(26) Harada, K.-I.; Ogawa, K.; Kimura, Y.; Murata, H.; Suzuki, M.; Thorn, P. M.; Evans, W. R.; Carmichael, W. W. *Chem. Res. Toxicol.* **1991**, *4*, 535−540.
(27) Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. *Chem. Res. Toxicol.* **1992**, *5*, 661−666.
(28) Kobayashi, J.; Sato, M.; Murayama, T.; Ishibashi, M.; Wälchi, M. R.; Kanai, M.; Shoji, J.; Ohizumi, Y *J. Chem. Soc., Chem. Commun.* **1991**, 1050−1052.
(29) Kobayashi, J.; Sato, M.; Ishibashi, M.; Shigemori, H.; Nakamura, T.; Ohizumi, Y. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2609−2611.
(30) Harada, K.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett.* **1995**, *36*, 1511−1514.
(31) Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, **36**, 5933−5936.
(32) Kühler, T. C.; Lindsten, G. R. *J. Org. Chem.* **1983**, *48*, 3589−3591.

NP960108L