Radiosumin B, an Unusual Dipeptide from the Cyanobacterium *Microcystis aeruginosa*

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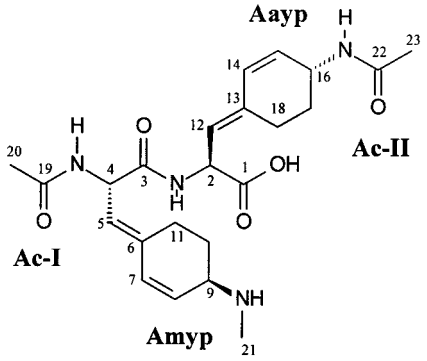
Radiosumin B (**1**), an *N*-methyl dipeptide containing two unusual amino acid residues, was isolated from the cyanobacterium *Microcystis aeruginosa* Kützing. The structure and stereochemical details were elucidated on the basis of 1D and 2D NMR data, MS data, and chemical degradation.

Freshwater cyanobacteria (blue-green algae) have proven to be a prodigious source of novel biologically active secondary metabolites. These compounds include hepatotoxins, such as the microcystins;[1] neurotoxins, such as anatoxin-A;[2] protease inhibitors, such as the cyanopeptolins;[3] and antitumor agents, such as the cryptophycins.[4,5] In 1997 a bloom of cyanobacteria in Pakowki Lake, Alberta, was linked to bird deaths.[6] The predominant cyanobacterium was identified as *Microcystis aeruginosa* Kützing,[7] a species that has often been associated with bird and livestock deaths.[8] There were also minor quantities of akinetes of *Aphanizomenon* sp.[7] During the routine analysis for microcystins, a novel dipeptide, radiosumin B (**1**), containing two unusual amino acid residues, was isolated. Described herein are details of the isolation and structure elucidation of **1**, which was found to be closely related to the known compound radiosumin (**2**), previously isolated from the cyanobacterium *Plectonema radiosum*.[9,10]



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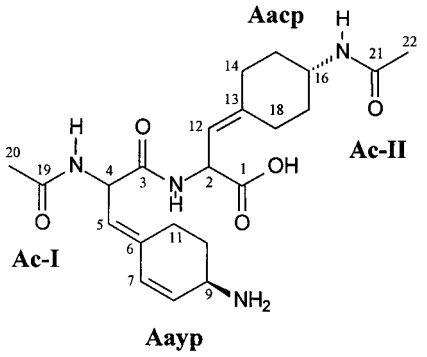

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A microalgal bloom, consisting mainly of the cyanobacterium *Microcystis aeruginosa* Kützing, was collected from Pakowki Lake, Alberta, using zooplankton nets. The cells (100 g) were frozen, lyophilized, and extracted repeatedly with MeOH. The combined MeOH extracts were concentrated in vacuo and partitioned between $CHCl_3$ and H_2O/MeOH (3:2) and the polar extracts reduced to dryness in vacuo to yield 1.82 g of a brown oil. Fractionation of the oil by C_{18} reversed-phase flash chromatography (gradient H_2O to MeOH) yielded a fraction that contained a mixture of radiosumin B and several other unidentified compounds (30 mg). Further purification of this fraction by gel permeation chromatography (LH-20), followed by C_{18} reversed-phase HPLC, provided pure radiosumin B (**1**) (11 mg) as a white optically active solid.

The HRLSIMS spectra of radiosumin B (**1**) gave an $[M + H]^+$ ion corresponding to the molecular formula of $C_{23}H_{33}N_4O_5$, which requires 10 degrees of unsaturation. Analysis of the ^{13}C NMR, DEPT, and HSQC experiments identified three methyl groups, comprising two acyl methyls and another attached to nitrogen, and four methylene groups. In addition, 10 methine carbons, of which six were olefinic, and six quaternary carbons, identified as four carbonyl and two olefinic carbons, were observed (NMR data summarized in Table 1). Five exchangeable ^1H NMR resonances at δ 8.23, 8.38, 8.41, 8.53, and 8.63, which were not correlated to carbons in the HSQC experiment, were identified as either amide or amine resonances. The UV absorption at λ_{max} 239 nm was consistent with the presence of a substituted diene in the molecule. Taken together, the data account for 8 degrees of unsaturation, and hence **1** must contain two rings.

Analysis of the COSY, HSQC, and HMBC spectra for **1** revealed that, in addition to two acetate groups, the molecule contained the known amino acid 2-amino-3-(4-amino-2-cyclohexen-1-ylidene)propionic acid (Aayp),[10] which was identified as follows. The COSY and TOCSY data identified two isolated spin systems. The first consisted of a correlation between the NH resonance at δ 8.38 (NH-2) and the methine resonance at δ 5.15 (H-2), which in turn was correlated to a resonance at δ 5.36 (H-12), thus establishing the N-2/C-2/C-12 connectivity. The second spin system contained a resonance at δ 6.14 (H-14) correlated with resonances at δ 5.69 (H-15), 4.44 (H-16), and 8.23

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Table 1. ^{1}H and ^{13}C Data and COSY and HMBC Correlations for Radiosumin B (**1**)[a]

position		δ_C	δ_H	COSY	HMBC[b]
1		173.8			H-2, 12
2		52.1	5.15 d (9.1)	NH-2, H-12	NH-2
NH-2			8.38 bs	H-2	
3		171.8			H-2, 4, NH-2
4		52.7	5.23 d (9.6)	NH-4, H-5	NH-4
NH-4			8.41 bs	H-4	
5		127.2	5.49 d (9.6)	H-4	H-4, 7
6		137.8			H-7, 8
7		136.6	6.39 dd (10.1, 1.9)	H-8	H-5
8		124.2	5.82 dd (10.0, 2.8)	H-7, 9	
9		55.9	3.87 m	H8, 10, NH-9	H-7, 10, 10′, 11
NH-9			8.53 bs, 8.63 bs	H-9, 21	
10		25.8	1.71 m	H-9, 10′, 11′	H-8
10′			2.21 m	H-10, 11′	
11		23.0	2.47 m	H-10, 11′	H-5, 7, 10′
11′			2.83 m	H-10′, 11	
12		123.0	5.36 d (9.0)	H-2	H-2,
13		140.0			H-14, 15
14		132.6	6.14 dd (10.1, 2.0)	H-15	
15		131.9	5.69 dd (9.8, 3.2)	H-14, 16	
16		46.6	4.44 m	H-15, 17, NH-7	H-14, 17, 17′, NH-16
NH-16			8.23 bs	H-16	
17		29.4	1.56 m	H-16, 17′, 18	H-15
17′			1.93 m	H-17, 18′	
18		23.9	2.34 m	H-17, 18′	H-12, 14, 17′
18′			2.66 m	H-17′, 18	
19	Ac(I)	172.7			H-20, 4, NH-4
20		22.3	1.97 s		
21		30.7	2.72 bs	NH-9	
22	Ac(II)	172.6			H-23, 16, NH-16
23		22.4	1.93 s		

[a] *J* values (in Hz) in parentheses. [b] ^{13}C NMR signal correlating with ^{1}H resonance.

(NH-16). Additional correlations between H-16 and two methylene resonances at δ 1.56 (H-17) and 1.93 (H-17′) and between H-17/H-17′ and the methylene resonances δ 2.34 (H-18) and 2.66 (H-18′) completed this spin system and established the C-14/C-15/C-16(N-16)/C-17/C-18 connectivity. An HMBC between the carbon resonance at δ 140.0 (C-13) and H-14 and H-15, between the carbon resonance at δ 29.4 (C-17) and H-15, and between the carbon resonance at δ 23.9 (C-18) and H-12 and H-14 established the C-12/C-13 connectivity in the conjugated diene and hence the connectivity of the cyclohexylidene ring. The downfield shift of C-16 (δ 46.6) and an HMBC between this resonance and the exchangeable proton resonance at δ 8.32 (NH-16) established it as the point of attachment of a nitrogen atom. These results, along with HMBCs between the carbonyl resonance at δ 173.8 (C-1) and H-2 and H-12, and an HMBC between the carbon resonance at δ 52.1 (C-2) and NH-2, completed the assignment of the planar structure of the Aayp moiety.

Identification of the novel amino acid 2-amino-3-(4-aminomethyl-2-cyclohexen-1-ylidene)propionic acid (Amyp), an N-Me derivative of Aayp, was established in a similar manner. The only difference was the presence of the NMe, which was confirmed by COSY correlations between two broad exchangeable singlet resonances at δ 8.53 and 8.63, assigned to the protonated NH_2-9, and the methine resonance at δ 3.87 (H-9) and the methyl resonance at δ 2.72 (H-21). The point of attachment of this NMe group was further confirmed by an HSQC between the carbon resonance at δ 30.7 (C-21) and H-21, along with an HMBC correlation between the carbon resonance at δ 55.9 (C-9) and the H-21 resonance, thus completing the C-9(N-9/C-21) connectivity.

HMBCs between the C-3 resonance (δ 171.8) and the NH-2 (δ 8.41) and the H-2 (δ 5.15) resonances together with the observation of a ROESY correlation between the H-4 (δ 5.23) and the NH-2 resonance established the amide linkage between the Aymp and Aayp residues. HMBCs between the carbonyl resonance at δ 172.7 (C-19) and resonances at δ 1.97 (Me-20), 8.41 (NH-4), and 5.23 (H-4) established the placement of one of the acetyl groups on N-4 of Amyp. The attachment of the remaining acetyl group to N-16 of Aayp was confirmed by HMBCs between the carbonyl resonance at δ 172.6 (C-22) and the resonances at δ 22.4 (Me-23), 8.23 (NH-16), and 4.44 (H-16).

The *Z* stereochemistry of the C7−C8 and C14−C15 double bonds in Amyp and Aayp, respectively, were based on the observed ROESY correlations observed between H-7/H-8 resonances in Amyp and between H-14/H-15 resonances in Aayp. The stereochemistry at the α-carbons of the two amino acid residues was determined by chemical degradation, followed by derivatization and analysis of the products. OsO_4−$NaIO_4$ oxidation of radiosumin B (**1**) followed by $NaBH_4$ reduction and subsequent acid hydrolysis yielded two serine residues. Derivatization with Marfey's reagent,[11] followed by HPLC analysis, established that both serine residues, and therefore both parent amino acids, had the L configuration based on a comparison to standards made from from L-Ser and DL-Ser. The optical rotations for both radiosumin B (**1**) and radiosumin (**2**)[10] had the same sign and a similar magnitude, $[\alpha]^{25}_D$ 70° and $[\alpha]^{20}_D$ 96° for **1** and **2**, respectively. On the basis of ^{1}H and ^{13}C NMR chemical shift comparisons and the similarity of the optical rotation data, the stereochemistry at the C-9 and C-16 centers of both amino acids in **1** were assumed to have the same configuration as in radiosumin (**2**), which had been previously established through synthesis.[12,13] Thus in **1** the stereochemistry at the C-9 and C-16 centers is proposed to be *R*.

Radiosumin B (**1**) showed no antibacterial or antifungal activity in our assays, although it is likely that it will be active against tyrpsin and plasmin, as was previously reported for **2**.[9,10] This is only the second report of these unusual radiosumin dipeptides from cyanobacteria. Their occurrence in *P. radiosum*,[9,10] and now in *M. aeruginosa*, suggests that this class of compound may occur in other genera (or species) of cyanobacteria.

Experimental Section

General Experimental Procedures. Normal and reversed-phase thin-layer chromatography (TLC) was carried out on commercial glass-backed Si gel 60 F254 (E. Merck, type 5554, 0.25 mm) and Whatman MKC18F reversed-phase TLC plates, respectively. TLC plates were eluted with 40:60 H_2O/MeOH, and the TLC spots were visualized by either UV light (254 nm) or a solution of vanillin in a sulfuric acid/EtOH mixture (6% vanillin w/v, 4% sulfuric acid, and 10% water v/v in EtOH). Flash chromatography was performed on 230−400 mesh C_{18} Si gel (Bakerbond, Si gel 60). Gel permeation chromatography was performed using Sephadex LH-20 resin. All solvents used were HPLC grade purchased from Fisher Scientific. H_2O was purified using a Waters Millipore system. UV spectra were recorded on a Pharmacia Biotech Ultrospec 2000 UV/visible spectrophotometer. IR spectra were obtained from small amounts of dried solid using a Bomem DA3.02 FTIR spectrometer equipped with a Spectrascope IR microscope. NMR samples (ca. 5 mg) were dissolved in CD_3OH in 5 mm tubes (Wilmad 535 PP), and spectra were recorded at 20 °C with a Bruker Avance/DRX 500 spectrometer at 500.13 MHz (^{1}H)

using a 5 mm inverse-geometry probe or at 125.77 MHz (^{13}C) using a 5 mm standard-geometry probe. Spectra were obtained under the following conditions: ^{1}H one-pulse, spectral width (SW) 10 330 Hz, 40° pulse, acquisition time (AQ) 1.58 s, delay after acquisition (D) 2s, processed with 0.1 Hz line broadening (LB) or with Lorentz−Gauss resolution enhancement and zero-filling; ^{13}C{^{1}H}-decoupled SW 29762 Hz, 40° pulse, AQ 2.2 s, D 0.1 s, Waltz decoupling, processing with zero filling and LB 1.0 Hz; ^{13}C DEPT 90 conditions were similar except that D was 2 s; ^{1}H 2D double-quantum filtered COSY, TOCSY, and NOESY SW5482 Hz, D 1s, 512 × 512 or 256 increments, 90° shifted sine-bell-squared apodization, zero-filled to 1024 in both dimensions during processing, mixing times 160 ms (TOCSY), 400 ms NOESY; ^{1}H/^{13}C HSQC and HMBC SW 5482 Hz (^{1}H), 19 483 or 25 154 Hz (^{13}C), 512 × 246 to 1024 increments, processing as for ^{1}H 2D above, HMBC long-range delay (D6) of 60 and 90 ms. Solvent suppression, where necessary, was by presaturation. Chemical shifts were referred to the solvent resonances (CHD_2OH at δ_H 3.30, $^{13}CD_3OH$ at δ_D 49.0 ppm). HRLSIMS and LRLSIMS were recorded using a Fisons VG AutoSpec mass spectrometer.

Plant Material. Algal samples of *M. aeruginosa* were collected using horizontal hauls of a 63 μm mesh 0.18 × 0.48 m zooplankton net, frozen, and later freeze-dried. All samples were supplied by Al Sosiak, Alberta Environmental Protection, Water Sciences Branch, Edmonton. Samples of the algal cells were deposited in the Nova Scotia Museum herbarium, reference number NSPM-007608P. No attempt was made to separate zooplankton from the algal material.

Isolation of Radiosumin B (1). The freeze-dried algal cells (100 g) were extracted repeatedly with MeOH (2 × 200 mL), and the combined extracts were concentrated in vacuo before being partitioned between $CHCl_3$ (5 × 50 mL) and H_2O/MeOH (3:2, 100 mL). The combined $CHCl_3$ extracts were back-extracted with fresh H_2O/MeOH (3:2, 3 × 50 mL). The combined aqueous extracts were reduced to dryness in vacuo to yield 1.82 g of brown oil. Fractionation of the oil by C_{18} reversed-phase flash chromatography (gradient H_2O to MeOH) gave a fraction (30 mg, eluting with 4:1 H_2O/MeOH) that contained a mixture of radiosumin B and several other unidentified compounds. This mixture was further fractionated by gel permeation (Sephadex LH20, open column) eluting with MeOH. Final purification of the radiosumin B fraction (15 mg) by C_{18} reversed-phase HPLC (CSC Sil 80A/ODS2, 5 μm, eluting with 4:1 0.05% TFA/MeOH) afforded radiosumin B as a white solid (11 mg), $[\alpha]^{25}_D$ 70° (*c* 0.36 MeOH); UV absorption in H_2O λ_{max} 239 nm (ϵ 15 000); IR (crystal) ν_{max} 3279, 3034, 2947, 1702, 1661, 1541, 1434, 1374, 1200, 1135 cm^{-1}; HRLSIMS *m*/*z* obsd 445.2550 $[M + H]^+$ (δ_m 0.5 ppm, for formula, calcd 445.2451); for ^{1}H and ^{13}C NMR data, see Table 1.

Oxidation by OsO_4 and Acid Hydrolysis of Radiosumin B (1). Radiosumin B (**1**) (1 mg, 0.002 mmol) was dissolved in distilled H_2O (1 mL) and OsO_4 (0.5 mg, 0.002 mmol) added. $NaIO_4$ (8 mg, 0.038 mmol) was carefully added to the solution with stirring at room temperature. After stirring for 80 min, an excess of $NaBH_4$ (2 mg, 0.053 mmol) was added, and stirring continued for a further 30 min. To the reaction mixture was added freshly distilled constant boiling HCl (1 mL), and the resulting solution was heated at 106 °C, with stirring, for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolyzate by repeated evaporation from H_2O (3 × 3 mL).

Derivatization of Amino Acids with Marfey's Reagent and HPLC Analysis.[11] Standards of L and DL-serine were prepared as follows: To each of two vials (1 mL), one containing 2 mmol pure l-serine standard in H_2O (80 μL) and the other containing 2 mmol of a 1:1 mixture of D/L-serine standard in H_2O (80 μL), was added *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA, 2.8 mmol in 170 μL of acetone) followed by 1 N $NaHCO_3$ (20 μL). The mixture was heated for 1 h at 40 °C. After cooling to room temperature, 2 N HCl (10 μL) was added and the resulting solution was filtered through a 4.5 μm filter and stored in the dark until HPLC analysis. To prepare FDAA derivatives of the amino acids in the hydrolyzate of the oxidation product of radiosumin B, an aliquot (90 μL) containing amino acid mixture (0.9 mg) was reacted with FDAA (2.86 mmol) in acetone (172 μL) as described above. An aliquot (10 μL) of the resulting mixture of FDAA derivatives was analyzed by reversed-phase HPLC. A linear gradient of (A) 9:1 triethylammonium phosphate (50 mM, pH 3.0)/MeCN and (B) MeCN, with 0% B at the start to 40% B over 60 min (flow rate 1 mL/min), was used to separate the FDAA derivatives, which were detected by UV at 340 nm. In all cases a peak was observed at 35.7 min, which was attributed to excess FDAA. The L-serine FDAA derivative eluted at 26.0 min, and the mixture of D/L-serine contained two peaks eluting at 25.9 and 26.6 min, corresponding to L- and D-serine, respectively. HPLC analysis of the FDAA-derivatized radiosumin B (**1**) hydrolyzate contained only one peak, eluting at 25.9 min, corresponding to L-serine.

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