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Isolation and structure determination of two new hydrophobic microcystins from *Microcystis* sp. (CAWBG11)

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ABSTRACT

Two new hydrophobic microcystins, microcystin-FA (**1**) and microcystin-WA (**2**), were isolated from the cyanobacterium *Microcystis* sp. (CAWBG11). The structures were deduced using one- and two-dimensional nuclear magnetic resonance spectroscopy and tandem mass spectrometry. The absolute stereochemistry of the amino acid residues in **1** and **2** was determined using the Advanced Marfey's method.

1. Introduction

Microcystins (MCs) are cyclic heptapeptides produced by cyanobacteria which can be toxic to animals upon ingestion. They are generally comprised of the unique β-amino acid, 3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), D-glutamic acid (Glu), D-alanine (Ala), *N*-methyldehydroalanine (Mdha), D-erythro-β-methylaspartic acid (Masp) and two variable L-amino acids (Welker and von Döhren, 2006). To date, at least 90 different microcystin congeners have been characterized (Pearson et al., 2010), mostly due to substitutions of the variable L-amino acids in positions two and four, although modifications have been reported for all of the amino acids (Rinehart et al., 1994). Microcystin congeners range in toxicity from non-toxic to highly toxic (Rinehart et al., 1994), according to their ability to inhibit the important eukaryotic regulatory enzymes, serine/threonine protein phosphatases 1 and 2A (An and Carmichael, 1994). Microcystins predominantly affect the liver cells of mammals as they cannot translocate the membranes of most tissues but are actively transported into hepatocytes (Runnegar et al., 1981). Inhibition of the hepatocyte protein phosphatases results in excessive signaling due to a lack of regulation via phosphatases. This may lead to cellular disruption due to actin filaments of the cytoskeleton becoming hyperphosphorylated (Falconer and Yeung, 1992), or cell proliferation and tumor promotion (Fujiki and Suganuma, 1993).

Microcystins are produced by a multitude of cyanobacterial genera including; *Anabaena*, *Anabaenopsis*, *Hapalosiphon*, *Microcystis*, *Nostoc* and *Planktothrix* (Chorus and Bartram, 1999). The production of microcystins is dependent on the presence of specific non-ribosomal peptide synthetase and polyketide synthase genes. The resulting multi-enzyme complex sequentially adds malonyl-CoA and amino acids onto phenylacetate to produce a peptide chain which is condensed to form a cyclic ring (Tillett et al., 2000).

During the chemical characterization of the cyanobacterial strain, *Microcystis* sp. (CAWBG11), 17 known microcystin congeners were observed, including MC-LR, MC-FR, MC-WR and MC-LA (Puddick, 2013), as well as two new microcystin congeners. Here, we report the spectroscopic characterization of these new microcystins; MC-FA (**1**) and MC-WA (**2**), isolated from *Microcystis* CAWBG11 (see Fig. 1).

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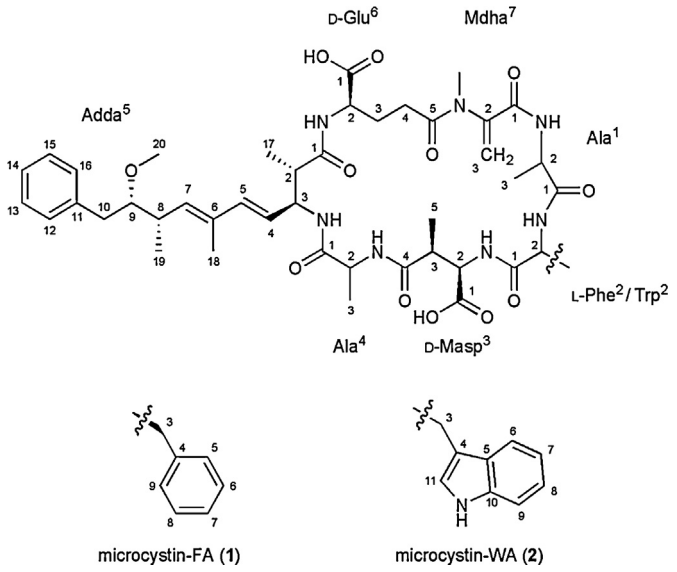

Fig. 1. Structures of microcystin-FA (**1**) and microcystin-WA (**2**).

2. Results and discussion

Microcystis CAWBG11 was isolated from a cyanobacterial bloom sample obtained from Lake Hakanoa (Huntly, New Zealand) in 2005 (Rueckert et al., 2007) and grown in MLA media (Bolch and Blackburn, 1996). Lyophilized cell material was sequentially extracted with 70% EtOH, then 100% MeOH. These extracts were fractionated using a combination of reversed-phase C_{18} and size-exclusion chromatography with final purification by HPLC, to yield **1** and **2** in 2.99×10^{-3}% and 1.04×10^{-3}% yields, respectively (from lyophilized material).

The structures of **1** and **2** were determined using a combination of amino acid analysis, MS and NMR spectroscopy. The HRESIMS spectrum for **1** established a molecular formula of $C_{49}H_{65}N_7O_{12}$. Advanced Marfey's amino acid analysis (Fujii et al., 1997a,b) of **1** indicated the presence of L-Ala, D-Ala, L-Phe, D-Glu, D-Masp, *N*-methylamine and an amino acid derivative with *m/z* 592, indicative of 3(*S*)-Adda (Fujii et al., 1997b). Interpretation of COSY, TOCSY, HSQC and HMBC spectra allowed for the assignment of the ^{1}H and ^{13}C signals for **1** (Table 1). The presence of the unusual amino acid Adda, which is found in microcystins and nodularins, was indicated by signals in the ^{1}H NMR spectrum for a monosubstituted aromatic ring (δ 7.18, 2H, d, H12/16; 7.25, 2H, dd, H13/15 and 7.16, t, H14), a trisubstituted diene system (δ 5.44, dd, H4; 6.26, d, H5 and 5.45, d, H7), a methoxyl group (δ 3.24, s, H20) and a downfield methyl signal (δ 1.63, s, H18; Botes et al., 1984). Connectivity between these ^{1}H signals was established using the 2-D correlations observed in the COSY and HMBC spectra (Table 1). Generally, the diene system in Adda is in the 4(*E*),6(*E*)-configuration, however, exposure to ultraviolet light has been shown to isomerize the double bonds to the *Z*-configuration (Kaya and Sano, 1998). The large coupling constant observed between the H4 and H5 signals (15.9 Hz) indicated that the C4 double bond was in the *E*-configuration. The H5-H7 correlation observed in the ROESY NMR spectrum of **1** (Appendix A8) was consistent with these protons being part of a transoid diene system (Harada et al., 1990).

The *N*-methylamine observed in the amino acid analysis suggested that Mdha could be present in **1** (Fujii et al., 1997b). This was confirmed by the observation of geminal alkene proton signals at 5.89 (H3A) and 5.45 ppm (H3B) and a *N*-methyl signal at 3.30 ppm (Harada et al., 1991; Namikoshi et al., 1992). As observed with other microcystins (Ooi et al., 1989; Park et al., 2001), the chemical shift of the carbonyl signal in the Mdha residue (165.9 ppm) was approximately 5–7 ppm upfield of that expected for a carbonyl adjacent to an amide. The small coupling constant observed in the H2 doublet of the D-Masp residue (3.9 Hz) indicated that it was in the β-orientation as is commonly observed in microcystins (Botes et al., 1984).

As an exchangeable NMR solvent was utilized (CD_3OD), the amide proton signals could not be used to establish connectivity between the amino acids in **1** (L-Ala, D-Ala, Phe, Glu, Masp, Mdha and Adda). The amino acid sequence of **1** was determined by ESI-CID-MS/MS, where the presence of Adda was indicated by the loss of 134 Da (*m/z* 810; Adda sidechain; Table 2) and 313 Da (*m/z* 631; entire amino acid; Diehnelt et al., 2006). The Adda′-Glu-Mdha fragment ion commonly observed in microcystins (*m/z* 375; Adda′ = Adda minus NH_2 and the $C_9H_{11}O$ sidechain; del Campo and Ouahid, 2010; Frias et al., 2006) was extended to include Ala (71 Da) and Phe (147 Da; Fig. 2A). This sequence was supported by a similar ion series containing Adda minus NH_3 (*m/z* 509, 580, 727) and was extended to include Masp (129 Da; Fig. 2B). The fragment ion series which began with Phe-Masp-Ala (*m/z* 348) and extended in the opposite direction to include a second Ala residue, gave the complete amino acid sequence of Adda-Glu-Mdha-Ala^1-Phe-Masp-Ala^4. A fragment where Mdha and H_2O were lost from the compound (*m/z* 843; Table 2) indicated that Adda and the Ala^4 residue were joined and that the structure was cyclic.

Comparison of the ^{1}H NMR chemical shifts of the two Ala methyl signals of **1** (1.25 and 1.30 ppm) with literature values for previously reported microcystins was used to assign each residue. Most microcystins contain a single Ala residue in position one (Ala^1), which yields methyl proton resonances around 1.29–1.37 ppm in CD_3OD (Beattie et al., 1998; Sano and Kaya, 1998; Sano et al., 2004). Therefore, the more downfield Ala methyl signal in **1** (1.30 ppm) was assigned as Ala^1 and the upfield signal (1.25 ppm) was assigned as the Ala^4. As a D-amino acid has consistently been observed in position one in other microcystins (Welker and von Döhren, 2006), the D-Ala of **1** was putatively assigned as Ala^1 and the L-Ala as Ala^4.

The HRESIMS spectrum for **2** was used to determine a molecular formula of $C_{51}H_{66}N_8O_{12}$. As for **1**, Advanced Marfey's amino acid analysis indicated the presence of L-Ala, D-Ala, D-Glu, D-Masp, *N*-methylamine and the *m/z* 592 derivative indicative of 3(*S*)-Adda. The majority of the ^{1}H and ^{13}C NMR signals observed for **2** (Table 3) were the same as those of **1**, except that the signals indicative of the Phe residue were absent. Instead, there were signals in the ^{1}H NMR spectrum indicative of a tryptophan (Trp) residue; an asymmetrically disubstituted aromatic ring (δ 7.31, d, H6; 7.07, dd, H7; 7.00, dd, H8 and 7.67, d, H9) and a downfield singlet at 7.29 ppm (H11). These signals were within 0.02 ppm of those reported previously for a Trp-containing microcystin (Namikoshi et al., 1992). Furthermore, these proton signals showed HMBC correlations to three quaternary carbons as would be expected for the indole of a Trp residue.

The ESI-CID-MS/MS spectrum for **2** indicated that the microcystin had the amino acid sequence Adda-Glu-Mdha-Ala^1-Trp-Masp-Ala^4 (Table 2). The fragment ion series starting with Adda′-Glu-Mdha (*m/z* 375) was extended to include Ala^1 and Trp (Fig. 3A). This sequence was supported by the ion series containing Adda minus NH_3 (*m/z* 509, 580, 766) which was extended to include Masp (Fig. 3B). A fragment ion series which began with Trp-Masp-Ala (*m/z* 387; Fig. 3B) and extended in the opposite direction to include a second Ala residue, gave the complete amino acid sequence of Adda-Glu-Mdha-Ala^1-Trp-Masp-Ala^4. As for **1**, a fragment where Mdha and H_2O were lost from the compound (*m/z* 882 in this case; Table 2) indicated that Adda and the Ala^4 residue were joined and that the structure was cyclic.

Table 1
NMR spectroscopic data (400 MHz; CD_3OD) for microcystin-FA (**1**).

Position[a]		δ_C, type	δ_H (J in Hz)	COSY	HMBC[b]
Ala[1]	1	173.8, C			
	2	49.1, CH[c]	4.45, q (7.3)[d]	Ala[1]-3	Ala[1]-1
	3	18.0, CH_3	1.30, d (7.3)	Ala[1]-2	Ala[1]-1, 2
Phe[2]	1	178.9, C			
	2	59.7, CH	4.35, m[c]	Phe-3	Phe-1
	3	37.9, CH_2	3.19 (2H), m	Phe-2	
	4	139.5, C			
	5/9	130.4, CH	7.37, d (7.8)	Phe-6/8	Phe-3, 7
	6/8	129.5, CH	7.24, dd (6.8, 7.8)[d]	Phe-5/9, 7	Phe-4
	7	127.7, CH	7.17, t (6.8)[d]	Phe-6/8	Phe-5/9
Masp[3]	1	nd			
	2	57.9, CH	4.41, d (3.9)[c]	Masp-3	
	3	41.6, CH	3.13, m	Masp-2, 5	
	4	179.1, C			
	5	15.7, CH_3	1.05, d (7.3)[d]	Masp-3	Masp-3, 4
Ala[4]	1	175.1, C			
	2	49.3, CH[c]	4.56, q (7.3)[d]	Ala[4]-3	Ala[4]-1
	3	17.5, CH_3	1.25, d (7.3)	Ala[4]-2	Ala[4]-1, 2
Adda[5]	1	176.8, C			
	2	45.5, CH	2.97, m	Adda-3, 17	
	3	56.7, CH	4.59, dd (9.7, 10.9)	Adda-2, 4	Adda-17
	4	126.7, CH	5.44, dd (10.9, 15.9)[d]	Adda-3, 5	Adda-5, 6
	5	138.8, CH	6.26, d (15.9)	Adda-4	Adda-3, 6, 7, 18
	6	134.0, C			
	7	137.1, CH	5.45, d (11.6)[d]	Adda-8, 18	Adda-5, 6, 8, 9
	8	37.8, CH	2.59, m	Adda-7, 9, 19	Adda-6
	9	88.5, CH	3.26, dt (5.9, 6.7)[d]	Adda-8, 10A, 10B	Adda-11, 19, 20
	10	39.1, CH_2	2.82, dd (4.7, 14.0)	Adda-9, 10B	Adda-8, 9, 11, 12/16
	11	140.7, C	2.67, dd (7.2, 14.0)	Adda-9, 10A	Adda-8, 9, 11, 12/16
	12/16	130.7, CH	7.18, d (7.8)[c]	Adda-13/15	Adda-10, 14
	13/15	129.3, CH	7.25, dd (7.3, 7.8)[c]	Adda-12/16, 14	Adda-11
	14	127.1, CH	7.16, t (7.3)[c]	Adda-13/15	Adda-12/16
	17	16.4, CH_3	1.06, d (6.8)[d]	Adda-2	Adda-1, 3
	18	13.0, CH_3	1.63, s	Adda-7	Adda-6, 7
	19	16.7, CH_3	1.02, d (6.8)	Adda-8	Adda-7, 8, 9
	20	58.8, CH_3	3.24, s		Adda-9
Glu[6]	1	nd			
	2	55.3, CH	4.33, m[c]	Glu-3A, 3B	
	3	29.5, CH_2	2.22, m	Glu-2, 4	
			1.96, m	Glu-2, 4	
	4	33.6, CH_2	2.55 (2H), m	Glu-3A, 3B	
	5	177.3, C			
Mdha[7]	1	165.9, C			
	2	146.6, C			
	3	114.4, CH_2	5.89, s	Mdha-3B	Mdha-1
			5.45, s[d]	Mdha-3A	Mdha-1
	N-CH_3	38.5, CH_3	3.30, s[c]		Mdha-2; Glu-5

[a] Position in structure indicated by the superscript number; nd = not detected.
[b] HMBC were optimized for 10 Hz and are from the proton(s) stated to the indicated carbon.
[c] Signals were overlapped and calibrated using the 2-D HSQC spectrum.
[d] Multiplicity and coupling constants were determined using 1-D selective TOCSY experiments.

Since 1995, the structures of many microcystins have been reported based solely on MS/MS data (for example, Bateman et al., 1995; del Campo and Ouahid, 2010; Ferranti et al., 2009; Frias et al., 2006; Robillot et al., 2000; Wood et al., 2008). Whilst this technique provides a good indication of the structure from a small amount of sample (ca. 1 ng), it does not definitively identify the amino acid constituents as it cannot distinguish between isometric substructures and provides no stereochemical information. Techniques such as NMR spectroscopy and amino acid analysis provide more definitive proof of structure, but require larger quantities (>0.1 mg) of purified material. In microcystins, the position seven amino acid is frequently Mdha, although the isometric amino acid; dehydrobutyrine (Dhb), has also been reported (Beattie et al., 1998; Sano et al., 1998; Sano and Kaya, 1998). The use of NMR spectroscopy during the present study supplied definitive evidence that Mdha was present in **1** and **2**. A recently described technique (Miles et al., 2013) which assesses the reaction rate of a thiol derivatization, has also been shown to be effective for the discrimination of these isometric moieties. When using this technique, a microcystin containing Mdha will readily react with β-mercaptoethanol under alkaline conditions, causing a mass increase of 78 Da (Miles et al., 2012). However, when Dhb is present, the reaction rate is hundreds of times slower (Miles et al., 2013). This thiol derivatization technique will provide a valuable method to distinguish between Mdha- and Dhb-containing analogs when characterizing microcystins from a small amount of sample.

Acid hydrolysis and amino acid analysis is generally used to determine the stereochemistry of amino acids present in microcystins, however, it is a destructive technique which consumes ca. 0.1 mg of the compound. In the present study, Advanced Marfey's amino acid analysis revealed the presence of both D- and L-alanine in **1** and **2**. The MS/MS analysis placed these amino acids in positions one and four, however, to conclusively establish the position, each stereoisomer would require further destructive analyses (Botes et al., 1984). Whilst these analyses were not performed during the present study, D-alanine was putatively placed in position one as all other microcystin congeners which

Table 2
Fragment ions observed for microcystin-FA (**1**) and microcystin-WA (**2**) by electrospray ionization collision-induced dissociation (ESI-CID).

Fragment assignment	1	2
M+H	944	983
M−H_2O+H	926	965
M−Mdha−H_2O+H	843	882
M−Adda sidechain+H	810	849
M−Adda sidechain−H_2O+H	792	831
M−Adda+H	631	670
M−Adda−H_2O+H	613	652
Adda-Glu-Mdha-Ala[1]-X-Masp−NH_3+H[a]	856	895
Adda-Glu-Mdha-Ala[1]-X−NH_3+H[a]	727	766
Adda-Glu-Mdha-Ala[1]−NH_3+H	580	580
Adda-Glu-Mdha−NH_3+H	509	509
Adda′-Glu-Mdha-Ala[1]-X+H[a]	593	632
Adda′-Glu-Mdha-Ala[1]+H[a]	446	446
Adda′-Glu-Mdha+H[a]	375	375
Mdha-Ala[1]-X-Masp-Ala[4]+NH_4[a]	519	558
Ala[1]-X-Masp-Ala[4]+NH_4[a]	436	475
X-Masp-Ala[1/4]+NH_4[a]	365	404
Mdha-Ala[1]-X-Masp-Ala[4]+H[a]	502	541
Ala[1]-X-Masp-Ala[4]+H[a]	419	458
X-Masp-Ala[1/4]+H[a]	348	387

[a] X = position two amino acid (**1** = Phe; 147 Da and **2** = Trp; 186 Da); Adda′ = Adda minus NH_2 and the $C_9H_{11}O$ sidechain.

have been structurally characterized to a similarly high degree contain D-amino acids in position one (for example, Botes et al., 1984; Christiansen et al., 2008; Namikoshi et al., 1992, 1995; Park et al., 2001; Sano and Kaya, 1998; Sivonen et al., 1992). Stereochemical elements of some of the unusual amino acids found in microcystins were also confirmed using NMR spectroscopy. Coupling constants observed in the ^{1}H NMR spectrum confirmed that D-Masp was in the β-orientation and that the C4 double bond of Adda was in the *E*-configuration. Furthermore, a correlation observed in the ROESY NMR spectrum confirmed that the C6 double bond was also in the *E*-configuration.

Unfortunately, the material isolated for structural characterization was insufficiently pure to proceed with toxicology or protein phosphatase inhibition studies, at the present time. However, the Adda-Glu portion of the microcystin structure, which has previously been shown to be important for protein phosphatase inhibition (Rinehart et al., 1994), was not modified in these congeners. Therefore it is likely that the two microcystins would inhibit protein phosphatases and pose a health risk to humans and animals.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AVIII-400 NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts were determined at 300 K and are reported relative to the solvent signal (CD_3OD; ^{1}H 3.31 ppm, ^{13}C 49.15 ppm). HRESIMS was performed on a Bruker MicrOTOF mass spectrometer. Amino acid and MS/MS analyses were performed on a Bruker AmaZon × ESI mass spectrometer coupled to a Dionex UltiMate 3000 HPLC system. UV data was determined using a Varian Cary 100 Scan UV–vis spectrophotometer over a wavelength range of 200–800 nm. Optical rotations of the purified microcystins were determined using an AUTOPOL IV polarimeter (Rudolph Research Analytical). Reversed-phased C_{18} separations were conducted using YMC-gel ODS-A (YMC) where solvent flow was assisted by compression with N_2 gas. Size-exclusion chromatography was conducted using Sephadex LH-20 (Pharmacia Fine Chemicals) where solvent flow was due to gravity. HPLC purification was performed using Waters 515 HPLC pumps coupled to a photodiode array detector (200–400 nm; Waters 2996) and an Econosil C_{18} Column (250 × 10 mm, 10-μ; Alltech).

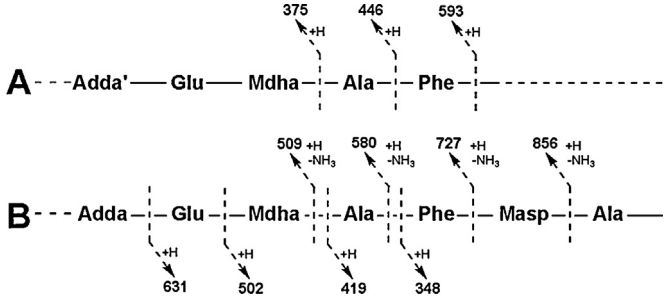

Fig. 2. Tandem mass spectrometry fragment ions indicating the amino acid sequence in microcystin-FA (**1**).

3.2. Cyanobacterial material

Microcystis CAWBG11 was isolated from a bloom sample obtained from Lake Hakanoa (Huntly, New Zealand) in 2005 (Rueckert et al., 2007). The culture is maintained alive and cryopreserved in the Cawthron Institute micro-algae culture collection (http://cultures.cawthron.org.nz). The 16S ribosomal RNA gene partial sequence and full 16S-23S rRNA intergenic spacer sequence are available (GenBank: EF634465).

Microcystis CAWBG11 was grown in 20 × 20 L plastic carboys, each containing 16 L of MLA media (Bolch and Blackburn, 1996). Cultures were grown at 18 °C under a 12:12 h light/dark cycle with a photon-flux of 100 μE m^{-2} s^{-1}. After 40 days, the cultures were harvested using plankton netting (11 μm mesh diameter). The concentrated cell material was lyophilized and stored at −20 °C until extracted.

3.3. Extraction and isolation procedure

Freeze-dried cells (76.9 g) were extracted in 7:3 EtOH/H_2O (5 × 800 mL). The remaining cell pellet was extracted in MeOH (5 × 250 mL). A voucher of the cellular material extracted (JP2-033-05) is held at the Department of Chemistry, University of Waikato, Hamilton, New Zealand. The crude extracts (5.6 g and 0.45 g, respectively) were evaporated and individually fractionated by reversed-phase C_{18} chromatography, where the microcystins of interest eluted between 3:7 and 1:1 MeOH/H_2O. These fractions were combined (331.8 mg) and separated on a reversed-phase C_{18} column acidified with 0.1% formic acid (FA; v/v) where the hydrophobic microcystins eluted with 7:3 MeOH/H_2O + 0.1% FA (v/v). This fraction (127.5 mg) was neutralized with K_2CO_3 (40 mg) in MeOH (0.6 mL) and separated on a reversed-phase C_{18} column. The fraction which eluted in MeOH/H_2O (1:3; 34.2 mg) was dissolved in MeOH and subjected to size exclusion chromatography which yielded three mixtures with varying proportions of **1**, **2** and MC-LA. The three mixtures (4.9 mg, 6.7 mg and 10.2 mg) were individually fractionated by isocratic HPLC using ACN:10 mM ammonium acetate (27:73). The dried samples were lyophilized then residual ammonium acetate removed by passing the sample, dissolved in 10% MeOH, through a plug of C_{18} material (200 mg) and eluting with 70% MeOH to yield **1** (2.3 mg) and **2** (0.8 mg).

Microcystin-FA (**1**): White amorphous solid; $[\alpha]^{20}_D$ −173° (*c* 0.06 g/100 mL, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.20), 238 (4.13) nm; ^{1}H and ^{13}C NMR data (CD_3OD) see Table 1; HRESIMS *m/z* 966.4550 $[M+Na]^+$ (calculated for $C_{49}H_{65}N_7O_{12}Na$, 966.4583).

Microcystin-WA (**2**): White amorphous solid; $[\alpha]^{20}_D$ −40° (*c* 0.04 g/100 mL, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.19), 224 (4.25), 238 (4.13) nm; ^{1}H and ^{13}C NMR data (CD_3OD) see Table 2;

Table 3
NMR spectroscopic data (400 MHz; CD_3OD) for microcystin-WA (**2**).

Position[a]		δ_C, type	δ_H (*J* in Hz)	COSY	HMBC[b]
Ala[1]	1	174.0, C			
	2	49.1, CH[c]	4.49, q (7.7)[d]	Ala[1]-3	Ala[1]-1
	3	18.0, CH_3	1.31, d (7.7)[d]	Ala[1]-2	Ala[1]-1, 2
Trp[2]	1	nd			
	2	58.2, CH	4.47, m	Trp-3A, 3B	
	3	28.0, CH_2	3.38, m	Trp-2	
			3.31, dd (1.7, 3.3)[d]	Trp-2	
	4	111.5, C			
	5	138.2, C			
	6	112.3, CH	7.31, d (8.7)	Trp-7	Trp-8, 10
	7	122.3, CH	7.07, dd (7.4, 8.7)	Trp-6, 8	Trp-5, 9
	8	119.8, CH	7.00, dd (7.4, 8.4)	Trp-7, 9	Trp-10
	9	119.1, CH	7.67, d (8.4)	Trp-8	Trp-5
	10	128.3, C			
	11	125.7, CH	7.29, s		Trp-4, 5
Masp[3]	1	nd			
	2	58.8, CH	4.36, d (4.0)[c]	Masp-3	
	3	41.5, CH	3.15, m	Masp-2, 5	
	4	179.1, C			
	5	16.3, CH_3	1.07, d (7.7)[d]	Masp-3	Masp-3, 4
Ala[4]	1	175.0, C			
	2	49.0, CH[c]	4.60, q (7.7)[d]	Ala[4]-3	Ala[4]-1
	3	17.4, CH_3	1.23, d (7.7)[d]	Ala[4]-2	Ala[4]-1, 2
Adda[5]	1	176.7, C			
	2	45.3, CH	2.98, m	Adda-3, 17	
	3	56.5, CH	4.63, m	Adda-2, 4	Adda-17
	4	126.9, CH	5.46, m	Adda-3, 5	Adda-5, 6
	5	138.7, CH	6.29, d (16.1)	Adda-4	Adda-3, 6, 7, 18
	6	134.0, C	–		
	7	137.1, CH	5.46, d (10.4)[d]	Adda-8, 18	Adda-5, 6, 8, 9
	8	37.8, CH	2.60, m	Adda-7, 9, 19	Adda-6
	9	88.5, CH	3.26, m	Adda-8, 10A, 10B	Adda-11, 19, 20
	10	39.1, CH_2	2.83, dd (4.6, 14.0)	Adda-9, 10B	Adda-8, 9, 11, 12/16
			2.68, dd (7.5, 14.0)	Adda-9, 10A	Adda-8, 9, 11, 12/16
	11	140.7, C			
	12/16	130.7, CH	7.19, d (8.1)[c]	Adda-13/15	Adda-10, 14
	13/15	129.3, CH	7.25, dd (7.4, 8.1)	Adda-12/16, 14	Adda-11
	14	127.2, CH	7.17, t (7.4)[c]	Adda-13/15	Adda-12/16
	17	16.2, CH_3	1.08, d (6.7)[d]	Adda-2	Adda-1, 3
	18	13.0, CH_3	1.64, s	Adda-7	Adda-6, 7
	19	16.6, CH_3	1.03, d (6.7)	Adda-8	Adda-7, 8, 9
	20	58.9, CH_3	3.25, s		Adda-9
Glu[6]	1	nd			
	2	55.9, CH	4.32, m	Glu-3A, 3B	
	3	30.5, CH_2	2.23, m	Glu-2, 4	
			1.89, m	Glu-2, 4	
	4	33.8, CH_2	2.55 (2H), m	Glu-3A, 3B	
	5	177.4, C			
Mdha[7]	1	166.0, C			
	2	146.4, C			
	3	113.8, CH_2	5.87, s	Mdha-3B	Mdha-1
			5.45, s	Mdha-3A	Mdha-1
	N-CH_3	38.3, CH_3	3.30, s[c]		Mdha-2; Glu-5

[a] Position in structure indicated by the superscript number; nd = not detected.
[b] HMBC were optimized for 10 Hz and are from the proton(s) stated to the indicated carbon.
[c] Signals were overlapped and calibrated using the 2-D HSQC spectrum.
[d] Multiplicity and coupling constants were determined using 1-D selective TOCSY experiments.

HRESIMS *m/z* 1005.4650 $[M+Na]^+$ (calculated for $C_{51}H_{66}N_8O_{12}Na$, 1005.4692).

3.4. Advanced Marfey's amino acid analysis

Purified microcystins were subjected to amino acid analysis according to the Advanced Marfey's method (Fujii et al., 1997a,b). 1-Fluoro-2,4-dinitrophenyl-5-leucine (FDLA) was synthesized according to the method of Marfey (1984), but using leucinamide (Bachem) instead of alaninamide. Both the D- and L-forms of the reagent were synthesized from the respective stereoisomers of leucinamide. Microcystins (100 μg) were dried at 35 °C under a stream of N_2, resuspended in 6 N HCl (0.5 mL) and incubated at 110 °C for 16 h. The HCl was removed at 35 °C under a stream of N_2. Hydrolyzates were resuspended in H_2O (105 μL) and aliquoted into two microcentrifuge tubes (50 μL each), to which 1 M $NaHCO_3$ (20 μL) and 1% L- or DL-FDLA (w/v; 100 μL) was added. The tubes were incubated at 40 °C for 1 h, before being quenched with 1 N HCl (20 μL). The derivatized hydrolyzates were diluted with HPLC-grade MeOH (810 μL), centrifuged (14,000 × *g*, 5 min; Eppendorf MiniSpin Plus) and the supernatant transferred to a septum-capped LC vial. The derivatized sample (20 μL) was analyzed by LC-MS using an Econosil C_{18} column (250 × 3.2 mm, 5-μ; Alltech) and a gradient of 25–75% (v/v) ACN + 0.1% (v/v) formic acid over 30 min. Eluting derivatives were detected by UV absorption (250–500 nm) and ESI MS (negative ion mode, *m/z* 300–1100). Retention

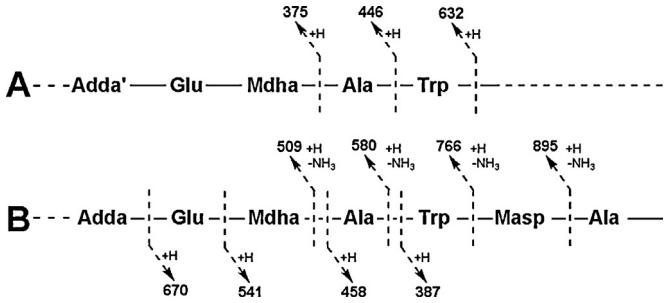

Fig. 3. Tandem mass spectrometry fragment ions indicating the amino acid sequence in microcystin-WA (**2**).

times (min) of the L-FDLA derivatives were as follows: **1**: D-Glu (14.3), L-Ala (15.8), D-Masp (15.9), D-Ala (18.6), L-Phe (21.3), *N*-methylamine (19.6), 3(*S*)-Adda (32.9); **2**: D-Glu (14.3), L-Ala (15.8), D-Masp (15.9), D-Ala (18.6), *N*-methylamine (19.6), 3(*S*)-Adda (32.9).

3.5. Liquid chromatography-mass spectrometry analysis.

Samples (20 μL) for LC-MS and LC-MS/MS were separated on a C_{18} column (Ascentis Express C_{18}, 100 × 2.1 mm, 2.7-μ; Supleco Analytical) at a flow of 200 μL/min using a gradient of 2% acetonitrile + 0.1% formic acid (v/v; solvent A) and 98% acetonitrile + 0.1% formic acid (v/v; solvent B) with the following program; the sample was loaded in 10% B; 10% B was held for 1 min and increased to 100% B over 12 min; 100% B was held for 2 min; the solvent concentration was returned to 10% B in 1 min and the column re-equilibrated for 4 min. The eluting compounds were ionized using a capillary voltage of 3.5 kV and a nebulizer pressure of 3.0 bar. Desolvation was accomplished with a nitrogen flow of 8 L/min at 220 °C. Mass spectra were acquired for negative ions over a range of *m/z* 100–2000. Daughter ion scans were gathered using the singly-protonated ions of the target compounds and collision-induced dissociation (collision amplitude of 1.0).

4. Conclusions

Two new microcystins, MC-FA (**1**) and MC-WA (**2**), were isolated from *Microcystis* CAWBG11 and structurally characterized using a combination of MS/MS, NMR spectroscopy and amino acid analysis. This in-depth characterization was used to definitively identify the amino acid constituents of these new microcystin congeners and to assign much of their stereochemistry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.07.011.

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