Microcystilide A: A Novel Cell-Differentiation-Promoting Depsipeptide from *Microcystis aeruginosa* NO-15-1840

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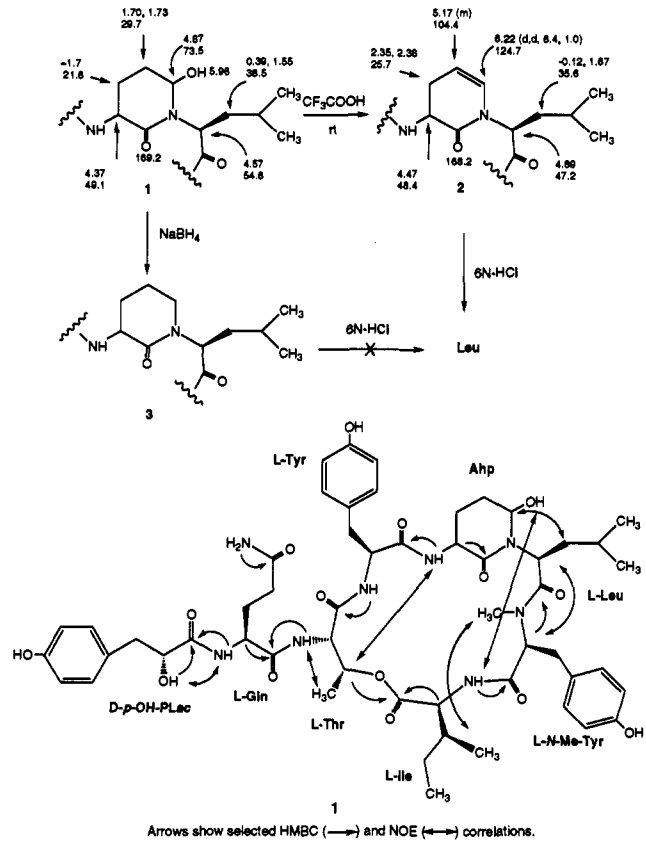
Microcystis aeruginosa is probably the most deleterious of freshwater bloom-forming cyanobacteria (blue-green algae), posing serious worldwide problems to human and livestock health. A series of hepatotoxic cyclic peptides, the microcystins, has been identified in a number of strains of *Microcystis* and some other cyanobacteria.[1] All microcystins contain a characteristic β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), or its derivatives, and act as strong protein phosphatase 1 and 2A inhibitors.[2] We have been studying the toxic components of *Microcystis aeruginosa* NO-15-1840[3] and have reported microcystin-RR and -LR (cyanoginosin-RR and -LR) as the major toxic components.[4] In this communication, we report the isolation and structural determination of a novel non-Adda-containing cyclic depsipeptide, microcystilide A, from the same organism.

Microcystilide A, **1**, was isolated from the methanol extract of the cultured cells (yield: 0.1% from the dried cells)[5] as an amorphous powder, $[\alpha]_D^{22}$ −23.8°, λ_{max} (log ϵ) 227 nm (4.28), 278 (3.52), 284 (3.44 sh), and FABMS (matrix, 1-thioglycerol + 1% *p*-toluenesulfonic acid monohydrate): *m/z* 1095.5057 [(M + Na)$^+$, calcd for $C_{54}H_{72}N_8O_{15}Na$ 1095.5014, Δ 3.9 ppm]. Amino acid analysis of the 6 N HCl hydrolysate of **1** by the PTC derivatization method (Waters PICO-TAG)[6] revealed 1 equiv each of glutamate (from glutamine), threonine, leucine, isoleucine, and tyrosine. The chiralities of the amino acids were all determined as all L by the HPLC analysis of the FDAA derivatives (Marfey's method).[7] In this analysis, the presence of 1 equiv of L-*N*-methyltyrosine was also confirmed. On the basis of ^{1}H–^{1}H COSY, RCT, and ^{1}H–^{13}C COSY NMR spectra of **1**, the presence of a *p*-hydroxyphenyllactate moiety was deduced. In fact, the ethereal extract of the acid hydrolysate of **1** afforded *p*-hydroxyphenyllactic acid, whose absolute configuration was determined as the D-form by the HPLC comparison of the *l*-menthyl ester with the authentic diastereomers.[8] For the aforementioned amino acid residues, the NMR signal assignments and connectivities were in accordance with their structures, with the exception of the NH proton signal of the leucine moiety, which was not observed in any of the spectra.

Treatment of **1** with trifluoroacetic acid overnight at room temperature afforded a dehydro derivative, **2**, $[\alpha]_D^{21}$ −49.1° (*c* = 0.16, MeOH); FABMS: *m/z* 1055.5105 [(M + H)$^+$, calcd for $C_{54}H_{71}N_8O_{14}$ *m/z* 1055.5089, Δ 1.5 ppm], which had a newly-formed disubstituted double bond. The NMR signals, δ 4.87 for ^{1}H and δ 73.5 for ^{13}C, which correlated with a methine group, were replaced with signals at δ 6.22 and 124.7 in **2**. The amino acid composition of its 6 N HCl hydrolysate was identical with that found for the parent compound, **1**. Large chemical shift changes were observed with the leucine moiety, with one of the methylene hydrogen signals shifted upfield 0.51 ppm, and the α-carbon signal shifted upfield 7.4 ppm. When **1** was reduced with $NaBH_4$ in MeOH, leucine was no longer detected in the acid hydrolysate of the resulting reduction product, **3**. On the basis of these observations, it was concluded that **1** has an amino acetal moiety, which involves the amino group of the leucine moiety (Chart I).

Chart I



Arrows show selected HMBC (⟶) and NOE (⟷) correlations.

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(3) This toxic strain was obtained from a commercial source (Carolina Biological Inc.) and has been in culture at University of Rhode Island since 1978. It was told that the strain has its origin in Ichimura Collection, Japan. It was cultured in Fitzgerald medium under fluorescent lighting, 8 h/16 h dark–light cycle at 25 °C.

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(5) In a typical isolation, the freeze-dried cells (36 g from 170 L of culture) were extracted with MeOH, and the extract was partitioned between 90% MeOH and *n*-hexane. The aqueous MeOH extract was fractionated on Sephadex LH-20 with MeOH, C18 cartridges with 70% MeOH, and a C18 column with 50% MeOH. The final purification by HPLC on Hamilton PRP-1 with 40% CH_3CN afforded 40 mg of **1**.

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(8) *p*-Hydroxyphenyllactic acid was treated with *l*-menthol in benzene with *p*-toluenesulfonic acid under reflux for 22 h. The reaction mixture was washed with a $NaHCO_3$ solution and H_2O, dried, and analyzed on LiChrosorb RP-18 (5 μm, 4.6 × 250 mm) using 45% CH_3CN as eluent at 227 nm. The D-isomer showed a retention time of 24.6 min and the L-isomer a retention time of 26.2 min at a flow rate of 1 mL/min.

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Dansylation followed by hydrolysis showed that there is no N-terminal in the molecule, and also the molecular formula supports a bicyclic structure. The sequence of the segments has been established with the help of COLOC, HMBC, NOESY, and ROESY NMR experiments.[9] The disappearance of leucine in the hydrolysate of the $NaBH_4$ reduction product supports the connection of 3-amino-6-hydroxypiperidone (glutamate δ-semialdehyde, Ahp) to leucine. The hydroxyl group of threonine was speculated as the site of the lactone closure, since the hydroxy proton signal was absent in the NMR spectra and the methine proton is shifted downfield (δ 5.47 ppm). Furthermore, an HMBC experiment confirmed the connectivity of the methine proton to the isoleucine carbonyl group. Several long-range cross peaks in the NOESY and ROESY spectra were observed, which made the initial assignment of the sequence rather difficult. However, once the structure was established, on the other hand, they provided interesting information as to the shape of the molecule. The molecule seems to have an elongated shape constricted in the middle by possible hydrogen bonding between the isoleucine NH hydrogen and the axial hydroxyl group of Ahp. As a result, an NOE was observed between the hydroxyl proton of Ahp and the isoleucine NH proton.[10] In addition, an NOE was observed between the Ahp NH and threonine 3-H.

The established structure of **1** has a resemblance to that proposed for dolastatin 13, a cytotoxic depsipeptide isolated from the sea hare, *Dolabella auricularia*, collected in the Indian Ocean,[11] although the latter has a quite different amino acid profile. Recently, a seemingly related compound, which has a different amino acid composition and a long-chain fatty acid, has been reported in *Microcystis* sp. in Japan.[12] There has been persisting speculation that a number of interesting compounds—many of them are peptides—found in *Dolabella* are derived from dietary algae. Interestingly enough, a derivative of the cyclic hepatotoxin, nodularin, which is closely related to microcystins,[13] has been found in a marine invertebrate.[14] A similar observation was also made with scytophycin-type compounds, which are also found in both marine sponges and freshwater blue-greens.[15]

Microcystilide A was first recognized as a component which causes convulsions or spasms to mice upon intaperitoneal injection.[16] The symptoms were distinctively different from those of the acute toxicity caused by the coexisting microcystins. The compound was found to be only weakly cytotoxic against HCT116 and HCTVP35 cell lines (IC_{50} 0.5 mg/mL), but found to be active in the cell differentiation assay using HL-60 cells at a concentration of 0.5 mg/mL.

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Supplementary Material Available: ^1H, ^{13}C, ^1H–^1H and ^1H–^{13}C COSY, ROESY, and HMBC spectra (20 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(9) ^1H–^1H COSY, ^1H–^{13}C COSY, RCT, COLOC, NOESY, and DEPT spectra were taken on a Bruker 300-Mz instrument, and HMBC and ROESY spectra on a Varian Unity 500-MHz instrument. ^1H NMR **1** (DMSO-d_6) OH-PLac δ 4.01 (ddd, J = 8.7, 5.9, and 3.5 Hz, H2), 5.51 (d, J = 5.9 Hz, 2-OH), 2.54 (dd, J = 14 and 8.7 Hz, H3), 2.87 (dd, J = 14 and 3.5 Hz, H3′), Gln δ 4.45 (dt, J = 5.0 and 8.1 Hz, H2), 1.75 (m, H3), 1.80 (m, H3′), 2.03 (m, H4), 2.05 (m, H4′), 7.78 (d, J = 8. 1 Hz, NH), 6.84 (br s, NH_2), 7.22 (br s, NH_2), Thr δ 4.57 (dd, J = 9.3 and 1 Hz, H2), 5.47 (dq, J = 1 and 6.5 Hz, H4), 1.14 (d, J = 6.5 Hz, H4), 8.06 (d, J = 9.3 Hz, NH), Tyr δ 4.44 (dd, J = 9.0 and 3.7 Hz, H2), 2.59 (dd, J = 14.5 and 9.0 Hz, H3), 3.22 (dd, J = 14.5 and 3.7 Hz, H3′), 8.51 (d, J = 9.0 Hz, NH), Ahp δ 4.37 (dt, J = 5.1 and 8.5, H2), 1.75 (m, H3), 2.5 (m, H3′), 1.7 (m, H4), 1.73 (m, H4′), 4.87 (br s, H5), 5.98 (d, J = 3 Hz, 5-OH), 7.33 (d, J = 9.3 Hz, NH), Leu δ 4.58 (dd, J = 10 and 2 Hz, H2), 0.39 (ddd, J = 13, 10, and 2 Hz, H3), 1.55 (ddd, J = 13, 10, and 2 Hz, H3′), 1.00 (m, H4), 0.49 (d, J = 6.5 Hz, 5-CH_3), 0.69 (d, J = 6.5 Hz, 5′-CH_3), *N*-MeTyr δ 4.88 (br d, J = 9 Hz, H2) 2.7 (m, H3), 3.06 (dd, J = 14 and 2 Hz, H3′), 2.68 (s, NMe), Ile δ 4.73 (dd, J = 9.6 and 4.5 Hz, H2), 1.75 (m, H3), 0.81 (d, J = 7.4 Hz, 3′-CH_3), 0.95 (m, H4), 1.2 (m, H4′), 0.78 (t, J = 7.3 Hz, H5), 7.47 (d, J = 9.6 Hz, NH). ^{13}C NMR **1** (DMSO-d_6) OH-PLac δ 173.4 (s, C1), 72.5 (d, C2), 39.6 (t, C3), Gln δ 171.7 (s, C1), 51.5 (d, C2), 28.4 (t, C3), 31.3 (t, C4), 174.2 (s, C5), Thr δ 168.9 (s, C1), 54.6 (d, C2), 72.0 (d, C3), 17.5 (q, C4), Tyr δ 170.0 (s, C1), 53.7 (d, C2), 35.2 (t, C3), Ahp δ 169.2 (s, C1), 49.1 (d, C2), 21.8 (t, C3), 29.7 (t, C4), 73.5 (d, C5), Leu δ 170.8 (s, C1), 47.7 (d, C2), 38.5 (t, C3), 23.6 (d, C4), 22.1 (q, C5), 23.9 (q, C5′), *N*-MeTyr δ 169.1 (s, C1), 60.8 (d, C2), 32.9 (t, C3), 30.3 (q, N-Me), Ile δ 172.3 (s, C1), 55.2 (d, C2), 38.5 (t, C3), 16.0 (q, C3′), 24.4 (t, C4), 11.4 (q, C5).

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(16) Intraperitoneal injection of 0.5–100-μg samples in 1% Tween 80 to 20-g male CD-1 mice caused spasms or convulsions, but not death. The animals recovered overnight.