A Partial Structure for the Toxin BE-4 from the Blue-green Algae, *Microcystis aeruginosa*

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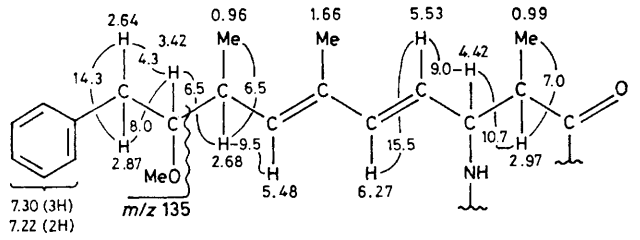
The amino acid sequence of a peptide fragment of a peptide toxin isolated from the blue-green algae, *Microcystis aeruginosa*, is D-Ala-L-Leu-*erythro*-β-methyl-D-isoAsp-L-Ala-Baa-D-Glu, where Baa is a novel β-amino acid; the toxin is monocyclic, and the position of an *N*-methyldehydroalanine residue has been tentatively established.

Several instances of animal poisoning in many parts of the world have been attributed to the sporadic blooms of toxic strains of the blue-green algae *Microcystis aeruginosa*.[1] Four toxin variants were isolated from a laboratory clone of *M. aeruginosa* forma *aeruginosa* cultured from a natural bloom in Witbank Dam.[2] Details of the absolute configurations of common amino acids contained in the four toxin variants have been reported.[3] We now report the identification of a novel β-amino acid, and an amino acid sequence of the toxin variant BE-4.

The reported[3] amino acid composition of BE-4 is (molar ratios in parentheses): *erythro*-β-methyl-D-Asp(1), D-Glu(1), D-Ala(1), L-Ala(1), L-Leu(1), and *N*-methyldehydroalanine (*N*-MeDha) (1). The peptide has a blocked *N*-terminus (no acetylation with Ac_2O–H_2O), and is resistant to enzymic digestion. The molecular weight is determined as 909 Daltons by fast atom bombardment mass spectrometry (f.a.b.m.s.) but neither this spectrum, nor the electron impact (e.i.) mass spectrum of permethylated BE-4, showed evidence of the sequence ions normally observed in the spectra of linear peptides.[4,5] On the basis of the above evidence, we conclude that BE-4 is likely to be a cyclic peptide. If the peptide is monocyclic, then the component(s) to be added to the already identified units has a mass of 313 Daltons. The identification of this component as a novel β-amino acid is presented.

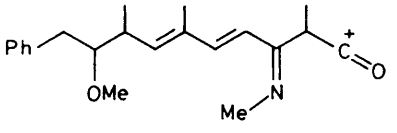
In addition to the presence of the above-mentioned amino acids, the 400 MHz ^1H n.m.r. spectrum of BE-4 in D_2O showed carbon-bound protons corresponding to the β-amino acid residue (**1**). The connectivity was established by spin decoupling experiments [*J* values indicated in (**1**)] and negative nuclear Overhauser effects (n.O.e.s) [{5.53} → 1.66 (−12%), {6.27} → 5.48 (−15%), and {5.48} → 6.27 (−15%)], the n.O.e.s being determined in $(CD_3)_2SO$ solution at 303 K.



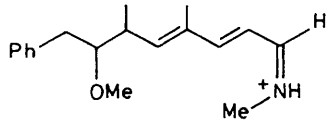
(1)

The nature of the functional groups in (1) was established by e.i. mass spectrometry. An abundant fragment ion at *m/z* 135 in the mass spectrum of BE-4 (or permethylated BE-4) is in accord with a methoxy substituent β to the phenyl group. This conclusion is consistent with the chemical shift of the adjacent proton (δ 3.42), and the observation of a methyl resonance at δ 3.35 (D_2O solution). Fragment ions in e.i. mass spectra of permethylated BE-4 before, and after, pulse hydrolysis (6 M HCl, 5 min, 100 °C, followed by acetylation) correspond to structures (2) and (3), respectively. These assignments are in accord with high resolution mass measurements, and the shifts to *m/z* 329 and 275 when CD_3I replaces CH_3I in the permethylation step.

Reduction of the *N*-MeDha residue in BE-4 with $NaBH_4$ (or $NaBD_4$) gives a product with a molecular weight increased by two (or three) Daltons, as established by f.a.b.m.s. The reduced toxin was subjected to pulse hydrolysis (6 M HCl, 5 min, 100 °C) and two major products, BE-4I and BE-4II, were isolated by reversed phase h.p.l.c.; their molecular weights (f.a.b.m.s.) were 812 and 794 Daltons, and both showed fragment ions corresponding to the loss of a *C*-terminal 'Glu' residue[5] (where 'Glu' represents either Glu or β-MeAsp, since these amino acids have the same molecular weights). These molecular weights were unchanged when reduction of BE-4 was carried out with $NaBD_4$. Thus, the residue which had been lost is reduced *N*-MeDha. F.a.b.m.s. analysis of the esterified (MeOH–HCl) or acetylated (Ac_2O–H_2O) products revealed that both have a free amino group; while BE-4I has three, and BE-4II has two, carboxylic acid groups. These results suggest that BE-4I is a linear peptide produced from hydrolysis (+18 Daltons) of monocyclic BE-4, with losses of the reduced *N*-MeDha unit (−85 Daltons) and methanol (−32 Daltons). The last loss is readily accommodated from (1). Product BE-4II is concluded to differ from BE-4I by dehydration involving one carboxy group.



(2): *m/z* 326.2112
(calc. 326.2120)



(3): *m/z* 272.2011
(calc. 272.2014)

BE-4I was acetylated [Ac_2O–$(CD_3CO)_2O$ (1 : 1) in H_2O] and then deuterio-permethylated. The e.i. mass spectrum of the product shows pairs of acylium sequence ions at *m/z* 131/134, 261/264, 424/427, 512/515, 810/813, and 973/976. These, in conjunction with the above f.a.b.m.s. data, define the sequence: Ac-Ala-Leu-'Glu'-Ala-Baa'-'Glu' where Baa' is the β-amino acid (1) minus methanol. A similar experiment on BE-4II gave the partial sequence Ac-Ala-Leu-'Glu'-Ala. . . .

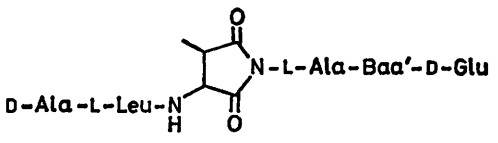
Analysis of the *N*-trifluoroacetyl isopropyl esters of the amino acids derived from acid hydrolysis of BE-4II, by a g.c.–m.s. system fitted with a chiral capillary column, showed D-Ala : L-Ala in the ratio 1 : 1.5. After one Edman degradation cycle on BE-4II, the f.a.b. mass spectrum of the remaining peptide[6] showed that *N*-terminal Ala was cleanly removed. Analysis of the remaining amino acid components of this peptide as above showed L-Ala as before, but no D-Ala was detected. Thus D-Ala is the *N*-terminal residue and L-Ala the fourth residue from the *N*-terminus. The second Edman cycle on BE-4I or BE-4II removed L-Leu, but the third Edman cycle failed as indicated by no change in the molecular weight as determined by f.a.b.m.s. Dansylation of this tetrapeptide, followed by hydrolysis, gave dansyl-β-MeAsp, establishing β-MeAsp as residue 3. Since it is not removed by the Edman procedure, it must be β-linked to the next residue. Thus, the structure of BE-4I (except the stereochemistry of Baa') is (4), where Glu is now restricted to Glu or isoGlu in the parent peptide.

D-Ala-L-Leu-*erythro*-β-methyl-D-isoAsp-L-Ala-Baa'-D-Glu

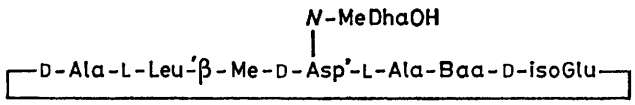
(4)

The degradation at the β-methyl Asp residue in BE-4II possibly involves the formation of a cyclic imide as in (5). This may well be an intermediate in the aspartyl → isoaspartyl rearrangement.[7,8] Therefore, the presence of β-methylisoAsp in BE-4 cannot be deduced from its presence in BE-4I.

To determine the positions of free carboxy groups in BE-4, its ^1H n.m.r. spectrum was recorded as a function of 'pH' (NaOD added to a solution in D_2O). Only the resonance of the C_α-proton of the Glu residue (δ 4.14) shifted significantly upfield (to δ 3.86) with increasing 'pH'. We conclude that the α-carboxy group of Glu is free, whereas the γ-carboxy group, and both the α- and β-carboxy groups of β-methylAsp (or β-methylisoAsp) are blocked. Since the molecular weight of the toxin requires two free carboxy groups in the structure, the n.m.r. data can be satisfied if the second carboxy group is attached to the α-carbon of *N*-MeDha (lacking an α-CH). This conclusion is additionally supported by the observation that $NaBH_4$-reduced BE-4 forms a dimethyl ester (0.02 M HCl–MeOH, followed by f.a.b.m.s.), whereas BE-4 itself forms a monomethyl ester; evidently under these conditions, the conjugate carboxy group is not esterified. Thus, (6) where 'β-Me-D-Asp' is either *erythro*-β-methyl-D-Asp or *erythro*-β-methyl-D-isoAsp, satisfies all the structural information we have for BE-4.



(5)



(6)

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