Article

MALDI-TOF Mass spectrometry of Cyanobacteria: a Global Approach to the Discovery of Novel Secondary Metabolites*

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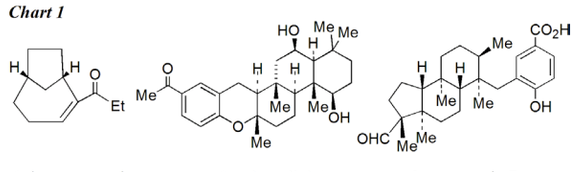
*This article, originally printed in the January issue of this *Journal* (**2008**, *72*, 25-28), was subject to major production errors with the loss of structural displays critical to its understanding. In fairness to the authors, and to you the reader, the article is reproduced here in full; the editors of *Chemistry in New Zealand* apologise unreservedly.

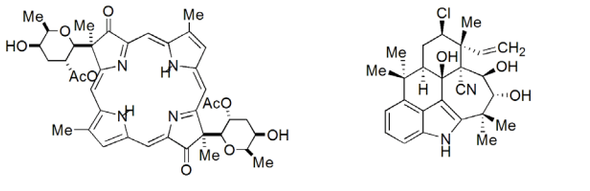
Cyanobacteria (blue-green algae) are a group of ancient prokaryotic organisms dating back between three and four billion years.[1] They have been attributed with oxygenating the earth's atmosphere[2] but, since the anthropogenic euthrophication of lakes, ponds and oceans, they have become synonymous with water hygiene issues.[3] This is due to the alteration of the nutrient composition of their habitat to one which is optimal for growth (or blooms). Cyanobacterial blooms may simply cause foul tastes and odours,[4] but they can also lead to the production of toxic secondary metabolites poisonous to humans and animals upon ingestion.[5] NZ has yet to suffer a human fatality, but the deaths of several dogs in Wellington was attributed to homoanatoxin-a **1** (Chart 1) from a *Phormidium* species.[6]

Although toxins are the most highly publicized cyanobacterial secondary metabolites, a vast array of compounds are produced which range in size, structure, and bioactivity. Terrestrial cyanobacteria have yielded diterpenes such as the anti-inflammatory tolypodiol **2**,[7] and the antimicrobial comnostins[8] such as comnostin B **3**, in addition to other unusual metabolites including tolyporphin A **4** (a porphyrin-like compound with multi-drug resistance reversal properties)[9] and the ambiguenes, *e.g.* ambiguene F **5**, which are antifungal chlorinated alkaloids (Chart 1).[10]

Chart 1



1, homoanatoxin-a **2,** tolypodiol A **3**, comnostin B



4, tolyporphin A **5**, ambiguene F

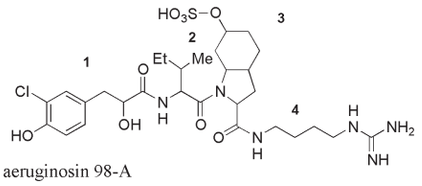
The major class of secondary metabolites produced by cyanobacteria is that of the oligopeptides, which are synthesised by non-ribosomal peptide synthetases.[11] These can be divided into six families depending on their structural characteristics,[12] namely the aeruginosins, the microginins, the anabaenopeptins, the cyanopeptolins, the microcystins, and the microviridins, as exemplified by metabolites **6-11** of Chart 2.

In the past, oligopeptides have been detected via enzyme-linked immunosorbent assays, enzyme inhibition assays, or according to their toxicity.[13] These assays have focused on obtaining quantitative data on the metabolites present, therefore the potential of these methods as discovery tools is limited. Analysis by high performance liquid chromatography (HPLC) is hindered by a lack of commercially available standards[14] so that time is wasted isolating known metabolites. Bioactivity-directed isolation has proved to be very effective in the past[8] but again limits the researcher to detecting molecules possessing a certain activity. More powerful still is liquid chromatography-mass spectrometry (LC-MS). Here, one can separate the components in a complex mixture and obtain their relative molecular masses. This allows one to assess the potential novelty of a compound according to both mass and elution time prior to large-scale purification and characterization. Most LC-MS instruments allow for tandem MS that enables structural clues to be deduced and the identity of known molecules to be confirmed.[15] However, separation by HPLC involves costly and time consuming sample preparation and, due to long run times, high throughput can be cumbersome. Analysis of cyanobacterial extracts by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS can provide comparable data to those from LC-MS but with far simpler sample preparation.

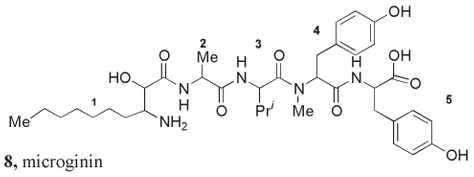
MALDI-TOF produces ions from laser irradiation of a sample co-crystallized with a matrix; the laser energy is absorbed and passed to the analyte molecules. This method of ionization predominantly produces singly protonated ions to *ca. m/z* = 5000, a range which encompasses the oligopeptides. Thus complex mixtures can be analyzed from a minute amount of sample without prior separation, and the relative molecular mass of each component present deduced from the protonated molecular ions.[16] Cyanobacterial extracts are assessed simply from mixing with the matrix, application of the mix to a target, and spectral recording.

The advantages described above make MALDI-TOF screening of cyanobacterial extracts particularly useful in the discovery of novel secondary metabolites. Due to the high sensitivity, low sample volumes, and speed of analysis, environmental samples can be assessed for the presence of novel compounds prior to culturing. Even single cyanobacterial colonies can be analysed by suspending them directly in matrix solution.[17] Novel compounds are easily detected using this method by comparing the

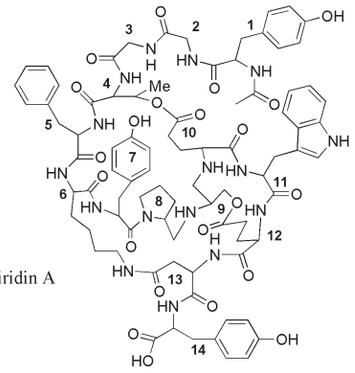
Chart 2



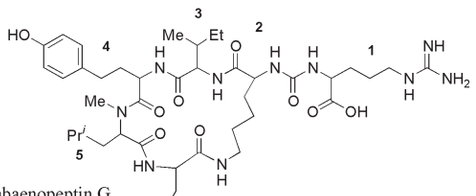
6, aeruginosin 98-A



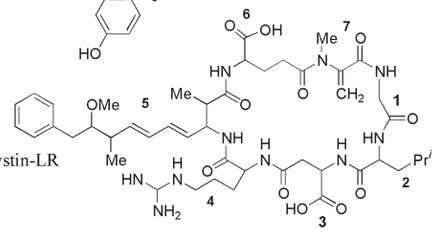
8, microginin



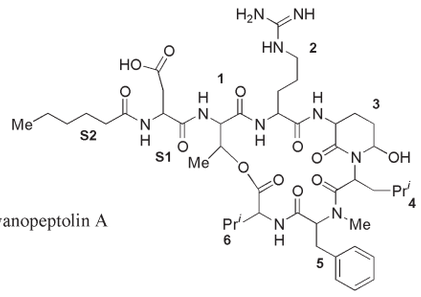
10, microviridin A



7, anabaenopeptin G



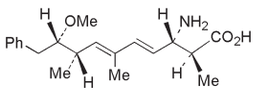
9, microcystin-LR



11, cyanopeptolin A

component masses recorded with those in an appropriate database. If the mass spectrometer is also equipped for the analysis of post source decay (PSD) species, it is then analogous to LC-MS with tandem MS, and the presence of known compounds can be confirmed from the masses of the fragment ions produced.

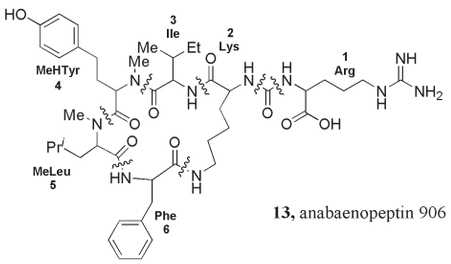
Since cyanobacterial oligopeptides are already well characterized, PSD allows for partial characterization of any novel compounds discovered. Often the subclass of oligopeptide present can be deduced by the presence of diagnostic fragment ions in the spectrum, e.g. the presence of a *m/z* = 135 ion ($PhCH_2CHOMe^{+\bullet}$) is characteristic of 2*S*,3*S*,8*S*,9*S*-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid (Adda, **12**), the unique amino acid found in microcystins.[16] The low mass daughter ions indicate the amino acids present in the molecule, while those at higher mass can indicate how the amino acids are joined together.



12, Adda

Oligopeptide characterization by MALDI-TOF MS has been undertaken successfully in Germany. Using the approach described above, von Dohren and co-workers were able to characterize a range of oligopeptides including aeruginosins, microginins, anabaenopeptins, and cyanopeptolins, whilst assessing the oligopeptide diversity of different cyanobacteria. They deduced structures for anabaenopeptin G, **7** (Chart 2) and anabaenopeptin 820 from analysis of the PSD fragments.[12,18,19]

The anabaenopeptins are cyclic peptides containing six amino acids. Each contains a D-lysine unit that has an ureido bond to a carbonyl that is linked to a side-chain amino acid. The D-lysine also forms a secondary peptide bond which encloses the ring. The CO-linked side-chain and the ring amino acids vary as does their degree of amino methylation.[11] There are 21 published structures of anabaenopeptins and these are listed in Table 1. The different compounds have varied biological activities including relaxation of norepinephrine-induced contraction,[20] protein phosphatase inhibition, and protease inhibition.[21-24] Like the German workers, we too have been able to deduce most of the structure of a new metabolite using MALDI-TOF MS, namely anabaenopeptin 906, **13**.



13, anabaenopeptin 906

An environmental sample containing cyanobacteria was collected from a North Island lake. The MALDI-TOF MS (Fig. 1) showed the presence of several known compounds as well as an unknown metabolite with *m/z* = 907. The PSD spectrum of this *m/z* = 907 ion is shown as Fig. 2 and the loss of 200 Da is clear. This is diagnostic for an anabaenopeptin possessing an arginine side-chain. The low mass/charge species indicate the presence of arginine (Arg; *m/z* = 70), lysine (Lys; *m/z* = 70, 84), isoleucine (Ile; *m/z* = 86), methylleucine (MeLeu; *m/z* = 100), and methylhomotyrosine (MeHTyr; *m/z* = 107, 164). Thus, five of the six amino acids present in the anabaenopeptins are identified, with the missing mass/charge entity correlating with that of a phenylalanine (Phe) residue. This

Table 1. Amino acid sequence of the known Anabaenopeptinsa

Compound	Mr (Da)	1	3	4	5	6	Ref.
Anabaenopeptin A	843	Tyr	Val	HTyr	MeAla	Phe	20
Anabaenopeptin B	836	Arg	Val	HTyr	MeAla	Phe	20
Anabaenopeptin C	808	Lys	Val	HTyr	MeAla	Phe	25
Anabaenopeptin D	827	Phe	Val	HTyr	MeAla	Phe	25
Anabaenopeptin E	850	Arg	Val	MeHTyr	MeAla	Phe	26
Anabaenopeptin F	850	Arg	Ile	HTyr	MeAla	Phe	26
Anabaenopeptin G	908	Arg	Ile	HTyr	MeLeu	Tyr	18
Anabaenopeptin G*	929	Tyr	Ile	HTyr	MeHTyr	Ile	23
Anabaenopeptin H	922	Arg	Ile	HTyr	MeTyr	Ile	23
Anabaenopeptin I	759	Ile	Val	HTyr	MeAla	Leu	22
Anabaenopeptin J	793	Ile	Val	HTyr	MeAla	Phe	22
Anabaenopeptin T	865	Ile	Val	HTyr	MeHTyr	Ile	24
Anabaenopeptin KT864	864	HArg	Ile	HTyr	MeAla	Phe	27
Anabaenopeptin 820	820	Arg	Val	HPhe	MeAla	Phe	12
Ferintoic Acid A	866	Trp	Val	HTyr	MeAla	Phe	28
Ferintoic Acid B	880	Trp	Ile	HTyr	MeAla	Phe	28
Nodulapeptin A	929	Ile	Met(O_2)	HPhe	MeHTyr	Ser(Ac)	29
Nodulapeptin B	913	Ile	Met(O)	HPhe	MeHTyr	Ser(Ac)	29
Oscillamide B	868	Arg	Met	HTyr	MeAla	Phe	21
Oscillamide C	956	Arg	Ile	HTyr	MeHTyr	Phe	21
Oscillamide Y	857	Tyr	Ile	HTyr	MeAla	Phe	21

[a]Numbering for anabaenopeptins amino acids is as for **7** of Chart 2; D-Lys is omitted as it is always present in position 2 in the known anabaenopeptins.

Table 2. Fragment ions of anabaenopeptin 906 observed by PSD.

m/z	Sequence
70	Arg/Lys-related ion
84	Lys-Immonium ion
86	Ile-Immonium ion
100	MeLeu-Immonium ion
107	Tyr-side chain
112	Arg-Immonium ion
129	Arg-Immonium ion
164	MeHTyr-Immonium ion
175	Arg + 2H
201	CO + Arg
275	MeLeu + Phe + H
305	Ile + MeHTyr + H
449	Arg + CO + Lys + Phe - CO + 2H
466	MeHTyr + MeLeu + Phe + H
579	Ile + MeHTyr + MeLeu + Phe + H
594	Lys + Phe + MeLeu + MeHTyr + 2H
603	Arg + CO + Lys + Phe + MeLeu + H
707	Lys + Ile + MeHTyr + MeLeu + Phe +2H
907	M + H

also matches well with anabaenopeptin G, **7** in that its mass is only 2 Da higher than the new **13**; it corresponds to the loss of a hydroxyl group from the tyrosine in position 5, and an additional amino methylation on HTyr in position 3.

The higher mass fragments provide the sequence of the ring amino acids in **13**. Thus, the *m/z* = 275 fragment shows that the Phe is joined to the MeLeu and the *m/z* = 449 fragment shows that the Phe is also attached to the Lys, thus placing it in either position 3 or 6. The *m/z* = 466 fragment can then be used to show that MeHTyr is attached to MeLeu as Phe is already attached to both Lys and MeLeu. This gives a final sequence of Ile-MeHTyr-MeLeu-Phe, and supports the presence of an Ile-MeHTyr fragment at *m/z* = 305.

None of the fragments observed confirm the order in which the amino acids are present in the ring and whether Ile or Phe is located at position 3. The structure proposed as **13** has been constructed according to the sequences of presently known anabaenopeptins, where Phe is commonly seen at position 6 and an aromatic amino acid, such as HTyr, is always at position 4. This illustrates the limitation in characterizing secondary cyanobacterial

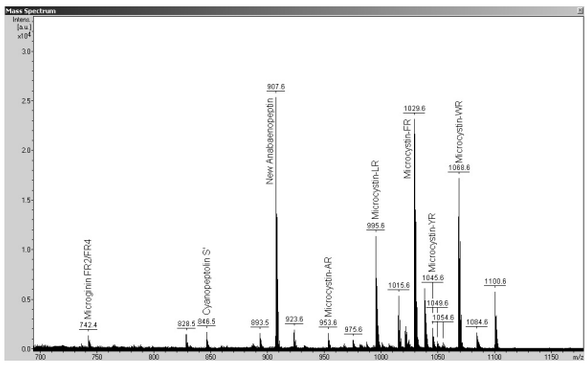


Fig. 1. MALDI-TOF MS of a NZ lake sample.

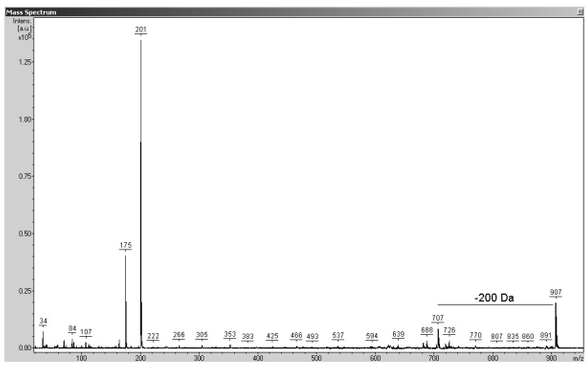


Fig. 2. The MALDI-TOF PSD spectrum of *m/z* = 907 from Fig. 1.

metabolites by MALDI-TOF MS as, at times, the complete structure cannot be elucidated and stereochemistry can never be deduced. Ultimately, full characterization of these novel compounds requires purification and NMR spectroscopic investigations.

The screening of cyanobacterial extracts for oligopeptides by MALDI-TOF MS is a very powerful technique that can lead to the discovery of new compounds. It is simple, quick and inexpensive. Its use requires only a minute amount of sample that gives a rapid assessment of the presence or absence of novel metabolites.

Acknowledgements

The contribution of Susanna Wood (Cawthron Institute, Nelson) is gratefully acknowledged.

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Dates of Note

April 22 marks the 150th anniversary of development of the fused salt electrolysis for commercial production of aluminium by ***Martin Killani***.

Born on 27 Apr 1913, ***Philip Hauge Abelson*** was the US physical chemist who proposed the gas diffusion process for separating ^{235}U from ^{238}U, which was essential to the development of the atomic bomb. Also on this day (in 1896) was born ***Wallace Hume Carothers***, the discovered of nylon.

Joel H. Hildebrand died on 30 Apr 1983. His monograph *Solubility* (1924; later editions, *Solubility of Non-Electrolytes*) was the classic reference for almost a half century.

John William Draper, the English-American chemist who pioneered in photochemistry, was born on 5 May 1811. He recognized that light initiated chemical reactions as molecules absorbed light energy.

May 15 marks the 150th anniversary of the death of 1858 ***Robert Hare***, the American chemist who devised the first oxyhydrogen blowpipe for the purpose of producing great heat. He melted sizeable quantities of platinum with this. His device is the precursor to the modern welding torch.

René-Just Haüy, the French mineralogist who founded of the science of crystallography through his discovery of the geometrical law of crystallization, died on 1 Jun 1822. In 1781, he saw an accidentally dropped calcite crystal break into rhombohedral pieces. Deliberately breaking various forms of calcite, he found the same result, concluding that all the molecules of calcite have the same form and it is only how they are joined together that produces different gross structures. He suggested that other minerals should show different basic forms. His theory predicted the correct angles of crystal faces in many cases.

June 19 is the 225th anniversary of the birth of ***Fredrich Wilhelm Sertürner***, the discoverer of morphine.

Richard Buckminster Fuller died on July 1 1983. He was the US engineer/architect who developed the geodesic dome.

Amedeo Avogadro published his memoir about the molecular content of gases on July 11 in 1811.