ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL COMPOUNDS SYNTHESISED BY *MICROCYSTIS* SP.

by

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A Thesis submitted for the degree of

Doctor of Philosophy

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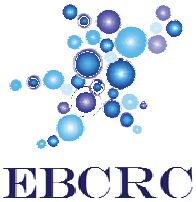

"Never regard study as a duty, but as the enviable opportunity to learn to know the liberating influence of beauty in the realm of the spirit for your own personal joy and to the profit of the community to which your later work belongs." Albert Einstein

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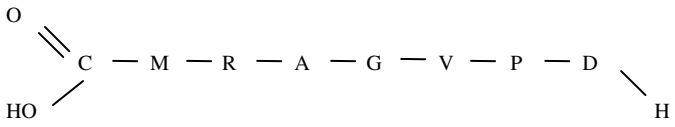
EXECUTIVE SUMMARY

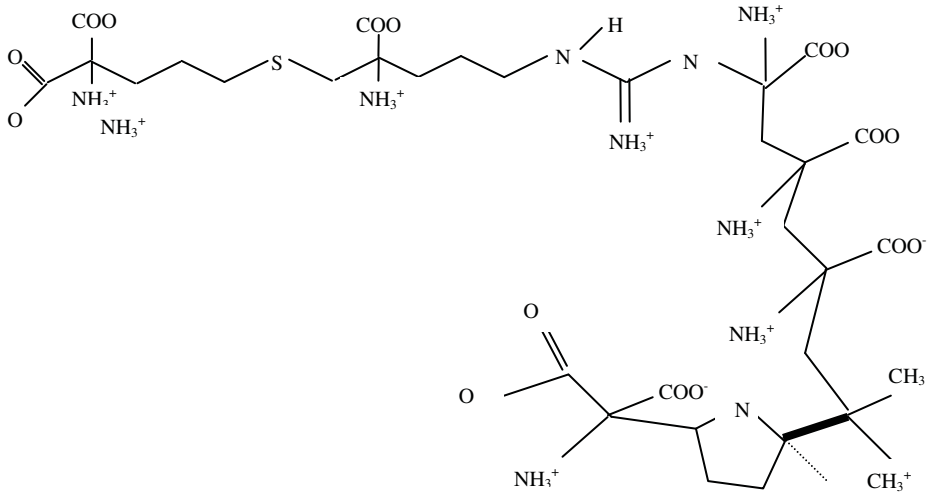
Cyanobacterial secondary metabolites, often identified as toxins such as microcystin, have also demonstrated biological functions including inhibition of bacterial and viral growth. In this study, 10 cyanobacterial strains were isolated from field sites around Adelaide and laboratory cultures and assessed for bioactivity against bacterial, viral and fungal pathogens. A comprehensive literature search identified a number of screening assays employed by research groups to identify cyanobacterial strains with biological activity. Within the review, methods to optimise extraction of the compounds were also noted. Combinations of extraction methods, solvents and assay procedures were investigated to optimise the success of this phase of the study. Bioactivity was confirmed by development of agar disc diffusion and microtitre plate assays to analyse cyanobacterial biomass extracts. Result of the assays indicated a methanolic extract of one species, *Microcystis flos-aquae* (Wittr Elenkin), inhibited growth of bacterial cells and viral infectivity and was selected for further analysis.

The bioactive compound was isolated by HPLC and mass spectrometric analysis. Separation of the bioactive extract into component peaks indicated only one that was likely to represent the metabolite of interest, at a retention time of approximately 18 min. A second profile was constructed of a methanolic extract of the same species in a later growth stage that did not inhibit growth of either the bacterial or viral test organisms. Comparison of the profiles exposed the absence of the peak at 18 min retention time in the second profile. Accumulation of the fraction was conducted using a semi-preparative HPLC column for analysis by mass spectrometry.

A sample of the isolated peptide was submitted to Proteomics International, a subsidiary of Murdoch University, WA, for identification and structural characterisation. Proteomics International analysed the data by electronspray ionisation time of flight mass spectrometry (LC/MS/TOF) followed by LC. *De novo* sequence analysis of the data was carried out using Analyst QS software; however, PI was unable to provide a readily interpretable, continuous amino acid sequence, despite their admission that some gaps in the fragmentation ladder corresponded to known amino acids. Interpretation of the data generated by Proteomics International by a research chemist within the University of Adelaide proposed the following amino acid sequence and subsequent structure for the compound:



(a)



(b)

Proposed (a) amino acid sequence and (b) structure for the bioactive compound isolated from non-toxic *M. flos-aquae***.**

Comparison of the proposed sequence with those contained in peptide databases was unable to classify the compound (B Neilan, personal communication, April 2008), suggesting the bioactive metabolite is perhaps previously undetected and therefore may be considered a novel compound, or has undergone a modification and is thus a variant of a known compound.

Taxonomic classification of the strain used during this study was completed by PCR amplification of 16S ribosomal RNA, using primers from alternative cyanobacterial sources. The sequence was classified in the following taxonomic hierarchy (with 100% assignment detail, for a confidence threshold of 95%):

Domain: Bacteria

Phylum Cyanobacteria

Class Cyanobacteria

Family Family 1.1

Genus *Microcystis*

This classification confirms that the species investigated during this research is of the genus *Microcystis*.

Synthesis of cyanobacterial metabolites is generally accepted to be a result of non-ribosomal synthetic pathways. The presence of non-ribosomal peptide synthetase and polyketide synthetase genes in *Microcystis flos-aquae* was confirmed by PCR amplification using degenerate primers from other cyanobacterial sources. Analysis of sequence data identified the presence of an NRPS gene demonstrating significant similarity (98%) to the NRPS cyanopeptolin gene of *Microcystis* sp. However, the PKS (polyketide) gene identified verified only a 63% similarity to a known sequence, that of the PKS (*mcyG*) gene of *M. aeruginosa* PCC 7806 (Koch). Results of the molecular investigation imply this compound may belong within the cyanopeptolin family.

Researchers have speculated that the majority of cyanobacteria possess genes for production of toxins, though in many instances the gene cluster may be incomplete or one or more genes may be absent or mutated. The presence of microcystin genes was confirmed by PCR amplification using primers from previously characterised cyanobacterial genes. Analysis of the sequence data identified the presence of several *mcy* genes generally found in toxic strains of cyanobacteria noted for synthesis of the toxin microcystin. The DNA sequences show significant similarity to the *mcyA*, *mcyC*, *mcyD* and *mcyE* genes described for *Microcystis* sp. and *Microcystis aeruginosa* PCC 7806. However, analysis of the sequence data for the *mcyB* gene revealed that this gene was not present. Further PCR amplification of the region between *mcyA* and *mcyC* using the reverse complements of the original primers indicated that a sequence was present that may have been a truncated variant of *mcy*B or another gene entirely. Time constraints prohibited submission of this region for sequence analysis.

The primary objective of this research project was to screen a field strain of cyanobacteria for synthesis of biologically active secondary metabolites, and to isolate those compounds using a combination of analytical chemistry and molecular biotechnology. This study forms part of a collaborative project between the University of Adelaide, South Australian Research and Development Institute (Aquatic Sciences) and the Environmental Biotechnology Cooperative Research Centre, entitled “P6:

Commercial scale integrated biosystems for organic waste and wastewater treatment for the livestock and food processing industries".

STATEMENT OF ORIGINALITY

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:…… Date:…..……

Kyleigh Jane Victory

LIST OF PUBLICATIONS

Conference Papers

K. J. Victory, D. M. Lewis, C. J. Thomas, K D King (2007). "**Isolation and analysis of pharmacologically-active peptides of *Microcystis flos-aquae*"** Chemeca 2007, Melbourne, Australia 23-26 September 2007 (oral presentation) ISBN 0 858 25844 7

K. J. Victory, D. M. Lewis, K. D. King, C. J. Thomas (2007). "**Development of analytical techniques for extraction of biologically active compounds from *Microcystis flos-aquae*"** International Society for Pharmaceutical Engineering, Gold Coast, Australia 2-4 September 2007 (Poster Presentation)

K J Victory, D M Lewis, C J Thomas, M Kumar, K D King (2006). **"Comparison of bioactivity of 3 strains of non-toxic *Microcystis* grown in different culture media"** 6th Asia-Pacific Conference Algal Biotechnology, Makati City, Philippines 12-15 October 2006 ISSN 0921-8971 (Print) 1573-5176 (Online) (Poster presentation)

K J Victory, D M Lewis, C J Thomas, J M Kumar, K D King (2005) "**Detection of antimicrobial metabolites Produced by microalgae and cyanobacteria"** Environmental Biotechnology Cooperative Research Centre Annual conference, Brisbane, Australia 30 November - 1 December 2005 (Oral presentation)

Victory K J, Lewis D M, Thomas C J, Kumar J M, King K D (2005). **"Detection of antimicrobial metabolites Produced by microalgae and cyanobacteria"** 8th International Phycological Congress, Durban, South Africa, 13-17 August 2005. (Oral presentation)

Victory K J, Lewis D M, Thomas C J, Kumar J M, King K D (2005). **"Analysis and Classification of Antimicrobial metabolites produced by microalgae and cyanobacteria"** 7th World Congress of Chemical Engineering, Glasgow, Scotland 10-14 July 2005. (Poster presentation) [7th World Congress of Chemical Engineering Proceedings, P20–012 (IChemE, ISBN 0 85295 494 8)]

Victory K J, Lewis D M, Thomas C J, Kumar J M, King K D (2004). **"Analysis and Classification of Antimicrobial metabolites produced by microalgae and**

cyanobacteria" Australasian Society for Phycology and Aquatic Botany Adelaide, Australia December 2004 (oral presentation) Australasian Society for Phycology and Aquatic Botany 19th Conference CSIRO Publishing

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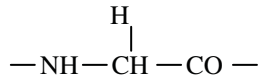
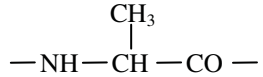
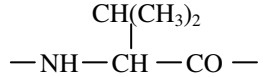
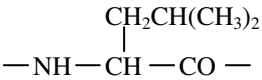
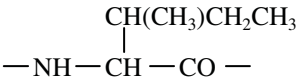
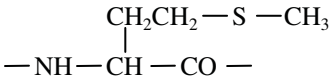
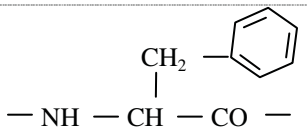
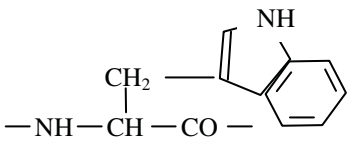
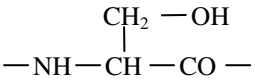
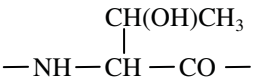
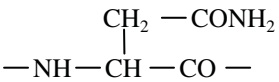
ABBREVIATIONS

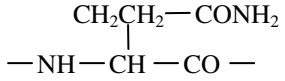
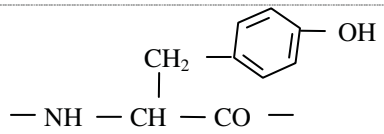
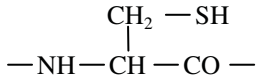
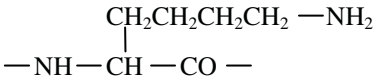
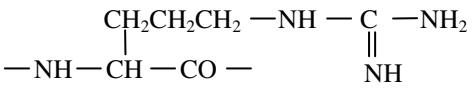
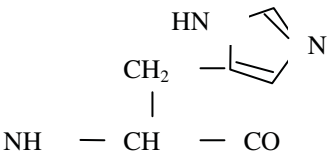
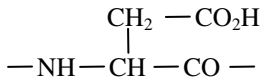
°C	degrees Centigrade
Å	Ångström
A. fumigatus	*Aspergillus fumigatus*
ACN	Acetonitrile
ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Amp	Ampicillin
Arg	Arginine
ASM	Artificial Seawater Media
B sub	*Bacillus subtilis*
bp	Base pairs
C. albicans	*Candida Albicans*
Ca	Calcium
CBD	Central Business District
cm	Centimetres
CO_2	Carbon dioxide
Da	Daltons
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DV	Dengue virus
E coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
EtOH	Ethanol
eV	Electron volts
GC	Gas Chromatography
Glu	Glutamine
HIV	Human Immunodeficiency Virus

HPLC	High Pressure Liquid Chromatography
hr	Hour
HSV	Herpes Simplex Virus
ID	Internal diameter
kb	Kilobases
LB	Luria Bertani
LC/MS	Liquid Chromatography Mass Spectrometry
LCPUFA	Long chain polyunsaturated fatty acids
LPS	Lipopolysaccharide
MDCK	Madin Darby Canine Kidney
MeOH	Methanol
mg	Micrograms
$MgCl_2$	Magnesium chloride
min	Minute
mL	Millilitres
mm	Millimetres
(m)V	(milli) Volts
mQ H_2O	Double distilled (Milli-Q®) water
MS	Mass Spectrometry
N	Nitrogen
NH3	Ammonia
NMR	Nuclear Magnetic Resonance
NOX	Nitrous oxides
NRPS	Non-ribosomal Peptide Synthetase
P	Phosphorous
PBR	Photobioreactor
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthetase
PS	Peptide Synthetase
PUFA	Polyunsaturated fatty acids
Q-STAR	Quadrapole time-of-flight mass spectrometer
R(n)F(n)	Run (n) Fraction (n)
RNA	Ribonucleic acid

RNAse	(Enzyme)
RSV	Respiratory Scyncytial Virus
RT	Room temperature
S aureus	*Staphylococcus aureus*
s	Seconds
SB	Stable bond
TAE	Tris-acetate-EDTA
T_d	Doubling time
TFA	Trifluoroacetic acid
TKN	Total Kjeldahl Nitrogen
T_m	Melting temperature (oligonucleotide primer)
TOF	Time-of-flight
µL	Microlitre
µm	Micron
UNSW	University of New South Wales
UV	Ultraviolet
v/v	Volume per volume basis
w/v	Weight per volume basis
×g	centrifugal force

Covalent structures of 20 common amino acids (Voet and Voet 2004)

Amino Acid	Abbreviation		Residue Mass (D)	Structure
	3 Letter	1 Letter		
Amino acids with non- polar side chains				
Glycine	GLY	G	57.0	
Alanine	ALA	A	71.0	
Valine	VAL	V	99.1	
Leucine	LEU	L	113.1	
Isoleucine	ILE	I	113.1	
Methionine	MET	M	131.1	
Proline	PRO	P	97.1	
Phenylalanine	PHE/	F	147.1	
Tryptophan	TRP	W	186.2	
Amino acids with uncharged polar side chains				
Serine	SER	S	87.0	
Threonine	THR	T	101.1	
Asparagine	ASN	N	114.1	

Amino Acid	Abbreviation		Residue Mass (D)	Structure
	3 Letter	1 Letter		
Glutamine	GLN	Q	128.1	
Tyrosine	TYR	Y	163.1	
Cysteine	CYS	C	103.1	
Amino acids with charged polar side chains				
Lysine	LYS	K	129.1	
Arginine	ARG	R	157.2	
Histidine	HIS	H	137.1	
Aspartic acid	ASP	D	114.0	
Glutamic acid	GLU	E	128.1