Isolation and Characterisation of Aeruginosin and Microviridin variants from *Microcystis species* CAWBG11

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Abstract

The cyanobacterial strain *Microcystis species* CAWBG11 was investigated in order to isolate and characterise bioactive oligopeptides such as aeruginosins and microviridins.

Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) Analysis of a methanol extract of a *Microcystis* CAWBG11 identified four known aeruginosin variants and four putatively new aeruginosin congeners as aeruginosins 618, 656A, 656B and 602B. Planar structures for these new congeners were proposed based on the MS/MS data, in conjunction with the sequence of the known aeruginosins.

Amongst the aeruginosins detected there were three pairs, each with the same molecular masses but exhibiting different retention times. Each pair was assigned as stereoisomers since they yielded identical tandem mass fragment ions.

The previously reported aeruginosin 602A was further characterised using the Advanced Marfey's Method of amino acid analysis and Nuclear Magnetic Resonance (NMR) spectroscopy.

Four new congeners of the cyclic microviridins; 1778A, 1778B, 1760 and 1764 were detected from a methanol extract of *Microcystis* CAWBG11. The sample was subsequently purified, enabling a planar structure for each congener to be proposed based on MS/MS data and amino acid analysis. The structure for microviridin 1778A was further investigated by NMR spectroscopy but was not fully elucidated due to time constraints.

The presence of the new aeruginosin and microviridin congeners indicated the diversity of the bioactive oligopeptides in the *Microcystis* strain.

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List of Abbreviations

ACN	Acetonitrile
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
aer	Aeruginosin synthetase gene
Agma	Agamatine
Ala	Alanine
Adc	1-(*N*-aminodino-Δ^3-pyrrolino)ethyl
Aeap	1-amino-2-ethoxy-3-aminopiperidine
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
ACE	Angiotensin-converting enzyme
Argal	Argininal
Arg	Arginine
Argol	Argininol
ABC	ATP-binding cassette transporter
Ccoi	2-carboxy-6-chlorooctahydroindole
Choi	2-carboxy-6-hydroxyoctahydroindole
Cleu	3-chloroleucine
COSY	Correlation Spectroscopy
HCCA	α-cyano-4-hydroxycinnamic acid
Cys	Cysteine
C	Celsius
Da	Dalton
^{o}	Degrees
DEPT	Distortionless Enhancement by Polarisation Transfer
Masp	D-erythro-β-methylaspartic acid
$FADH_2$	Falvin adenine dinucleotide
FA	Formic acid
FDLA	1-difluoro-2,4-dinitrophenyl-5-leucine amide
GC	Gas Chromatography
Glu	Glutamic acid
g	Gram/s
IC_{50}	Half-maximal inhibitory concentration
Hz	Hertz

HMBC	Heteronuclear Multiple-Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
HPLC	High Performance Liquid Chromatography
HRFAB	High Resolution Fast-Atom Bombardment
Has	Homoaspartic acid
h	Hour/s
HLeu	3-hydroxyleucine
Hpla	Hydroxyl-phenyl lactic acid
Ile	Isoleucine
K	Kelvin
k	Kilo (10^3)
Leu	Leucine
LC	Liquid Chromatography
L	Litre/s
Lys	Lysine
MS	Mass Spectrometry
m/z	Mass to charge ratio
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time of Flight
MeOH	Methanol
CD_3OD	Methanol-d_4
Me	Methyl
Mdha	*N*-methylhydroalanine
m	Metre/s
μ	Micro (10^{-6})
MCs	Microcystins
mdn	Microviridin synthetase gene (as defined by Zeimert *et al*.)
mvd	Microviridin synthetase gene (as defined by Philmus *et al*.)
m	Milli (10^{-3})
min	Minute/s
M	Molar or moles per litre
N	Nano (10^{-9})
NRPS	Non-Ribosomal Peptide Synthetase
nd	Not detected or not determined
NMR	Nuclear Magnetic Resonance
ppm	Part per million

PAP	Papain
Phe	Phenylalanine
Plas	Phenyllactic acid-2-*O*-sulfate
PCP	Peptidyl carrier protein
PLM	Plasmin
PKS	Polyketide sythetase
RP	Reversed-Phase
RNA	Ribonucleic acid
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
Ser	Serine
sp.	Species
Thr	Threonine
THB	Thrombin
TOCSY	Total Correlation Spectroscopy
TFA	Trifluoroacetic acid
TRP	Trypsin
Tyr	Tyrosine
Val	Valine
Xyl	Xylopyranose

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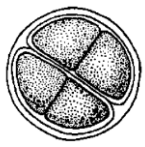
Chapter 1: Introduction

1.1 Cyanobacteria

Cyanobacteria are ancient photosynthetic prokaryotes found in almost every conceivable environment on Earth. They comprise parts of many terrestrial and aquatic ecosystems and are some of the oldest known organisms on the planet, originating 3.5 billion years ago.[1] Cyanobacteria are commonly known as blue-green algae due to the bluish cell colour that results from a high concentration of phycocyanin[2] and were apparently the first photosynthetic organisms capable of utilising water for the generation of cellular energy through photosynthesis.[3] They contribute significantly to the nitrogen economy of terrestrial and aquatic habitats through their ability to fix atmospheric nitrogen into forms which can be utilised by other organisms.[4]

There are five morphological orders of Cyanobacteria (Figure 1.1) which differ with respect to their size, shape and 16S ribosomal RNA sequences. Orders *Nostocales* and *Stigonematales* are monophyletic while orders *Chroococcales*, *Oscillatoriales* and *Pleurocapsales* are polyphyletic (being a taxonomic group that includes members, as genera or species, from different ancestral lineages).[5]



Order *Chroococcales*

Order *Pleurocapsales*

Order *Oscillatoriales*

Order *Stigonematales*

Order *Nostocales*

Figure 1.1: Microscopic view of the five morphological orders of Cyanobacteria. Adapted from Figure 2.1 of Reference 6.

Orders *Chroococcales* and *Pleurocapsales* are both unicellular but many strains of these orders can aggregate into irregular colonies. Both *Chroococcales* and *Pleurocapsales* have the ability to form baeocytes (internal spores) or endospores respectively. The remaining three cyanobacterial orders; *Oscillatoriales*, *Nostocales* and *Stigonematales* are multicellular and filamentous forms with trichomes (chains of cells). Order *Oscillatoriales* consists of uniseriated (arranged in one row) and unbranched trichomes with identical cells while orders *Nostocales* and *Stigonematales* consist of heterogeneous cellular trichomes with the presence of heterocytes (thick walled, transparent cells; capable of nitrogen fixation) and akinetes (large, thick walled cells which contain reserve materials for survival under unfavourable conditions).[6]

Under favourable eutrophic conditions, cyanobacteria multiply to form planktonic blooms and dense benthic mats that hamper recreation by diminishing water clarity and producing bad odours and tastes. These blooms are sources of a variety of bioactive secondary metabolites including some compounds that are harmful to humans and animals (Table 1.1).[3]

The cyanobacterial genera most commonly associated with the ability to produce toxic substances include; *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis*, *Nodularia*, *Nostoc* and *Planktothrix*.[7] These secondary metabolites endanger other organisms including humans when consumed in drinking water or by physical contact during recreational activities. However, they are also a source of new pharmaceutical lead compounds due to their chemical diversity and biological activities.[8] Cyanotoxins fall into various categories such as hepatotoxic, neurotoxic and those that cause dermal toxicity.[1] Based on their structure, cyanotoxins can be divided into three groups: cyclic peptides, alkaloids and lipopolysaccharides.

Table 1.1: Several classes of cyanotoxins and the cyanobacterial genera responsible for their production.

Cyanotoxin Class	Cyanotoxin Family	Primary target (in mammals)	Genera
Cyclic peptide	Microcystins	Liver	*Microcystis, Anabaena, Oscillatoria (Planktothrix),*[a] *Nostoc, Hapalosiphon, Anabaenopsis*
	Nodularin	Liver	*Nodularia*
Alkaloid	Anatoxin-a	Nerve synapse	*Anabaena, Aphanizomenon, Oscillatoria (Planktothrix)*[a]
	Anatoxin-a(S)[b]	Nerve synapse	*Anabaena*
	Aplysiatoxins	Nerve synapse	*Lyngbya, Schizothrix, Oscillatoria (Planktothrix)*[a]
	Cylindrospermopsins	Liver	*Cylindrospermopsis, Aphanizomenon, Umezakia*
	Lyngbyatoxin-a	Skin, gastro-intestinal tract	*Lyngbya*
	Saxitoxins	Nerve axon	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides		Potential irritants. Affect any exposed tissue	All

[a] *Planktothrix* was originally classified into the genus *Oscillatoria*, [b] Anatoxin-a(S) is an unique phosphate ester of a cyclic *N*-hydroxyguanine. Adapted from Table 3.1 of Reference 3.

1.2 Cyanobacterial Peptides

Cyanobacterial secondary metabolites display a vast diversity of structures. Among the known structures, the most commonly observed are linear or cyclised chains of amino acids called oligopeptides or cyanopeptides.[9] These small compounds are mostly produced by freshwater planktonic cyanobacteria such as *Anabaena*, *Microcystis* and *Planktothrix*[10] and are primarily produced by the non-ribosomal peptide synthetase (NRPS) pathway, the polyketide synthetase (PKS) pathway or a hybrid of the two pathways.[11] Cyanopeptides are grouped into classes with conserved structures which only differ by either exchange of an amino acid or modifications including methylation, glycosylation and halogenation.[9]

More than 600 cyanobacterial peptides have been described from more than 30 genera of all of the five morphological orders of cyanobacteria, however their

function in the producing cells has not yet been unravelled.[12]

Seven classes of cyanobacterial oligopeptides and their main bioactivities are reviewed in Table 1.2. They have attracted the attention of researchers over the past two decades based on the acute toxicity of some of these metabolites, their effect on human and animal health and the therapeutic use of several of these secondary metabolites.[13]

Table 1.2: The seven major oligopeptide classes and their main bioactivities.

Oligopeptides	Structure	Bioactivity (Inhibitors)
Anabaenopeptins	Cyclic	Protein phosphatase, carboxypeptidase-A
Cyanopeptolins	Cyclic	Proteases
Cyclamides	Multicyclic	Proteases
Microcystins	Cyclic with Adda[a]	Protein phosphatases 1 and 2A
Microginins	Linear	Proteases, angiotensin-converting enzyme
Aeruginosins	Linear	Proteases
Microviridins	Cyclic	Proteases, tyrosinase

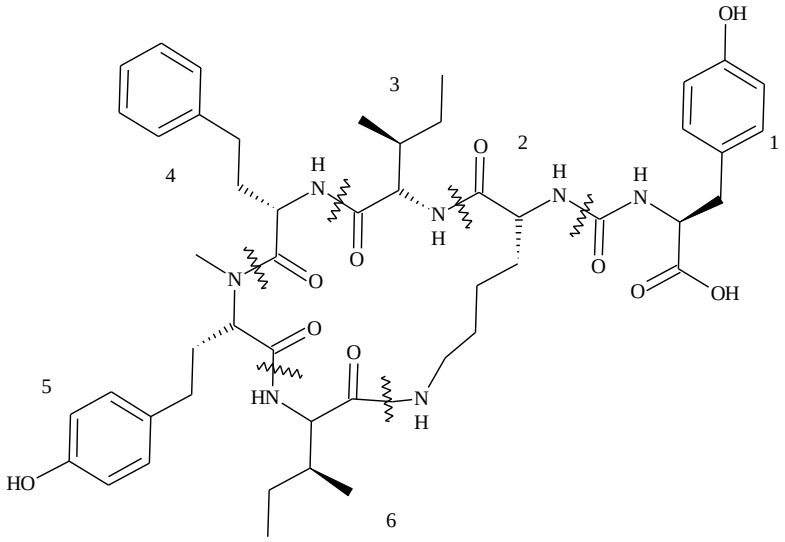
[a] 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid.
Adapted from Reference 11.

The focus of this research is the isolation and characterisation of cyanopeptides from a *Microcystis species* (CAWBG11) isolated from a cyanobacterial bloom sample obtained from Lake Hakanoa in 2005.[14] These cyanopeptides include linear and cyclic peptides such as aeruginosin and microviridin which were detected during the course of Dr. Jonathan Puddick's research in our group.[15] As a result of the previous investigation of these *Microcystis* samples, there were semi-purified samples with putative new congeners of aeruginosin and microviridin available, on which this research project is based. The main task in the current research is to structurally characterise some of the new congeners of aeruginosin and microviridin.

1.2.1 **Anabaenopeptins**

Anabaenopeptins are a group of cyclic hexapeptides which are characterised by the presence of D-lysine (Lys) in the second position and the formation of a ring through the carboxyl group of the position six amino acid and the primary ε-amine

of the Lys.[9] The α-amine of Lys is linked to a side chain amino acid (position one) through an ureido bridge,[16] such as in Anabaenopeptin MM913 (**1**).[17]



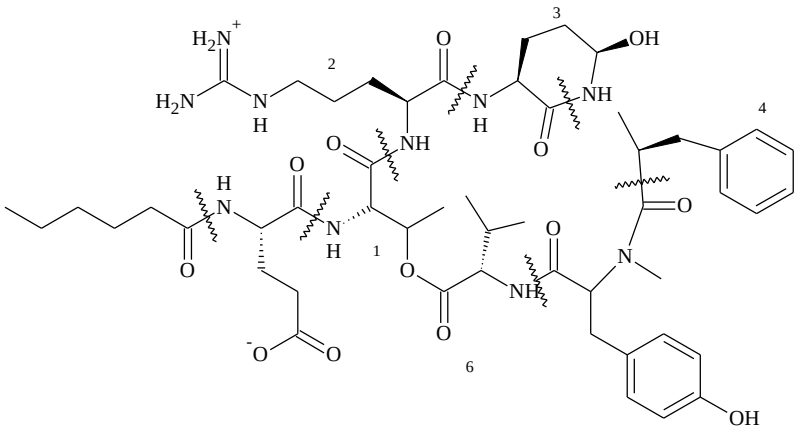
(**1**)

At least fifty two known variants of anabaenopeptins[9,18] have been isolated and characterised from various cyanobacterial strains such as; *Aphanizomenon flos-aquae*,[19] *Anabaena sp.*,[20] *Microcystis aeruginosa*,[21] *Oscillatoria agardii*,[22] *Nodularia spumigena*,[23] *Lyngbya sp.*[18] *Siliquariaspongia mirabilis*,[24] *Schizothrix sp.*[25] and also from the marine sponge *Theonella swinhoei*.[26] The genus *Theonella* has been shown to be one of the most prolific sources of bioactive natural products, some of which are almost certainly biosynthesised by symbiotic bacteria or cyanobacteria.[26] All of the anabaenopeptins which have been characterised to a sufficient level contain D-Lys whilst the other amino acids are all in the L-configuration and most of the variants tested are carboxypeptidase-A inhibitors.[17]

1.2.2 **Cyanopeptolins**

Cyanopeptolins are cyclic depsipeptides with the unusual structural element of 3-amino-6-hydroxy-2-piperidone.[9] The peptide ring is cyclised by an ester bond between the carboxyl group of the position six amino acid and the β-hydroxyl group of the position one threonine (Thr)[27] such as in cyanopeptolin 1020 (**2**).[28] The side chain of the cyanopeptolins is attached via the amino group

of the position one Thr and consists of at least two amino acids and either an aliphatic fatty acid or a glyceric acid unit at the N-terminus. All of the amino acids of the cyanopeptolins presently described are in the L-configuration.[9]

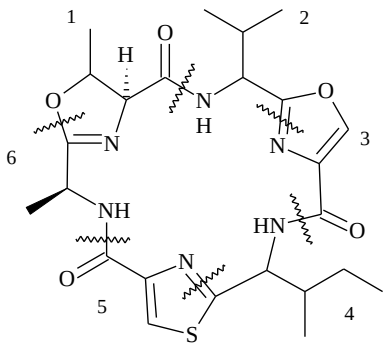


(**2**)

At least one hundred and twenty three known variants of cyanopeptolins[9,29] have been characterised which were isolated from various cyanobacterial strains including; *Aphanocapsa sp.*,[30] *Planktothrix agardhii*,[31] *Anabaena strain*,[32] *Microcystis sp.*,[33] *Nosctoc sp.*,[34] *Scytonema hofmanni*,[35] *Chroococcales cyanophyceae*[36] and *Symploca sp.*[37]

1.2.3 **Cyclamides**

Cyclamides are a class of cytotoxic, cyclic hexapeptides with three azole or azoline rings, particularly thiazole and oxazole.[9] They are characterised by a repeating motif of nitrogen and oxygen or sulphur containing, five-membered ring amino acids, with small, non-polar side chains,[38] such as in aerucyclamide C (**3**).[39] The formation of azole moieties is thought to be by dehydration and reduction of serine (Ser), Thr and cysteine (Cys) residues.[9]

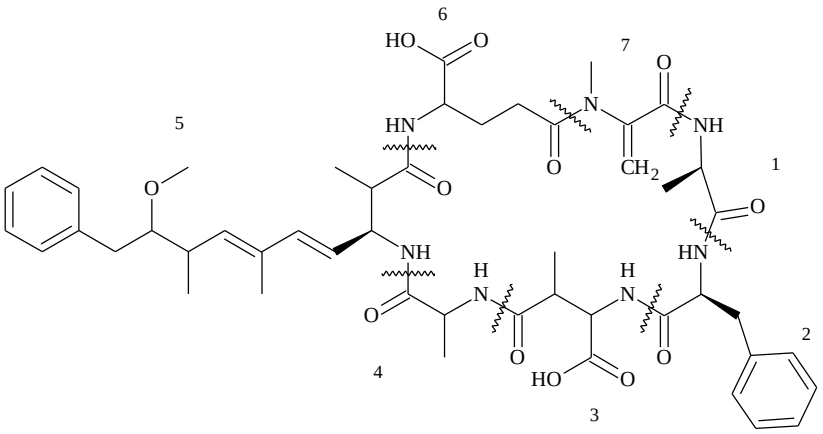


(**3**)

At least forty nine known variants of cyclamides[9,40] have been isolated and characterised from various cyanobacteria including *Microcystis sp.*,[40] *Oscillatoria sp.*,[41] *Lyngbya sp.*,[42] *Stigonema dendroideum*,[43] *Nostoc sp.*,[44] *Westiellopsis prolifica*[45] and *Lissoclinum bistratum* which is a marine tunicate with a cyanobacterial symbiont.[46] The cyclamides are divided into two subgroups; the first group are those that are derived from cyclisation of a hexapeptide containing two Cys and one Thr/Ser[40] such as the tenuecyclamides,[47] nostocyclamides[44] and dendroamides.[43] The second group are those derived from one Cys and two Thr/Ser moieties,[40] including examples such as the raocyclamides,[48] microcyclamide[49] and aerucyclamide.[39]

1.2.4 **Microcystins**

Microcystins (MCs) are cyclic peptides which are generally composed of the unique β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), D-glutamic acid (Glu), *N*-methyldehydroalanine (Mdha), D-alanine (Ala), D-erythro-β-methylaspartic acid and two variables L-amino acids,[9] such as in (MC-FA) (**4**).[14] Generally there are D-amino acids in positions one, three and six whilst positions two and four are occupied by variable L-amino acids and position seven is often occupied by Mdha.[15]



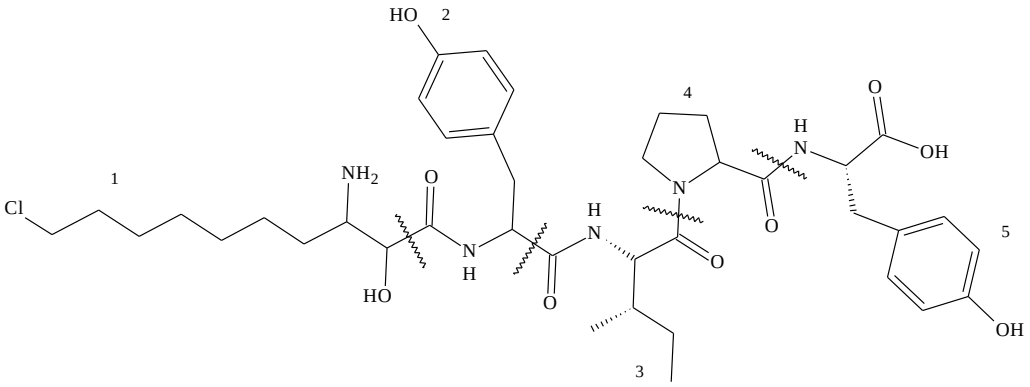
(**4**)

At least one hundred and twenty nine variants[15] of microcystins have been characterised from many cyanobacterial genera including *Anabaena*,[50] *Anabaenopsis*,[51] *Aphanizomenon flos-aquae*,[52] *Aphanocapsa*,[53] *Arthrospira*,[54]

Cylindrospermopsis,[55] *Fischerella*,[56] *Hapalosiphon*,[57] *Microcystis*,[58] *Nostoc*,[59] *Oscillatoria*,[60] *Phormidium*,[61] *Planktothrix*,[62] *Radiocystis*,[63] *Synechocystis*[64] and *Woronichinia*.[15]

1.2.5 **Microginins**

Microginins are a class of linear lipopeptides that contain four to six amino acid residues. They are characterised by a decanoic acid derivative, 3-amino-2-hydroxy-decanoic acid and a predominance of a tyrosine (Tyr) unit at the C-terminus,[65] such as in microginin GH78 (**5**).[66]



(**5**)

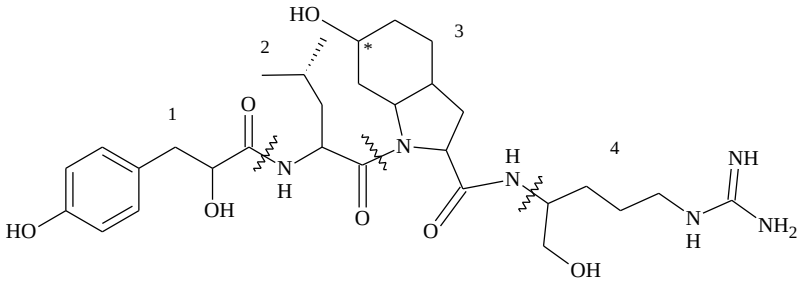
These compounds are of interest to many researchers because they are able to inhibit enzymes such as Leucine (Leu) amino peptidase M and the angiotensin-converting enzyme (ACE). Inhibitors of ACE are the most effective treatments for hypertension and congestive heart failure or are target compounds in the discovery of novel antihypertensive agents.[67]

To date, more than thirty variants of microginins [67] have been isolated and characterised from various cyanobacteria such as; *Microcystis sp.*,[68] *Nostoc sp.*[69] and *Oscillatoria agardhii.*[70]

1.2.6 **Aeruginosins**

Aeruginosins are a group of linear modified tetrapeptides which are defined by hydroxyl-phenyl lactic acid (Hpla) at the N-terminus, varying hydrophobic amino acids in position two, 2-carboxy-6-hydroxyoctahydroindole (Choi) in the third

position and an arginine (Arg) derivative at the C-terminus,[71] such as in aeruginosin 298A (**6**).[72]



(**6**)

The phenolic ring of Hpla is typically substituted with either hydroxyl groups or halogen substituents. Position two of the peptide is commonly occupied by a small, non-polar amino acid such as Leu or *allo*-isoleucine (Ile),[71, 72] however Tyr and phenylalanine (Phe) have also been reported.[73,21] The Choi moiety can be hydroxylated, sulphated or chlorinated at C6 (*) and the modified Arg C-terminus can be agmatine (Agma), arginol (Argol), argininal (Argal) and/or an amine.[9]

The biological activities of aeruginosins are very dependent upon the modifications at Hpla, Choi and the C-terminus.[38] Aeruginosins with an Argal or Agma at position four and a sulphate ester in Hpla or Choi are more potent protease inhibitors than those which contain an Argol or amine C-terminus.[74]

1.2.6.1 **Structural Diversity of Aeruginosins.**

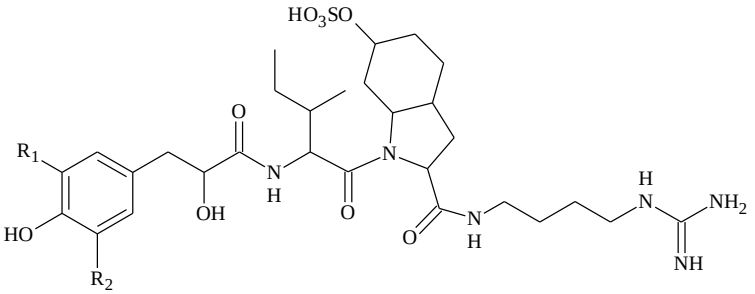
More than thirty eight aeruginosin congeners (Appendix A.1) have been isolated from various cyanobacterial strains including *Microcytis aeruginosa*,[71] *Microcystis viridis*,[73] *Oscillatoria sp.*,[75] *Nodularia spumigena*[76] and also from sponges such as *Lamellodysidea chlorea*[77] and members of the *Dysideidae* family.[78] The D-configuration is the most common for the amino acid in the second position, however exceptions have been observed in aeruginosins SF608, NAL2 and NOL6 where an L-amino acid was present.[76, 79]

The majority of the known aeruginosins (60%) were reported from *Microcystis aeruginosa* and the remainder were from the other cyanobacterial strains stated

above. The following discussion of aeruginosin structural diversity is based on aeruginosins reported from the same cyanobacterial strain.

The discovery of aeruginosin metabolites from *Microcystis aeruginosa* began in the early 1990s when Murakami and co-workers isolated aeruginosin 298A (**6**) from NIES-298 (Microbial Culture Collection, Japan) to consist of Hpla, Leu, Choi and Argol with the L-configuration for Leu[71] which was later revised to the D-configuration.[80] Aeruginosin 298A (**6**) actively inhibits the proteases thrombin (THB) and trypsin (TRP) at IC_{50} values of 0.3 μg/mL and 1 μg/mL respectively, but not other proteases such as papain (PAP), chymotrypsin (CTRP), elastase (ELT) and plasmin (PLM).[81]

In the following year, the same group isolated aeruginosins 98A (**7**) and 98B (**8**) which contained a *meta*-halogenated-Hpla, Ile, sulphated Choi and Agma.[82] Similar variants were also isolated in 1999 by Ishida and co-workers as 98C (**9**) and 101 (**10**), together with others such as 298B (**11**), 89A (**12**) and 89B (**13**).



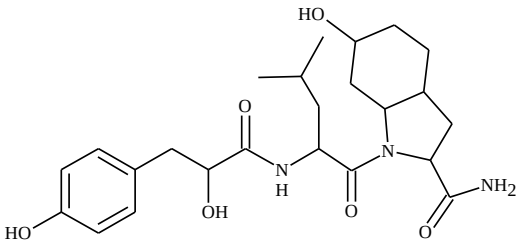
(**7**) R_1 = Cl, R_2 = H

(8) R_1 = R_2 = H

(9) R_1 = Br, R_2 = H

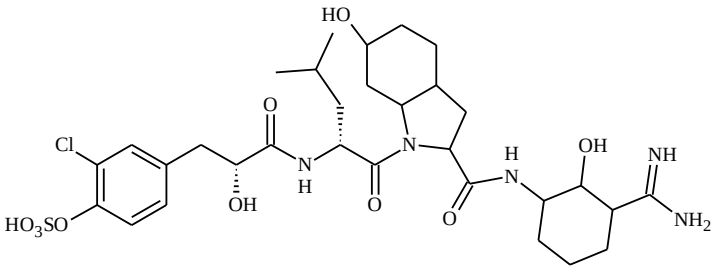
(**10**) R_1 = R_2 = Cl

The structure of aeruginosin 298B (**11**) resulted from lack of the Argol moiety which is present in aeruginosin 298A (**6**). The amino acid in position two was originally assigned as L-Leu [83] but was later revised to the D-configuration.[80]



(**11**)

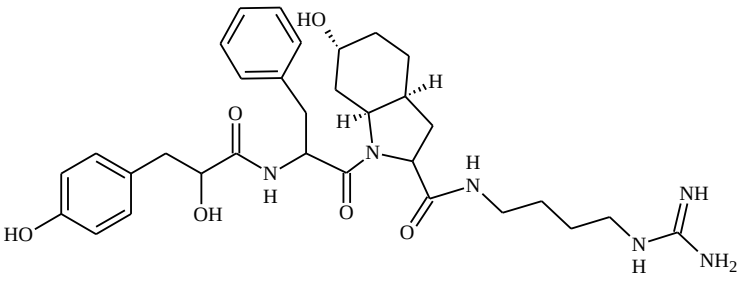
Aeruginosins 89A (**12**) and 89B (**13**) are stereoisomers which contain chloro-sulphated Hpla, Leu and Choi and different conformations of Argal at the C-terminus.



(**12**) (*2S*)

(**13**) (*1S,2R*)

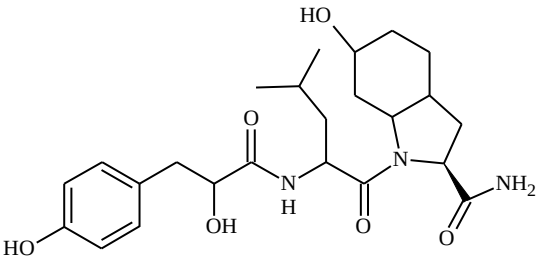
Another variant, known as SF608 (**14**), was isolated by Carmeli and Banker with a composition of L-Hpla, L-Phe, L-Choi and Agma. The presence of the L-Phe residue is an exception to the usually observed D-amino acid in position two.[79]



(**14**)

In 2001, a second aeruginosin lacking the usual guanidine-containing C-terminus, aeruginosin EI461 (**15**) was isolated from a *Microcystis aeruginosa* strain

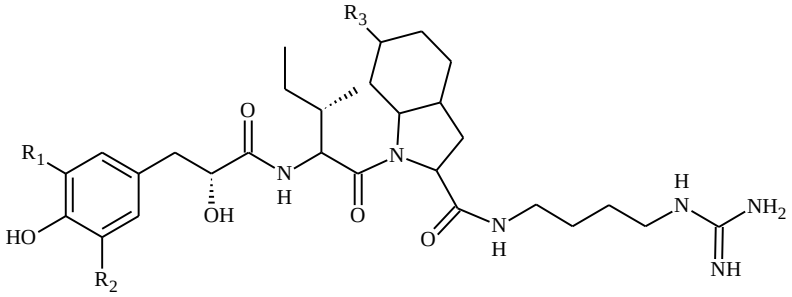
collected from Einan Reservoir in Israel by Carmeli *et al*.[84] The structure was very similar to aeruginosin 289B (**11**) but with different stereochemistry at the Choi moiety. Aeruginosin EI461 (**15**) was determined to be the only member of the aeruginosins with an anti-relationship between H-2 and H-3a/H-7a of the Choi moiety.[84]



(**15**)

Raveh and Carmeli isolated two more aeruginosins; KY642 (**16**) and KY608 (**17**) in 2008 from a cyanobacterial bloom collected at a water reservoir near Kfar-Yehoshua, Valley of Armageddon, Israel. These variants were very similar to aeruginosin 101(**10**) but with an hydroxyl-C6 instead of a sulphate on the Choi moiety.[85]

In 2012, Carmeli and co-workers isolated five similar halogenated aeruginosins from a bloom collected at Kibbutz Geva, Israel and designated the new constituents as aeruginosins GE686 (**18**), GE730 (**19**), GE766 (**20**), GE810 (**21**) and GE642 (**22**).[72]



(**16**) $R_1 = R_2 = Cl$, $R_3 = OH$

(**17**) $R_1 = Cl$, $R_2 = H$, $R_3 = OH$

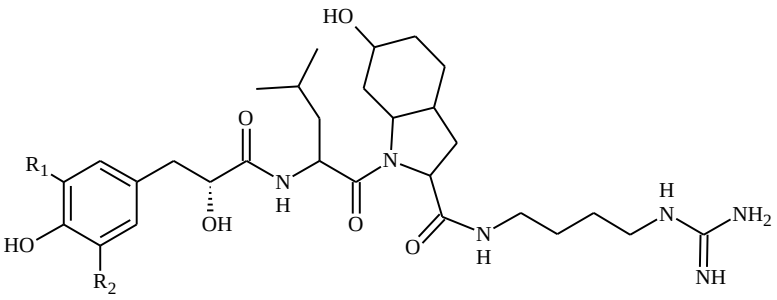
(**18**) $R_1 = Cl$, $R_2 = Br$, $R_3 = OH$

(**19**) $R_1 = R_2 = Br$, $R_3 = OH$

(**20**) $R_1 = Cl$, $R_2 = Br$, $R_3 = OSO_3H$

(**21**) $R_1 = R_2 = Br$, $R_3 = OSO_3H$

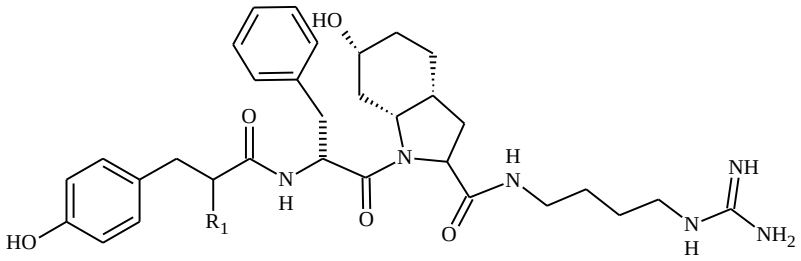
Aeruginosin GE 642 (**22**) differs from the other metabolites isolated from the Kibbuts Geva bloom material by the inclusion of Leu at position two instead of Ile.



(**22**) $R_1 = R_2 = Cl$

In the same year, Lifshits and Carmeli isolated another five aeruginosins; KT608A (**23**), KT608B (**24**), KT650 (**25**), KT554 (**26**) and GH553 (**27**) from a bloom collected at Lake Kinneret, Israel.[21]

Aeruginosins KT608A (**23**) and KT608B (**24**) were composed of D-Phe, D-Choi and Agma but contained L- and D-stereoisomers of the Hpla moiety respectively. Aeruginosin KT 650 (**25**) differed by acetylation of the Hpla C2-OH.

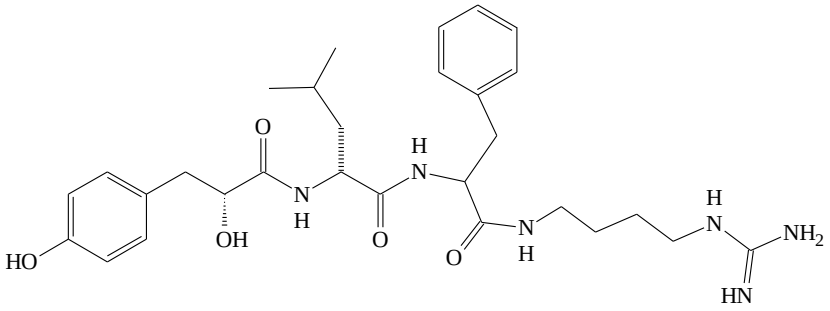


(**23**) $R_1 = OH$ (L-Hpla)

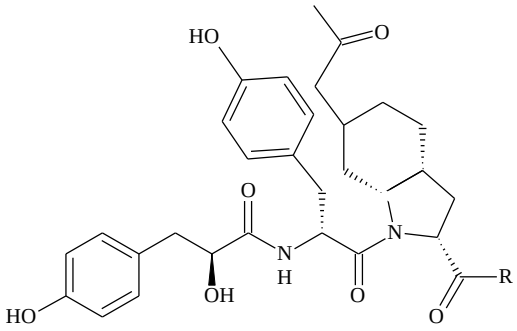
(**24**) $R_1 = OH$ (D-Hpla)

(**25**) $R1 = OOCCH_3$ (D-Hpla)

Aeruginosin KT 554 (**26**), a pseudoaeruginosin, contains Phe in position three instead of Choi and aeruginosin GH553 (**27**) is very similar to 298B (**11**) except that the usual guanidine-containing C-terminus is replaced with an acetylated Choi and a Tyr at the second position.[21]

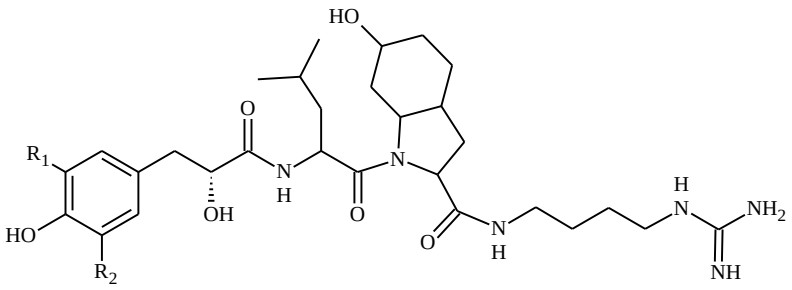


(**26**)



(**27**)

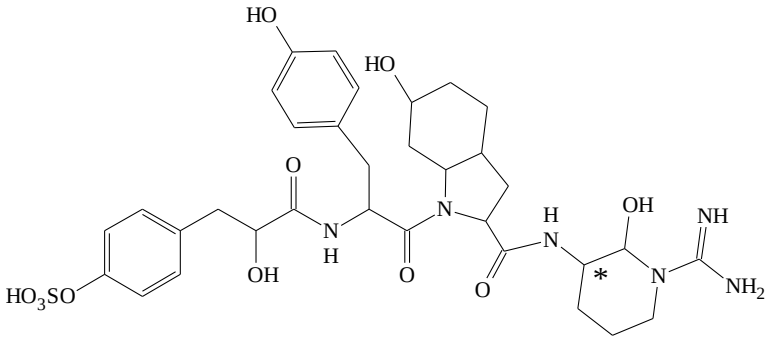
The most recent report of aeruginosins from *Microcystis aeruginosa* was in early February 2013 by Carmeli and co-workers who isolated aeruginosin IN608 (**28**) and IN652 (**29**). The cyanobacterial strain was isolated from a bloom collected at Durgakund water reservoir in Varanasi, India and both aeruginosins are very similar to aeruginosin GE642 (**22**) and only differ by halogenation at the Hpla moiety. Both compounds are inhibitors of the proteolytic enzyme TRP.[86]



(**28**) R_1 = Cl, R_2 = H

(**29**) R_1 = Br, R_2 = H

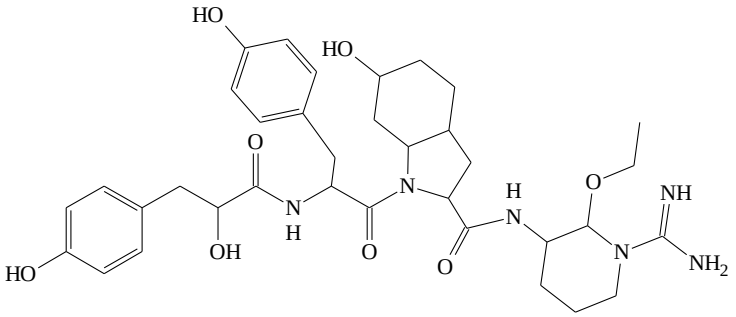
To date, only three aeruginosins have been isolated from *Microcystis viridi*s. In 1996, Matusda *et al*. reported the isolation of aeruginosins 102A (**30**) and 102B (**31**) which contained a sulphated Hpla, Tyr, Choi and Argal. These two compounds were isomers with differing stereochemistry of the C-terminus.[73]



(**30**) L-Argal (*3S**)

(**31**) D-Argal (*3R**)

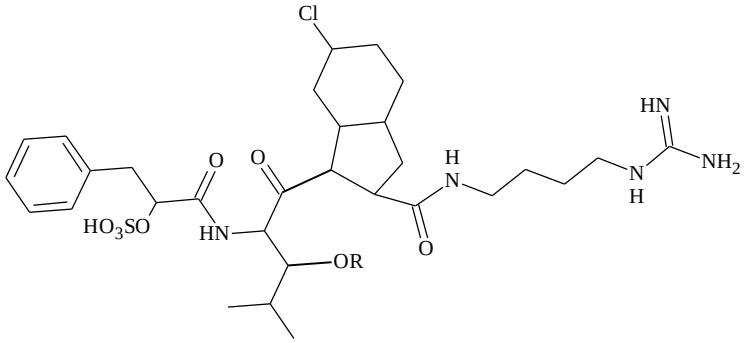
In 1998, Murakami and co-workers isolated and characterised aeruginosin 103A (**32**) which had a structure similar to that of 102A (**30**) but with an unique 1-amidino-2-ethoxy-3-aminopiperidine (Aeap) subunit which contains a cyclic ethyl hemiaminal.[87]



(**32**)

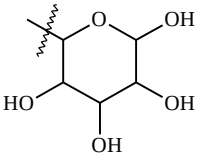
There have been several aeruginosins isolated from *Oscillatoria agardhii*. The first, aeruginosins 205A (**33**) and 205B (**34**) were isolated in 1997 from Kasumiguara Lake, (Japan) by Shin and co-workers and contained an unusual linear glucopeptide. They comprised phenyllactic acid-2-*O*-sulphate (Plas) in position one, 3-hydroxyleucine (HLeu) and xylopyranose (Xyl) in position two respectively, 2-carboxy-6-chlorooctahydroindole (Ccoi) in position three and

Agma at the C-terminus. The presence of HLeu is very rare in naturally occurring metabolites[88] and both aeruginosins were found to have opposite stereochemistry at the Plas and HLeu.[75]

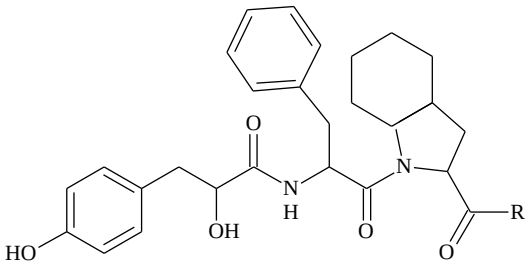


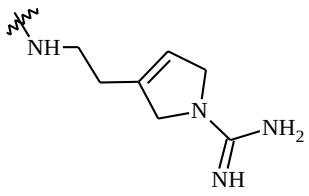
(**33**) L-Plas, *2R,3S,2'S* (Hleu)

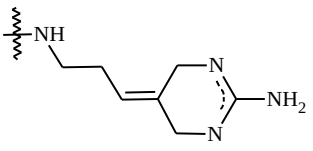
(**34**) D-Plas, *2S,3R,2'R* (Hleu)

R = 

In the same year, Konetschny-Rapp and co-workers isolated and characterised oscillarin from *Oscillatoria agardhii* collected from Lake Kasumiguara (Japan). The metabolite was initially determined to comprise of D-Plas, D-Phe, L-Choi and a cyclic guanidine (**35**)[89] but later revised to consist of a 1-(*N*-amidino-Δ^3-pyrrolino)ethyl subunit (Adc) (**36**) at the C-terminus.[90]

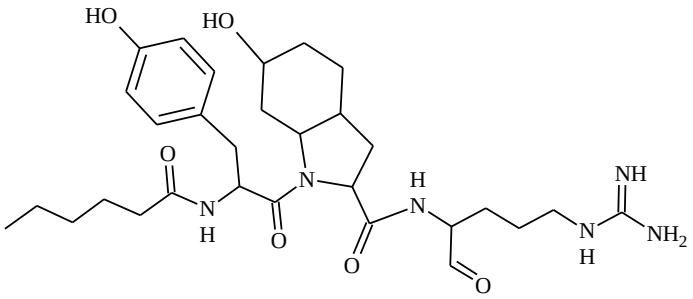


(**35**) R = 

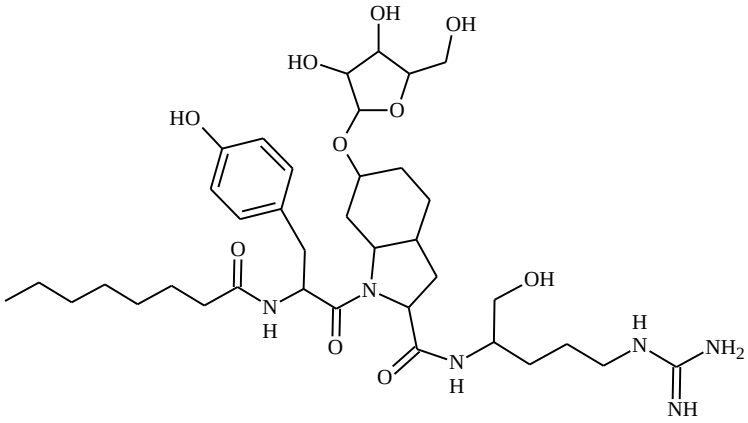
(**36**) R = 

In September 2013, two new aeruginosins were reported from the filamentous, diazotrophic *Nodularia spumigena* by Fewer *et al*. These aeruginosins; NAL2 (**37**) and NOL6 (**38**) were Ser protease inhibitors and comprised a rare N-terminal short fatty acid chain. In addition NOL6 (**38**) also containing a rare pentose sugar

modification on the Choi. These were the first aeruginosins reported with a short fatty acid chain at the N-terminus instead of Hpla or Plas.[76] Additionally, these metabolites contained a position two amino acid in the L-configuration as with SF608 (**14**).[76]

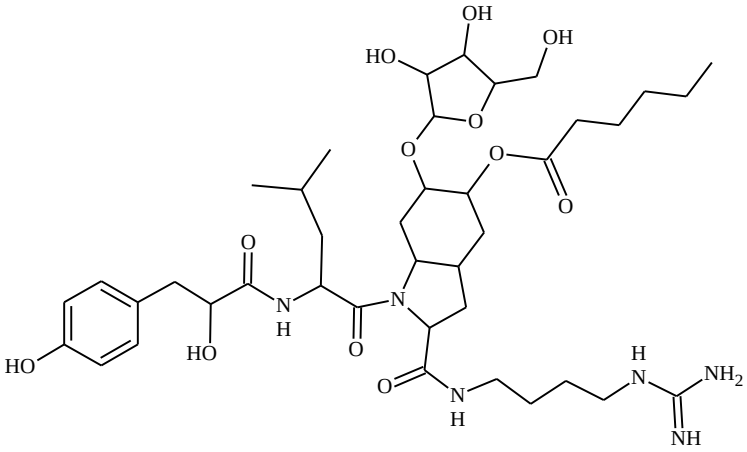


(**37**)



(**38**)

Other interesting aeruginosin structures have been identified, including the first aeruginosin to contain both a fatty acid and a carbohydrate at the Choi moiety; Aer856 (**39**). This was also the first aeruginosin to be found in the genus *Nostoc* and the sample was obtained by Kapuścik and co-workers (2013) from the forest soil of the Krušné mountains, Czech Republic. The peptidic sequence of Aer856 (**39**) was D-Hpla, D-Leu, 5-OH-Choi, Agma, with hexanoic and mannopyranosyl uronic acid moieties linked to Choi. The compound displays remarkable anti-inflammatory activity with no cytotoxic or barrier disruption effects, which makes it an interesting immunomodulatory agent.[91]

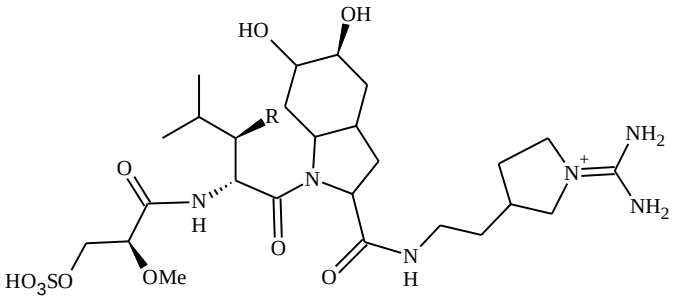


(**39**)

There have been a several aeruginosin-like metabolites isolated from marine sponges such as *Lamellodysidea chlorea* and members of the family *Dysideidae* found in North Queensland, Australia.[77,78] These bioactive metabolites were believed to be produced by symbiotic cyanobacteria.[92]

In 2002, Quinn and co-workers isolated dysinosin A (**40**) as a potent inhibitor of the blood coagulation cascade factor VIIa2 and a peptidic THB inhibitor with significant structural similarities to the aeruginosins. It was composed of an Adc subunit as in oscillarin (**37**), but with an additional hydroxyl group at C5 of the Choi moiety and the N-terminal residues were D-Leu and a sulphated glyceric acid derivative which has not been reported in known aeruginosins.[78]

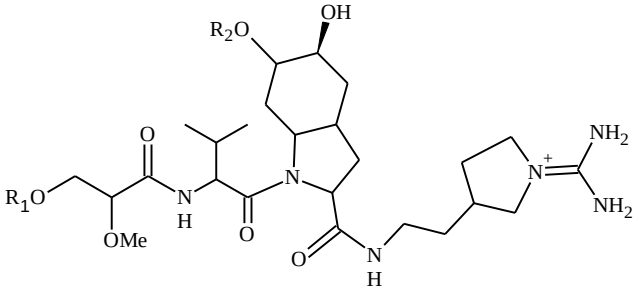
In 2003, a similar metabolite was isolated; chlorodysinosin A (**41**) which contained *2S,3R*-3-chloroleucine. It was a potent inhibitor of the Ser proteases; THB, factor VIIa and factor Xa, which are critical enzymes in the process leading to platelet aggregation and fibrin mesh formation in humans.[93]



(**40**) R = H

(**41**) R = Cl

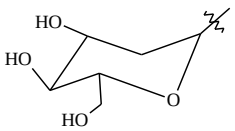
In 2004, three similar metabolites were isolated from the sponge *Lamellodysidea chlorea* by the same group, which were designated as dysinosin B-D (**42**-**44**) and are inhibitors of THB and the blood coagulation cascade Ser proteases factor VIIa.[77]



(**42**) $R_1 = SO_3^-$, R_2 = A

(**43**) R_1 = H, $R_2 = SO_3^-$

(**44**) R_1 = H, R_2 = H

A = 

The isolation and structural characterisation of the aeruginosin variants above were determined using common methods such as octadecylsilyl flash chromatography, reversed-phase (RP) high performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS), high resolution fast-atom bombardment mass spectrometry (HRFABMS), high resolution x-ray, 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, amino acid degradation and derivatisation.[94]

1.2.6.2 **Inhibitory Activities of Aeruginosins.**

The members of the aeruginosin family are potent inhibitors of the Ser proteases TRP, THB and PLM (Table 1.3). Ser proteases are involved in a number of vital physiological processes and are important in the complex blood coagulation cascade.[76] The modification of the C-terminus plays a significant role for the bioactivities of the aeruginosins. Aeruginosins which lack a guanidine-containing C-terminus such as aeruginosins 298B (**11**), EI461 (**16**) and GH553 (**27**) show no Ser protease inhibition. Metabolites which contain Argal are stronger protease inhibitors compared to those which contain Agma and Argol.[87] They are more potent against THB and thus the aldehyde of Argal is very important to inhibit THB.[95] The bulky Argal ethyl aminal structure of the Aeap unit in 103A (**32**) reduced the TRP inhibitory activity.[87]

Table 1.3: Inhibitory activities of aeruginosins against thrombin, trypsin, plasmin and FVIIa.

Aeruginosin	**Bioactivities (IC_{50} µg/mL)**			
	Thrombin	**Trypsin**	**Plasmin**	**FVIIa**
298A (**6**)	0.3	1	>10	-
98A (**7**)	7	0.6	6	-
98B (**8**)	10	0.6	7	-
98C (**9**)	3.2	3	3.3	-
101 (**10**)	3.3	3.9	5	-
298B (**11**)	>100	>100	>100	-
89A (**12**)	0.03	0.4	0.02	-
89B (**13**)	0.05	6.6	0.46	-
SF608 (**14**)	-	0.5	-	-
EI461(**15**)	-	15%	-	-
KY642 (**16**)	-	1.4	-	-
KY608 (**17**)	-	1.7	-	-
GE686 (**18**)	12.8	3.2	-	-
GE730 (**19**)	12.9	2.3	-	-
GE766 (**20**)	>45.5	18.2	-	-
GE810 (**21**)	>45.5	12.2	-	-
GE642 (**22**)	>45.5	18.2	-	-
KT608A (**23**)	30%[a]	1.9	-	-
KT608B (**24**)	<50%	1.3	-	-
KT650 (**25**)	<50%	19.9	-	-
KT554 (**26**)	-	45.5	-	-
GH553 (**27**)	-	45.5	-	-
IN608 (**28**)	-	4.3	-	-
IN652 (**29**)	-	4.1	-	-
102A (**30**)	0.04	0.2	0.3	-
102B (**31**)	0.1	1.1	0.8	-
103A (**32**)	9	51	68	-
205A (**33**)	1.5	0.07	-	-
205B (**34**)	0.07	0.07	-	-
Oscillarin (**35**)	0.018	0.024	>260	2.5
NAL2 (**37**)	nd[b]	nd	nd	-
NOL6 (**38**)	nd	nd	nd	-
Aer856 (**39**)	-	-	-	-
DysA (**40**)	0.29	-	-	0.21
Cl-DysA (**41**)	0.004	0.025	-	0.02
DysB (**42**)	0.13	-	-	0.07
DysC (**43**)	0.34	-	-	0.07
DysD (**44**)	>3.3			0.86

[a] inhibit at concentration of 45.5µg/L, [b] not detected.
Similar to Table 1 of Reference 88.

The compounds with 6-OH Choi are more potent toward TRP and THB compared to those containing a 6-sulphated Choi such as aeruginosin GE686, GE730 and GE 642 (**18**-**20**).[72] However, the halogen substituent of the Hpla moiety does not influence the potency of the aeruginosins such as GE686 (**18**), GE730 (**19**), IN688 (**29**) and IN652 (**30**).[86] The aeruginosins from sponges which contained an octahydroindole system and lacked a sulphated group such as dysinosin D (**44**) were less potent against FVIIa and THB.[77]

1.2.6.3 **Biosynthesis of Aeruginosins.**

In 2009, Ishida *et al.* provided the first insight into the molecular basis of the biosynthetic pathway of aeruginosin by analysing and comparing the aeruginosin synthetase (*aer*) gene clusters from *Microcystis sp.* and *Planktothrix agardhii*.[96] It was indicated that the biosynthesis of aeruginosin was very much dependent on the specific NRPS and PKS genes, yet only a few studies have been carried out to date.[96]

The *aer* gene cluster had sequences from strains of *Planktothrix agardhii* CYA126/8[97] and *Microcystis* strains PCC 7806,[98] NIES-843[99] and NIES-98.[96] The entire gene cluster (*aerA*-*N*) totals a size of 25-27 kb and was similarly organised between different cyanobacterial strains. The first three NRPS modules encoding *aerA*, *aerB* and *aerG* were coding for hybrid NRPS/PKS enzyme complex. The regions coding for tailoring enzymes such as halogenases and sulphotransferases depend upon the individual cyanobacterial strains.[96]

In *Planktothrix agardhii* CYA126/8, the biosynthesis started with the activation of an aryl acid; phenylpyruvate, the keto group of which was reduced by the ketoreductase domain of the first module (*aerA*). Similarly, in *Microcystis* strains, the biosynthesis was assumed to start with an hydroxy-phenylpyruvate which was reduced to Hpla (Figure 1.2).[96]

The putative Falvin adenine dinucleotide ($FADH_2$)-dependent halogenase gene *aerJ*, was responsible for the formation of the non-, mono- and di-halogenated variants of the Hpla moieties of *Microcystis* strains[12] and also at the Choi moiety from *Oscillatoria agardhii*.[75] The presence or absence of this halogenase gene

was one of the reasons for the structural diversity observed in aeruginosins. Although only a few studies have been carried out, the halogenated aeruginosins are known to be isolated from *Microcystis species*, thus it was assumed that *aerJ* was a characteristic of the *Microcystis* strain.[12]

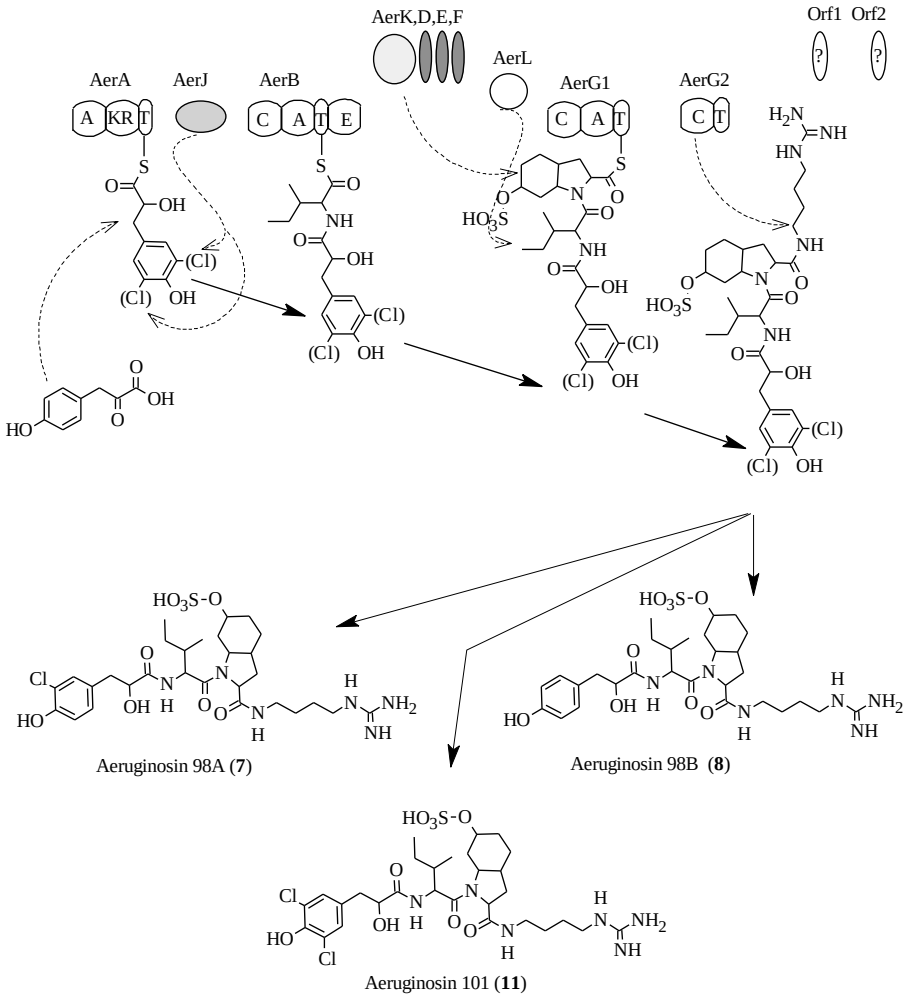


Figure 1.2: Schematic representing the biosynthetic pathways for the production of aeruginosins 98A (**7**), 98B (**8**) and 101 (**10**) in *Microcystis* strain NIES-98. Adapted from Reference 96.

The condensation, thiolation and epimerisation domains present in *aerB*, incorporated the amino acid at the second position into the structure. Invariably the conformation of this epimerisation domain was consistent with the D-configuration of the position two amino acids incorporated in most aeruginosins.[97]

The genes responsible for Choi formation in *Microcystis* were *aerD-F* and *aerK*

whereas in *Planktothrix*, these genes are located in *aerC*. [97] Different pathways of Choi formation were assumed to operate in each strain. The presence of *aerK* was essential for the synthesis of aeruginosin only in *Microcystis* yet the precursor for Choi has not been defined.[96] Ishida *et al*. (2007) proposed that biosynthesis of Choi in a *Planktothrix* strain involved oxidation of arogenate (pretyrosine) by *aerC*, followed by cyclisation via Michael addition in *aerE* to produce vinylogous β-keto acid which was then decarboxylated by *aerD* to yield a bicyclic amino acid which may be reduced by *aerF* to the Choi moiety.[97]

The sulphotransferase *AerL* was responsible for the sulphonation of the aeruginosins produced by *M. aeruginosa* NIES-98 and NIES-843. The sulphonation at Hpla and Choi were different in each strain. The Choi sulphonation was likely to be accomplished after the formation of the Choi-phosphopanthetheine thioester.[12]

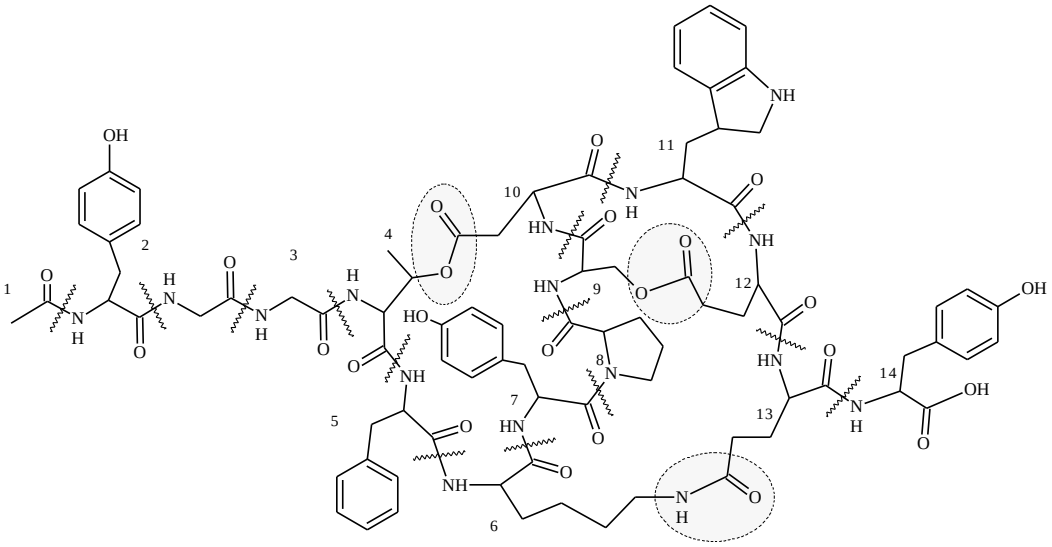
At the incorporation of the last moiety into the aeruginosin molecules, the Agma was bound to the peptidyl carrier protein (PCP)-bound carboxyl group of Choi and release of the peptide was triggered by amide bond formation between the PCP-bound carboxyl group for Choi and the primary amine of Agma.[97]

The glycosyl transferase genes present in the *aer* gene cluster in *Planktothrix agardhii* were absent from the gene cluster found in the *Microcystis* strain, hence the aeruginosins produced were not glycosylated. However glucosylation of aeruginosin had been observed exclusively and frequently in *Planktothrix* strains, indicating a possible genus-specific association of this gene with the *aer* gene cluster.[96]

1.2.7 **Microviridins**

Microviridins are tricyclic depsipeptides comprising thirteen or fourteen amino acids, seven of which are conserved; Thr [4], Lys [6], Tyr [7], Proline (Pro) [8], Ser [9], Aspartic acid (Asp) [10] and Glu [12],[9] as observed in microviridin A (**45**).[100] They are the largest known cyanobacterial oligopeptides[9] and are one of the most intriguing classes of peptides, with a cage-like structure and highly unusual tricyclic architecture with ω-ester and ω-amide bonds between the

alcoholic, acidic and amine side chains of several of the conserved residues.[100]



(**45**)

The main peptide ring consists of seven amino acids with a peptide (amide) bond between the 6-amino group of Lys [6] and the sidechain carboxy group of Glu, Asp or homoaspartic acid (Has) [13]. Di-cyclisation of the structure occurs through an ester bond between the 4-carboxyl group of Asp [10] and the hydroxyl group of Thr [4]. The final ring is formed through another ester bond between the side chains of Ser [9] and Glu [12].[9] These bonds are indicated by circles on microviridin A (**45**) above. The ester linkages can undergo partial methylation or methanolysis which contributes to several of the reported structures for microviridins.[101]

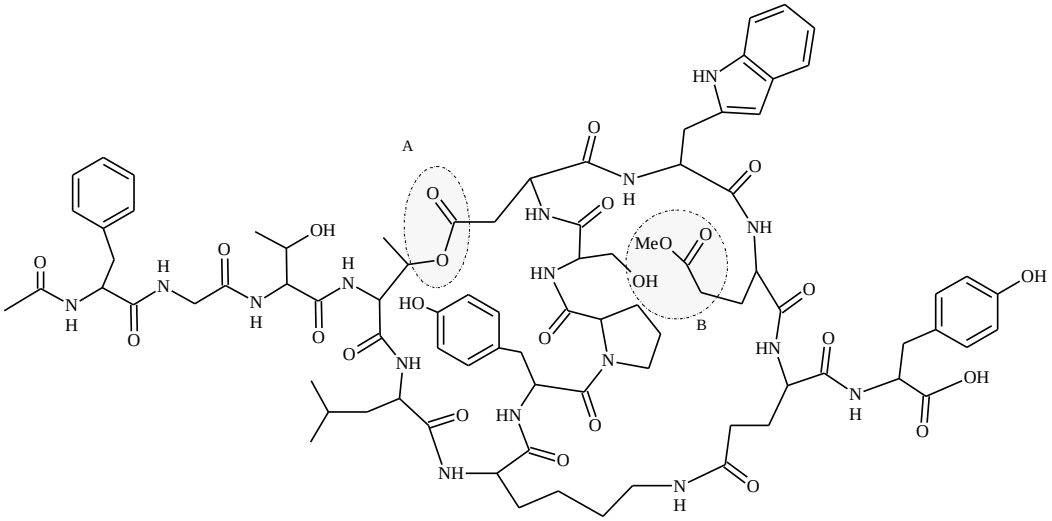
1.2.7.1 **Structural Diversity of Microviridins.**

To date, fifteen microviridin congeners (Appendix A.2) have been isolated from various cyanobacterial genera such as *Microcystis*, *Oscillatoria* and *Nostoc*.[100-105] The structural diversity of microviridins was due to variation of the amino acids incorporated at the non-conserved positions together with the presence or absence of the two ester linkages between the amino acids at positions four/ten and positions nine/twelve. The amide bond formed between Lys and the amino acid in position thirteen is conserved in all of the known structures of the metabolites. To date, all of the amino acids incorporated into microviridins have been in the L-conformation.[9]

The majority of the isolated microviridins (seven) were obtained from *Microcystis aeruginosa*.[100-103] Only one microviridin has been reported from *Microcystis viridis*,[104] five congeners have been isolated from *Oscillatoria agardhii*[105-107] and two have been reported from *Nostoc minutum*.[108]

Microviridin A (**45**) was isolated from a *Microcystis viridis* strain obtained from Kasumigaura Lake, Japan in 1990 by Ishituska and co-workers. It was the first tricyclic depsipeptide with fourteen amino acids to be reported from natural sources and strongly inhibited the Ser protease, tyrosinase.[104]

Five years later, microviridins from *Microcystis aeruginosa* were initially reported by Okino *et al*. as microviridin B (**46**) and C (**47**).



(**46**) A = √, B = x

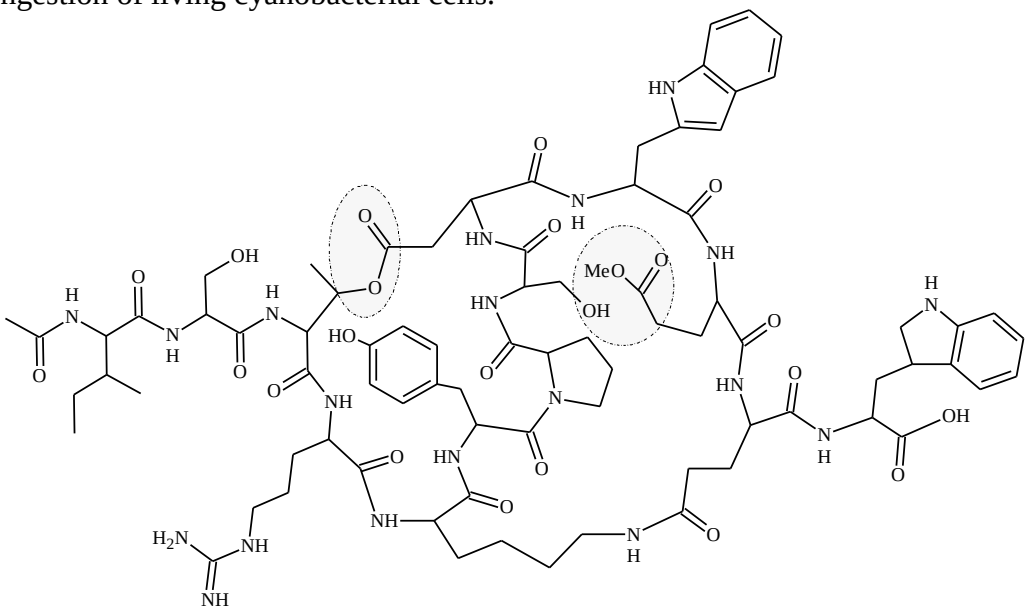
(**47**) A = √, B = √

x = ester linkage absent, √ = ester linkage present

These two microviridins also comprised fourteen amino acids but as tricylic and bicyclic depsipeptides respectively. Microviridin C (**47**) had no ester linkage between Ser in position ten and Glu in position twelve. They were potent inhibitors of ELT with potential for use as therapeutic agents of pulmonary emphysema.[102]

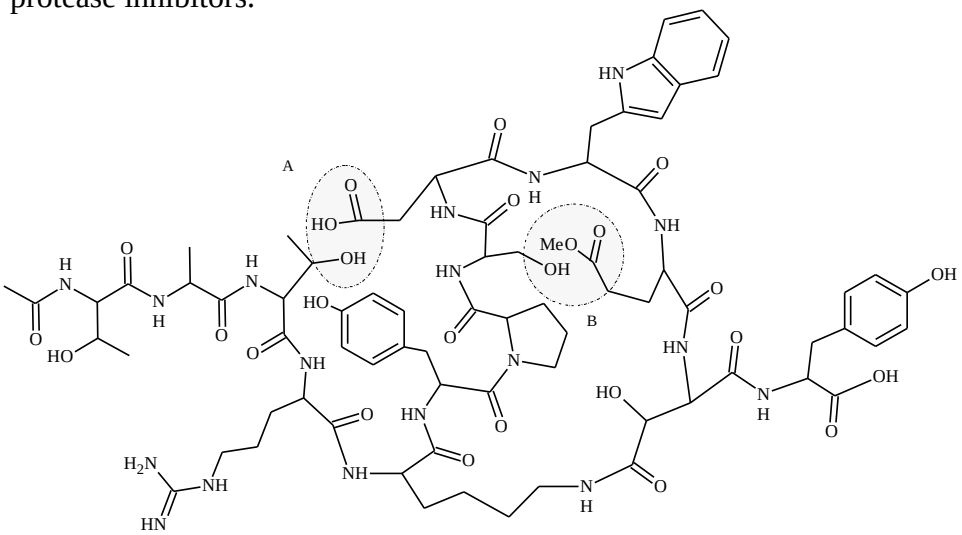
Microviridin J (**48**) was isolated in 2003 by Rohrlack *et al*. from a bloom collected at Malpas Dam, Australia. It was a tricyclic depsipeptide with thirteen

amino acids and was an inhibitor of porcine TRP, bovine CTRP and daphid TRP-like protease. It also caused lethal molting disruption in *Daphnia spp*., upon ingestion of living cyanobacterial cells.[101]



(**48**)

In 2006, Reshef and Carmeli isolated microviridins SD1684 (**49**), SD1634 (**50**) and SD1652 (**51**) from a bloom collected in 1998 from a pond in the Dan District sewage treatment plant, Shofdan, Israel. These microviridins were the first to contain a non-proteinogenic amino acid in their structure; L-threo-β-hydroxy Asp in position thirteen. They each have thirteen amino acids in the L-configuration and are differentiated by the two ester linkages; SD1684 (**49**) contains no ester linkages, making it a monocyclic peptide, SD1634 (**50**) is tricyclic and SD1652 (**51**) is a bicyclic peptide. All three microviridins were mild Ser protease inhibitors.[103]



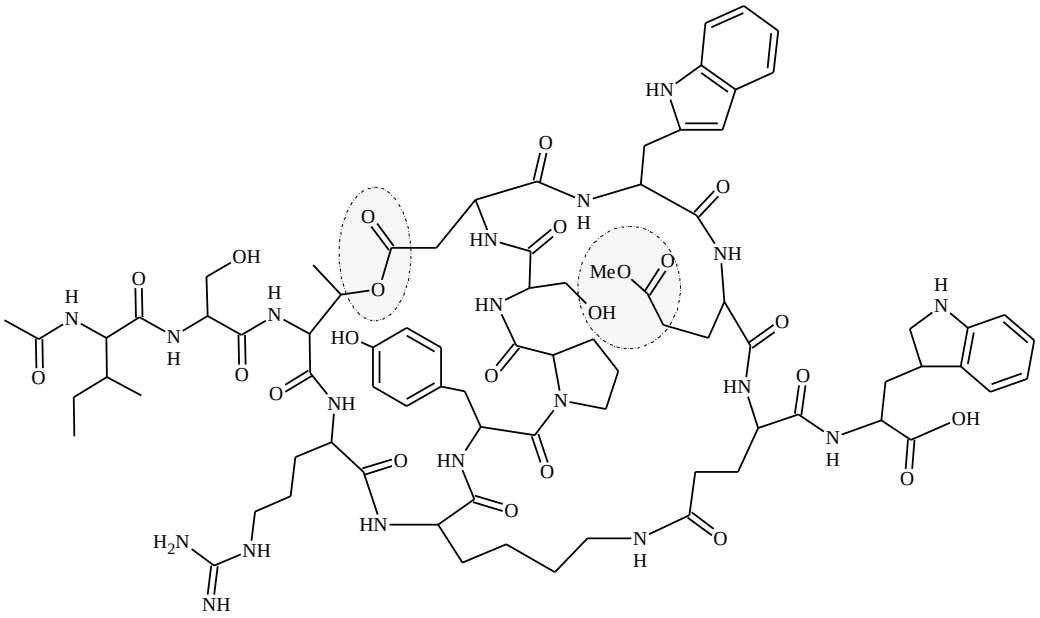
(**49**) A = x, B = x

(**50**) A= √, B = x

(**51**) A= x, B = √

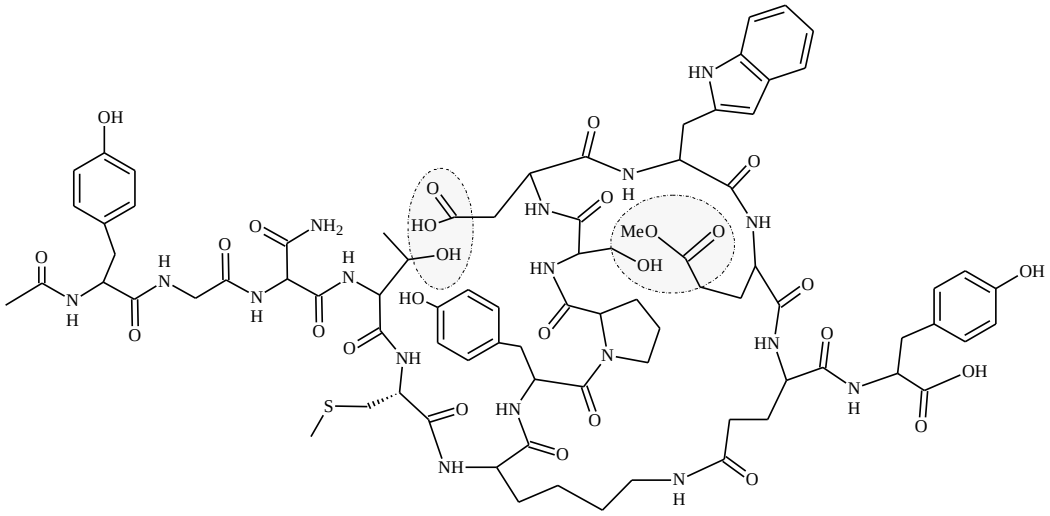
x = ester linkage absent, √ = ester linkage present

In 2010, microviridin L (**52**) was isolated from a bloom of *Microcystis aeruginosa* from Lake Kasumigaura, (Japan) by Zeimert and coworkers. It was a tricyclic peptide with fourteen amino acids which inhibited TRP. [100]

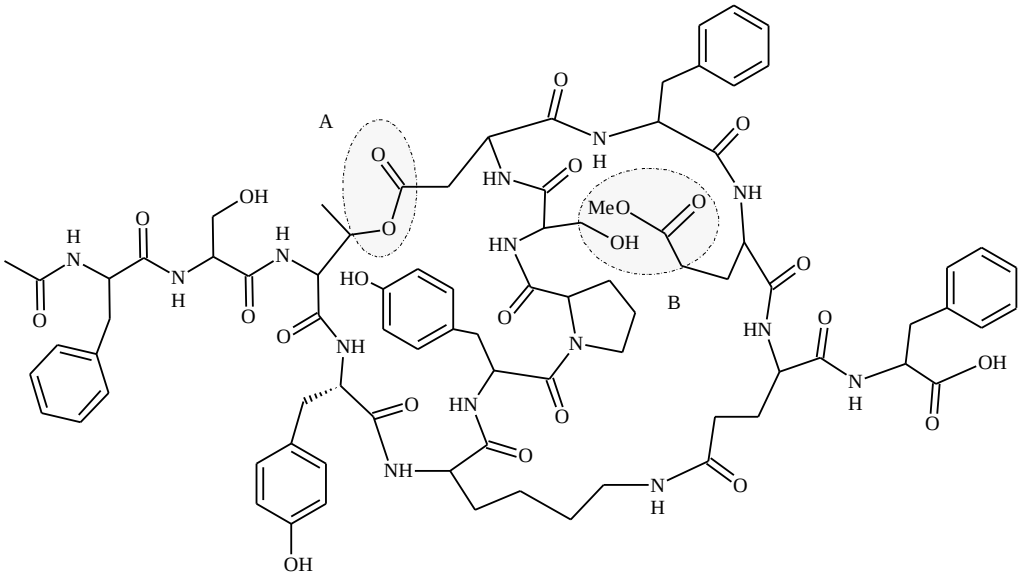


(**52**)

Microviridin variants were isolated from *Oscillatoria agardhii*, including microviridins D-F (**53)** - (**55**) discovered by Shin *et al*. (1996) from a bloom obtained from the NIES-collection, (Microbial Culture Collection, National Institute for Environmental Studies, Japan). Microviridin D (**53**) was a bicyclic depsipeptide with fourteen amino acids which differs from E (**54**) and F (**55**) by the amino acids in the first three positions and at position five and eleven. All are potent inhibitors of Ser protease and D (**53**) and E (**54**) also inhibited CTRP while F (**55**) did not at 100 µg/mL.[105]



(**53**)

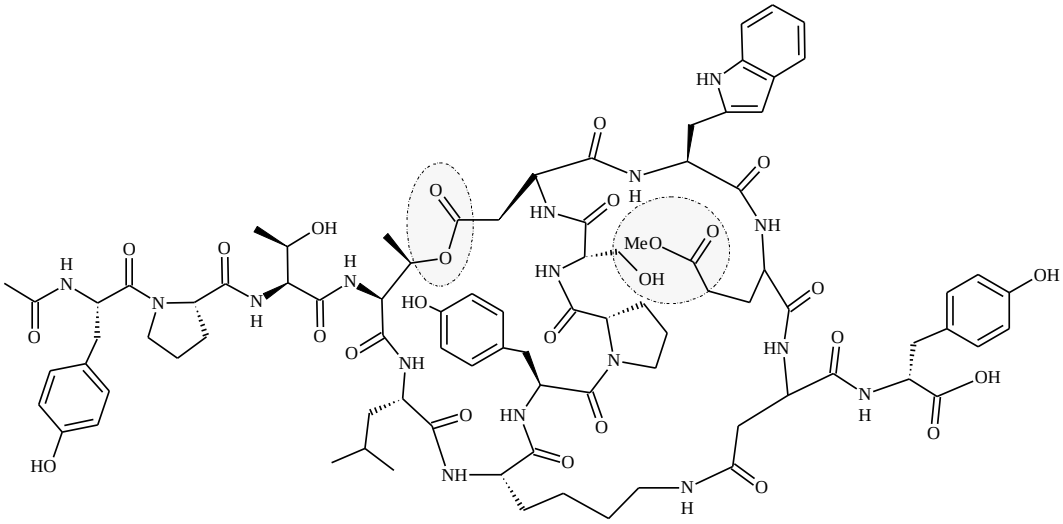


(**54**) A= x, B = √

(**55**) A=√, B = √

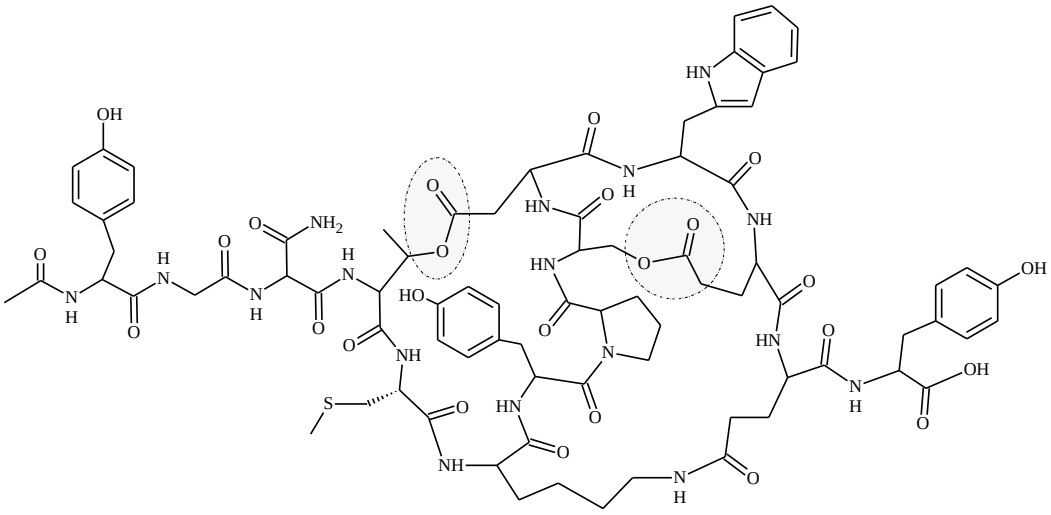
x = ester linkage absent, √ = ester linkage present

In 2001, Fujii *et al*. reported the isolation of microviridin I (**56**) from non-toxic *Oscillatoria agardhii* strains; 97 (Lake Maarianallas, Findland) and CYA 128 (Lake Vesijärvi, Findland). It was a depsipeptide with fourteen amino acids which inhibits ELT with an IC_{50} value of 0.34 µg/mL.[107]



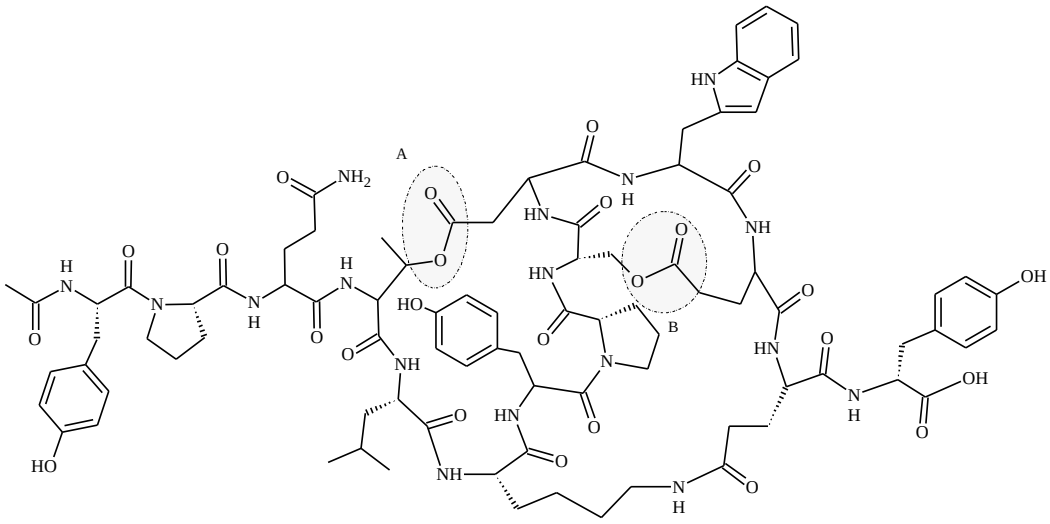
(**56**)

Microviridin K (**57**) was discovered in 2009 from *Oscillatoria (Planktothirix) agarhdii* NICA-CYA 126/8 by Philmus and co-workers. Its amino acid composition was very similar to that of microviridin D (**53**) with both ester linkages being present. It was presumed that microviridin K (**57**) is a methanolysis product, so biological activities of this metabolite were not determined.[106, 109]



(**57**)

The only microviridins isolated from *Nostoc minitum*, microviridins G (**58**) and H (**59**) were reported by Murakami and co-workers in 1997. They both comprised the same fourteen amino acids but microviridin H (**59**) lacked the ester linkages between the position nine Ser and the position twelve Glu. Both metabolites were found to be potent inhibitors of ELT.[108]



(**58**) A= √, B = √

(**59**) A= √, B = x

x = ester linkage absent, √ = ester linkage present

Some microviridin congeners lack an ester linkage or contain a methyl ester. At times, these latter congeners have been shown to be a result of the isolation and purification processes when using methanol as a solvent.[110] Rohrlack *et al*. indicated that incubation of metabolites with methanol at high temperature resulted in methylation of one of the ester linkages.[101] To avoid these processes, the sample should be kept at 5 ºC and either 50% methanol (MeOH)[101] or a more hindered alcohol solvent such as isopropanol should be used for extraction and a lipophilic solvent such as acetonitrile (ACN) should be used for RP chromatography. However this does not disprove the potential for these variants to occur naturally.[110]

The isolation and structural characterisation of the above microviridins were determined with common spectroscopic and chemical analyses such as HPLC, gel column filtration chromatography, RP FABMS, NMR spectroscopy, gas chromatography mass spectrometry (GC MS) and amino acid analysis.

1.2.7.2 **Inhibitory Activities of Microviridins.**

The amino acid composition of the metabolite is very important for the biological activities of the microviridins (Table 1.4). The significance of the amino acid at the fifth position, between Thr and Lys was highlighted when comparing the structures of the known microviridins and their biological activities.

Microviridins with Leu in position five (microviridins B (**46**), C (**47**), F (**55**), I (**56**), G (**58**) and H(**59**)) are potent inhibitors of ELT, while the presence of Methionine (Met), Tyr or Phe in the same position resulted in weak (microviridins D (**53**), E (**54**), F (**55**) or no inhibitory effects on ELT (microviridin A (**45**)).[107, 108] In addition, absence of a hydrophilic amino acid residue in position three (microviridins A (**45**), SD1684 (**49**), SD1634 (**50**), SD1652 (**51**) and K (**57**)) resulted in no ELT inhibition. The ester linkage with the hydroxyl group of Ser was shown to not be important for ELT inhibition (microviridin A (**45**)).[108]

Table 1.4: Inhibitory activities of isolated microviridins against elatase chymotrypsin and trypsin.

Microviridin	**Inhibitory Activity (IC_{50} µM)**		
	Elastase	**Chymotrypsin**	**Trypsin**
A (**45**)	>58[a]	>58	>58
B (**46**)	0.026	1.5	34
C (**47**)	0.048	2.8	18
J (**48**)	>6	1.7	20-90
SD1684 (**49**)	nd[b]	inactive[c]	inactive
SD1634 (**50**)	nd	15.7	8.2
SD1652 (**51**)	nd	inactive	inactive
L(**52**)	nd	nd	nd
D (**53**)	0.388	0.7	>55
E (**54**)	0.360	0.7	>60
F (**55**)	0.034	>59	>59
I (**56**)	0.193	nd	nd
K (**57**)	>58	42	5.8
G (**58**)	0.010	0.78	>55
H (**59**)	0.017	1.6	>54

[a] no inhibitory activity has been detected below this value, [b] not determined,[c]inactive at 45µM. Adapted from Table 3 of Reference 100.

Microviridin J (**48**) has an Arg in position five which may account for the strong TRP inhibitory action while at the same time negating its inhibitory effect on

ELT.[101] A similar effect may occur in microviridins SD1684 (**49**), SD1632 (**50**) and SD1652 (**51**) with the Arg residue. As only SD1632 (**50**) displays TRP inhibition, and since microviridins SD1684 (**49**) and SD1652 (**51**) have no ester bond between the Asp [10] and Thr [4], this indicates that this ester linkage is important for TRP-inhibition.[103]

In addition to the main acid at the fifth position, a hydrophilic residue at the position just before Thr and the ester linkage between Thr and Asp appear to be crucial for inhibitory effects on ELT.[108]

1.2.7.3 **Biosynthesis of Microviridins.**

The genetic basis of microviridin production was initially assumed to be by a NRPS pathway as with the cyanopeptolins.[111] However, two groups which independently studied microviridin biosynthesis using different approaches detected that microviridins were produced via a ribosomal assembly route with subsequent tailoring functions to produce the final structure.[106, 112] Zeimert and co-workers pursued a strategy of heterologous expression of the microviridin cluster from *Microcystis aeruginosa* NIES 298[112] in *E. coli* while Philmus and co-workers pursued *in vitro* reconstitution of parts of the pathways from *Planktothrix agardhii* CYA 126/8.[112]

The gene cluster for the biosynthesis of microviridin B from *Microcystis aeruginosa* NIES298 (*mdn*) was about 6 kb and contained five genes (*mdnA*-*E*). It consisted of two genes encoding adenosine triphosphate (ATP)-grasp type ligases (*mdnB* and *mdnC*), a gene coding for a putative peptide precursor (*mdnA*), a gene for an ATP-binding cassette (ABC) transporter (*mdnE*) and an *N*-acetyltransferase of the Gcn5-related *N*-acetyltransferase (GNAT) family (*mdnD*).[112] The steps involved in the biosynthesis of microviridin B are shown in Figure 1.3.

The *mdnA* gene consisted of a leader peptide and a core peptide which encoded the precursor molecule for microviridin production.[112] The presence of N-terminal leader peptides is a common feature for ribosomal peptides, which is very important for their processing and cellular export by a transporter peptidase.[112,113]

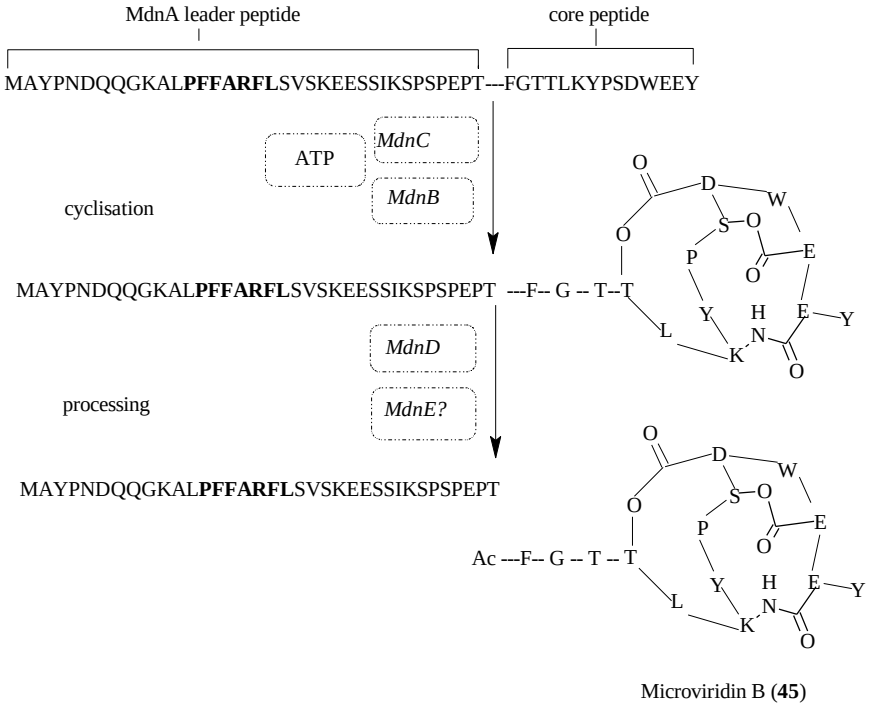


Figure 1.3: Schematic representating the biosynthesis of microviridin B (**45**) in *Microcystis aeruginosa* NIES298. (Letters represent amino acids using the common code). Adapted from Reference 110.

Weiz *et al*. (2011) investigated the role of the leader peptide in the biosynthesis of microviridin and deduced that there was a strictly conserved motif "PFFARFL" in all of the leader peptides of the identified gene clusters for microviridin (highlighted in the leader peptide of Figure 1.3). This conserved motif served as a recognition motif for the ATP-grasp gene which was essential for the post-translational modification of microviridin.[106, 113] The presence of the Arg residue in the motif was crucial for the activity of the ATP-grasp like enzyme introducing the lactam function or cyclic amide.[112]

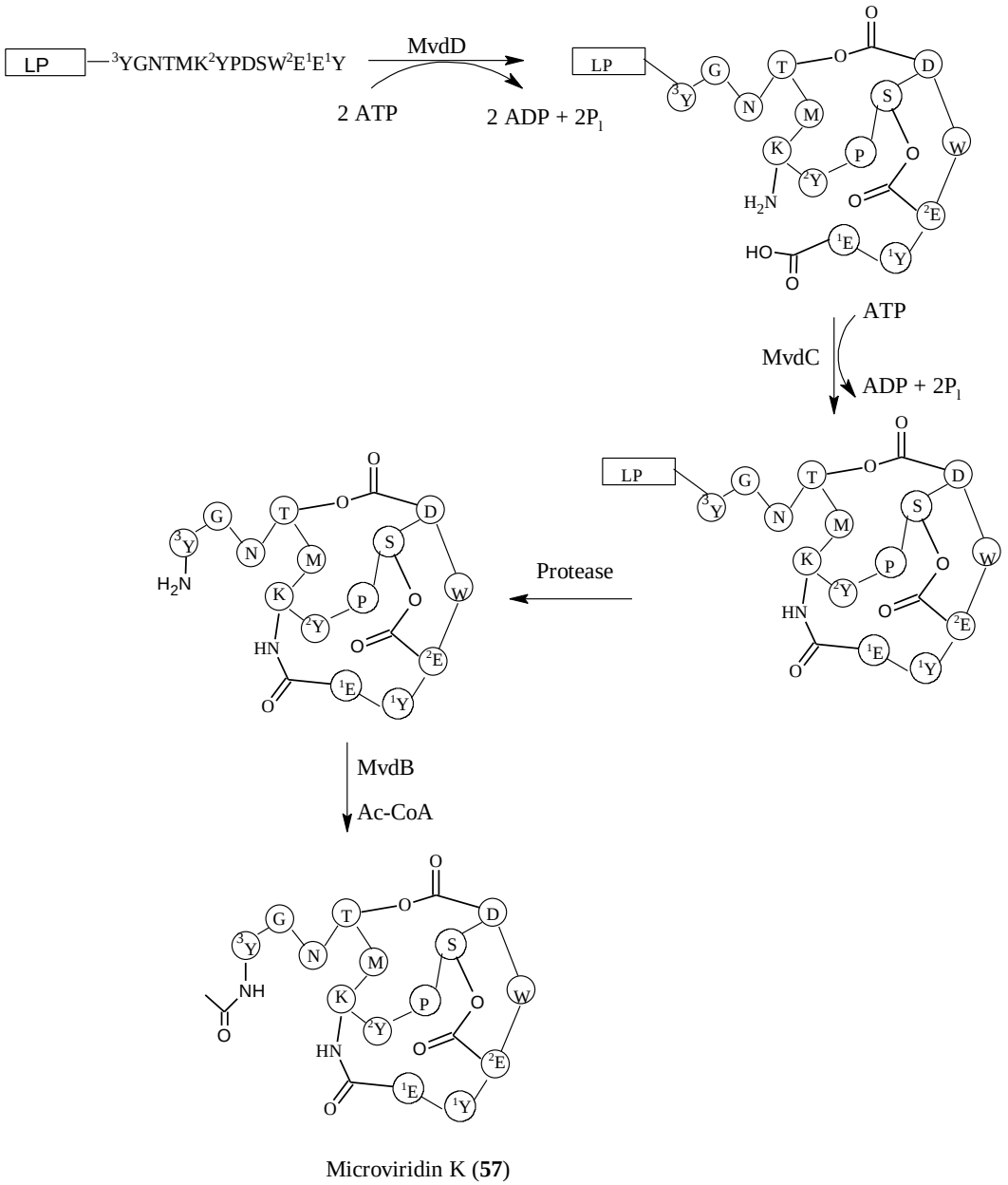
The MdnB and MdnC ligases belong to a family of enzymes with ATP-grasp fold. These enzymes were used to form the peptide bond by conducting an ATP-dependent carboxylate-amine thioligase activity upon primary and secondary amines.[114] The MdnB and MdnC ligases catalysed the ring closure in microviridin biosynthesis, for example, the MdnC introduced the two rings, starting with the larger one. The activity of MdnB relied on MdnC activity and can establish the peptide bond only once the ester bond formations were completed. MdnB and

MdnC cyclised the ring with leader peptide intact prior to its cleavage.[112] MdnD then acetylates the completely processed microviridins at the N-terminus.[106] The ABC-transporter encoded by *mdnE* was assumed to be involved in proteolytic cleavages and translocation of microviridin B.[112] It strongly influences the stability of the putative biosynthetic complex, which could be due to the membrane and ATP-binding domains anchoring the putative microviridin complex.[113]

Philmus and co-workers suggested an orthologous gene cluster for the biosynthesis of microviridin K in *Planktothrix agardhii* CYA126/8. The biosynthetic gene cluster encoded as *mvdA-F* was designated as two structural genes of microviridin prepeptides (*mvdE* and *mvdF*), two genes for grasp-like ligases (*mvdC* and *mvdD*), one gene for an acetyl transferase (*mvdB*) and an ABC transporter (*mvdA*).[106, 113] Three of the four post-translational modifications required to convert linear peptide *mvdE* into microviridin K are indicated in Figure 1.4.[106]

The prepeptide of microviridin K was encoded by *mvdE* which required proteolytic processing as part of the post-translational modification process. It is composed of a leader peptide and a core peptide (YGNTMKYPSDWEEY). The gene *mvdF* appeared to encode another putative microviridin, however this microviridin was not detected in extracts of *P. agardhii.*[106]

MvdD catalysed the two ester linkages by formation of a carboxylated-phosphate mixed anhydride with release of adenosine diphosphate (ADP), followed by the release of phosphate upon esterification.[106] The macrocyclisation of the peptide depends upon the two ATP-grasp-type ligases; MvdD and MvdC.[110] The amide bond is formed by MvdC after the two ester linkages have been formed. The cleavage of the leader peptide requires a protease which is not present in the *mvd* gene cluster and the terminal acetylation is achieved by the acetyltransferase MvdB.[106]



Microviridin K (**57**)

Figure 1.4: Schematic of the biosynthetic steps for production of microviridin K (**57**) in *Planktothrix agardhii* CYA126/8 (Letters represent amino acids using the common code). Adapted from Reference 108.

The structural modifications of the microviridin introduced during the maturation were the formation of the two ester bonds and one amide bond. Further maturation of the peptide was achieved by cleavage of the leader peptide and N-terminal acetylation.[110] The different approaches of the two research groups have labelled the biosynthetic gene clusters differently but with a common indication of the presence of a ribosomally-produced precursor peptide, grasp-like ligases, an ABC transporter and an *N*-acetyltransferase.[100]

Chapter 2: Aeruginosin Metabolites from *Microcystis* CAWBG11.

2.1 Introduction

Aeruginosins are a group of structurally diverse bioactive oligopeptides produced by bloom-forming cyanobacteria of the genera *Microcystis*, *Planktothrix*, *Nodularia* and *Nostoc*. In addition, they are also produced by symbiotic cyanobacteria of marine sponges *Lamellodysidea chlorea* and by members of the *Dysideidae* family.[13-18,78,91] Aeruginosins are tetrapeptides containing the N-terminal Hpla, an unusual Choi moiety at position three, a C-terminal Arg derivative and a variable hydrophobic amino acid in position two (see Section 1.2.6).[9]

The metabolites are inhibitors of the Ser proteases; TRP, THB and PLM.[88] Thirty eight aeruginosin variants have been isolated and characterised since the 1990s, however, additional cyanopeptides belonging to the aeruginosin family such as aeruginosins 586, 602, 670 and 678 are not included in the known lists as they have not been fully characterised or biologically tested against Ser proteases.[11, 115, 116]

In the current chapter, *Microcystis* CAWBG11 was screened for known and new aeruginosins. Four known aeruginosins 298A (**6**), 298B (**11**), EI461 (**15**) and 602A (**60**)[95, 115, 117] were detected in a methanol extract of *Microcystis* CAWBG11, as well as four new aeruginosins which were characterised by tandem mass spectrometry (MS/MS) and Advanced Marfey's amino acid analysis. In addition, the known aeruginosin 602A (**60**)[115] was characterised by NMR spectroscopy to confirmed the putative structure proposed by Welker *et al*. [115].

2.2 Characterisation of aeruginosin-like compounds from *Microcystis* CAWBG11.

The LC-MS analysis of a methanol extract of *Microcystis* CAWBG11 indicated the presence of various ions at *m/z* 462, 603, 605, 619 and 657 (Figure 2.1; Table 2.1) which were within the mass region of the aeruginosins. Initially discrimination of known from new aeruginosin congeners was achieved by comparison of molecular masses of the detected ions with those of known

aeruginosins. The confirmation of the known aeruginosins with molecular mass 461Da (A and B), 602ADa (A) and 604 Da was done by MS/MS data analysis. The aeruginosins with molecular masses 602 Da (B), 618 Da, and 656 Da (A and B) were potential canditates.

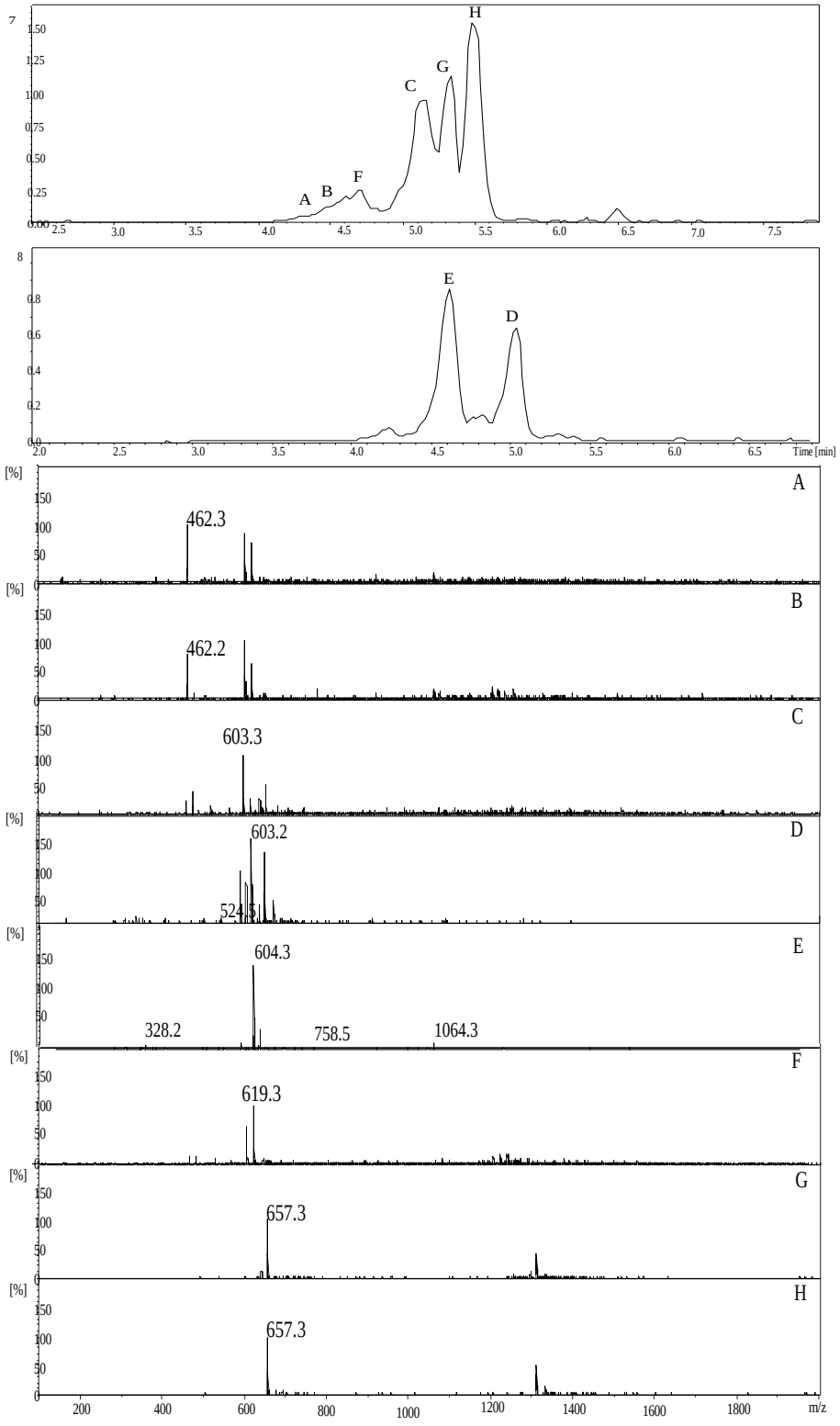


Figure 2.1: LC-MS base peak ion chromatogram of a methanol extract from *Microcystis* CAWBG11 for the retention region of aeruginosin-like compounds.

The tandem mass spectral analysis of each compound confirmed all were aeruginosins as they produced diagnostic fragments commonly observed in aeruginosins[115] such as *m/z* 70 (Arg fragment $C_4H_8N^+$), *m/z* 86 (Leu or Ile immonium ions), *m/z* 140 (Choi immonium ion) and *m/z* 250 (Hpla-Leu – CO + H) (Appendix B.5). There were several aeruginosins with the same molecular mass (461, 602 and 656 Da) but with different retention times.

Table 2.1: Molecular masses and retention times of aeruginosin analogues found in *Microcystis* CAWBG11.

Compound	Aeruginosin	M_r[a] (Da)	RT[b] (min)	Reference
A	298B (**11**)	462.3	4.2	95
B	EI461B (**15**)	462.5	4.4	117
C	602A (**60**)	602.3	5.0	115
D	602B (**61**)	602.4	4.9	This study
E	289A (**6**)	604.3	4.5	71
F	618 (**62**)	618.2	4.6	This study
G	656A (**63**)	656.3	5.2	This study
H	656B (**64**)	656.3	5.4	This study

[a] molecular mass rounded to one decimal place, [b] retention time,

2.2.1 Characterisation of aeruginosins 298B (11) and EI461 (15) by tandem mass spectrometry.

The MS/MS analysis of two ions with the same molecular mass; *m/z* 462 Da shared similar fragmentation patterns (Table 2.2; Appendix B.3).
The fragment ions demonstrated that these compounds had an amino acid sequence of Hpla-Leu-Choi with NH_2 at the C-terminus. This matches the structure of the previously reported aeruginosins 298B (**11**) and EI461(**15**).[95,117]
It was noted that aeruginosins 298B (**11**) and EI461(**15**) were stereoisomers consisting of the same amino acid compositions but with different stereochemistry at the Hpla and the Choi moieties.[95,117] This has also been reported for other aeruginosins; 89A/89B (**12**/**13**),[95] KT608A/KT608B (**23**/**24**)[21] and 205A/205B (**33**/**34**)[75] which might well explain why some of the aeruginosins

detected from *Microcystis* CAWBG11 have the same mass but different retention times.

Table 2.2: Assignment of fragment ions from tandem mass spectrometry for aeruginosins 298B (**11**) and EI461 (**15**).

Fragment Ion Assignment	***m/z* for 298B (11) and EI461 (15)**
Hpla-Leu-Choi-NH_2 – H_2O + H	445
Hpla-Leu-Choi + H	417
Hpla -Leu + H	278
Hpla-Leu – CO + H	250
Hpla-OH + H	185
Choi +H	167
Choi immonium ion	140
Leu immunion ion	86

2.2.2 **Characterisation of aeruginosin 602A (60) and 602B (61) by tandem mass spectrometry.**

The MS/MS spectrum of the ion with molecular mass *m/z* 603 (Figure 2.2) indicated that it was the known but incompletely characterised aeruginosin 602 reported by Welker *et al*. (2006),[115] as the fragment ions detected matched those reported previously. Also present was another compound with the same mass but a different retention time by reversed phase column chromatography (Table 2.1)

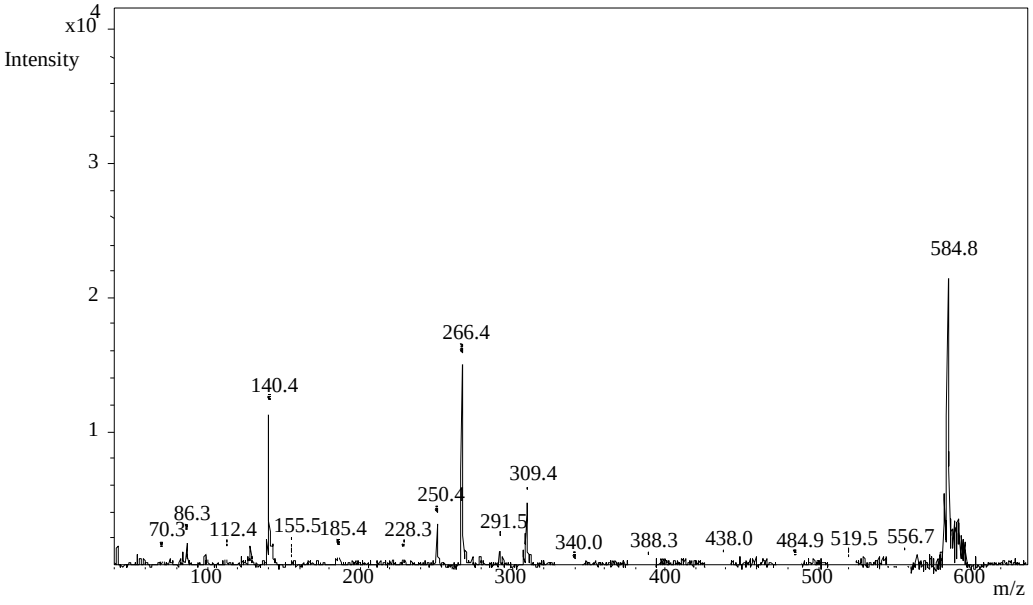


Figure 2.2: The tandem mass spectrum of aeruginosin 602A (**60**).Fragmentation was induced by matrix-assisted laser desorption/ionisation post-source decay.

This compound had identical MS/MS fragmentation pattern and therefore the two aeruginosins were designated as aeruginosins 602A (**60**) and 602B (**61**). Aeruginosin 602A (60) was previously characterised by MS/MS only. In this instance, a sufficient quantity of aeruginosin 602A (60) was purified in order to further characterise the compound by NMR spectroscopy and by amino acid analysis. An attempt to separate aeruginosin 602B (61) from other sample components by HPLC chromatography was not successful.

2.2.1 **Characterisation of aeruginosin 298A (6) by tandem mass spectrometry.**

The MS/MS spectrum of the *m/z* 605 ion (Figure 2.3) shared common fragments diagnostic of aeruginosins *m/z*; (86, 100, 140, 250 and 266). In addition to these, the fragment ions *m/z* 133 (Choi-Argol $-NH_2$ + H), *m/z* 155 (Argol fragment) and *m/z* 100 (Argol fragment) indicated that the amino acid sequence for this compound was Hpla-Leu-Choi-Argol. An aeruginosin with the same amino acid sequence had been previously reported as 298A (**6**), due to limited information gathered on *m/z* 605, it is highly likely that this is the known aeruginosin 289A (**6**).

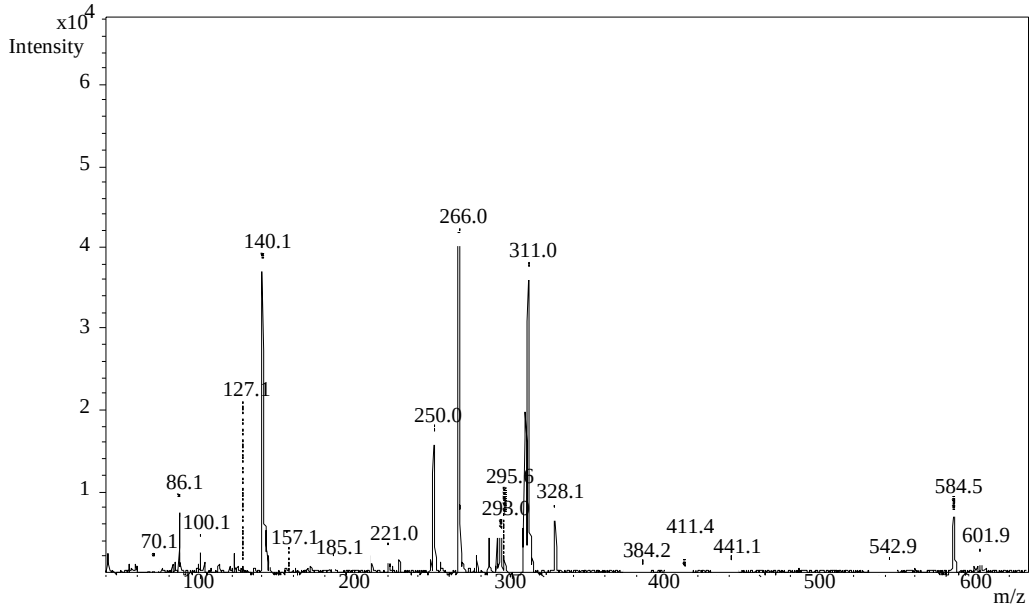


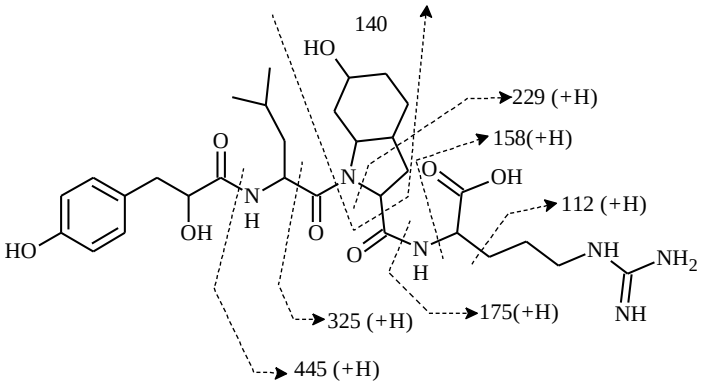
Figure 2.3: Tandem mass spectrum of aeruginosin 298A (**6**). Fragmentation induced by matrix-assisted laser desorption/ionisation post-source decay.

An attempt to isolate aeruginosin 298A (**6**) from other sample components was carried out using reversed-phase HPLC but this was unfortunately not successful.

2.2.2 **Characterisation of aeruginosin 618 (62) by tandem mass spectrometry.**

Based on the matrix-assisted laser desorption/ionisation post-source decay (MALDI PSD) - MS/MS spectrum and the fragmentation patterns commonly observed in the presently characterised aeruginosins, a planar structure for aeruginosin 618 was proposed (Figure 2.4a). The main fragment ion (*m/z* 325) resulted from the loss of Leu-Hpla and NH_3. Also observed was a Choi immonium ion (*m/z* 140) diagnostic for aeruginosins. The Arg y-ion (*m/z* 175) indicated that the C-terminus of this aeruginosin was Arg, a modification which has not been observed before. An ion series extending from *m/z* 175 confirmed that the amino acid sequence for aeruginosin 618 (**62**) was; Hpla-Leu-Choi-Arg (Figure 2.4).

a)



b)

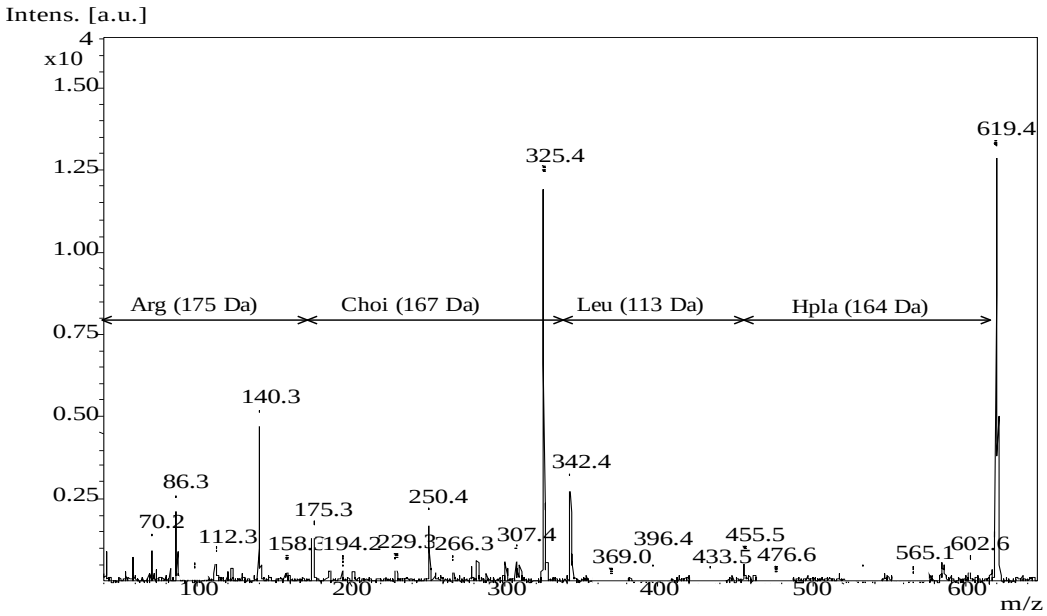


Figure 2.4: **a)** Proposed planar structure and **b)** the tandem mass spectrum of aeruginosin 618 (**62**). Fragmentation was induced by matrix- assisted laser desorption/ionisation post-source decay.

Unfortunately, the presence of phthalate plasticiser in the fractionated sample made the structural characterisation of aeruginosin 618 (**62**) by NMR spectroscopy too difficult to complete at the present time.

2.2.3 **Characterisation of aeruginosin 656A (63) and 656B (64) by tandem mass spectrometry.**

The MS/MS spectrum of aeruginosin 656A (**63**) was very similar to that of aerugisnosin 618 (**62**) as it contained *m/z* 70, 86 and 140. However the main fragmentation ion of 656A (**63**) was *m/z* 362 which results from the loss of Leu, Hpla and H_2O + 2H (Table 2.3, Appendix B.2). The presence of an ion with molecular mass *m/z* 196 indicated that the C-terminus of 656A contained an extra 38 Da compared to aeruginosin 618 (**62**). The composition of the ion *m/z* 196 could not be further determined by tandem mass spectrometry at the present time. Based on the gathered MS/MS information, a planar structure for 656A (**63**) was proposed (Figure 2.5).

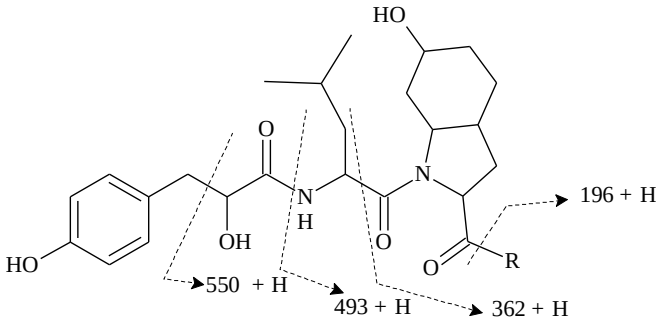
Table 2.3: Assignment of fragment ions from tandem mass spectrometry for aeruginosins 618 (**62**) and 656A (**63**).

Fragment Ion Assignment	***m/z* for** 618 (**62**)	***m/z* for** 656A (**63**)
M + H	619	657
M – H_2O + H	602	
M – Hpla + H	455	493
M – Hpla – Leu + H	342	380
M – Hpla – Leu – H_2O + 2H		362
M – Hpla – Leu – H_2O + H	325	
Choi-Argol – CH_3N_2 – H_2O + H	266	
Hpla-Leu – CO + H	250	250
C-term[a] + C_3H_3O + H	229	
C-term + H	175	196
C-term – NH_2 + H	158	180
Choi immonium ion	140	140
Dehydrated Choi immonium ion		122
C-term fragment ($C_5H_{10}N_3$)	112	
Leu immonium ion	86	86
C-term fragment ($C_4H_8N^+$)	70	70

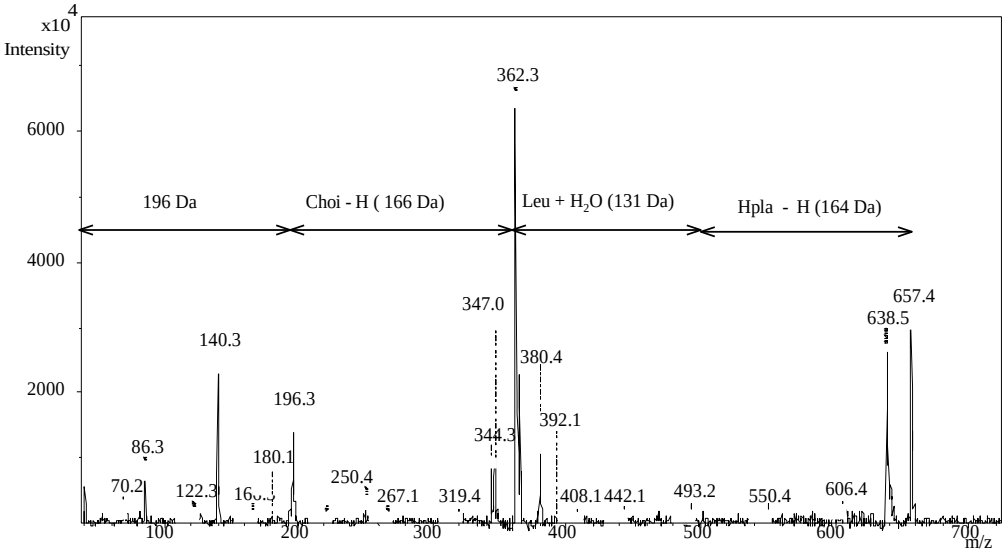
[a] C-terminus with Arg for aeruginosin 618 (**62**), 196 Da for aeruginosin 656A (**63**).

Figure 2.5: a) Proposed planar structure and **b)** the tandem mass spectrum of aeruginosin 656A (**63**).Fragmentation induced by matrix-assisted laser desorption/ ionisation post-source decay.

a)



b)



A compound, 656B (**64**) with the same mass as 656A (**63**) but with a different retention time on reversed-phase C_{18} chromatography was also detected in the LC-MS analysis of *Microcystis* CAWBG11. The LC-MS/MS fragmentation for this compound (Appendix B.2) was the same as that of aeruginosin 656A (**63**) suggesting that the two compounds have the same amino acid sequence but potentially differ by some stereochemical element.

2.3 **Advanced Marfey's Method of amino acid analysis of 656A (63), 656B (64) and 602A (60).**

The amino acid analyses of aeruginosins 656A (**63**), 656B (**64**) and 602A (**60**) indicated that the Leu residue in position two of each compound was in the

D-configuration (*m/z* 424; 28.7 min; Figure 2.6). The determination of the configuration of the amino acid present was possible by derivatising standard Leu with L- and D-1-difluoro-2,4-dinitrophenyl-5-leucine amide (FDLA) (Appendix C.2). It was not possible to determine the configuration of the Hpla, Choi and C-terminal amino acids during the period of this study due to time constraints.

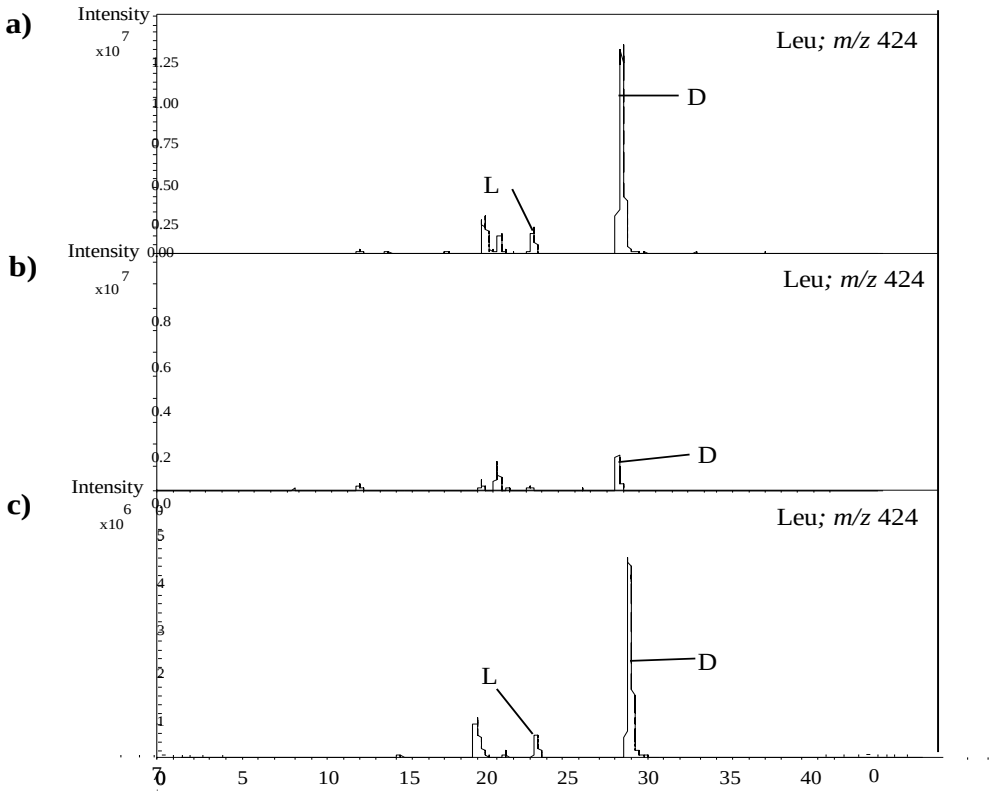


Figure 2.6: Advanced Marfey's method of amino acid analysis of hydrolysate of aeruginosins **a)** 656A (**63**), **b)** 656B (**64**) and **c)** 602A (**60**) with L-FDLA.

2.4 **NMR spectroscopic characterisation of aeruginosin 602A (60).**

In the current study, a sufficient quantity of aeruginosin 602A (**60**) was isolated to enable structural characterisation by NMR spectroscopy. In a Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY) spectrum (Appendix B.10) correlations confirmed the amino acid sequence determined during the MS/MS analysis (Hpla-H2 →Leu-H2; Leu-H3 → Choi-H7'; Choi-H3 → Argal-H2); Figure 2.7. Assignment of ^{1}H and ^{13}C NMR signals for 602A (**60**) (Table 2.4) was accomplished by interpretation of Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Heteronuclear Single Quantum Coherence

(HSQC) and Heteronuclear Multiple-Bond Correlation (HMBC) NMR experiments.

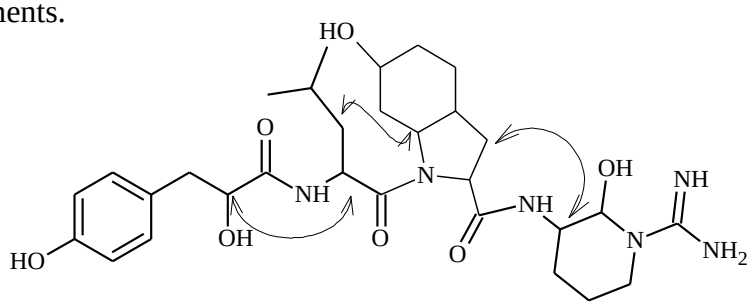


Figure 2.7: Proposed structure of aeruginosin 602A (**60**) with selected ROESY correlations (arrows).

Table 2.4: Nuclear magnetic resonance spectral data of 602A (**60**) in CD_3OD.

Position		^1H	Multiplicity[a] (*J* in Hz)	^{13}C	COSY	HMBC
Hpla	1	-		174.5		
	2	4.23	m	74.0	Hpla 3a, 3b	Hpla-1, 4
	3a	2.98	dd (14.7, 4.8)	40.8	Hpla-2	
	3b	2.82	m			
	4	-		129.4		
	5/9	6.68	m	116.1		Hpla-4, 7
	6/8	7.05	dd (8.68, 1.99)	131.9		Hpla-3, 4, 7
	7	-		157.4		
	2-OH	nd[b]				
Leu	1	-		nd		
	2	4.67	t (3.6,1.4)	50.9	Leu-3	
	3a	1.29	m	42.4	Leu-2, 4	Leu-4, 5
	3b	1.45	m			
	4	1.31	m	26.1	Leu-5, 3	
	5	0.91	m	22.1	Leu-4	
	5'	0.88	m	23.9	Leu-4	
	N-H	nd	d			
Choi	1	-		nd		
	2	4.43	t (8.2)	62.7	Choi-3	Choi-1,3
	3a	2.00	m	32.1		
	3b	2.09	m			
	3'	2.40	m	38.2	Choi-4a	
	4a	1.58	m	20.3	Choi-5	Choi-3
	4b	1.60	s			
	5	2.22	m	32.1	Choi-6	
	6	4.08	s	66.6	Choi-4, 5, 7	Choi-4, 7'
	7a	2.82	m	34.5		
	7b	1.83	s			
	7'	4.24	m	56.6	Choi-7a	
Argal	1	5.35	d (3.2)	78.3		
	2	3.85	m	54.0	Argal-3	
	3a	2.12	m	31.7	Argal-4	
	3b	2.00	m			
	4	1.56	m	27.1	Argal-5	
		1.63	m			
	5	3.14	d (1.09)	42.6		
		3.20	d (1.27)			
	6	-		nd		
	2-NH	nd	s			
	1-OH	nd				

[a]Multiplicity of proton signal; m = multiplet, d = doublet, s = singlet, [b]not detected.

The Hpla moiety of 602A (**60**) (Figure 2.8) gave rise to proton signals at, 2.82, 2.98, 4.23, 6.68 and 7.05ppm. (Appendix B.5, Appendix B.6) The chemical shifts of the signals at 7.05 and 6.68 ppm were indicative of an aromatic ring. Integration of the signals showed that each signal was due to two protons, indicating that the ring was symmetrically substituted and that the H5/H9 and H6/H8 protons were in equivalent chemical environments. The 6.68 ppm signal was assigned as the H5/H9 protons due to the vicinal coupling to H6 (7.05 ppm) and long range coupling (^4J) to the H3 methylene (2.82 and 2.98 ppm) and the 7.05 ppm signal was assigned as the H6/H8 due to being coupled to H5 (8.68 Hz) and to H9 (1.99 Hz).

a) **b)**

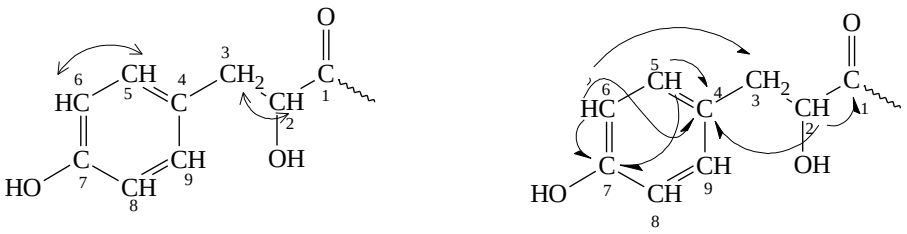


Figure 2.8: **a)** The COSY and **b)** HMBC correlations within the Hpla moiety of aeruginosin 602A (**60**).

The HMBC spectrum for 602A (**60**) (Appendix B.12) contained correlations from H6 to C3 (40.8 ppm), C4 (129.4 ppm), C5 (116.1 ppm) and C7 (157.3 ppm). Similar HMBC correlations were seen from H5 to C4 and C7. The HSQC spectrum (Appendix B.11) revealed that the protons attached to C3 were in two different chemical environments; 2.98 ppm (H3a) and 2.82 ppm (H3b) due to restricted rotation. The multiplicity of the H3a signal was a doublet of doublets due to germinal coupling to H3b (14.7 Hz) and vicinal coupling to H2 (4.23 Hz). The multiplet at H3b showed similar correlations to H3a and to H2 and could also experience long range coupling to H5 as indicated in the COSY spectrum (Appendix B.8). The chemical shift of H2 indicated the effect of the electron withdrawing hydroxyl group attached to it and the adjacent carbonyl group. The presence of the neighbouring carbonyl was indicated by HMBC correlations from H2 to C1 (74.5 ppm).

A correlation in the ROESY spectrum (Appendix B.10) from Hpla-H2 (4.23 ppm) to a one proton triplet at 4.67 ppm indicated connectivity with the second amino acid. The HSQC spectrum established that this proton signal correlated to a methine carbon at 50.9 ppm. The chemical shift of the proton signal (4.67 ppm) was typical of a methine adjacent to a carbonyl and an amide group. The multiplicity of the proton signal (triplet) indicated that a methylene group was adjacent, hence this was assigned as H2 of the Leu residue.

Within the Leu moiety (Figure 2.9), H2 showed a COSY correlation to two non-equivalent H3 protons (1.29 and 1.45 ppm). The HSQC spectrum indicated that these signals corresponded to the methylene carbon at 42.4 ppm. The two H3 proton signals were both multiplets, each with an integral value of one proton. The COSY spectrum indicated that H3a (1.45 ppm) was coupled to H4 (1.31 ppm), whilst the HSQC spectrum showed that H4 was attached to a methine carbon which resonated at 26.1 ppm. Correlations in the COSY and HMBC spectrum also supported this substructure (Figure 2.9).

a) **b)**

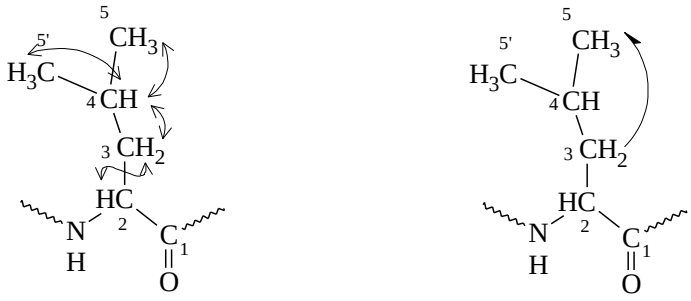


Figure 2.9: **a)** The COSY and **b)** HMBC correlations within the Leucine residue of aeruginosin 602A (**60**).

The Distortionless Enhancement by Polarisation Transfer (DEPT)-135 spectrum (Appendix B.7) indicated that there were two methyl carbons in the compound and these were assigned as C5 (22.1 ppm) and C5' (23.9 ppm) of the Leu residue. The HSQC spectrum revealed that the H5 and H5' protons resonated as multiplets at 0.91 and 0.88 ppm respectively. Whilst the H5 and H5' signals overlapped, integration of the spectral region revealed the six protons of the two methyl groups. The HMBC lacked correlations from H2 or H3 to the C1 of the Leu moiety; hence the chemical shift for C1 was not determined.

A correlation in the ROESY spectrum from Leu H3 (4.67 ppm) to a proton multiplet at 4.24 ppm yielded the connection to the Choi moiety. The HSQC spectrum indicated that the proton (4.24 ppm) was correlated to a methine carbon at 56.6 ppm. The DEPT-90/135 spectra indicated that the Choi moiety contained four methine carbons. The carbon signal at 56.6 ppm with an attached proton at 4.24 ppm could represent either C2 or C7' as both carbons neighbour the -**N**R- group. However H2 would be more downfield as it is also next to a carbonyl group. The COSY spectrum indicated that H7' was coupled to a multiplet signal at 2.82 ppm.

a) **b)**

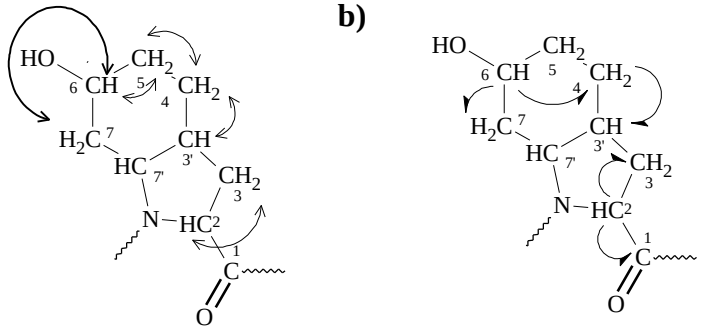


Figure 2.10: a) The COSY and **b)** HMBC correlations of the Choi moiety of 602A (**60**).

The HSQC spectrum indicated that the 2.82 ppm proton signal correlated to a methylene carbon which resonated at 34.5 ppm. It also revealed that the chemical shift of the other proton attached to it was a singlet at 1.83 ppm. The proton signal at 2.82 ppm also showed a COSY coupling to a signal at 4.08 ppm. The HSQC spectrum revealed that this singlet was correlated to a methine carbon at 66.6 ppm, the downfield chemical shift of which indicated the presence of the electron withdrawing group (OH) adjacent to it. This led to the assignment of C6 (66.6 ppm), C7 (34.5 ppm) and C7' (56.6 ppm). In addition, the COSY and TOCSY (150 ms) spectra (Appendix B.8, Appendix B.9) showed correlations between H6 (4.08 ppm) and H4a (1.60 ppm), H4b (1.58 ppm), H5a (2.24 ppm), H7a (1.89 ppm) and H7b (2.82 ppm). The COSY spectrum also contained a correlations from a triplet signal at 4.43 ppm (H2) to a signal at 2.40 ppm (H3') and to a multiplet signal at 2.00 ppm (H3a). The HSQC spectrum revealed that this proton triplet corresponded to a methine carbon (C2; 62.7 ppm). The HMBC spectrum

displayed a correlation from 4.43 ppm signal to a carbonyl group C1 (173.3 ppm). The correlations supported the substructure on Figure 2.10.

A ROESY correlation from Choi-H2 to a multiplet signal at 3.85 ppm correlated to a carbon signal at 54.0 ppm (by HSQC), indicated connection to a signal at C2 of the Argal moiety. The COSY spectrum showed a correlation from the H2 proton signal to multiplet signals at 5.35 ppm, 2.12 ppm and 1.56 ppm and the HSQC spectrum indicated the corresponding carbons as C1 (78.3 ppm), C3 (3.17 ppm) and C4 (27.1 ppm) respectively.

a) **b)**

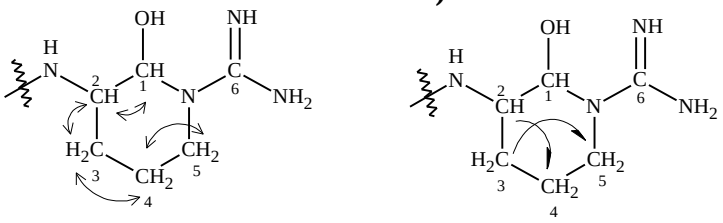


Figure 2.11: **a)** The COSY and **b)** HMBC correlations within the Argininal moiety of 602A (**60**).

In addition, the COSY spectrum contained a correlation from H3 (2.12 ppm) to a multiplet signal at 1.63 ppm (H4b) and to doublets at 3.14 ppm and 3.20 ppm. The downfield chemical shifts at 3.14 and 3.20 are typical chemical shifts of protons with an adjacent amine group; hence they were assigned as H5a and H5b and were correlated to a carbon signal at 42.6 ppm by the HSQC spectrum. The HMBC spectrum lacked any correlation from H5 to C6 so the chemical shift for C6 was not able to be determined at the present time. The correlation supported the substructure on Figure 2.11.

It is possible that there were impurities present in the sample. There were indications of the presence of other solvents apart from water, CD_3OD and HOD (3.31 ppm, 4.78 ppm). A proton singlet at 8.53 ppm was correlated to a carbon at 170.5 ppm with a ^1J coupling constant of 190 Hz, typical of a carbonyl proton from a formic acid or formate salt. Another carbon signal at 99.6 ppm did not theoretically fit into any of the carbon environments of the aeruginosin compound. However if the formate acid/salt was present than this could be a part of the salt as

it is a typical H-C(O)-O-**C**HR carbon. This formic acid/formate salt could have come from the formic acid used during isolation of the compound.[15] The presence of these solvents peaks could contribute to overlap in the NMR spectra.

2.5 **Future Work.**

Due to time constraints, a number of areas of interest remained unexplored but would form a basis for future research. Most of the aeruginosins detected are assumed to be stereoisomers of each other due to their different retention times but similar MS/MS fragmentation patterns. Identification of the structural differences in these will be very helpful for other researchers.

Plasticiser is a common problem, especially when working with semi-purified and purified samples of organic extract. Development of an effective method to totally remove plasticiser from precious sample would be very useful. If that is achievable, then further structural characterisation of aeruginosins 618 (**62**) and 656A (**63**) would be possible.

The structural characterisation and chemical properties of aeruginosin 602A (**60**) were not fully determined, so in order to fully elucidate the structure, further work is needed such as X-ray crystallography, biological activity testing and synthesis.

Chapter 3: Microviridin Metabolites from *Microcystis* CAWBG11.

3.1 Introduction

Microviridins are a group of tricyclic depsipeptides produced by cyanobacteria,[105] comprised of either thirteen or fourteen amino acids, seven of which are conserved. They are initially produced on the ribosome as a linear peptide which is folded into a tricyclic structure by grasp-like ligase enzymes to form the two esters and one amide bond.[110] The linkages are formed between the ω-carbonyl groups of Asp and Glu and ω-amino and hydroxyl groups of Lys and Ser or Thr respectively.[112] Final maturation of the microviridin occurs with release of the leader peptide and acetylation of the N-terminus.[110] Microviridins reported to date contain only L-amino acids[118] and due to their ability to inhibits specific proteases, are of pharmacological relevance.[113]

Four putative new microviridins were detected in *Microcystis* CAWBG11 with molecular masses 1778A (**65**) and 1778B (**66**), 1760 (**67**) and 1764 (**68**) (Figure 3.1). They were separated and purified using size exclusion chromatography and RP HPLC (Appendix C.1,Table 3.1). The microviridin judged the most pure by HPLC, 1778A (**65**), was subjected to NMR spectroscopy and amino acid analysis to determine the structure of the compound. The tandem mass spectral and amino acid analyses of the three other microviridins 1778B (**66**), 1760 (**67**) and 1764 (**68**) were used to propose their putative structures.

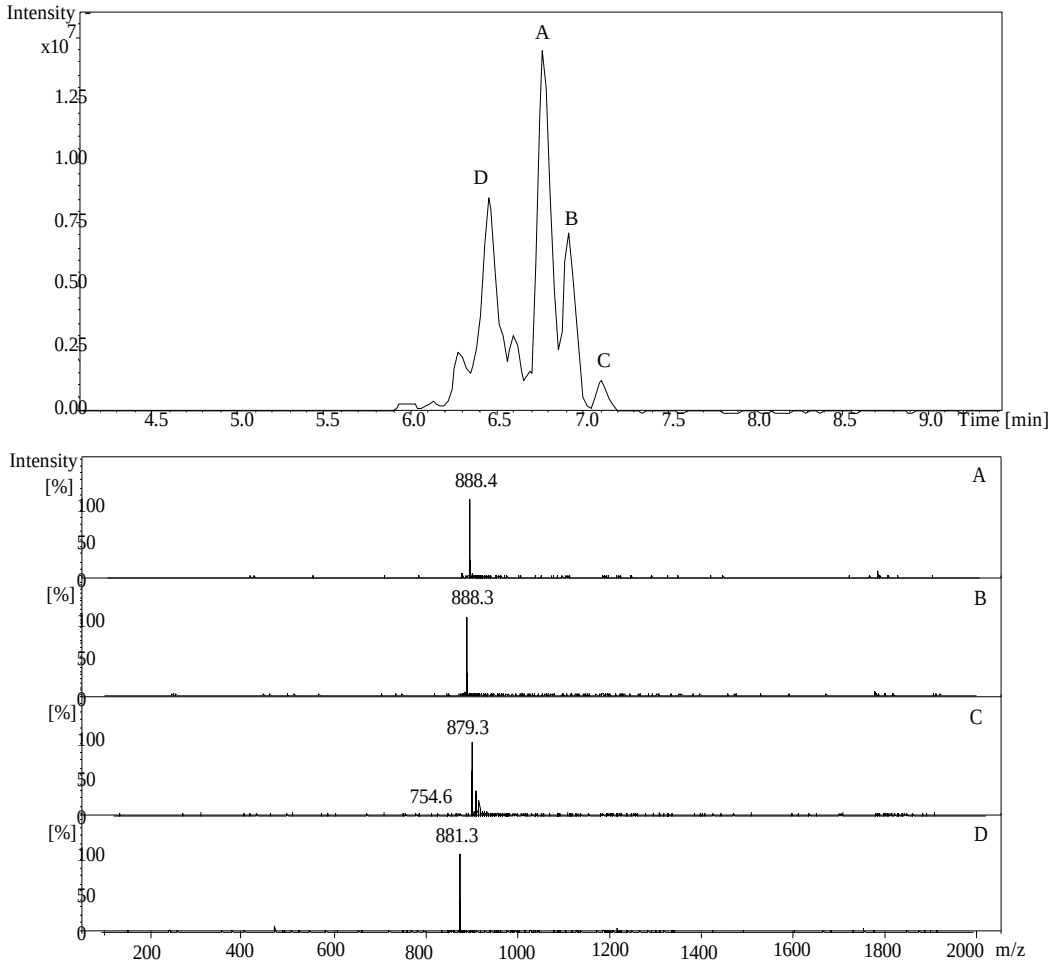


Figure 3.1: LC-MS base peak ion chromatogram of a methanol extract from *Microcystis* CAWBG11 for the retention region of microviridin-like compounds.

Table 3.1: Molecular masses and retention times of microviridin analogues found in *Microcystis* CAWBG11.

Compound	Microviridin	$[M + 2H]^{2+}$ [a]	M_r (Da)[b]	RT(min)[c]
A	1778A (**65**)	888.4	1778.6	6.8
B	1778B (**66**)	888.3	1778.8	7.0
C	1760 (**67**)	879.3	1760.6	7.1
D	1764 (**68**)	881.3	1764.2	6.5

[a]doubly charged ions to one decimal place, [b] molecular mass rounded to one decimal place and [c]retention time.

3.2 **Characterisation of microviridin 1778A (65).**

Based on the interpretation of the LC-MS/MS spectra, NMR spectroscopic and chemical analyses, a planar structure was proposed for microviridin 1778A (**65**) (Figure 3.2). The amino acids at positions one, two and three were assigned from the LC-MS/MS spectrum as glycine (Gly) and two Phe residues but the sequence could not be determined from NMR spectroscopy. In addition, LC-MS/MS spectra confirmed that the amino acid at position five was Met, as reported in microviridins D (**53**) and K (**57**).[105, 112] The Tryptophan (Trp) was assumed to be at position eleven as is observed in almost all the known variants except for microviridins E (**54**) and F (**55**).[105] The amino acids at positions thirteen and fourteen were assigned as Asp and Tyr respectively.

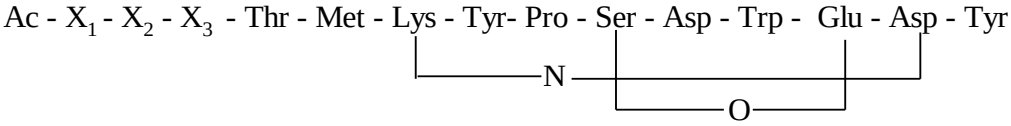


Figure 3.2: The proposed planar structure for microviridin 1778A (**65**). The amino acids X_1, X_2 and X_3 are Phe (×2) and Gly with the sequence yet to be determined.

3.2.1 **Characterisation of microviridin 1778A (65) by tandem mass spectrometry.**

The LC-MS/MS analyses as in Figure 3.3 yielded partial information about the amino acid composition of microviridin 1778A (**65**). The loss of the 218 Da (Tyr–OH) - *y ion* from the parent ion resulted in a fragment ion *m/z* 1559, an indication that the C-terminus was of Tyr. Similarly, the loss of Glu-Asp-Tyr-OH (418 Da) resulted in a fragment ion of *m/z* 1359, which could represent the last three amino acids at positions twelve to fourteen of the compound. The 147 Da loss in the higher mass region indicates the present of Lys (side chain) at position five while in the lower mass region an ion at *m/z* 147 Da (neutral loss) indicated the presence of 2 × Phe. The loss of 181 Da (Tyr +H_2O) shows that there was a free hydroxyl group in the compound. The presence of Met in the compound was determined by the loss of 150 Da (*y ion*) and the loss 57 Da indicated the presence of Gly.

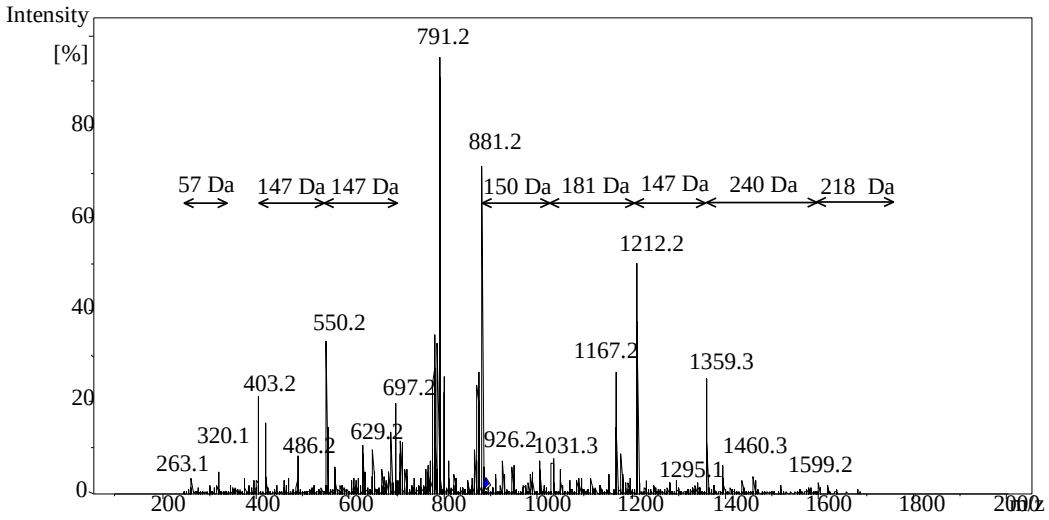


Figure 3.3: The LC-MS/MS spectrum of doubly charged ion *m/z* 888.4 (microviridin 1778A (**65**)).

3.2.2 **Advanced Marfey's Method of amino acid analysis for 1778A (65).**

The presence and stereochemistry of many of the amino acids in microviridin 1778A (**65**) were determined by Advanced Marfey's amino acid analysis. The LC-MS analysis of the L-FDLA derivative of hydrolysed peptide (Appendix C.3) indicated: L-Asp (*m/z* 426; 14.6 min), L-Glu (*m/z* 440; 15.5 min), L-Lys (*m/z* 733; 31.1 min), L-Phe (*m/z* 458; 23.9 min), L-Ser (*m/z* 398; 14.2 min), L-Pro (*m/z* 408; 17.6 min), L-Thr (*m/z* 412; 13.9 min), L-Gly (*m/z* 368; 17.5 min) and L-Tyr (*m/z* 474; 19.2 min). The identity and stereochemistry of the amino acids were determined by comparing standard amino acids derivatised with L-FDLA and analysed in the same manner (Appendix C.2).

All of the detected amino acids were in the L-conformation as reported in the previously characterised microviridin variants. Among these were the conserved amino acids such as Lys, Thr, Tyr, Pro, Ser, Asp and Glu. The peak for the Phe derivative was as twice as intense as that of the other amino acids, which indicates that there were two Phe residues present in microviridin 1778A. It was not possible to determine the stereochemistry of Trp as it is degraded during the hydrolysis and there was no Met standard available at the time of the experiment to confirm the suggestion from the tandem mass spectral analysis.

3.2.3 **NMR spectroscopic characterisation of microviridin 1778A (65).**

Analysis of COSY, HSQC, HMBC and ^{13}C NMR spectra of 1778A (**65**) provided information about the sequence of amino acids in the compound (Table 3.2), however due to time constraints and a complex spectrum on an instrument of insufficient resolution, information about the order of the amino acids in microviridin 1778A (**65**) was restricted. Since CD_3OD solvent was used, amide proton chemical shifts were not visible in the spectra.

Table 3.2: Nuclear magnetic resonance spectral data of microviridin 1778A (**65**) in CD_3OD.

Position		^1H	Multiplicity[a] (*J* in Hz)	^{13}C	COSY	HMBC
Ac	1			172		
	2	1.93	s	22.2		Ac-1
Thr	1			170.6		
	2	4.31	d	58.6	Thr -3	Thr-1
	3	4.12	m	67.0	Thr-4, 2	
	4	1.15	d	18.4	Thr-3	Thr-3,2
Phe	1	nd[b]				
	2	nd				
	3	nd				
	4			155.9		
	5/9	6.99	d (8.0)	118.3	Phe-6,8	Phe-4
	6/ 8	7.13	m	126.3	Phe-5,8,7	
	7	7.18	m	128.3		
Tyr	1	nd				
	2	nd				
	3	nd				
	4			136.9		
	5,9	7.05	d (8.8)	129.9	Thr-6,9	Thr-4
	6,8	7.55	d (7.8)	118.1	Thr-5,9	Thr-7
	7			155.9		
Trp	1	nd				
	2'			136.6		
	3'	7.08	s	118.6	Trp-4'	Trp-2',9'
	4'	7.33	D (8.8)	112.1	Trp-3',5'	Trp-3'
	5'	7.15	m	126.3	Trp-6',4'	
	6'	6.72	m	115.2	Tpr-7'	Trp-5'
	7'	6.68	d	114.8		Trp-6',8'
	8'			156.1		
	9'					
Asp	1			170.0		
	2	4.46	m	55.7	Asp-3	Asp-1

	3	3.06	m	38.4	Asp-2	
		2.84	m			
	4			172.3		
Lys	1			175.7		
	2	4.7	m	55.7		
	3	1.95	m	21.1	Lys-4	Lys-1
	4	2.21	m	31.7	Lys-5	
	5	1.44	m	28.1	Lys-6	
	6	1.29	s	22.3	Lys-5	

[a] Multiplicity of proton signal; m = multiplet, d = doublet, s = singlet, [b] not detected.

The DEPT-135 spectrum (Appendix C.5) indicated that there were two methyl carbons in the compound with signals at 22.2 ppm and 18.4 ppm. The HSQC spectrum (Appendix C.6) indicated that these methyl carbons correspond to a singlet at 1.93 ppm and a doublet at 1.12 ppm respectively and each integrated as three protons. The HMBC NMR spectrum (Appendix C.8) showed that the methyl carbon associated with the proton signal was attached to a quaternary carbon at 172.0 ppm and no other correlations were detected, hence this was assigned as the acetyl group at the N-terminus.

The other methyl would be expected to be due to H4 of the position four Thr, one of the conserved amino acids in the known microviridin variants. The multiplicity of the doublet signal indicated that the neighbouring carbon was a methine, which was confirmed by the COSY spectrum (Appendix C.7) as it contained a correlation to a multiplet signal at 4.12 ppm (H3). The chemical shift of H3 was typical of a methine proton with a hydroxyl group attached to it. In addition, the 4.12 ppm multiplet was also coupled to a doublet at 4.31 ppm (H2) and the HSQC spectrum (Appendix C.6) indicated that these methine protons were attached to carbons which resonated at 67.0 ppm (C3) and 58.6 ppm (C2) respectively. The HMBC spectrum indicated that H2 was correlated to a quaternary carbon at 170.6 ppm (C1). These correlations were indicated in the substructure on Figure 3.4.

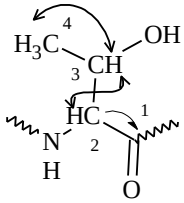


Figure 3.4: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the threonine for microviridin 1778A (**65**).

The presence of aromatic rings in the microviridin was indicated by downfield signals in the ^{1}H NMR spectrum (Appendix C.4). Close examination of the COSY and HMBC NMR spectra indicated correlations within the aromatic rings, enabling assignment of three aromatic amino acids; Tyr, Phe and Trp which were expected to contain two, three and five different proton environments respectively. However, correlations from the aromatic protons to the aliphatic protons were not able to be detected due to poor resolution in the HMBC experiment.

From the COSY spectrum, within the aromatic ring of Tyr, a proton with a doublet signal at 7.55 ppm (7.8 Hz) was correlated to another doublet signal at 7.05 ppm (8.8 Hz) as shown in Figure 3.5. The HSQC spectrum indicated that these protons were attached to C6/C8 (118.1 ppm) and C5/C9 (129.9 ppm) respectively. In addition, HMBC and DEPT-135 spectra showed that the methine protons of H6/H8 and H5/H9 showed correlations to quaternary carbons with chemical shifts of 155.9 ppm and 136.9 ppm respectively. The downfield chemical shift at 155.9 ppm was typical of a quaternary carbon with a hydroxyl group attached and hence was assigned as C7. The other quaternary carbon was then assigned as C4 (136.9 ppm). These correlations were indicated in the substructure Figure 3.5. The correlations from other parts of Tyr were not evident from the COSY and HMBC NMR spectra.

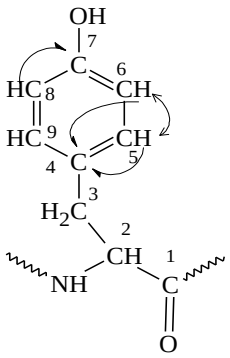


Figure 3.5: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the tyrosine aromatic ring for microviridin 1778A (**65**).

The correlations between the three proton environments of the Phe aromatic ring were evident from the COSY spectrum; a doublet at 6.69 ppm (H5/H9), multiplet at 7.13 ppm (H6/H8) and multiplet at 7.18 ppm (H7) as indicated in the substructure Figure 3.6. The HSQC spectrum showed that these methine protons

corresponded to carbon signals at 118.4 ppm, 126.3 ppm and 128.3 ppm respectively. The HMBC indicated that the quaternary carbon at 155.9 ppm (C4) was adjacent to H5/H9.

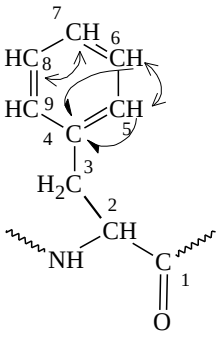


Figure 3.6: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the phenylalanine aromatic ring for microviridin 1778A (**65**).

The proton environments of the Trp ring was also determined to consist of a singlet at 7.08 ppm (H3'), a doublet at 7.33 ppm (H4'), multiplets at 7.25 ppm (H5') and 6.62 ppm (H6') and a doublet at 6.68 ppm (H7') from correlations in the COSY spectrum. The HSQC and DEPT-135 spectra identified the corresponding carbons of the above protons as methine carbons at chemical shifts of 118.6 ppm (C3'), 111.2 ppm (C4'), 126.3 ppm (C5') 115.2 ppm (C6') and 114.9 ppm (C7') respectively.

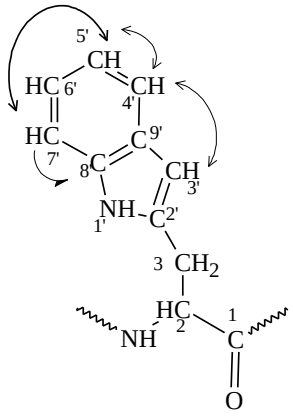


Figure 3.7: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the tryptophan aromatic ring for microviridin 1778A (**65**).

The HMBC spectrum indicated that one of the methine carbons (C7') was adjacent to a quaternary carbon at 156.1 ppm C8'. HMBC correlations to the other C2' and C9' were not clearly indicated in the HMBC spectrum and similarly no further correlation was observed from the protons of the aromatic ring to the methylene

protons at C3 and C2 of Tyr. These observed correlations supported the substructure (Figure 3.7).

Several other amino acids were able to be assigned from the COSY and HMBC spectra but full confirmation of the identities of each was not possible. The presence of Lys was deduced by interpretation of correlations of four consecutive methylene protons and one methine proton from the COSY spectrum. The methine signal H2 (4.7 ppm) is coupled to a multiplet at 1.95 ppm (H3). This multiplet was further split by other multiplets at 2.21 ppm (H4) and 1.44 ppm (H5) and H5 was also coupled to a triplet at 1.29 ppm (H6). The HMBC spectrum indicated that H3 correlated to 175.7 ppm (C1) but was not clear enough to further confirm the connection suggested by the COSY spectrum to the rest of Lys residue. The HSQC spectrum facilitated the assignment of the corresponding methine and methylene carbons as 55.7 ppm (C2), 21.1 ppm (C3), 31.7 ppm (C4), 28.1 ppm (C5) and 22.3 ppm (C6). The correlations supported the substructure depicted in Figure 3.8.

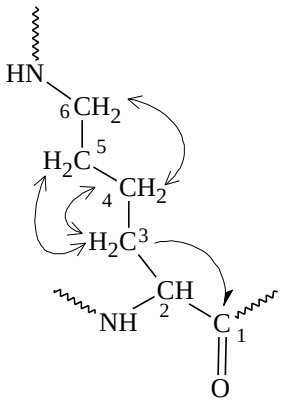


Figure 3.8: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the lysine residue for microviridin 1778A (**65**).

Similarly, the correlations within the Asp residue were deduced from the COSY spectrum which indicated that the 4.46 ppm signal (H2) was correlated to the 3.06 ppm (H3a) and 2.84 ppm (H3b) signals. The carbons to which each proton was attached were determined from the HSQC spectrum as 55.7 ppm (C2) and 38.4 ppm (C3) respectively. The HMBC spectrum indicated that H2 was correlated to a quaternary carbon signal at 170.0 ppm (C1) while H3 correlated to another quaternary signal at 172.4 ppm (which could be Asp C4). These correlation indicated the substructure Figure 3.9.

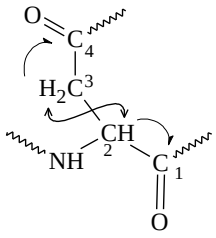


Figure 3.9: The COSY (two headed arrow) and HMBC (one headed arrow) correlations within the aspartic acid residue for microviridin 1778A (**65**).

3.3 **Characterisation of microviridin 1778B (66).**

Based on the similarity in terms of LC-MS/MS and amino acid analyses of the microviridin congener to that of 1778A (**65**), a planar structure was proposed for 1778B (**66**). The proposed structure for 1778B (**66**) was assumed to be very similar to that of 1778A (**65**) (Figure 3.10) but could differ in some stereochemical aspect which could cause them to elute at different times. Unfortunately the experimental results were not sufficiently clear to propose anything further.

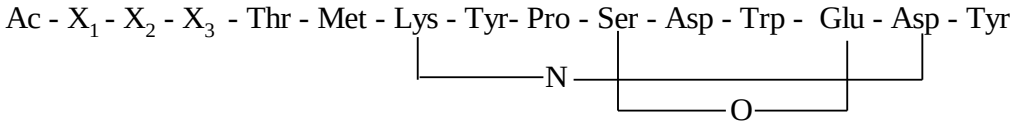


Figure 3.10: The proposed planar structure for microviridin 1778B (**66**). The amino acids X_1, X_2 and X_3 are Phe (×2) and Gly with the sequence yet to be determined.

3.3.1 **Characterisation of microviridin 1778B (66) by tandem mass spectrometry.**

The doubly charged ion at *m/z* 888.4 (microviridin 1778B (**66**)) was present in *Microcystis* CAWBG11. This compound had a MS/MS fragmentation pattern

(Figure 3.11) identical to that of microviridin 1778A (**65**) but eluted from a reversed-phase C_{18} HPLC column at a different retention time (Table 3.1).

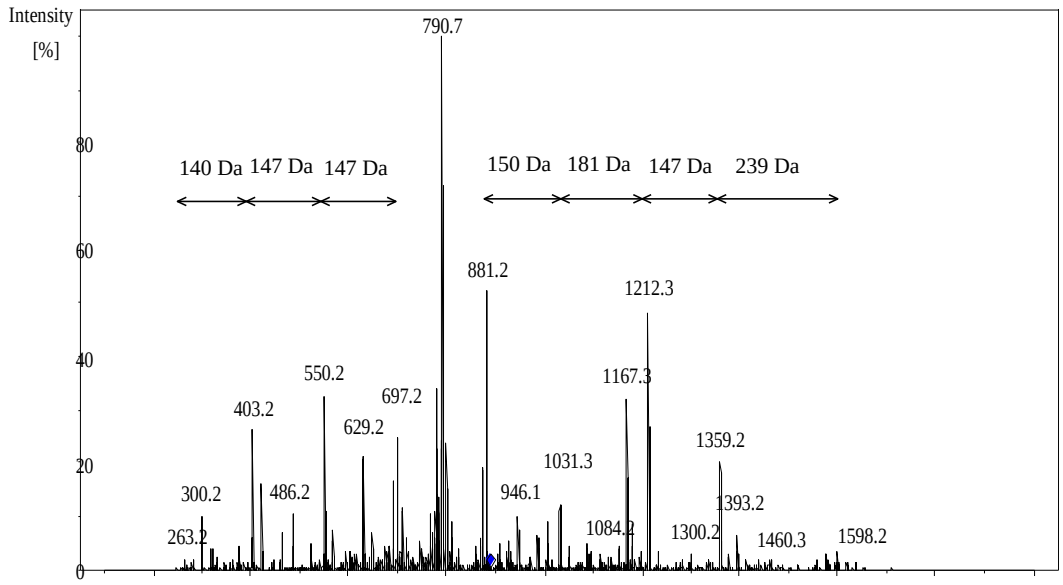


Figure 3.11: The LC-MS/MS spectrum of doubly charged ion *m/z* 888.4 (microviridin 1778B (**66**).

3.3.2 **Advanced Marfey's Method of amino acid analysis for microviridin 1778B (66).**

The Advanced Marfey's amino acid analysis of 1778B (**66**) indicated the presence of L-Asp (*m/z* 426; 14.6 min), D-Glu (*m/z* 440 ; 16.5min), L-Lys (*m/z* 733: 31.1 min), L-Phe (*m/z* 458; 23.9 min),L-Ser (*m/z* 398; 14.2 min), L-Pro (*m/z* 408; 17.8 min), L-Thr (*m/z* 412; 14.1 min) and L-Gly (*m/z* 368; 17.5 min) (Appendix C. 9).

3.4 **Characterisation of microviridin 1760 (67).**

The fragment ions from the MS/MS spectrum of microviridin 1760 (**67**) were very similar to those of 1778A (**65**) and 1778B (**65**). This was an indication of a similar planar structure. However the molecular mass of microviridin 1760 (**67**) was 18 Da less than that of the 1778A (**65**) and 1778B (**65**). An ester bond was established due to loss of 18 Da as indicated by the differences in molecular masses of 1778A (**65**) and 1760 (**67**) between the Thr and Asp as shown in

Figure 3.12. The amino acid analysis indicated the same amino acids present as per 1778A (**65**) and 1778B (**65**), hence a planar structure of microviridin 1760 was established (Figure 3.12).

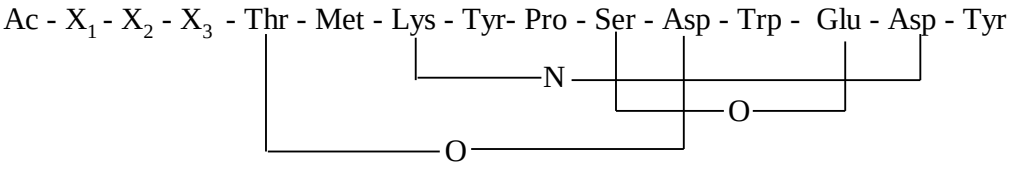


Figure 3.12: The proposed planar structure for microviridin 1760 (**67**). The amino acids X_1, X_2 and X_3 are Phe (×2) and Gly with the sequence yet to be determined.

3.4.1 **Characterisation of microviridin 1760 (67) by tandem mass spectrometry**

The MS/MS spectrum of microviridin 1760 (**67**) (Figure 3.13) showed common fragmentation ions and fragment losses to that of microviridins 1778A (**65**) and 1778B (**65**).

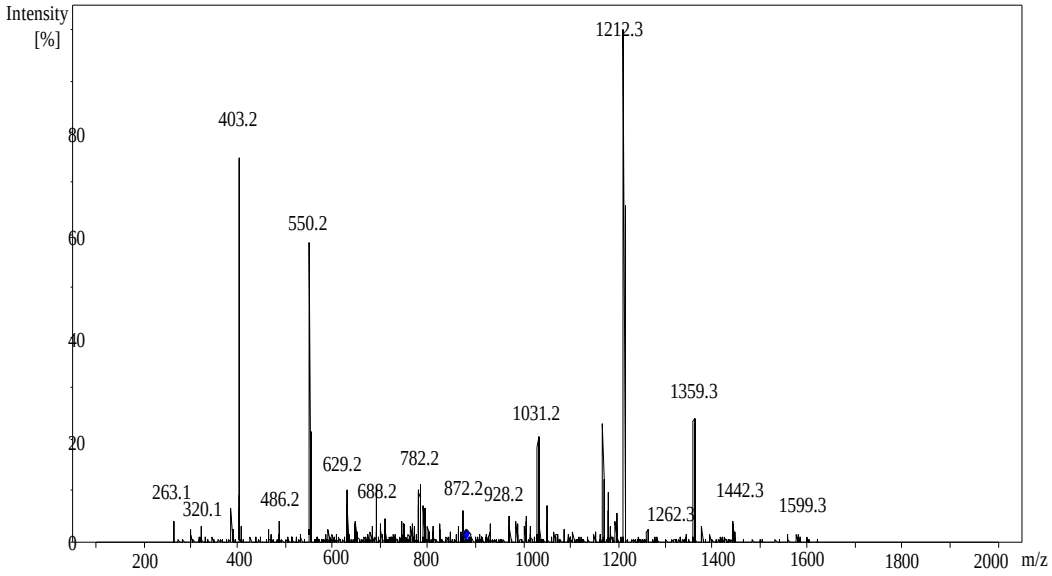


Figure 3.13: The LC-MS/MS spectra of doubly charged ion *m/z* 879.3 (microviridin 1760 (**67**)).

The major fragment ion of 1760 (**67**) was the fragment ion *m/z* 1212 which was also present in 1778A (**65**) and 1778B (**66**) and the molecular mass of microviridin 1760 (**67**) was 18 Da (H_2O) less than that of microviridin 1778A (**65**) which indicated that it has one less ester bond at the position four Thr and position ten Asp. As observed in the known microviridins (Appendix A.2), the most common loss of 18 Da to form an ester linkage occurred at the position four Thr and the position ten Asp. The Glu at position twelve was observed to have been methylated during methanolysis and would result in loss of 32 Da (MeO + H) when formed an ester linkage to Ser at position nine.

3.4.2 **Advanced Marfey's Method of amino acid analysis for microviridin 1760 (67).**

The amino acid analysis of 1760 (**67**) indicated the presence of the same amino acids as in microviridin 1778A (**65**) and 1778B (**66**): L-Asp (*m/z* 426; 14.6 min), L-Glu (*m/z* 440 ; 15.6 min), L-Lys (*m/z* 733; 31.1 min), L-Phe (*m/z* 458; 23.9 min), L-Ser (*m/z* 398; 14.2 min), L-Pro (*m/z* 408; 17.8min), Thr (*m/z* 412; 14.1 min) and L-Gly (*m/z* 368; 17.5min) (Appendix C.10).

3.5 **Characterisation of microviridin 1764 (68).**

A planar proposed structure of microviridn 1764 (**68**) given in Figure 3.14 was based on the MS/MS data and amino acid analysis. The MS fragment ions indicated the presence of Lys, Phe, Trp (×2) and Thr. In addition the amino acid analysis indicated that the first three amino acids (X_1-X_3) were Phe, Ala and Gly but the sequence is yet to be determined. The amino acid at position five was a Trp, indicated by the LC-MS/MS and due to overall molecular mass; the acid residue at position thirteen was Glu. It was assumed that the two ester linkages were formed as there was not loss of water indicated in the LC-MS/MS spectra.

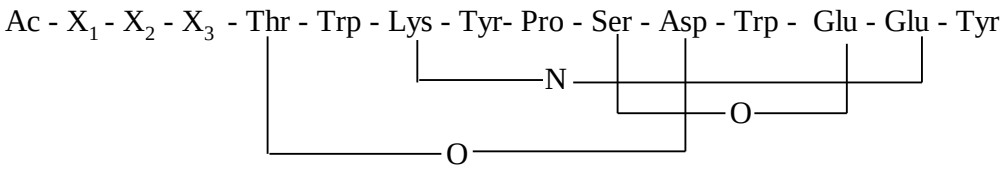


Figure 3.14: The proposed planar structure for microviridin 1764 (**68**).

3.5.1 **Characterisation of microviridin 1764 (68) by tandem mass spectrometry.**

The LC-MS/MS spectrum of 1764 (**68**) showed different fragmentation ions from the three earlier compounds as seen in Figure 3.15. However, a similar loss of 147 Da from the higher mass region and only one 147 Da loss in the lower mass region indicated the presence of Phe and Lys in the compound. The presence of 2 × Trp was indicated by the loss of 162 Da and the loss of 101 Da could indicate the presence of Thr. There was no indication of the presence of Met as seen in the earlier microviridins.

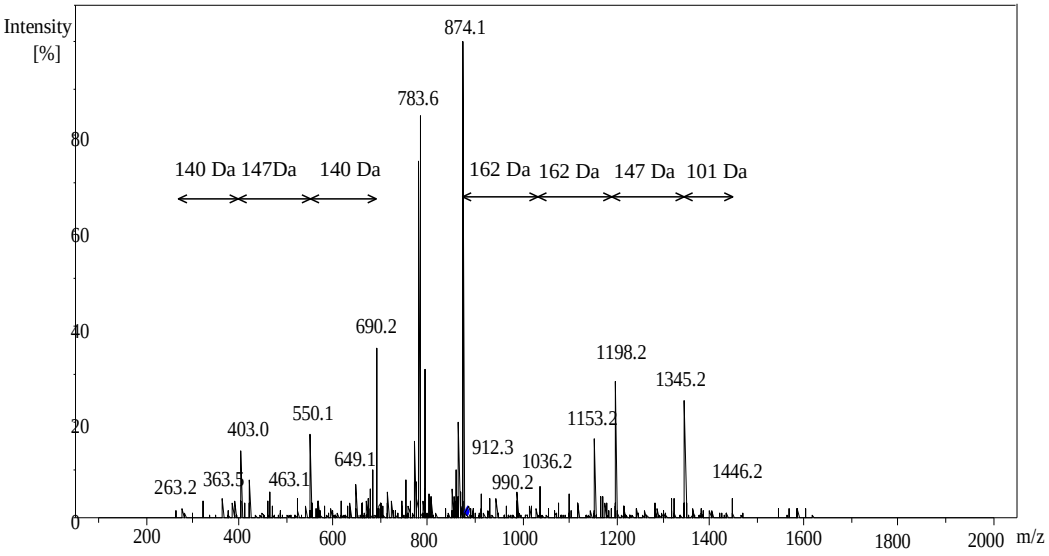


Figure 3.15: The LC-MS/MS spectrum of doubly charged ion *m/z* 881.3 (microviridin 1764 (**68**)).

3.5.2 **Advanced Marfey's Method of amino acid analysis for microviridin 1764 (68).**

The amino acid analysis of 1764 (**68)** indicated the presence of the majority of the amino acids present in the first three microviridins but with the additional of Ala: L-Ala (*m/z* 382; 18.0 min), L-Asp (*m/z* 426; 14.7 min), L-Glu (*m/z* 440; 15.2 min), L-Lys (*m/z* 733; 31.0 min), L-Phe (*m/z* 458; 24.0 min), L-Ser (*m/z* 398; 14.0 min), L-Pro (*m/z* 408; 17.4min), L-Thr (*m/z* 412; 13.9 min) and L-Gly (*m/z* 368; 17.5min) (Appendix C. 11).

3.6 **Future Work**

The new microviridin variants detected were putatively characterised according to the partial information gathered. In order to fully characterise these metabolites, the isolation and characterisation of these variants needs to be completed and may require isolation and purification of further material to provide enough sample.

To collect more structural information by NMR spectroscopy, a higher resolution NMR spectrometer was required to resolve the midfield methine and methylene signals. Alternatively, more information could be gathered if more time was invested in experiments which allow signal assignment using the present instrument including resolved HQSC and HMBC and selective TOSCY experiments. The experiments should be repeated with a non-exchangable solvent such as DMSO-d_6 or acetone-d_6, in order to gather information on connectivity of the amide protons. All of the amide protons were exchanged in CD_3OD in the current work, making it difficult to deduce the amide protons in the compound.

The stereochemistry of Trp needs to be determined which requires hydrolysis under different conditions in order to avoid the degradation of Trp. Similarly, the stereochemistry of Met could also be determined if a standard Met acid was available.

Chapter 4: Experimental

4.1 Commonly Used Solvents and Solutions

- MilliQ with sensitivity > 18MΩ.cm was purified on an E-Pure still (Barnstead).
- Methanol for LH-20 / bench columns only (not HPLC) was drum-grade and distilled before use.

Table 4.1: Solvents and their sources that were commonly used in this research.

Solvent	Source
Formic Acid (FA)	Ajax Chemicals
HPLC- grade acetonitrile (ACN)	Honeywell International
HPLC-grade Methanol (MeOH)	Ajax Chemicals
Methanol-d_4 (CD_3OD)	Sigma-Aldrich
Trifluoroacetic acid (TFA)	Acros Organics

Table 4.2: List of common solutions used in this research.

Solutions	Composition
10% Formic Acid	11.4 mL of 88% formic acid (10%; v/v) - Make up to 100 mL with MilliQ water and filter (0.45 μm)
LC-MS Solvent A	2% ACN + 0.1% FA - 5 mL of 10% FA (0.1%; v/v) - 10 mL of HPLC-grade ACN (2%; v/v) - Make up to 500 mL with MilliQ water
LC-MS Solvent B	98% ACN + 0.1% FA - 5 mL of 10% FA (0.1% ; v/v) - 5 mL of deionised water (2% ; v/v) - Make up to 500 mL with HPLC-grade ACN.
LC-MS Solvent C (Column wash)	60% HPLC-grade acetonitrile in MilliQ water
LC-MS Solvent D (Needle wash)	50% HPLC-grade methanol in MilliQ water
MALDI Matrix Solution	2:1:1 MeOH/ ACN / 0.1%TFA - 0.250 mL of MeOH (50%; v/v) - 0.125 mL of acetonitrile (25%; v/v) - 0.125 mL of 0.1% (v/v) TFA (0.025%; v/v) - Solution was used for one week then discarded and fresh solution prepared
MALDI Wash Solution	0.100 mL of 1% (v/v) TFA (0.1%; v/v) 0.900 mL of 11.1 mM $NH_4H_2PO_4$ (10 mM) - Stored at 4 °C and discarded after one month
Isocratic HPLC Solvent	60% MeOH + 0.01% TFA in deionised water -600 mL HPLC-grade MeOH - 100 μL TFA -Make up to 1L with MilliQ water
Gradient HPLC	
Solvent A	H_2O + 0.05% TFA
Solvent B	ACN + 0.05% TFA

4.2 Commonly Used Experimental Techniques

Centrifugation was carried out using an Eppendorf MiniSpin Plus centrifuge. Samples and solvents were sonicated at ambient temperature in an ultrasonic bath (Aqua, Barnstead International). Lyophilisation of samples was conducted using a FreeZone6 freeze-drier (Labconco).

4.3 Commonly Used Fractionation Techniques.

All the fractions were collected in conical flasks and concentrated under vacuum using a rotary evaporator (Büchi) at 35ºC. Fractions were then resuspended in MeOH, transferred into a glass scintillation vial (21 mL) and dried down under a stream of nitrogen at 35ºC.

4.3.1 Size Exclusion Chromatography

Size exclusion column chromatography was conducted using LH-20 material (Sephadex LH-20, Pharmacia Fine Chemicals). The samples were dissolved in minimal MeOH, and then loaded onto the column head and eluted with 100% MeOH.

4.3.2 Isocratic High Performance Liquid Chromatography.

Isocratic HPLC was performed using HPLC pumps (Waters 515) arranged to deliver different volumes of solvent. The eluting compounds were detected by a Photodiode Array Detector (PDA; Waters 996, 200-400 nm). Repeated injections of sample (0.120 mL) were fractionated on a C_{18} column (Econosil C_{18} 10-µ, 250 × 10 mm; Alltech) using a mixture of 60% ACN and 60% MeOH + 0.01%TFA at a flow rate of 5 mL/min.

4.3.3 Gradient High Performance Liquid Chromatography

Gradient HPLC was performed using two HPLC pumps (Waters 515) controlled by Empower Pro (Waters). Eluting compounds were detected by a PDA detector; (200-400 nm; Waters 2996). Samples were dissolved in HPLC-grade MeOH (1.2 mL) and syringe filtered (0.2 µm). Repeated injections of samples (100 µL) were

fractionated on a C_{18} HPLC column (Econosil C_{18} 10-µm, 250 × 10 mm; Alltech using the H_2O→ACN + 0.05% TFA gradient at a flow rate of 5 mL/min.

4.4 Characterisation Techniques Used.

4.4.1 Liquid Chromatography Mass Spectrometry

Samples were separated on an HPLC system (UltiMate 3000, Dionex) which was directly coupled to an electrospray ionisation (ESI)-ion trap mass spectrometer (AmaZon X; Bruker Daltonics). The dual system was contolled by Hystar software (Bruker Daltonics). Samples (20 µL) were separated on a C_{18} column (Ascentis Express C_{18}, 100 × 2.1 mm , 2.7-µm; Supleco Analytical) using the gradient outlined in Table 4.3 at a flow rate of 0.2 mL/min with a column temperature of 40 ºC.

Table 4.3: HPLC gradient for the LC-MS analysis.

Time (min)	%A (98:2 H_2O:ACN) + 0.1% FA	%B (2:98 H_2O:ACN) + 0.1% FA
0	90	10
1	90	10
13	0	100
15	0	100
16	90	10
20	90	10

Tandem mass spectra were gathered using the protonated or doubly protonated ions of the desired compound. The *m/z* of the resulting fragment ions were recorded. Chromatograms and spectra were reviewed in DataAnalysis (Bruker Daltonics) where each chromatogram and spectrum was smoothed and peaks automatically selected using the Apex algorithm.

4.4.2 Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry

Samples were analysed using an AutoFlex II MALDI-TOF mass spectrometer (Bruker Daltonics) operated with FlexControl (Bruker Daltonics). Samples were

prepared for MALDI-TOF analysis by resuspending in MeOH and mixing of the sample (1 µL) with matrix solution (1 µL) on a MALDI target (600 µm anchorchip). Mass calibration was performed using a peptide calibration standard which was prepared in the same manner as the samples.

4.4.3 **Nuclear Magnetic Resonance Spectroscopy**

Nuclear magnetic resonance spectra were recorded on a Bruker AVIII-400 NMR spectrometer. Chemical shifts were determined at 300 ºK and are reported relative to the solvent signal (CD_3OD; ^{1}H 3.31 ppm, ^{13}C 49.15 ppm). Standard pulse sequences were used for HSQC, HMBC, COSY, TOCSY and ROESY experiments. TOCSY experiments were conducted with mixing times optimised for the detection of short, medium and long range couplings. HMBC spectra were acquired using parameter sets optimised for $^{2\text{-}3}J$ ^{1}H-^{13}C correlations.

4.4.4 **Amino acid analysis.**

Semi-purified and purified aeruginosin and microviridin samples were subjected to amino acid analysis by the Advanced Marfey's method.[16, 94] The L- and D-FDLA used was synthesised by Dr. Jonathan Puddick during the course of his research.[15]

The sample was resuspended in MeOH and 100 µL was removed to a Reacti vial or boil-proof centrifuge tube and dried under a stream of nitrogen at 35 ºC. The dried sample was resuspended in 6M HCl (0.5 mL), sealed and incubated at 110 ºC for 16 h. The HCl was removed at 35 ºC under a stream of nitrogen before the hydrolysate was resuspended in water (110 µL) and aliquoted into two 1.5 mL centrifuge tubes (50 µL each) to which 1M $NaHCO_3$ (20 µL) and L- or D-FDLA (100 µL at 1% in MeOH) was added. The tube was then vortexed and incubated for 1 h at 40 ºC. The reaction was quenched using 1M HCl (20 µL), diluted with MeOH (810 µL), centrifuged (14,500 rpm, 5 min) and 980 µL was transferred into a septum-capped LC-vial.

The resulting derivative was analysed by LC-MS. The derivatised hydrolysate (20 µL) was separated on a C18 column (Econosil C18, 250 × 3.2 mm, 5-µm; Alltech) using the $H_2O \rightarrow$ACN gradient outlined in Table 4. 4 (600 µL/min; 40 °C).

Eluting derivatives were detected by ultraviolet absorption (250-500 nm) and ESI MS (*m/z* 300-500). Negative ions were assessed using a capillary voltage of 3.5 kV and a nebuliser pressure of 1.5 bar.

Table 4. 4: HPLC gradient for the Advanced Marfey's method of amino acid analysis.

Time (min)	**%A (98:2 H_2O/ACN + 0.1% Formic Acid)**	**%B (2:98 H_2O/ACN + 0.1% Formic Acid)**
0	75	25
1	75	25
31	35	65
33	0	100
35	0	100
38	75	25
45	75	25

4.4.5 **Assessment of the Fraction Composition**

Sample fractions were assessed using LC-MS. Sample was resuspended in HPLC-grade MeOH (500 µL) and an aliquot (5 µL) was syringe filtered (0.2 µm) into a septum-capped LC vial. The filter and syringe were then washed with 50% HPLC-grade MeOH (995 µL; v/v). Negative ions were assessed over a 100-2,000 *m/z* range and the resulting ion-chromatograms were integrated using QuantAnalysis (Bruker Daltonics).

4.5 **Work Described in Chapter 2**

4.5.1 Isolation of aeruginosin compounds.

Samples were isolated via a series of chromatography columns as in Appendix C.1. The sample was dissolved in MeOH (0.6 mL) and loaded straight onto an LH-20 column. The vials were rinsed with distilled water (0.3 mL) and the rinsings were loaded onto the column head. Fractions were eluted with 100% MeOH, concentrated down by rotary evaporation and dried under a stream of nitrogen gas at 35 ºC. They were assessed by LC-MS using the protocol described in Section 4.4.1.

4.5.2 Solid Phase extraction of aeruginosin 656A (**63**).

Solid-phase clean-up was performed using a glass Pasteur pipette lightly plugged with cotton wool and filled with C_{18} material (500 mg; YMC-gel ODS-A; YMC). The solid-phase clean up column was primed with 100% MeOH + 0.01%TFA then equilibrated with 20% MeOH + 0.01% TFA (5 mL). Sample was resuspended in 20% MeOH (0.3 mL 20% MeOH + 0.01% TFA). The sample was loaded onto the solid-phase column and allowed to flow through. It was washed with 20%, 40%, 60% × 2 and 100% MeOH + 0.01% TFA (5 mL each) and each fraction was collected (fractions 1-5) in scintillation vials. Samples were rotary evaporated and dried under a stream of nitrogen gas at 35 ºC and were assessed by LC-MS using the protocol described in Section 4.4.1.

4.5.3 Isocratic HPLC of aeruginosins.

Sample (11 mg) was suspended in HPLC-grade MeOH (250 µL) and was diluted with MeOH + 0.01% TFA (250 µL). It was then filtered into another scintillation vial through a syringe filter (0.2 µm). The original flask was rinsed with MeOH + 0.01% TFA (4 × 125 µL) and the rinsings were filtered into the solution making up a total of 1 mL. The solution was then subjected to isocratic HPLC fractionation using the protocol described in Section 4.3.2 with a 2 mL injection loop. Repeated isocratic HPLC was carried out with the same protocol but with 55% MeOH + 0.01% TFA as solvent. The fractions were assessed with LC-MS using the protocol described in Section 4.4.1.

4.5.4 NMR characterisation of Aeruginosin 602A (**60**).

Sample was vacuum dried for at least two hours to minimise the presence of water in the compound. It was then dissolved in CD_3OD, transferred into the NMR tube and several experiments were conducted using a Broadband Inverse Probe and the protocol described in Section 4.4.3.

4.5.5 Amino acid analysis of aeruginosin-like compounds.

The chemical analysis of each aeruginosin compound was determined using the protocol described in Section 4.4.4.

4.6 Work Described in Chapter 3

4.6.1 Isolation of microviridin-like compounds by LC-MS.

Sample was isolated via a series of chromatography columns as in Appendix B.1. The sample was dissolved in methanol (0.6 mL) and a few drops of deionised water and loaded onto the head of an LH-20 column. The flask was rinsed with distilled water (0.3 mL) and the rinsings loaded onto the column. The column was eluted with 100% MeOH. The collected fractions were concentrated by rotary evaporation and dried under a stream of nitrogen gas at 35 ºC and assessed with LC-MS using the protocol described in Section 4.4.1.

4.6.2 Gradient HPLC

Sample was resuspended in HPLC-grade MeOH (250 µL) and diluted with water + 0.05% TFA (250 µL) then filtered into a scintillation vial through a syringe filter (0.2 µm). The vial was rinsed with water + 0.05% TFA (4 × 150 µL) and the rinsings filtered. The solution was subjected to gradient HPLC with a 200 µL injection loop using the protocol described in Section 4.3.3 and the gradient profile in Table 4.5.

Table 4.5: HPLC gradient for fractionation of microviridin compounds.

Time (min)	**%A H_2O + 0.05% TFA**	**%B ACN + 0.05% TFA**
0	90	10
3	90	10
6	69	31
26	69	31
33	0	100
36	0	100
37	90	10
40	90	10

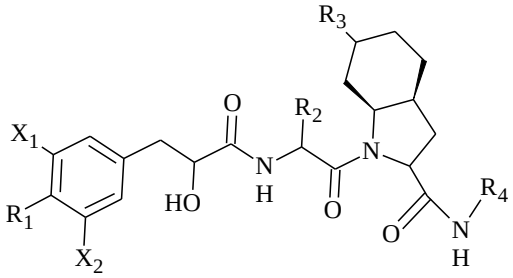
Fractions were analysed by LC-MS using the protocol described in Section 4.4.1.

4.6.3 Amino acid analysis of microviridin-like compounds.

The chemical analysis of each microviridin compound was determined using the protocol described in Section 4.4.4.

Appendices

Appendix A.1: Amino acid sequence and biological activities of the known aeruginosins.

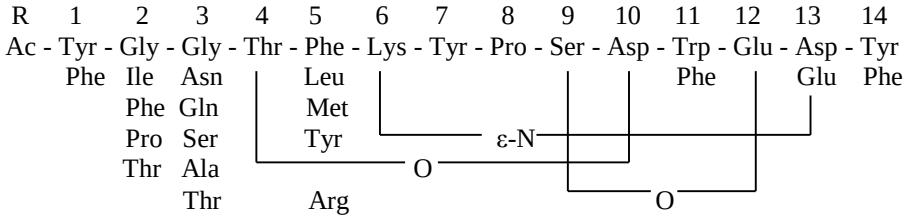


Aeruginosin[a]	M_r[b]	1: Hpla			2: Hydrophobic amino acid	3: Choi	4: Arginine derivative	Biological Activities[g]	Reference
		X_1	**R_1**	**X_2**	**R_2**	**R_3**	**R_4**		
298A (**6**)	604.4	H	OH	H	Leu	OH	Argol	THB, TRP	71
98A (**7**)	688.4	Cl	OH	H	allo-Ile	OSO_3H	Agma	TRP, THB, PLM	82
98B (**8**)	654.4	H	OH	H	allo-Ile	OSO_3H	Agma	TRP, THB, PLM	82
98C (**9**)	732.2	Br	OH	H	allo-Ile	OSO_3H	Agma	TRP, THB, PLM	83
101 (**10**)	722.2	Cl	H	Cl	allo-Ile	OSO_3H	Agma	TRP, THB, PLM	83
298B (**11**)	461.2	H	OH	H	Leu	OH	H_2	TRP, THB, PLM, PAP	83
89A (**12**)	716.2	Cl	OSO_3H	H	Leu	OH	Argal (2*S*)	TRP, THB, PLM, PAP	83
89B (**13**)	716.2	Cl	OSO_3H	H	Leu	OH	Argal(1*S*,2*R*)	TRP, THB, PLM, PAP	83
SF608 (**14**)	608.3	H	OH	H	L-Phe	OH	Agma	TRP	79
EI461 **(15**)	461.2	H	OH	H	Leu	OH	H_2	TRP	117
KY642 (**16**)	643.3	Cl	OH	Cl	Ile	OH	Agma	TRP	85
KY608 (**17**)	643.3	Cl	OH	H	Ile	OH	Agma	TRP	85

GE686 (**18**)	689.2	Cl	OH	Br	allo-Ile	OH	Agma	TRP, THB	72
GE730 (**19**)	733.2	Br	OH	Br	allo-Ile	OH	Agma	TRP, THB	72
GE766 (**20**)	768.2	Cl	OH	Br	allo-Ile	OSO_3H	Agma	TRP, THB	72
GE810 (**21**)	810.1	Br	OH	Br	allo-Ile	OSO_3H	Agma	TRP, THB	72
GE642 (**22**)	643.3	Cl	OH	Cl	Leu	OH	Agma	TRP, THB	72
KT608A (**23**)	608.3	H	OH	H	Phe	OH	Agma	TRP	21
KT608B (**24**)	608.3	H	OH	H	Phe	OH	Agma	TRP	21
KT650 (**25**)	650.3	H	OH	H	Phe	OH	Agma	TRP	21
KT554 (**26**)	554.3	H	OH	H	Leu	H	Agma	TRP	21
GH553 (**27**)	553.2	H	OH	H	Tyr	CO_2CH_3	H_2	TRP	21
IN608 (**28**)	608.3	Cl	OH	H	Leu	OH	Agma	TRP	86
IN652 (**29**)	652.3	Br	OH	H	Leu	OH	Agma	TRP	86
102A (**30**)	732.3	H	OSO_3H	H	Tyr	OH	L-Argal	THB, TRP, PLM	73
102B (**31**)	732.3	H	OSO_3H	H	Tyr	OH	D-Argal	THB, TRP, PLM	73
103A (**32**)	680.4	H	OH	H	Tyr	OH	Aeap	THB	87
205A (**33**)	804.3	H	H	H	D-Xyl-HLeu (2*R*,3*S*)	Cl	Agma	TRP, THB	75
205B (**34**)	804.3	H	H	H	D-Xyl-HLeu (2*S*,3*R*)	Cl	Agma	TRP, THB	75
Oscillarin (**36**)	612.2	H	H	H	Phe	OH	Adc	TRP, THB	90
NAL2 (**37**)	587.2		Butanoic acid		Tyr	OH	Argol		76
NOL6 (**38**)	589.4		Decanoic acid		Tyr	O-pentose	Argol		76
Aer856 (**39**)	865.4	H	OH	H	Leu	ManA, HA	Agma		91
DysA (**40**)	632.1		O-Me-O'-SO_3H[c]		Leu	OH	Adc	THB	78
Cl-DysA[d,e] (**41**)	666.5		O-Me-O'-SO_3H[c]		Cleu	OH	Adc	THB	93
Dys B[d,e,f] (**42**)	780.3		O-Me-O'-SO_3H[c]		Val	$OC_6H_6O_4$	Adc	THB	77
Dys C[d,e] (**43**)	618.2		O-Me-O'-SO_3H[c]		Val	OH	Adc	THB	77
Dys D[d,e] (**44**)	539.3		O-Me-O'-H[c]		Val	OH	Adc	THB	77

[a] Order of aeruginosins according to aeruginosin 298A, [b] molar mass to one decimal place, [c] glyceric acid instead of Hpla, [d]Ccoi instead of Choi, [e]Plas instead of Hpla, [f]Dysinosin, [g]Thrombin, Trypsin, Plasmin, Papain.

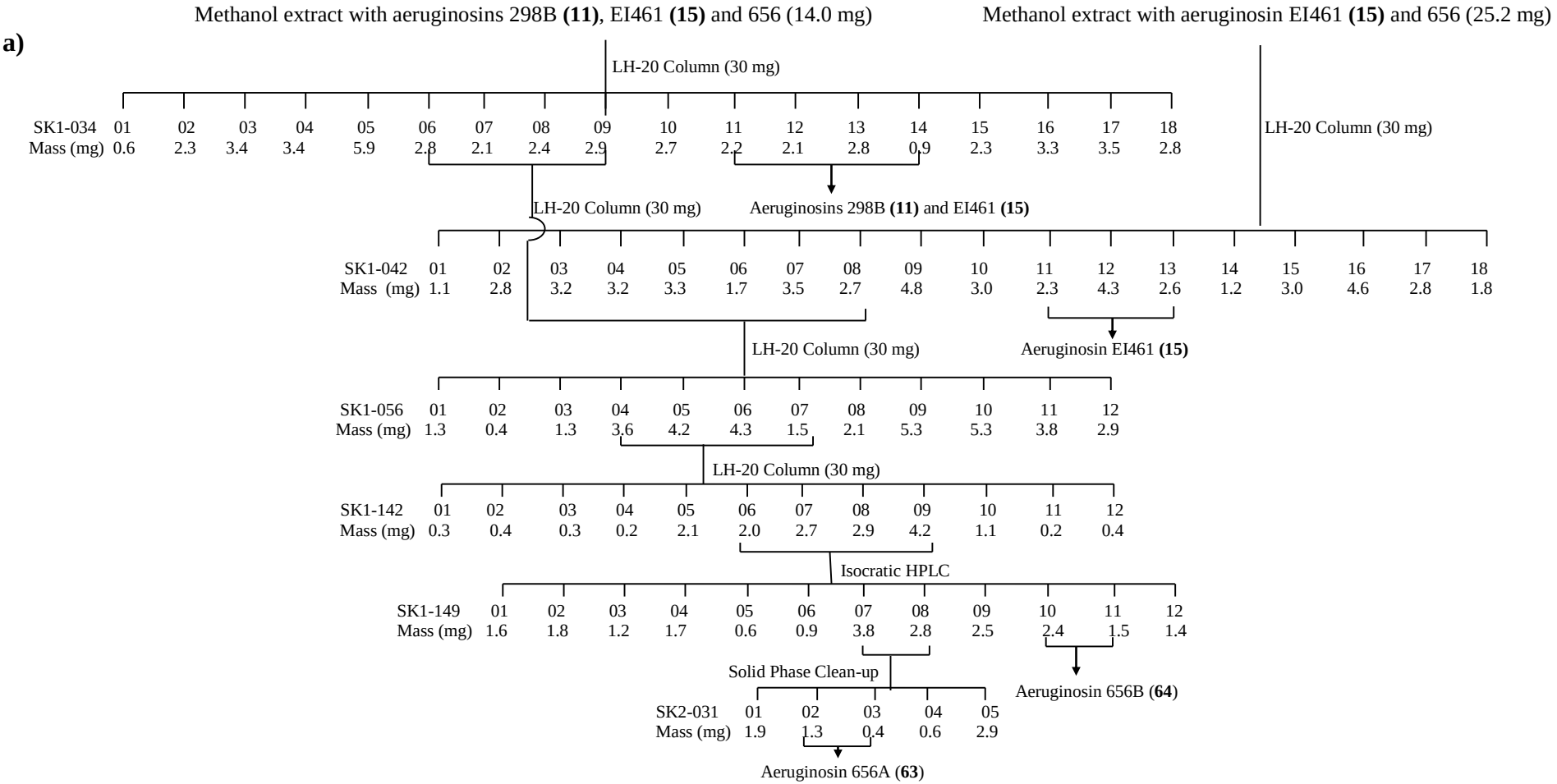
Appendix A.2: Amino acid sequence of the known microviridins.



Microviridin	*Mr*[a]	Sequence of amino acids[b]															References
		R	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
A (**45**)	1728.7	Ac[c]	Try	Gly	Gly	Thr	Phe	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Tyr	104
B (**46**)	1722.7	Ac	Phe	Gly	Thr	Thr	Leu	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Tyr	102
C (**47**)	1754.8	Ac	Phe	Gly	Thr	Thr	Leu	Lys	Tyr	Pro	Ser-OH	Asp	Trp	Glu-OMe	Glu	Tyr	102
J (**48**)	1683.7	Ac		Ile	Ser	Thr	Arg	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Trp	101
SD 1684 (**49**)	1684.7	Ac		Thr	Ala	Thr-OH	Arg	Lys	Tyr	Pro	Ser	Asp-OH	Trp	Glu-OMe	Has	Tyr	103
SD 1634 (**50**)	1634.7	Ac		Thr	Ala	Thr	Arg	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Has	Tyr	103
SD 1652 (**51**)	1652.7	Ac		Thr	Ala	Thr-OH	Arg	Lys	Tyr	Pro	Ser	Asp-OH	Trp	Glu	Has	Tyr	103
L (**52**)	1684.9	Ac	Tyr	Gly	Gly	Thr	Phe	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Tyr	100
D (**53**)	1801.7	Ac	Tyr	Gly	Asn	Thr	Met	Lys	Tyr	Pro	Ser-OH	Asp	Trp	Glu-OMe	Glu	Tyr	105
E (**54**)	1664.7	Ac		Phe	Ser	Thr	Tyr	Lys	Tyr	Pro	Ser-OH	Asp	Phe	Glu-OMe	Asp	Phe	105
F (**55**)	1682.7	Ac		Phe	Ser	Thr-OH	Tyr	Lys	Tyr	Pro	Ser-OH	Asp-OH	Ph	Glu-OMe	Asp	Phe	105
I (**56**)	1764.7	Ac	Tyr	Pro	Thr	Thr	Leu	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Asp	Tyr	107
K (**57**)	1769.7	Ac	Tyr	Gly	Asn	Thr	Met	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Tyr	109
G (**58**)	1805.8	Ac	Tyr	Pro	Gln	Thr	Leu	Lys	Tyr	Pro	Ser	Asp	Trp	Glu	Glu	Tyr	108
H (**59**)	1837.8	Ac	Tyr	Pro	Gln	Thr	Leu	Lys	Tyr	Pro	Ser-OH	Asp	Trp	Glu-OMe	Glu	Tyr	108

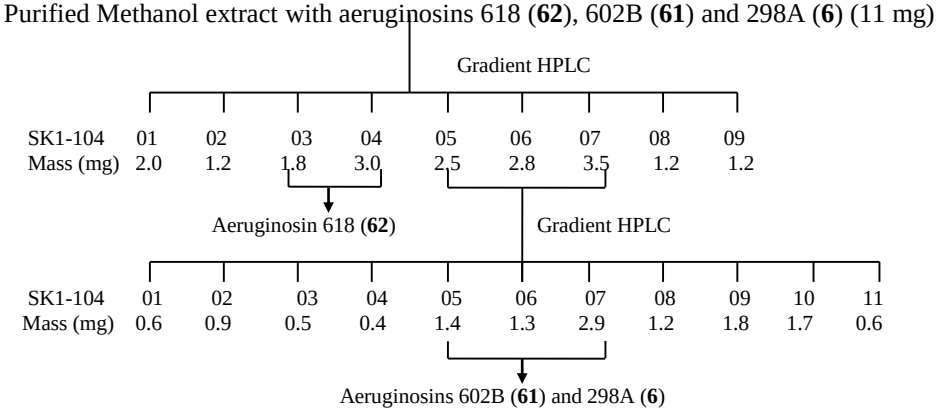
[a] molar mass to one decimal place, [b]sequence of amino acids according to microviridin A, [c]acetic acid

Appendix B.1: Separation tree for fractionation of aeruginosin metabolites from *Microcystis species* CAWBG11.



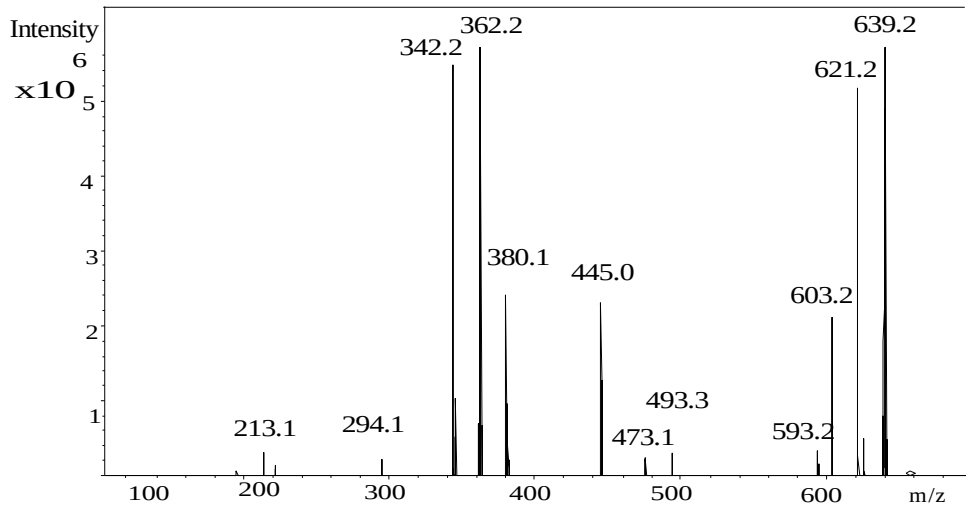
b)

Semi Purified Methanol extract with aeruginosins 618 (**62**), 602B (**61**) and 298A (**6**) (11 mg)

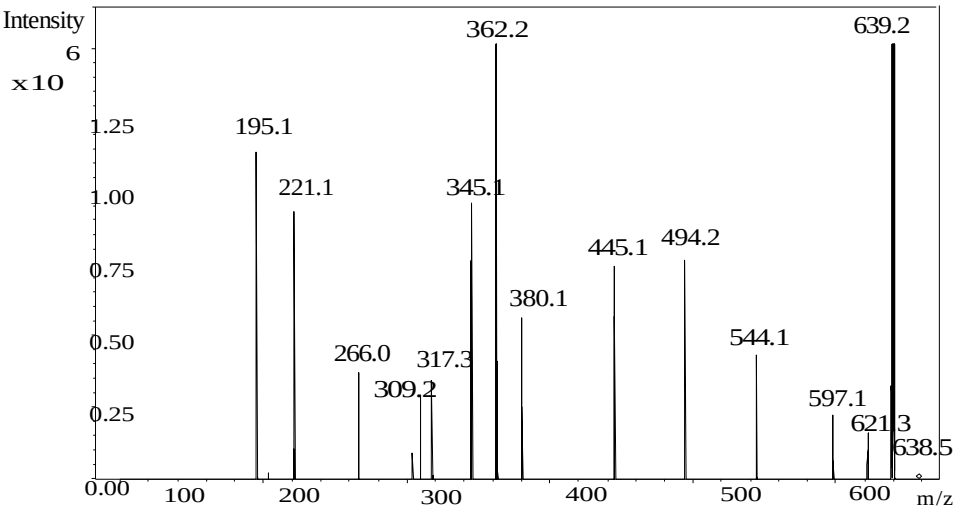


Appendix B.2: The LC-MS/MS spectrum of **a)** aeruginosin 656A (**63**) and **b)** 656B (**64**).

a)

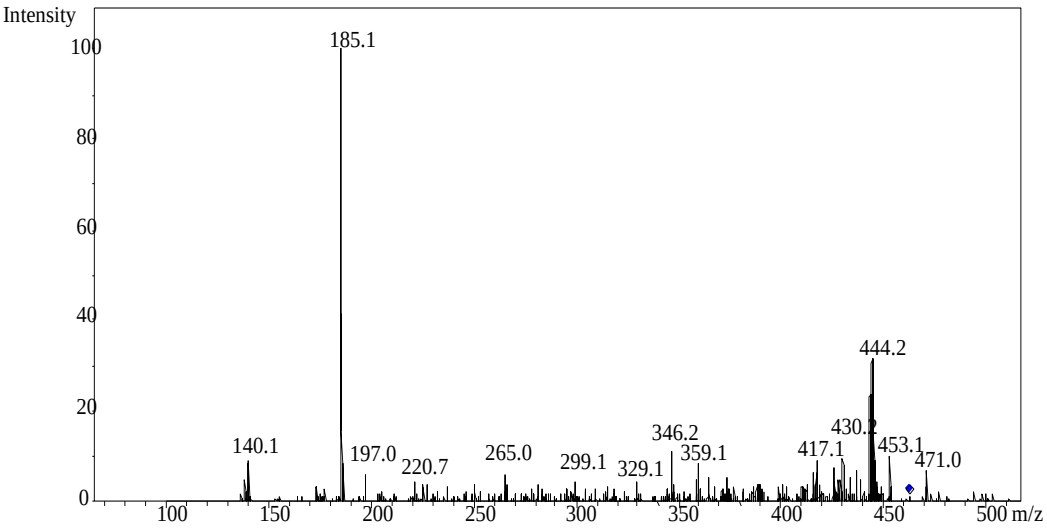


b)

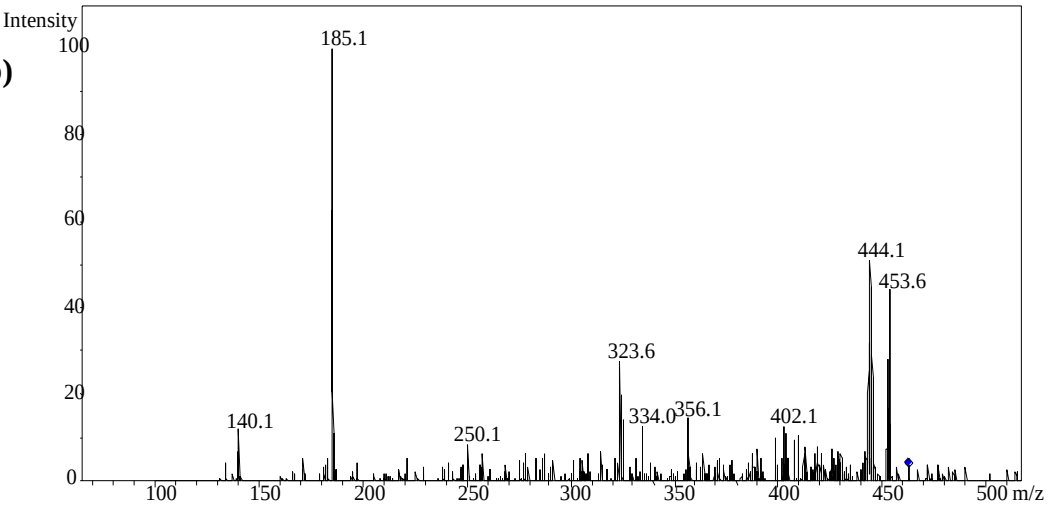


Appendix B.3: Tandem mass spectrum of aeruginosin **a)** 298B (**11**) and **b)** EI461B (**15**). Fragmentation was induced by matrix-assisted laser desorption post-source decay.

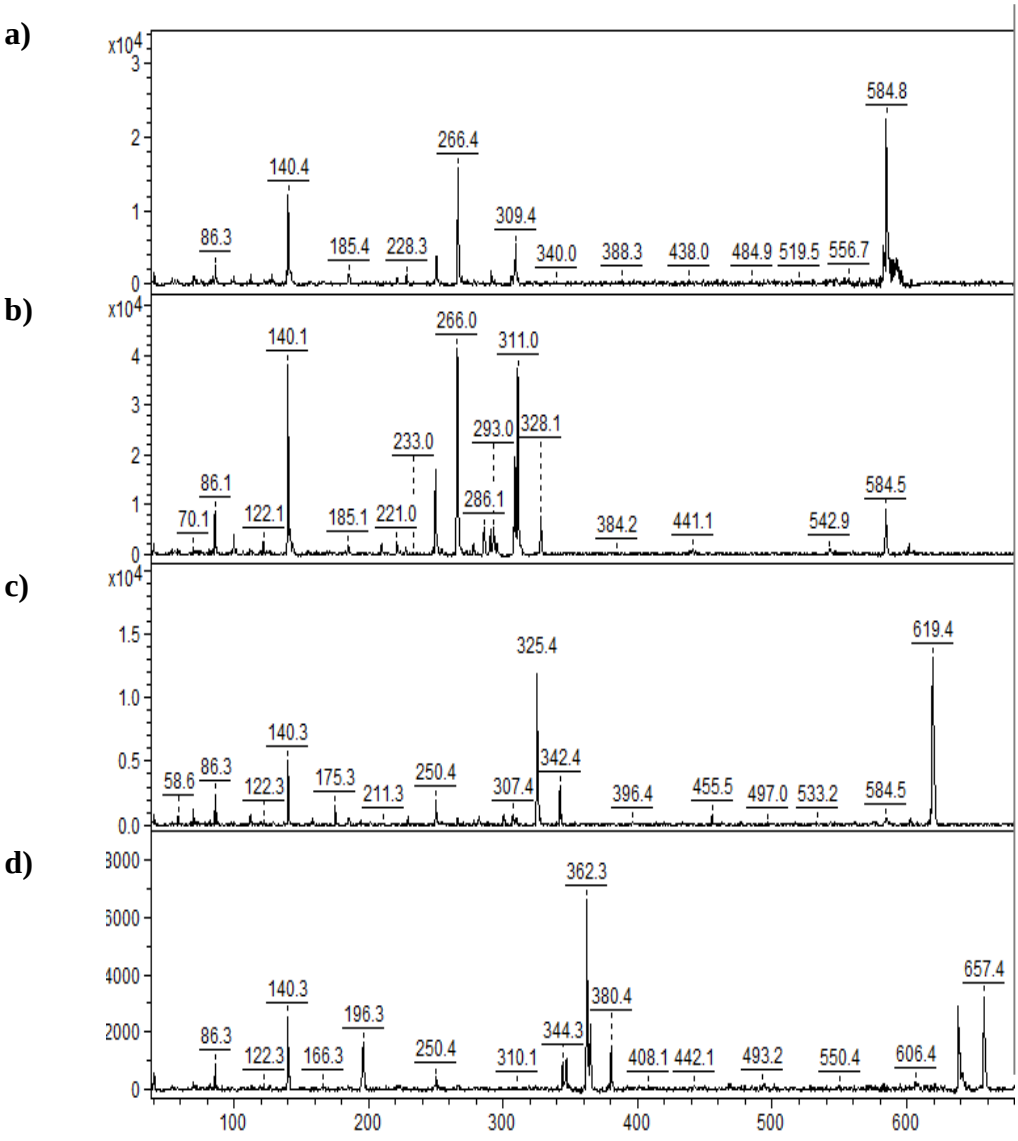
a)



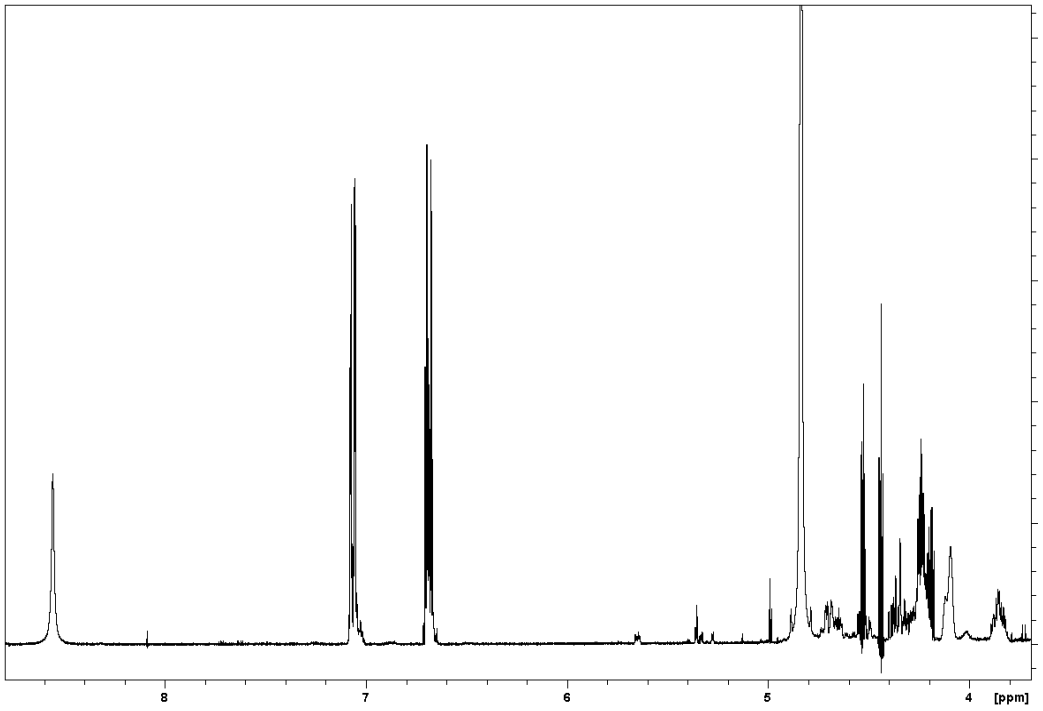
b)



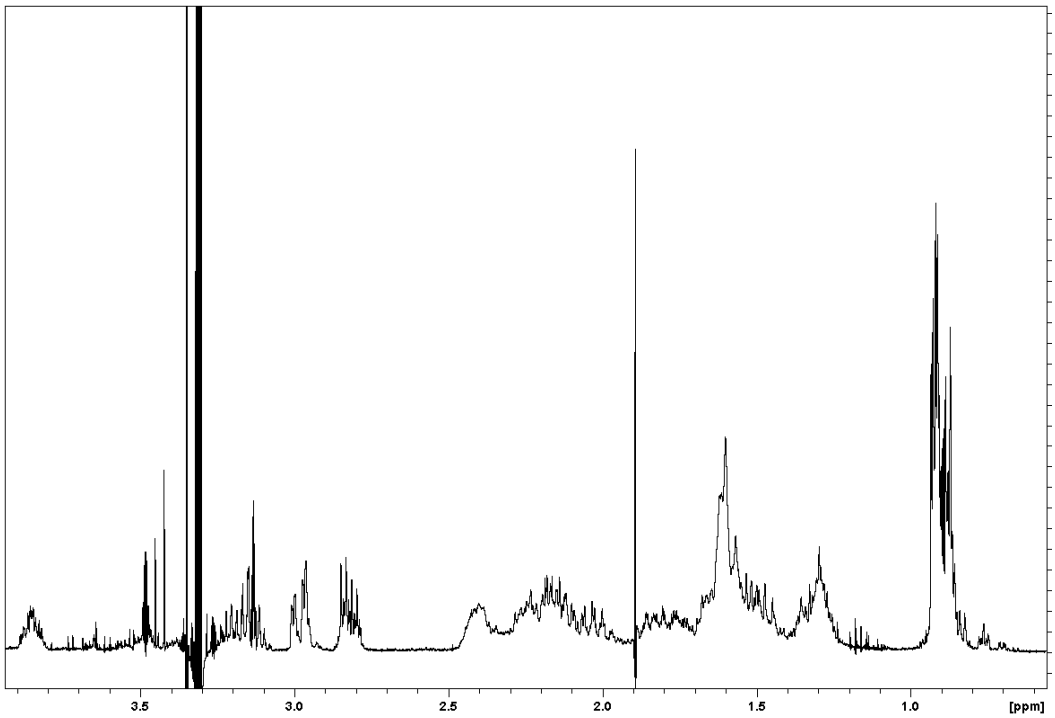
Appendix B.4: Tandem mass spectrum of aeruginosins **a)** 602A (**60**) **b**) 298A (**6**) **c)** 618 (**62**) and **d)** 656A (**63**).



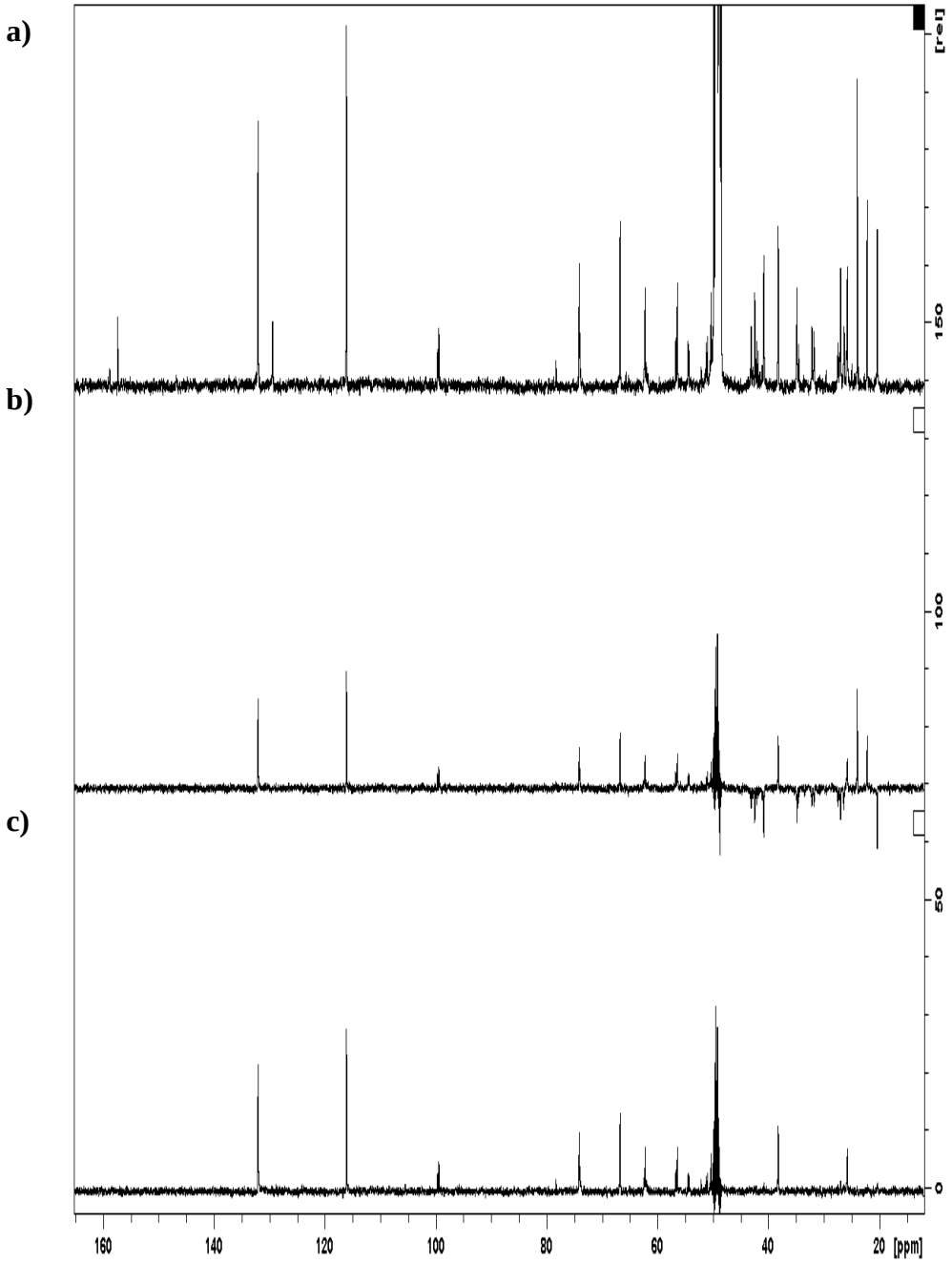
Appendix B.5: Downfield region of the ^{1}H NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



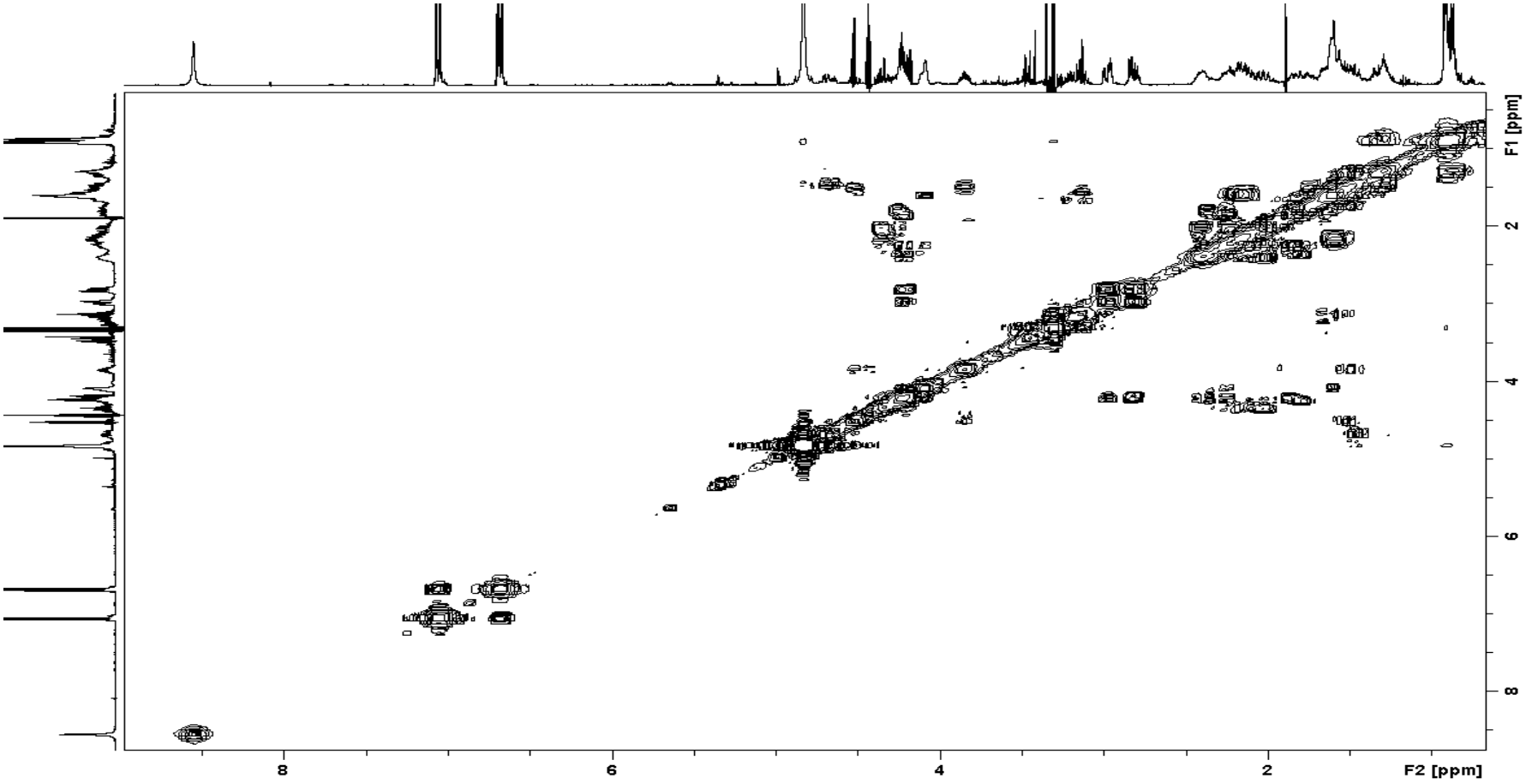
Appendix B.6: Upfield region of the ^{1}H NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



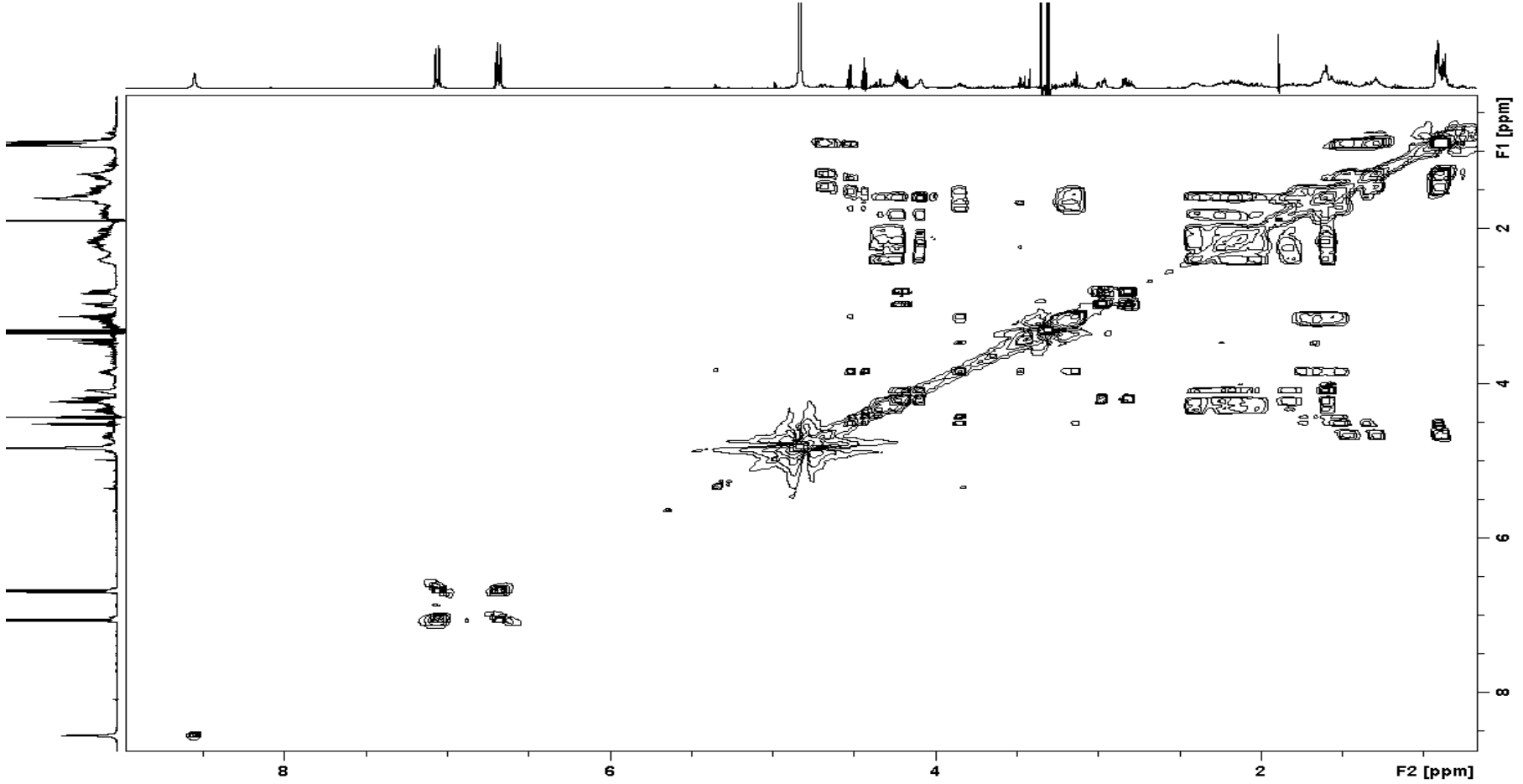
Appendix B.7: Spectra for **a)** ^{13}C NMR, **b)** DEPT-135 and **c)** DEPT-90 of aeruginosin 602A (**60**) in CD_3OD.



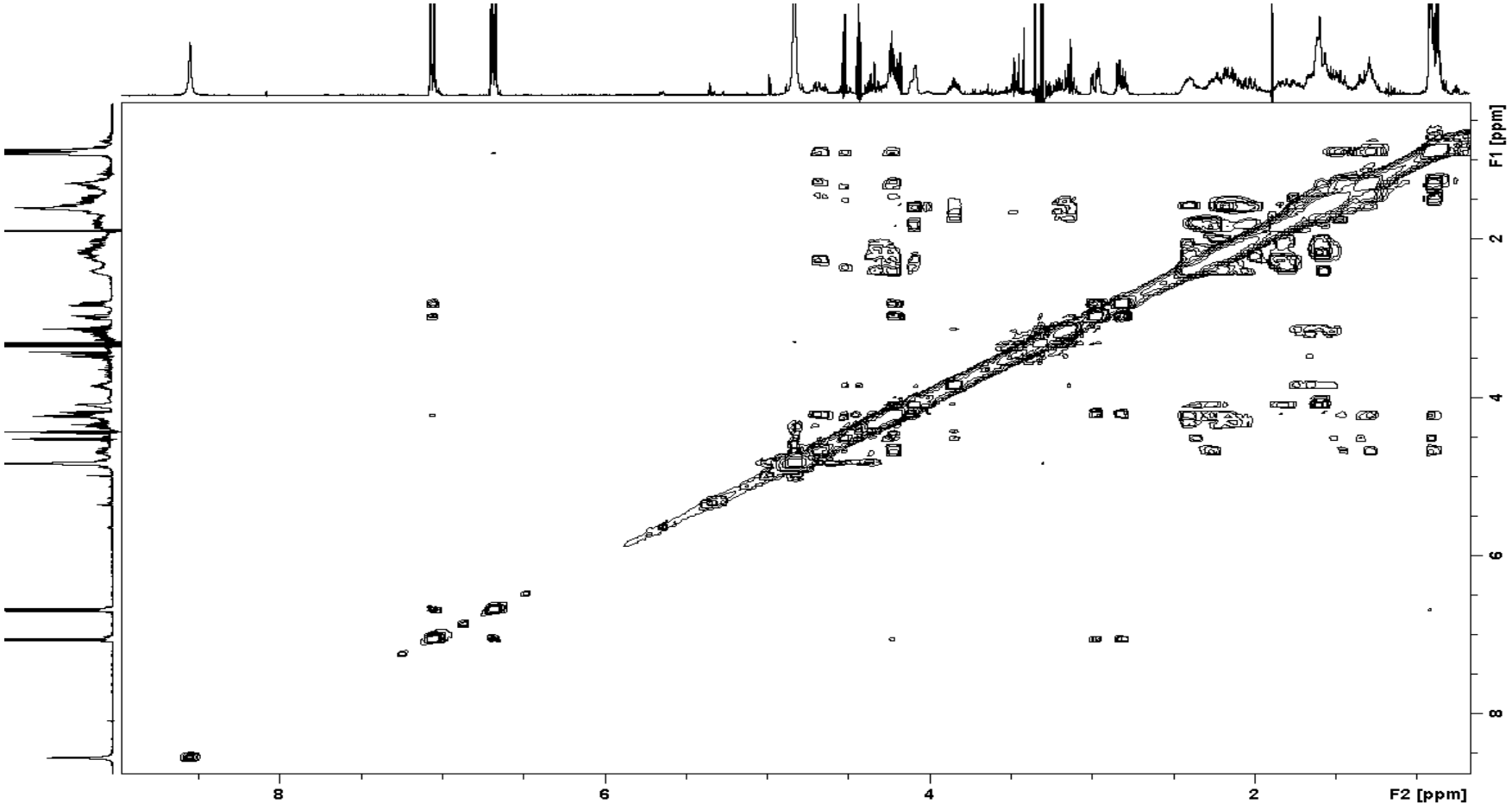
Appendix B.8: COSY NMR spectrum of aeruginosin 602A (**60**) in CD_3OD



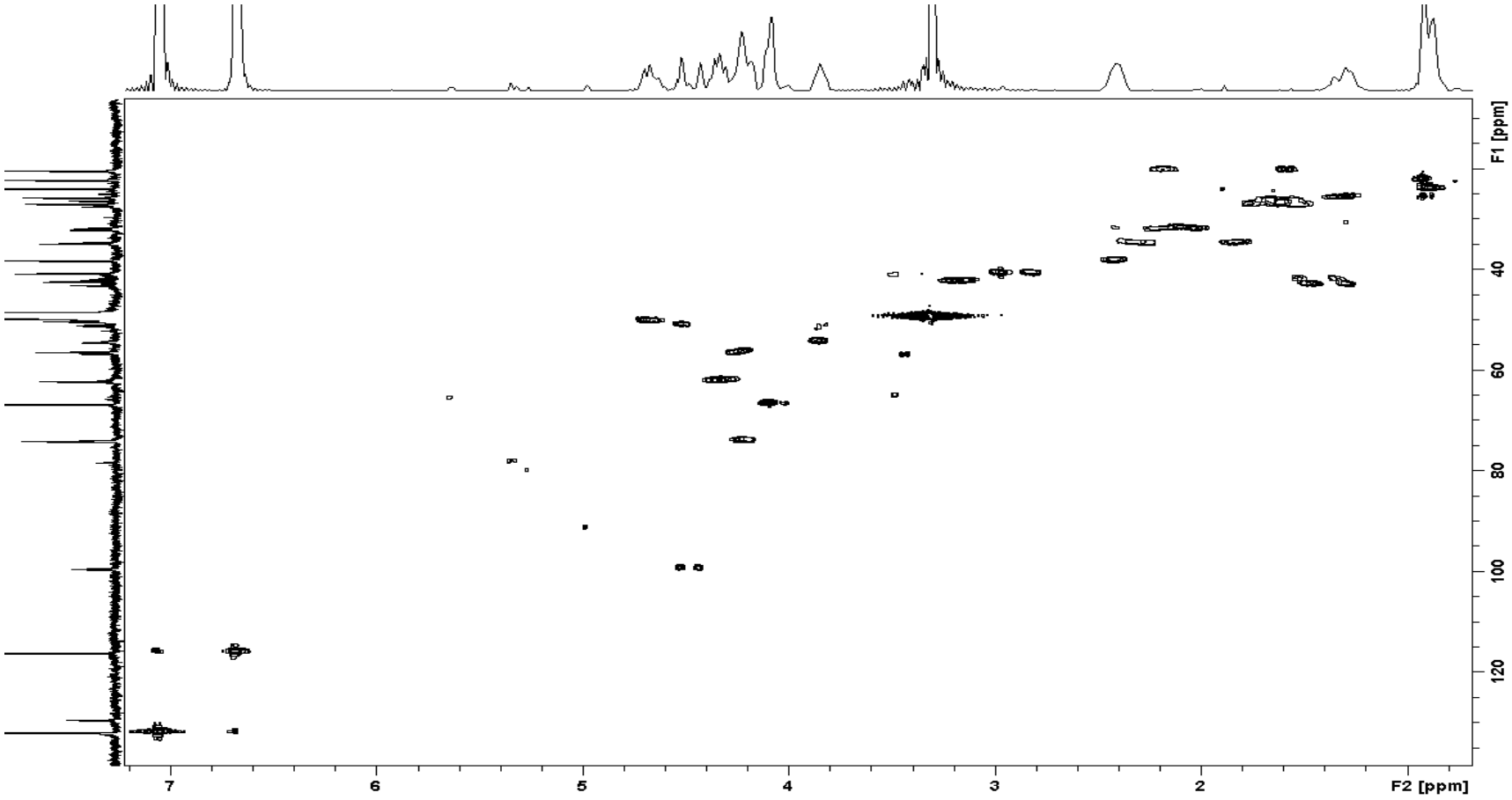
Appendix B.9: TOCSY (150 ms) NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



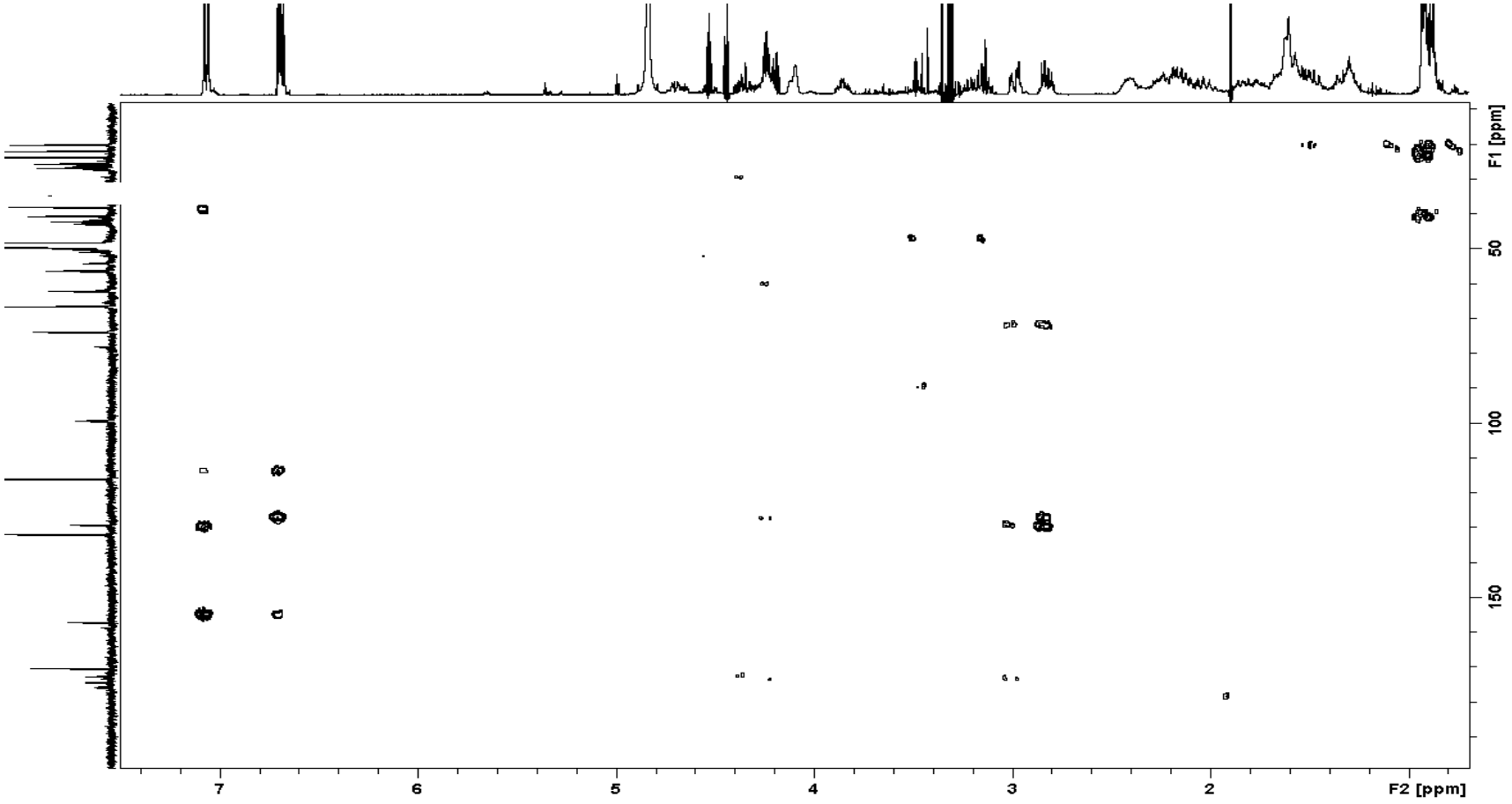
Appendix B.10: ROESY NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



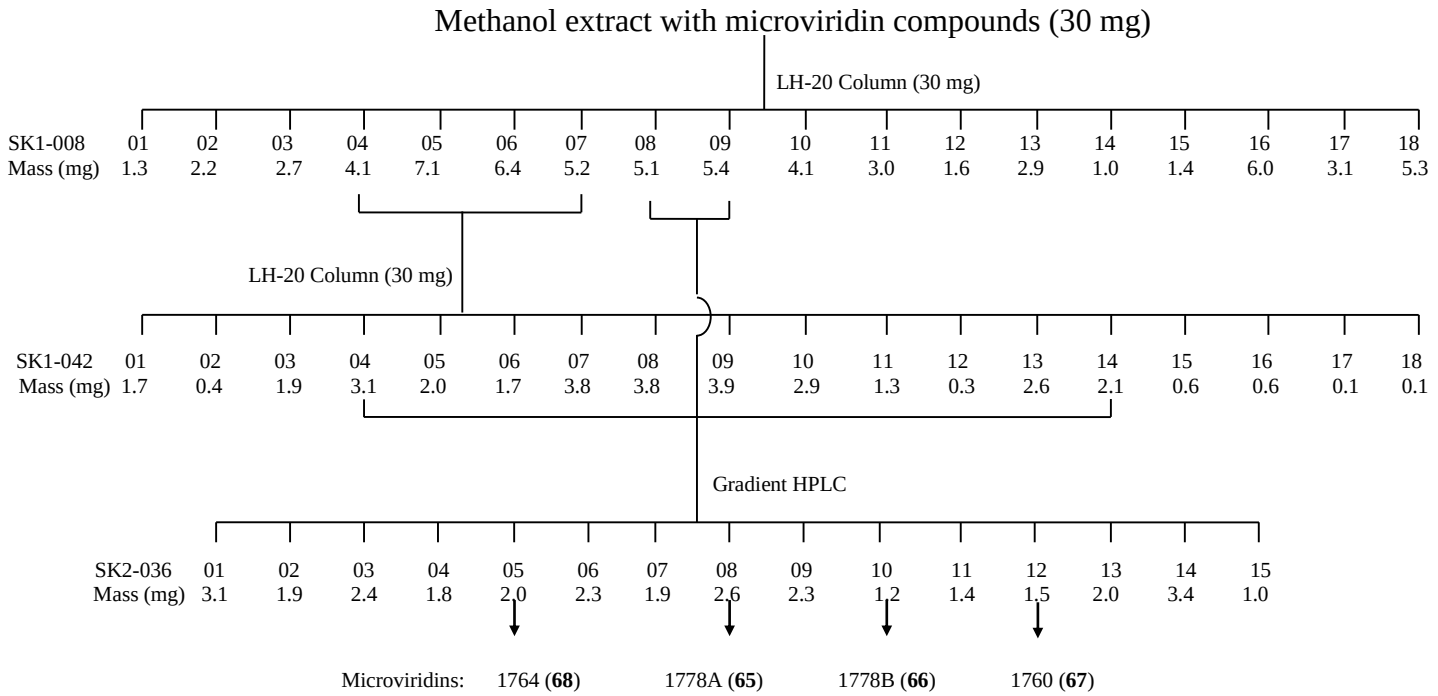
Appendix B.11: HSQC NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



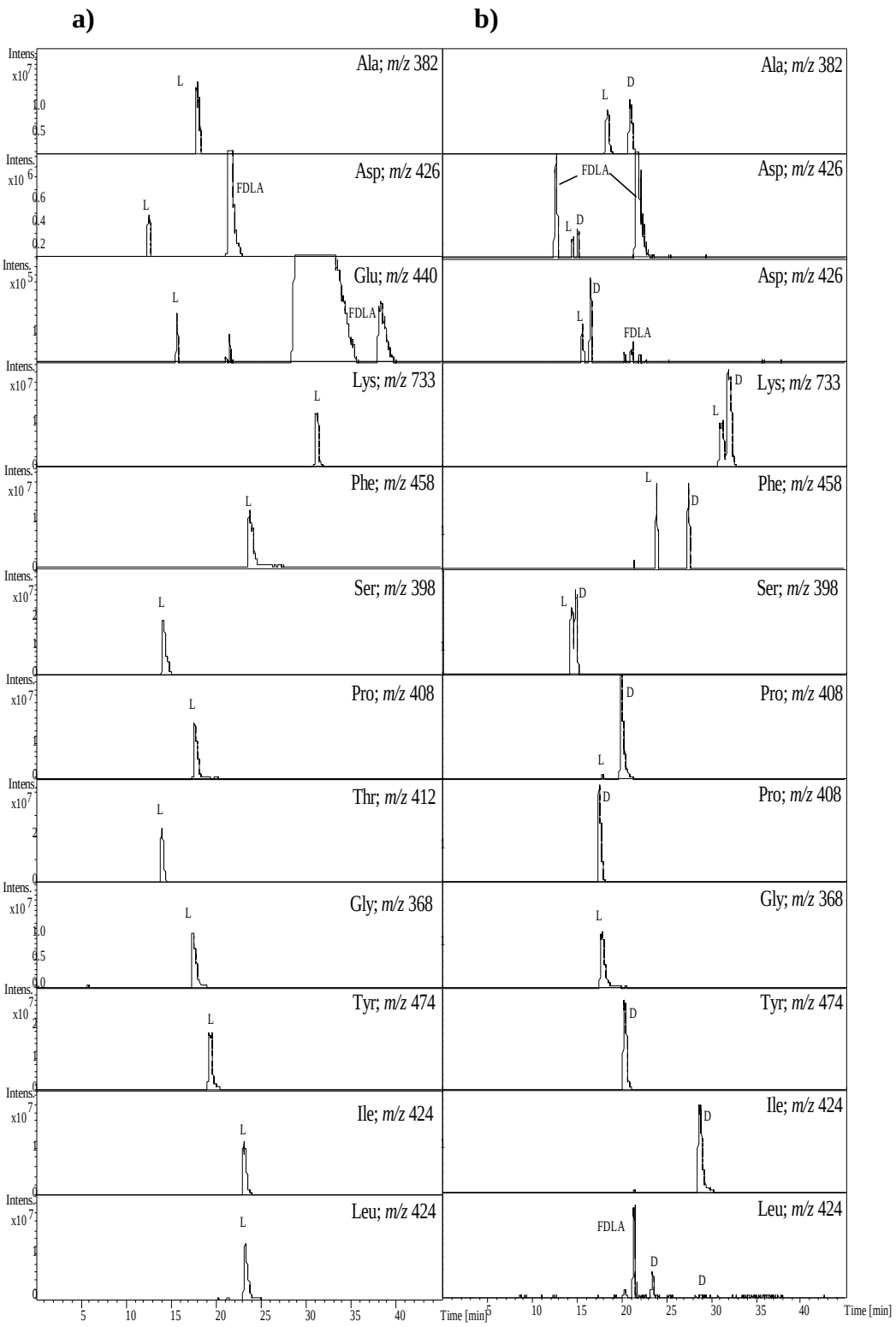
Appendix B.12: HMBC NMR spectrum of aeruginosin 602A (**60**) in CD_3OD.



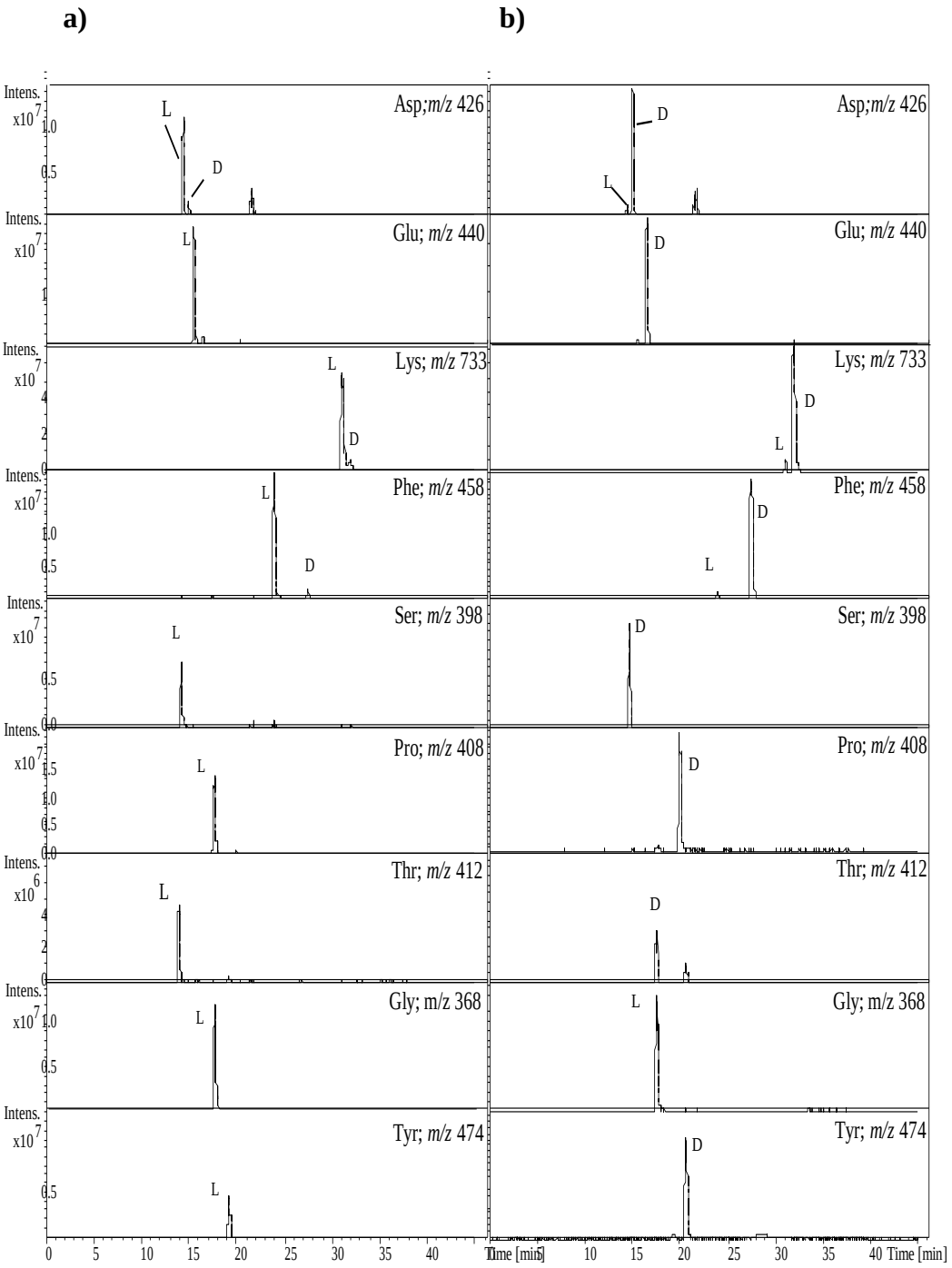
Appendix C.1: Separation tree for fractionation of microviridin metabolites from *Microcystis species* CAWBG11.



Appendix C.2: Extracted ion chromatograms from Advanced Marfey's amino acid analysis using **a)** L-FDLA and **b)** D-FDLA standard amino acid.

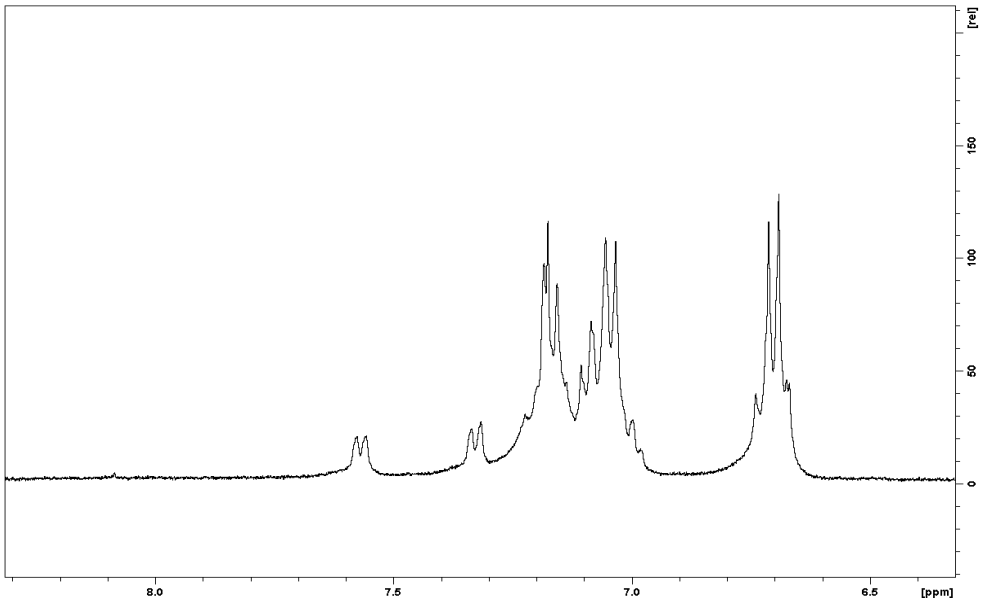


Appendix C.3: Extracted ion chromatograms from Advanced Marfey's amino acid analysis using **a)** L-FDLA and **b)** D-FDLA for 1778A (**65**).

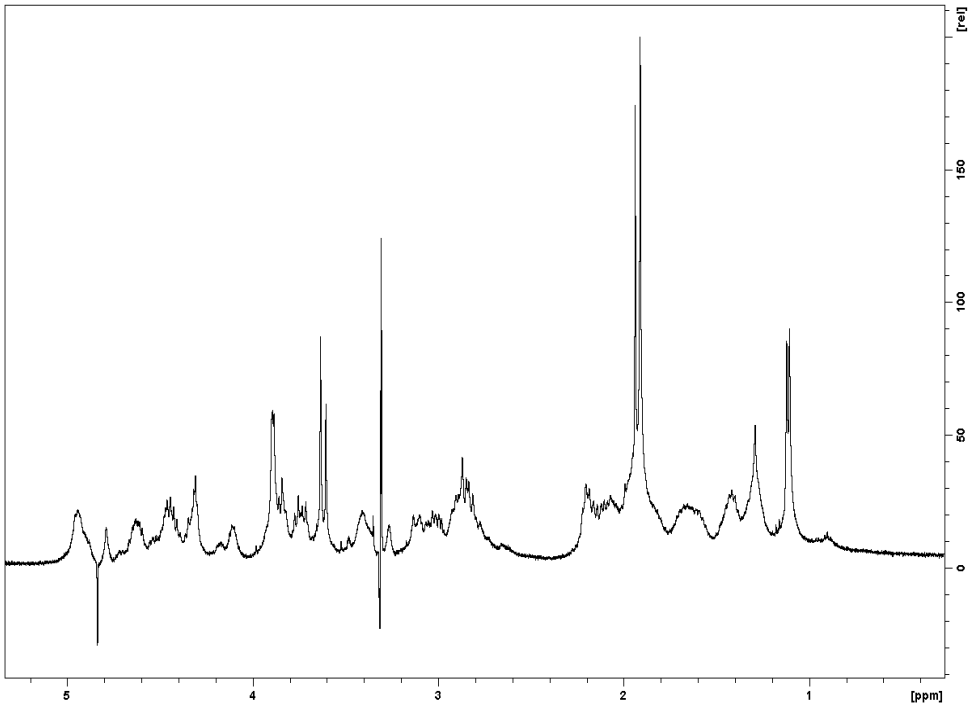


Appendix C.4: The ^{1}H NMR spectrum **a)** downfield region and **b)** upfield region of microviridin 1778A (**65**) in CD_3OD.

a)



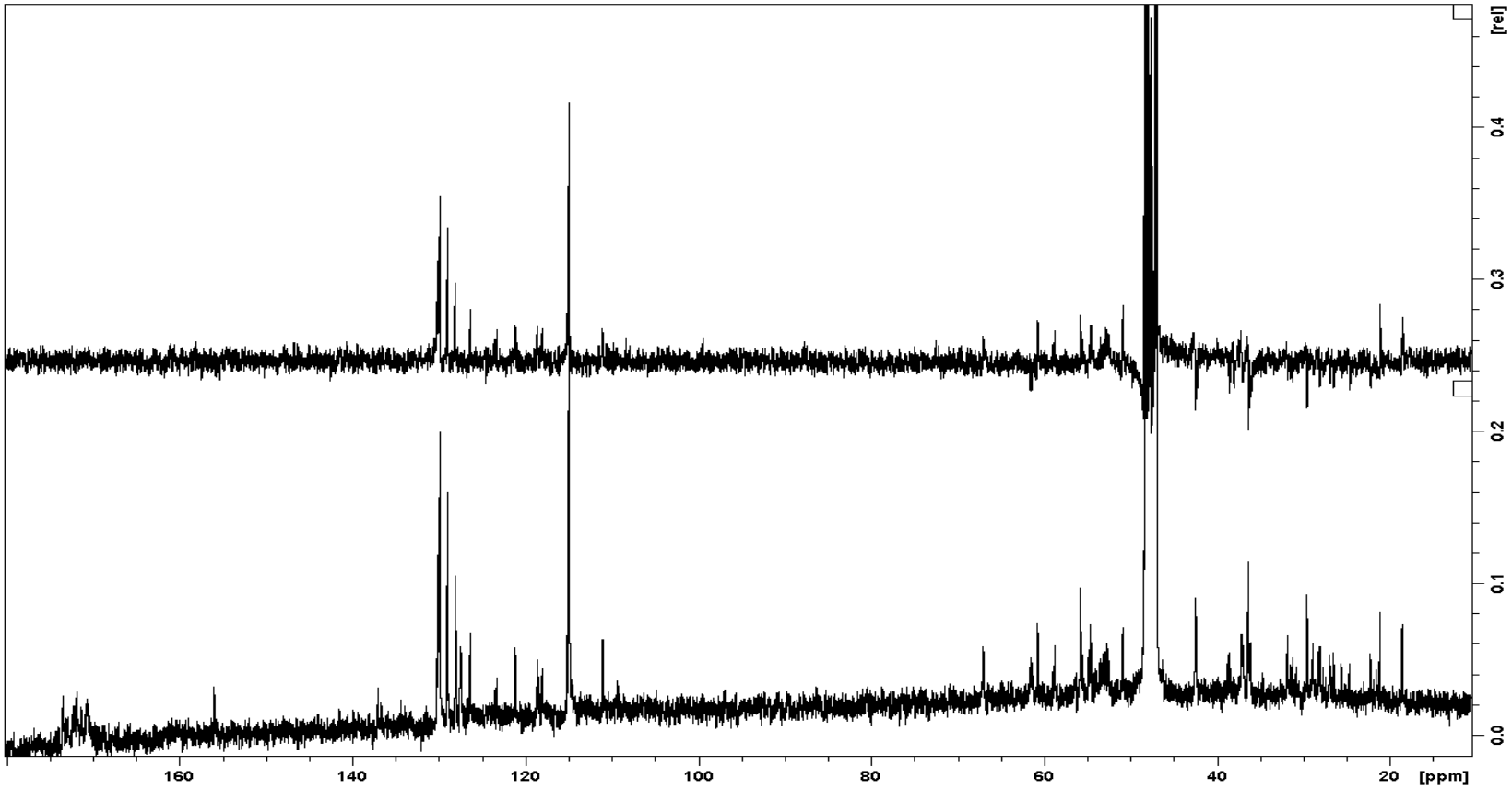
b)



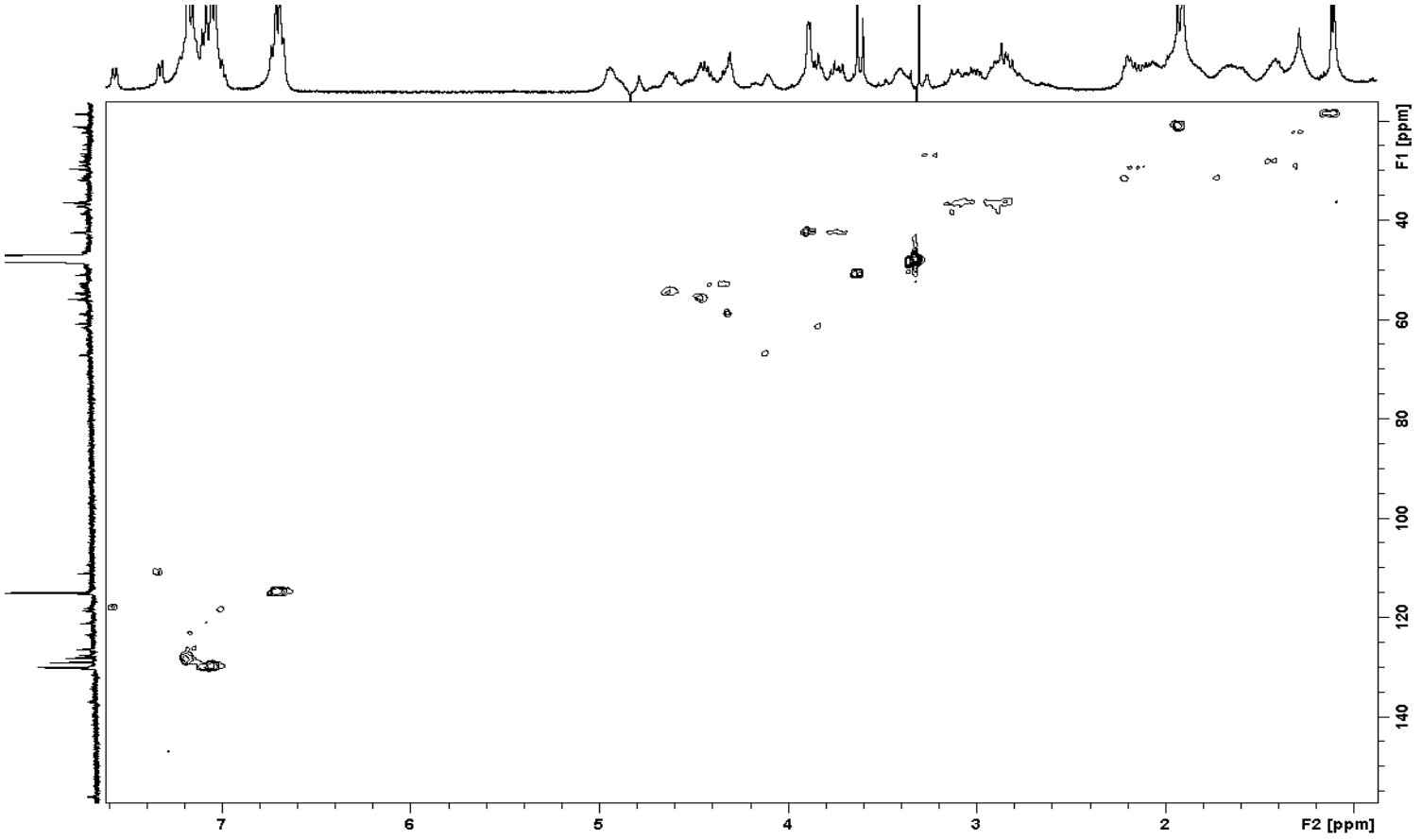
Appendix C.5: a) The DEPT-135 and **b)** ^{13}C NMR spectrum of microviridin 1778A (**65**) in CD_3OD.

a)

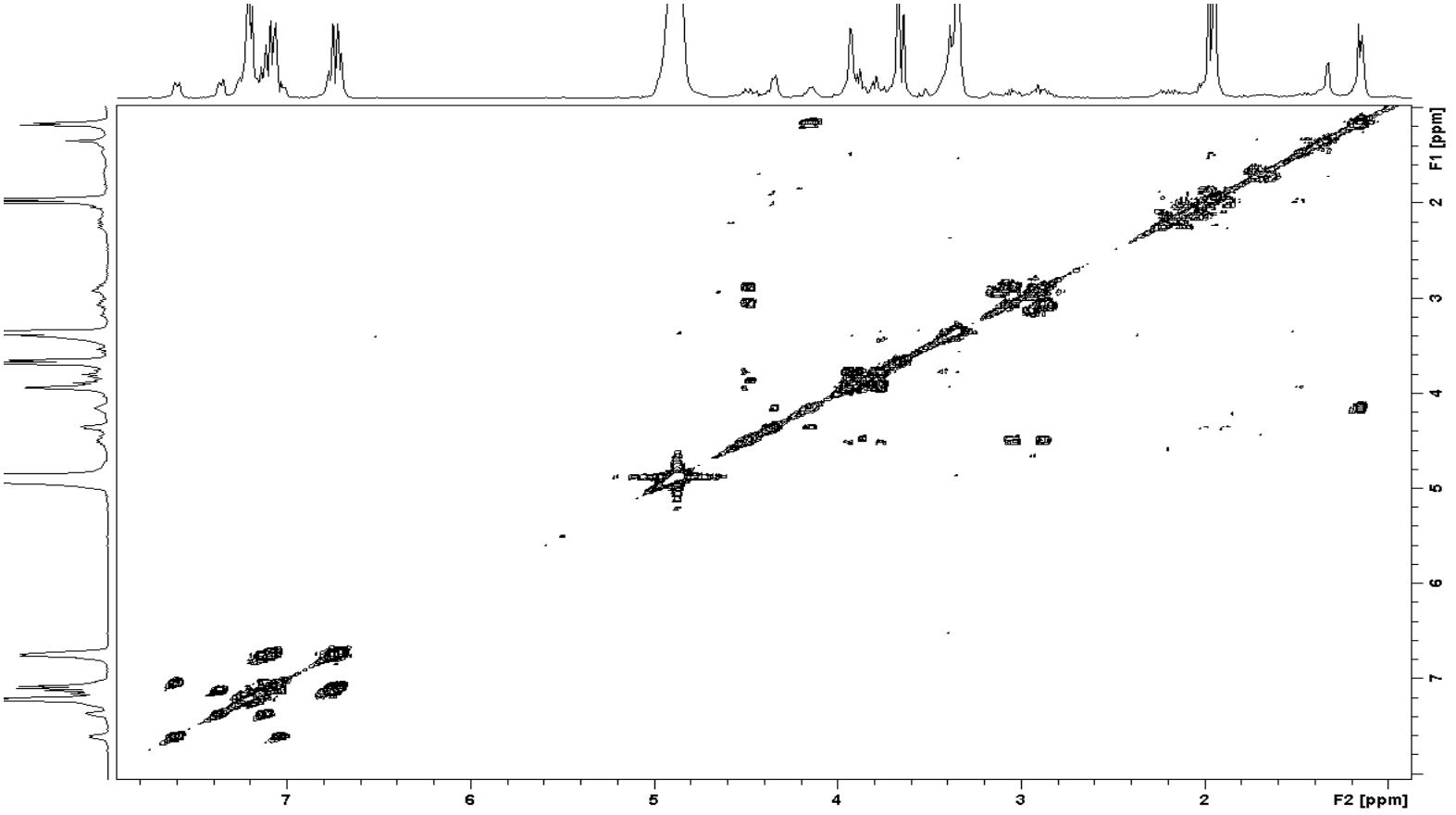
b)



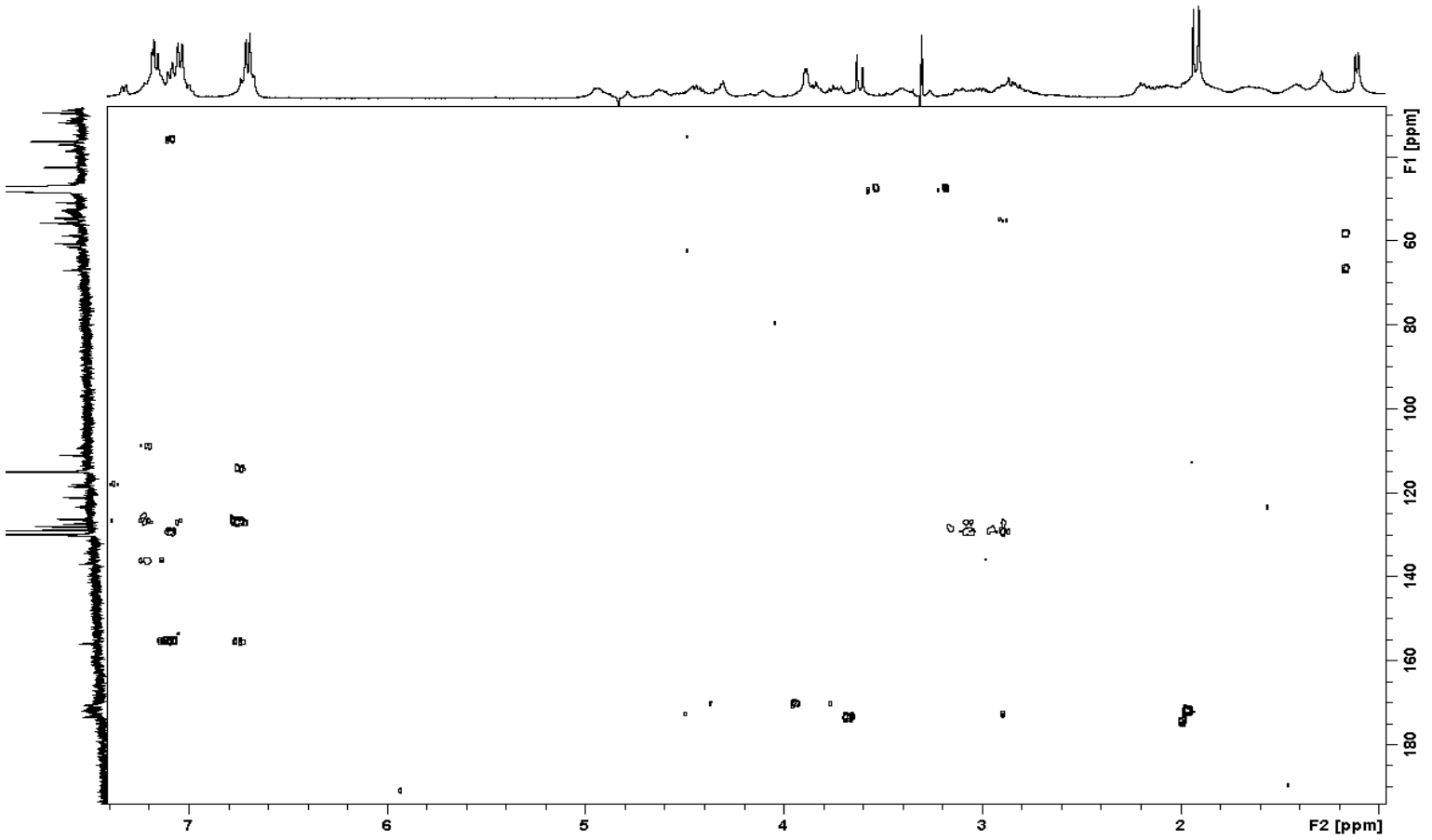
Appendix C.6: The HSQC NMR spectrum of microviridin 1778A (**65**) in CD_3OD.



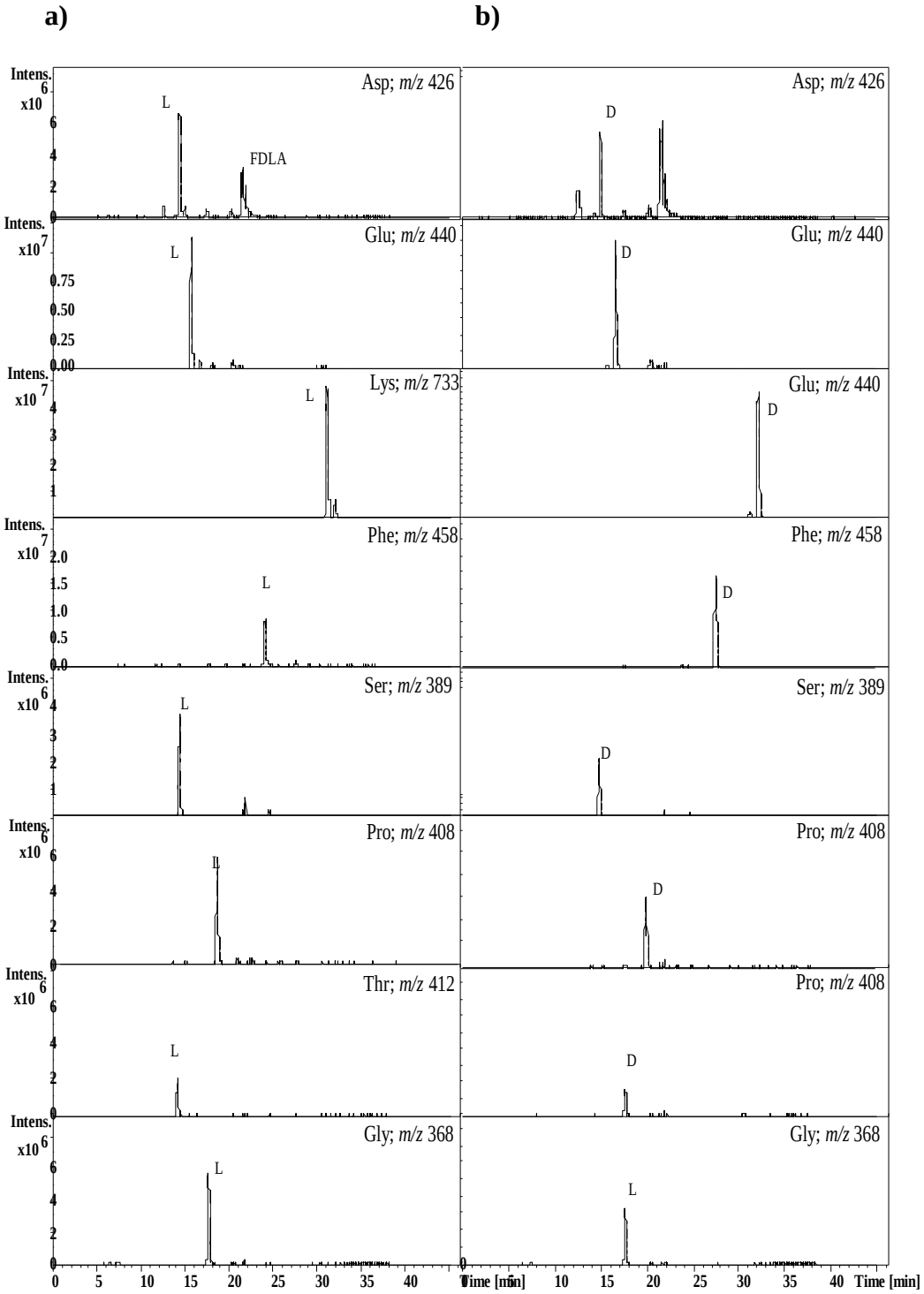
Appendix C.7: The COSY NMR spectrum of microviridin 1778A (**65**) in CD_3OD.



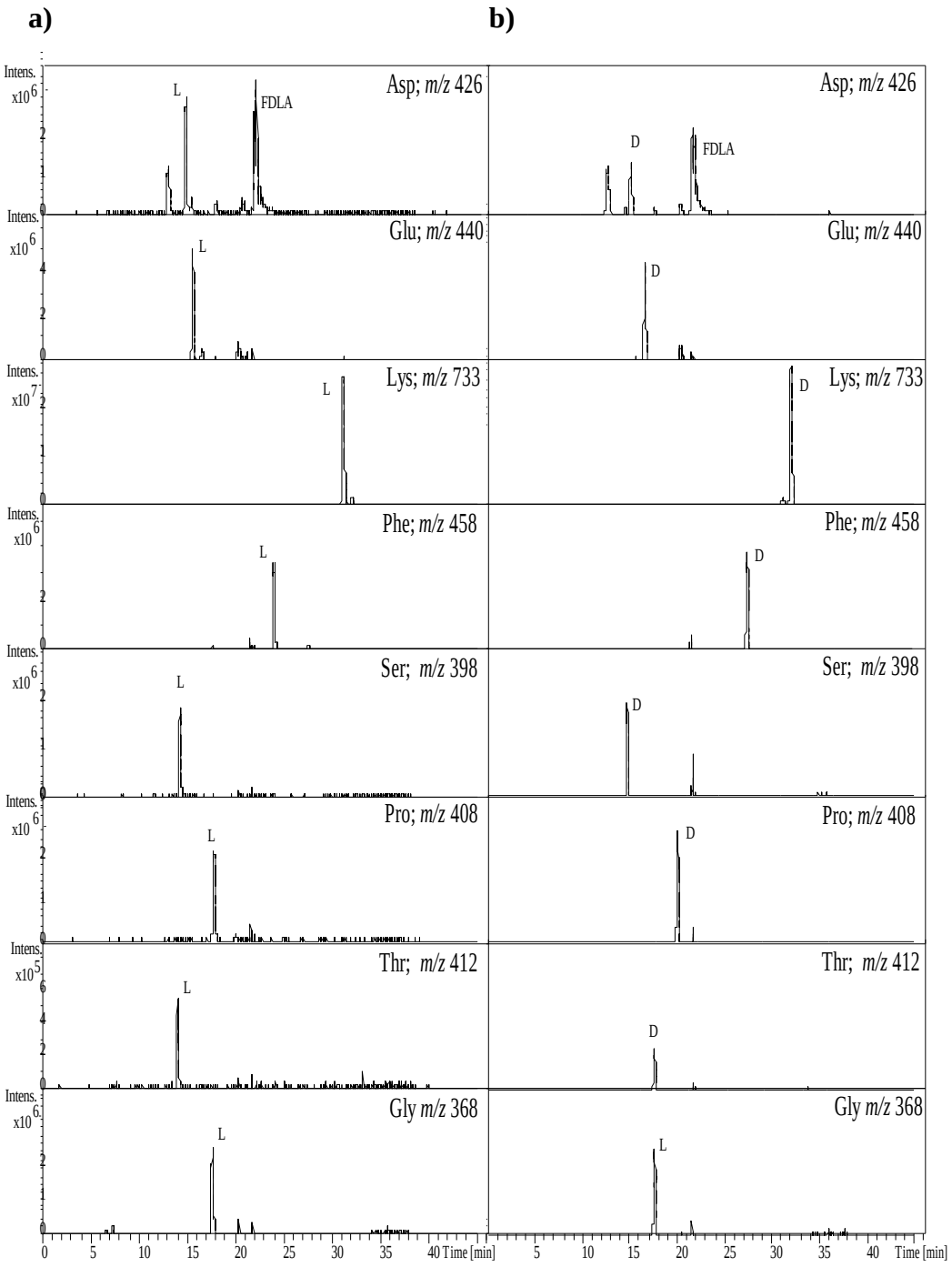
Appendix C.8: The HMBC NMR spectrum of microviridin 1778A (**65**) in CD_3OD.



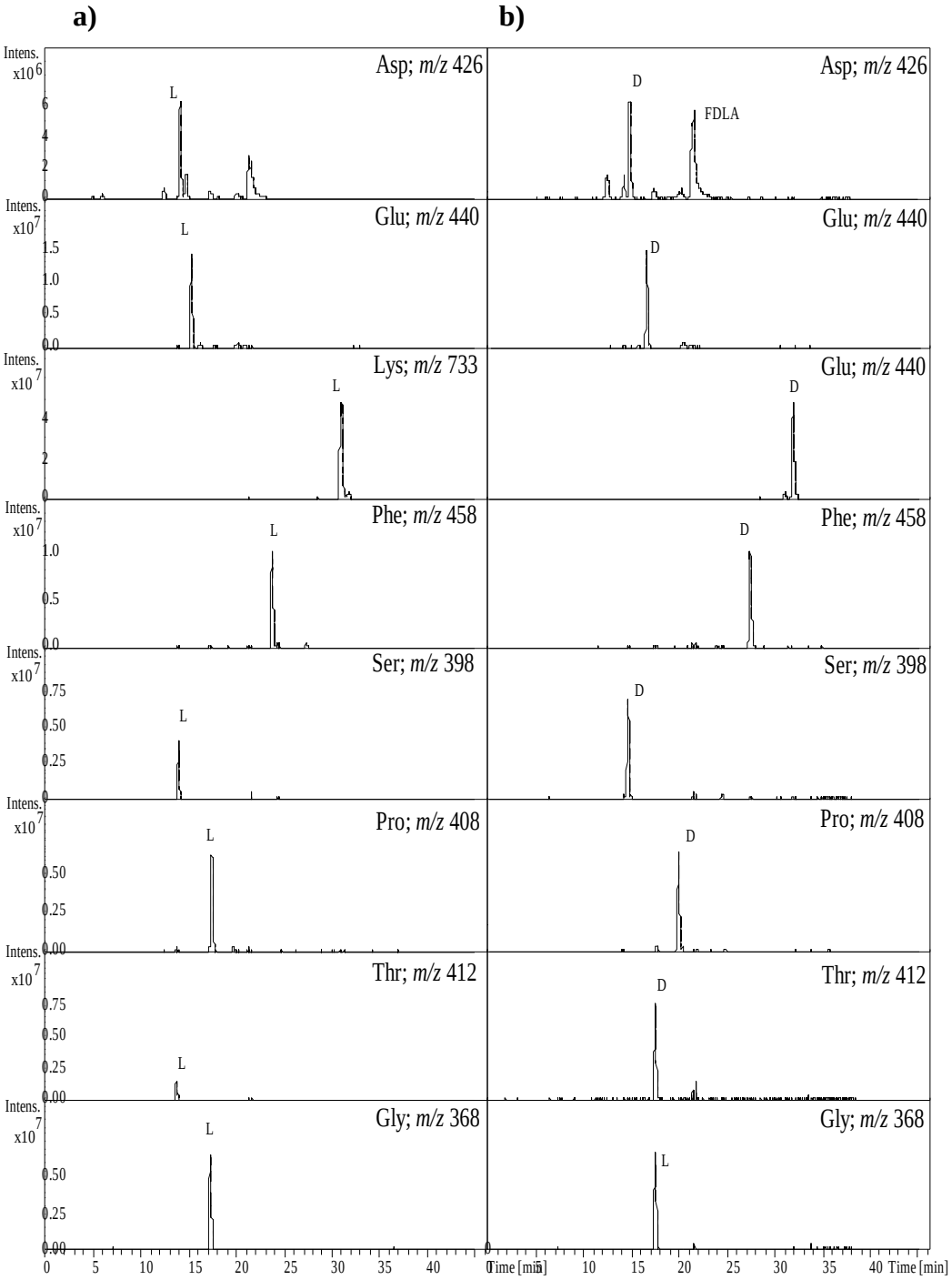
Appendix C. 9: Extracted ion chromatograms from Advanced Marfey's amino acid analysis using **a)** L-FDLA and **b)** D-FDLA for 1778B (**66**).



Appendix C.10: Extracted ion chromatograms from Advanced Marfey's amino acid analysis using **a)** L-FDLA and **b)** D-FDLA for 1760 (**67**).



Appendix C. 11: Extracted ion chromatograms from Advanced Marfey's amino acid analysis using **a)** L-FDLA and **b)** D-FDLA for 1764 (**68**).



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