

***Characterisation of small cyclic peptides with antilisterial and
antimalarial activity***

by

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The crest of the University of Stellenbosch, featuring a shield with various symbols, a crown on top, and a banner at the bottom with the motto 'PACIS SCIENTIAE LIBERTAS'.

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Declaration

I, *Leussa Nyango-Nkeh Adrienne* hereby declare that the entirety of the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it for obtaining any qualification at any university.

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.....

Date

Summary

Antimicrobial peptides (AMPs) are currently the most researched group of compounds for new antimicrobial drugs especially with the rise in resistance to almost all available drugs by public health relevant pathogens. In this study we set out to characterise small cyclic AMPs in terms of their activity towards human pathogens *Listeria monocytogenes*, a food-borne pathogen causing listeriosis and *Plasmodium falciparum*, a parasite that causes malaria respectively, each a threat to public health.

One of the small cyclic peptide libraries examined is the tyrocidines (Trcs) and analogues, which are cyclic decapeptides [*cyclo*-(D-Phe-Pro-(Phe/Trp)-D-Phe/DTrp)-Asn-Gln-(Tyr/Phe/Trp)-Val-(Orn/Lys)-Leu] produced by the Gram-positive bacteria *Bacillus aneurinolyticus* as part of the tyrothricin complex which is non-ribosomally synthesised during sporulation. Previous research found that the six major Trcs were active against *Listeria monocytogenes* and *Plasmodium falciparum* and it was found that the identity of the aromatic residues in the aromatic dipeptide unit has an important role in activity. We set out to extend the qualitative structure to activity relationship (QSAR) studies using more Trc analogues and small synthetic Arg- and Trp-rich cyclic peptides (RW-peptides) in a bid to establish essential structural motifs and pre-requisites for activity. Eight natural and three synthetic Trc analogues and fifteen RW-peptides were either naturally or by chemical synthesis produced and characterised in terms of chemical character and biological activity. The Trcs were significantly more active than RW peptides, although much more haemolytic and thus toxic. Results indicated the relevance for hydrogen bonding with an aromatic amino acid residue for selective activity towards the leucocin A resistant *L. monocytogenes* B73-MR1. However, structural properties favouring a tighter membrane interaction hindered the Trc mode of action (MOA). We determined that Gln⁶ and hydroxyl group of Tyr⁷ may be involved in interaction with the putative target in *L. monocytogenes*. There was also need for an amphipathic balance between hydrophobicity and size/steric parameters for optimal activity. From our QSAR studies we predict as lead peptide for a future library of antilisterial Trcs: *cyclo*(VOMe₃LfPWfNQY). Furthermore, the antilisterial activity of the Trcs was found to be predominantly lytic and salt tolerant while RW-peptides were non-lytic and sensitive to Ca²⁺. We confirmed that Ca²⁺ enhanced Trc antilisterial activity with Ca²⁺ increasing the Trc anti-metabolic activity, but conversely inducing a non-lytic mechanism of action. From

model membrane studies, we propose that the calcium induced Trc non-lytic MOA could be due to detrimental lipid demixing, presence of a Trc sensitive Ca^{2+} -induced non-membrane target in the prematurely calcium induced intracellular anaerobic form of *Listeria monocytogenes*, and/or the Trc- Ca^{2+} complexes may inhibit key components such as membrane bound electron transport system or bacterial dehydrogenases.

We confirmed, as previously found, that the Trcs have potent antimalarial activity that is sequence specific and non-lytic. The RW-peptides had very weak activity, but our results again indicated that more hydrophobic and haemolytic peptides tend to be more active, particularly the RW-peptide containing the Trp analogue β -(benzothien-3-yl)-alanine (Bal). A novel finding was that one of the more polar Trc C analogues, namely tryptocidine C (Tpc C), in contrast to Trc C showed potent antimalarial activity indicating the specific sequence and the role of the Trp⁷ in activity. From these results a proposed lead peptide for future research is *cyclo*[VOLfP(Bal)fNQ(Bal)]. Furthermore, in our search for the Trc and Tpc C target(s) we employed high resolution fluorescence microscopy. Results show that Trc led to disorganisation of neutral lipid structures and chromatin halting growth in late trophozoite/early schizont stages. This indicated that membrane structures containing neutral lipids, as well as chromatin may be targeted by the Trcs. Another novel finding in our studies was that chloroquine (CQ) resistance not only correlated with resistance to Trcs, but the Trcs and CQ were found to be antagonistic towards each other's activity. This indicated a shared target and we propose the food vacuole as another of the Trc targets in *P. falciparum*.

Opsomming

Antimikrobiese peptiede (AMPe) is tans die mees nagevorsde groep verbindings in die soeke na nuwe antimikrobiese middels, veral weens 'n toenemende weerstandigheid van patogene in die openbare gesondheidssektor teen alle beskikbare middels. Die doel van hierdie studie was om klein, sikliese AMPe in terme van hul aktiwiteit teenoor twee menslike patogene wat 'n bedreiging vir openbare gesondheid is, *Listeria monocytogenes*, 'n voedsel-oordraagbare patogeen wat listeriose veroorsaak, asook *Plasmodium falciparum*, die parasiet verantwoordelik vir malaria, te karakteriseer.

Een van die klein, sikliese peptiedbiblioteke wat ondersoek is, is die tyrocidines (Trcs) en analoë (sikliese dekapeptiede [*siklo*-(D-Phe-Pro-(Phe/Trp)-D-Phe/DTrp)-Asn-Gln-(Tyr/Phe/Trp)-Val-(Orn/Lys)-Leu]). Hierdie peptiede deur die Gram-positiewe bakterie *Bacillus aneurinolyticus* word wat nie-ribosomaal gesintetiseer as deel van die tirotrisien kompleks word tydens sporulasie. Vorige navorsing het gewys dat die ses hoof Trcs teen *Listeria monocytogenes* en *Plasmodium falciparum* aktief is en dat die identiteit van die aromatisiese residu in die aromatisiese dipeptiede-eenheid 'n belangrike rol speel in die Trc-aktiwiteit. Ons het gepoog om die kwalitatiewe struktuur-aktiwiteit-verwantskap (QSAR) studies uit te brei deur meer Trc analoë en klein sintetiese Arg- en Trp-ryke sikliese peptiede (RW-peptiede) te gebruik en sodoende essensiële struktuur-motiewe en voorvereistes vir aktiwiteit vas te stel. Agt natuurlike en drie sintetiese Trc analoë, asook vyftien RW-peptiede is of deur natuurlike of chemiese sintese geproduseer en gekarakteriseer in terme van chemiese karakter en biologiese aktiwiteit. Die Trcs het beduidend meer aktiwiteit as RW-peptiede getoon, maar is ook meer hemolities en dus meer toksies. Die resultate dui op die belang van waterstofbinding met 'n aromatisiese aminosuurresidu vir die selektiewe aktiwiteit teenoor die leucocin A weerstandige *L. monocytogenes* B73-MR1. Strukturele eienskappe wat tot 'n sterker membraan-interaksie lei, verhinder egter die werkingsmeganisme. Ons het vasgestel dat Gln en die hidroksielgroep van Tyr betrokke kan wees in die interaksie met die vermeende teenmiddelsteiken in *L. monocytogenes*. 'n Balans tussen amfipatiese/hidrofobiese en grootte/steriese parameters is ook noodsaaklik vir optimale aktiwiteit. Vanuit ons QSAR studies word die peptied *siklo*-(VOMe₃LfPWfNQY) as die voorloper vir 'n toekomstige peptiedbiblioteek van antilisteriale Trcs voorgestel. Verder is daar gevind dat die antilisteriese aktiwiteit van die Trcs oorwegend lities en sout-verdraagsaam is,

terwyl die RW-peptiede nie-lities en Ca^{2+} sensitief is. Ons het bevestig dat Ca^{2+} die Trc antilisteriese aktiwiteit verbeter, deur die Trc se antimetaboliese aktiwiteit verhoog, maar terselfdertyd 'n nie-litiese werkingsmeganisme induseer. Vanuit model-membraan studies word voorgestel dat Trc se nie-litiese werkingsmeganisme, soos teweeggebring deur Ca^{2+} , die gevolg kan wees van nadelige lipied vermenging, die teenwoordigheid van 'n kalsium geïnduseerde Trc-sensitiewe nie-membraan teiken in 'n vervroegde kalsium geïnduseerde intrasellulêre anaerobiese vorm van *Listeria monocytogenes*, en/of dat die Trc- Ca^{2+} komplekse belangrike komponente soos 'n membraan-gebonde elektron transport sisteem of bakteriële dehidrogenases inhibeer.

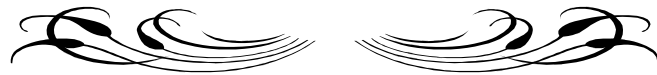
Daar is ook bevestig, soos voorheen gevind, dat die Trcs kragtige, antimalaria aktiwiteit besit wat volgorde-spesifiek en nie-lities is. Die RW-peptiede het swak aktiwiteit getoon, maar ons resultate het weereens bewys dat peptiede wat meer hidrofobies en hemolities is, meer aktief is, veral die RW-peptiede wat die Trp analoog β -(bensoteïen-3-iel)-alanien (Bal) bevat. 'n Nuwe bevinding is dat een van die meer polêre Trc C analoë, genaamd triptosidien C (Tpc C), in teenstelling met Trc C, sterk antimalaria aktiwiteit het, wat 'n aanduiding is van die spesifieke volgorde en die rol van die Trp⁷ in aktiwiteit. Vanuit hierdie bevindinge word die peptied *siklo*-(VOLFP(Bal)fNQ(Bal)) as 'n voorloper vir toekomstige navorsing aangedui.

Vir ons soeke na die Trc en Tpc C teiken(s), het ons hoë resolusie fluoressensie mikroskopie aangewend. Resultate toon dat Trc tot die ontwrigting van 'n neutrale lipied strukture en chromatien lei en sodoende groei beperk in die laat trofosoïet/vroeë skisont fases. Dit het aangedui dat die membraanstrukture wat neutrale lipiede bevat, sowel as chromatien, deur die Trcs geteiken word. 'n Verdere nuwe bevinding in hierdie studie was dat chloroquine (CQ) weerstandigheid nie net korreleer met weerstandigheid teen Trcs nie, maar dat die Trcs en CQ antagonisties optree teenoor mekaar se aktiwiteite. Dit dui op 'n gemeenskaplike teiken en die kosvakuool as 'n addisionele Trc teiken in *P. falciparum* word voorgestel.



“It always seems impossible until it's done!”

Nelson Mandela



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Table of Contents

List of abbreviations and acronyms	xiv
Preface	xxii
Chapter 1	1.1
Part I: Literature Review Small cyclic antimicrobial peptides as antilisterial compounds	1.1
1.1. Introduction.....	1.1
1.2. <i>Listeria monocytogenes</i>	1.1
1.2.1. Cell structure	1.2
1.2.2. Pathogenesis of listeriosis	1.2
1.2.3. <i>Listeria</i> control strategies	1.3
1.2.4. Listerial antibiotic resistance.....	1.5
1.3. Peptide antibiotics and antimicrobial peptides	1.7
1.4. Small cyclic antimicrobial peptides targeting <i>Listeria</i>	1.11
1.4.1. The tyrocidines and analogues	1.12
1.4.2. Model Trp-Arg rich cyclic hexapeptides.....	1.18
1.5. Concluding remarks.....	1.22
1.6. References.....	1.23
Chapter 2	2.1
Production, purification and characterisation of selected natural and synthetic Trcs	2.1
2.1. Introduction.....	2.1
2.2. Materials	2.6
Bacterial strains:	2.6
Research materials:.....	2.6
2.3. Methods	2.7
2.3.1. Optimisation of Trc analogue production	2.7
2.3.2. Purification of the Trcs.....	2.12
2.3.3. Characterisation of purified Trc analogues	2.13
2.4. Results and Discussion	2.15
2.4.1. Manipulation of production and composition of Trc analogues by <i>B. aneurinolyticus</i> ATCC 8185 cultures.....	2.15
2.4.2. Optimisation for high yield of single Trc analogues from culture extracts.....	2.21

2.4.3.	Purification and characterisation of Trcs and analogues	2.27
2.4.4.	Homology models of newly purified Trc analogues	2.42
2.5.	Conclusions	2.46
2.6.	References.....	2.47
Chapter 3.....		3.1
Role of antimicrobial peptide structure on antilisterial activity: tyrocidine and <i>cyclo</i> (RRRWFW) analogues		3.1
3.1.	Introduction.....	3.1
3.2.	Materials	3.3
3.3.	Methods	3.4
3.3.1.	Bacteria culturing	3.4
3.3.2.	Peptide preparation.....	3.4
3.3.3.	Determination of antilisterial activity of peptides	3.5
3.3.4.	Analysis of dose-response data	3.6
3.3.5.	Qualitative Structure-to-Activity Relationship (QSAR) analyses.....	3.6
3.4.	Results and Discussion: Part I - Tyrocidine analogues.....	3.7
3.4.1.	<i>L. monocytogenes</i> strain susceptibility to the tyrocidines	3.8
3.4.2.	Comparative activity analyses of tyrocidine analogues.....	3.10
3.4.3.	QSAR and PCA analyses of the Trc libraries	3.16
3.5.	Results and Discussion: Part II - <i>cyclo</i> (RRRWFW) analogues	3.22
3.5.1.	<i>L. monocytogenes</i> strain susceptibility towards RW-peptides	3.26
3.5.2.	Structure-activity analyses of c-WFW and analogues.....	3.28
3.5.3.	QSAR and PCA analyses	3.34
3.6.	Conclusions	3.40
3.7.	References.....	3.43
3.8.	Addendum	3.51
3.8.1.	Influence of culture storage on sensitivity to peptides	3.51
3.9.	Supplementary data	3.54
Chapter 4		4.1
Activity and salt-tolerance of tyrocidines, cyclic decapeptides from <i>Bacillus aneurinolyticus</i> , and their analogues towards <i>Listeria monocytogenes</i> as target.....		4.1
4.1.	Introduction.....	4.1

4.2.	Materials	4.3
4.3.	Methods	4.3
4.3.1.	Bacteria culturing	4.3
4.3.2.	Peptide preparation.....	4.4
4.3.3.	Growth and metabolism inhibition assays.....	4.4
4.3.4.	Membrane permeabilisation/lysis assays.....	4.5
4.3.5.	Influence of EDTA on Trc activity.....	4.6
4.3.6.	Analyses of data	4.7
4.3.7.	Fluorescence microscopy	4.8
4.3.8.	Vesicle interaction studies.....	4.8
4.3.9.	Fluorescence spectroscopy	4.9
4.3.10.	Light scattering assays.....	4.9
4.4.	Results and Discussion	4.10
4.4.1.	Influence of metal cations on antilisterial activity of the cyclic peptides.....	4.10
4.4.2.	Influence of metal cations on membrane activity of the cyclic peptides.....	4.14
4.4.3.	Antilisterial activity of tyrocidines in combination with EDTA and divalent cations	4.17
4.4.4.	Fluorescent microscopy.....	4.20
4.4.5.	Interaction of tyrocidines with metal cations	4.22
4.4.6.	Vesicle interaction studies.....	4.26
4.5.	Conclusions	4.27
4.6.	References.....	4.29
Chapter 5		5.1
Part II: Literature Review: Small cyclic antimicrobial peptides as anti-malarial compounds ...		5.1
5.1.	Introduction.....	5.1
5.2.	<i>Plasmodium falciparum</i>	5.2
5.2.1.	Malaria pathogenesis	5.2
5.2.2.	Malaria related modifications of the red blood cell membrane.....	5.4
5.2.3.	Anti-malarial strategies	5.5
5.3.	Potential of antimicrobial peptides as antimalarial agents	5.9
5.3.1.	Antiplasmodial antimicrobial peptides.....	5.9
5.3.2.	Possible mechanisms of antiplasmodial activity	5.10
5.4.	Concluding remarks.....	5.14
5.5.	References.....	5.15

Chapter 6	6.1
Role of antimicrobial peptide structure on antimalarial activity: tyrocidine and <i>cyclo</i> (RRRWFW) analogues	6.1
6.1. Introduction.....	6.1
6.2. Materials	6.4
6.3. Methods	6.5
6.3.1. Parasite culturing.....	6.5
6.3.2. Peptide preparation.....	6.7
6.3.3. Determination of antimalarial and haemolytic activities of peptides.....	6.7
6.3.4. Determination of toxicity	6.8
6.3.5. Assessment of dose-response data	6.8
6.3.6. Interaction between chloroquine and selected tyrocidine analogues.....	6.9
6.4. Results and Discussion: Part I – <i>cyclo</i> (RRRWFW) analogues	6.11
6.4.1. Antimalarial and haemolytic activities.....	6.11
6.5. Results and Discussion: Part II – tyrocidine A and C analogues.....	6.14
6.5.1. Antimalarial activity and cytotoxicity	6.14
6.5.2. Evaluation of antimalarial activity of tyrocidines in combination with chloroquine	6.23
6.6. Conclusion	6.24
6.7. References.....	6.25
6.8. Addendum	6.33
 Chapter 7	 7.1
Investigation of tyrocidine and tryptocidine antiplasmodial mechanism of action using light and fluorescence microscopy	7.1
7.1. Introduction.....	7.1
7.2. Material and methods	7.2
7.2.1. Materials.....	7.2
7.2.2. Methods.....	7.3
7.3. Results and discussion	7.5
7.3.1. Effect of tyrocidine and tryptocidine on intra-erythrocytic life cycle parasite stages	7.6
7.3.2. Visualization of the effect of tryptocidine C on parasite neutral lipids.....	7.12
7.4. Conclusion	7.14
7.5. References.....	7.14

Chapter 8	8.1
Summary, conclusions and outlook.....	8.1
8.1. Introduction.....	8.1
8.2. Summary of findings and future prospects	8.2
8.2.1. Production of selected natural Trcs	8.2
8.2.2. QSAR of antilisterial activity of small cyclic peptides	8.3
8.2.3. Salt sensitivity and tolerance of antilisterial activity of the cyclic peptides.....	8.4
8.2.4. Antimalarial activity of small cyclic peptides	8.6
8.3. Last word	8.7
8.4. References.....	8.7

List of Abbreviations and Acronyms

(Q)SAR	(qualitative) structure to activity relationship
[M]	molecular ion
μ	growth rate
1MeW	1-methyl tryptophan
5MeW	5-methyl tryptophan
ACN	acetonitrile
ACTs	artemisinin-based combination therapies
AMP(s)	antimicrobial peptide(s)
A _p	activity product
APAD	acetylpyridine adenine dinucleotide
ATCC	American type culture collection
ATP	adenosine triphosphate
<i>B. aneurinolyticus</i>	<i>Bacillus aneurinolyticus</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
b3hW	L-beta-homotryptophan
Bal	beta-(benzothien-3-yl)-alanine
BHI	brain heart infusion
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
cl/cp	ratio of concentration of lipid to concentration of peptide
CQ	chloroquine
CQR	chloroquine resistance
CQS	chloroquine sensitive
CSP	circumsporozoite protein
DDT	dichloro-diphenyl-trichloroethane
Dha	2, 3-dehydroalanine
Dhb	2, 3-dehydrobutyrine

DLP	defensin-like protein
DMEM	Dulbecco's modified Eagle's medium
DMPC	dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DNA	deoxyribonucleic acid
DPC	diphosphatidylcholine
DPPE	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DPPG	1,2-dipalmitoyl- <i>sn</i> -glycero-3-[phospho- <i>rac</i> -(1-glycerol)]
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
ESMS	electrospray mass spectrometry
FIC	fractional inhibition concentration
FICI	fractional inhibition concentration index
Fpa	fluorophenylalanine
fs	femtosecond
FT-IR	Fourier transform infrared spectroscopy
GMCS	global malaria control strategies
GRAS	generally recognized as safe
Grm	linear gramicidin
GS	gramicidin S
HDA	histone deacetylase
HC ₅₀	peptide concentration leading to 50% haemolysis
HEPES	hydroxyethyl piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IC ₅₀	peptide concentration leading to 50 % microbial growth inhibition
IC _F	inhibitory concentration factor
IC _{max}	peptide concentration leading to maximal microbial growth inhibition
IEPM	infected erythrocyte plasma membrane
Igl	alpha-(2-indanyl) glycine
IL-1	interleukin-1
Inl	internalin

iRBC	infected red blood cell
IRS	indoor residual spraying
ITN	insecticide treated nets
LAB	lactic acid bacteria
LB	Luria Bertani
LC	liquid chromatography
LC ₅₀	peptide concentration leading to 50% cytotoxicity
LCMS	liquid chromatography mass spectrometry
LDH	lactose dehydrogenase
L-PrAsn	L- <i>N</i> ^δ -propylasparagine
LPS	lipopolysaccharide
LUV	large unilamellar vesicles
M	molar
<i>M. luteus</i>	<i>Micrococcus luteus</i>
<i>m/z</i>	mass over charge ratio
MALDI	matrix-assisted laser desorption/ionization
MDG	millennium development goal
MIC	minimum inhibitory concentration
MOA	mode of action
M _r	molar mass
MS	mass spectrometry
MV	molecular volume
NBT	nitro blue tetrazolium
NCTC	national collection of type cultures
NFG	tryptophan-N-formylated gramicidin
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NRPSs	non-ribosomal peptide synthethases
NuB	nutrient broth
OD	optical density
Orn	ornithine

O	ornithine
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. malariae</i>	<i>Plasmodium malariae</i>
<i>P. ovale</i>	<i>Plasmodium ovale</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCA	principal component analysis
PCP-domain	peptidyl carrier protein domain
PCS	photon correlation spectroscopy
PE	phosphatidylethanolamine
PES	phenazine ethosulfate
PfATPase	<i>Plasmodium falciparum</i> adenosine-triphosphatase calcium pump
<i>Pfcr1</i>	<i>Plasmodium falciparum</i> chloroquine resistance gene
<i>PfCRT-</i>	<i>Plasmodium falciparum</i> chloroquine resistance protein
<i>Pfmdr</i>	<i>Plasmodium falciparum</i> multi-drug resistance gene
<i>PfMDR-</i>	<i>Plasmodium falciparum</i> multi-drug resistance protein
Phc A	phenycidine A
Phc(s)	phenycidine(s)
PI	phosphatidylinositol
PIP2	phosphatidylinositol-4,5-bisphosphate
pM	picomolar
POPC	palmitoyloleylphosphatidylcholine
POPE	palmitoyloleylphosphatidylethanolamine
POPG	palmitoyloleylphosphatidylglycerol
PPan	phosphopantotheine
Prf	protein release factor
PS	phosphatidyl serine
PTS	phosphotransferase system
PVM	parasitophorous vacuole membrane
RBC	red blood cell

R _f	retention factor
RMSD	root-mean-square deviation
RNA	ribonucleic acid
RP-HPLC	reverse phase high performance liquid chromatography
RPMI	Roswell park memorial institute medium
R _t	retention time of analyte in column chromatography
RW-peptides	Arg- and Trp-rich peptides
SASA	solvent accessible surface area
SAV	solvent accessible volume
SCSA	side chain surface area
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SM	sphingomyelin
SP	sulfadoxine/pyrimethamine
spp.	species in plural
Srf	surfactin
sTpc A	synthetic tryptocidine A
sTrc A(Q-O)	synthetic tyrocidine A with glutamine to ornithine substitution
sTrc AOMe ₃	synthetic tyrocidine A with trimethylated ornithine
Tcn	tyrothricin
TE	thioesterase
TFA	trifluoroacetic acid
TGS	tryptone glucose and salts culture medium
TGYM	tryptone glucose yeast extract milk
TLC	thin layer chromatography
TNF	tumour necrosis factor
TOF	time of flight
Tpc C	tryptocidine C
Tpc(s)	tryptocidines(s)
Trc A	tyrocidine A
Trc B	tyrocidine B

Trc C	tyrocidine C
Trc(s)	tyrocidine(s)
TSB	tryptone soy broth
UPLC	ultra performance liquid chromatography
UV	ultraviolet
WHOPES	World Health Organisation Pesticide Evaluation Scheme

Preface

Malaria is a serious health threat that has been around for ages and is still present with us today especially in sub-Saharan Africa, but also in Latin America and Asia where millions of people die or are affected by the disease particularly children under the age of five. With recent reports signalling imminent clinical resistance towards artemisinin, the major component of the current therapy of choice, the ACTs (artemisinin-based combination therapies), there is an urgent need for new drugs that act *via* mechanisms to which the parasite will not develop resistance. Antimicrobial peptides (AMPs) act principally through membrane lysis in addition to other mechanisms of action. It is difficult for microorganisms to develop resistance to these unspecific and combined mechanisms. Some of these AMPs have been shown to have antimalarial activity. One group will be the subject of this research project; the tyrocidines (Trcs) which are cationic, amphipathic, cyclic decapeptides produced by *Bacillus aneurinolyticus* formerly known as *Bacillus brevis*.

The major tyrocidines (Trcs) of the tyrothricin complex have also shown significant activity against *Listeria monocytogenes*. This food borne pathogenic bacteria which causes listeriosis has equally been a health scourge and threat to food safety mainly due to drug and disinfectant resistance by the pathogen. With the favourable attributes of the Trcs recognised, it is important to improve the collective understanding of their structure to activity relationship (SAR). We therefore, set out to produce a wider array of tyrocidine analogues by a variety of methods in high purity in order to characterise them and evaluate their structure to activity relationship (SAR). In a bid to develop bio-preservatives based on the structure of active Trcs we shall further verify the role of salts of metal cations, usually abundant in food processing and preservation settings, in modulating the antibacterial activity and mode of action of Trcs with emphasis on the previously observed non-lytic mode of action induced by calcium chloride. We would also be investigating the possible mechanisms of action of the Trcs towards these two microbial targets using a variety of state-of-the-art techniques which could enable the rational design of more specific analogues and peptide mimics.

In addition to the Trcs we also investigated the activities of another group of small cyclic peptides that are possible antimalarial and antilisterial candidates, the synthetic analogues of the

hexapeptide *cyclo*(RRRWFW) or RW-peptides. They share the predominant aromatic residue and cationic character of the Trcs yet they are known to have a primarily non-lytic mode of action. Characterisation of these libraries of small cyclic AMPs will enable us to gain insight into the structural motifs and prerequisites necessary for antimicrobial activity and selectivity.

The goal of this study is to contribute to the global effort towards the eradication of malaria, increase in food safety and reduction of the incidence of food borne diseases caused by *Listeria* by selecting for and characterizing cyclic peptide analogues with improved *in vitro* activity and selectivity to serve as lead compounds in drug development studies against drug-resistant forms of *P. falciparum* and *L. monocytogenes*.

To achieve this goal, the following objectives were set to:

1. To produce, purify and characterise the major and novel tyrocidines and analogues via
 - optimized production by *Bacillus aneurinolyticus* ATCC 8185 (Chapter 2)
 - HPLC, ESMS, UPLC-MS and molecular modelling (Chapter 2);
2. To characterise small cyclic peptides rich in Arg and Trp via HPLC and modelling (Chapter 3);
3. To determine the *in vitro* antilisterial activities of cyclic peptides with detailed QSAR analyses to establish essential structural motifs and prerequisites for activity (Chapter 3);
4. To verify the salt-tolerance of selected tyrocidine and *cyclo*(RRRWFW) analogues towards *Listeria monocytogenes* as target and the calcium chloride induced non-lytic mechanism of action of the tyrocidines (Chapter 4);
5. To determine antiplasmodial (*Plasmodium falciparum* strains) and cytolytic (human erythrocytes and COS-1 cells) activity of tyrocidines and *cyclo*(RRRWFW) analogues (Chapter 6);
6. To establish possible mode of action of tyrocidines towards *Plasmodium falciparum* using light and fluorescence microscopy (Chapter 7).

This dissertation consists of two parts with Part I focusing on *L. monocytogenes* as target for the cyclic peptides in this study. The literature survey of Part I is given in Chapter 1 and the experimental chapters consist of Chapters 2-4. Part II of this dissertation focuses on *P. falciparum* as target with the literature survey given in Chapter 5 and the experimental chapters

consisting of Chapters 6 and 7. The conclusions and future prospects of this study are given in Chapter 8. This dissertation was structured to consist of relatively independent chapter units so as to ease the publication of findings and all attempts to minimise inevitable repetitions were made.

Outputs of PhD study

Presentations at professional meetings

2013: Oral presentation at 6th MIM Pan-African Malaria Conference which was held in Durban, South Africa, 6 – 11 October 2013 on “*Anti-malarial activity and structure-activity relationships of the tyrocidines, cyclic decapeptides from Bacillus aneurinolyticus*”

2013: Poster presentation at 6th MIM Pan-African Malaria Conference which was held in Durban, South Africa, 6 – 11 October 2013 on “*The relationship between the insecticide dichloro-diphenyl-trichloroethane and chloroquine in Plasmodium falciparum resistance*”

2013: Oral presentation for PhD progress lecture to the Department of Biochemistry Forum on “*The role of antimicrobial peptide structure on antilisterial activity: tyrocidine and cyclo(RRRWFW) analogues*” on the 25th July 2013 at the University of Stellenbosch, South Africa

2012: Poster presentation at the third International Symposium on Antimicrobial Peptides June, 13-15, 2012, Lille (Villeneuve d'Ascq), France on “*Activity and salt-tolerance of tyrocidines, cyclic decapeptides from Bacillus aneurinolyticus, and their analogues towards Listeria monocytogenes as target.*”

2012: Oral presentation at the 23rd congress of the South African Society of Biochemistry and Molecular Biology and Federation of African Societies of Biochemistry and Molecular Biology conference, Drakensberg, South Africa (29 January - 1 February 2012) on “*Antilisterial activity of synthetic tyrocidine analogues and natural tyrocidines, cyclic decapeptides from Bacillus aneurinolyticus, for structure-activity relationships*”.

Publications

- Vosloo JA, Stander MA, Leussa A N-N, Spathelf BM, Rautenbach M, (2013) Manipulation of the tyrothricin production profile of *Bacillus aneurinolyticus*, *Microbiology* 159, 2200–2211

- Leussa A N-N, Rautenbach M, QSAR and PCA analysis of the tyrocidines towards leucocin A sensitive and resistant *Listeria monocytogenes*. Submitted to *Chemical Biology & Drug Discovery*, CBDD-RA-01-14-1898.
- Leussa A N-N, Rautenbach M, New tyrocidine analogues show activity against intraerythrocytic *Plasmodium falciparum* and do not act by lysis of the infected erythrocyte. *Malaria Journal* (Manuscript in preparation)
- Rautenbach M, Leussa A N-N, Spathelf BM, Bhattacharya B, Dathe M, Influence of Mg^{2+} and Ca^{2+} on the activity of tyrocidines towards *Listeria monocytogenes*. *BBA-Biomembranes* (Manuscript in preparation)

Chapter 1

Part I: Literature Review

Small cyclic antimicrobial peptides as antilisterial compounds

1.1. Introduction

The on-going emergence of resistance by pathogenic microorganisms to currently available drugs has fuelled the search for the next generation of antimicrobial drugs¹⁻⁴. Compounds isolated from nature form a rich database of structures that could be optimized to develop more active drugs⁵. Out of this assortment of natural compounds available, the antimicrobial peptides (AMPs) are one of the most researched groups for alternative and/or additional drugs because of their unique killing mechanism and minimal toxicity to normal mammalian cells^{3,6-23}. In order to carry out rational design of novel drug leads from AMPs active against resistant pathogens, there is need to increase knowledge of their mechanisms of action and the specific properties relevant to their activity and selectivity^{10,24,25}. This review is intended to summarize the main facts around listeriosis and to discuss the prospective use of AMPs as antilisterial therapeutics and/or food preservatives with emphasis on the small cyclic peptides.

1.2. *Listeria monocytogenes*

Listeria monocytogenes is a non-sporulating, flagellated and Gram-positive rod bacterium²⁶⁻²⁹. The organism is a ubiquitous saprophyte in soil occurring primarily in decaying plant materials^{26,28,30-32}. It is facultatively anaerobic and can grow at temperatures ranging from -0.4 to 50 °C^{26,33,34} and a pH range from 4.3 to 9.6³⁵⁻³⁷. *L. monocytogenes* is moreover a facultative intracellular pathogen occurring mostly in domestic animals such as cattle, sheep, goats, and poultry, and occasionally in wild animals^{26,35}. It is an enteric pathogen which means that it resides in the gastrointestinal tract and has been isolated from animal and human waste^{26,28,38}.

Since its emergence as a public health concern in the second half of the 1980s, several epidemic outbreaks of human listeriosis have been recorded especially in North America and Europe^{26,32,39-41}. The latest outbreak which occurred in May 2013 in the USA has been linked to infected cheese resulting in human death and product recall⁴². Human listeriosis, mainly caused by *L. monocytogenes*, is characterized by severe infections, like meningitis and septicaemia, principally in newborns and adults with compromised immunity^{26,39}. Contaminated food and

generally industrially produced, refrigerated ready-to-eat products are implicated^{26,28,32}. Therefore, listeriosis also has an economic effect as food manufacturers register massive losses during product recalls in food-borne outbreaks. *Listeria* spp. are therefore a serious threat to food safety, especially because of their tolerance of high salt concentrations and relatively low pH environments, as well as their ability to replicate at refrigeration temperatures^{32,43}. Moreover, these bacteria have developed resistance to currently available disinfectants and antibiotics^{26,44-46}. The other members of the genus *Listeria* include *L. innocua* (non-pathogenic), *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi*, and *L. murrayi*²⁶.

1.2.1. Cell structure

The cellular structure of *L. monocytogenes* consists of a thick cell wall surrounding the cytoplasmic membrane. The cell wall is mostly made up of carbohydrates in the form of peptidoglycan, teichoic acids and lipoteichoic acids which are covalently linked to each other^{26,47}. The peptidoglycan, which forms 35% of the dry weight of isolated dry cell walls, consists of cross-linked meso-diaminopimelic acid²⁷. Teichoic acids are polymers formed from glycerol or ribitol, neutral sugars, N-acetylamino sugars, and phosphate. The teichoic acids are covalently bonded to a particular site on the peptidoglycan. The lipoteichoic acids are the only amphipathic polymers at the cell surface. They are covalently bonded to the last phosphomonoester of the teichoic acids through their glycolipid portion. The lipid moiety serves to anchor the polymer chain to the cytoplasmic membrane. The major component of the cytoplasmic membrane of *L. monocytogenes* is phospholipids alongside a smaller amount of glycol- and neutral lipids²⁷. The major phospholipids involved are anionic and include bis(phosphatidyl)glycerol or cardiolipin, L-lysylcardiolipin, phosphatidylglycerol (PG), L-lysylphosphatidylglycerol and a phospholipid, referred to as bis(phosphatidylglycerol) phosphate⁴⁸⁻⁵⁰.

1.2.2. Pathogenesis of listeriosis

Transmission of *L. monocytogenes* can be direct from contaminated animals to farm workers and veterinarians through skin lesions²⁶. However, contaminated industrially produced, refrigerated ready-to-eat food products such as vegetables, soft cheeses and dairy products, pâtés and sausages, smoked fish and egg products, as well as seafood are generally the vehicle of infection for sporadic and epidemic listeriosis^{26,32,43,51,52}. This fact was established following a number of epidemic outbreaks in North America and Europe from 1983^{26,32,43,53}. Ruminants get infected by eating spoiled silage³². Due to the ubiquitous presence of *Listeria* spp. in the rural environment they contaminate the raw materials used to prepare industrially processed foods including the production plants⁵⁴.

Human listeriosis mostly occurs in very young or old individuals, as well as in persons with an underlying disease state associated with suppression of their T-cell-mediated immunity and this includes conditions such as neoplastic disease, pregnancy, diabetes mellitus, alcoholism, HIV, cardiovascular and renal collagen diseases, and hemodialysis failure ²⁶. Upon entry into the gastrointestinal tract from contaminated food, the pathogenesis of listeriosis is contributed by several factors ^{26,32}. The most important factor is the intracellular growth capacity of *L. monocytogenes* using its virulent cell surface proteins: internalin A (InlA) and internalin (InlB), a hemolysin (listeriolysin O), two phospholipases, and a protein (ActA) responsible for intracellular motility ^{26,29,55}. The bacteria penetrate intestinal epithelial cells and macrophages through the phenomenon of parasite-directed endocytosis ²⁶ using the internalin proteins ^{29,56}. The hemolysin and a phosphatidylinositol-specific phospholipase enable the escape of the listerial cells from the internalized phagosome ^{29,57,58}. The bacteria replicate within the host while moving towards the cell membrane where they induce invagination of the membrane ^{29,59,60}. Consequently the listerial cells become surrounded by a double membrane ²⁹. A phosphatidylcholine-specific phospholipase enables the escape of the listerial cells from the double membranous vacuole before reinvasion of new cells ^{29,57,61,62}. Invasion of neighbouring enterocytes results in enteritis ³². It has also been demonstrated that listerial cells can translocate to deeper organs without prior intraepithelial replication in the gut ^{32,63}. After crossing the intestinal barrier, the cells are transported by lymph or blood to the mesenteric lymph nodes, spleen, and liver ^{32,63,64}. In the absence of appropriate immune response in the liver, such as with immune compromised individuals, unlimited replication of *L. monocytogenes* in the liver parenchyma could lead to the release of the organism into the circulation ³². Being a multisystemic pathogen, *L. monocytogenes* can contaminate a wide range of host tissues and cause septicaemia ³². Nevertheless, *L. monocytogenes* seems to have a pathogenic tropism to the pregnant uterus and the central nervous system leading to abortion, still birth, and meningitis as the major clinical manifestations of the disease ³².

1.2.3. Listeria control strategies

1.2.3.1. Management of listeriosis

In the late nineteenth century Pasteur, Joubert, von Freudenreich and a host of other scientists demonstrated that microbes released substances (antibiotics) capable of therapeutic action for infections by other microbes ⁶⁵. The development of antibiotics contributed in significantly increasing life expectancy and is perhaps one of humanity's greatest triumphs ^{66,67}. Generally, *Listeria* spp. are susceptible *in vitro* to antibiotics that are effective against other Gram-positive

bacteria with the exclusion of the newer cephalosporins and fosfomycin^{45,68-70}. However, only few antibiotics can kill the intracellular stage of these bacteria⁴⁵. The sporadic nature and rarity of human listeriosis makes prospective clinical studies on the best antibiotic treatment unavailable, thus choice of the optimal therapy relies on intermittent observations and impressions⁴⁵. Penicillin derivatives are important for treatment of all forms of listeriosis⁴⁵. Ampicillin has also been successfully used in treating adults and newborns^{45,71}. Penicillin and ampicillin (β -lactams) act via high affinity binding to and inhibition of the membrane enzyme PBP3 (penicillin binding protein 3) which is involved in the final stage of peptidoglycan synthesis leading to lethal effect on the bacterial cell⁴⁵. However, ampicillin is not reliably bactericidal towards *L. monocytogenes* but combination with gentamicin, an aminoglycoside (acts by irreversibly binding the 30S bacterial ribosomal subunit thus inhibiting protein synthesis), increases the therapeutic effect in *in vitro* and animal experiments⁷². Therefore, penicillin G or ampicillin in combination with gentamicin is recommended as the current therapy of choice^{73,74}. Co-trimoxazole has been recommended for patients allergic to β -lactams as second choice⁷⁵⁻⁷⁷ treatment of intracranial and extracranial symptoms. Co-trimoxazole for example trimethoprim has also been used alone after parenteral treatment to prevent relapse⁷⁸ or in combination with ampicillin⁴⁵. Even though vancomycin has been suggested as a substitute of ampicillin or co-trimoxazole⁷⁹ it is associated with contradictory results such as treatment failures⁸⁰ and the development of listerial meningitis during vancomycin therapy⁸¹. Listeriosis has been observed to be fatal in more than 30% of patients even with rational antibiotic therapy^{45,74}. It has been suggested that this high lethality is due to the facultative intracellular location of *Listeria* where they are hidden from the high concentrations of antibacterial agents in extracellular environment⁸². In addition, antibiotics do not easily penetrate the brain where some of the bacteria may be located⁷⁴. It is speculated that it is important to include rifampicin in the treatment regimen to completely eradicate intracellular bacteria hidden, for example, inside parenchymal cells of the brain^{45,83}.

1.2.3.2. Food preservation

The principal goal of food preservation is to reduce proliferation of microorganisms while food is in storage thereby extending the shelf life and decreasing hazard from food consumption⁸⁴. Food preservation approaches that can be used individually or in combination include physical removal of the microorganisms via centrifugation or filtration, use of low and high temperature, decreasing water availability, use of chemicals such as sulphur dioxide, organic acids and nitrates, and radiation techniques⁸⁴. In order to satisfy the demands of present day food consumers in terms of reduced use of chemical preservatives and harsh heat treatment so as to

achieve safe, more nutritious and fresh-tasting food, a class of AMPs called bacteriocins are increasingly employed as natural food preservatives^{85,86}. The class I bacteriocin (classification according to molecular weight and chemistry), nisin⁸⁷⁻⁸⁹ have been shown to have antilisterial activity and is commonly used and has been given the GRAS (generally recognized as safe) status for food in the United States at levels of 5.5-6.9 mg nisin per kg of food^{9,18,90}. However, this group of peptides may have been compromised as there is emerging resistance against class I and IIa bacteriocins (class IIa bacteriocins and nisin are antilisterial through interaction with lipid II, a peptidoglycan precursor⁹¹). There have been several reports of resistance by some *Listeria* strains to nisin and class IIa bacteriocins⁹¹⁻¹⁰³.

1.2.4. Listerial antibiotic resistance

Due to the knowledge that *L. monocytogenes* in contaminated animal products is the main route to acquire listeriosis, efforts to control the disease have also focused on antibiotic treatment of animals^{44,104}. Antibiotics are moreover randomly used in animal breeding¹⁰⁵. The extensive use of antibiotics in both humans and animals as well as use of sanitizers or disinfectants in food processing or equipment cleaning has led to the development of resistance by food-borne pathogens including *L. monocytogenes* to currently available antibiotics, sanitizers and disinfectants^{39,44,104,106}. Generally, bacteria acquire resistance through either of the following mechanisms¹⁰⁷:

- 1) Cross-resistance arising from chromosomal mutation
- 2) Transfer of resistance genes from one microorganism to another via plasmids (conjugation or transformation), transposons (conjugation), integrons and bacteriophages (transduction).

The resistance genes protect bacteria from antimicrobial agents through numerous types of biochemical resistance mechanisms^{107,108}:

- 1) Interfering with cell wall synthesis rendering some antibiotics inactive, e.g., β -lactams¹⁰⁹
- 2) Modification of antibiotic target through inhibition of protein synthesis e.g., macrolides and tetracyclines^{110,111} or meddling with nucleic acid synthesis, e.g., fluoroquinolones¹¹²
- 3) Modification of cell permeability by alterations in outer membrane, e.g., aminoglycosides or using novel membrane transporters, e.g., chloramphenicol¹¹³.
- 4) Through metabolic pathway “bypass” or inhibition, e.g., trimethoprim-sulfamethoxazole¹¹⁴.

In 1988 was the first report of *L. monocytogenes* strains resistant to >10 μ g of tetracycline per mL and isolation of a clinical multiresistant *L. monocytogenes* strain in France¹¹⁵. From then, several strains of *Listeria* spp. that are resistant to one or more antibiotics have been isolated and

described^{39,105}. Although the number of *Listeria* spp. resistant to antibiotics (excluding tetracycline) remains low, the appearance of multiresistant strains makes doubtful the general claim that *Listeria* is a bacterial genus consistently susceptible to antibiotics¹⁰⁵. A food-borne strain of *L. monocytogenes* resistant to trimethoprim³⁹, a clinical multiple antibiotic (including gentamicin) resistant isolate of *L. monocytogenes*¹¹⁶ and resistance to streptomycin, erythromycin, kanamycin, sulfamethoxazole, or rifampin^{39,105,117} have been observed. It is debated that antibiotic resistance in *Listeria* could be transferred by genes found on plasmids and transposons from saprophytic or commensal isolates to human isolates of *L. monocytogenes*^{26,44-46}. Self-transferable plasmids suggested to originate in *Enterococcus-Streptococcus* have been implicated in multiple antibiotic resistance¹¹⁵ and resistance to tetracycline^{39,105} in *L. monocytogenes* in addition to conjugative transposons. The digestive tract of humans and animals has been proposed to be where *Listeria* spp. acquire these conjugative plasmids and transposons from *Enterococcus-Streptococcus*^{105,118}. This rise in resistance to conventional antibiotics is now a major public health concern^{18,39,105}.

There are also several reports of resistance by *L. monocytogenes* strains to nisin and other bacteriocins^{89,95,97,103,119} with the term “resistance” referring to the ability of strains to grow at the highest bacteriocin concentration available. The proposed nisin resistance model of *L. monocytogenes* ATCC 700302 by Crandall & Montville⁹³ (reviewed by Kaur *et al.*¹²⁰) included three factors: 1) alteration of peptidoglycan composition¹²¹ possibly leading to increased binding of divalent cations that interfere with the cationic peptide; 2) phospholipid content changes leading to modified membrane electric charge which inhibits pore formation; and 3) decreasing membrane fluidity which prevents peptide insertion and association. The proposed mechanisms of resistance to class IIa bacteriocins specifically leucocin A resistance in *L. monocytogenes* B73-MR1 involve absence of a putative mannose-specific PTS enzyme IIAB subunit¹⁰⁰, up-regulating the synthesis of a putative β -glucoside-specific PTS enzyme II (EII^{Bgl}) and a phospho- β -glucosidase, as well as increased membrane fluidity by increasing levels of desaturated and short-acyl-chain phosphatidylglycerols in the membrane^{89,92,95}. Furthermore, bacteriocin cross-resistance in which resistance to a bacteriocin leads to resistance to other bacteriocins of the same class or in other classes reduces the efficiency of bacteriocins⁹⁴.

In response to the emergence of resistance to currently available drugs and disinfectants by *L. monocytogenes*, other antimicrobial peptides are investigated for antilisterial activity to complement or replace the bacteriocins as natural food preservatives or serve as therapeutic agents. Those that have shown significant activity include AMPs of animal origin, human defensins^{122,123}, protamine^{122,123}, plectasin, novicidin, novispirin G10¹²³ and magainin¹²², the

plant-derived thionin and snakina¹²² and bacterial subtilosin from *Bacillus amyloliquefaciens*¹²⁴. Spathelf and Rautenbach¹²⁵ observed that the six major tyrocidines from commercial tyrothricin had significant lytic and growth inhibitory activity towards *L. monocytogenes*. The leucocin A resistant strain, *L. monocytogenes* B73-MR1 was more susceptible than the sensitive *L. monocytogenes* B73 strain. Therefore, peptide antibiotics and AMPs are a promising new generation of antibiotics in drug discovery to combat resistant pathogens^{8,9,14,126–128}.

1.3. Peptide antibiotics and antimicrobial peptides

Peptide antibiotics have an average molecular mass ranging from 270 (bacilysin) to approximately 4500 (licheniformin)¹²⁹ and are also classified as antimicrobial peptides (AMPs) defined as “small, cationic, amphiphilic peptides, characterized by microbicidal activity against bacteria, fungi, viruses and other pathogens”¹³⁰. AMPs vary extensively according to their sequences but they can be grouped according to their secondary structure under four main categories namely those containing either α -helix, β -sheet, extended coil or loops^{13,15}. They are also grouped as linear or cyclic peptides (including cyclic lipopeptides) and could be of natural or synthetic origins (Table 1.1).

These peptides are attractive as substitutions for classic antibiotics because of their fast and effective microbicidal action combined with a decreased likelihood for microbes to develop resistance to their non-specific membrane-mediated mechanism of action^{9,12,16–22}. Their molecular simplicity also makes them appealing for development of future antibiotics¹³¹.

The nature of the antimicrobial peptide, the characteristic of the cell membrane and the metabolic state of the target cells all contribute to determine the mechanisms of action of antimicrobial peptides and hence their activity^{12,132–137}. The physicochemical parameters related to peptide structure that control their activity include conformation, hydrophobicity, charge distribution, comparative size of the polar/nonpolar face, and amphipathicity^{10,12,135,136,138–150}. These parameters are relevant to different steps in the multi-step mode of action of AMPs^{146,147,151}. Moreover AMP activity is conditioned by environmental factors like ionic strength and pH^{134,145} which would influence peptide aggregation and interaction with charged membrane surfaces of target cells^{145,152–155}. Important to the activity and selectivity of AMPs is their cationic and amphiphilic nature as these factors are relevant for the initial interaction with microbial membranes^{131,180}. Accumulation at polyanionic microbial cell surfaces is ensured by the overall positive charge of the AMPs^{12,141,144,146,147,181} because microbial membranes contain acidic polymers, such as lipopolysaccharide (LPS) (in Gram-negative bacteria)¹⁸² and wall-associated teichoic or teichuronic acids (in Gram-positive bacteria)¹⁸³. The AMPs eventually

reach the cell membrane through self-promoted uptake, a hypothesis proposed by Hancock and Chapple⁸. Upon making contact with the anionic surface of the external lipid layer of the cytoplasmic membrane, these peptides firstly interact with the polar head groups of membrane phospholipids through their hydrophilic face and then the hydrophobic face interacts with fatty acyl chains for membrane insertion¹³¹. Peptide accumulation at the membrane and ensuing permeation therein are therefore influenced by peptide amphipathicity and hydrophobicity^{144,146,147,165,184-190}.

Following insertion into the membrane, AMPs cause an array of structural distortions or damages to the membrane architecture using the following possible mechanisms adapted from¹³¹.

- 1) Carpet-like mechanism in which peptide molecules gather parallel to the surface and have a detergent-like effect on the membrane via thinning^{9,182};
- 2) “Torroidal-pore or wormhole model” which proposes that peptide molecules self associate to form transient pores^{187,189,191};
- 3) The insertion of peptides into the membrane could lead to the formation of a water core lined by both the inserted peptides and the lipid head groups resulting from an induced bending of lipids near peptide aggregates which leads to disruption of the membrane barrier function¹⁸⁸.
- 4) The peptides could also translocate across the cytoplasmic membrane and act on internal targets through unique mechanisms^{9,22,25,108,184,192,193} for example, the proline hinge of some buforin II analogues enabled them to penetrate the cytoplasmic membrane without permeabilisation and determined their antimicrobial potency¹⁹⁴. The Pro-rich AMP apidaecin undergoes membrane translocation via a permease/transporter-mediated mechanism¹⁹⁵.

Table 1.1 Natural and synthetic antimicrobial peptides classified according to secondary structure. Standard one-letter amino acid code was used in the primary structure of the peptides with the following additions: lower case letters represent the D-enantiomers; all other amino acids are L-form. O represents ornithine. Kyn: kynurenine; n: D-Asn; a: D-Ala; s: D-Ser, f: D-Phe, y: D-Tyr, and MeE: methyl glutamate. The subscript numbers show cysteine residues that are joined by disulfide bridges.

Structural group	Examples	Origin	Primary structure	Source
α -helical peptides	Magainin 2	Frog	GIGKFLHSAKKFGKAFVGEIMNS	156
	Penetratin	Drosophila	RQIKIWFQNRRMKWKK	157,158
	Temporin L	Frog	FVQWFSKFLGRIL	159
	NLK-18	Synthetic	YKLLKLLLPKLLKGLLFKL-NH ₂	160
	MSI-78	Synthetic	GIGKFLKKAKKFGKAFVKILKK-NH ₂	161
β -sheet peptides	Protegrin-1	Pig	RGGRLC ₁ YC ₂ RRRFC ₁ VC ₂ VGR-NH ₂	162
	(KIGAKI) ₃ -NH ₂	Synthetic	KIGAKIKIGAKIKIGAKI-NH ₂	163
Extended peptides	Indolicidin	Cattle	ILPWKWPWWPWR-NH ₂	164,165
	Histatin-5	Human	DSHAKRRHHGYKRKFHEKHSHRGY	166
	CP-11	Synthetic	ILKKWPWWPWRK	167
Disulphide bonded β -turn loop peptides	Bactenecin	Cattle	RLC ₁ RIVVIRVC ₁ R	168,169
	Lactoferricin	Cattle	FKC ₁ RRWQWRMKKLGAPSITC ₁ VRRAF	170,171
	BacP ₃ R-V	Synthetic	RRRLC ₁ PIVIRVC ₁ RR	172
N \rightarrow C cyclic peptides	Gramicidin S	Gram + Bacteria	<i>cyclo</i> (VOLfPVOLfP)	173
	GS10	Synthetic	<i>cyclo</i> (VKLyPVKLyP)	174
	Tyrocidine C	Gram + Bacteria	<i>cyclo</i> (VOLFPWWNQY)	175
N \rightarrow C cyclic lipopeptides	Iturin A	Gram + Bacteria	<i>cyclo</i> (amino-octadecanoyl-NynQPnS)	176,177
	Daptomycin	Gram + Bacteria	<i>cyclo</i> (n-decanoyl-WnDTGODaGs-(2S,3R)3-MeE-Kyn)	178,179

In order for the AMPs to target intracellular *Listeria* infection they must be able to translocate over membranes. Several Arg-rich peptide groups have been shown to translocate across cellular as well as nuclear membranes. These include HIV-1 peptide Tat (48-60) and analogues, NLS peptides, RNA-binding peptides, DNA-binding peptides and polyarginine and arginine-rich antimicrobial peptides¹⁹⁶. In addition to the Arg residues, the two Trp residues of penetratin, a *Drosophila* antennapedia peptide, have been shown to be relevant in the translocation of the peptide across cell membranes¹⁵⁸. Studies with the cathelicidin-derived peptide tritrpticin which is rich in all three residues (Pro-, Arg- and Trp-rich) indicated that it acts by both membrane depolarization and secondary intracellular targeting¹⁹⁷. However, when the two Trp residues of tritrpticin were replaced with Phe residues, the resulting analogue lacked membrane

permeabilising activity and translocated the membranes for improved antibacterial action ¹⁹⁷. The mechanism of uptake of Arg-rich peptides and penetratin involves endocytosis mainly by macropinocytosis, clathrin-mediated endocytosis and caveolae/lipid-raft-mediated endocytosis ^{198,199}. On the other hand histatins translocate fungal cell membranes by binding to a receptor and induce non-lytic loss of ATP from metabolically active cells ²⁰⁰. Non-lytic mechanisms of antimicrobial action of the internalized peptides involve modification of cell division via the cytoplasmic membrane septum formation, inhibition of cell-wall synthesis, inhibition of nucleic acid synthesis, inhibition of protein synthesis or enzymatic activity inhibition, cell cycle disruption and consequent production of reactive oxygen species ¹⁰⁸. The study reported in this dissertation includes a group of synthetic cyclic peptides rich in Arg and Trp residues (RW-peptides). The fact that their primary mode of action is not *via* membrane lysis attests to the role played by Arg and Trp in promoting translocation of AMPs across cell membranes. By comparing their antilisterial activity to that of the tyrocidines (Trcs), which also have Trp and Phe residues could assist in distinguishing which moiety of the Trcs is relevant for possible membrane translocation and interaction with the purported intracellular target(s).

The potential of AMPs to be a new class of antimicrobial agents is demonstrated in for example the extensive use of gramicidin S and polymyxin B as non-prescription topical agents and use of tyrothricin, consisting of the Trcs and linear gramicidins ²⁰¹, in non-prescription lozenges, ear drops and topical antibiotic creams ^{9,23}. Vancomycin, a cyclic glycopeptide has been in clinical use for several years and is considered the ‘antibiotic of last resort’ ⁶⁶. Also the lipopeptide daptomycin is one of the new classes of antibiotics used in clinical setting with systemic application ¹¹. However, challenges towards clinical application of antimicrobial peptide therapies include possible *in vivo* proteolytic degradation of peptides in relation to pharmacokinetics for systemic applications; incomplete information about toxicity; maintaining the high microbicidal activity at physiological conditions of salt, pH, and serum; as well as relatively high development and manufacturing costs ²⁰². Nevertheless, synthetic peptides which are stable, more cost-effective and have broad-spectrum activity are being developed to overcome these previous obstacles ^{22,23}. Efforts have seen several peptide leads at different levels of pre-clinical and clinical trials to treat a variety of bacterial, fungal, parasitic, and viral infections ^{9,11,22,23}. Examples include the magainin peptide/pexiganan acetate which is currently for the second time in phase III clinical trials for the treatment of diabetic foot ulcers and is developed by the companies Dipexium Pharma, MacroChem and Genaera ²². The modified R-type bacteriocins avidocin and purocin from *Pseudomonas aeruginosa* are in preclinical trials by AvidBiotics (S. San Francisco, California) for their potential as narrow spectrum antibiotics for

human health and food preservation ²². There is emphasis on fully optimising AMPs before moving to pre-clinical and clinical testing ²².

As mentioned above, bacteriocins have been seen as the most promising natural food-preservatives ^{85,86}. Bacteriocins are gene-encoded peptides produced by lactic acid bacteria or LAB ²⁰⁵ and are variable in their structure, functions, ecology and inhibition spectra ⁸⁵. Three main classes of bacteriocins have been identified on the basis of their biochemical and genetic properties ²⁰⁵. Class I bacteriocins are the lantibiotics, which are post-translationally modified small peptides containing atypical amino acid residues like lanthionine and methyl lanthionine. Class II bacteriocins are not modified and can be sub-classified as: class IIa (pediocin-like bacteriocins and possess antilisterial activity), class IIb (two-peptide bacteriocins) and class IIc (other one-peptide bacteriocins or non-pediocin-like bacteriocins). Class III bacteriocins include large, heat-labile proteins which show antimicrobial activity. However, the class I bacteriocin, nisin, the main bacteriocin used in food preservation, has the following the shortcomings as concluded by Mendoza *et al.* ²⁰⁹: it is unstable at neutral to alkaline pHs, its antimicrobial activity is reduced when it is included in complex foods, its solubility is low over the physiological pH range, and its activity is restricted to Gram-positive bacteria. In addition, the resistance by some *Listeria* spp. to a number of Class I and class II bacteriocins is currently a concern that must be addressed ⁹¹⁻¹⁰³.

Although previous studies indicated the potent antilisterial activity of the cyclic decapeptides tyrocidines especially towards the leucocin A resistant strain of *L. monocytogenes* ¹²⁵, only six analogues were tested whereas more than twenty natural analogues have been identified ²¹⁰. Studies with AMPs such as the cyclic RW-peptides and Trcs have reported that it is mainly the amphipathic balance of the different peptide analogues that are relevant to their antibacterial activity ^{125,203,204}. However, more analogues need to be evaluated for activity as well as to determine their stability, salt tolerance and toxicity with the aim of employing them in food preservation against drug and disinfectant resistant pathogens. There is also need to test for more analogues to establish the structural motifs that are required for optimal activity and selectivity.

1.4. Small cyclic antimicrobial peptides targeting *Listeria*

The flexibility of linear peptides causes the existence of a variety of possible conformations which leads to decreased selectivity and specificity of most linear peptides for their receptors ²¹¹. Cyclisation through peptide (amide or disulfide) bond formation is employed as one strategy to constrain the peptides ²¹¹. The advantages especially of small cyclic peptides over their linear analogues include: stability, resistance of proteolytic degradation, improved receptor selectivity,

better bioavailability and available conformational proximity for receptor binding^{211,212}. This Part I of the research project focussed on the tyrocidines (Trcs), small cyclic decapeptides and model Trp/Arg-rich cyclic hexapeptides in an attempt to identify essential structural motifs and pre-requisites for their antilisterial activity (Chapters 3 and 4).

1.4.1. The tyrocidines and analogues

The Trcs are cyclic decapeptide antibiotics^{65,213,214} produced by the bacteria *Bacillus aneurinolyticus* formerly known as *Bacillus brevis* as part of the tyrothricin complex together with gramicidins, a family of analogous peptides^{129,215–217}. Trcs are positively charged at neutral pH but do not have an overt amphipathic structure²¹⁸ and can thus be classified as cationic antimicrobial peptides¹²⁸. They are not gene-encoded but are produced via special thiotemplate enzymatic systems in aerobic sporulating soil bacteria *Bacillus aneurinolyticus*¹²⁶. Together with the gramicidins they form the tyrothricin complex, an alcohol-soluble, water-insoluble fraction thus named by René Dubos in 1939^{201,210,219–221}. They were discovered based on the observation that some bacilli exhibited antagonistic bactericidal activity to other microorganisms²¹⁹. Tyrothricin was eventually employed as the earliest antibiotic in clinical practice though discovered a decade after penicillin²¹⁰. The tyrocidines are strictly considered peptide antibiotics, but differ from conventional antibiotics in that they possess fast killing kinetics via pore-forming mechanisms in membranes unlike the slower antibiotic mechanism of primarily targeting a metabolic enzyme¹⁸. Their positive charge provides for selectivity and broad spectrum activity as they preferentially interact with bacterial membranes that are negatively charged as opposed to the neutral membranes of normal vertebrate cells¹⁴. Hence, resistance to Trcs will necessitate major modification of the lipid composition of the microbial membranes. Incidentally, no resistance has been reported to the tyrocidines²²². It is this unique mode of action shared with most AMPs that prompted the characterization of the antilisterial activity of the tyrocidines.

Previous research has already demonstrated the *in vitro* antilisterial and anti-malarial activity of the tyrocidines and also highlighted that their activity is related to the structure of the analogues^{45,125,223,224}. However, the tyrocidines also have high haemolytic activity which has halted their intravenous use²²². Knowledge of the structure-activity relationship of these type of cyclic peptides has been useful for separation of the haemolytic and antibacterial activity²²². For example synthetic analogues of gramicidin S (GS) in which the D-His and Lys residues replaced D-Phe and Orn retained good antibacterial activity with reduced haemolytic activity^{7,174}. Other GS analogues with increased ring size from 10 to 14 showed that perturbation of the amphipathic moment also resulted in suppressed haemolytic activity²²⁵. However, similar perturbations did

not improve selectivity in the decapeptide GS10_0 (*cyclo*[VKLhPVKKhP]) and analogues as they resulted in considerably reduced antimicrobial activity along with the suppressed hemotoxicity²²⁶.

1.4.1.1. Structural properties

Both the gramicidin and tyrocidine fractions of tyrothricin are a composite mixture of peptides²¹⁰. Nine linear gramicidins and 28 different tyrocidines have been identified from tyrothricin²¹⁰. The sequences of tyrocidine A^{227,228}, B²²⁹ and C²³⁰ were established over 50 years ago. In the major three tyrocidines A, B, and C, the phenylalanine residue(s) in the aromatic dipeptide unit is replaced by tryptophan residue(s). The Orn residue is sometimes substituted with Lys (Figure 1.1) while the Val and Leu residues are sometimes substituted by other aliphatic amino acids in variants of the tyrocidines. When the invariable Tyr is substituted with Trp or Phe the tyrocidine-like peptides are named tryptocidines and phenycidines (as designated by our group)¹²⁵. The primary structure of the tyrocidines is comparable other peptides such as GS (*cyclo*[VOLfPVOLfP])¹⁷³ and streptocidins (with major analogues (A-D) consisting of the structure *cyclo*[F¹P²L³W⁴/F⁴N⁵Q⁶Y⁷/W⁷V⁸O⁹L¹⁰])²³¹.

Under physiological conditions the cyclic decapeptide tyrocidine A (*cyclo*[f¹P²F³f⁴N⁵Q⁶Y⁷V⁸O⁹L¹⁰]) is basic and has an antiparallel β -pleated sheet conformation with β I and β II' turns stabilised by four backbone hydrogen bonds^{218,232–236}. The backbone conformation is conserved in the other major tyrocidines²³⁷ and this backbone conformation is mainly responsible for the tendency of tyrocidines to form homo- and heteropolymers (or aggregates) in aqueous solution^{217,218,230,237,238}. The occurrence of D- and L-amino acids is possibly essential for the spatial distribution of the hydrophilic and hydrophobic moieties of tyrocidines²³⁹. The residues Gln⁶ and Tyr⁷ do not fit into either side of the antiparallel sheet structure, but form a type II' β -turn with little contribution to the amphipathicity of the molecule²⁴⁰. The tyrocidines share the Val⁸Orn⁹Leu¹⁰D-Phe¹Val² pentapeptide sequence and backbone conformation with GS (Table 1.1) which is a cyclic decapeptide produced by *Aneurinibacillus migulanus* ATCC 9999^{16,135,232,241–244}.

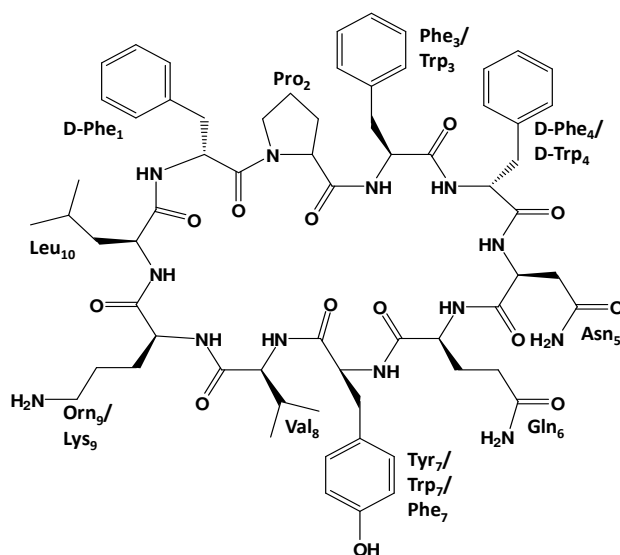


Figure 1.1 Structure of tyrocidine A showing possible residue substitutions in major analogues

1.4.1.2. Biosynthesis

In 1964, Mach and Tatum²⁴⁵ observed that the synthesis of the different tyrocidine analogues could be determined by the environmental concentration of constituent amino acids and attributed this fact to a low specificity of the enzyme systems which can incorporate certain structurally similar amino acids. In their case, the addition of Phe to the culture led to an almost exclusive synthesis of tyrocidine A at the expense of tyrocidines B and C. Adding Trp to the minimal medium compromised the synthesis of the Phe-rich forms of tyrocidine and a new tyrocidine was produced instead, tyrocidine D (renamed tryptocidine C), which contains three Trp and no Phe. In the presence of both Phe and Trp, the four tyrocidine analogues were synthesized. This phenomenon can be exploited to achieve high yields of specific analogues in high purity for use in bioassays.

The onset of tyrocidine production occurs when logarithmic growth turns into the stationary phase immediately before the start of spore formation^{246–251} and continues during spore formation. Lee *et al.*²⁵² observed the coincidence between the early stages of sporulation of *Bacillus brevis* 8185, the increase in uptake capacity for tyrocidine-constituent amino acids and tyrocidine production, and suggested that accumulation of amino acids and the synthesis of tyrocidine occurred in forespores. It has been observed that tyrocidine production rate is rapid and only occurs within a short period such as in the case of *Bacillus brevis* 10068 where synthesis started round about 26 hours following inoculation²⁵³.

The tyrocidines fall under the class of microbial peptides that are non-ribosomally synthesized which includes among others, daptomycin, gramicidin S, isopenicillin N, surfactin, iturin A,

rapamycin, bleomycin and cyclosporine^{254–256}. Their synthesis is catalysed by the modularly organized non-ribosomal peptide synthetase (NRPS)-mega-enzymes, which also determine the sequence and chemical identity of the final peptide by the order of their catalytic units^{254–262}. The biosynthesis of the tyrocidines requires three complementary enzyme fractions of molecular weights 100 , 230 , and 444 kilodaltons²⁴⁶. These correspond to three peptide synthetases, Tyc A, Tyc B, and Tyc C (tyrocidine synthetases 1, 2, and 3) which activate 1, 3, and 6 amino acids respectively²⁴⁷.

A “multiple carrier model” has been proposed for NRPS enzymology²⁶³. Modules are formed by catalytic domains, each in charge of a particular synthetic step in peptide synthesis^{247,257,263–266}. In each module, an adenylation domain (A-domain, about 500 amino acids) is responsible for the selection of a specific substrate (amino or carboxy acid), producing an aminoacyl AMP-mixed anhydride through ATP hydrolysis^{247,254}. The 4'-phosphopantotheine (PPan) prosthetic group that is attached to the peptidyl carrier protein (PCP) or thiolation domain, binds the reactive aminoacyl AMP-mixed anhydride via a thiol moiety^{267,268}. The next step involves the formation of a peptide bond between two bound flanking PCP intermediates catalyzed by the condensation domain (C-domain, about 450 amino acids)^{254,268}. This is followed by translocation of the ensuing peptidyl intermediate down the assembly line for successive condensation and modification steps making the upstream PPan-PCP available for subsequent reloading reaction²⁵⁴.

Supplementary domains located within the NRPS elongation module include the epimerization (E) domains that are found downstream of the PCP^{254,269,270}. They catalyse the conversion of L-configured amino acids to their corresponding D-isomers when bound to the cofactor PPan²⁵⁴. The final step involves the release of the peptide product from the NRPS-template by the thioesterase domain (TE-domain, ~280 amino acids), linked to the terminal module of Tyc C synthetase^{271–274}. The reaction involves the translocation of the completed peptide chain from the last PCP to the active site Ser of the TE, producing a peptidyl-acyl-enzyme intermediate (acyl-O-TE)²⁵⁴. Therefore, catalysis involves a sequential acylation and deacylation of the active-site Ser residue²⁵⁴. In the case of Trcs, the TE domain catalyzes a macrocyclisation using an intramolecular nucleophilic attack producing a cyclic lactam²⁵⁴. Tyrocidine synthetase 3 (723,577 Da) is composed of six modules and harbours the TE domain at its C-terminal end^{247,254}. The NRPS assembly is found at the cytoplasmic membrane of the producing *Bacillus*. It is speculated that this sub cellular localization is necessary for the released cyclic Trc to be transported out by a committed ABC transporter, maintaining a low intracellular concentration in the organism as part of its self-defence^{247,275}.

1.4.1.3. Mode of action

Like most AMPs, tyrocidines can induce cell lysis, but lysis has been demonstrated also to be a resultant process caused by autolytic enzymes²⁰¹. Trc activity against bacteria was attributed to the inactivation of the glucose dehydrogenase system which consequently affected metabolic activity^{201,219}. Trc was observed to specifically and reversibly interact with and inhibit acetylcholinesterase found in excitable membranes²⁷⁶. This interaction was dependant on the positive charge of the Trc. A similar Trc inhibition was found in the same study with β -galactosidase, a soluble cytoplasmic enzyme²⁷⁶. Trcs are able to bind to DNA in the producer strains as non-specific repressors leading to inhibition of transcription²⁷⁷⁻²⁷⁹, and their antimicrobial action could also involve a similar mechanism²²⁴. The sequence, specifically the identity of the aromatic dipeptide unit, of the six major tyrocidines from tyrothricin was observed to be relevant only to growth inhibitory activity against three Gram-positive bacterial strains including two *Listeria* strains. Conversely cell lysis, was independent of sequence, supporting the hypothesis of an alternative sequence dependant target or mode of action (MOA)¹²⁵. Nevertheless, the β -sheet structure and related amphipathicity of the Trcs, specific dimer form of the Trcs^{ref} and the analogous GS predisposes them for interaction with bacterial membranes. The hydrophilic and cationic residues will bind to the anionic phospholipids while the lipophilic groups should insert into hydrophobic core of the lipid bilayer to form a non-selective porous channel leading to membrane destabilization, loss of structural integrity and eventually membranolysis^{16,280,281}.

1.4.1.4. Structure - activity relationships

Correlation of the growth inhibitory activity of these tyrocidines against Gram-positive bacteria with several physicochemical parameters like solution amphipathicity, theoretical lipophilicity, side-chain surface area and mass-over-charge ratio similarly indicated that Orn was more important for activity than Lys¹²⁵. However, it was the Trcs containing Trp (Trc C, Trc C₁, Trc B and Trc B₁) that were more active than the more hydrophobic Phe-containing (A and A₁) peptides. Moreover, the results indicated that the aromatic residue on position 3 had a major influence on activity. This agreed with previous studies by Danders *et al.*²¹⁵ who showed that substitution of this hydrophobic residue at position 3 in Trc A with Val led to a decrease of its antibacterial activity, as well as considerable decrease in its capacity to inhibit both active transport in membrane vesicles and *in vitro* transcription. Doubly charged Trc A with a D-Phe⁴ to Orn substitution was also found to be less active in relation to the parent Trc A. They suggested that such residue replacements may intervene with the topographical distribution of

the hydrophobic and hydrophilic regions of Trc A proposed by Kuo and Gibbons²³². In contrast, Marques *et al.*¹⁶ recorded a 2- to 8-fold increase in the minimum inhibitory concentration (MIC) for two Trc A analogues with increased charge towards a range of pathogenic bacteria. In one of the two analogues, Gln⁶ was replaced with a Lys while in the other analogue an additional D-Phe⁴ to D-Lys⁴ substitution was made. However, just a D-Phe⁴ to D-Lys⁴ substitution led to a loss of activity. They concluded that there is a preference for increased amphipathicity within a range for improved antibacterial activity, but did not detect an obvious inclination for increasing hydrophilicity or hydrophobicity in improving antibacterial activity. In addition, increasing the charge in certain analogues improved their activity against the Gram-negative bacteria *Escherichia coli* towards which Trcs are normally not active^{125,282}. Qin *et al.*²²² also demonstrated that an Ala for Gln⁶ and D-Phe⁴ substitution in Trc A each led to a significant improvement of the therapeutic index without affecting the antimicrobial potency. The selectivity index was significantly further enhanced by up to 140-fold through the introduction of the cationic amino acid residual Orn, Lys, or Arg to position 6 by increasing the antibacterial potency. In a further study, they showed that replacing either or both D-Phe⁴ and Phe³ with two small aliphatic amino acids other than Ala or one aliphatic and one aromatic residue improved the therapeutic index and antibacterial activity²⁴⁰. They attributed this to the loss of hydrophobicity which would reduce interaction with mammalian cells²⁴⁰. In a previous study by Kohli *et al.*²⁸³ replacing D-Phe⁴ with a charged residue like D-Arg, D-Lys, or D-Orn also improved the selectivity index. They also indicated the relevance of the cyclic nature of Trc A by noting that the synthetic linear analogue was inactive. Joo *et al.*²⁸² designed and synthesized a 400-member combinatorial library of Trc A analogues by randomly replacing the Val⁸ and Orn⁹ with 20 L-amino acids. They identified that Pro⁸-Lys⁹ and Lys⁸-Lys⁹ analogues with an L- rather than a D-epimer of L-N^δ-propylasparagine (L-PrAsn) at position 5 led to improved activity against both Gram-positive and Gram-negative bacteria. This method was later used to develop a larger library of Trc A analogues (1716 analogues) by randomising positions 4, 6, and 10²¹⁴. An analogue with D-Arg, Gly and L-4-fluorophenylalanine (Fpa) at positions 4, 6 and 10 respectively was identified to be 4-8 times less toxic than the parent compound to human erythrocytes while maintaining antibacterial activity.

These previous studies show the possibility to significantly improve the therapeutic index of the Trcs notably Trc A through structural modification indicating charge and hydrophobicity as the most crucial factors for antimicrobial activity.

1.4.2. Model Trp-Arg rich cyclic hexapeptides

Efforts to understand the mechanism of action of AMPs and reduce unwanted toxicity, as well as production costs have involved combinatorial library synthesis strategies and development of shorter analogues that retain activity, in order to increase information about structure-activity relationships^{9,131}. This is possible because the AMP sequence in itself is not central to its antimicrobial activity and cytotoxicity as much as certain biophysical features of its structure that are maintained in shorter oligomers^{9,131}. These physicochemical and structural factors include cationicity, hydrophobicity, amphipathicity, structural propensity, etc. A delicate balance of these structural parameters is necessary to optimise the antibacterial potency and minimize host cell toxicity²²¹. It has also been demonstrated that two types of amino acid side chains are crucial for antimicrobial activity. The first type is the positively charged side chains of Arg (R), Lys (K), and His (H), because of their role in facilitating peptide interactions with negatively charged membranes and/or cell walls of bacteria, as well as lipopolysaccharide²⁸⁴. Second, the bulky non-polar side chains of Pro (P), Phe (F), and Trp (W), are relevant in forming lipophilic interactions that eventually bring about membrane disruption^{285,286}. According to the Liu-Deber hydrophobicity scale²⁸⁷, the aromatic side chain of Trp is considered to be the most hydrophobic of these residues.

Small cyclic antimicrobial peptides which predominantly contain the residues Arg, Trp, and Pro have received great attention as lead compounds²⁸⁸⁻²⁹¹. The foundation of this interest is their presence as small motifs in larger natural compounds with antimicrobial activity^{139,292} such as the Arg and Trp-rich antimicrobial peptide lactoferricin, a 25-residue cyclic peptide from degradation product of lactoferrin which is glycoprotein that functions in iron binding^{170,171} (Table 1.1). Other Arg and Trp-rich antimicrobial peptides include tritrpticin (VRRFPWWPFLRR) which was recognized as a cDNA stretch in a porcine cathelicidin with broad spectrum antimicrobial activity²⁹³, indolicidin (Table 1.1) which was isolated from bovine neutrophils^{164,165}. Even short chain sequences derived from the pharmacophore of these peptides as small as trimers (WRW and RWR) retained antimicrobial activity^{285,290}. A group of synthetic hexapeptides that share the cationic, amphipathic and aromatic character of the Trcs and thus are potential antilisterial and antimalarial candidates are the analogues of *cyclo*(RRRWWF) or RW-peptides (Fig. 1.4). Following the discovery through screening of synthetic combinatorial libraries of the linear analogue (Ac)-RRWWRF-NH₂²⁹⁴ and subsequent cyclization that improved antibacterial activity¹⁴⁹ they have been subjected to several activity and biophysical studies^{149,203,204,295-300}, but not yet evaluated for their activity against the intracellular pathogens *L. monocytogenes* and *Plasmodium falciparum*. Remarkably the most active and parent analogue

cyclo(RRRWFW) has been found to be primarily non-membranolytic¹⁴⁹. The Arg provides the cationic character and hydrogen bonding capacity while the hydrophobic bulky amino acid Trp is preferentially located in the bilayer interfacial region, permitting an extended attachment and a deeper membrane insertion^{296,301}.

1.4.2.1. Structural properties

The parent RW-peptide *cyclo*(RRRWFW) or c-WFW (Fig. 1.2) is a cyclic analogue¹⁴⁹ of the linear peptide (Ac)-RRWWRF-NH₂²⁹⁴. Within a membrane-mimetic environment, the linear analogue assumes an amphipathic structure³⁰².

An analogue of c-WFW, c-RW (*cyclo*[RRWWRW]) was observed to possess two β -turn motifs using solution NMR spectroscopy²⁹⁶. Likewise nuclear magnetic resonance (NMR) studies showed that in a membrane-mimicking environment, c-WFW assumes a structure with two regular β -turns in which the side chains of the clustered aromatic residues of c-WFW are positioned in the hydrophobic section while the polar backbone and the charged residues are found in the lipid head group segment²⁹⁷. The analogue c-RW 2 (*cyclo*[RRRWFW]) was observed to have one regular and one irregular β -turn structure²⁹⁷. Therefore, the peptide backbone is a flexible scaffold that can adapt in order to generate an amphipathic molecule that permits proper orientation of the side chains²⁹⁷.

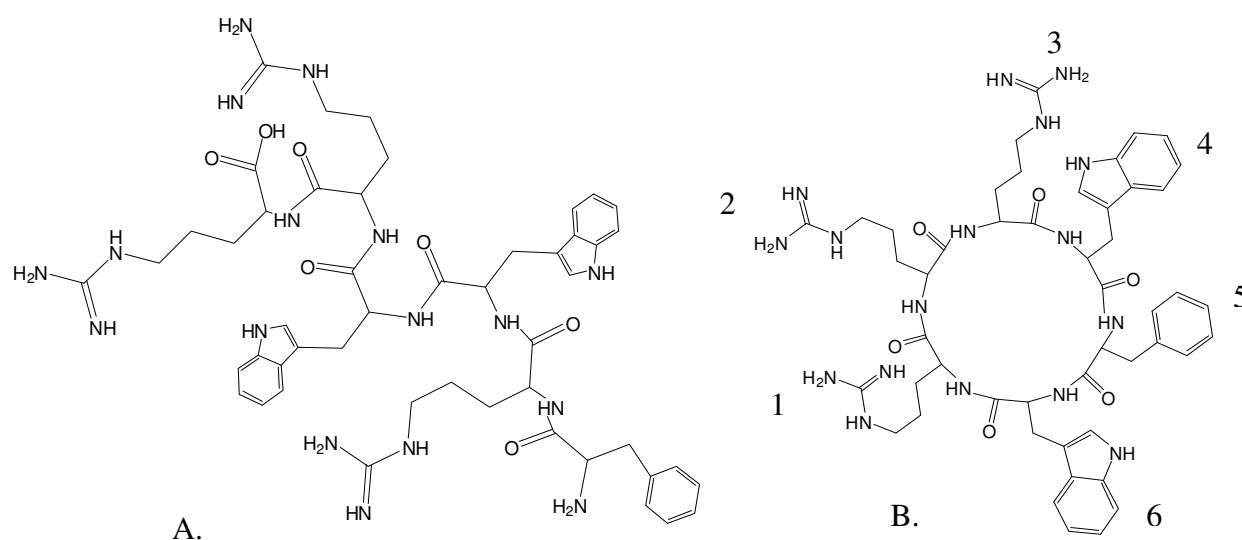


Figure 1.2 Primary structures of **A.** (Ac)-RRWWRF-NH₂ and **B.** linear peptide cyclic hexapeptide *cyclo*(RRRWFW). Structures were drawn using ChemDraw Ultra 10.0 (CambridgeSoft[®], UK)

1.4.2.2. Mode of action

The peptides c-WFW and c-WWW fulfil the structural prerequisites for membrane activity (positively charged and amphipathic). When c-RW was made to interact with detergents and lipids, it switched from the flexible structure observed in water to an amphipathic ordered structure²⁹⁷. Circular dichroism (CD) of c-WFW in negatively charged POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1)-glycerol] small unilamellar vesicles, Gram positive bacteria model membrane phospholipids, suggested insertion of the hydrophobic cluster into the lipid acyl chain region resulting from reorientation of the side chains with respect to the backbone²⁰⁴. A similar observation was made for c-WRW (*cyclo*[RWRWRW]). In contrast, the c-WWW (*cyclo*[RRRWWW]) analogue was observed to be more stable with reduced influence in the presence of POPG, trifluoroethanol (H-bond stabilising solvent), guanidinium-hydrochloride (denaturant) and increased temperature, on its secondary structure²⁰⁴. Reducing the ring size by omission of one R residue in c-WFW 5 resulted in large negative ellipticity values indicative of a more constrained structure than c-WFW whereas increasing the cycle size to 10 and 12 did not significantly affect the CD spectra²⁰⁴. Contrary to the negative ellipticity minimum of c-WFW at 202 nm (associated with backbone peptide bonds), the analogue c-KRK (*cyclo*[KRKFW]) registered a positive ellipticity under 200 nm indicating a change in the backbone conformation³⁰³.

It was also observed that the reversed phase HPLC retention times, t_R , which measures hydrophobicity and amphipathicity was reduced when W was replaced with F in the cyclic hexapeptides, when the positions of R and W were scrambled²⁰⁴, as well as replacement of R by K in the analogue c-KRK³⁰³. On the contrary, increase in ring size from 6 to 12 residues²⁰⁴, the absence of an R residue in c-WFW5²⁰⁴, as well as replacement of R by K in the sequence of the parent c-WFW in analogues c-RKR, c-RKK, c-KKR and c-KKK³⁰³ increased the t_R . These results led to the suggestion that the different analogues probably bind to and insert into lipid bilayers at varying depths²⁰⁴.

Based on molecular dynamic simulations, the peptide backbone supposedly lied parallel to the lipid bilayer surface, with the cationic Arg residues interacting with lipid phosphates, and the aromatic residues buried within the acyl chain region of DPPC (1,2-dipalmitoyl-sn-glycero-3-phospho-choline) liposomes²⁹⁹. However, it has been shown that membrane activity of RW-peptides is not their preferred mode of action²⁰⁴. The analogue c-WFW did not permeabilise POPG : 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-ethanoleamine (POPE) mixed liposomes (1:3 ratio) at a peptide to lipid ratio of 8:1 or induced influx of the membrane impermeable fluorescent stain propidium iodide into cells²⁰⁴. This peptide was also bactericidal towards *E.*

coli D21 at a MIC (maximum inhibitory concentration) of $8 \mu\text{M}$ ²⁰⁴. It was observed that c-WFW induces morphological changes in *E. coli* DH5 α (Dr. Dathe, personal communication).

Using DSC (differential scanning calorimetry) and FT-IR (Fourier transform infrared) analyses, it was shown that c-RW and Ac- RW (linear analogue) bring about a demixing in 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPG/DPPE) model membranes resulting in the development of two domains, a peptide-rich DPPG-peptide domain and a peptide-poor DPPE-enriched region. However, in DPPG/1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) membranes, the peptides only induced a considerable down-shift in the transition temperature of the mixtures³⁰⁰. c-WFW was observed to induce aggregation of POPE/POPG (1/3) vesicles (lipid concentration = $20 \mu\text{M}$)²⁰⁴. According to Junkes *et al.*²⁰⁴ the possible MOA of c-WFW involves a non-lytic mechanism related to the reorganization of functionally relevant lipid domains, lipid demixing and phase changes connected to changes in the function of bacterial membrane components. These may result in membrane translocation of the peptides which potentially interact with cytoplasmic components such as DNA/RNA. Lys-analogues of this peptide investigated by Scheinpflug *et al.*³⁰³ are thought to have a similar non-permeabilising mode of action. In the same study, it was observed that introduction of fluorophores within the hydrophobic cluster of c-WFW led to a change in the mode of action which relied on increasing hydrophobicity and led to membrane lysis³⁰³.

1.4.2.3. Structure-activity relationships

Quantitative structure-activity studies have indicated that charge and multiple Trp residues are required for antimicrobial activities of RW-peptides with the possibility of replacing Trp side chains by analogues with bulkier side chains^{138,304,305}. In shorter peptides with respect to cationic and lipophilic residues, the composition rather than the order of the amino acids is important for antibacterial activity^{138,290,306}. Exchanges between W and F in the hydrophobic region while maintaining the aromatic cluster and the cyclic backbone will not influence the two regular β -turns unlike ring size modification and scrambling of R and W residues which are expected to affect the backbone structure²⁰⁴ and may affect activity. Reducing the ring size by omission of one R residue in c-WFW 5 resulted in a more constrained structure than c-WFW whereas increasing the cycle size to 10 and 12 did not significantly affect the secondary structure as determined by the CD²⁰⁴. On the contrary, increase in ring size from 6 to 12 residues and the absence of an R residue in c-WFW5 increased the t_R ²⁰⁴, therefore increased hydrophobicity and haemolytic activity (this study, Chapter 6).

For example, scrambling of residues in c-WRW resulted reduced HPLC retention times, t_R , which measures hydrophobicity and amphipathicity and a shallower insertion of the peptide into model lipid bilayers in comparison to c-WWW and c-WFW as observed using W fluorescence spectroscopy²⁰⁴. c-RW analogues in which Trp was replaced with a lipophilic β -(2-naphthyl)alanine (Nal) or a bulky non-aromatic amino acid residue, bicyclo[1.1.1]pentane revealed higher bactericidal activity, while substitution with Tyr or Phe eradicated the activity^{291,292,307,308}. In contrast, the introduction of the bulky fluorophore, carboxyfluorescein within the *cyclo*(RRRWK[Fluos]) and *cyclo*(RRWWK[Fluos]) analogues led to a loss in activity due to the decrease in overall charge and modification of the peptide hydrophobicity and amphipathicity as reflected by an increase in the HPLC t_R ³⁰³. Single amino acid substitutions or replacement of L-amino acid residues by D- enantiomers have been shown to improve or eliminate the antimicrobial activity of c-WFW towards *E.coli*^{291,292,307,308}.

Arg has also been shown to be the preferred cationic residue over Lys in terms of antimicrobial activity^{306,309}, while Trp was observed to be the more relevant lipophilic residue in comparison to either Phe or Tyr^{290,291,310}. Substituting tryptophan with tyrosine or arginine with lysine does not affect peptide structure but changes the activity significantly^{291,296}. The guanidinium group of Arg is regarded as a more diffused positive charge than the single amine of K, which potentially enhances electrostatic interactions between peptides and the negatively charged bacterial membrane surface^{311,312}. Then again, the bulkier W side chain may guarantee a more efficient contact with membrane surfaces than F, P, or Y^{138,313}. Electrostatic effects, including dipole and quadrupole moments of Arg and Trp side chains, may contribute to the hydrogen bonding ability once the peptides make contact with membranes^{285,312,314,315}.

Analogues of c-WFW could thus in themselves serve as antilisterial drug leads or help us to understand the details of the mechanisms that govern selectivity of cyclic, amphipathic, and cationic peptides with a β -sheet conformation.

1.5. Concluding remarks

The motivation for this study is to further investigate the structural scaffold of the tyrocidines and the RW-peptides for the design of peptides improve the selectivity index toward bacteria, specifically *L. monocytogenes* (Chapter 3) and *Plasmodium falciparum* (Chapters 5 and 6). Moreover, the precise mechanism of tyrocidine action against the pathogens in this study is not fully understood and there is the possibility of alternative target(s)/MOA²²⁴ other than the membrane that needs to be investigated (Chapters 4 and 7). Identification of the target(s) could enable the rational design of lead compounds with improved selectivity indices based on the

most active tyrocidine analogue to combat drug-resistant *L. monocytogenes* and *P. falciparum*. However, in order to conduct these studies meticulous preparation of the peptide libraries was necessary and will be reported in Chapter 2.

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Chapter 2

Production, purification and characterisation of selected natural and synthetic Trcs

2.1. Introduction

The tyrocidines (Trcs) are cyclic decapeptides produced by *Bacillus aneurinolyticus* formerly known as *Bacillus brevis*¹ as part of the tyrothricin complex². The tyrothricin complex which consists of both the cationic cyclic Trcs and neutral linear gramicidins is mainly produced by *B. aneurinolyticus* cultures in late logarithmic growth phase¹⁻³. With the basic sequence of *cyclo*[f¹P²X³x⁴N⁵Q⁶Y⁷V⁸X⁹L¹⁰], the major Trcs only vary in three residue positions Trp^{3,4}/Phe^{3,4} and Lys⁹/Orn⁹⁴⁻⁸. Analogues are named with respect to the identity of the aromatic residue at position 7: Tyr (Trcs) or Trp (tryptocidines) or Phe (phenycidines) (Fig. 2.1).

As antimicrobial peptides (AMPs), the most striking character of Trcs include broad spectrum antimicrobial activity, selectivity, rapid action, and reduced likelihood of resistance development⁹. The major Trcs of the tyrothricin complex have shown considerable antilisterial¹⁰, antimalarial¹¹ and antifungal¹² activities. Like other AMPs, the Trcs have also shown pathogen selectivity due to their cationic nature which allows preferable interaction with more negatively charged bacterial cell membranes thus discriminating between pathogens and plant/animal hosts with neutral membranes^{11,13-15}. Hence, they represent a promising group of compounds in the fight against antimicrobial resistance to currently available drugs. Rational design to improve activity and selectivity of the Trcs requires establishment of structure to activity relationships (SAR). In order to achieve this aim it will be necessary to analyze as many structural analogues of the Trcs as possible.

Trcs were discovered in the acid precipitate of the culture medium of the aerobic sporulating soil bacteria *Bacillus aneurinolyticus*¹. The tyrocidine producers were originally referred to as the Dubos strain of *Bacillus brevis*¹⁶. However, it was demonstrated that *B. aneurinolyticus* is a distinct group with comparable phenotype¹⁷ and phylogenetics¹⁸ to the *B. brevis* group. Nevertheless, due to the fact that taxonomical descriptions of *B. aneurinolyticus* relied on only few strains¹⁹ this name is not official nor is it included in the “Approved lists of bacterial names”²⁰. Shida *et al.* have proposed a revival of the name *B. aneurinolyticus*²¹. The same

authors later suggested that the *B. brevis* cluster of bacteria be referred to as *Brevibacillus* gen. nov. and that bacteria of the *B. aneurinolyticus* cluster be referred to as *Aneurinibacillus* gen. Nov²². In this study, to avoid further confusion, we will be referring to the tyrothricin/tyrocidine producers as *B. aneurinolyticus*.

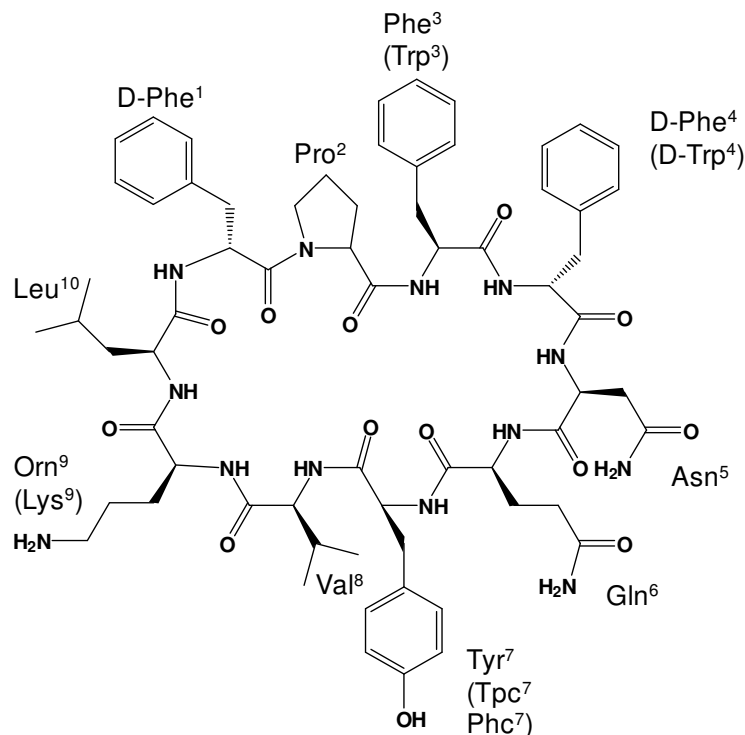


Figure 2.1 Primary structure of Trc A. The residues in brackets show that amino acid substitutions in other Trcs, the phenycidines and tryptocidines. Standard three letter amino acid abbreviations used for residues (Orn represents ornithine). The order of incorporation during biological synthesis is used to number the residues; optional residues are presented in brackets. Adapted from Rautenbach *et al.*¹¹.

Together with the gramicidins the tyrocidines form the tyrothricin complex, an alcohol-soluble, water-insoluble fraction thus named by René Dubos in 1939^{1-3,8,23}. These compounds were discovered based on the observation that some bacilli exhibited antagonistic bactericidal activity to other microorganisms². Tyrothricin was eventually employed as the earliest antibiotic in clinical practice though discovered a decade after penicillin⁸.

Trcs get produced by *B. aneurinolyticus* when the cultures start sporulating²⁴. Trcs interact with DNA possibly via a charged amino acid causing helical unwinding at low concentration and forming nuclease-resistant complexes with DNA at higher concentration²⁴. They are thought to function as a DNA regulatory factor involved in regulating the onset of sporulation by selectively inhibiting RNA synthesis during cell differentiation²⁴⁻³¹. This was supported by the

fact that addition of tyrocidine (Trc) induced sporogenesis and accompanied the synthesis of gramicidin in vegetative cells removed from a rich medium and submitted to severe nitrogen starvation^{24,26}. The gramicidins possibly counteract the inhibitory activity of the Trcs *in vivo* by influencing the DNA-Trc interaction²⁴. It has also been proposed that Trc packing of DNA may be beneficial in the ability of the spores to resist negative environmental factors²⁴. However, studies with mutant strains of *B. aneurinolyticus* defective in Trc production but capable of producing heat resistant spores indicate that in this strain at least Trc is not required for sporulation or spore resistance and thus its precise function is unknown in the producer organism^{25,29}.

There is a general limitation in terms of cost and efficiency of production of cyclic AMPs³² and the Trcs are no exception. Recombinant DNA technology as an option is specifically appropriate to produce large peptides such as insulin^{33,34} and is not very efficient in production especially of non-ribosomal peptides like the Trcs³⁵⁻³⁸. Although automated chemical synthesis is a viable method for production of small and medium size peptides³⁹ which make up the bulk of the peptides of pharmaceutical interest³⁴ it is very expensive⁴⁰. Previous work in our group employed natural analogues isolated from commercially available tyrothricin but Spathelf⁴¹ expressed difficulty in isolating certain Trc analogues that have shown promising activity namely tryptocidine B, tryptocidine C, and phenycidine A (latter named by our group) in sufficient quantity and/or purity from this source. More analogues could be isolated from the tyrothricin complex in *B. aneurinolyticus* fermentation broth as revealed in a study by Tang *et al.*⁸ in which 28 possible Trc analogues were identified. There is need for efficient strategies to produce the rare and potentially useful natural Trc analogues in sufficient amounts and purity for bioactivity analyses and physicochemical characterization so as to improve their inherent activity or bring about new activities.

In the past many groups achieved high yields of tyrothricin from cultures of producer strains⁴²⁻⁴⁶ and later production was achieved in cell-free preparations containing the Trc synthetase enzyme complex⁴⁷⁻⁵². Varying nutrients in the culture medium brought about changes in the secondary metabolism of *B. aneurinolyticus* leading to modulation of Trc production^{4,44,46,48,53}. This could be useful in cost-effective production of rare analogues in high yields and purity. Studying the modulation of the production of Trcs by changing the culture conditions of the producer strains of *B. aneurinolyticus* would be useful to induce the synthesis of novel Trcs and analogues⁴. Trc production by *B. aneurinolyticus* is governed by nitrogen supplementation with urea⁴⁴ and amino acids^{4,5,46} as well as carbon supplementation⁴⁶. It is also known that different *Bacillus*

strains produce different ratios of gramicidin to Trcs. *B. aneurinolyticus* ATCC 8185 is an ideal strain to principally produce Trcs with little or no gramicidins⁵³. The various strains also synthesize different major Trcs such as the *B. aneurinolyticus* (formerly *B. brevis*) DSM-5618 strain whose major Trc analogue produced is Trc C⁵³ and the *B. aneurinolyticus* (formerly *B. brevis*) ATCC strain 10068 that synthesises a broad spectrum of Trcs⁴. Focusing on nitrogen supplementation, Stokes and Woodward⁴⁶ showed that tyrothricin production in submerged aerated cultures was generally favourable in the presence of single amino acids as opposed to complex nitrogenous substances. They also observed that supplementation of culture media with specific amino acids used led to varying yields and compositions of tyrothricin. Baron⁴⁴, whose patented protocol is used by the Sigma company (St. Louis, USA) for tyrothricin production, observed that the addition of urea or its derivatives to the culture medium, containing nutrient mineral salts, glucose, a single amino acid or its salt, substantially boosted the yield of Trc. Baron⁴⁴ proposed that the presence of the urea or its derivatives also act in preventing contamination of the products and also suggested using submerged cultures with sufficient aeration for maximum tyrothricin production. However, Vogt *et al.*⁵³ suggested that sufficiently anaerobic conditions are required for gramicidin and Trc production. In addition, the investigators observed that production of the maximum amounts of tyrothricin were in media like peptone from *Escherichia coli* and yeast, peptone from *E. coli* or a mixture of twenty amino acids. Pipecolic acid, a proline analogue had been reported to selectively block the synthesis of Trc⁴⁷. However, Vogt *et al.*⁵³ found a similar Trc to gramicidin ratio irrespective of whether pipecolic acid was present or not in the culture medium. Nevertheless, use of pipecolic acid as sole nitrogen source inhibited growth of *B. aneurinolyticus*. Celtone, a commercially available rich medium, obtained from bacterial and yeast cultures, was shown to inhibit the growth of the *B. aneurinolyticus* ATCC 8185 strain⁵³. Martek, another commercially available medium acquired by partial hydrolysis of algal proteins, led to growth but not tyrothricin production⁵³.

Some researchers have demonstrated that the amino acid sequence of Trc produced by cultures was changed by the amino acid composition of the medium^{4,5,48}. This phenomenon is thought to be due to the low specificity of the enzyme systems in charge of incorporating some structurally related amino acids⁴. Experiments carried out by Mach and Tatum⁴ revealed that adding L-Phe to the culture medium led to an approximately exclusive production of Trc A at the expense of the other analogues. Likewise, addition of L-Trp led to an exclusive synthesis of Trc D (renamed by our group to tryptocidine C) with similar antibiotic activity as the A, B, and C analogues. In the presence of both L-Phe and L-Trp, all four forms (Trc A-D) were produced. Fujikawa *et al.*

⁴⁸ observed that the composition of the Trc synthesized by a partially purified Trc synthetase enzyme preparation of *B. brevis* ATCC 8185 was modified by changing the amino acids present during the incubation period. They were able to produce Trcs A, B, C, and tryptocidine (Tpc) C, as well as Trc E (renamed by our group to phenycidine A), Trc A₁ (Orn residue in Trc A was replaced by Lys) and isoleucyl-Trc A (Leu residue in Trc A was replaced by Ile) *in vitro*. The production of these Trcs was directly influenced by the amino acid concentrations in the reaction mixture. Moreover, certain unnatural amino acid analogues, such as 5-methyltryptophan, *p*-fluorotryptophan, thienylalanine and *p*-fluorophenylalanine were incorporated into Trc ⁴⁸.

In this study we used analogous amino acid supplementation in culture medium for the Trc producer of *B. aneurinolyticus* ATCC 8185 to develop a cheaper and more sustainable production process for the rare Trc analogues. A number of rare analogues and the major Trcs in commercial tyrothricin were isolated with established high performance liquid chromatography (HPLC) ⁵⁴ and analysed with analytical HPLC ⁵⁴, ultra performance liquid chromatography (UPLC) ⁵⁵ and electrospray mass spectrometry (ESMS) ⁵⁴.

High purity Trcs and analogues produced will be required for the analysis of their structure, biological activity and qualitative structure-to-activity relationships (QSAR) (Chapter 3). The Trcs for this study can be grouped into two libraries namely the Trc A library and Trc C library (Table 2.1), based on the identity of their aromatic dipeptide unit. Selected tryptocidines (Tpc) and a phenycidine (Phc), where the aromatic amino acid, Tyr⁷, was substituted with Trp or Phe respectively, would allow us to assess the role of the Tyr in the peptide activity (Table 2.1). The cationic residue would be assessed in terms of size, hydrogen bonding ability and integration into membranes, as Spathelf & Rautenbach ¹⁰ found lower antilisterial activity for the Lys-analogues, than the Orn-analogues. Following comparable antibacterial activity to the parent gramicidin S (GS) of analogues containing N-methyl groups including trimethylated-Orn analogues against Gram-positive bacteria ⁵⁶, we included a synthetic Trc A analogue, sTrc AOMe₃, containing a trimethylated-Orn residue (Table 2.1). From the reported improved activity of Trc A towards *Bacillus subtilis* ¹³, after charge increase with Gln to Orn substitution, this Trc A analogue (sTrcA(Q-O)), was also included. It should be noted that Trc B doubles as both a Trc A and a Trc C analogue.

We determined a number of structural parameters on the purified peptides for the qualitative structure-to- activity relationship (QSAR) analysis (refer to Chapter 3 for the QSAR study). The solution phase hydrophobicity of the Trcs and analogues were determined with reverse-phase HPLC (RP-HPLC) which separates the peptides based on differences in hydrophobicity. Time-

of-flight electrospray mass spectrometry (TOF-ESMS) was employed for the determination of monoisotopic mass and mass/charge (m/z) ratio. Homology modelling with YASARA 9.10.5[©] was used to elucidate *in silico* physicochemical parameters related to the three-dimensional structure of the Trc analogues.

Table 2.1 Summary of tyrocidines and analogues in this study.

Peptide	Abbr.	Sequence	Theoretical mono-isotopic mass (M_r)	Reference
Tyrocidine A	Trc A	<i>cyclo</i> -(VOLfPFfNQY)	1269.6568	⁵⁷
Tyrocidine A ₁	Trc A ₁	<i>cyclo</i> -(VKLfPFfNQY)	1283.6703	⁵⁸
Trc A with trimethylated Orn	Trc AOMe ₃	<i>cyclo</i> -(VO(CH ₃) ₃ LfPFfNQY)	1312.7090	This study
Trc A with Gln to Orn substitution	Trc A(Q-O)	<i>cyclo</i> -(VOLfPFfNOY)	1255.6754	¹³
Phenycidine A (Tyrocidine E)	Phc A	<i>cyclo</i> -(VOLfPFfNQF)	1253.6600	Named by our group, ⁸
Tryptocidine A	Tpc A	<i>cyclo</i> -(VOLfPFfNQW)	1292.6706	⁸
Tyrocidine B	Trc B	<i>cyclo</i> -(VOLfPWfNQY)	1308.6655	⁵⁷
Tyrocidine B ₁	Trc B ₁	<i>cyclo</i> -(VKLfPWfNQY)	1322.6812	⁵⁸
Tyrocidine C	Trc C	<i>cyclo</i> -(VOLfPWwNQY)	1347.6764	⁵⁷
Tyrocidine C ₁	Trc C ₁	<i>cyclo</i> -(VKLfPWwNQY)	1361.6921	⁵⁸
Tryptocidine B	Tpc B	<i>cyclo</i> -(VOLfPWfNQW)	1331.6793	⁸
Tryptocidine C (Tyrocidine D)	Tpc C	<i>cyclo</i> -(VOLfPWwNQW)	1370.6924	⁸

2.2. Materials

Bacterial strains: *Brevibacillus parabrevis* 8185 (referred to as *Bacillus aneurinolyticus* ATCC 8185 in this study) cultures were supplied by the American Type Culture Collection (Manassas, VA, USA). *Micrococcus luteus* NCTC 8340 cultures were obtained from National Collection of Type Cultures (Porton Down, Salisbury, United Kingdom).

Research materials: Deep 96-well plates, tryptone soy broth (TSB), Luria Bertani broth (LB), peptone, tryptone, yeast extract, glucose, agar, diethyl ether, sodium chloride, hydrochloric acid, L-Lys and L-Arg were obtained from Merck (Darmstadt, Germany). L-Cys, L-Phe, L-Tyr and L-Trp were supplied by Sigma Aldrich (Steinheim, Germany). Nutrient broth (NuB) was supplied by Unipath Ltd (Basingstoke, England). Ultra pure urea was got from ICN Biomedicals Inc. (Aurora, USA). Tween 20 was supplied by Fluka (Buchs, Switzerland), skimmed powdered milk

was from Clover (Roodepoort, South Africa), standard untreated, non-sterile polystyrene 96-well flat bottom plates and culture dishes were from Greiner bio-one (Frickenhausen, Germany) and Lasec (Cape Town, South Africa) respectively. Falcon[®] tubes were from Becton Dickson Labware (Lincoln Park, USA). Acetonitrile (HPLC-grade, far UV cut-off) and methanol (>99.9%) were from Romill Ltd. (Cambridge, UK). Analytical grade water was prepared by filtering water from a reverse osmosis plant through a Millipore-Q[®] water purification system (Milford, USA). Gramicidin S (GS), trifluoroacetic acid (TFA; >98%) and commercial tyrothricin were made available by Sigma (St. Louis, USA). Synthetic Trcs were supplied by GL Biochem (Shanghai) Ltd., China. Diethyl ether was provided by Merck (Darmstadt, Germany). Acetone was provided by Saarchem (Krugersdorp, RSA). The Nova-Pak[®] C₁₈ (5 µm particle size, 60 Å pore size, 150 mm × 3.9 mm) reverse-phase analytical column, the Nova-Pak[®] C₁₈ (6 µm particle size, 60 Å pore size, 7.8 mm × 300 mm) semi-preparative HPLC column and an ACQUITY UPLC[®] bridged ethyl hybrid (BEH) C₁₈ (1.7 Mm particle size, 2.1 mm × 100 mm) column were from Waters Millipore (Milford, USA).

2.3. Methods

2.3.1. Optimisation of Trc analogue production

2.3.1.1. Preparation of pre-cultures of *Bacillus aneurinolyticus* ATCC 8185

The culture stocks were preserved as lyophilized powder using 10% *m/v* skimmed milk in analytical quality water as lyophilisation buffer. Culturing was done using normal sterile techniques. Tryptone glucose yeast extract milk (TGYM) broth (0.5 g peptone, 0.25 g yeast extract, 0.1 g glucose and 0.1% *m/v* skimmed milk in 100 mL water, pH 7.0) was used to rehydrate the cultures before streaking them onto TGYM agar plates (0.5 g peptone, 0.25 g yeast extract, 0.1 g glucose, 0.1% *m/v* skimmed milk, and 1.5 g agar in 100 mL water, pH 7.0) and incubating for 48 hours at 37 °C. The choice of this pre-culture medium was justified by the works of Baron⁴⁴ and Fujikawa *et al.*⁴⁹. Single colonies were prepared by diluting a small amount of the culture from the plates with sterile broth and carrying out serial dilutions using the TGYM broth. Each sample (50 µL) was spread onto TGYM agar plates and incubated for 48 hours at 37 °C. Culturing of the bacteria for Trc production was adapted from the method of Baron⁴⁴. A single colony was pre-cultured by adding it to 5 mL of TGYM broth and incubating at 37 °C for 24 hours while constantly shaking at 20 × g.

2.3.1.2. Preparation of *Micrococcus luteus* NCTC 8340 cultures

M. luteus NCTC 8340 from freezer stocks was cultured using standard sterile methods on Luria Bertani (LB) agar (1 g tryptone, 0.5 g yeast extract, 1 g NaCl, and 2 g agar in 100 mL water) for 48 hours at 37 °C. Three to five selected colonies were grown for 16 hours at 37 °C in LB broth (1 g tryptone, 0.5 g yeast extract, 1 g NaCl in 100 mL water) with constant shaking at 15 × g to an optical density (OD) of 0.8 at 620 nm. These cultures were sub-cultured in TSB: 500 µL of cultures into 20 mL of TSB medium (3 g TSB powder dissolved in 100 mL of water) and incubated at 37 °C for 5-6 hours to an OD of 0.6 at 620 nm.

2.3.1.3. Radial diffusion assay to select colonies for optimum antibiotic production

Because colonial variation in the yield of Trcs is typical for the *B. aneurinolyticus* ATCC 8185 strain, there was need to select colonies that optimally produced Trcs. Radial diffusion assay was used for this purpose. It was executed according to the methods of Du Toit and Rautenbach⁵⁹ and Lehrer *et al.*⁶⁰ with modifications. *M. luteus* cultures (1 mL) in tryptone soy broth (TSB) at OD 0.6 was diluted with 9 mL of a sterilized gel solution at 45 °C (prepared using 1% *m/v* powdered TSB medium, 1% *m/v* low electro-endosmosis type agarose and 0.02% *v/v* Tween 20 in water). After homogenizing the mixture, it was poured onto sterile Petri dishes on a level surface and allowed to set for 30 minutes. Single colonies of *B. aneurinolyticus* ATCC 8185 were spotted equal distances apart onto the gel. A duplicate of the *B. aneurinolyticus* colonies was grown on a TGYM agar plate from which the selected colony was picked for propagation. The plates were incubated overnight at 37 °C in order to observe the antibacterial activity production of the different spotted colonies. The colony with the broadest inhibition zone selected for tyrothricin production was further cultivated on TGYM agar plates and monitored by assaying for antibiotic production as above.

2.3.1.4. The influence of supplemented nitrogen source on the growth of *B. aneurinolyticus* ATCC 8185

To investigate the influence of the amino acids and their concentration on the growth of *B. aneurinolyticus* ATCC 8185, the pre-culture was prepared as described above and then diluted four times using NuB. The amino acids chosen for supplementation were specifically the variable amino acids in the identified Trc analogues and other amino acids known to play a role during Trc synthesis by the multi-enzyme complex (Phe, Tyr, Trp, Lys, Cys, Met, β-Ala, and D/L-Phe). Specialised plates (96-deep well plates) were used for the culturing of the Trc

producers. The culture medium (total volume = 500 μL) supplemented with single amino acids at concentrations ranging from 0.01 – 1% *m/v* and 0.1% *m/v* urea was transferred into the deep wells of the plates in quadruplicate for each amino acid concentration.

To examine the influence of the concentration of urea on the growth of the Trc producer, NuB supplemented with a two-fold dilution series of urea ranging from 1 to 0.008% *m/v* were used as culture media. Each urea concentration was assayed in quadruplicate. For two columns of each plate, the 4 first wells contained only NuB while the 4 last wells contained NuB + 0.1% *m/v* urea. One of these columns was used as sterility control and did not receive any bacterial culture. The other was used to examine the effect of the absence of urea and amino acid on the growth of the bacteria.

The diluted pre-cultures (10 μL) were added to each well in the plate excluding those for sterility control. The homogenized cultures (200 μL) was immediately transferred into corresponding wells in a standard 96-well microtiter plate and light dispersion of the cultures was read every hour for 24 hours at 595 nm using the Bio RadTM microtiter plate reader. The data was analysed using GraphPad Prism® 4.03 (GraphPad Software, San Diego, USA) to determine the growth rates of the cultures.

2.3.1.5. High throughput analysis of the influence of nitrogen supplementation on the composition and antibacterial activity of produced Trc complex

A protocol was developed to investigate the influence of the concentration of the above amino acids on the composition as well as the antibacterial activity of Trcs from the culture extracts of *B. aneurinolyticus* ATCC 8185 against *M. luteus* NCTC 8340. For this purpose 96-deep well plates were used for the culturing of the Trc producers. The pre-culturing was done as mentioned above and then diluted four times using NuB. The culture medium supplemented with the highest concentration of a single amino acid (Phe, Tyr, Trp, Lys, Cys, Met, β -Ala, and D/L-Phe) and 0.1% *m/v* urea was added to the top wells of the plates and diluted down in the lower wells using NuB supplemented with 0.1% *m/v* urea. The supplemented nitrogen source (specific amino acid, amino acid mixture or urea) ranged from 1 – 0.008% *m/v* and the volume of culture medium in the wells was 300 μL . The amino acid concentrations were prepared in quadruplicates and 10 μL of the diluted pre-cultures was added to each well. For positive control, one column received no amino acid supplementation and another column serving as negative (sterility) control received no bacteria. The plates were then covered and incubated for 36 hours at 37 °C with constant shaking at 15 \times g. Each well's content was then acidified to a pH

of 4.7 with HCl and allowed to stand without stirring for 24 hours. The plates were then centrifuged at 2200×g for 60 minutes and the supernatant discarded. The pellet in each well was re-suspended in 200 µL of methanol and sonicated for 15 minutes and the 200 µL of analytical quality water was added to each well and the plate was again sonicated for another 15 minutes. The plates were again centrifuged at 2200×g for 30 minutes. The supernatant (10 µL) from each well was then transferred carefully to a sterilized standard 96-well plate containing the indicator organism, *M. luteus* for the investigation of antibacterial activity. An adaptation of the broth dilution method described by Du Toit and Rautenbach⁵⁹ and Lehrer *et al.*⁶⁰ was used. The *M. luteus* culture was diluted using TSB to an OD = 0.2 at 620 nm (5×10^5 CFU/mL) and 90 µL of this culture was transferred into each well of a sterilized 96-well plate except for those in the first column. The wells for sterility control received only 90 µL of TSB and 0.5% v/v methanol or sterile filtered analytical quality water. In the growth controls no Trc extract was added to the *M. luteus* cultures; 0.5% v/v methanol or water was added instead and other wells all received the supernatants (10 µL) from the deep well culture extracts. The microtiter plates were incubated at 37 °C for 16 hours. Subsequently, the growth inhibition was analyzed by spectrophotometry at 595 nm using a BioRad™ microtiter plate reader followed by data analysis using GraphPad Prism® 4.03 (GraphPad Software, San Diego, USA) as described by Du Toit and Rautenbach⁵⁹. The percentage growth inhibition was calculated using the following generic equation:

$$\% \text{ growth inhibition} = 100 - \frac{100 \times (A_{595} \text{ of well} - \text{Average } A_{595} \text{ of background})}{\text{Average } A_{595} \text{ of growth wells} - \text{Average } A_{595} \text{ of background}} \quad (1)$$

For the analysis of the Trc composition of the culture extracts, the supernatants derived from the *Bacillus* cultures were pooled together from the four wells for each amino acid concentration and transferred to glass vials. The solvent was evaporated under vacuum and the residual content was suspended in 50% v/v acetonitrile in analytical quality water for analytical HPLC (1.0 mg/mL) and ESMS (200 µg/mL) analyses.

2.3.1.6. Manipulation of Trc production by *B. aneurinolyticus* ATCC 8185 cultures

B. aneurinolyticus ATCC 8185 pre-cultures (5 mL) were transferred to 200 mL culture medium in 1 L Erlenmeyer flasks. The culture medium was made of nutrient broth (1.3 g NuB powdered medium in 100 mL water) supplemented with 0.1% m/v urea. The cultures were incubated at 37 °C with constant shaking at 15 × g for 36 hours. The pH of the cultures was adjusted to 4.7 with HCl and allowed to stand without stirring for 24 hours. The acidified media was centrifuged at 12 000 × g at 4 °C for 20 minutes. The precipitate was extracted three times with 20 mL

methanol by adding the methanol to the precipitate and then sonicating for 15 minutes in conical flasks at room temperature before centrifuging for 8 minutes at $2200 \times g$ in glass test tubes. The combined extracts were distilled at $64\text{ }^{\circ}\text{C}$ to remove the methanol before lyophilizing the residue overnight. The dried precipitate was washed thrice with 2 mL portions of diethyl ether and lyophilized again overnight. The extracted Trcs thus obtained were analysed by thin layer chromatography (TLC) against Trcs extracted from a commercial tyrothricin after dissolving them in acetone:diethyl ether (1:1) mixture. Subsequently, semi-preparative HPLC was done at 4 mg/mL in 50% acetonitrile diluted with analytical grade water. The major fractions were collected and analysed by electrospray mass spectrometry (ESMS) at a concentration of 200 $\mu\text{g/mL}$. The procedures for these analyses are detailed below.

To investigate the influence of the presence of amino acids in the culture medium on the Trc production, the above methodology was used with the exception that the culture media were supplemented with 1% *m/v* of single amino acids. In addition to the amino acids mentioned in *Section 2.3.1.5* influence of D-Phe was also examined. The influence of 1:1 *m/m* combinations of pairs of some amino acids to final concentration of 1% *m/v* was also investigated notably:

Lys + Phe/Trp

Tyr + Trp/Phe

Phe + Trp

We also investigated the following combinations with each amino acid at 0.5% *m/v*:

Lys + Phe + Tyr/Trp

Lys + Tyr + Trp

The influence of the presence or absence of 0.1% *m/v* urea in the culture medium was investigated as well following the same methodology used for amino acids. The culture media chosen for investigation included:

NuB + 0.3% Trp

NuB + 0.5% *m/v* Phe + 0.5% *m/v* Tyr

NuB + 0.5% *m/v* Lys + 0.5% *m/v* Phe + 0.5% *m/v* Tyr

The cultures were prepared in triplicate with 20 mL culture media spiked with 45 μL pre-culture and grown at $37\text{ }^{\circ}\text{C}$ in 50 mL Falcon™ tubes for 36 hours. Analytical HPLC and ESMS were used to analyse the Trc peptide composition of the culture extracts.

2.3.2. Purification of the Trcs

The peptides of interest in this study for purification included the Trcs and analogues from the commercial tyrothricin complex and from the tyrothricin isolated from culture extracts of the *B. aneurinolyticus* ATCC 8185 (Table 2.1). Some synthetic Trc analogues to be used in structure-to-activity relationship analyses were also purified and/or analysed.

Isolation of the Trcs from the tyrothricin complex was by the organic extraction method of Hotchkiss and Dubos⁶¹ previously used by Rautenbach *et al.*¹¹. Weighed tyrothricin dry powder was washed thrice in clear glass test tubes with a 1:1 mixture of acetone and ether (5 mL for 5 mg tyrothricin in each test tube). Following sonication, the precipitate which contained the Trcs was collected by centrifugation at $700 \times g$ for 8 minutes. The supernatant was decanted and the residue was dried under vacuum. The supernatant contained most of the neutral gramicidin fraction while the pellet contained most of the basic Trc fraction.

A preliminary analysis of purity of the crude extracts was done by thin layer chromatography (TLC) on aluminium backed Kieselguhr 60-F₂₅₄ TLC plates developed in a butan-1-ol:acetic acid:water (10:2:3) solvent. Samples were diluted to 1 mg/mL with 50% *v/v* acetonitrile in water and 5-10 μ L spotted on the silica plate for TLC. The TLC plates were visualised under ultraviolet light and also by spraying with ninhydrin. The samples used were: Trc extract (Trc), gramicidin supernatant extract (Grm), and commercial tyrothricin (Tcn). The gramicidin was expected as a spot near the solvent front (retention factor, $R_f = 0.9$) while the Trc fraction was expected at $R_f = 0.6$ ⁶².

Purification was done using the RP-HPLC purification methodology developed and optimised by our group⁵⁴ previously used to purify the six major Trcs from commercial tyrothricin¹¹. Prior to the semi-preparative HPLC analysis, the isolated Trc complex was dissolved to a concentration of 10 mg/mL in 50% *v/v* acetonitrile and centrifuged for 10 minutes at $8600 \times g$ to remove any particulate. A semi-preparative C₁₈ Nova-Pak HR column (6 μ m particle size, 60 Å pore size, 7.8 mm \times 300 mm) was used with two Waters 510 pumps, a MAXIMA controller system, a Waters Model 440 detector and WISP 712 sample processor. A solvent gradient (3 mL/min flow rate) was generated using eluant A (0.1% *v/v* TFA) and eluant B (90% *v/v* acetonitrile and 10% *v/v* eluant A). The chromatography was done at 35 °C and visualised using an inline Waters Model 440 detector at 254 nm. Peptide fractions from multiple 100 μ L sample injections were collected, lyophilized and stored dry at -10 °C for further analyses.

Table 2.2 HPLC purification and analysis gradient program with eluant A = 0.1% v/v TFA and eluant B = 90% v/v acetonitrile and 10% v/v eluant A; as defined by the Waters gradient control curve type ^{11,54}.

Time (min)	% eluant A	% eluant B	Curve type
0.0	50	50	-
0.5/1.0*	50	50	-
23.0	20	80	non-linear
24.0	0	100	-
26.0	0	100	-
30.0	50	50	linear
35.0	50	50	-

* 0.5 min for semi-preparative RP-HPLC, 1.0 min for analytical RP-HPLC

For the fractions in which the separation was observed to be unsatisfactory on the semi-preparative column, a further purification was done on a reverse-phase analytical C₁₈ Nova-Pak[®] HPLC column (5 µm particle size, 60 Å pore size, 150 mm × 3.9 mm) used with the system, solvents and gradient program described above for the semi-preparative HPLC, but with a 1.0 mL flow rate and 50 µL injection per chromatographic run. The gradient program employed for the HPLC is detailed in Table 2.2.

2.3.3. Characterisation of purified Trc analogues

2.3.3.1. Analytical HPLC

The purified fractions obtained from HPLC were further analyzed to determine their purity by analytical HPLC as previously described ⁴¹. Identical methodology (Table 2.2) and system were used for the analytical HPLC, but with 1.0 mL flow rate; 20 µL injection at lower peptide concentrations were injected on a C₁₈ Nova-Pak[®] HPLC column (5 µm particle size, 60 Å pore size, 150 mm × 3.9 mm). The concentration of the peptide samples was 100 mg/mL to 1.0 mg/mL, depending on peptide to limit aggregation. Analytical HPLC analyses retention time results were also used as a QSAR parameter to describe the solvent hydrophobicity of the Trcs and analogues.

2.3.3.2. ESMS and UPLC-MS analysis of purified peptides

ESMS was used to determine the identity (M_r), purity and m/z parameters of each of the purified peptides and peptide fractions. TOF-ESMS was done on a Waters Quadrupole TOF Synapt G2. Sample injections (3 µL) of purified (200 µg/mL) samples were used for direct mass analysis via a Waters Acquity UPLC[™] into a Z-spray electrospray ionization source. Samples were subjected to a capillary voltage of 3.0 kV with an ionization source temperature of 120 °C and

cone voltage of 15 V. The data was acquired in positive mode in the first mass analyzer (MS₁) through mass over charge ratio (m/z) = 300–2000. All peptide samples were dissolved in 50% *v/v* acetonitrile diluted with analytical quality water.

For UPLC-MS, the sample injection volume was 3 μ L of 200 μ g/mL peptide in water with less than 5% acetonitrile. Chromatography was done over 17 minutes on an Acquity UPLC[®] BEH C₁₈ column with 1% *v/v* formic acid in water (eluant A) and acetonitrile (eluant B) as the two mobile phases (Table 2.3). Detection of chromatography was done in-line with ESMS as described above.

Multi-protonated spectra in centroid mode were deconvoluted using the QTOF transform algorithm of Mass Lynx V4.1 software with calculated range from 300-4000 atomic mass units, maximum 4 charges. Multi-protonated spectra in continuum mode were deconvoluted using the MaxEnt 3 algorithm of Mass Lynx V4.1 software with calculated range from 300-6500 atomic mass units, maximum 10 charges, 50 iterations and auto peak width determination.

Table 2.3 UPLC analysis gradient program for the analysis of Trcs and analogues (method developed by M. Rautenbach and M.A. Stander, Department of Biochemistry, Stellenbosch University).

Time (min)	% eluant A	% eluant B
0.0	100	0
0.5	100	0
12.0	42	58
13.0	10	90
13.5	10	90
14.0	10	90
17.0	100	0

2.3.3.3. Fluorescence spectroscopy

Fluorescence measurements using the model RF-5301PC spectrofluorophotometer (Shimadzu, Japan) were performed to compare Trc A from commercial and culture extracted tyrothricin. The chemically synthesized Tpc A which is the only Trc A analogue with a Trp was used as control peptide. The samples were excited at 280 and 295 nm with the emission spectra recorded between 280 and 450 nm in 0.2 nm steps. Slit widths of 5 nm were utilized for both excitation and emission⁴¹.

2.3.3.4. Homology modelling

Homology modelling was performed as previously described⁴¹, using as starting point a low energy model of Trc C created using two dimensional nuclear magnetic resonance nuclear

Overhauser enhancement (2D-NMR NOE) constraints during a combined study with the group of Prof. Graham Jackson (University of Cape Town). The major Trcs isolated from commercial tyrothricin have previously been modelled by M. Rautenbach using YASARA 9.10.5© software^{41,63,64}. In this study, the structure of Tpc C was obtained from transformation of low energy structures of Trc C while those of Tpc A, Phc A, Trc AOMe₃, and Trc A(Q-O) were obtained from mutating low energy structures of Trc A. The derived structures were submitted to YASARA molecular dynamic (MD) simulations for 200 fs at 298 K followed by *in vacuo* energy minimisations using the YASARA 2 force field⁶⁴. This was followed by MD simulations for 400 fs at 333 K and by minimisations to assess if the structures had reached a global minimum. A structure was regarded as a representative structure when 10 near identical structures in terms of backbone structure, overall structure (RMSD < 1 Å from average) and calculated energy were obtained from consecutive simulation runs and energy minimisations. The solvent accessible volume (SAV) and solvent accessible surface area (SASA) for each peptide were determined from their ten lowest energy structures using YASARA 9.10.5©⁴¹.

2.4. Results and Discussion

2.4.1. Manipulation of production and composition of Trc analogues by *B. aneurinolyticus* ATCC 8185 cultures

2.4.1.1. Colony selection for optimum tyrothricin production

The radial diffusion assays of *B. aneurinolyticus* ATCC 8185 single colonies against *M. luteus* NCTC 8340 showed antibiotic tyrothricin production as evidenced by the clear growth inhibition zones observed (Fig. 2.2).

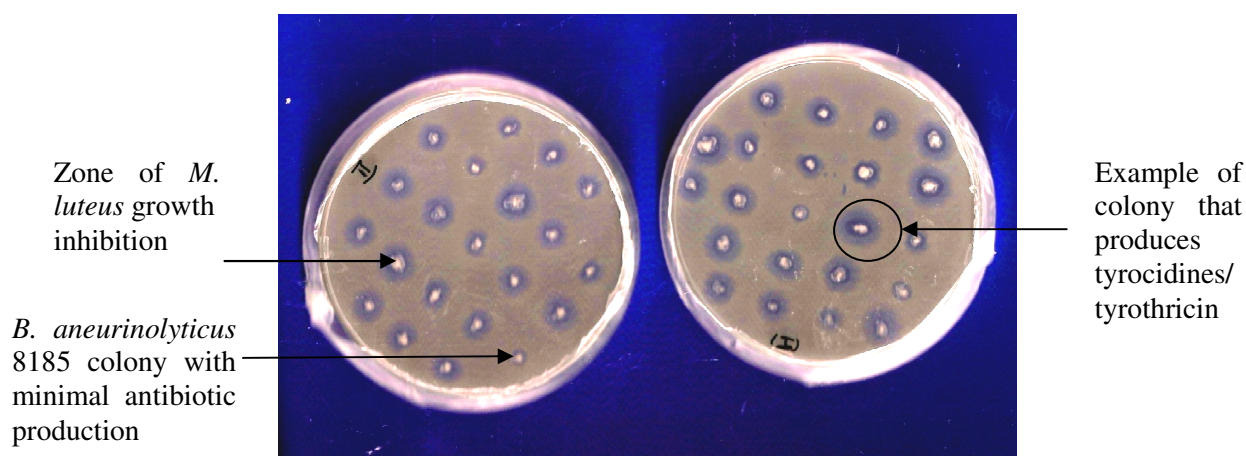


Figure 2.2 Gel diffusion assays for tyrothricin production on *M. luteus* lawn. The inhibition zones (clear zones) were created by the antibiotic tyrothricin complex produced by colonies of *B. aneurinolyticus* ATCC 8185.

Colonies that gave the largest inhibition zones were selected for further propagation to produce Trcs. An average yield of 500-700 mg crude Trc was obtained per litre of *B. aneurinolyticus* ATCC 8185 culture. This is in accordance with the expected yield of 500 mg/L tyrothricin produced estimated by Dubos and Hotchkiss².

Eighty percent of the tyrothricin extract is expected to be Trc⁵³. Thin layer chromatography of samples isolated from the culture extracts revealed a ninhydrin active spot with the same R_f value ($R_f = 0.6$) to that of the Trcs extracted from the commercial tyrothricin (results not shown).

2.4.1.2. Influence of urea supplementation on the growth of and Trc production by *B. aneurinolyticus* ATCC 8185

The selected *B. aneurinolyticus* ATCC 8185 colonies grew well in NuB culture medium with or without 0.1% *m/v* urea (Fig. 2.3A) as additional nitrogen source, although urea supplementation induced a slight decrease in the specific growth rate from 0.41 to 0.33 (/hour) (Fig. 2.3B). However, the final biomass yields were similar with and without urea in culture medium (not shown).

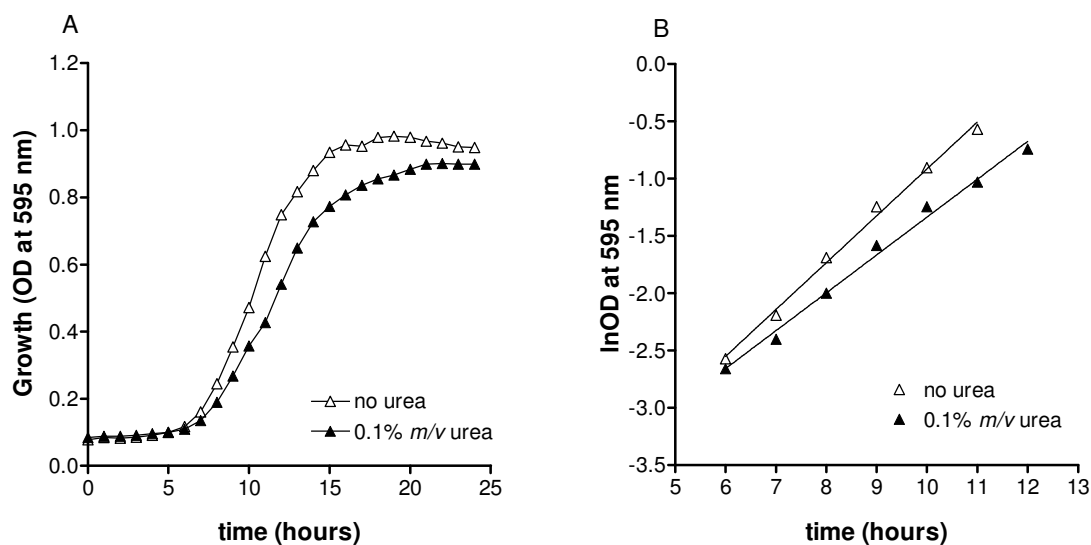


Figure 2.3 Comparison of **A.** Growth curves of *B. aneurinolyticus* ATCC 8185 cultures in nutrient broth (NuB) in the presence or absence of 0.1% urea over 24 hours. Each data point represents the mean of 3-4 measurements. **B.** Specific growth rate difference during the exponential growth phase of *B. aneurinolyticus* ATCC 8185 induced by supplementation with 0.1% *m/v* urea.

Using semi-preparative RP-HPLC seven main fractions were purified from the Trcs isolated from the cultures grown in NuB in the presence (Fig. 2.4) or absence (results not shown) of 0.1% *m/v* urea. ESMS analyses of the purified fractions revealed that fraction 1 did not contain any

Trcs. This fraction contains a yellow pigment from the culture co-extracting with the Trcs. ESMS of the other fractions revealed that fractions 2, 3 and 5 consisted of Trc C, B, and A respectively. Fraction 6 also contained Trc A while fraction 4 contained Trc B and Tpc C. The seventh fraction was composed of Phc A and Tpc A (identities confirmed by MS-MS analysis by our group).

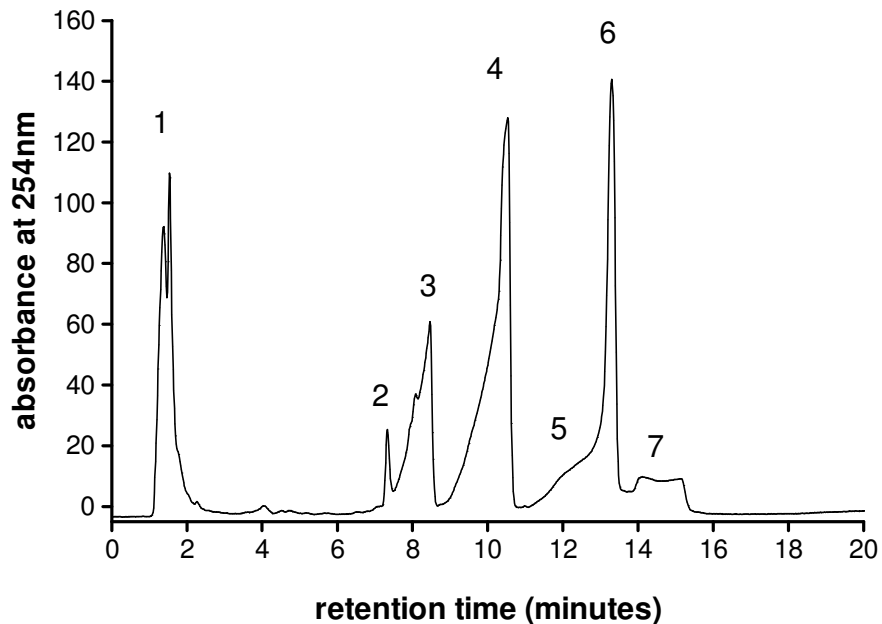


Figure 2.4 Semi-preparative RP-HPLC chromatogram of Trcs extracted from *B. aneurinolyticus* ATCC 8185 grown in NuB + 0.1% *m/v* urea showing 7 fractions purified (1-7)

Examination of the influence of urea concentration on the antibacterial activity of Trc extracts indicated that the optimal urea concentration was 0.1% *m/v* (Fig. 2.5) in accordance with Baron⁴⁴. Since the urea over 1 to 0.008% *m/v* did not prevent growth of bacteria its effect is probably linked to the regulation of the Trc synthesis mechanism.

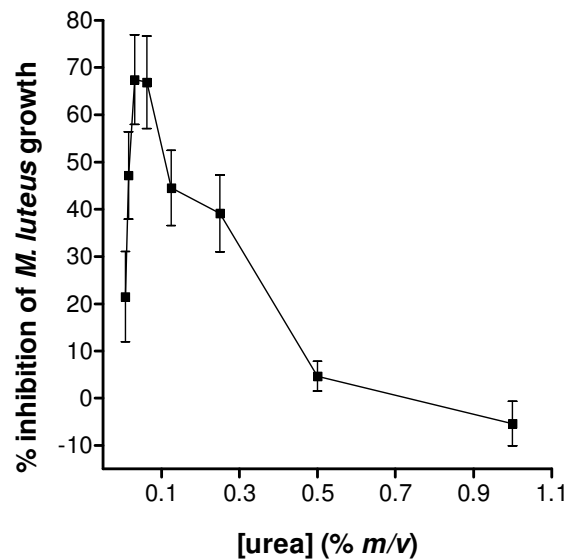


Figure 2.5 Effect of urea concentration on the extracted antibacterial activity of the crude Trc culture extracts. Each data point represents the mean of quadruplicate measurements

2.4.1.3. Influence of amino acid supplementation on the growth of and Trc production of *B. aneurinolyticus* ATCC 8185

Supplementation with different amino acids had different effects on the growth rate of the cultures depending on the amino acid concentration (Fig. 2.6). The general tendency was of decrease in growth rate with increase in amino acid concentration perhaps as a result of the amino acids at high concentration.

Stokes & Woodward⁴⁶ also observed that tyrothricin production in submerged, aerated cultures was inhibited following supplementation with Trp at a concentration of 0.5% *m/v*. All cultures showed least growth at highest amino acid concentration (1% *m/v*) while optimum growth was mostly observed at 0.1% *m/v* except for Trp and Met for which it was observed at 0.001% *m/v*. Phe had the least inhibitory effect on growth of producer as opposed to D/L-Phe that rapidly decreased the growth rate. Studies on the *B. aneurinolyticus* ATCC 10068 strain also showed growth inhibition by increasing concentrations of Trp and Phe with the effect of Phe being observed only from 13 hours⁵⁵.

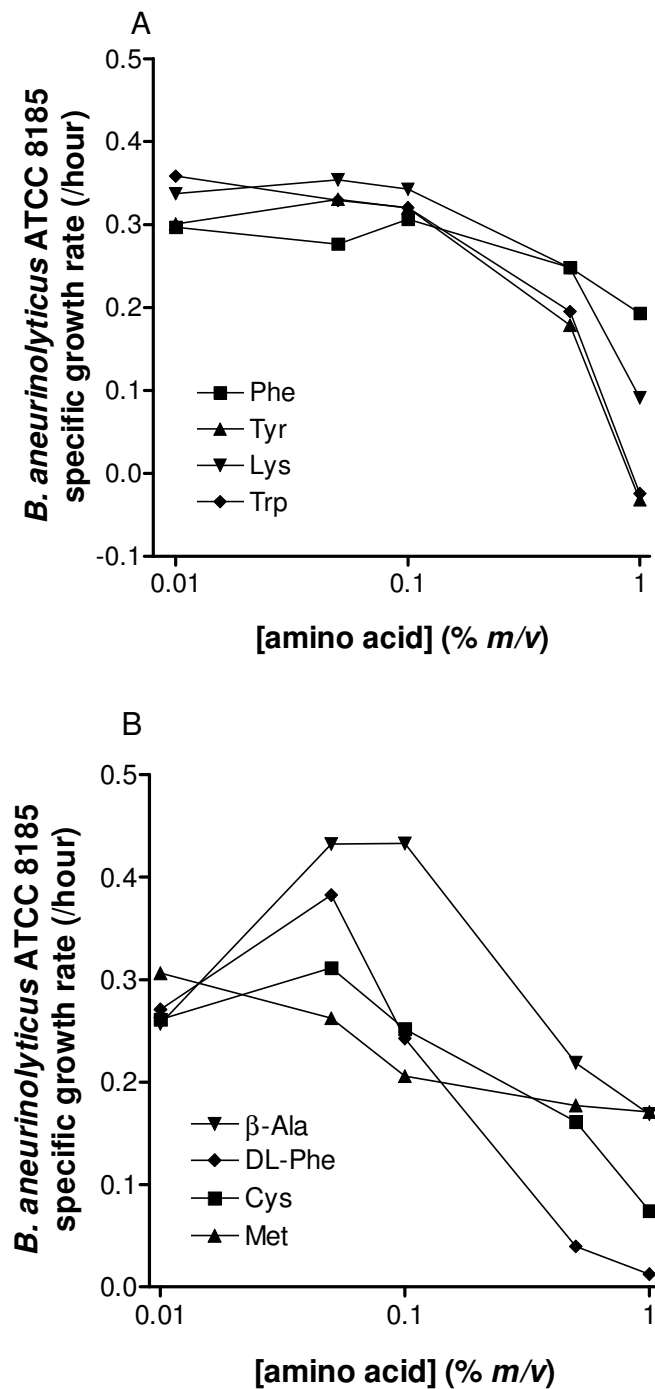


Figure 2.6 Influence of amino acid concentration on the specific growth rate of *B. aneurinolyticus* ATCC 8185 cultures in nutrient broth (NuB) in the presence of 0.1% m/v urea over 24 hours (left). Specific growth rate (μ) was computed as the change in $\ln OD_{595}$ over the change in time during the exponential growth phase over 24 hours. Each data point represents the mean of quadruplicate measurements.

The relative amount and/or composition of the Trc analogues produced varied with the concentration of the added amino acid (Fig. 2.7). Trp particularly influenced the composition of

Trcs produced as seen in Fig. 2.7A wherein as the concentration of added Trp decreased, there was an increase in the range of and/or amount of Trcs produced. Increasing concentrations of Trp also led to increase production of gramicidins (Fig. 2.7A). Unlike Trp, the concentration of Phe (Fig. 2.7B) and the other amino acids (results not shown) only influenced the relative amounts of the Trcs produced. However, up to 0.05% *m/v* Phe and DL-Phe supplementation favoured the synthesis of Trc A, Phc A and to a lesser extent Trc B (Trc A analogues) and concomitant inhibition of gramicidin production. The Trc analogues produced in the presence of the other amino acids did not differ from those in the non-supplemented culture extract (results not shown). Similar observations were made by Vosloo *et al.*⁵⁵ in terms of effect of varying concentrations of Trp and Phe added to the culture medium of *B. aneurinolyticus* ATCC 10068.

The change in Trc profile was similar with the Trp-containing peptides forming with Trp supplementation and Phe-containing peptides forming with Phe supplementation, even though different culture medium of tryptone, glucose and salts (TGS)⁴⁵ was used and the producer strain was different. However, Vosloo *et al.*⁵⁵ observed a broader array of Trc analogues such as the Lys analogues of the major Trcs as well as other minor tryptocidines. Rao *et al.*⁵¹ previously observed that *B. aneurinolyticus* ATCC 10068 responded more readily to modifications in amino acids than *B. aneurinolyticus* ATCC 8185 even though the effect was evaluated in cell-free extracts and similar culture medium was used for both strains. Our results are in accordance also with previous work by other authors^{4,5,65} who demonstrated preferential synthesis of Trc A by *B. aneurinolyticus* ATCC 10068 cultures upon addition of Phe to the culture medium while supplementing with Trp led to preferred production of Tpc C (formerly known as Trc D). Similar results were obtained in cell-free production systems^{48,51}. It has been suggested that the fact that only Trp addition seemed to cause the exclusive production of Tpc C is due to very high Trp affinity of the enzyme synthetase complex^{37,51}.

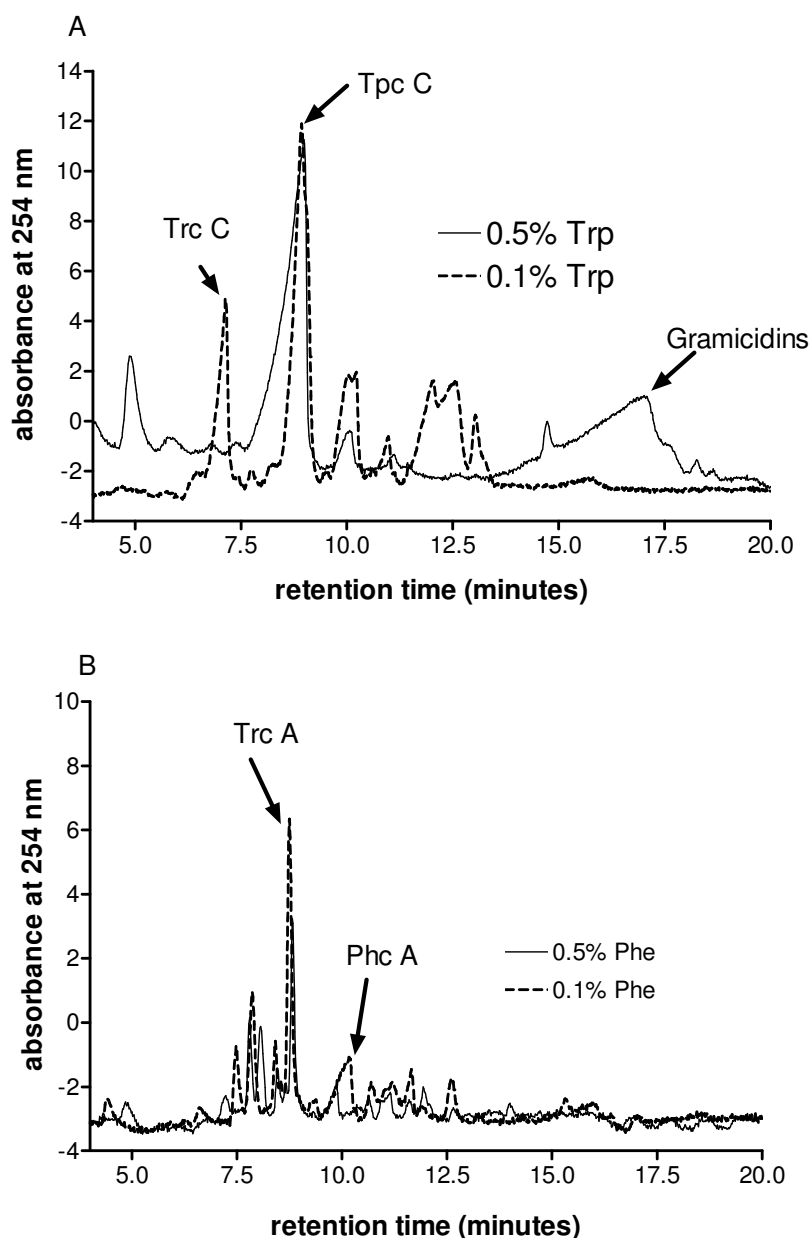


Figure 2.7 HPLC chromatography showing the influence of concentration of **A.** Trp and **B.** Phe on the composition of Trcs produced by *B. aneurinolyticus* ATCC 8185 cultures. The identity of fractions was confirmed by TOF-ESMS.

2.4.2. Optimisation for high yield of single Trc analogues from culture extracts

Following the results from the previous section and the goal being to increase the yield of single analogues, we opted to supplement the cultures with higher amino acid concentrations individually and in combinations, followed by isolation of the Trcs and purification of the major fractions. The identity of components of the purified fractions was confirmed by TOF-ESMS and comparison of the observed molecular masses to the list of identified Trcs by Tang *et al.*⁸.

2.4.2.1. Influence of amino acid supplementation

The amino acids chosen for supplementation were specifically the variable amino acids in the identified Trc analogues and other amino acids known to play a role during Trc synthesis by the multi-enzyme complex (Phe, Tyr, Trp, Lys, Cys, Met, β -Ala, and D/L-Phe). Table 2.4 summarises the modulation of Trc analogue production under specified amino acid supplementation.

Table 2.4 Summary of Trc analogue production by *B. aneurinolyticus* ATCC 8185 cultures as modulated by amino acid supplementation. Where more than one amino acid is mentioned, they were used at 0.5% *m/v* each while single amino acids were at 1% *m/v*. Standard three letter abbreviations are used for amino acids. The '+' indicates the confirmed presence of Trc or analogue. The *m/z* of the unknowns is given as observed in the TOF ESMS data.

Amino acids added to NuB culture medium	Identity of Trcs and analogues								
	Trc A	Trc B	Trc C	Tpc A	Tpc B	Tpc C	Phc A	Phc C or Tpc B	<i>m/z</i> of other compounds/peptides observed
Phe	+						+		
D-Phe									
DL-Phe	+						+		1263.18
Tyr	+	+	+	+	+	+			
Trp					+	+			
Lys	+	+	+						
Cys	+	+		+	+				1302.59
β -Ala	+	+	+	+	+	+			1302.20
Met	+			+					
Lys + Phe	+						+		
Lys + Trp	+			+	+	+			1229.16
Tyr + Trp						+			
Tyr + Phe	+						+		
Phe + Trp	+				+	+		+	1226.06
Lys + Phe + Tyr	+						+		
Lys + Phe + Trp						+		+	1378.50, 1384.59**
Lys + Tyr + Trp						+			

**Tryptocidine analogue with Lys², Trp¹⁰ identified, but not named by Tang *et al.*⁸.

The extract of 1% *m/v* β -Ala- and Tyr-supplemented cultures contained all of the major Trcs and most of the minor peptides, while Lys seemed to favour the production only of the major Trcs, although at lower yield. None of the Lys-containing major Trc analogues were found in any of the preparations. In the presence of Cys the production of Trc C, Tpc C, and Phc A was inhibited. Met induced the preferential production of Trc A with Tpc A. D-Phe completely

inhibited Trc production unlike the Phe or DL-Phe that induced production of Trc A and Phc A. Addition of Tyr to Phe with or without Lys, however, led to preferential production of Trc A over Phc A. Lys influenced Trp supplementation by inducing production of more analogues like Trc A and Tpc A.

Supplementing with Tyr and Trp in a 1:1 ratio as well as a combination of equal amounts of Lys, Tyr and Trp led to exclusive production of Tpc C though at lower yield than when Trp alone was added. Phe and Trp induced production of Tpc B and inhibition of Phc A production. Addition of Lys to Phe and Trp shifted production mainly towards Tpc C. Some unknown analogues were also produced though in very small amounts (Table 2.4).

It has been demonstrated in previous studies that the enzymes involved in the incorporation of the aromatic amino acids have low affinity and will incorporate the aromatic amino acids with related structures according to availability^{4,5}. However, some of these amino acids have higher affinity for certain enzyme domains than others which will explain their preferential abundance in the final Trc product. Mootz & Marahiel³⁷ observed that the adenylation domain of the *B. aneurinolyticus* ATCC 8185 in charge of integrating either Phe or Trp at position 4, had a fivefold higher affinity for Trp. This explains the preferential tendency to produce Tpc C with three Trp residues in the presence of the other aromatic amino acids. Therefore, the affinity of the synthetase enzyme complex for the amino acid supplement also determines the nature of Trcs produced⁴. It also seems that the hydrophobic amino acids play a role in determining the preferential affinity of the enzymes that incorporate the aromatic amino acids. The absence of Trc production in the presence of D-Phe indicates that L-Phe is a rate limiting factor in the production of Trcs and this is contrary to the results of Mach and Tatum who observed no difference in using either D- or L-Phe⁴. These results indicate the need for further detailed investigation of the influence of the role played by amino acids in the tyrocidine production but were enough to inform of which amino acids could provide large yield of rare analogues like Tpc C and Phc A.

A pilot experiment was performed to improve the yield of Trc A and Tpc C produced in cultures supplemented with combinations of Lys + Phe + Tyr and Lys + Phe + Trp respectively by varying the relative concentration of each amino acid. Several combinations of varying concentrations of each amino acid were used to supplement the *B. aneurinolyticus* ATCC 8185 cultures.

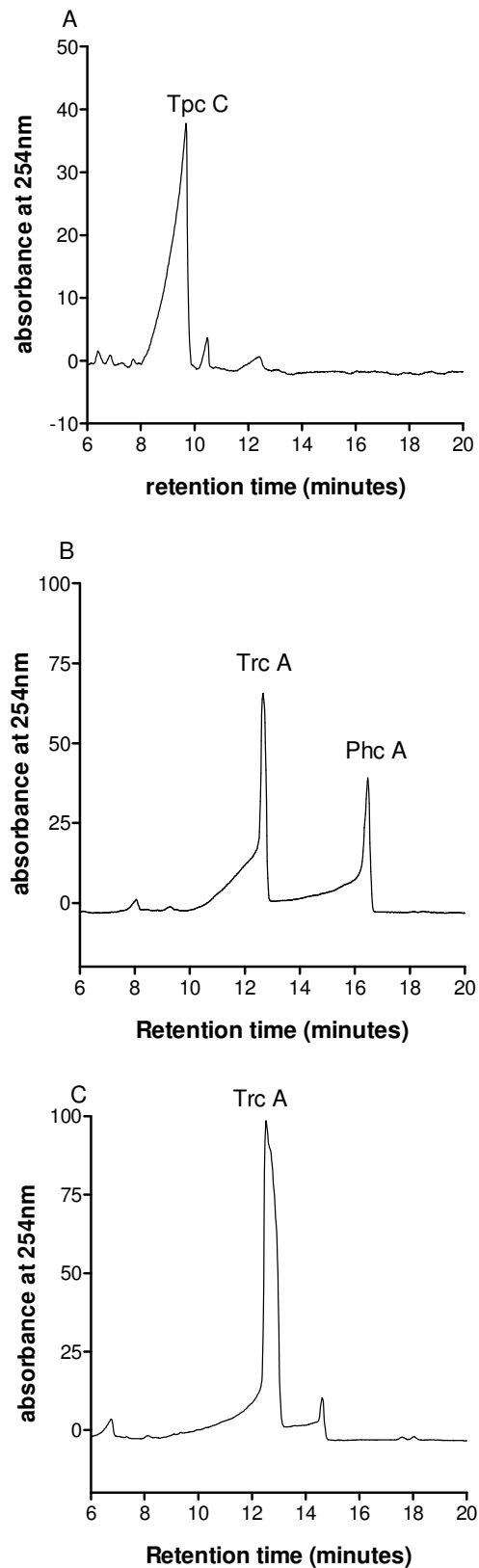


Figure 2.8 Semi-preparative RP-HPLC chromatogram of tyrocidine analogues isolated from extracts of *Bacillus aneurinolyticus* ATCC 8185 cultured in nutrient broth (NuB) supplemented with **A.** 0.3% *m/v* Trp **B.** 1% *m/v* Phe **C.** 0.5% *m/v* Lys + 0.5% *m/v* Phe + 0.5% *m/v* Tyr

The isolated Trcs from the culture extracts were analysed as previously described using HPLC and TOF-ESMS and their antibacterial activity against *M. luteus* was determined as previously described. None of the preparations led to production of a single analogue (results not shown). Comparison of activity against *M. luteus* showed large variations. The activity variation of the isolated Trcs could be due to differences in the proportions of the different analogues produced, as well as non-antibiotic contaminants co-extracted.

Based on the above results, supplementation with Trp was chosen for high yield production of Tpc C (Fig. 2.8A); Phe (Fig. 2.8B) or Phe + Tyr supplementation for production of Phc A along with Trc A; Phe + Tyr + Lys for production of Trc A (Fig. 2.8C). Trp at 0.3% *m/v* was the chosen concentration for supplementation because results indicated that a comparable yield of Tpc C was produced as that with 1% *m/v* supplementation. There was a difference in retention time for Trc A and Phc A in Figs 2.7B and 2.8B probably due to minor differences in HPLC conditions and amount of material separated, specifically impurities that could change elution of the peptides due to interference with matrix interaction.

2.4.2.2. Influence of urea supplementation

As previously observed (Fig. 2.3B), the presence of urea seemed to reduce the growth rate of the Trc producers. We investigated if this had any impact on the yield of Trcs produced upon supplementation with the selected amino acids Trp, Phe + Tyr or Phe + Tyr + Lys for production of Tpc C, Phc A and Trc A respectively. Urea supplementation at 0.1% *m/v* was added to cultures along with the specific amino acids. The average yield obtained for all the conditions of amino acid supplementation selected in *Section 2.4.2.1* was unchanged except for addition of 0.5% *m/v* Lys + 0.5% *m/v* Phe + 0.5% *m/v* Tyr for preferential production of Trc A which gave a significantly higher yield in the absence of urea than with 0.1% *m/v* urea as determined by Student t-test (Fig. 2.9). This is contrary to the recommendations made by Baron ⁴⁴, who claimed that the presence of urea or its derivatives in the culture media led to a substantial increase in the amount of Trcs produced in the cultures. However, deep tank fermentation was utilised, which could influence the yield and account for the disparity of the observations. Baron obtained an average yield of 150 mg/L of Trc in the presence of 0.1% *m/v* urea which was double the average yield obtained in its absence.

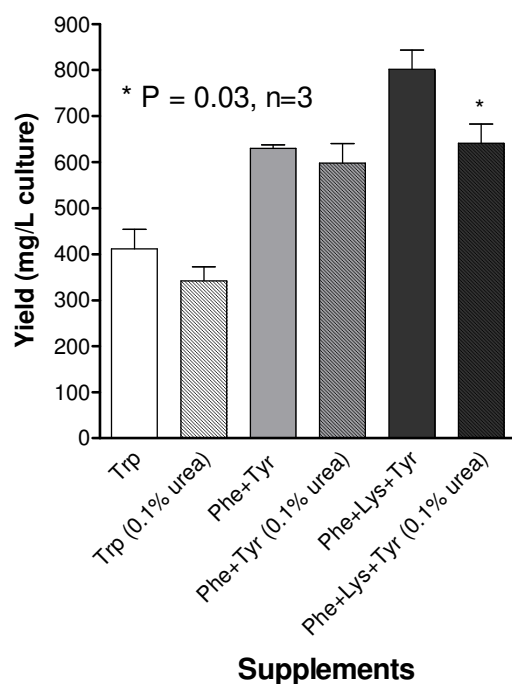


Figure 2.9 Effect of 0.1% *m/v* urea on the yield of tyrocidine analogues produced by *B. aneurinolyticus* ATCC 8185 in NuB culture media supplemented with amino acids.

Table 2.6 Effect of nitrogen supplementation on composition of Trcs produced by *B. aneurinolyticus* ATCC 8185 cultures

Nitrogen supplement in culture media	Total yield (mg/L ± SEM) n = 3	Major <i>m/z</i> signals	Peptide identity	% Signal in ESMS
Phe+Tyr + urea	598.3 ± 42.06	1254.38	Phc A	20
Phe+Tyr	630.0 ± 7.64	1270.34	Trc A	100
		1292.31	Trc A + Na	55
		1309.30	Trc B	trace
Trp + urea	341.7 ± 30.60	1293.36	Tpc A/PhcB	trace
Trp	411.7 ± 42.26	1332.32	Tpc B	trace
		1371.30	Tpc C	100
Phe + Tyr + Lys + urea	641.7 ± 41.47	1254.39	Phc A	20
Phe + Tyr + Lys	801.7 ± 41.67	1270.37	Trc A	100
		1310.30	Trc B	trace

This could be accounted for by the difference in the depth of the fermentation media which will influence oxygenation which could in turn affect nutrient supply to the cultures. *B. aneurinolyticus* cultures will initiate antibiotic production under conditions of nutrient limitation^{25,49,65} and so the increased oxygenation in shallow cultures may not be favourable for the high yield production of Trcs. As previously demonstrated in *Section 2.4.1.1* the particular colony selected for Trc production may also have a large influence on the yield. As shown in Table 2.6 the addition of urea did not affect the composition of the Trc analogues produced.

2.4.3. Purification and characterisation of Trcs and analogues

From the previous sections we identified three supplementation regimens that gave high yield of some rare Trc analogues notably Trc A, Phc A and Tpc C which are preferentially produced in *B. aneurinolyticus* ATCC 8185 cultures grown in nutrient broth medium supplemented with either an equimolar combination of Phe, Tyr and Lys to a total amino acid concentration of 1.5% *m/v* for Trc A or 1% *m/v* Phe for Phc A or 0.3% *m/v* Trp for Tpc C. Other Trc analogues (Trc A₁, B, B₁, C, and C₁) were purified from commercially obtained tyrothricin. Some of the synthetic Trc analogues were also purified and/or analysed. Following isolation of tyrocidines from the tyrothricin from both culture and commercial sources through organic extraction, purification was done using the RP-HPLC⁵⁴ previously used to purify the six major Trcs from commercial tyrothricin¹¹. The purity of the fractions was verified by analytical HPLC⁴¹, ESMS⁴¹ and UPLC-MS⁵⁵.

2.4.3.1. Purification from supplemented *B. aneurinolyticus* ATCC 8185 culture extracts

From the culture extract following supplementation with 0.3% *m/v* Trp, we purified Tpc C which could be identified from its TOF-ESMS spectra showing the singly charged molecular ion ($m/z = 1371.6957$) and singly charged sodium adduct ($m/z = 1393.6774$) (Fig. 2.10).

Purified Tpc C eluted from the analytical C₁₈ RP-HPLC column at 9.52 minutes and from the UPLC column at 8.13 minutes (Fig. 2.11). Its purity was 97% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

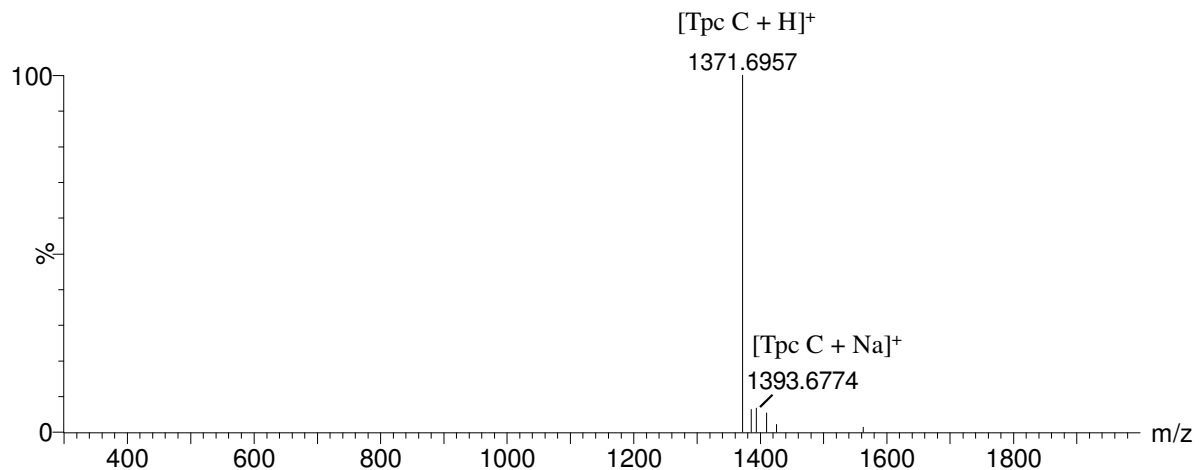


Figure 2.10 TOF-ESMS analysis of purified Tpc C. The recorded positive mode spectrum from 300-2000 atomic mass units (amu). The singly charged molecular ion ($[\text{Tpc C} + \text{H}]^+$) and singly charged sodium adduct ($[\text{Tpc C} + \text{Na}]^+$) are indicated.

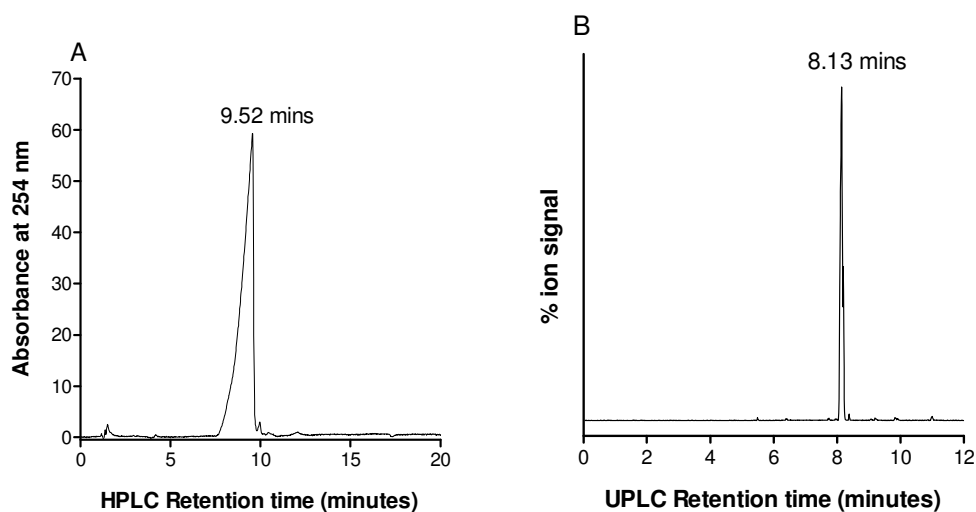


Figure 2.11 **A.** Analytical C_{18} RP- HPLC chromatogram of purified Tpc C at $200 \mu\text{g/mL}$ ($50 \mu\text{L}$ injection volume) **B.** UPLC chromatogram of Tpc C preparation.

From the culture extract following supplementation with $0.5\% m/v$ Phe + $0.5\% m/v$ Tyr + $0.5\% m/v$ Lys, we purified Trc A as the major peak. It could be identified from its TOF-ESMS spectra showing the singly charged molecular ion ($m/z = 1270.6613$) and singly charged sodium adduct ($m/z = 1292.6399$) (Fig. 2.12).

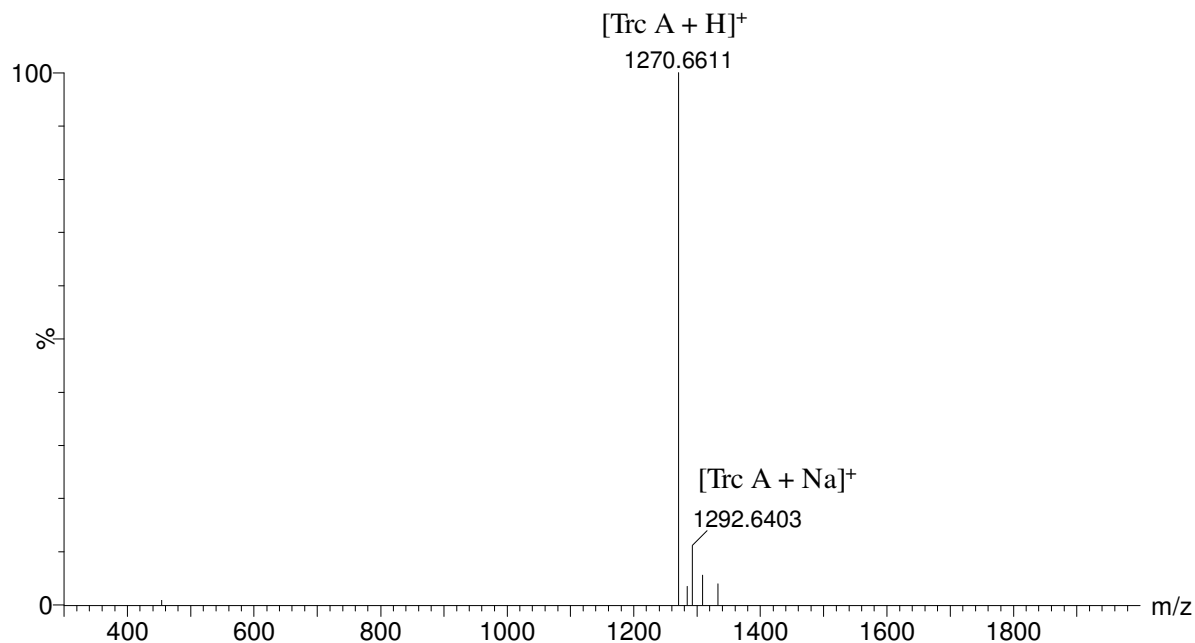


Figure 2.12 TOF-ESMS analysis of purified Trc A. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[\text{Trc A} + \text{H}]^+$) and singly charged sodium adduct ($[\text{Trc A} + \text{Na}]^+$) are indicated.

The retention time of purified Trc A was 11.26 minutes on the analytical C_{18} RP-HPLC column and 9.22 minutes on the UPLC column (Fig. 2.13). Its purity was 97% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

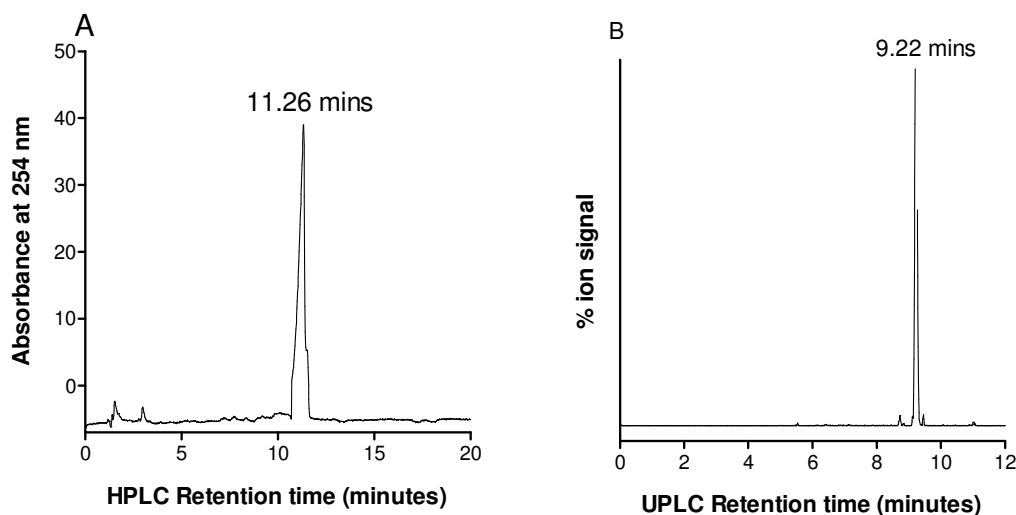


Figure 2.13 **A.** Analytical C_{18} RP- HPLC chromatogram of purified Trc A at 100 $\mu\text{g}/\text{mL}$ (20 μL injection volume) **B.** UPLC chromatogram of Trc A preparation

Trc A could also be purified from commercial tyrothricin, but this fraction was frequently contaminated with Tpc A which was difficult to get rid of as revealed from fluorescence spectroscopy (Fig. 2.14) and MS analyses. We also observed presence of the linear gramicidin A from the MS spectra co-eluting with Trc A (not shown).

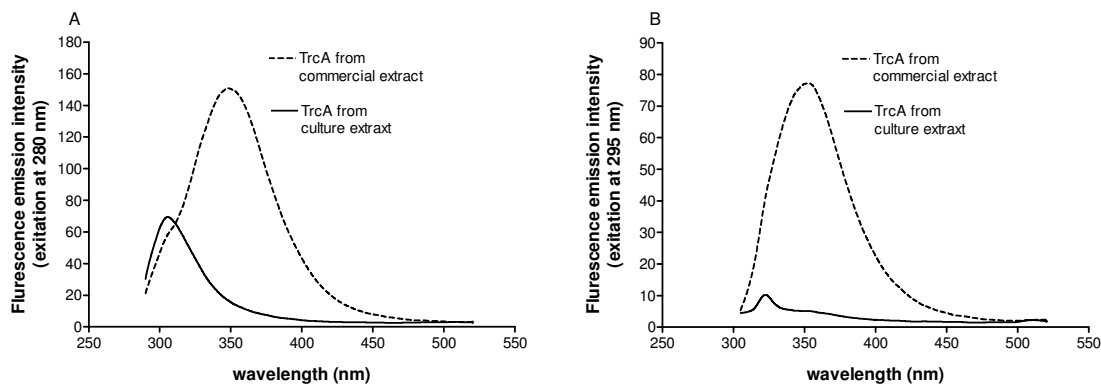


Figure 2.14 Comparison of fluorescence emission spectra of Trc A from commercial extracts and culture extracts in an aqueous environment **A**. Excitation at 280 nm yields the combined emission of Tyr and tyrosinate **B**. Excitation at 295 yields the emission of tyrosinate

Hence production of Trc A from the supplemented culture extracts offered the advantage of fewer purification rounds using RP-HPLC and a higher purity Trc A preparation.

Tyrothricin derived from culture extracts following supplementation with 1% *m/v* Phe showed two main peaks. The first fraction contained Trc A while the second fraction contained Phc A. Phc A was identified from its TOF-ESMS spectra showing singly charged ($m/z = 1255.6670$) molecular ions and singly charged potassium adduct ($m/z = 1292.6232$) (Fig. 2.15).

The high aggregation tendency of this very hydrophobic peptide was observed as there was an initial peak that eluted before 5 minutes on the analytical C_{18} RP-HPLC column. The retention time of purified Phc A was 13.61 minutes on the analytical C_{18} RP-HPLC column and 9.49 minutes on the UPLC column (Fig. 2.16). Its purity was 90% as determined from analysis of the UPLC results.

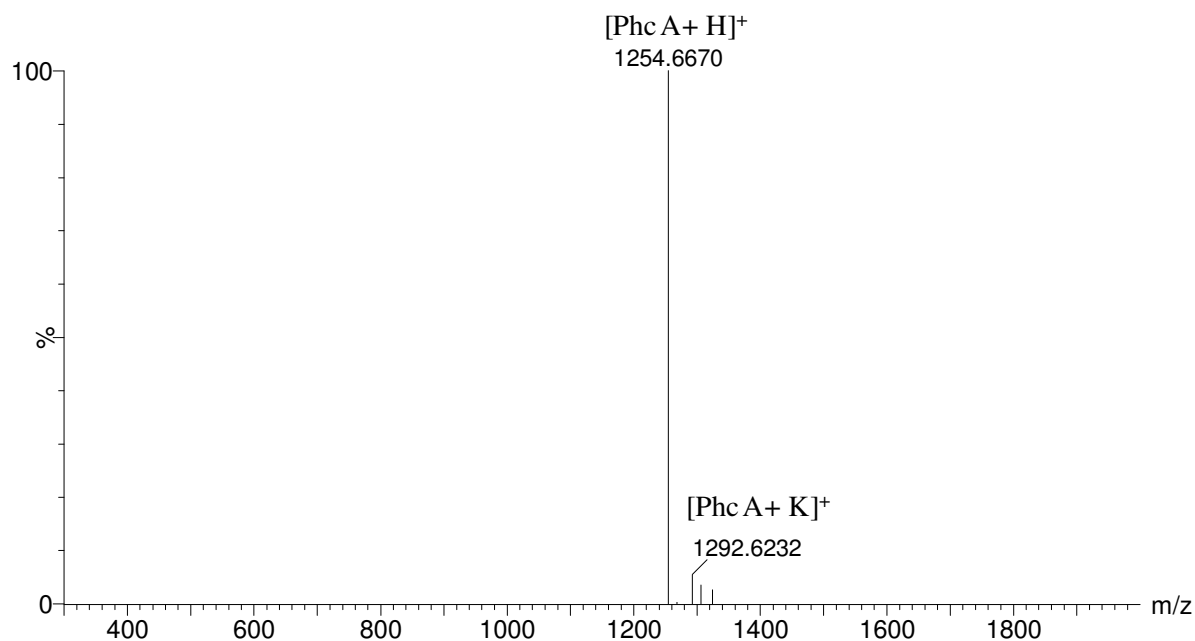


Figure 2.15 TOF-ESMS analysis of purified Phc A. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[\text{Phc A} + \text{H}]^+$) and singly charged potassium adduct ($[\text{Phc A} + \text{K}]^+$) are indicated.

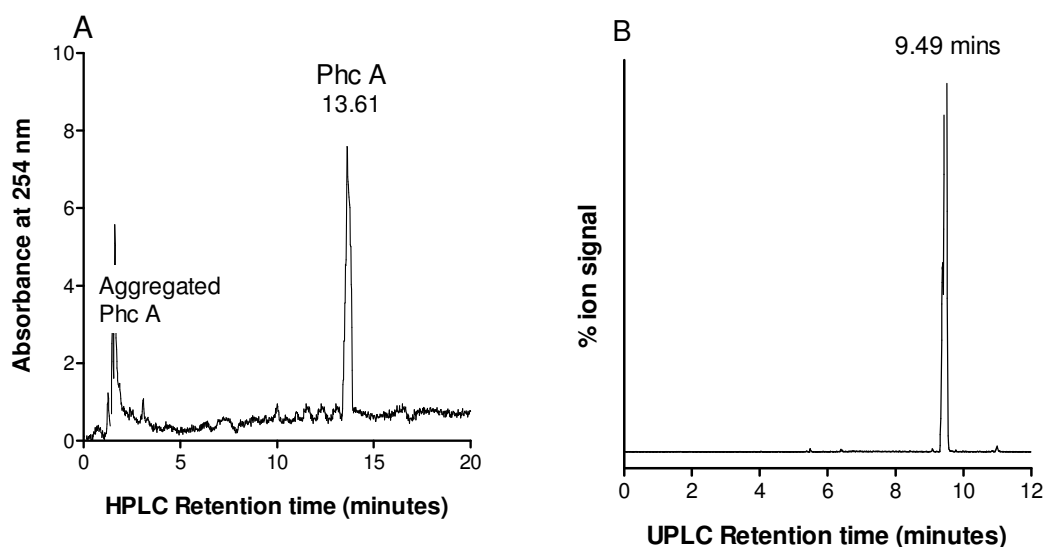


Figure 2.16 **A.** Analytical C_{18} RP- HPLC chromatogram of purified Phc A at $200 \mu\text{g/mL}$ ($50 \mu\text{L}$ injection volume) **B.** UPLC chromatogram of Phc A preparation

2.4.3.2. Purification of Trcs from commercial tyrothricin

The rest of the natural Trc analogues of interest in this study (Table 2.7) were obtained from commercial tyrothricin. Organic extraction of 300 mg commercial tyrothricin gave a yield of 185

mg (62%) Trcs corresponding to the expected yield of 60%⁶¹. The semi-preparative C₁₈-RP-HPLC of the crude Trc extract from the commercial tyrothricin complex gave 6 major fractions (Fig. 2.17). Analytical RP-HPLC and ESMS of the purified fractions showed that fraction A contained Trc C₁, fraction B constituted mainly of Trc C, fraction C was shown to contain a mixture of Trc B₁ and Trc B contaminated with their analogues Trc B₁' and Trc B' respectively with inverted aromatic dipeptide unit. Fraction D was found to contain Trc B and Trc B', fraction E was shown to contain a mixture of peptides including Trc A and Trc A₁ and fraction F contained a mixture of Trc A, Trc A₁ and Tpc A. Fractions C, D, E and F were subjected to further purification by analytical HPLC. Following this Trc A₁, Trc B and Trc B₁ were successfully purified. Commercially obtained gramicidin S (GS) which will be used as reference peptide in most assays was also identified using TOF-ESMS (m/z of singly charged molecular ion = 1141.7167 and m/z of doubly charged molecular ion 571.3670).

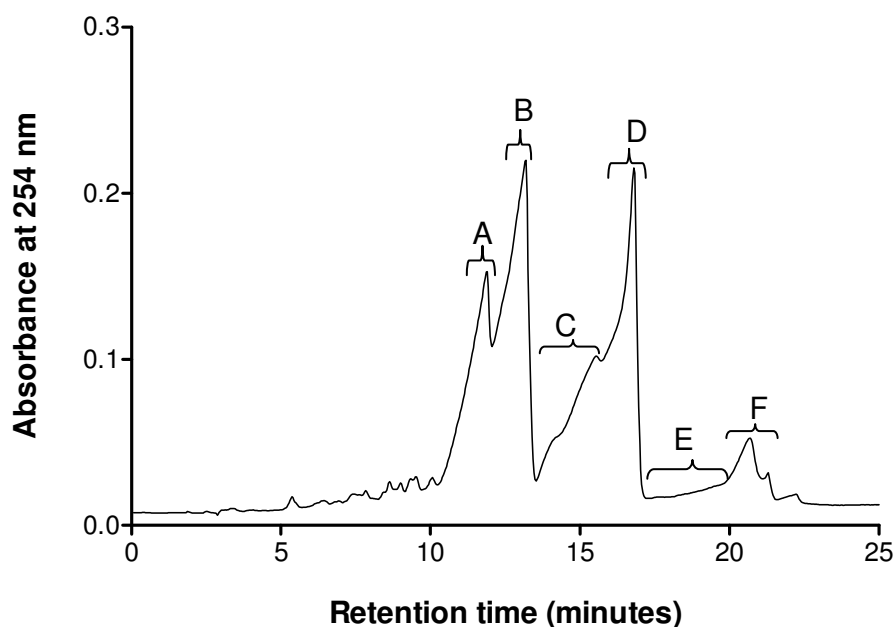


Figure 2.17 Semi-preparative RP-HPLC chromatogram of Trcs in commercial tyrothricin showing the six major fractions (A to F) that was collected.

Trc C₁ was purified from fraction A. It could be identified from its TOF-ESMS spectra showing the singly charged molecular ion ($m/z = 1362.6975$) as well as the sodium ($m/z = 1384.6797$) and potassium adducts ($m/z = 1400.6497$) (Fig. 2.18).

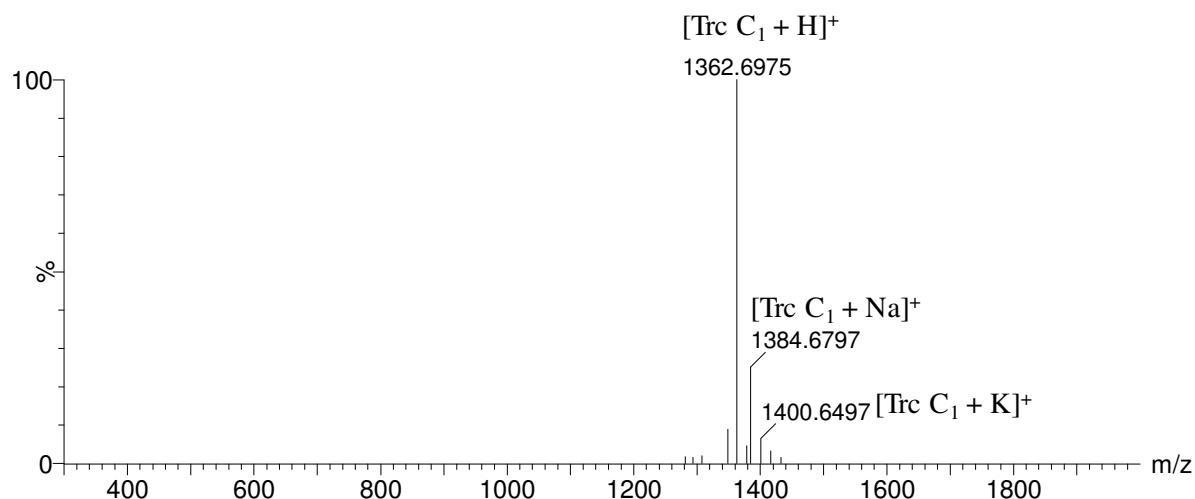


Figure 2.18 TOF-ESMS analysis of purified Trc C₁. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ([Trc C₁ + H]⁺) and singly charged sodium ([Trc C₁ + Na]⁺) and potassium ([Trc C₁ + K]⁺) adducts are indicated.

The retention time of purified Trc C₁ was 7.31 minutes on the analytical C₁₈ RP-HPLC column and 7.79 minutes on the UPLC column (Fig. 2.19). Its purity was 98% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

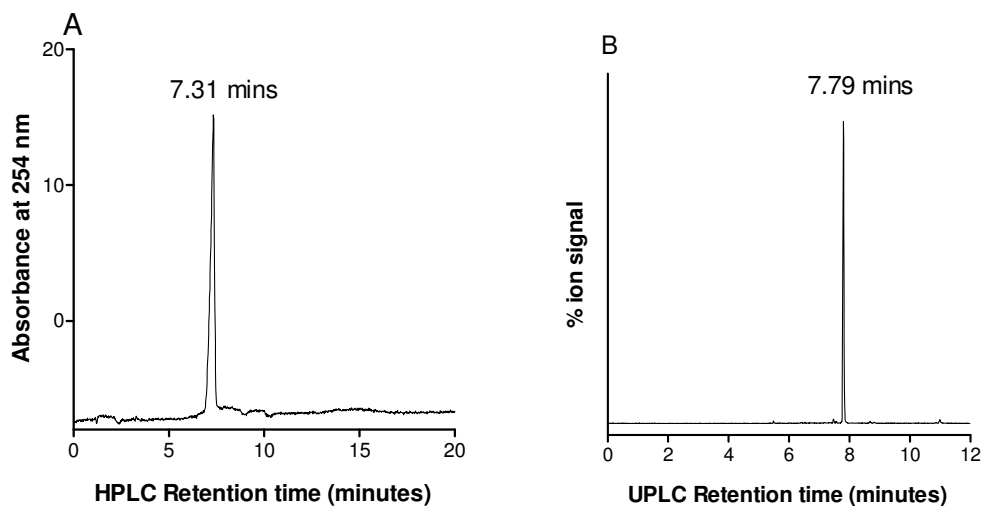


Figure 2.19 **A.** Analytical C₁₈ RP- HPLC chromatogram of purified Trc C₁ at 500 µg/mL (50 µL injection volume) **B.** UPLC chromatogram of Trc C₁ preparation

Trc C was purified from fraction B. It could be identified from its TOF-ESMS spectra showing the singly charged molecular ion ($m/z = 1348.6824$) (Fig. 2.20). The retention time of purified Trc C was 7.71 minutes on the analytical C₁₈ RP-HPLC column and 8.10 minutes on the UPLC

column (Fig. 2.21). Its purity was 94% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

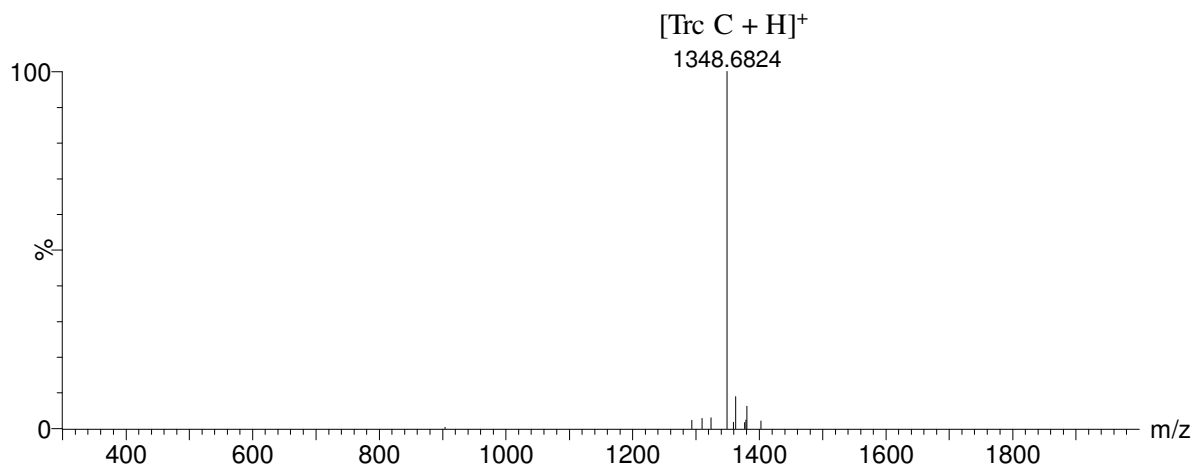


Figure 2.20 TOF-ESMS analysis of purified Trc C. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[\text{Trc C} + \text{H}]^+$) is indicated.

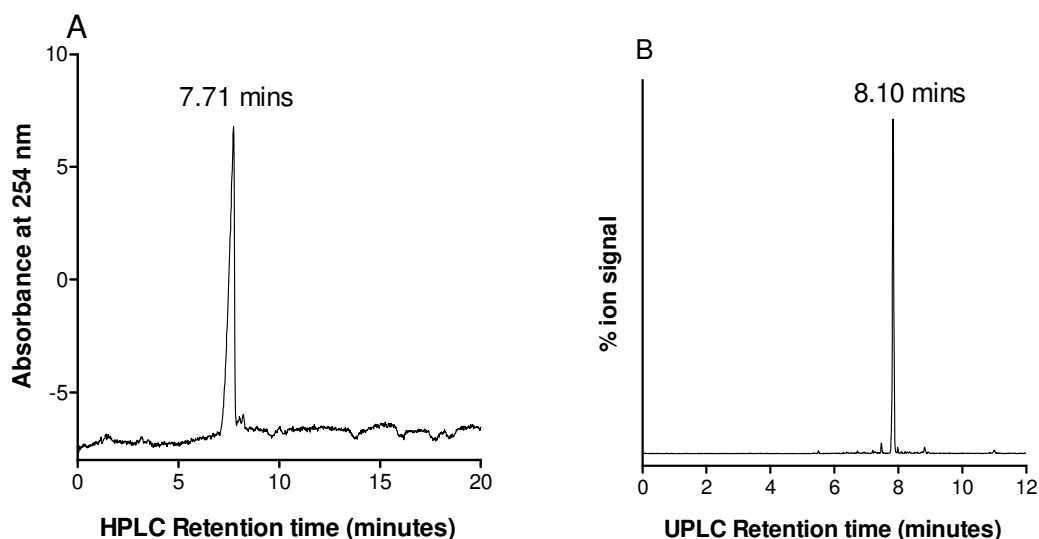


Figure 2.21 **A.** Analytical C_{18} RP- HPLC chromatogram of purified Trc C at 500 $\mu\text{g/mL}$ (50 μL injection volume) **B.** UPLC chromatogram of Trc C preparation.

Trc B₁ was not successfully purified to >90% from fraction C as it contained about 20% amounts of Trc B₁' and Trc B. Exhaustive purification of Trc B₁ from a similar fraction by our group yielded a purity >90%. Trc B₁ could be identified from its TOF-ESMS spectra showing the singly ($m/z = 1323.6958$) and doubly charged molecular ions ($m/z = 662.3505$) (Fig. 2.22).

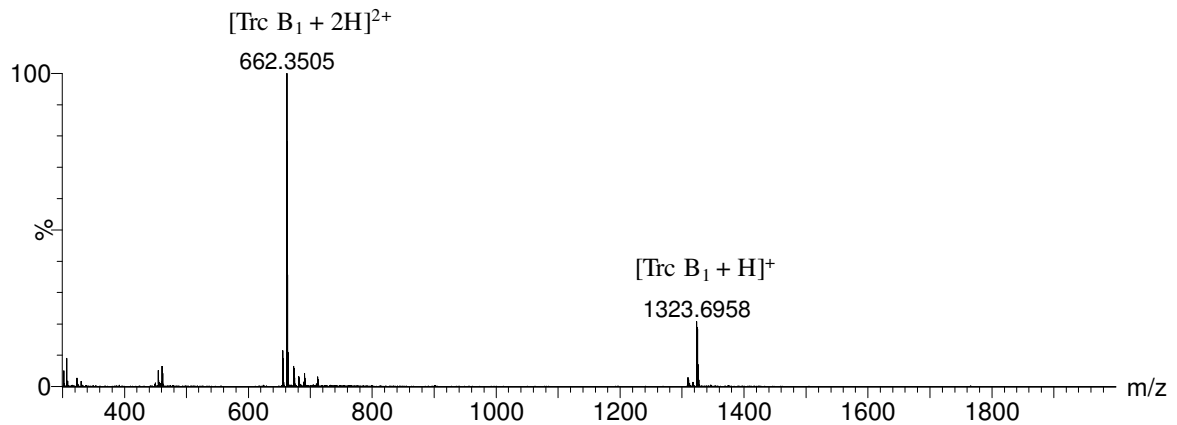


Figure 2.22 TOF-ESMS analysis of purified Trc B₁. The recorded positive mode spectrum from 300-2000 amu. The doubly charged molecular ion ($[\text{Trc B}_1 + 2\text{H}]^{2+}$) and singly charged molecular ion ($[\text{Trc B}_1 + \text{H}]^+$) are indicated.

The retention time of purified Trc B₁ was 9.28 minutes on the analytical C₁₈ RP-HPLC column and 8.74 minutes on the UPLC column (Fig. 2.23). Its purity was 90% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

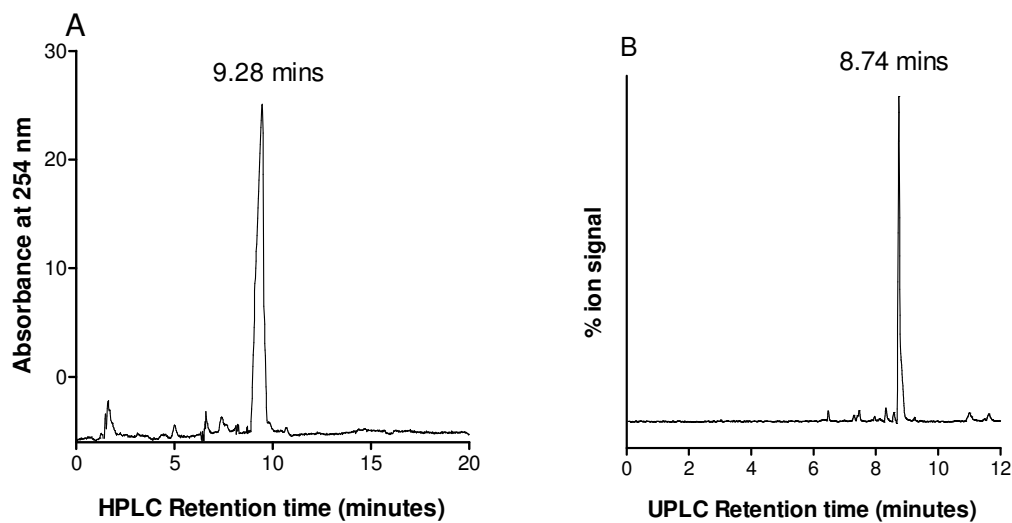


Figure 2.23 **A.** Analytical C₁₈ RP- HPLC chromatogram of purified Trc B₁ at 250 $\mu\text{g}/\text{mL}$ (50 μL injection volume) **B.** UPLC chromatogram of Trc B₁ preparation

Trc B was only purified from fraction D to 76%, and as with Trc B₁ also contained the related peptides, which was in this case Trc B₁ and Trc B'. Exhaustive purification of Trc B from a similar fraction by our group yielded a purity >90%. Trc B could be identified from its TOF-ESMS spectra showing the singly ($m/z = 1309.6499$) and doubly ($m/z = 655.3290$) charged molecular ions (Fig. 2.24).

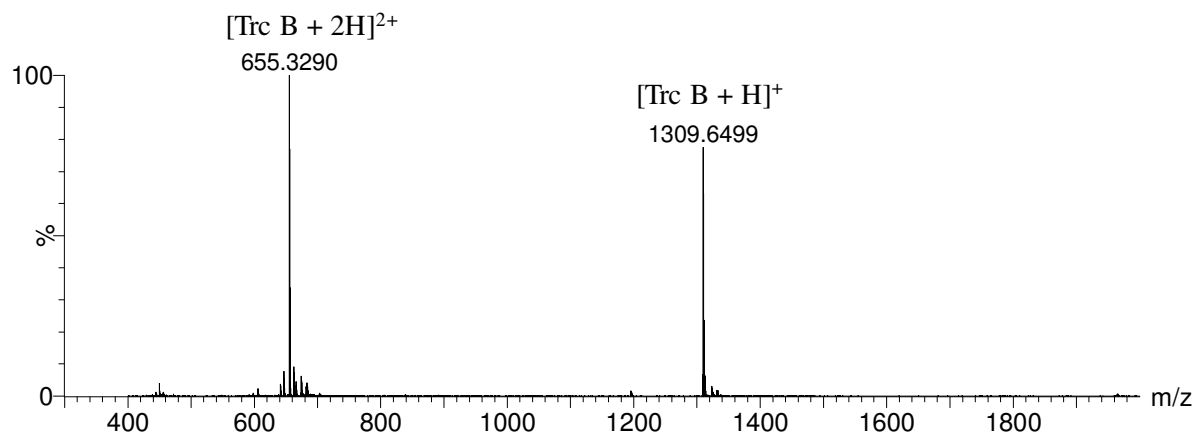


Figure 2.24 TOF-ESMS analysis of purified Trc B. The recorded positive mode spectrum from 300-2000 amu. The doubly charged molecular ion ($[\text{Trc B} + 2\text{H}]^{2+}$) and singly charged molecular ion ($[\text{Trc B} + \text{H}]^+$) of are indicated.

The retention time of purified Trc B was 9.56 minutes on the analytical C_{18} RP-HPLC column and 8.38 minutes on the UPLC column (Fig. 2.25). Its purity was 91% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

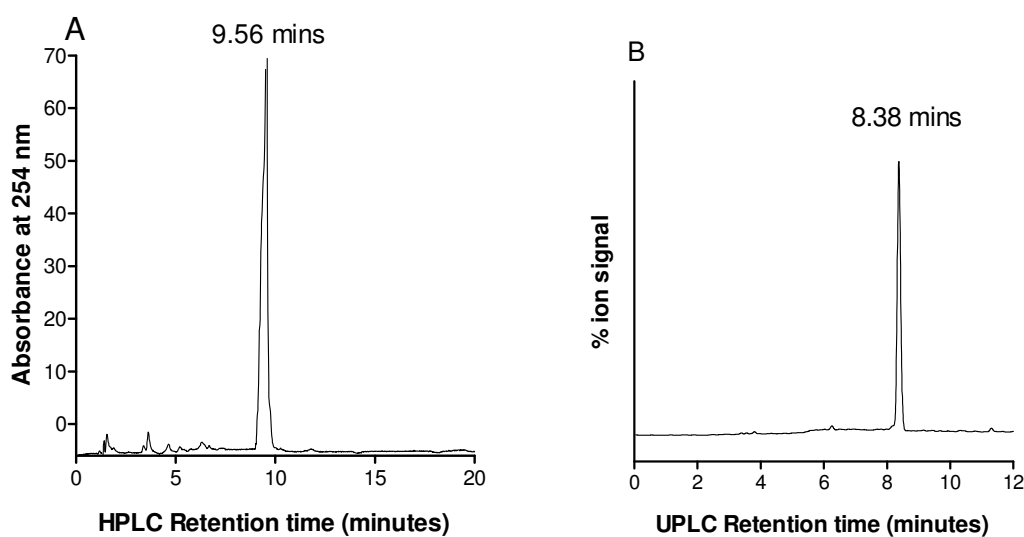


Figure 2.25 **A.** Analytical C_{18} RP- HPLC chromatogram of purified Trc B at 500 $\mu\text{g}/\text{mL}$ (50 μL injection volume) **B.** UPLC chromatogram of Trc B preparation

Trc A₁ was purified from fraction E. It could be identified from its TOF-ESMS spectra showing the singly charged molecular ion ($m/z = 1284.6760$) and singly charged sodium ($m/z = 1306.6543$) and potassium ($m/z = 1322.6252$) adducts (Fig. 2.26).

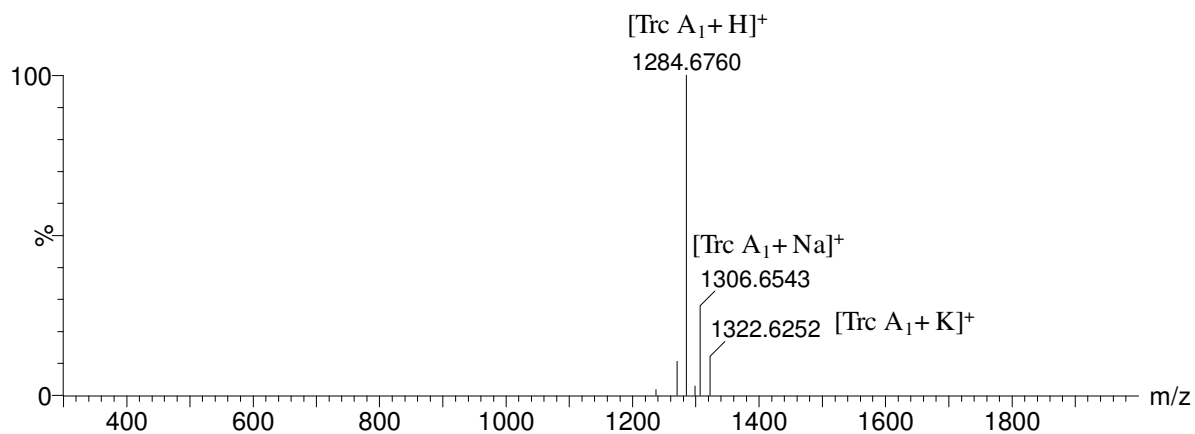


Figure 2.26 TOF-ESMS analysis of purified Trc A₁. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[\text{Trc A}_1 + \text{H}]^+$) and singly charged sodium ($[\text{Trc A}_1 + \text{Na}]^+$) and potassium ($[\text{Trc A}_1 + \text{K}]^+$) adducts are indicated.

The retention time of purified Trc A₁ was 11.12 minutes on the analytical C₁₈ RP-HPLC column and 9.11 minutes on the UPLC column (Fig. 2.27). Its purity was 98% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

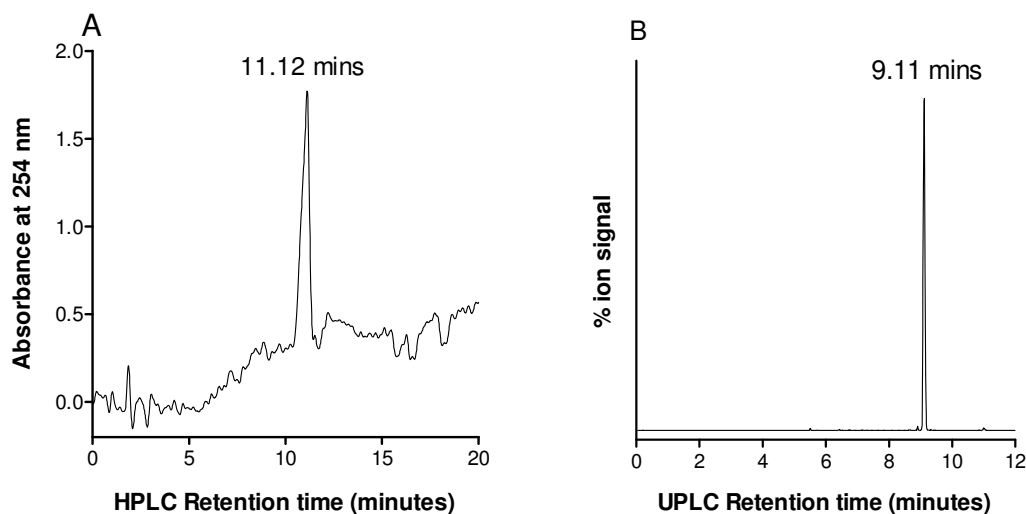


Figure 2.27 **A.** Analytical C₁₈ RP- HPLC chromatogram of purified Trc A₁ at 100 $\mu\text{g/mL}$ (20 μL injection volume) **B.** UPLC chromatogram of Trc A₁ preparation

2.4.3.3. From chemical synthesis

Among the synthetic Trc analogues, sTrc A(OMe₃) was not satisfactorily pure so it was purified by semi-preparative RP-HPLC to >95% purity. The synthetic analogues of Trc A and Phc A eluted earlier (6.33 and 8.80 mins respectively) than the natural analogues (11.26 and 13.61

minutes respectively) which revealed that there was an interference with at least their hydrophobicity during the chemical synthesis process (not shown). This could affect the mechanism of interaction of the synthetic analogues with targets; hence focus was on analysis of the natural analogues.

The synthetic Trc A analogue with two Orn residues could be identified from its TOF-ESMS spectra showing the singly ($m/z = 1256.6832$) charged molecular ion (Fig. 2.28).

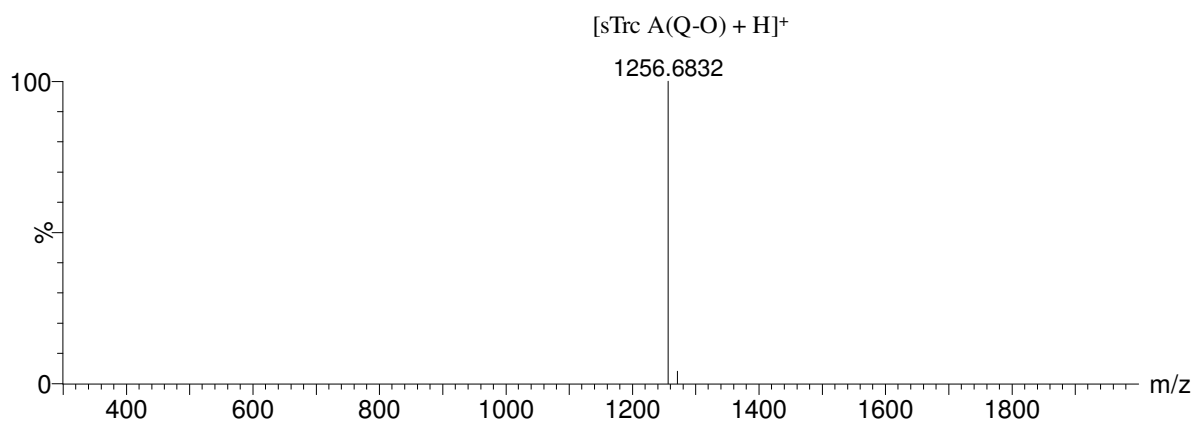


Figure 2.28 TOF-ESMS analysis of purified sTrc A(Q-O). The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[sTrc A(Q-O) + H]^+$) is indicated.

The retention time of purified sTrc A(Q-O) was 6.73 minutes on the analytical C₁₈ RP-HPLC column and 7.05 minutes on the UPLC column (Fig. 2.29). Its purity was 94% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

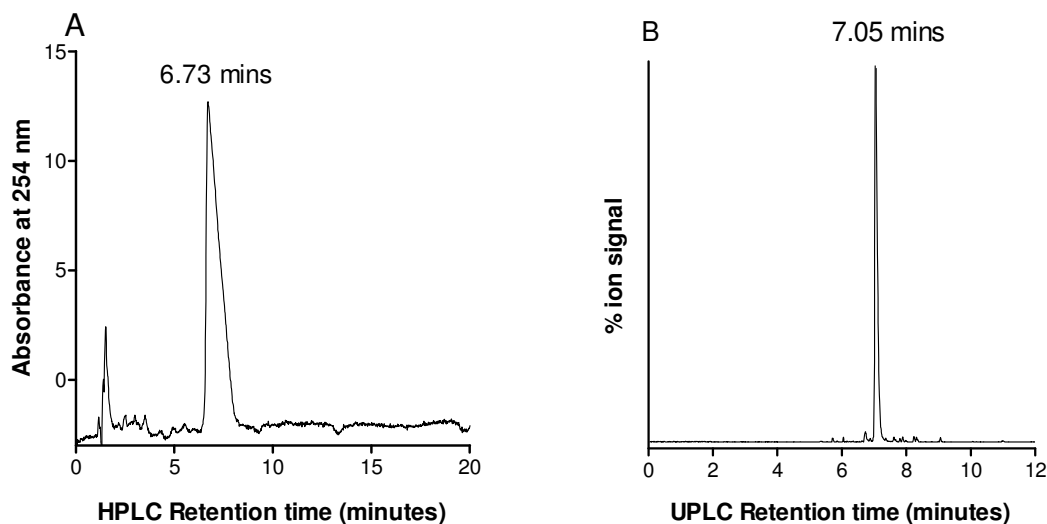


Figure 2.29 A. Analytical C₁₈ RP- HPLC chromatogram of purified sTrc A(Q-O) at 200 µg/mL (50 µL injection volume) B. UPLC chromatogram of sTrc A(Q-O) preparation

The synthetic Trc A analogue with a trimethylated Orn residue could be identified from its TOF-ESMS spectra showing the singly ($m/z = 1312.7084$) charged molecular ion (Fig. 2.30).

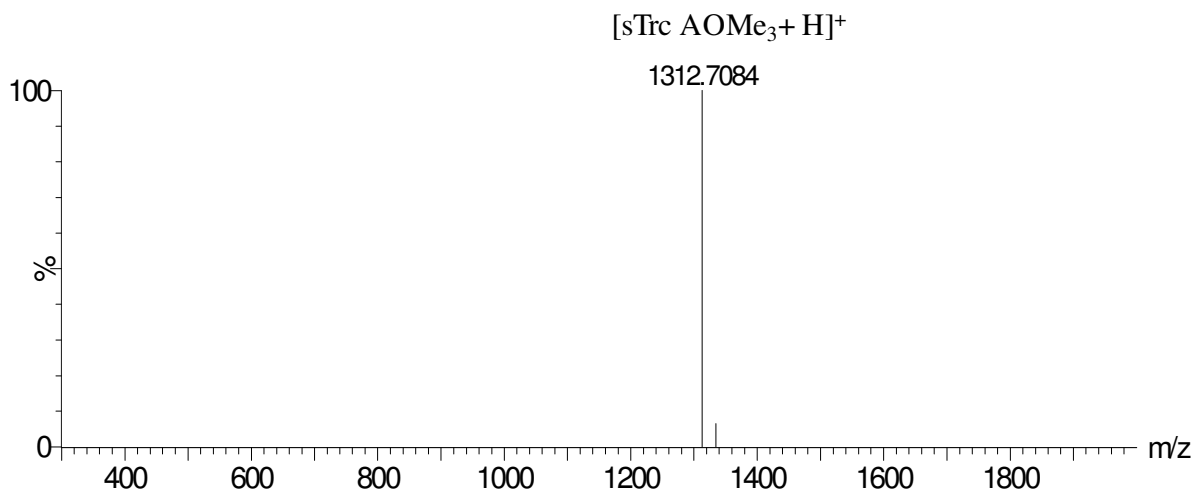


Figure 2.30 TOF-ESMS analysis of purified sTrc AOMe₃. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[sTrc AOMe_3 + H]^+$) is indicated.

The retention time of purified sTrc AOMe₃ was 10.04 minutes on the analytical C₁₈ RP-HPLC column and 9.04 minutes on the UPLC column (Fig. 2.31). Its purity was 96% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

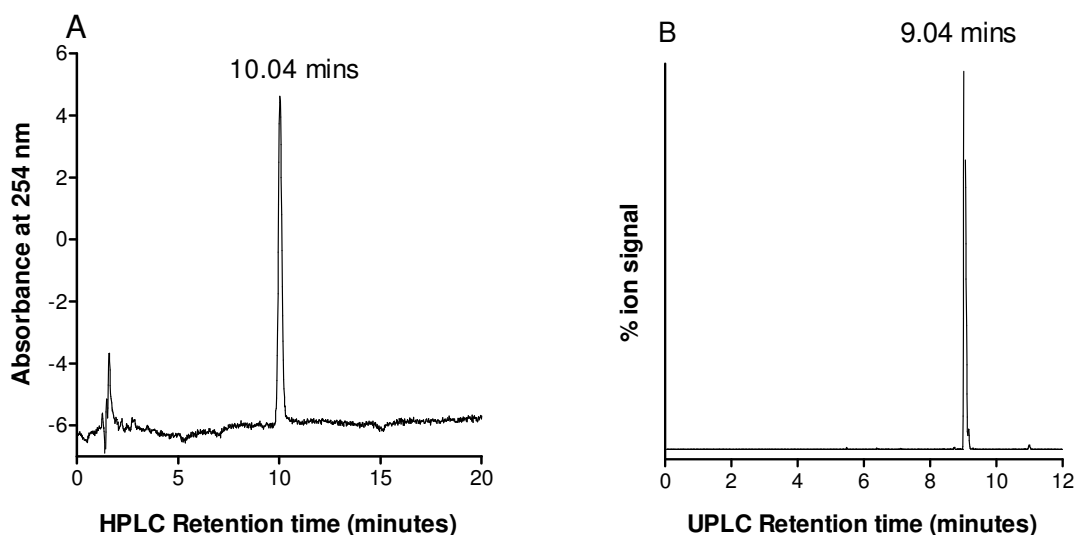


Figure 2.31 A. Analytical C_{18} RP- HPLC chromatogram of purified sTrc AOMe₃ at 200 $\mu\text{g/mL}$ (50 μL injection volume) B. UPLC chromatogram of sTrc AOMe₃ preparation

The synthetic Tpc A analogue could be identified from its TOF-ESMS spectra showing the singly ($m/z = 1293.6755$) charged molecular ion (Fig. 2.32).

The retention time of purified sTpc A was 13.58 minutes on the analytical C_{18} RP-HPLC column and 9.50 minutes on the UPLC column (Fig. 2.33). Its purity was 97% as determined from analysis of the UPLC results and >95% as determined from analytical HPLC.

The results of the purification and characterisation of the Trc analogues in this study are summarised in Table 2.5. The qualitative structure-to-activity relationship (QSAR) between the structure of the Trcs and their observed antimicrobial activities will be investigated in Chapter 3 with the aim of estimating the optimal/minimal structural determinants of activity necessary to facilitate the design of peptide derivatives and mimics for therapeutic evaluation^{11,66}. The HPLC R_t will be used as previously^{10,11,67,68} to depict the peptide hydrophobic character and potential membrane activity. The m/z will be used to derive the M_r parameter which will demonstrate peptide size factors that may affect target interaction.

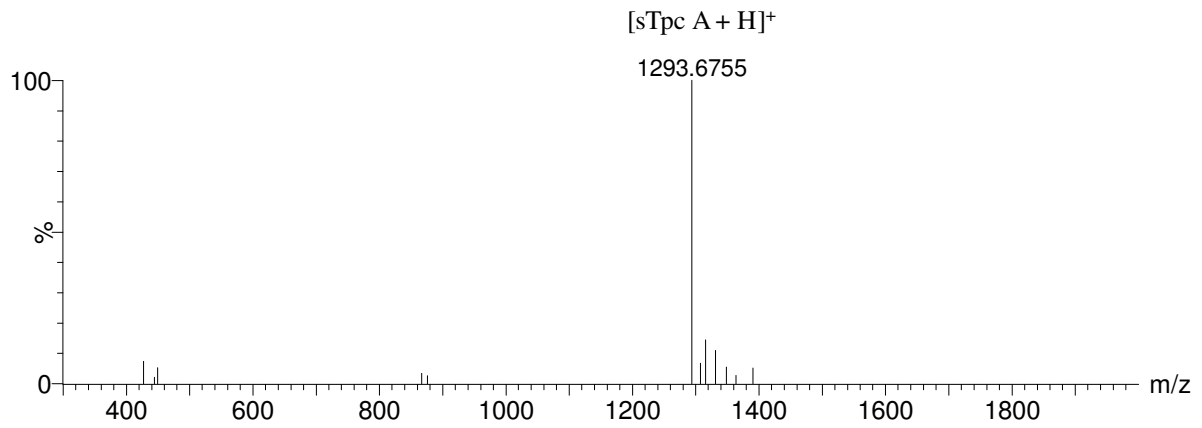


Figure 2.32 TOF-ESMS analysis of purified sTpc A. The recorded positive mode spectrum from 300-2000 amu. The singly charged molecular ion ($[sTpc\ A + H]^+$) is indicated.

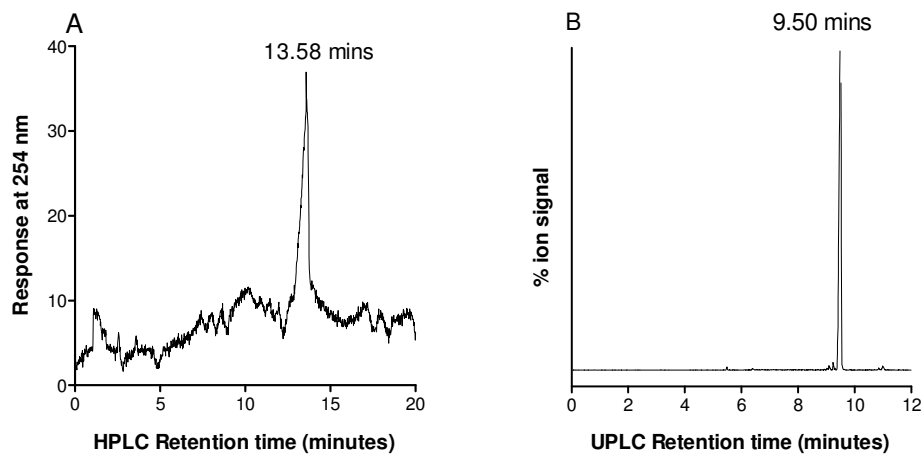


Figure 2.33 **A.** Analytical C_{18} RP- HPLC chromatogram of purified sTpc A at 100 $\mu\text{g/mL}$ (50 μL injection volume) **B.** UPLC chromatogram of sTpc A preparation

Table 2.5 Summary of characterisation of purified Trcs analogues in this study.

Peptide	HPLC R _t (mins.)	% HPLC purity	UPLC R _t (mins.)	% UPLC purity	Experimental M _r (Theoretical M _r)	Experimental <i>m/z</i> (Theoretical <i>m/z</i>) of singly charged molecular ion
From commercial tyrothricin						
Trc C ₁	7.31	>95	7.80	98	1361.6897 (1361.6921)	1362.6975 (1362.6999)
Trc C	7.71	>95	7.84	94	1347.6746 (1347.6764)	1348.6824 (1348.6842)
Trc B ₁	9.28	>95	8.31	90	1322.6826 (1322.6812)	1323.6958 (1323.6890)
Trc B	9.56	>95	8.39	91	1308.6337 (1308.6655)	1309.6499 (1309.6733)
Trc A ₁	11.12	>95	9.12	98	1283.6674 (1283.6703)	1284.6760 (1284.6781)
From amino acid supplemented culture extracts of <i>B. aneurinolyticus</i> 8185						
Tpc C	9.52	>95	8.13	97	1370.6949 (1370.6924)	1371.6957 (1371.7002)
Trc A	11.26	>95	9.23	97	1269.6605 (1269.6568)	1270.6611 (1270.6624)
Phc A	13.61	>90	9.49	90	1253.6663 (1253.6600)	1254.6670 (1254.6675)
From chemical synthesis						
sTrc A(Q-O)	6.73	>95	7.04	94	1255.6754 (1255.6754)	1256.6832 (1256.6832)
sTrc AOMe ₃	10.04	>95	9.04	96	1311.7006 (1311.7337)	1312.7084 (1312.7415)
sTpc A	13.58	>95	9.50	97	1292.6677 (1292.6706)	1293.6755 (1293.6784)

2.4.4. Homology models of newly purified Trc analogues

We used a low energy model of Trc C, created from 2D-NMR NOE constraints (model courtesy of Prof. G. Jackson, University of Cape Town, Cape Town, South Africa), as the basic three-

dimensional structure for the homology modelling of the new Trc analogues. Previous modelling experiments by M. Rautenbach (University of Stellenbosch, Stellenbosch, South Africa) produced homology models for GS and the six major Trcs⁴¹ which were mutated to generate the new structures in this study. Four internal hydrogen bonds between Phe³-NH:O-Leu¹⁰, Leu¹⁰-NH:O-Phe³ and Val⁸-NH:O-Asn⁵ and Asn⁵-NH-O-Val⁸ are expected to stabilise the β -sheet structures of the Trcs, two of which are in the conserved VOLFP pentapeptide moiety as predicted for Trc A by Gibbons *et al.*⁶⁹. The low energy model of all the new analogues showed at least three matching hydrogen bonds (Fig. 2.34). These bonds were also found in the structure of GS and those of major Trcs⁴¹. Only Tpc A and Trc A(Q-O) structures exhibited all four internal hydrogen bonds. We observed that the lowest energy structures of the other three peptide structures lacked the hydrogen bond between Asn⁵-NH:O-Val⁸. Therefore, all predicted model structures were similar in their backbone conformations and therefore their secondary structures. They all showed a β -sheet with two strands made of Val⁸-O⁹-Leu¹⁰ and Ar³-D-Ar⁴-Asn⁵, where O stands for either Orn or trimethylated-Orn and Ar denotes Trp or Phe. The adjoining β -turns are made of Gln/Orn⁶-Ar⁷ and D-Phe¹-Pro² where Ar is Tyr, Phe or Trp. This agrees with the predicted structures of the major Trcs⁴¹ and the type I β -turn/type II β -turn/ β -pleated sheet conformation indicated for Trc A in solution⁶⁹⁻⁷¹.

From the ten lowest energy structures (RMSD < 1Å from average) of each peptide we determined the solvent accessible area (SASA) and volume (SAV) *in silico* using YASARA 9.10.5[©] (Table 2.6). SASA and SAV are size parameters that are also useful in calculating hydrophobic burial⁷² and as a measure of the probable magnitude of binding-induced changes⁷³ such as during target interaction making them also relevant in QSAR studies. Their relationship to the hydrophobicity of molecules is through computation of the logarithm of the partition coefficient^{74,75}. Phc A had the least SASA and SAV values while Trc C₁ had the largest values (Table 2.6).

Table 2.6 Summary of information derived from molecular models of tyrocidine analogues using YASARA 9.10.5[©]

Peptides	Observed hydrogen bonding of backbone	Solvent accessible surface area (SASA, Å ²)	Solvent accessible volume (SAV, Å ³)
Trc A*	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1199	2680
Trc A ₁ *	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1208	2715
Phc A	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1120	2591
sTpc A	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵ Asn ⁵ -NH- O-Val ⁸	1122	2631
sTrc AOMe ₃	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1170	2741
sTrc A(Q-O)	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵ Asn ⁵ -NH- O-Val ⁸	1122	2605
Trc B*	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1217	2741
Trc B ₁ *	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵ Asn ⁵ -NH- O-Val ⁸	1231	2777
Trc C*	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1267	2833
Trc C ₁ *	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1269	2854
Tpc C	Phe ³ -NH-O-Leu ¹⁰ Leu ¹⁰ -NH-O-Phe ³ Val ⁸ -NH-O-Asn ⁵	1193	2772

*Results from Spathelf⁴¹

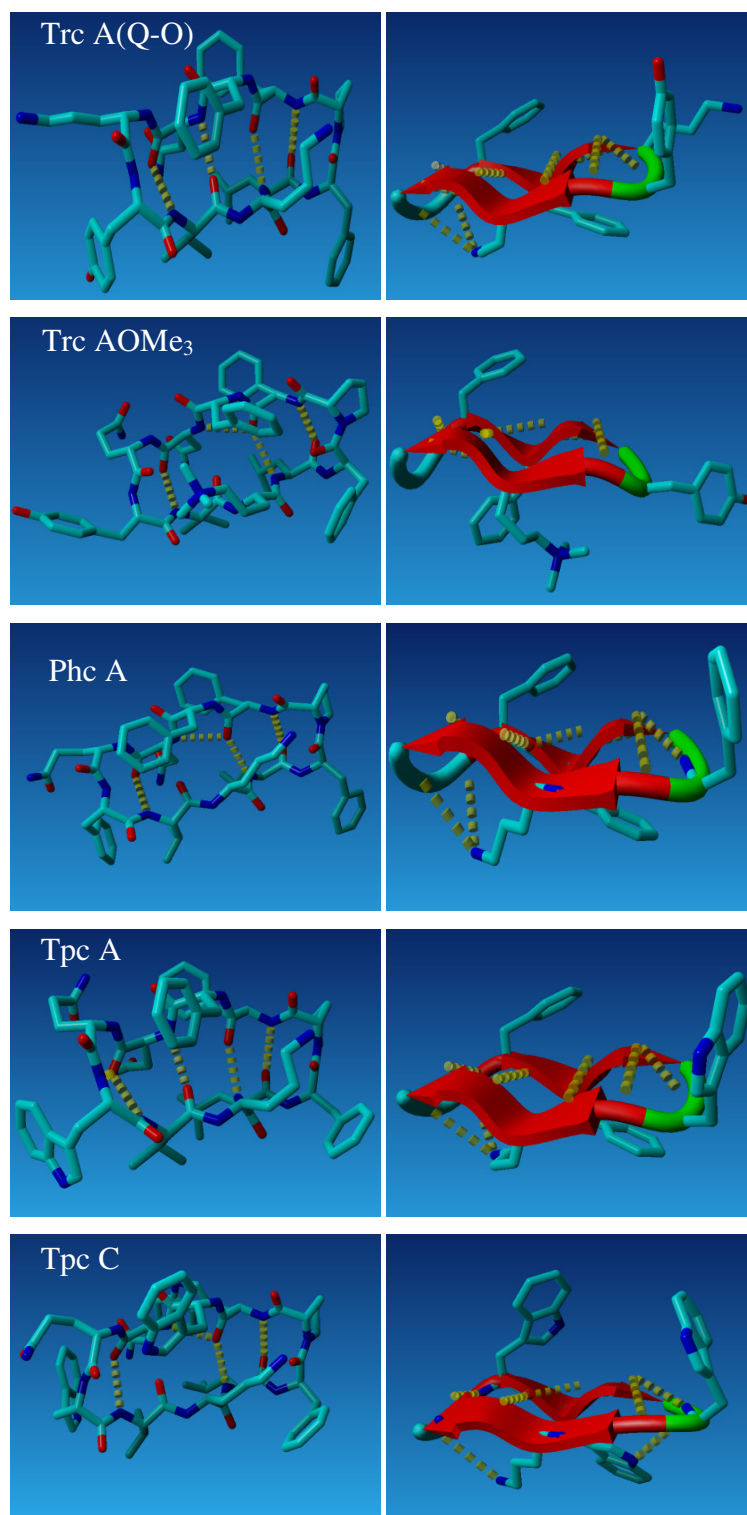


Figure 2.34 Examples of low energy structures of the novel Trc analogues in this study. The left panels show hydrogen bonding pattern within the cyclic backbone and the right panels show the secondary structure (β -sheet structure shown as a red ribbon, β -turn as green tube) and the side chains of the variable amino acids.

2.5. Conclusions

Bacillus aneurinolyticus strains ATCC 8185 effectively produce Trcs that inhibit the growth of bacteria such as *M. luteus* NCTC 8340. We successfully manipulated *B. aneurinolyticus* ATCC 8185 cultures for tailored Trc production to ease extraction and purification and ensure high yields of single peptides (tryptocidine C, Trc A and phenycidine A). The RP-HPLC purification methodology established by our group enabled the purification of the six major Trcs (Trc A₁, B, B₁, C and C₁) from a commercial tyrothricin complex. This methodology was also used for the purification of the synthetic trimethylated Trc A. Nine natural Trcs and three synthetic analogues were purified. The purity of the collected fractions was confirmed using analytical RP-HPLC, UPLC-MS and TOF-ESMS. The use of these analytical tools together with the modification of the culture media of the Trc producer *Bacillus* have enabled the purification of Tpc C, Phc A and Trc A in milligram quantities. It is worth noting that the successful purification to 97% and 90% of Tpc C and Phc A respectively was an important result as this had proven difficult in previous work⁴¹. The low energy homology models of Tpc C, Phc A, Tpc A, Trc AOMe₃, and Trc A(Q-O) were determined for the first time in this study. All predicted models were highly similar to models of the other tyrocidine analogues previously determined⁴¹ in both their backbone conformations and secondary structures. Chemical characterization of the purified peptides using analytical HPLC and TOF-ESMS, as well as molecular modelling of the peptides allowed us to derive structural parameters (HPLC retention time, molecular mass, solvent accessible surface area, solvent accessible volume) that will be useful for qualitative structure-to-activity analyses (QSAR) to predict structural motifs and pre-requisites of tyrocidine structure that are relevant to antimicrobial activity (Chapter 3).

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Chapter 3

Role of antimicrobial peptide structure on antilisterial activity: tyrocidine and *cyclo*(RRRWF_n) analogues

3.1. Introduction

Listeriosis has received increased attention from the 1980s due to the rise in the number of human cases in several countries as well as evidence for food borne transmission ¹. *Listeria monocytogenes* is the major species pathogenic to humans causing this opportunistic infection with a case fatality of 20 to 40% in immunocompromised subjects, infants, pregnant women, and senior citizens, thus a public health threat ¹⁻⁴. The disease is characterised by abortion, septicemia and meningoenzephalitis with listerial meningitis ranking among the most lethal infections ^{3,5}. *Listeria* spp. contamination also has a serious economic impact due to the loss incurred by the food industry during outbreaks from execution of the WHO recommendation to “withdraw from the market any foods which have been demonstrated to be causally associated with human cases of listeriosis” ⁶. The current therapy of choice is ampicillin and penicillin, often in combination with gentamicin because most β -lactam antibiotics are only bacteriostatic to *Listeria* spp. ^{7,8}. However, use of these drugs does not always result in successful treatment and there is a general tendency of resistance by *Listeria* spp. to antibiotics and disinfectants ^{1,3,4}. This concern propelled the search for new antimicrobials ⁹.

Cationic peptides with antimicrobial activity also known as “natural antibiotics” have been investigated for food processing and preservation to control harmful organisms such as *L. monocytogenes* ^{2,10}. Three main groups of antimicrobial peptides (AMPs) can be distinguished: the gene-encoded heat-stable prokaryotic bacteriocins ^{9,11}, ribosomally produced eukaryotic AMPs ¹²⁻¹⁴ and the non-ribosomally synthesized AMPs ^{10,15}. Bacteriocins produced by lactic acid bacteria (LAB) have been of particular interest in the food industry for use as natural preservatives ¹⁶. Their recent classification groups them into two major classes ^{16,17}: class I or lantibiotics and class II or nonlantibiotics. The two classes differ in whether they possess or lack post-translationally modified amino acids such as 2, 3-dehydroalanine (Dha), 2, 3-dehydrobutyrine (Dhb), and the distinctive lantionine and methylantionine residues ^{11,17}. Class I peptide bacteriocins are further divided into type A or B in terms of their structures; type A have a flexible structure in solution and include nisin, subtilin and Pep5 while type B are rigid and globular ^{17,18}. Class II bacteriocins are also sub-classified into classes IIa (pediocin-like which

possess the conserved N-terminal consensus sequence YGNGVXCXK), IIb (two-peptide bacteriocins), IIc (leaderless peptide bacteriocin), and IId (circular bacteriocins). Nisin and class IIa bacteriocins are particularly interesting for their antilisterial activity^{19–21}. Nisin is the only bacteriocin accorded the status of GRAS (generally recognized as safe) in the United States for use in food at levels of 5.5–6.9 mg nisin per kg of food and licensed as food preservative in over 45 countries^{2,21,22}. Nevertheless, the shortcomings of nisin include the fact that it is unstable and less soluble above pH 7.0 and its antimicrobial activity, which is limited to Gram-positive bacteria, reduces when it is integrated in complex foods². Leucocins such as leucocin A produced by *Leuconostoc gelidum* UAL-187 are class IIa bacteriocins reported to inhibit *L. monocytogenes*^{23–25}. Leucocin A is a 37 residue-peptide predicted to consist of three short β -strands at the N-terminus and an amphiphilic helical region in the C-terminus. Between the two sections, the sequence fragments containing Cys-9 and Cys-14 form two β -strands connected by a short six-member disulfide loop or turn connecting Cys-9 and Cys-14^{23,26}. Bacteriocins act primarily via membrane pore formation as their net positive charge and amphipathic structure assists in their interaction with negatively charged microbial membranes or other cellular targets^{9,27}. Secondary cellular targets including lipid II targeted by mersacidin and mesentericin Y to inhibit cell-wall synthesis, bacterial RNA synthesis inhibited by microcin J25 and bacterial phospholipase A₂ inhibited by duramycin-C as well as more than one mechanism (nisin induces membrane pore formation and inhibits cell-wall synthesis) are also employed for killing bacteria^{9,28–30}. It has been proposed that class IIa bacteriocins require a mannose phosphotransferase system (PTS) permease as cell surface target molecule for their activity^{21,31,32}.

Despite the multi-targeted mode of action of the bacteriocins, there are several reports of resistance by *L. monocytogenes* strains to class I (i.e. nisin) and class IIa bacteriocins^{21,33–36} with the term “resistance” referring to the ability of strains to grow at the highest bacteriocin concentration available. The proposed nisin resistance model of *L. monocytogenes* ATCC 700302 by Crandall & Montville³⁷ reviewed by Kaur *et al.*³⁸ included three factors: (i) alteration of peptidoglycan composition³⁹ possibly leading to increased binding of divalent cations that interfere with the cationic peptide; (ii) phospholipid content changes leading to modified membrane electric charge which inhibits pore formation; and (iii) decreasing membrane fluidity which prevents peptide insertion and association. The proposed mechanisms of resistance to class IIa bacteriocins specifically leucocin resistance in *L. monocytogenes* B73-MR1 involve absence of a putative mannose-specific PTS enzyme IIAB subunit⁴⁰, up-regulating the synthesis of a putative β -glucoside-specific PTS enzyme II (EII^{Bgl}) and a phospho- β -glucosidase, as well as increased membrane fluidity by increasing levels of desaturated and

short-acyl-chain phosphatidylglycerols in the membrane^{21,36,41}. Furthermore, bacteriocin cross-resistance in which resistance to a bacteriocin leads to resistance to other bacteriocins of the same class or in other classes reduces the efficiency of bacteriocins¹⁶. There is therefore, a need for other antimicrobial compounds to be used in isolation or in combination with nisin or other bacteriocins as food preservatives. Katla *et al.*²¹ proposed that bacteriocins should be part of a multiple preservative principle system referred to as “hurdles”.

There are several records of high antilisterial activity by AMPs. Their most attractive attributes include broad spectrum antimicrobial activity, selectivity, rapid action, and reduced likelihood of resistance development¹⁵. For example, the alpha-defensins from rhesus macaque leukocytes have been shown to be active against *L. monocytogenes* strain EGD at 7-15 µg/mL in a study by Tang *et al.*⁴³. López-Solanilla *et al.*⁵ found that human defensins, thionins, protamine and magainin had significant antilisterial activity with a minimum inhibitory concentration (MIC) of 2-5 µg/mL. More recently, Spathelf & Rautenbach⁴⁴ demonstrated significant activity by the major tyrocidines (Trcs) of the tyrothricin complex; cyclic decapeptides produced by *Bacillus aneurinolyticus*, against the leucocin A resistant strain, *L. monocytogenes* B73-MR1. Testing more Trc analogues is needed to improve the understanding of the structure-to-activity relationship (SAR) of the Trcs. Possible cyclic peptide candidates for antilisterial activity, sharing the predominant aromatic residue and cationic character with the Trcs, are the analogues of synthetic hexapeptide (Ac)-RRWRF-NH₂ (Ac-RW) which was identified as an AMP by Blondelle and Houghten⁴⁵ through synthetic combinatorial libraries screening. Dathe *et al.*⁴⁷ obtained *cyclo*(RRRFW) (c-WFW) by head-to-tail cyclisation of the synthetic hexapeptide (Ac)-RRWRF-NH₂ (Ac-RW). This resulted in a 62-fold increase in activity against *Escherichia coli* and 8-fold increase against *Bacillus subtilis*⁴⁸.

This study aims to determine, compare and contrast the *in vitro* antilisterial activities of purified Trc analogues with *cyclo*(RRRFW) and analogues (RW-peptides) against the leucocin A sensitive (B73) and resistant (B73-MR1) strains of *L. monocytogenes* and to find correlation between biophysical/theoretical structural parameters and bioactivity parameters through qualitative structure-to-activity relationship (QSAR) analyses. Characterisation of these libraries of small cyclic AMPs will enable us to gain insight into the structural motifs and prerequisites necessary for antilisterial activity and selectivity.

3.2. Materials

Tyrothricin (extracted from *Bacillus aneurinolyticus*), gramicidin S (GS) (from *Brevibacillus brevis* Nagano), Corning Incorporated[®] cell culture cluster non-pyrogenic polypropylene

microtiter plates and trifluoroacetic acid (TFA, >98%) were obtained from Sigma (St. Louis, USA). Acetonitrile (ACN) (HPLC-grade, far UV cut-off) came from Romill Ltd. (Cambridge, UK). Culture dishes were obtained from Lasec (Cape Town, South Africa) and microtitre plates (Nunc™-Immuno Maxisorp) were from AEC Amersham (Kyalami, South Africa). Brain heart infusion (BHI) and BHI agar were supplied by Biolab Diagnostics (Midrand, South Africa). To obtain analytical grade water, water was filtered from a reverse osmosis plant via a Millipore Milli-Q water purification system (Milford, USA). Ethanol (>99.8%) was supplied by Merck (Darmstadt, Germany). Falcon® tubes were from Becton Dickson Labware (Lincoln Park, USA). Promega (Madison, USA) supplied the Cell Titer-Blue™ Cell Viability Assay kit. The synthetic tyrocidines were supplied by GL Biochem (Shanghai) Ltd., China. The Nova-Pak® C₁₈ (5 µm particle size, 60 Å pore size, 150 mm × 3.9 mm) reverse-phase analytical column, the Nova-Pak® C₁₈ (6 µm particle size, 60 Å pore size, 7.8 mm × 300 mm) semi-preparative HPLC column and an ACQUITY UPLC® bridged ethyl hybrid (BEH) C₁₈ (1.7 Mm particle size, 2.1 mm × 100 mm) column were from Waters Millipore (Milford, USA). Dr Margitta Dathe from Leibniz Institute of Molecular Pharmacology (FMP), Berlin, Germany supplied the pure (>90%) synthetic cyclic Arg- and Trp-rich peptides (RW-peptides).

3.3. Methods

3.3.1. *Bacteria culturing*

Normal sterile techniques were used to culture *L. monocytogenes* strains B73, a leucocin A-sensitive wild-type meat-isolate²⁵ and B73-MR1, a leucocin A resistant mutant of B73⁴⁰. The bacteria were cultured on BHI agar from freezer stocks for 24 hours at 37 °C followed by culture of selected colonies at 37 °C in BHI broth to log phase for growth inhibition studies. Subsequently the cultures were sub-cultured in BHI broth to an OD₆₂₀ of 0.4 and diluted to OD₆₂₀ of 0.2 (6.7×10⁸ colony forming units per mL) prior to use in assays. Extreme care was taken in the storage and culturing conditions of the target strains in this study as *L. monocytogenes* tends to adapt to different conditions and in the process its peptide susceptibility changes. Details on this aspect are given in the addendum under *Section 3.8.1*.

3.3.2. *Peptide preparation*

The major Trcs from the commercial tyrothricin were purified and characterised as previously described in Chapter 2 according to the methods of Rautenbach *et al.*⁴⁹ and Spathelf & Rautenbach⁴⁴. The other natural analogues (Tpc C, TrcA and Phc A) were extracted from

cultures of *B. aneurinolyticus* ATCC 8185 (supplied by the American Type Culture Collection (Manassas, VA, USA)), purified and characterised as previously described^{44,49}. Following peptide isolation by established reverse-phase high performance liquid chromatography (RP HPLC)^{44,49}, the peptides were analysed for purity and integrity using analytical HPLC, ultra performance liquid chromatography (UPLC) and time-of-flight electrospray mass spectrometry (TOF-ESMS) according to the methods of Rautenbach *et al.*⁵⁰.

The purified peptides were subsequently used to analytically prepare stock solutions of 2.00 mM (most synthetic peptides) or 1.00 mM (natural peptides and GS) or 1.00 mg/mL (Trc mixture) with 40% *v/v* ethanol in analytical grade water (Trcs) or with analytical grade water (synthetic RW-peptides and GS). Subsequently for broth microdilution assays, the stock solution was used to construct two-fold dilutions in polypropylene 96 multi-well plates using the culture medium (BHI broth).

The effect of low pH due to residual trifluoroacetic acid on peptide activity was counteracted by repeatedly lyophilizing the preparations to remove residual trifluoroacetic acid and to obtain a pH 7 ± 1 for 200 μ M peptide in analytical quality water.

3.3.3. Determination of antilisterial activity of peptides

The activity of the peptides was analysed for inhibition of growth of the bacteria cultures. The broth microdilution assay for growth inhibition was carried out as previously described^{51,52}. The diluted cell suspension (90 μ L) was transferred into each well in separate sterile microtiter plates followed by the addition of 10 μ L of peptides from the dilution series accordingly or solvent devoid of peptide as control. The microtiter plates were sealed and incubated subsequently at 37 °C for 16 hours, after which the light dispersion of the wells was obtained spectrophotometrically at 595 nm using a Biorad model 680 microplate reader. This was used to compute the percentage growth inhibition (equation 1) as previously described⁵³.

$$\% \text{ growth inhibition} = 100 - \frac{100 \times (A_{595} \text{ of well} - \text{Average } A_{595} \text{ of background})}{\text{Average } A_{595} \text{ of growth wells} - \text{Average } A_{595} \text{ of background}} \quad (1)$$

After reading the light dispersion of the wells at 595 nm, the assay for activity proceeded with the addition of 10 μ L of CellTiter-Blue™ reagent to every well and incubation for an additional hour. Resazurin (blue, absorption maximum at 605nm) in the CellTiter-Blue™ is reduced to resofurin (pink, absorption maximum at 573nm) in viable and respiring cells⁵⁴. The plates were read spectrophotometrically at 570 nm and 600 nm using a Biorad microtiter plate reader and the percentage metabolism inhibition was computed using equation 2.

$$\% \text{ inhibition} = 100 - \frac{100 \times (A_{570}/A_{600} \text{ of well} - \text{Average } A_{570}/A_{600} \text{ of background})}{\text{Average } A_{570}/A_{600} \text{ of growth wells} - \text{Average } A_{570}/A_{600} \text{ of background}} \quad (2)$$

3.3.4. Analysis of dose-response data

GraphPad Prism 4.03 (GraphPad Software, San Diego, USA) was used to plot the dose–response curves. The data was analysed by carrying out non-linear regression and sigmoidal curves were fitted (having variable slope and a constant difference of $100 \pm 10\%$ between the top and bottom plateau).

Equation 2 from Rautenbach *et al.*⁵³ was used to fit the dose-response curves:

$$Y = \text{bottom} - (\text{top} - \text{bottom}) / (1 + 10^{\log \text{IC}_{50} \times \text{Activity slope}}) \quad (3)$$

Top and bottom are defined as the experimental percentage inhibition in the presence of high peptide concentrations and in the absence of peptide, respectively; $\log \text{IC}_{50}$ is the x-value that denotes the response halfway between the top and bottom and the activity slope, which is related to the Hill slope, defines the slope of the curve. IC_{max} , related to the minimal inhibitory concentration (MIC), was computed from the x-values at the intercept between the slope and the top plateau⁵². The IC_{50} , defined as the peptide concentration leading to 50% growth inhibition, the IC_{max} , the inhibition concentration factor (IC_F , concentration factor that describes increase from minimum to maximum inhibition)⁵³, and a factor defined in this study, the activity product ($A_P = \text{IC}_{50} \times \text{IC}_{\text{max}}$) were chosen as parameters to characterise and compare activity of the Trcs. We used the IC_F parameter because for comparison of antimicrobial peptide activity it is crucial to describe activity by more than one parameter⁵³. Parameters such as IC_F can provide information about peptide mechanism of action and compound parameters like the A_P are useful in instances where the IC_{50} and IC_{max} give different trends as is yields a single factor for comparing the peptides. Activity at a fixed concentration of 25 μM was rather used to compare the RW-peptides.

3.3.5. Qualitative Structure-to-Activity Relationship (QSAR) analyses

QSAR analyses consisted of investigating the correlation between different physicochemical properties of the purified peptides and their antilisterial activity. The physicochemical properties that were considered include: analytical HPLC retention time (R_t) and a number of parameters describing the chemical properties of the peptides, as well as molecular mass (M_r) and a number of parameters describing the size of the peptides. The theoretical parameters, lipophilicity (π_{FP}),

^{55,56}, Q/L ⁵⁵, hydrophathy ⁵⁷, hydrophobicity ⁵⁸, interphase properties ⁵⁹, molecular volume (MV) ⁶⁰ and side-chain surface area (SCSA) ^{55,61} of each peptide were additive parameters obtained from the summation of the parameters of all the constituent amino acids of the particular peptide. Side-chain surface areas (SCSA) were computed from Connolly surfaces ⁶¹. The solvent accessible surface area (SASA) and volume (SAV) as well as molecular volume (for RW-peptides) were determined *in silico* from the ten lowest energy structures (RMSD < 1Å from average) of each peptide with YASARA 9.10.5[©]. Principal component analysis (PCA) was done using *STATISTICA* version 11, series 0112 (StatSoft[®], Tulsa, OK, USA) on all structural and activity parameters against both strains of *L. monocytogenes* to identify clusters of peptides with similar characteristics as well as structural parameters that are important for antilisterial activity.

3.4. Results and Discussion: Part I - Tyrocidine analogues

We divided the tyrocidines into two libraries namely the Trc A library and Trc C library (Table 3.1), on the grounds of the identity of their aromatic dipeptide unit and the lower antilisterial activity found previously for the Phe-Phe containing Trc A *versus* that of Trp-Trp containing Trc C ⁴⁴. We included selected tryptocidines (Tpc) and a phenycidine (Phc), where the aromatic amino acid, Tyr⁷, was substituted with Trp or Phe respectively, to assess the role of the Tyr in the peptide activity (Table 3.1). The cationic residue was assessed in terms of size, hydrogen bonding ability and integration into membranes, as Spathelf & Rautenbach ⁴⁴ found lower antilisterial activity for the Lys-analogues, than the Orn-analogues. Following comparable antibacterial activity to the parent gramicidin S (GS) of analogues containing N-methyl groups including trimethylated-Orn analogues against Gram-positive bacteria ⁶², we included a synthetic Trc A analogue, sTrc AOMe₃, containing a trimethylated-Orn residue (Table 3.1). From the reported improved activity of Trc A towards *Bacillus subtilis* ⁶³, after charge increase with Gln to Orn substitution, this Trc A analogue (sTrc A(Q-O)), was also included. It should be noted that Trc B doubles as both a Trc A and a Trc C analogue. The structural parameters of all the Trcs are detailed in Chapter 2 and Table S3.1 in supplementary data.

Table 3.1 Description of tyrocidine A and C analogues

Type	Identity	Sequence	Source	Abbreviation
Trc A analogues	Tyrocidine A	<i>cyclo</i> [f ¹ P ² F ³ f ⁴ N ⁵ Q ⁶ Y ⁷ V ⁸ O ⁹ L ¹⁰]	Natural	Trc A
	Tyrocidine A ₁	<i>cyclo</i> [fPFfNQYVKL]	Natural	Trc A ₁
	Tyrocidine B	<i>cyclo</i> [fPWfNQYVOL]	Natural	Trc B
	Phencycline A	<i>cyclo</i> [fPFfNQFVOL]	Natural	Phc A
	Tryptocidine A	<i>cyclo</i> [fPFfNQWVOL]	Synthetic	sTpc A
	Trc A-trimethylated Orn	<i>cyclo</i> [fPFfNQYVO(Me) ₃ L]	Synthetic	sTrc AOMe ₃
	Tyrocidine A-Gln to Orn	<i>cyclo</i> [fPFfNOYVOL]	Synthetic	sTrc A(Q-O)
Trc C analogues	Tyrocidine C	<i>cyclo</i> [fPWwNQYVOL]	Natural	Trc C
	Tyrocidine B	<i>cyclo</i> [fPWfNQYVOL]	Natural	Trc B
	Tyrocidine B ₁	<i>cyclo</i> [fPWfNQYVKL]	Natural	Trc B ₁
	Tyrocidine C ₁	<i>cyclo</i> [fPWwNQYVKL]	Natural	Trc C ₁
	Tryptocidine C	<i>cyclo</i> [fPWwNQWVOL]	Natural	Tpc C

3.4.1. *L. monocytogenes* strain susceptibility to the tyrocidines

The Trc A and Trc C analogues (Table 3.1) were evaluated for their antilisterial activity against both the leucocin A sensitive strain *L. monocytogenes* B73 and the resistant strain *L. monocytogenes* B73-MR1. GS with 50% sequence identity to the Trcs and with known lytic activity was included as a reference peptide. In general, both strains were sensitive to the Trcs tested with IC₅₀ values ranging from 5.5 to 27 µM (Table 3.2). The observed growth inhibition activities of the major Trcs purified from commercial tyrothricin (A₁, B, B₁, C, and C₁) and Trc A from culture extracts of *B. aneurinolyticus* ATCC 8185 as well as GS were similar to those previously reported⁴⁴ with GS again more active than the Trcs against both strains (Table 3.2). The Trc analogue with the lowest IC₅₀ was Trc B (IC₅₀ = 7.4 µM) against *L. monocytogenes* B73 and Trc C (IC₅₀ = 5.5 µM) against *L. monocytogenes* B73-MR1.

When there was a significant difference of the Trc activity in terms of IC₅₀ values towards the two strains, the activity was generally greater against the resistant strain than against the sensitive strain (Fig. 3.1A and Fig. 3.2, refer to Tables S3.2 and S3.3 in supplementary data for statistical analysis data). Spathelf & Rautenbach⁴⁴ made a similar observation on the higher susceptibility of the resistant strain to the Trcs and GS. The IC_{max} values, however, revealed that there was increased susceptibility of the sensitive strain (B73) over the resistant strain (B73-MR1) for the more hydrophobic Phc A and Trc A (Fig. 3.3A).

Table 3.2 Summary of the antilisterial activity parameters of the Trc mixture, Trc A analogues, Trc C analogues and GS toward the leucocin A sensitive strain (B73) and the leucocin A resistant strain (B73-MR1) of *Listeria monocytogenes*. Trc mixture's concentrations are given in $\mu\text{g/mL}$ (top row) and those of all other peptides are given in μM . Every value denotes the average \pm standard error of the mean (SEM) of n biological repeats (number of repeats given in brackets), with triplicate technical repeats per assay. Trc B is shaded in grey to indicate that it belongs both to the Trc A analogues above it and Trc C analogues below it.

Peptide	<i>L. monocytogenes</i> B73		<i>L. monocytogenes</i> B73-MR1	
	IC ₅₀ \pm SEM (n)	IC _{max} \pm SEM (n)	IC ₅₀ \pm SEM (n)	IC _{max} \pm SEM (n)
GS	4.2 \pm 0.14 (9)	6.0 \pm 2.90 (9)	3.9 \pm 0.15 (3)	5.40 \pm 0.27 (3)
Trc mix	10.4 \pm 1.30 (10)	20.7 \pm 2.90 (10)	7.6 \pm 0.36 (5)	12.0 \pm 2.00 (5)
Trc A	12.2 \pm 0.09 (5)	21.0 \pm 2.30 (5)	8.8 \pm 0.24 (3)	26.0 \pm 0.89 (3)
Trc A ₁	18.0 \pm 4.10 (3)	32.0 \pm 7.04 (3)	14.0 \pm 1.50 (3)	29.0 \pm 5.20 (3)
sTrc AOMe ₃	12.0 \pm 0.68 (3)	18.0 \pm 0.31 (3)	5.7 \pm 0.40 (3)	11.0 \pm 0.94 (3)
sTrc A(Q-O)	27.0 \pm 2.30 (3)	37.0 \pm 2.50 (3)	25.0 \pm 1.70 (3)	43.0 \pm 7.20 (3)
Phc A	15.0 \pm 0.26 (4)	20.3 \pm 0.20 (3)	14.0 \pm 2.00 (3)	35.0 \pm 3.30 (3)
sTpc A	23.0 \pm 2.80 (4)	52.0 \pm 5.40 (4)	11.0 \pm 0.98 (3)	40.8 \pm 5.05 (3)
Trc B	7.4 \pm 0.31 (7)	16.2 \pm 2.25 (7)	5.9 \pm 0.28 (7)	14.2 \pm 1.46 (7)
Trc B ₁	10.7 \pm 0.82 (8)	25.1 \pm 1.67 (8)	10.4 \pm 1.24 (6)	26.1 \pm 4.26 (6)
Trc C	9.0 \pm 1.20 (3)	22.0 \pm 4.30 (3)	5.5 \pm 0.60 (3)	14.0 \pm 0.85 (3)
Trc C ₁	10.6 \pm 1.90 (3)	28.0 \pm 1.50 (3)	10.6 \pm 1.20 (3)	31.0 \pm 3.01 (3)
Tpc C	16.0 \pm 1.50 (4)	39.0 \pm 1.80 (4)	14.0 \pm 1.70 (3)	33.0 \pm 7.01 (3)

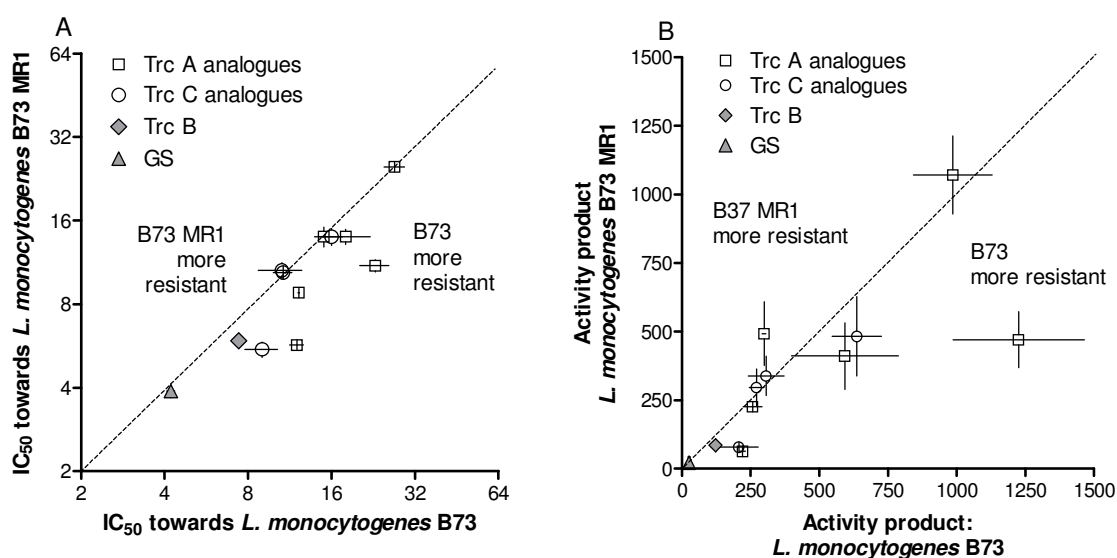


Figure 3.1 Comparison of the susceptibility of the leucocin A sensitive *L. monocytogenes* B73 and leucocin A resistant *L. monocytogenes* B73-MR1 strains to Trc A and Trc C analogues according to **A.** IC₅₀ values (Table 3.2) and **B.** activity product calculated as $A_p = IC_{50} \times IC_{max}$ for each peptide. GS was included as reference peptide.

The difference in sensitivity of the two *Listeria* strains has been attributed to differences in membrane structures and metabolism^{40,41,64}. Resistance to leucocin A by B73-MR1 has been associated with the absence of a putative mannose-specific phosphotransferase system protein suggesting glucose as a preferred carbohydrate source in this strain⁴⁰. Spathelf & Rautenbach⁴⁴ proposed that the known interference of Trcs with glucose metabolism in Gram-positive organisms⁶⁵ could account for their greater activity towards *L. monocytogenes* B73-MR1. Also, the increased fluidity of the cytoplasmic membrane of *L. monocytogenes* B73-MR1 known to be rich in desaturated and short-acyl-chain phosphatidylglycerols⁴¹ in comparison to that of the B73 strain would interfere with the hydrophobic interactions of the Trcs. It is possible that the two smallest peptides, Phc A and Trc A being rich in Phe, a residue without hydrogen bond capability among the aromatic amino acid residues, are able to insert deeper across the more rigid membrane of the B73 strain resulting in its greater sensitivity to these analogues. In contrast, sTpc A, which differs from Phc A and Trc A by having a Trp rather than a Phe or Tyr at position 7, is more active against B73-MR1 than B73 (Figs. 3.2 and 3.3).

Katla *et al.*²¹, after testing some bacteriocins against 200 *L. monocytogenes* strains, also noted strain-to-strain differences in susceptibility and recommended that these differences have to be considered should bacteriocins be used as bio-preservatives. In addition, we have found that conditions of bacteria culturing could influence their susceptibility to the Trcs, specifically leading to a lowered susceptibility after long term culturing at 37 °C (refer to Addendum *Section 3.8.1*).

3.4.2. Comparative activity analyses of tyrocidine analogues

3.4.2.1. Tyrocidine A analogues

The activity of the Trc A analogues against each strain gave different trends as illustrated in Fig. 3.3. In order to assess both the IC₅₀ and IC_{max} parameters we defined the activity product (A_P) which is calculated from IC₅₀ × IC_{max}. Based on the A_P against *L. monocytogenes* B73, the trend was (Trc A, Trc B, sTrc AOMe₃, Phc A) > Trc A₁ > (sTpc A, sTrc A(Q-O)) (Figs. 3.2 and 3.3, Supplementary data Table 3.2). The groupings of the peptides indicate statistically similar activities (refer to Fig. 3.3 and Supplementary data Table 3.2). This shows that going from the structure of Trc A (*cyclo*[fPFfNQYVOL]) activity was maintained with an Orn⁹ to trimethylated-Orn⁹ substitution in sTrc AOMe₃, with a Phe³ to Trp³ substitution in Trc B and a Tyr⁷ to Phe⁷ substitution in Phc A. However, activity decreased significantly when Orn⁹ was replaced by a Lys⁹ in TrcA₁, and when Tyr⁷ was substituted by Trp⁷ in sTpc A. Moreover, increasing the

charge by increasing the number of Orn residues in sTrc A(Q-O) with a Gln⁶ to Orn⁶ substitution led to a substantial loss of activity (Table 3.2 and Fig. 3.3A).

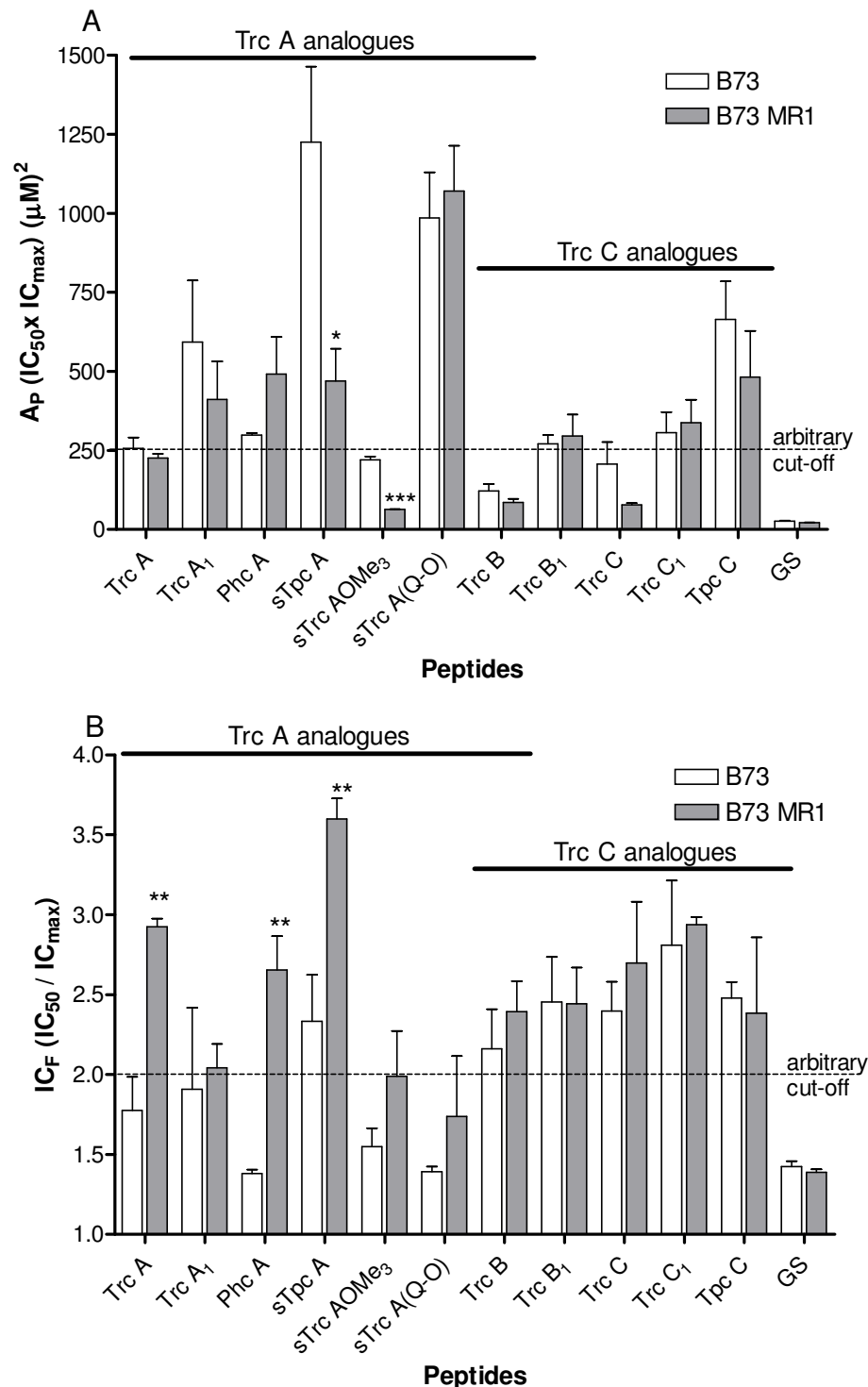


Figure 3.2 Comparison of the activity in terms of **A.** activity product ($A_p = IC_{50} \times IC_{max}$) for each peptide and **B.** inhibition concentration factor ($IC_F = IC_{max}/IC_{50}$) of the Trc A and Trc C analogues towards the leucocin A sensitive strain *L. monocytogenes* B73 and leucocin A resistant strain *L. monocytogenes* B73-MR1. Bars represent the average of 3-9 biological repeats (each consisting of triplicate technical repeats) and standard error of the mean. Statistical comparison of activity towards the two strains was done with the Student t-test (*** $P < 0.0001$; ** $P < 0.005$; * $P < 0.05$).

The following activity sequence was observed in terms of the A_P towards the resistant *L. monocytogenes* B73-MR1: (Trc A, Trc B, sTrc AOMe₃) > (Trc A₁, Phc A, sTpc A) > sTrc A(Q-O) (Figs. 3.2 and 3.3, Table 3.2, Table S3.2 in supplementary data). Similar to the B73 strain, the activity of the parent peptide Trc A was maintained with the Orn⁹ to trimethylated-Orn⁹ substitution in sTrc AOMe₃ and increased with the Phe³ to Trp³ substitution in Trc B. Activity was likewise decreased following Orn⁹ to Lys⁹ substitution in Trc A₁, Tyr⁷ to Trp⁷ substitution in sTpc A and with a Gln⁶ to Orn⁶ substitution in sTrc A(Q-O). The difference was that in this strain substitution of Tyr⁷ by Phe⁷ led to loss of activity. As mentioned above, the differences in membrane structures of the two strains could account for this difference.

Using a cut-off point of 250 μM^2 for the A_P , the most active Trc A analogues against *L. monocytogenes* strains B73 and B73-MR1 were identified as Trc A, Trc B and sTrc AOMe₃ (Fig. 3.3B). Employing a cut-off of 2.5 for the inhibition concentration factor ($\text{IC}_F = \text{IC}_{\text{max}}/\text{IC}_{50}$) which describes the concentration factor that describes fold increase peptide concentration between minimum inhibition and maximum inhibition⁵³, all peptides were active against *L. monocytogenes* strains B73. However, for activity towards *L. monocytogenes* B73-MR1, sTrc AOMe₃ and Trc B were identified as the most promising analogues (Fig. 3.3B). sTrc AOMe₃ was the only synthetic analogue to show comparable activity to the active natural analogues towards both strains of *L. monocytogenes*. The known consequences of Orn trimethylation include: 1) preserving the overall charge, 2) higher bulkiness and hydrophobicity of the side chain, and 3) quaternary amino group with no hydrogen bonding ability⁶⁶. However, according to the reverse phase (C₁₈) HPLC retention times, the trimethylation of Orn⁹ did not lead to an overall increased hydrophobicity, as the trimethylated analogue eluted earlier than Trc A (Supplementary data Table 3.1). This observation was similar to that made by Fernández-Reyes *et al.*⁶⁶ about Lys-trimethylated analogues of the cecropin A-melittin hybrid peptide. A study by Kawai *et al.*⁶² on GS analogues containing N-methyl groups including trimethylated-Orn analogues, indicated that these analogues showed comparable antibacterial activity against Gram-positive bacteria as the parent GS. The loss of hydrogen bonding activity may lead to a weaker electrostatic binding to the target(s), but increased hydrophobicity and bulkiness could allow deeper more disruptive imbedding into the membrane.

The N^δ-trimethyl Orn group in sTrc AOMe₃ would be involved in electrostatic interactions with anionic phospholipids, but could have weaker interaction due to the loss of hydrogen bonding character, possibly supporting the Trc mode of action.

Increase in the bulkiness of the charged group through the replacement of Orn⁹ by Lys⁹ in TrcA₁, led to a decrease in activity against both strains when compared to the activity of the parent Trc A. Lys, with a butylene moiety in its side chain, has been shown to carry out “snorkeling” into the membranes, leading to a tighter membrane interaction than Orn, which does not translate into higher activity against *L. monocytogenes*^{43,49}. This is an indication that tighter binding to the listerial membrane (or cell wall) may hamper the mode of action of Trcs. From these and previous results⁴⁴ it is thus clear that the sequence of preference for cationic amino acid is as follows: Orn(Me)₃ > Orn > Lys.

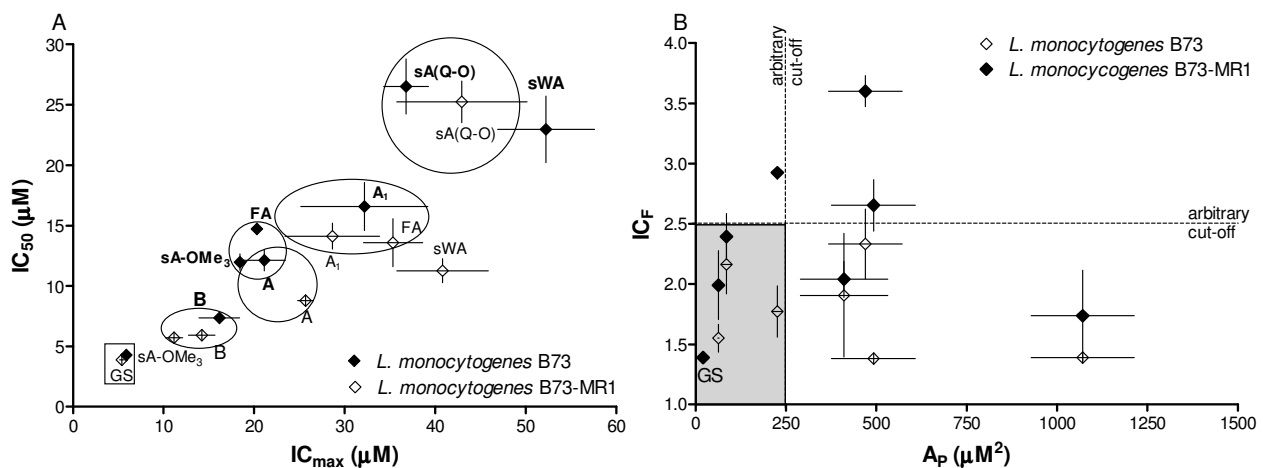


Figure 3.3 Comparison of the Trc A analogues in terms of activity against the leucocin A sensitive *L. monocytogenes* B73 and leucocin A resistant *L. monocytogenes* B73-MR1 strains with **A.** IC₅₀ versus IC_{max} and **B.** A_p versus IC_F. Circled groups in **A.** have similar activities based on results of the Newman-Keuls multiple comparison test for IC₅₀ values (Supplementary data Table 3.2). The shaded area in **B.** indicates the most active peptides selected using cut off values of IC_F = 2.5 and A_p = 250 µM². Abbreviations used are; A: Trc A, B: Trc B, A₁: Trc A₁, SA-OMe₃: sTrc AOMe₃, FA: Phc A, sWA: sTpc A and sA(Q-O): sTrc A(Q-O).

Increasing the charge of Trc A by Gln⁶ to Orn⁶ replacement resulted in a pronounced decrease in activity. In contrast, Gln to Orn/Lys/Arg substitution in Trc A resulted in improved activity towards *Bacillus subtilis*⁶³. This is possibly related to the fact that in highly negatively charged membranes, such as in the case of *L. monocytogenes* known to be rich in phosphatidylglycerols⁴¹, the increase in charge of the peptide leads to an increase in electrostatic interaction with the membrane and possibly trapping and reduced activity⁶⁷. Danders *et al.*⁶⁸ also found a Trc A analogue with two Orn residues, the second of which was at the aromatic position 7, to be inactive against *Bacillus* species. We observed a decrease in HPLC retention time and thus hydrophobicity for sTrc A(Q-O) compared to the parent Trc A, corresponding with the loss in activity. Alternatively, the polar Gln⁶ may be important to binding to the listerial target. This result confirmed that there is a more complex relationship between peptide charge and activity

than a membrane affinity relationship, especially because increasing charge (Q) is always linked to reduced peptide hydrophobicity (H) and a modification of the hydrophobic moment (μ)⁶⁷. Kohli *et al.*⁶⁹ observed an increased selectivity for bacterial membranes when charge was increased by substituting D-Phe⁴ in Trc A with positively charged amino acids. In another study by Marques *et al.*⁷⁰, increase in charge with a resulting loss of hydrophobicity, however, led to up to eight fold increase in the activity of Trc A analogues against Gram-positive bacteria where Gln⁶ was replaced by Lys. Yet, activity was lost when charge was increased with D-Phe⁴ to D-Lys⁴ substitution. These studies strongly indicated, as we also observed, that simply increasing charge does not always result in improved antibacterial activity, especially as different target cells have different sensitivities.

Substitution of the Tyr at position 7 in Trc A with Trp⁷ to form Tpc A decreased the activity significantly in both strains. There seems to be a preference for either a Tyr or Phe in position 7 indicating that a bulky Trp may have some steric interference with the target interaction. However, in the *L. monocytogenes* B73-MR1 strain, the requirement for a Tyr at position 7 seems more critical, with both the Trp⁷ and Phe⁷ substitution, leading to lower activity, indicating more selective target specificity. Therefore, the weakly acidic hydroxyl group of Tyr may be important for the mechanism of action of the Trcs against the resistant B73-MR1 strain. It has been shown that the resistant strain has cells with a more fluid membrane due to modification in membrane phosphatidylglycerols containing shorter, unsaturated acyl chains⁴¹. Given that Trp and Tyr both form hydrogen bonds and that Phe is essentially hydrophobic in nature with a purely aromatic hydrocarbon side chain, it can be suggested that in the more fluid membrane of the resistant strain, Trc activity modulated by hydrogen bonding is critical with a preference for a less bulky interacting group. However, in the sensitive strain with a more rigid membrane, it is the size of the aromatic side chain that may be more important than hydrogen bonding, since Phe and Tyr both contain side chains with a phenyl ring. The aromatic residues (Trp, Phe and Tyr) are therefore important in target interaction due to their significant impact on the hydrophobicity and have been proposed to account for/contribute to membrane integration of the Trcs⁴⁴.

Activity of Trc A analogues against *L. monocytogenes* is therefore, sensitive to the amphipathic balance, with hydrophobic interactions being essential while an increase of electrostatic interactions leads to a decrease in Trc A activity. Trapping of the Trc A(Q-O) and TrcA₁ in the membrane could counteract their mode of action, while in sTrcAOMe₃ the more bulky trimethylated Orn probably led to binding that enhances the mode of action. A further prerequisite seems to be size and hydrogen bonding ability of residue 7 with Tyr preferred above

Phe and Trp. The aromatic amino acid at position 3 had been previously shown to be critical for antilisterial activity⁴⁴ and this was again demonstrated by the increase in IC₅₀ activity against both strains when the Phe³ of Trc A was replaced by Trp³ in Trc B. However, this preference is offset by the trimethylation of Orn, indicating that a bulky side chain in close proximity to the Phe can also lead to good activity.

3.4.2.2. Tyrocidine C analogues

The Trc C analogues showed the same trend of activity against both *L. monocytogenes* B73 and B73-MR1 strains (Fig. 3.2). The A_P values decreased in the following order: (Trc B, Trc B₁, Trc C, Trc C₁) > Tpc C. According to the cut-off point for the A_P set earlier at 250 μM², the most active Trc C analogues were Trc B and Trc C towards both strains (Figs. 3.2 and 3.4B). The IC_F cut-off value of 2.5 designates Trc B and Trc C towards *L. monocytogenes* B73 and Trc B towards *L. monocytogenes* B73-MR1 as the peptides with best activities (Fig. 3.4B).

These results indicated that from the parent peptide Trc C (*cyclo*[fPWwNQYVOL]), activity was preserved following D-Trp⁴ to D-Phe⁴ substitution either alone in Trc B or in combination with Orn⁹ to Lys⁹ substitution in Trc B₁. However, as with the Trc A analogues, replacement of Tyr⁷ by Trp⁷ in Tpc C also led to a loss of activity. Although increasing the bulkiness of the charged group by replacing Orn⁹ with Lys⁹ in Trc C₁ in comparison with Trc C did not show any significant difference in A_P, there was decrease in IC₅₀ and IC_{max} activity in *L. monocytogenes* strain B73-MR1 (refer to Table 3.2 and S3.3), as found for the Trc A analogues. A similar observation was made for this substitution comparing Trc B₁ and Trc B (refer to Table 3.2 and S3.3). The proposed tighter membrane interaction of Lys due to the “snorkel effect”⁷¹ could account for the decreased antilisterial activity, as was observed for Trc A₁. There is little change in activity between Trc C and Trc B with Orn⁹ as cationic residue or between Trc B₁ and Trc C₁ with Lys⁹. This suggests that the size of the cationic residue more than the size of the side chains in the aromatic dipeptide is critical in membrane interaction particularly in the more fluid membrane of *L. monocytogenes* B73-MR1. Changing the aromatic residue at position 7 from Tyr as in Trc C to Trp as in Tpc C resulted in a significant decrease in activity. A similar decrease in activity was observed for Trc A when the Tyr at position 7 was replaced by Trp. This indicated strongly that the identity of the aromatic group at position 7 and possibly the hydroxyl group of Tyr is critical for the activity of the Trcs against the resistant *Listeria* strain.

Therefore, the character of the charged amino acids (Lys or Orn) in the conserved V(K/O)LfP pentapeptide moiety in addition to the aromatic amino acids in the variable pentapeptide moiety

modulate the efficacy of the Trc activity by influencing membrane activity or binding to a different target. Similar observations were made by Spathelf & Rautenbach⁴⁴.

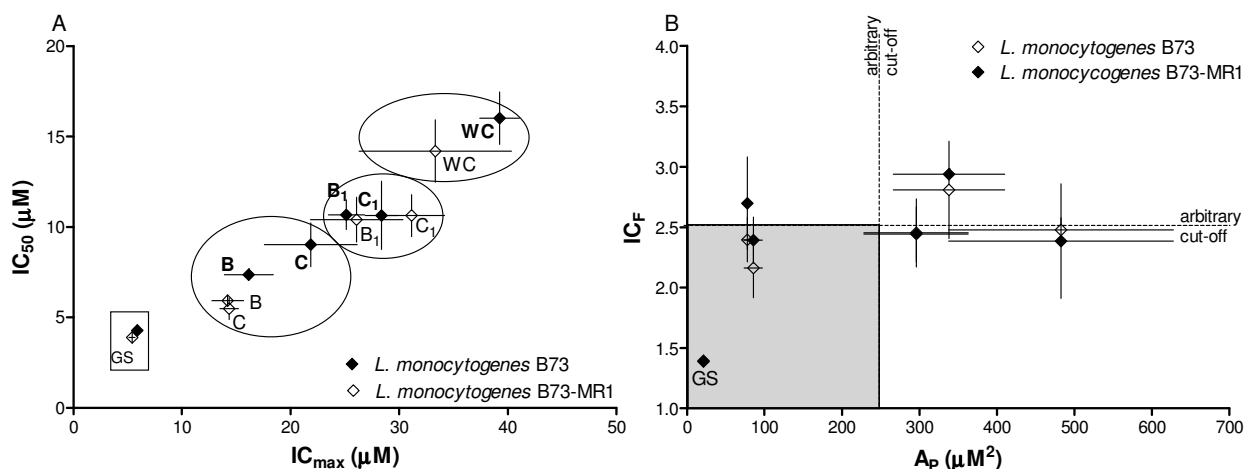


Figure 3.4 Comparison of the activity of Trc C analogues against the leucocin A sensitive *L. monocytogenes* B73 and leucocin A resistant *L. monocytogenes* B73-MR1 strains with **A.** IC_{50} versus IC_{max} and **B.** A_P versus IC_F . Circled groups in **A.** have similar activities based on results of the Newman-Keuls multiple comparison test for IC_{50} values (Supplementary data Table 3.3). The shaded area in **B.** indicates the most active peptides selected using a cut off value of $IC_F = 2.5$ and $A_P = 250 \mu M^2$. Abbreviations used: B: Trc B, B₁: Trc B₁, C: Trc C, C₁: Trc C₁ and WC: Tpc C. GS was included as reference peptide.

3.4.3. QSAR and PCA analyses of the Trc libraries

The QSAR between the structure of the Trc and its observed antilisterial activity was investigated with the aim of estimating the optimal/minimal structural determinants of activity necessary to facilitate the design of peptide derivatives and mimics for therapeutic evaluation^{49,72}. Experimental and theoretical structural parameters (refer to Table S3.1 in supplementary data) for each of the analogues were correlated with the observed biological activity parameters (IC_{50} , IC_{max} , IC_F and/or A_P). The HPLC R_t , hydrophathy, lipophilicity, hydrophobicity, interphase properties and Q/L were used as previously^{44,49,55,58,59,73,74} to depict the peptide hydrophobic character and potential membrane activity. SASA and SAV are size parameters that are also useful in calculating hydrophobic burial⁷⁵ and as a measure of the probable magnitude of binding-induced changes⁷⁶. They are related to the hydrophobicity of molecules through computation of the logarithm of the partition coefficient^{77,78}. The molecular mass, SCSA and MV were used to demonstrate side-chain and peptide size factors that may affect target interaction. All data were fitted to linear, quadratic, hyperbolic and exponential equations and only correlations with $R^2 \geq 0.50$ were considered as significant QSAR trends (refer to Table 3.4 in supplementary data). In addition, multivariate QSAR through principal component analysis

(PCA) was performed to verify/identify relevant molecular descriptors of the peptides that affect their antilisterial activity.

Two dimensional QSAR trends were generally weak, indicating a strong selection of specific sequence combinations. In general, correlation of activity against B73-MR1 with the size and chemical character parameters yielded larger and/or more significant correlation coefficient (R^2) values than that for B73 (Table S3.4 and Fig. 3.5A). This could indicate that the more fluid membrane of B73-MR1 is more sensitive to changes in the peptide structure and character. Most correlations between amphipathicity/ lipophilicity/hydrophobicity and antilisterial activity led to either no trend or quadratic (2^{nd} order) trends with $R^2 < 0.50$ except for the Σ (hydropathy) where quadratic trends generally gave $R^2 > 0.50$ (Table S3.4). There was a distinct relevance of the aromatic side chain at position 7 as the peptides were separated into two groups with those containing Tyr as one group and those containing instead Trp or Phe in another group when the hydropathy parameter was considered (Fig. 3.5A).

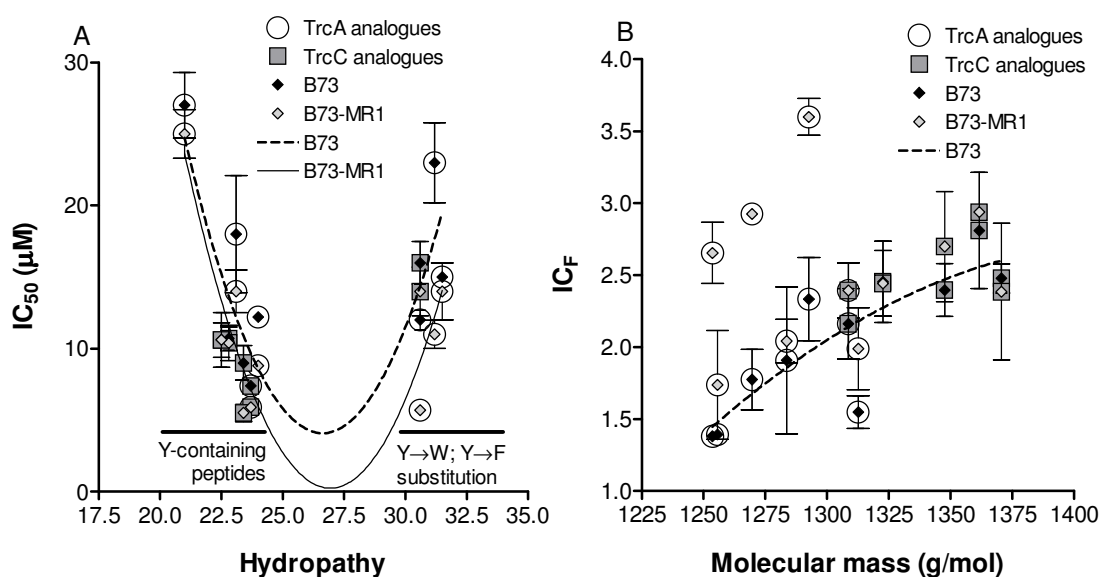


Figure 3.5 Relationships between **A.** IC_{50} and Σ (hydropathy) **B.** IC_{50} and M_r fit to quadratic (2^{nd} order polynomial) equation. Each data point represents the mean of at least 3 biological repeats, with 3–5 technical repeats per assay \pm SEM. Refer to Table S3.4 for correlation coefficient (R^2) values and trends and Table 3.2 for details on activity parameters.

The only significant QSAR trend for both strains was observed for correlation between the IC_{50} and the Q/L parameter (Table S3.4). Therefore, hydrophobicity does play a role in the modulation of Trc activity and the identity of the aromatic residue in position 7 affects this relationship. Similarly, when considering size parameters mostly weak trends were found. Only correlations between the size parameters SASA, SAV and especially M_r , and activity (IC_{50} or

IC_F) resulted in good quadratic trends towards *L. monocytogenes* B73, implying that the peptide size are highly relevant to activity in *L. monocytogenes* B73 with the more rigid membrane (Fig. 3.5B, Table S3.4). Quadratic correlations, although weaker were also obtained for correlation between activity (IC₅₀ or A_P) towards *L. monocytogenes* B73-MR1 and size parameters, M_r and SAV (Table S3.4).

The molecular descriptors representing bulk (size) and chemical character of the peptides (Table S3.1 in supplementary data) together with four activity parameters (IC₅₀, IC_{max}, IC_F and A_P) for both listerial strains were studied by the multivariate data analysis technique using the PCA projection method. This QSAR technique described by Strøm *et al.*⁷⁹ offers the advantage of estimating QSAR by simultaneously taking into account several activity parameters for a relatively small group of peptides. Two PCAs were done; in the first PCA we included all eighteen variables while in the next PCA we excluded the compound variables A_P and IC_F comparing the remaining sixteen variables.

When all eighteen variables were considered, the first two principal components (factors) explained ~60% of the total variance (factor 1 = 39% and factor 2 = 23%). The resulting t₁t₂ score plot (Fig. 3.6A) clustered the peptides into distinct groups which corresponded to the Trc A analogues and Trc C analogues along the PC1 scale. However, the Trc A analogue sTrc AOMe₃ clustered with the Trc C analogues (Fig. 3.6A). We also observed closer association between the analogues that have either Orn or Lys as charge groups i.e. Trc A₁/Trc A, Trc B₁/Trc B and Trc C₁/Trc C along PC1 with sTrc AOMe₃ associating with the Trc B₁/Trc B pair. Thus the “PC1” variable appeared to be strongly associated with the identity of the variable aromatic dipeptide unit not distinguishing based on the identity of the charged group. The second principal component “PC2” seemed to be strongly associated with the activity of the peptides as the analogues were separated according to their observed antilisterial activity along the PC2 scale.

The p₁p₂ loading plot (Fig. 3.6A) grouped together the size parameter M_r with the theoretically derived size parameter MV, which correlated with the size parameters derived from *in silico* molecular modelling SASA and SAV. On the p₁p₂ loading plot the M_r, MV, SASA and SAV had a significant negative correlation with the IC₅₀, IC_{max} and A_P of *L. monocytogenes* B73-MR1 along with the IC₅₀ and A_P of *L. monocytogenes* B73. The theoretical parameter SCSA, however, did not correlate with the other size parameters. It was observed to positively correlate with the IC_{max} of *L. monocytogenes* B73 and both showed a negative correlation to the hydrophobicity parameters. The experimentally determined HPLC R_t, as expected, grouped with the theoretically derived hydrophobicity and interphase property parameters. The IC_F variables for both strains along with the lipophilicity and hydrophobicity did not contribute significantly to the

variance of the parameters on this plot according to the limit of the correlation coefficient ($R^2 = 0.50$). Based on the bi-plots (loading and score plots superimposed) the grouping of the peptides along the “PC1” variable could be explained by the negative correlation between size and the magnitude of most activity parameters of the peptides with the larger peptides (containing Trp-Trp dipeptide unit) showing higher activity than the smaller peptides (with Phe-Phe dipeptide unit). The smaller peptides are also the more hydrophobic ones. The Trc A analogue with trimethylated Orn clustered with the Trc C analogues due to its higher activity which as previously mentioned was comparable to that of the most active Trc C analogues.

The t_{13} score plot (Fig. 3.6B) with factor 3 of 17%, clustered the peptides into distinct groups which corresponded to the Orn-containing analogues and the analogues with Orn to Lys substitution clustered into a separate group along the PC3 scale. The analogue sTrc A(Q-O) was separated from the rest of the Orn-containing peptides. Thus the “PC3” variable seems to be strongly associated to the identity of the charged group. There was also a separation of the Trc A analogues in which the Tyr was substituted by either Trp or Phe from Trc A as well as separation of Trc C with a Trp residue at position 7 from the rest of the Tyr-containing Trc C analogues along the PC3 scale. Based on the p_{13} loading plot, the segregation of the peptides along PC3 can be mainly explained by the hydrophobicity and lipophilicity which in the p_{13} plot had $R^2 = 0.88$ and 0.49 respectively. The IC_F towards both *L. monocytogenes* strains as well as the IC_{max} towards *L. monocytogenes* B73, hydrophobicity, SCSA and interphase properties did not significantly contribute to the variance on this plot.

With the exclusion of the A_P and IC_F parameters and considering sixteen variables, the magnitude of the first two principal components changed (factor 1 = 42% and factor 2 = 25%). The separation of the peptides on the t_{12} score plot along the PC1 and PC2 scales was similar to that described above (not shown). The hydrophobicity was the only variable that did not significantly contribute to the variance in this plot. The t_{13} score plot was also similar to that obtained with inclusion of the compound activity parameters (not shown).

The PCA results in Fig. 3.6 show, as expected from our basic trend analyses, the importance of the SAV and SASA to the modulation of antilisterial activity against B73-MR1 as these structural parameters correlate negatively to the activity parameters. Results depicted in Fig. 3.6 further reveal that the separation of peptides according to activity along PC2 in the t_{12} score plot is contributed by the following structural parameters: SASA, SAV, M_r and MV. The success of the multivariate QSAR was validated by the high correlation between most of the actual variables and those predicted by the models as evidenced by the R^2 values in Table 3.3. In this

PCA, each variable was considered the dependant variable and estimates were calculated from the PCA models.

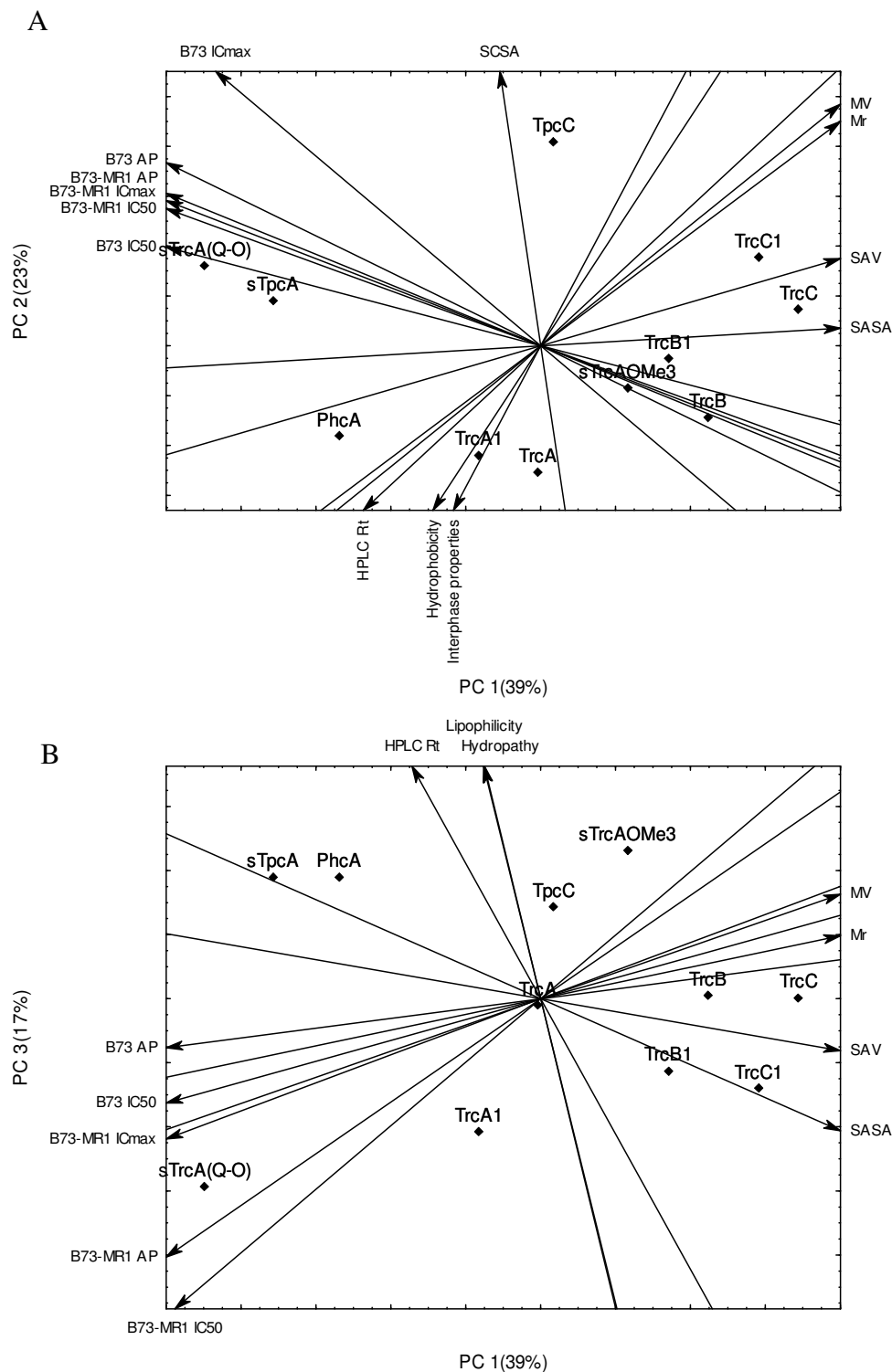


Figure 3.6 **A.** PCA bi-plot showing the relationship between the variation in the 18 structural and antilisterial activity parameters and the relationship between the 11 Trc analogues along factor 1 and 2. **B.** Bi-plot showing the relationship between the variation in the 18 structural and antilisterial activity parameters and the relationship between the 11 Trc analogues along factors 1 and 3. Parameters with $R^2 < 0.5$ are not indicated on the graphs.

Table 3.3 Correlation coefficients (R^2) for agreement between actual variables and PCA model-predicted variables used in the PCA QSAR analyses of the tyrocidine library

Variables	PCA Model fit	
	R^2 p ₁ p ₂ plot	R^2 p ₁ p ₃ plot
Activity parameters		
B73 IC ₅₀	0.904	0.866
B73-MR1 IC _{max}	0.747	0.676
B73 IC _{max}	0.673	0.348
B73-MR1 IC ₅₀	0.666	0.809
B73 A _p	0.797	0.612
B73-MR1 A _p	0.775	0.818
B73 IC _F	0.152	0.259
B73-MR1 IC _F	0.003	0.158
Size parameters		
M _r	0.936	0.552
MV	0.847	0.489
SCSA	0.556	0.263
SASA	0.848	0.943
SAV	0.943	0.863
Chemical parameters		
HPLC R _t	0.412	0.570
Lipophilicity	0.336	0.492
Hydropathy	0.097	0.933
Hydrophobicity	0.707	0.177
Interphase properties	0.754	0.288

To further interpret the QSAR results for the Trcs it is assumed that their initial association with the membrane, driven principally by electrostatic interactions and membrane integration through hydrophobic interactions, are the essential steps in spite of the specific subsequent mechanism of action or interaction with a molecular target⁴⁴. From our QSAR analysis the aromatic amino acids (Trp, Phe and Tyr) in the Trc analogues are pivotal in determining the activity. Phe has a greater lipophilicity compared to Trp and has also been found to integrate the membrane deeper while the indole and phenol analogues (Trp and Tyr) are shallower^{80,81}. On the other hand, Trp which is the largest of the aromatic amino acids should display better anchoring also as a result of the formation of hydrogen bonds between its NH-group and lipid carbonyl groups⁸². However, a very tight membrane association does not always translate into better activity and there is need for an optimal amphipathicity which still allows for efficient membrane integration and self-assembly into active lytic complexes and/or translocation to an internal molecular target^{44,70}. This was also illustrated by the PCA analyses showing Tpc C with three Trp residues as an

outlier. Analysis of the structure-activity relationship of a group of Trc A analogues by Marques *et al.*⁷⁰ revealed that though there was a preference for increased amphipathicity, it was not clear if either hydrophilicity or hydrophobicity was preferred to improve antibacterial activity.

The cationic amino acids in the conserved V(K/O)LfP pentapeptide also contribute in modulating the efficacy of the Trc. The side chains of Lys and Orn differ by having either ϵ - or γ -amino groups ($pK_a = 10.54$ vs. $pK_a = 10.76$) respectively⁸³ and in terms of hydropathy (-9.9 and -9.0 respectively)⁵⁷. The longer Lys side-chain has a tighter binding due to a “snorkel effect” where the longer butylene chain can fold into a cis-conformation and associate with the acyl chains, while the amino group remains linked with the head groups in the aqueous phase⁷¹. It is assumed that the optimal amphipathicity is achieved by Phe and/or Lys contributing to tighter membrane binding and Trp and/or Orn amending this binding, probably by limiting peptide trapping⁴⁴.

3.5. Results and Discussion: Part II - *cyclo*(RRRWFW) analogues

The RW-peptides are a library of synthetic cyclic derivatives of the hexapeptide (Ac)-RRWWRF-NH₂ (R-, W-rich or RW-peptides), carrying substitutions of Trp and/or Arg (Table 3.4). The RW-peptides share some characteristics with the Trcs such as cyclic, aromatic and cationic character, as well as their active conformation which consists of two β turns, which is the smallest possible β -sheet^{47,84,85}. In contrast to the Trcs, the RW-peptides have been found to have reduced haemolytic activity^{48,85,86}.

After showing that the aromatic motif and cationic residues modulate activity of *c*-WFW [*cyclo*(RRRWFW)], *c*-WFW analogues with altered hydrophobicity, dipole and quadrupole moments, hydrogen-bonding ability, amphipathicity, and ring size were synthesized by substitution of Trp and Arg with analogues in order to discover interaction sites and to explain the mode of action^{48,85,87}. Examples of such modifications are shown in Fig. 3.7. The cyclic model peptides, *c*-WFW and analogues, consist of four groups of structural peptide analogues (Table 3.4). The aromatic substitutions included unnatural amino acids such as 5-methyl-DL-tryptophan (5MeW), 1-methyl-L-tryptophan (1MeW), α -(2-indanyl) glycine (Igl) and β -(benzothien-3-yl)-alanine (Bal) with subtle chemical differences to Trp (Table 3.5 and Fig 3.8). The β -amino acid b3hW (L- β -homotryptophan) was also incorporated to increase the size of the backbone cycle^{85,88}. Analogues investigated in this project would be derived from four structural changes to the two domains of the parent sequence. Modifications will include:

- 1) Alteration of the hydrophobic domain by replacing Phe with Trp, some unnatural amino acids and Trp analogues (Table 3.5 and Fig 3.8)
- 2) Modification of the cationic domain by replacing Arg with Lys
- 3) Scrambling the residues to upset the clustering of the hydrophobic and cationic domains
- 4) Increasing ring size through increase in number of residues in both domains

Table 3.4 Descriptions of groups of analogues of *cyclo*(RRRWFW)

Group	Domain altered	Analogue Sequence	Name	Amino acid substitution
1	Hydrophobic	c(RRRWFW)	c-WFW	Parent compound
		c[RRR(b3hW)F(b3hW)]	c-b3hW	W to b3hW
		c(RRRWWW)	c-WWW	F to W
		c(RRRWIW)	c-WIW	F to Igl
		c[RRR(1MeW)F(1MeW)]	c-1MeW	W to 1MeW
		c[RRR(5MeW)F(5MeW)]	c-5MeW	W to 5MeW
		c[RRR(Bal)F(Bal)]	c-Bal	W to Bal
2	Polar	c(RRRWFW)	c-WFW	Parent sequence
		c(KRKWFW)	c-KRK	R to K
		c(KKWWKF)	c-KW	R to K
		c(RRRWWW)	c-WWW	Parent sequence
		c(KKKWWW)	c-KWW	R to K
3	Polar and hydrophobic (ring size)	c(RRRWFW)	c-WFW	Parent sequence
		c(RRRRWFWF)	c-WFW8	Addition of R and F
		c(RRRRRWFWFW)	c-WFW10	Addition of RR and FW
		c(RRRRRRWFWFWF)	c-WFW12	Addition of RRR and FWF
4	Polar and hydrophobic (destruction of clusters)	c(KKKWWW)	c-KWW	Parent sequence
		c(KWKWKW)	c-WKW	Altered sequence
		c(RRRWWW)	c-WWW	Parent sequence
		c(RWRWRW)	c-WRW	Altered sequence

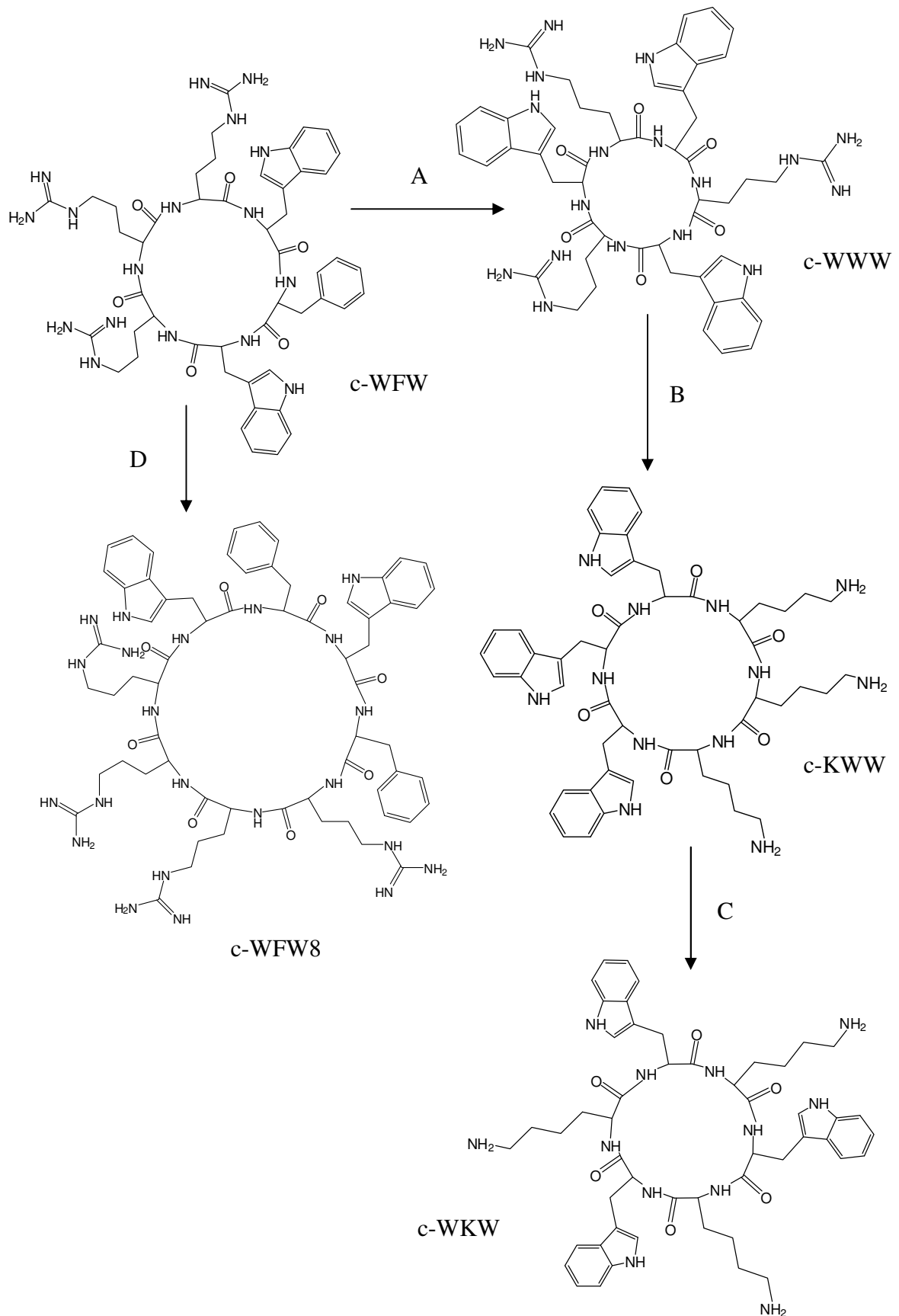
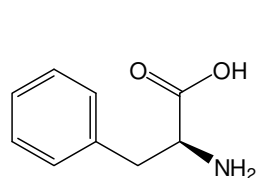


Figure 3.7 Chemical alteration of the structure of *cyclo*(RRRFWW) to yield analogues. **A.** Change in hydrophobic domain to *cyclo*(RRRWWW) **B.** Change in cationic domain to *cyclo*(KKKFWW) **C.** Scrambling of residues to *cyclo*(KWKWKW) **D.** Increase in peptide size to *cyclo*(RRRRWFWF).

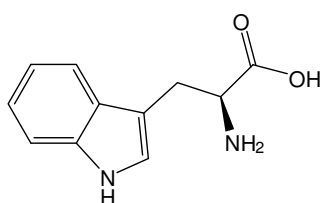
Table 3.5 Differences in physicochemical properties between Trp and unnatural Trp analogues

	H- bonding	Dipole moment	Quadrupole moment	Amphipathicity	Hydrophobicity
<W	1MeW, Bal, Igl	Bal, Igl	Igl	Bal, Igl	Igl
=W	b3hW, 5MeW	b3hW, 5MeW, 1MeW	b3hW, 5MeW, 1MeW, Bal	b3hW, 5MeW, 1MeW	
>W					b3hW, Bal, 1MeW, 5MeW

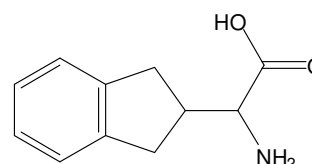
Note. Table adapted from “Cationic Antimicrobial Peptides: Thermodynamic Characterization of Peptide-Lipid Interactions and Biological Efficacy of Surface-Tethered Peptides” by M. Bagheri, 2010, *PhD thesis*, p. 24⁸⁸.



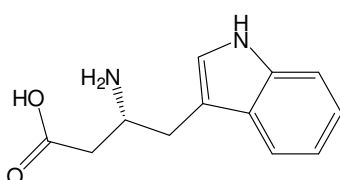
Phenylalanine



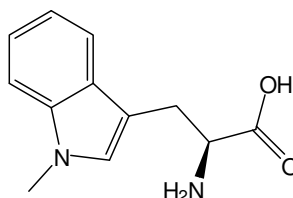
Tryptophan



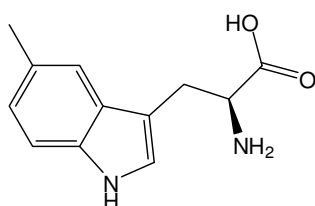
Alpha-(2-indanyl) glycine (Igl)



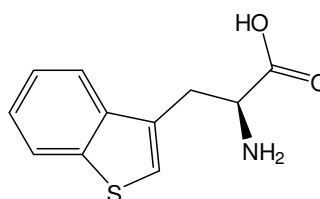
L-beta-homotryptophan (b3-hW)



1-methyl-L-tryptophan (1MeW)



5-methyl-L-tryptophan (1MeW)



Beta-(benzothien-3-yl)-alanine (Bal)

Figure 3.8 Structures of amino acid residues employed in the hydrophobic domain of the RW-peptides

Trp has a dipole moment of ~ 2.1 D in magnitude, running from N-1 in the five-membered ring to C-5 in the six-membered ring of the indole ring⁸⁹ with hydrogen bonding capacity⁸⁸. The

aromatic ring is closer to the backbone in Igl which is not supposed to lie at the lipid-water interfacial region ⁸⁸. In the analogue b3hW, the size and flexibility of the backbone ring are increased while hydrogen bonding ability is maintained ⁸⁸. The hydrogen bonding ability is eradicated in the analogue 1MeW due to the presence of the more hydrophobic and bulky methyl group. Nevertheless, its hydrophobicity is higher than that of Trp while the amphipathicity and direction and magnitude of the dipole moment (~2.2 D) are similar to those of Trp ⁸⁹. The analogue 5MeW retains the hydrogen bonding ability, the quadrupole moment (aromaticity) and the dipole moment of Trp ^{89,90}. Although, the size of the indole ring is the same as that of the ring in the analogue Bal, the presence of the sulphur atom with reduced electronegativity in the five-member ring, decreases the dipole moment compared to that of Trp. This results in a loss of amphipathic structure and hydrogen bonding capacity ⁹¹. Bal is also more hydrophobic than Trp ⁸⁸. The changes in the hydrophobic domain may lead to different conformations from the parent cyclic peptide (c-WFW) with altered degree of amphipathicity in the lipid-bound peptide which would affect antimicrobial activity and selectivity ⁸⁸.

3.5.1. *L. monocytogenes* strain susceptibility towards RW-peptides

Out of the 15 synthetic analogues of *cyclo*(RRRWFW) evaluated for their antilisterial activity against the leucocin A sensitive *L. monocytogenes* B73, seven peptides were active namely: c-WFW, c-WWW, c-WFW8, c-KWW, c-5MeW, c-Bal and c-1MeW (Table 3.6) although they were significantly less active than the Trcs. This could be related to the fact that membrane permeabilisation is not the preferred mode of action of the RW-peptides ⁸⁶.

The active RW-peptides were further evaluated for their activity against the leucocin A resistant strain *L. monocytogenes* B73-MR1. When there was a significant difference between the susceptibility of the two strains, B73-MR1 was more susceptible to the RW-peptides tested, except for c-Bal (Fig. 3.9). Although the five-member ring of the unnatural amino acid Bal is equal in size with the indole ring in Trp, Bal has a lower dipole moment due to the less electronegative sulphur in the ring (Table 3.5). Bal loses the amphipathic structure and hydrogen bonding characteristics of Trp even though it is more hydrophobic ⁸⁸. The observed increased sensitivity of the B73 strain to c-Bal is consistent with the activity of the Trc analogue, Phc A, which possesses Phe in place of Tyr; Phe having less hydrogen bonding capacity than both Tyr and Trp. Thus differences in membrane composition and/or metabolism of the two *Listeria* strains ^{40,41,92} influence their susceptibility to the RW-peptides.

Table 3.6 Summary of the growth inhibitory activity parameters of the *cyclo*(RRRWFW) analogues, toward the leucocin A sensitive strain of *Listeria monocytogenes* (B73) and the leucocin A resistant strain of *L. monocytogenes* (B73-MR1). Concentrations are given in μM . Every value denotes the average of **n** biological repeats (number of repeats given in brackets), with 3 technical repeats per assay \pm SEM given in 2 significant figures. Refer to Table 3.4 for groupings of peptides and Tables S3.5 -S3.7 for statistical analyses of data.

Peptide	<i>L. monocytogenes</i> B73		<i>L. monocytogenes</i> B73-MR1	
	IC ₅₀ \pm SEM (n)	IC _{max} \pm SEM (n)	IC ₅₀ \pm SEM (n)	IC _{max} \pm SEM (n)
c-WFW	27.0 \pm 0.50 (5)	38.0 \pm 1.03 (5)	25.0 \pm 2.50 (3)	38.0 \pm 0.28 (3)
c-b3hW	> 100 (3)	> 100 (3)	nd	nd
c-WWW	23.0 \pm 1.80 (4)	30.4 \pm 4.70 (4)	14.0 \pm 0.27 (3)	20.0 \pm 0.65 (3)
c-WIW	> 100 (3)	> 100 (3)	nd	nd
c-1MeW	32.0 \pm 1.50 (3)	52.0 \pm 4.30 (3)	27.0 \pm 2.40 (3)	40.3 \pm 2.30 (3)
c-5MeW	27.0 \pm 0.90 (3)	43.0 \pm 5.02 (3)	17.0 \pm 0.40 (3)	23.0 \pm 1.20 (3)
c-Bal	19.0 \pm 1.50 (5)	29.0 \pm 4.20 (5)	27.0 \pm 1.10 (3)	37.0 \pm 1.60 (3)
c-KW	> 100 (3)	> 100 (3)	nd	nd
c-KWW	78.0 \pm 14.00(4)	93.0 \pm 2.40 (4)	63.0 \pm 4.70 (3)	86.0 \pm 7.70 (3)
c-KRK	> 100 (3)	> 100 (3)	nd	nd
c-WFW8	36.0 \pm 6.20 (4)	> 100	17.0 \pm 0.33 (3)	26.0 \pm 3.50 (3)
c-WFW10	> 100 (3)	> 100 (3)	nd	nd
c-WFW12	> 100 (3)	> 100 (3)	nd	nd
c-WKW	> 100 (3)	> 100 (3)	nd	nd
c-WRW	> 100 (3)	> 100 (3)	nd	nd

nd = not determined

Membrane activity is not the only mode of action of the RW-peptides, but it contributes to their activity against Gram-positive bacteria⁴⁸. The RW-peptides share similar secondary β -structure and amphipathic character with the Trcs which accounts at least in part for the membrane activity^{85,88,93}. It is possible that Bal which is less capable of hydrogen bond formation in comparison to Trp is able to insert deeper across the more rigid membrane of the B73 strain resulting in its greater sensitivity to c-Bal. Similarly Phc A which was more active against this strain possesses Phe with less hydrogen-bonding ability. This indicates that membrane insertion rather than hydrogen bonding with a peptide probably at the cell wall affects susceptibility of *L. monocytogenes* B73.

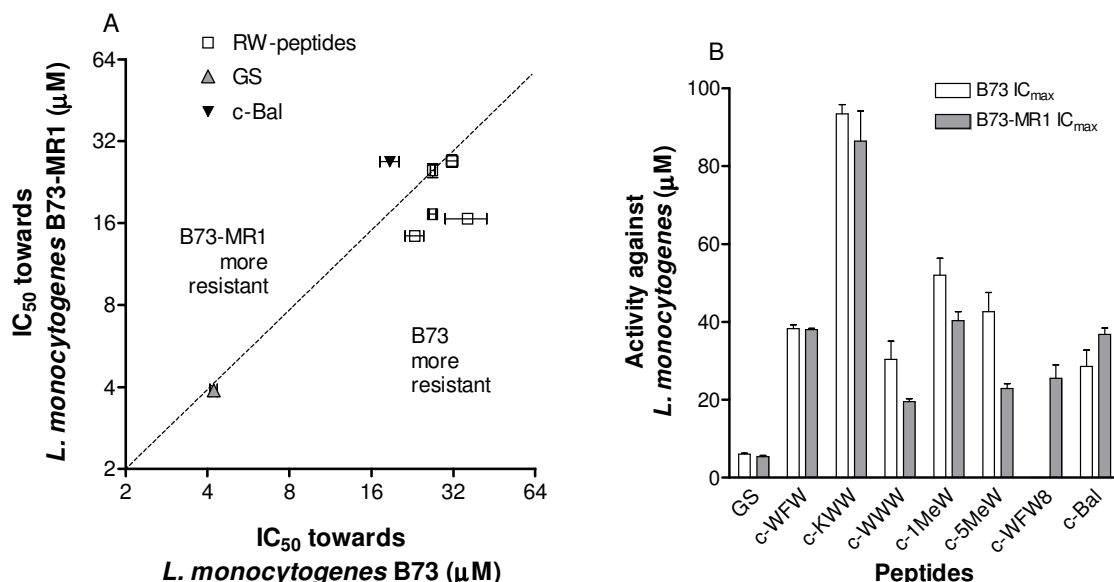


Figure 3.9 Comparison of **A.** IC_{50} and **B.** IC_{max} of the active RW-peptides and gramicidin S for growth inhibitory activity towards the leucocin A sensitive *L. monocytogenes* B73 and leucocin A resistant *L. monocytogenes* B73-MR1. Parameter data are given in Table 3.6. The IC_{max} of c-WFW8 was $>100 \mu\text{M}$ and is not shown in **B.**

3.5.2. Structure-activity analyses of c-WFW and analogues

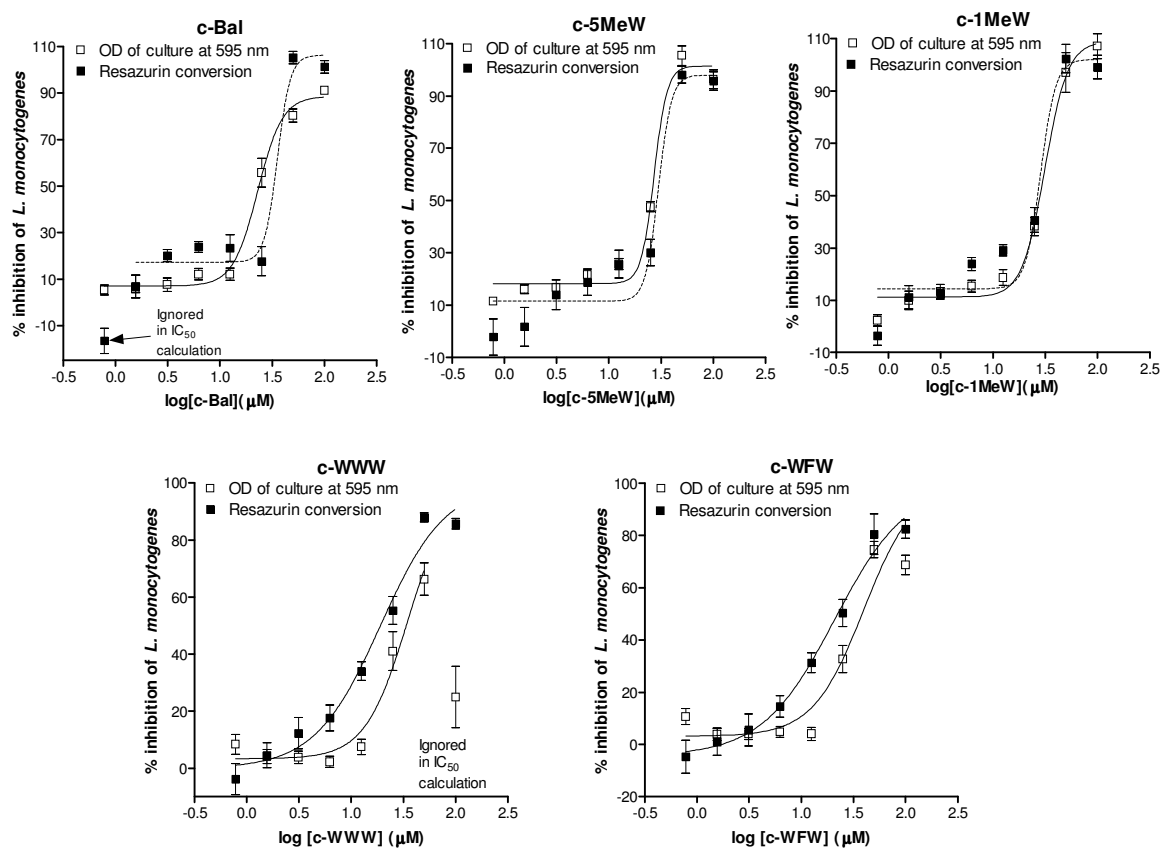
The most active peptide against *L. monocytogenes* B73 was found to be c-Bal with an IC_{50} of $19.0 \mu\text{M}$. Due to the fact that IC_{50} and/or IC_{max} values could not be derived from data for all the RW-peptides (Fig. 3.10), growth and metabolism inhibitory activities at $25 \mu\text{M}$ were considered. Activity at $25 \mu\text{M}$ gave the following trend: c-Bal $>$ c-WFW8 $>$ c-5MeW $>$ (c-1MeW, c-WWW) $>$ c-WFW $>$ (c-WFW10, c-KRK) $>$ (c-WKW, c-KWW, c-KW, c-WIW, c-b3hW, c-WRW) $>$ c-WFW12 (Table 3.6 and Table S3.5-S3.7). Some of the peptides lost activity at higher concentration (not shown) probably due to loss of “active peptide” monomers as a result of self-association into higher order structures⁹⁴. Such was the case for c-WFW8 and c-WWW both of which are more hydrophobic according to HPLC R_t , than the parent compound c-WFW (refer to Table S3.8 in supplementary data).

3.5.2.1. Group 1: Modification of the hydrophobic domain

In this group, we investigated the effect of the modification of the hydrophobic domain while maintaining number of residues and the polar domain unchanged. The dipole and quadrupole moments of the Trp residue and its hydrogen-bonding ability with both water and polar lipid head groups are said to account for its propensity to be located at the membrane interface^{85,95}. Trp differs from the unnatural residues Igl, b3hW, 5MeW, 1MeW, and Bal in terms of hydrophobicity, electron distribution, hydrogen-bonding ability, amphipathicity, and flexibility

in the hydrophobic domain of c-WFW (Table 3.5). Comparing the growth inhibition activity at 25 μM against *L. monocytogenes* B73 using the Newman-Keuls multiple comparison test, the following sequence was obtained: c-Bal > c-5MeW > (c-WWW, c-1MeW) > c-WFW > (c-WIW, c-b3hW) (Table 3.6 and Table S3.5). Dose response data depicting the antilisterial growth and metabolic activity of the peptides are shown in Figs. 3.10 and 3.13A.

Active peptides



Inactive peptides

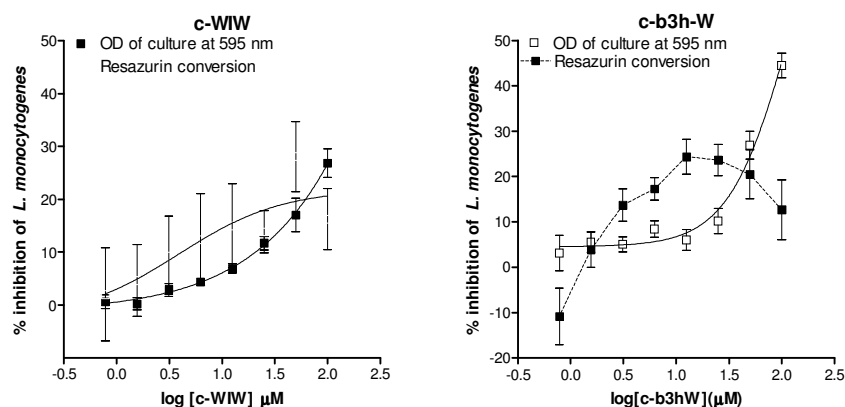


Figure 3.10 Representative dose–response curves of the active and inactive RW-peptides in group 1. Activity was measured as percentage of both growth and metabolism inhibition. Each data point represents the average of at least six determinations \pm SEM.

It can be observed that activity of the parent compound was improved by either replacing the Phe with a Trp or substituting the two Trp residues with the unnatural amino acid Bal (β -(benzothien-3-yl)-alanine) in c-Bal and the Trp methylated at either the nitrogen atom in position 1 or the carbon at position 5 of the indole ring in c-1MeW or c-5MeW. Activity was lost when Igl (α -(2-indanyl) glycine) replaced the Phe residue in c-WIW or when the two Trp residues were replaced by b3hW (L- β -homotryptophan). In Igl the aromatic ring is closer to the backbone⁸⁸. Although c-WIW has similar hydrophobicity to c-WFW according to R_1 values (Table S3.8), the fact that Igl is not positioned at the lipid-water interfacial region⁸⁸ could account for loss in activity against *L. monocytogenes* B73 of c-WIW.

Size and flexibility of the backbone ring are increased in b3hW while hydrogen bonding ability is maintained. Even though two extra methylene groups in c-b3hW resulted in slight increase in hydrophobicity compared to c-WFW, the decreased activity of c-b3hW is probably related to an increase in size and flexibility of the backbone ring which leads to a change in the amphipathic conformation of c-WFW by disturbing the β -turn motifs⁹⁹ and affects membrane interaction⁸⁸. Previously, c-b3hW showed weakened interaction with neutrally charged 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles and decreased haemolytic activity and activity against *B. subtilis*⁸⁵.

The analogues 5MeW and 1MeW are similar to Trp in terms of dipole and quadrupole moments. The effect of methylation in 1MeW is the obstruction of the hydrogen-bonding ability of Trp by the more hydrophobic and bulky methyl group⁸⁸. Nevertheless, 1MeW and Trp share the same amphipathicity and dipole moment (~ 2.2 D) with similar direction and magnitude (Table 3.5). 1MeW is also more hydrophobic than Trp⁸⁸. The presence of either 1MeW or 5MeW resulted in increased hydrophobicity and maintenance of or increase in antilisterial activity compared to that of c-WFW. This reveals the role of hydrophobicity over hydrogen-bonding ability in activity towards *L. monocytogenes* B73. In previous studies, c-1MeW and c-5MeW showed the same activity as c-WFW against *B. subtilis*, but were more haemolytic⁸⁵; we found the activity trend as c-1MeW > (c-5MeW; c-WFW). This result also indicated that the hydrogen bonding character of Trp may, similar to what we found for the Trcs, influence activity negatively.

The Bal-containing peptide, which was the most active in this group, was also the most hydrophobic and due to the less electronegative sulphur atom, the dipole moment of Bal is lower than that of Trp and Bal does not possess the hydrogen-bonding ability of Trp (Table 3.5). This side chain also lacks an amphipathic structure⁸⁵. This analogue has been shown to increase anti-*B. subtilis* activity compared to the parent compound⁸⁸. Our results also agree with the higher

antimicrobial activity observed for a 15-residue Bal-modified lactoferricin derivative over that of the parent peptide ⁹¹.

Substituting the Phe in c-WFW with a Trp led to a gain in activity. This particular residue is probably important in anchoring and localisation properties of the indole ring as a result of the formation of hydrogen bonds between its NH-group and lipid polar head groups and its larger size ⁸², similar to the one Trp residue in the highly antilisterial Trc B. Phe, however, integrates the membrane deeper than Trp ^{70,81}, which could signify that anchoring may not explain the increase in activity.

The critical role of the hydrophobicity of Trp residues was supported by the significant enhancement in the antilisterial activities against *L. monocytogenes* B73 of c-Bal, c-5MeW, c-1MeW and c-WWW. Reduced activity of c-b3hW also emphasized the importance of the increased size and flexibility of the backbone ring in c-b3hW which leads to a change in the amphipathic conformation of c-WFW by disturbing the β -turn motifs ⁹⁹. This substantiates the proposal by Wessolowski *et al.* ⁹⁷ that optimization of electrostatic and hydrophobic interactions of AMPs requires the formation of an amphipathic structure since interaction with the cell membrane is the fundamental step in antimicrobial activity, despite the specific details of the mode of action of the individual peptides.

The activity spectrum of the active members of this group in terms of the activity at 25 μ M towards *L. monocytogenes* B73-MR1 was (c-WWW, c-5MeW) > (c-Bal, c-WFW) > c-1MeW. Unlike for the leucocin A sensitive strain, methylation at position 1 of the indole ring reduces activity against the resistant strain compared to the parent compound. This correlates well with the results for the Trcs that indicated that hydrogen bonding with an aromatic residue is critical for susceptibility of *L. monocytogenes* B73-MR1. Likewise, unlike the gain in activity against *L. monocytogenes* B73 when Bal (which also lacks the hydrogen-bonding ability of Trp) replaced Trp, in B73-MR1 activity of c-Bal was not different from that of c-WFW.

3.5.2.2. Group 2: Modification of the polar domain

The polar domain was altered by consistently replacing the charged residue Arg with Lys at selected positions. The R_t values of all Lys-containing analogues show only minor differences from the Arg-containing analogues (Table S3.8). Substituting two of the Arg residues with Lys from c-WFW to c-KRK resulted in a loss of activity (Figs. 3.11 and 3.13B, Table 3.6 and Table S3.6). This is in contrast to previous results that showed increased antimicrobial activity for c-KRK ⁹⁸. Scheinpflug *et al.* ⁹⁸ observed that the CD spectrum of c-KRK was different from that of the rest of the peptides indicating a change in the backbone ring conformation. This

conformation may reduce interaction with the membrane or intracellular target of c-WFW in *L. monocytogenes* B73.

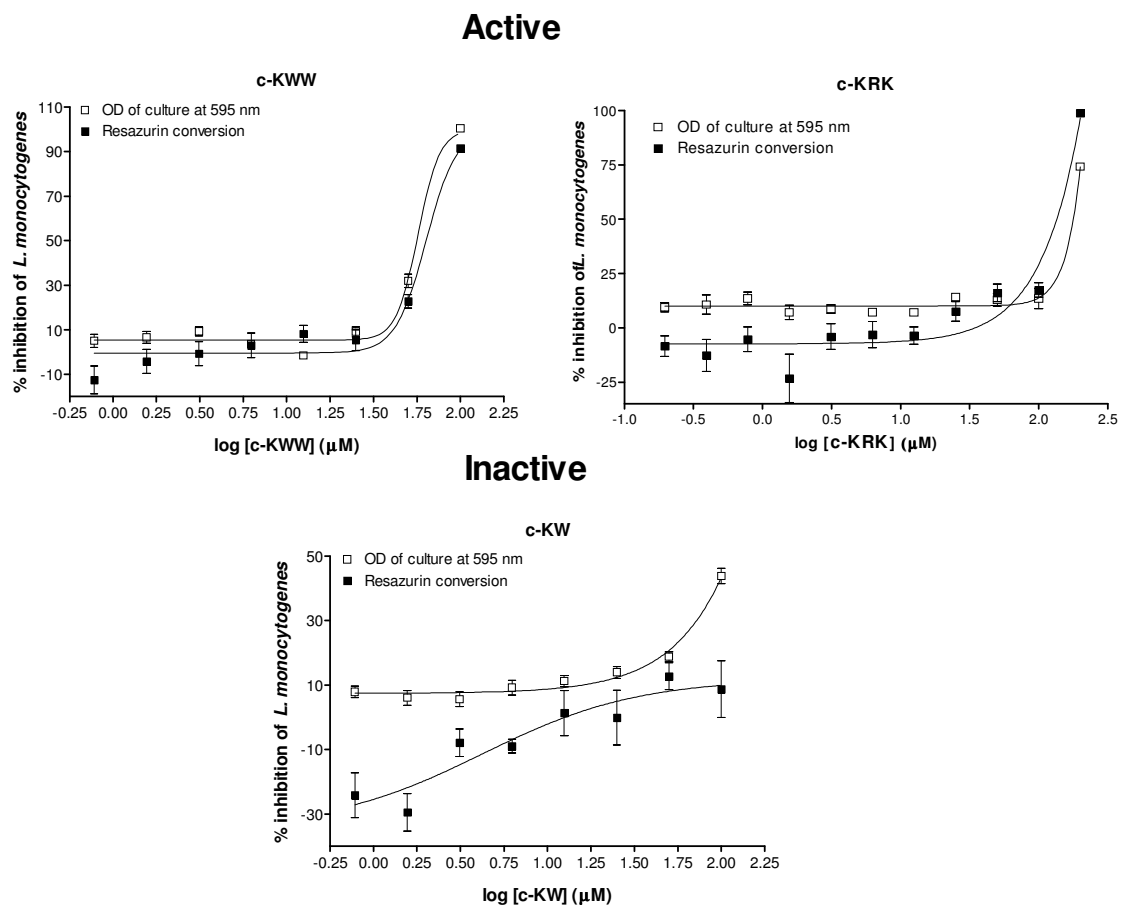


Figure 3.11 Representative dose–response curves of the active and inactive RW-peptides in group 2. Activity was measured as percentage of both growth and metabolism inhibition. Each data point represents the average of at least six determinations \pm SEM.

Replacement of all three Arg residues with Lys and a disruption of the aromatic cluster by one of the Lys in the case of c-KW led to a decrease in activity too. In previous work, this analogue also showed decreased bactericidal and haemolytic activity in comparison to another structural analogue of c-WFW with which it had comparable size of the hydrophilic and hydrophobic areas as well as net charge, c(RRWRF) or c-RW¹⁰⁰. This was attributed to the decrease in the ability of Lys with three hydrogen-bond donors to interact with hydrogen-bond acceptors of lipid head groups. In contrast, the guanidine fraction of Arg residues possesses five hydrogen-bond donors¹⁰⁰. There was a similar loss of activity from c-WWW to c-KWW when all the Arg side chains were replaced by Lys. In other studies, Arg-containing analogues have been observed to show higher membrane permeability and/or activity against Gram-positive and/or Gram-negative bacteria than Lys-containing analogues of the AMPs RLA¹⁰¹, tritrypticin¹⁰², batenecin 5, lactoferrin B and a cyclic β -stranded synthetic peptide^{103–105}. Replacement of Orn by Lys or Arg

for GS left the activity unaltered¹⁰⁶. However, the same substitution showed decreased activity against Gram-negative bacteria for protegrin-1¹⁰⁷.

3.5.2.3. Group 3: Modification of number of residues

In this group, there was consistent increase in ring size. Both the polar and hydrophobic domains were extended by increasing the number of aromatic and charged residues while maintaining the amphipathic equilibrium⁸⁶. The hydrophobicity according to HPLC R_t was increased continuously (Table S3.8). The aim was to gain insight on the peptide structural designs that are relevant to antilisterial activity⁸⁶. The overall results showed that increasing peptide size and hydrophobicity led to loss of activity against *L. monocytogenes* B73, however, at 25 μ M the activity of c-WFW8 was significantly higher than that of c-WFW (Figs. 3.12 and 3.13C and Table S3.7 in supplementary data).

Our results contradict the results of Junkes *et al.*⁸⁷ who found a correlation between both haemolytic activity and activity against *B. subtilis* and *E. coli* with increase in peptide ring size. It was established in this study that the large rings have as primary mode of action, membrane permeabilisation unlike c-WFW which had little bilayer-disturbing and membrane permeabilising activity. Despite the increase in hydrophobicity with increase in peptide size, there was also increase in charge which could lead to an increase in electrostatic interaction with the anionic listerial membrane and possibly trapping, consequently reducing antilisterial activity⁶⁷. Studies by Dathe *et al.*⁴⁷ established that highly anionic lipid bilayers and bacterial target membranes counteract the improved activity of cyclic analogues of (Ac)-RRWWRF-NH₂ due to shallow partitioning of the peptides into the membrane. A similar observation was made for Trc A analogues in Section 3.4.2.1 above, though increased charge was accompanied rather by a decrease in hydrophobicity compared to the parent compound.

3.5.2.4. Group 4: Destruction of polar and hydrophobic clusters

Here the cationic and hydrophobic clusters were destroyed resulting in a loss of amphipathicity as reflected by the low R_t values. There seems to be a requirement for the aromatic and charged residues to be clustered together for optimal activity as there was a general loss of activity for c-WRW compared to c-WWW as well as c-KWW compared to c-WKW (Fig. 3.13D).

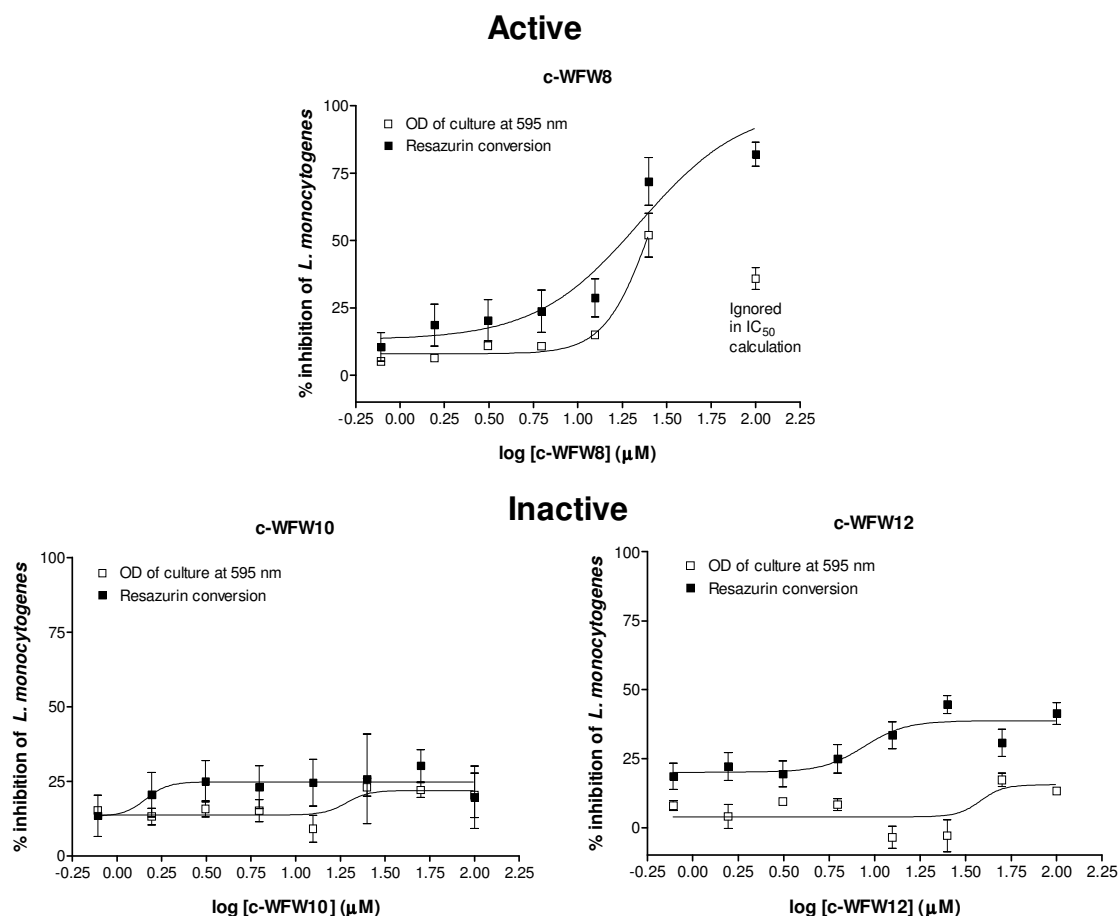


Figure 3.12 Representative dose–response curves of the active and inactive RW-peptides in group 3. Activity was measured as percentage of both growth and metabolism inhibition. Each data point represents the average of at least six determinations \pm SEM.

3.5.3. QSAR and PCA analyses

HPLC R_t values and molecular masses of the RW-peptides along with some structural parameters derived for the analogues (Supplementary data Table S3.8) were evaluated for correlation with the observed growth inhibitory activity at 25 μ M and 100 μ M. Data were fitted to Gaussian distribution, hyperbolic, exponential decrease and increase, sigmoidal, 1st, 2nd and 3rd order polynomial equations and correlations with $R^2 \geq 0.50$ were considered as significant QSAR trends (Fig. 3.14).

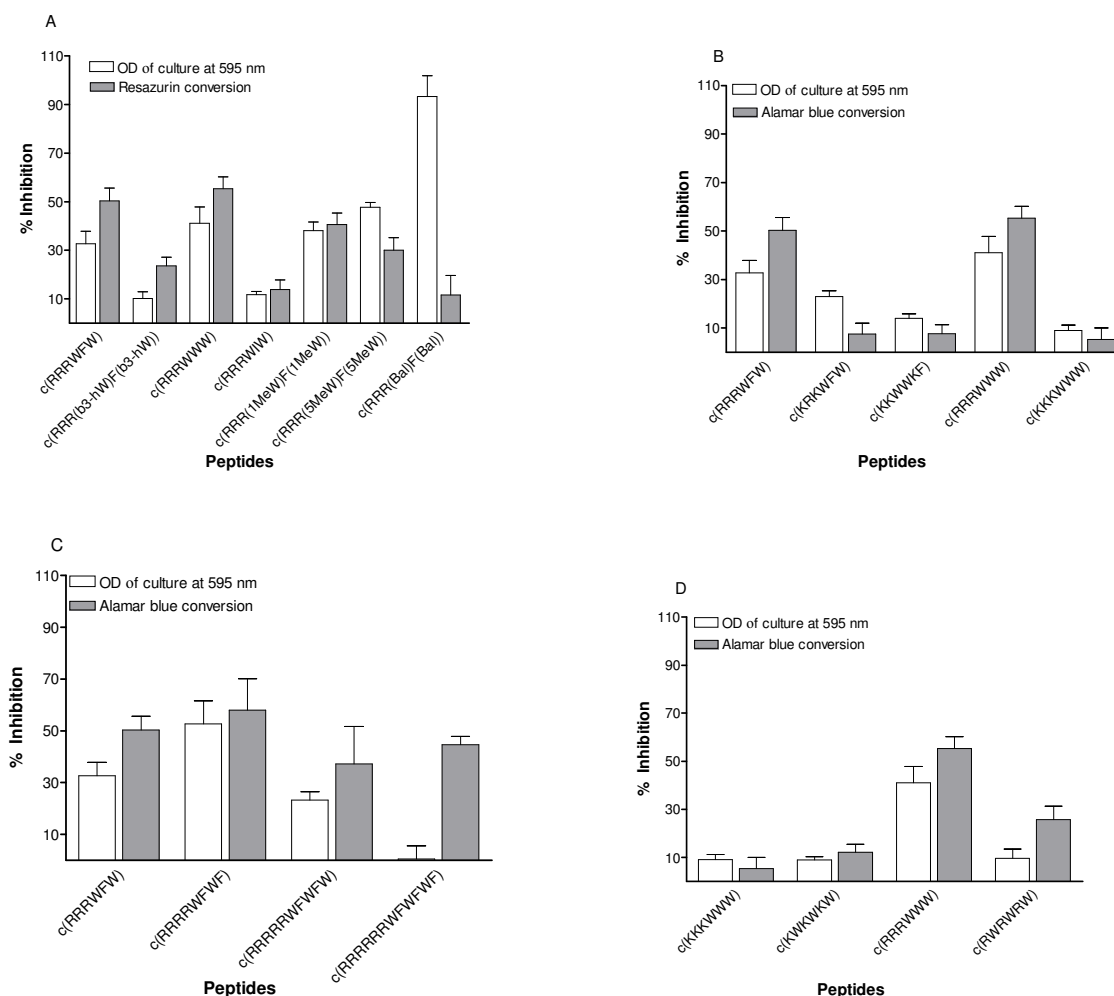


Figure 3.13 Comparison of the growth and metabolism inhibitory activity of four groups of synthetic cyclic analogues of (Ac)-RRWWRF-NH₂ (RW-peptides) at 25 μM against *L. monocytogenes* B73. Refer to Table 3.4 for more details on the peptide structures and Table 3.6 for their activity parameters **A**. Peptides differ with respect to aromatic residues in the hydrophobic domain; **B**. the polar domain was modified by R to K substitution in selected positions; **C**. group of sequences with increasing ring size. Both the polar and hydrophobic sequence regions are larger; **D**. effect of destruction of the cationic and hydrophobic clusters.

Significant correlations were obtained between activity at 25 μM and HPLC R_t, interphase properties, M_r and MV with R² values between 0.5-0.6 (Fig. 3.14, also refer to Table S3.8 for parameters). However, interphase properties, M_r and MV data for c-RW had to be excluded from analyses due to the same magnitude of the structural parameter as that of c-WWW, but total loss of activity which affected the trends.

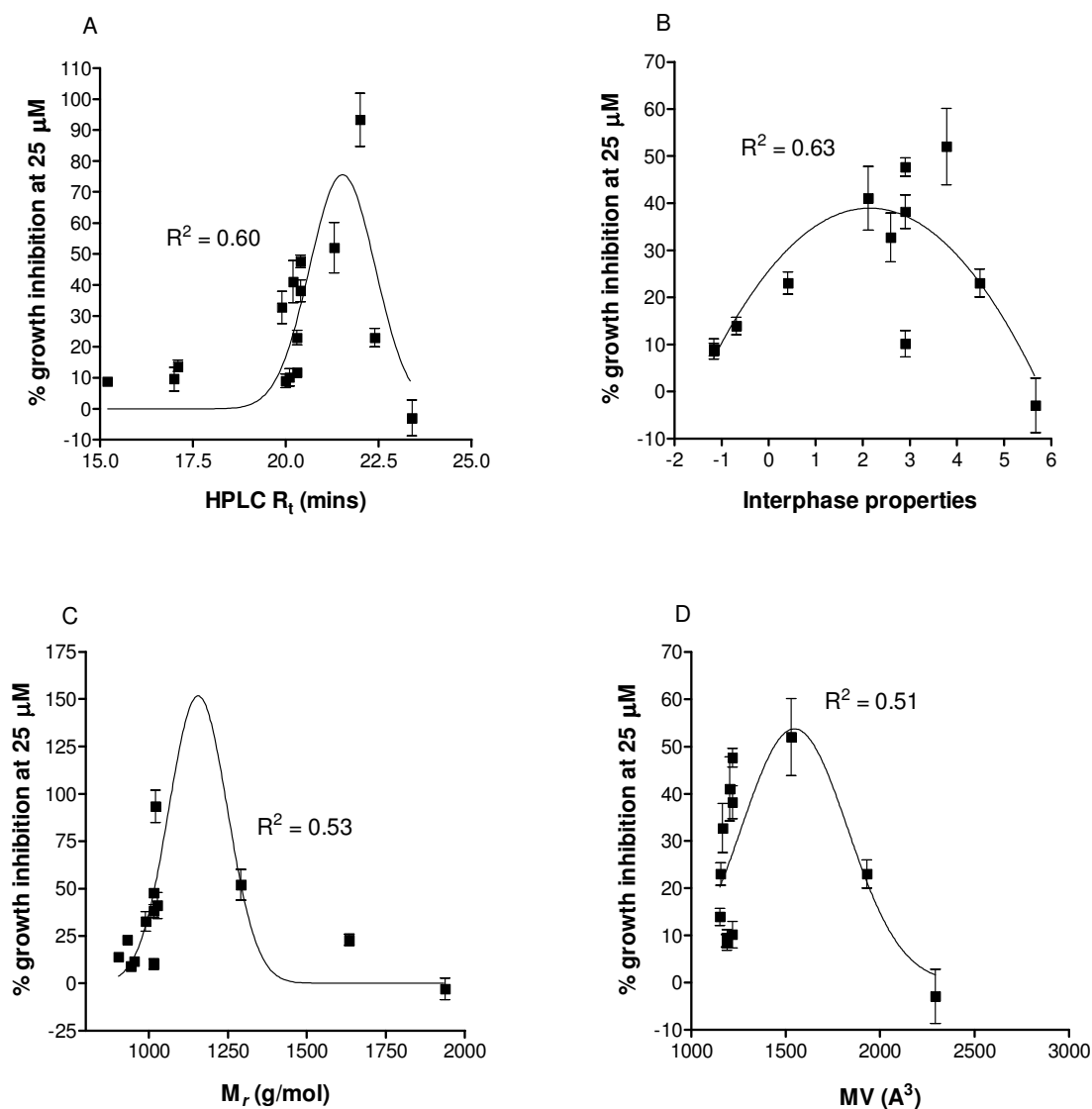


Figure 3.14 Relationship between growth inhibition of *L. monocytogenes* B73 and **A.** HPLC R_t **B.** Σ (interphase properties) **C.** molecular mass (M_r) **D.** Σ (molecular volume) fit to Gaussian distribution and 2nd order polynomial equations. Each data point represents the mean of at least 3 biological repeats; with 9–15 measurements \pm SEM. Correlation coefficient values are indicated.

The correlation between activity and retention behaviour in RP-HPLC followed a Gaussian distribution with activity increasing as R_t increased to an optimal value and then decreasing with further increase in R_t (Fig. 3.14A). Activity and interphase properties gave a significant correlation with a quadratic equation again indicating an optimal amphipathicity (Fig. 3.14B). These two correlations indicated that antilisterial activity is highly dependent on amphipathicity. Similar trends were observed between activity and the size parameters M_r and MV (Fig. 3.14 C, D). The 2D QSAR analyses did not give significant correlation between activity and the other structural parameters evaluated based on the limit of $R^2 \geq 0.50$ (results not shown). Therefore,

both size and hydrophobicity/amphipathicity are relevant in modulating antilisterial activity of RW-peptides.

As with the Trc library, we carried out multivariate data analysis using the PCA projection method for the molecular descriptors representing bulk (size) and chemical character of the peptides (Table S3.8) together with two activity parameters i.e. growth inhibitory activity at 25 μM (A25) and 100 μM (A100) against *L. monocytogenes* B73. We carried out two PCA; in the first PCA ten RW-peptides were evaluated due to exclusion of the analogues with unnatural Trp analogues and in the second PCA all the peptides were evaluated, but some parameters which could not be obtained for certain analogues with unnatural amino acids were excluded. A total of thirteen and seven parameters were considered in the first and second PCA respectively (Fig. 3.15 and Table 3.7).

Considering only the peptides with natural amino acids, the score plot t_1t_2 (Fig. 3.15A) showed that the first two principal components or factors explained 85% of the total variance among the analogues (factor 1 = 73%, factor 2 = 12%). Along the PC 1 scale, the peptides were segregated according to the number of residues with all hexapeptides grouped in the extreme right of the scale while the largest peptide with 12 residues c-WFW 12 was found at the extreme left (Fig. 3.15A). Separation of peptides along the PC 2 scale seemed to be according to activity as the most active analogues c-WFW8, c-WFW and c-WWW clustered together in the top right quadrant on this scale (Fig. 3.15A).

Based on the loading plot p_1p_2 the size parameters determined experimentally (M_r) along with the computed MV as well as those determined *in silico* (MV, SAV and SASA) correlated positively. The MV estimated using YASARA 9.10.5[©] was very closely associated with that derived from theoretical values of the constituent amino acids. The SCSA was the only size parameter that did not associate with the others, however, it had a negative correlation with the interphase properties parameter as well as the HPLC R_t (Fig. 3.15A). A similar observation was made with the Trc library analyses in *Section 3.4.3*. Lipophilicity correlated negatively with hydrophobicity and hydrophathy, but positively with the size parameters. This is probably due to an increase in the number of the highly lipophilic Phe residues not only increasing the size by also the hydrophobicity^{80,81}. Therefore, distribution of the peptides along the PC 1 scale can be explained by the negative correlation between size and hydrophobicity/hydrophathy parameters. As predicted activity at 25 μM (A25) had great influence in the top right quadrant along the PC 2 scale in which the most active peptides formed a cluster (Fig. 3.15A). In the bi-plot of the first

(PC 1) and third (PC 3) principal components, comparable trends were observed, but it was rather the activity at 100 μ M (A100) that had a significant correlation coefficient (not shown).

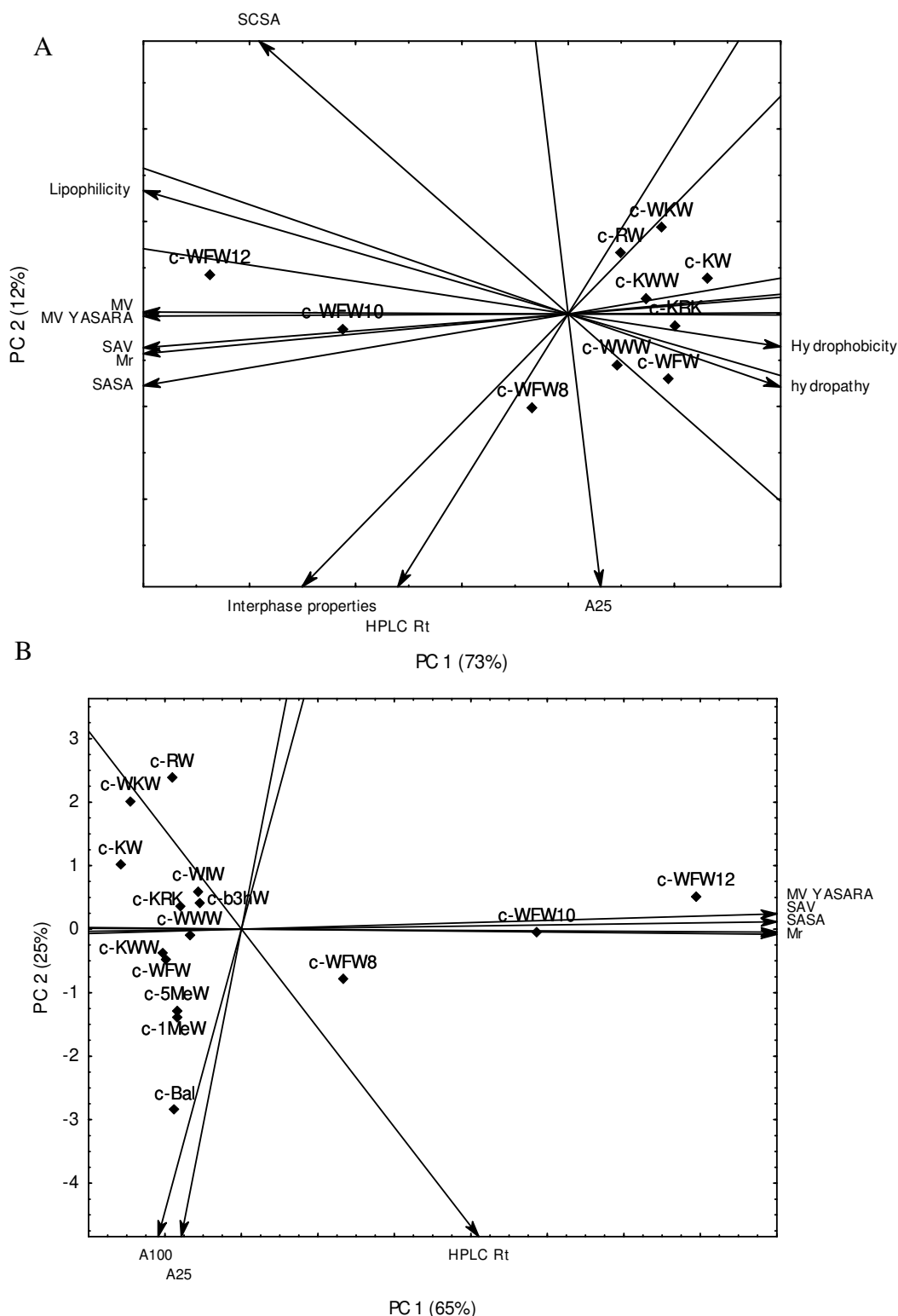


Figure 3.15 **A.** PCA bi-plot showing the relationship between the variation in the 13 structural and antilisterial activity parameters and the relationship between 10 RW-peptides (excluding analogues with unnatural Trp analogues) along factor 1 and 2. **B.** Bi-plot showing the relationship between the variation in 7 structural and antilisterial activity parameters and the relationship between all 15 RW-peptides along factors 1 and 2.

When all 15 RW-peptides were considered based on seven variables, PC 1 and PC 2 accounted for 90% of the total variance (Fig. 3.15B). From the t_1t_2 plot, distribution along the PC 1 scale was related once more to the number of residues with the hexapeptides clustered in the left half of the scale. Along the PC 2 scale the hexapeptides in which the aromatic and charged residues were scrambled clustered together in the bottom left quadrant. The peptides with methylated indole rings also associated closely to each other. The analogue c-Bal was an outlier in the group of hexapeptides. Based on the p_1p_2 plot, we observed clustering of all size parameters which explained peptide distribution along the PC 1 scale (Fig. 3.15B). The two activity parameters were closely associated to each other and correlated positively with the HPLC R_t .

Antilisterial activity of the RW-peptides is therefore, influenced by clustering of aromatic and cationic residues and total number of residues which all impact on both the chemical and structural parameters of the peptides. All determined physicochemical parameters gave high correlation coefficients in both PCAs (Table 3.7).

Table 3.7 Correlation coefficients (R^2) for agreement between actual variables and PCA model-predicted variables used in the PCA QSAR analysis of the RW-peptides

Variables	PCA Model fit	
	R^2 p ₁ p ₂ plot without peptides with unnatural Trp analogues	R^2 p ₁ p ₂ plot all peptides
Activity parameters		
A25	0.854	0.728
A100	0.201	0.727
Size parameters		
M_r	0.992	0.986
MV	0.984	0.990
MV yasara	0.989	na
SCSA	0.778	na
SASA	0.962	0.985
SAV	0.983	0.992
Chemical parameters		
HPLC R_t	0.852	0.864
Lipophilicity	0.990	na
Hydrophathy	0.830	na
Hydrophobicity	0.819	na
Interphase properties	0.845	na

As was previously observed by Junkes *et al.*⁸⁷, adjoining cationic and hydrophobic residues and a ring size of six amino acids caused maximal activity towards *Listeria* and alterations in amino acid arrangement and ring size probably lead to differences in the mode of action. Antimicrobial peptides carry out their antibacterial activity by lysis and/or alternative target inhibition that is frequently indicated by data captured within the dose-response according to Rautenbach *et al.*⁹⁴. The RW-peptides showed different dose-responses (Figs. 3.10-3.12) and antilisterial activity of most of the RW-peptides we analysed do not depend on membranolytic action. Previous experiments for membrane permeability have shown that the most active RW-peptides (c-WFW and c-WWW) do not rely primarily on membrane lysis for activity^{47,87,108}. Membrane activity of these peptides has been associated to lipid demixing¹⁰⁹⁻¹¹¹ resulting in interference with some cell regulatory functions¹¹² or packing defects leading to translocation of polar compounds into the cytoplasm¹¹³.

Phosphatidyl ethanolamine (PE)-rich membrane domains as in Gram-positive bacteria can also favour membrane destabilization because of the negative curvature propensity of the lipids¹¹⁰. The other mode of action which may involve peptides crossing the plasma membrane is supported by the high content in W and R residues for the active peptides which has been suggested to favour peptide translocation across the cell membrane^{87,114}. The observed biomass stress, which corresponds to observed “growth” with metabolism inhibition (Fig. 3.13), could be due to the formation of biofilms and exopolysaccharide formation leading to increased light scattering that is measured as growth.

3.6. Conclusions

Due to the public health and economic threat of *Listeria*^{1,2,4} coupled with a general tendency of resistance by *Listeria* spp. to antibiotics and disinfectants^{1,3,4} as well as the limitations of nisin², other AMPs are investigated for use in isolation or in combination with nisin or other bacteriocins as food preservatives. Recognition of the key structural parameters for the optimization of peptide activity toward the resistant strains of this pathogen provides the basis for the design of highly selective antimicrobial compounds.

In general the Trcs and RW-peptides were more active against the leucocin A resistant *L. monocytogenes* B73-MR1, than the sensitive B73 strain. The results from all the peptide libraries tested revealed the relevance of hydrogen bonding with an aromatic amino acid residue for activity towards B73-MR1 while for B73, the overall peptide size was more important. Hydrogen bonding may either result in an inactive conformation or retain the peptide in the cell wall/membrane, limiting its mode of action. The observed difference in susceptibility between

the strains to these peptides can be explained by the difference in their membrane composition, B73-MR1 having a more fluid and anionic membrane⁴¹. Since the effect of peptides on cells involves at least in part their interaction with membrane lipids, the lipid composition of membranes significantly impacts on their specificity¹¹⁵.

We employed a new activity parameter, the activity product ($A_P = IC_{50} \times IC_{max}$) and the inhibition concentration factor ($IC_F = IC_{50}/IC_{max}$) which improved selecting the most active Trc analogues, while still incorporating conventional inhibition parameters. Our results agreed with Spathelf & Rautenbach⁴⁴ on the higher activity of the tyrocidines (Trcs) containing Trp³ in the aromatic dipeptide unit and either Orn⁹/Lys⁹ as cationic residue. Re-evaluation of Trc A, Trc C, Trc B and their Lys analogues confirmed their low micromolar activity (IC_{50} s = 7-14 μ M and MICs <35 μ M). We also found that the Orn⁹ containing Trcs were generally more active than the Lys⁹ analogues. The most active Trc was the analogue of both Trc A and Trc C, namely Trc B with IC_{50} at 7 μ M and IC_{max} at 16 μ M. Modifying Trc C to Tpc C (Tyr⁷ to Trp⁷ substitution) resulted in reduced activity ($IC_{max} = 39 \mu$ M). Likewise Trc A to Phc A (Tyr to Phe substitution) modification yielded a higher IC_{50} of 15 μ M. However, the slightly lower IC_{max} at 20 μ M of Phc A to that of Trc A indicated a role of the size of the aromatic residue in the NQX sequence of the variable pentapeptide possibly due to size and dipolar/induced dipolar potential. This is the first evidence that the antilisterial activity of the tyrocidines may rely on a Tyr or Phe in the variable pentapeptide moiety. A novel synthetic analogue of Trc A, sTrc AOMe₃, with comparable activity to that of Trc A at IC_{50} of 12 μ M, but a lower IC_{max} of 18 μ M rivalling that of Trc B was identified. The presence a bulky charged residue in proximity to Phe³ can thus alleviate the negative effect of Phe³ on antilisterial activity. Also, despite the loss of hydrogen bonding in the N^δ-trimethyl Orn, the increased hydrophobicity probably enables deeper and more disruptive membrane interaction than the “snorkeling” Lys analogue. However, the synthetic analogue of Trc A with charge increase due to Gln⁶ substitution to Orn⁶ in the NQY sequence decreased the antilisterial activity, indicating a delicate amphipathic balance and possibly a role for the Gln⁶ in antilisterial activity. The results therefore, revealed that a small group such as Tyr or Phe at position 7 of the Trcs and possibly the Gln⁶ are probably involved in target interaction for activity against *Listeria*. Moreover the character of the aromatic dipeptide unit as well as the cationic residue in the conserved V(K/O)LfP pentapeptide are critical residues within the Trc structure for antilisterial activity. The aromatic dipeptide unit has a major influence on the hydrophobicity and size of the tyrocidine/analogue. Increase in charge, hydrophobicity and size parameters that lead to tighter membrane interaction tend to hamper the Trc mechanism of action by possibly trapping the peptides and preventing either the formation of lytic complexes or

translocation to target site(s). Multivariate QSAR models derived for the Trcs accurately predicted most of the structural and activity parameters and indicated the relevance of both hydrophobicity and overall size parameters for antilisterial activity. The most important hydrophobicity parameter were the hydrophathy, hydrophobicity and interphase properties while the SASA, SAV, M_r and molecular volume (MV) were the best steric/size parameters associated with antilisterial activity. From this study we predict that viable lead structures for antilisterial activity in the next generation tyrocidine library would be *cyclo*[VOMe₃LfPWfNQ(Y/F)].

Seven of the RW-peptides tested were active against *L. monocytogenes* B73 with $IC_{50} < 100 \mu\text{M}$; four having an $IC_{50} < 30 \mu\text{M}$ and $IC_{\text{max}} < 50 \mu\text{M}$. The template *cyclo*(RRRWFW) was found to have good activity ($IC_{50} = 27 \mu\text{M}$, $IC_{\text{max}} = 38 \mu\text{M}$). A slight improvement in activity was achieved with the substitution of Phe by Trp ($IC_{50} = 23 \mu\text{M}$, MIC = 30 μM) indicating that this residue is not constrained by size. A further increase in activity ($IC_{50} = 19 \mu\text{M}$, $IC_{\text{max}} = 29 \mu\text{M}$) was found with the substitution of Trp by Bal (β -(benzothien-3-yl)-alanine) to form *cyclo*[RRR(Bal)F(Bal)]. This indicated that a more hydrophobic aromatic sequence could be beneficial for antilisterial activity. Methylation of tryptophan residues increased the activity, indicating that the tryptophan side chain size may also be a factor. Substitution of the aromatic residues with analogues revealed the relevance of interaction at the membrane interface in which size, amphipathicity, hydrophobicity, and flexibility are all factors. Clustering of aromatic residues, a preference for Arg over Lys as cationic residue and a ring size of six residues (18 atoms) were also shown to be essential for their antilisterial activity, correlating with previous results obtained by Junkes *et al.*⁸⁷. Any change in the ring size, such as introducing beta-amino acids (beta-homotryptophan) or including more amino acids lowered or eradicated the antilisterial activity. Scrambling, and even minor changes in the sequence also eradicated the activity. Two-dimensional QSAR confirmed with good correlation the significance of hydrophobicity (HPLC R_t and interphase properties) and steric/size parameters (MV and molecular mass) to activity. These results were in agreement with previous results on other Gram-positive targets and underline the fact that a fine amphipathic balance is necessary to maintain activity in the RW-peptides. Similarly to the Trcs, multivariate QSAR models derived for the RW-peptides accurately predicted most of the structural and activity parameters and indicated the relevance of both hydrophobicity and overall size parameters for antilisterial activity. As with the 2D QSAR, the PCA projection technique indicated interphase properties and HPLC R_t as the most important parameters associated with antilisterial activity.

Based on the pivotal structural features identified in the peptide libraries, especially those that account for strain susceptibility and selectivity, lead peptides can be designed with desirable and

predictive effect against *Listeria* and other similar drug resistant pathogens. There is nevertheless the need to design and test more analogues in order to design more specific peptides and mimics.

3.7. References

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3.8. Addendum

3.8.1. *Influence of culture storage on sensitivity to peptides*

The transition by pathogenic *Listeria* from the environmental habitat to the mammalian host is accompanied by a temperature up-shift ensued by an up regulation of virulence factors (including surface-associated proteins) that could affect their susceptibility to AMPs⁵; hence it was important to investigate the influence of temperature on the antilisterial activity of the Trcs.

3.8.1.1. *Methodology*

The effect of storage of *L. monocytogenes* B73 pre-cultures at varying temperatures and for varying lengths of time on the growth inhibition activity of the Trc mixture extracted from commercial tyrothricin, as described in Chapter 2, was investigated. Gramicidin S (GS) was used as reference peptide. Following culturing of bacteria on BHI agar from freezer stocks for 24 hours at 37 °C, the cultures were stored separately at 4 ± 2 °C, 22 ± 2 °C, and 37 ± 2 °C for 3 and 6 days. Selected colonies were later cultured in BHI broth at 37 °C to log phase and sub-cultured to OD₆₂₀ of 0.4 for use in growth inhibition assays as described below. The sub-cultured cell suspensions at mid-log phase were diluted to OD₆₂₀ of 0.2 using BHI broth.

3.8.1.2. *Results and discussion*

Although storage at 4 ± 2 °C and 22 ± 2 °C for 3 and 6 days and at 37 ± 2 °C for 3 days did not significantly alter the activity of Trc mix, it was noted that prolonged storage of the pre-cultures to 6 days at 37 ± 2 °C increased the resistance of the bacteria to Trc mix's growth inhibitory activity (Fig. 3.16) as demonstrated by a significant increase in the IC₅₀ value. The same observation was made for GS' growth inhibition activity (see Table 3.8 and 3.9).

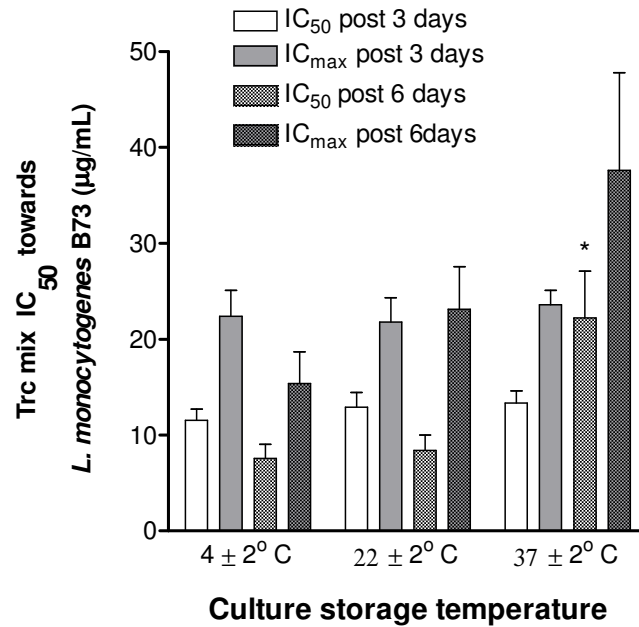


Figure 3.16 Influence of pre-culture storage conditions (time and temperature) on antilisterial activity (IC₅₀ and IC_{max}) of Trc mix from commercial tyrothricin against *L. monocytogenes* B73. Parameter data are given in Addendum Table 3.9 and 3.10. According to the Newman-Keuls multiple comparison test the IC₅₀ value of only post 6 days pre-culture storage at 37 °C was significantly different (* P < 0.05).

López-Solanilla *et al.*⁵ demonstrated a thermoregulated susceptibility of pathogenic *Listeria* to potato defensin mediated by protein release factor (Prf) A. PrfA which is a transcription factor and the central virulence regulator is the only known regulatory mechanism in pathogenic *Listeria* that is affected by altering temperature from 20 °C to 37 °C. PrfA-dependent genes are repressed at low temperature but they are induced at 37 °C⁵. It is possible that this could account for the observed difference in the susceptibility of *L. monocytogenes* B73 to the Trcs and GS following 6 days pre-incubation at 37 °C seen from our results. This could reflect a response aimed at improving survival of *Listeria* during their environmental saprophytic life⁵.

Table 3.8 Summary of the influence of storage conditions (time and temperature) of *L. monocytogenes* B73 pre-cultures on activity parameters of Trc mix and GS. Trc mixture's concentrations are given in $\mu\text{g/mL}$ (top two rows) and those of GS are given in μM . Every value denotes the average of n biological repeats (number of repeats given in brackets); with 3–5 technical repeats per assay \pm SEM.

Peptide	Pre-culture storage time (days)	Temperature of storage ($^{\circ}\text{C}$)	$\text{IC}_{50} \pm \text{SEM}$ (n)	$\text{IC}_{\text{max}} \pm \text{SEM}$ (n)
Trc mix	3	4 ± 2	12.0 ± 1.20 (5)	22.0 ± 2.70 (5)
		22 ± 2	13.0 ± 1.60 (4)	22.0 ± 2.50 (4)
		37 ± 2	13.0 ± 1.30 (3)	24.0 ± 1.50 (3)
	6	4 ± 2	7.5 ± 1.50 (3)	15.0 ± 3.30 (3)
		22 ± 2	8.4 ± 1.60 (3)	23.0 ± 4.40 (3)
		37 ± 2	22.0 ± 5.00 (3)	38.0 ± 10.2 (3)
GS	3	4 ± 2	4.1 ± 0.09 (5)	5.6 ± 0.11 (5)
		22 ± 2	4.2 ± 0.08 (4)	5.8 ± 0.12 (4)
		37 ± 2	4.1 ± 0.06 (4)	5.4 ± 0.09 (4)
	6	4 ± 2	4.5 ± 0.12 (3)	6.2 ± 0.11 (3)
		22 ± 2	4.6 ± 0.19 (3)	7.0 ± 0.36 (3)
		37 ± 2	15.0 ± 4.50 (3)	22.0 ± 6.80 (3)

Table 3.9 Summary of the P-values from the Newman-Keuls multiple comparison test on two listerial growth inhibition parameters (Table 3.8). The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05 . In each cell, the first value corresponds to the P-value for IC_{50} and the second value corresponds to that of IC_{max}

	Post 3 days 4 ± 2 $^{\circ}\text{C}$	Post 3 days 22 ± 2 $^{\circ}\text{C}$	Post 3 days 37 ± 2 $^{\circ}\text{C}$	Post 6 days 4 ± 2 $^{\circ}\text{C}$	Post 6 days 22 ± 2 $^{\circ}\text{C}$
Post 3 days 22 ± 2 $^{\circ}\text{C}$	ns ns				
Post 3 days 37 ± 2 $^{\circ}\text{C}$	ns ns	ns ns			
Post 6 days 4 ± 2 $^{\circ}\text{C}$	ns ns	ns ns	ns ns		
Post 6 days 22 ± 2 $^{\circ}\text{C}$	ns ns	ns ns	ns ns	ns ns	
Post 6 days 37 ± 2 $^{\circ}\text{C}$	0.05 ns	0.05 ns	0.05 ns	0.01 ns	0.01 ns

3.9. Supplementary data

Table S3.1 Summary of selected physicochemical parameters of the Trc A and Trc C analogues investigated for antilisterial activity. A number of parameters were taken from literature; HPLC retention times are from the analytical HPLC; SASA and SAV were determined *in silico* using YASARA 9.10.5[©].

Peptides	HPLC R _t (mins)	Q/L ⁵⁵	Σ (Lipophilicity) (π _{FP}) ^{55,*}	Σ (Hydrophathy) ⁵⁷	Hydrophobicity ⁵⁸	Interphase properties ⁵⁹	Molecular mass (M _r) (g/mol)	Σ (Molecular volume) * (MV, Å ³) ⁶⁰	Σ (Side-chain surface area) * (SCSA, Å ²) ¹¹⁶	Solvent accessible surface area (SASA, Å ²)	Solvent accessible volume (SAV, Å ³)
Trc A	11.26	0.105	9.55	24	-7.78	4.29	1269.661	1582.2	721.9	1199	2680
Trc A ₁	11.12	0.123	8.16	23.1	-1.34	4.33	1283.667	1609.2	741.4	1208	2715
Phc A	13.61	0.096	10.38	31.5	-9.41	4.68	1253.666	1578.5	786.9	1120	2591
sTpc A	13.58	0.092	10.84	31.2	-8.58	4.20	1292.668	1616.4	815.8	1122	2631
sTrc AOMe ₃	10.04	na	10.48	30.6	-5.77	4.47	1311.701	1659.5	850.2	1170	2741
sTrc A(Q-O)	6.73	0.197	10.17	21.0	-9.41	3.48	1255.675	1580	796.2	1122	2605
Trc B	9.56	0.1	10.01	23.7	-10.80	3.81	1308.634	1620.1	750.8	1217	2741
Trc B ₁	9.28	0.116	8.62	22.8	-13.44	3.85	1322.683	1647.1	770.3	1231	2777
Trc C	7.71	0.096	10.47	23.4	-13.82	3.33	1347.675	1658	779.7	1267	2833
Trc C ₁	7.31	0.11	9.08	22.5	-16.46	3.37	1361.690	1685	799.2	1269	2854
Tpc C	9.52	0.085	11.76	30.6	-17.64	3.24	1370.695	1692.2	873.6	1193	2772

* Missing amino acid parameters (P) were calculated as follows:

$$P_{Om} = P_{Lys} - P_{Leu} + P_{Val}; P_{Trimethylated Om} = P_{Om} + 3 \times (P_{Ala} - P_{Gly})$$

na: not available

Table S3.2 Summary of the P-values from the Newman-Keuls multiple comparison test on two listerial growth inhibition parameters (Table 3.2) for Trc A analogues. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05. In each cell, the first value corresponds to the maximum P-value for *L. monocytogenes* B73 and the second value corresponds to that of *L. monocytogenes* B73-MR1.

		sTpc A	Trc A	Trc A₁	sTrc AOMe₃	Trc B	sTrc A (Q-O)
Phc A	IC_{max}	0.001 ns	ns 0.05	0.01 ns	ns 0.001	ns 0.001	0.001 ns
	IC₅₀	0.001 0.05	ns 0.001	ns ns	ns 0.001	0.01 0.001	0.001 0.001
sTpc A	IC_{max}		0.001 0.001	0.001 0.01	0.001 0.001	0.001 0.001	0.001 ns
	IC₅₀		0.001 0.01	0.001 0.01	0.001 0.001	0.001 0.001	0.05 0.001
Trc A	IC_{max}			0.001 ns	ns 0.01	ns 0.01	0.001 0.001
	IC₅₀			0.001 0.001	ns 0.01	ns 0.01	0.001 0.001
Trc A₁	IC_{max}				0.01 0.001	0.001 0.001	ns 0.01
	IC₅₀				0.01 0.001	0.001 0.001	0.001 0.001
sTrc A OMe₃	IC_{max}					ns ns	0.001 0.001
	IC₅₀					ns ns	0.001 0.001
Trc B	IC_{max}						0.001 0.001
	IC₅₀						0.001 0.001

Table S3.3 Summary of the P-values from the Newman-Keuls multiple comparison test on two listerial growth inhibition parameters (Table 3.2) for Trc C analogues. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05. In each cell, the first value corresponds to the maximum P-value for *L. monocytogenes* B73 and the second value corresponds to that of *L. monocytogenes* B73-MR1.

		Tpc C	Trc C₁	Trc C	Trc B₁
Trc B	IC _{max}	0.001 0.001	0.01 0.001	ns ns	ns 0.01
	IC ₅₀	0.01 0.001	ns 0.001	ns ns	ns 0.001
Tpc C	IC _{max}		0.001 ns	0.001 0.001	0.001 ns
	IC ₅₀		0.001 0.01	0.001 0.001	ns 0.001
Trc C₁	IC _{max}			ns 0.001	ns ns
	IC ₅₀			ns 0.001	ns ns
Trc C	IC _{max}				ns 0.001
	IC ₅₀				ns 0.001

Table S3.4 Summary of the most prominent QSAR trends for correlation of the physicochemical parameters of the tyrocidines with antilisterial activity (IC_{50} , IC_{max} , IC_F and A_P) as determined from data fitted to polynomial equations. Only trends with correlation coefficients of $R^2 \geq 0.5$ were considered significant. Q = quadratic trend; – = no trend observed with fit $R^2 \geq 0.5$

Activity parameters toward <i>Listeria monocytogenes</i> strains		HPLC R_t (mins)	Q/L	Σ (Lipophilicity)	Σ (Hydrophathy)	Σ (Hydrophobicity)	Σ (Interphase properties)	Molecular mass (g/mol)	Σ (MV) (\AA^3)	Σ (SCSA) (\AA^2)	SASA (\AA^2)	SAV (\AA^3)
IC_{50} (μM)	B73	–	0.50 Q	–	0.61 Q	–	–	–	–	–	–	–
	B73-MR1	–	0.68 Q	–	0.76 Q	–	–	0.57 Q	–	–	–	–
IC_{max} (μM)	B73	–	–	–	–	–	–	–	–	–	–	–
	B73-MR1	–	–	–	0.58 Q	–	–	–	–	–	–	–
IC_F	B73	–	–	–	–	0.55 Q	–	0.73 Q	–	–	–	0.58 Q
	B73-MR1	–	–	–	–	–	–	–	–	–	–	–
A_P (μM^2)	B73	–	–	–	–	–	–	–	–	–	0.51 Q	–
	B73-MR1	–	0.71 Q	–	0.81 Q	–	–	0.58 Q	–	–	–	0.50 Q

Table S3.5 Summary of the P-values from the Newman-Keuls multiple comparison test on % growth inhibition of *L. monocytogenes* B73 at 25 μ M for Group 1 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value >0.05

	c-b3hW	c-WWW	c-WIW	c-1MeW	c-5MeW	c-Bal
c-WFW	0.001	0.001	0.001	0.05	0.001	0.001
c-b3hW		0.001	ns	0.001	0.001	0.001
c-WWW			0.001	ns	0.01	0.001
c-WIW				0.001	0.001	0.001
c-1MeW					0.01	0.001
c-5MeW						0.001

Table S3.6 Summary of the P-values from the Newman-Keuls multiple comparison test on listerial % growth inhibition of *L. monocytogenes* B73 at 25 μ M for Group 2 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value >0.05

	c-WFW	c-KRK	c-KW	c-KWW
c-WWW	0.001	0.001	0.001	0.001
c-WFW		0.01	0.001	0.001
c-KRK			0.01	0.001
c-KW				0.01

Table S3.7 Summary of the P-values from the Newman-Keuls multiple comparison test on listerial % growth inhibition of *L. monocytogenes* B73 at 25 μ M for Group 3 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value >0.05

	c-WFW8	c-WFW10	c-WFW12
c-WFW	0.001	0.001	0.001
c-WFW8		0.001	0.001
c-WFW10			0.001

Table S3.8 Summary of selected physicochemical parameters of the RW-peptides investigated for antilisterial activity. A number of parameters were taken from literature; HPLC retention times are from the analytical HPLC; M_r are from ESMS; SASA, SAV and molecular volume were determined *in silico* using YASARA 9.10.5[©].

Peptide	HPLC R_t (mins)	π (Lipophilicity) (π_{FP}) ^{55*}	Q/L ⁵⁵	ω (Hydrophathy) ⁵⁷	ω (Hydrophobicity) ⁵⁸	ω (Interphase properties) ⁵⁹	ω (Side-chain surface area) * (SCSA, Å ²) ¹¹⁶	ω (Molecular volume) * (MV, Å ³) ⁶⁰	YASARA Molecular volume (Å ³)	M_r (g/mol)	Solvent accessible surface area (SASA, Å ²)	Solvent accessible volume (SAV, Å ³)
c-WKW	15.2	3.78	1.26	-14.4	-11.07	-1.16	635.4	1189.2	822.99	943.8	1052.17	2136.62
c-WRW	17	3.72	1.24	-16.2	-13.35	2.12	482.4	1203.6	859.95	1027.2	996.48	2151.07
c-KW	17.1	3.32	1.11	-10.7	-8.05	-0.68	606.5	1151.3	794.26	904.1	1003.32	2042.6
c-WFW	19.9	3.26	1.09	-12.5	-10.33	2.60	453.5	1165.7	792.17	989.5	1061.89	2171.2
c-KWW	20	3.78	1.26	-14.4	-11.07	-1.16	635.4	1189.2	822.99	943.2	1056.26	2141.78
c-b3hW	20.1	4.22	0.71	-13.3	-15.61	2.90	491.1	1219.1	867.44	1015.6	1070.73	2200.9
c-WWW	20.2	3.72	1.24	-16.2	-13.35	2.12	482.4	1203.6	859.95	1027.2	1041.12	2169.22
c-KRK	20.3	3.3	1.1	-11.3	-8.81	0.41	555.5	1156.1	806.58	931.5	1077.16	2124.48
c-WIW	20.3	na	na	na	na	na	na	na	864.31	954.5	1078.11	2190.96
c-1MeW	20.4	4.22	0.71	-13.3	-15.61	2.90	491.1	1219.1	867.88	1015.6	1061.98	2195.09
c-5MeW	20.4	4.22	0.71	-13.3	-15.61	2.90	491.1	1219.1	874.5	1015.6	1060.17	2201.67
c-WFW8	21.3	4.04	1.01	-14.2	-11.76	3.78	585.4	1529	1089.14	1291.5	1327.34	2776.98
c-Bal	22	na	na	na	na	na	na	na	848.16	1021.5	1044.86	2178.58
c-WFW10	22.4	5.28	1.06	-19.6	-16.21	4.49	746.2	1930.2	1375.79	1633.9	1593.75	3478.08
c-WFW12	23.4	6.06	1.01	-21.3	-17.64	5.67	878.1	2293.5	1633.71	1937.3	1782.6	4027.57

* Missing amino acid parameters (P) were calculated as follows:

$$P_{b3hW} = P_{1MeW} = P_{5MeW} = P_{Trp} + (P_{Ala} - P_{Gly})$$

na: not available

Chapter 4

Activity and salt-tolerance of tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*, and their analogues towards *Listeria monocytogenes* as target

4.1. Introduction

The cyclic amphipathic decapeptides, tyrocidines (Trcs), are antimicrobial peptides (AMPs) produced by *Bacillus aneurinolyticus* as part of the tyrothricin complex¹. The major Trcs include Trc A/A₁, B/B₁, and C/C₁. In the analogous tryptocidines (Tpc) and phenycidines (Phc) (named by our group) the invariable aromatic amino acid Tyr is substituted with Trp and Phe respectively. They are effective against Gram-positive bacteria *in vitro*¹⁻⁴ and in the past have been limited to topical treatment of infections by these bacteria due to their systemic toxicity⁵. Notably, the six major tyrocidines (A, A₁, B, B₁, C, C₁) showed significant activity against the leucocin A resistant (B73-MR1) and sensitive strains (B73) of the Gram-positive bacteria *L. monocytogenes* (Spathelf & Rautenbach⁴ and Chapter 3 of this study). This pathogen is the causative agent of listeriosis which is a food-borne opportunistic infection. It is fatal in 20 to 40% of cases in immunocompromised subjects, infants, pregnant women, and senior citizens⁶⁻⁹. It is thus a public health and food safety threat¹⁰.

Listeria spp. are known to be resistant to antibiotics^{6-8,11-13}, disinfectants¹⁴⁻¹⁶ and even to nisin which is the most widely used bacteriocin in food preservation against listerial growth¹⁷⁻²⁰. Trcs are therefore, being investigated for the development of potential bio-preservatives against *Listeria* since microorganisms may not easily develop resistance to their rapid and principal membranolytic mode of action (MOA)^{21,22}. However, this pathogen is very resilient having the ability to grow at temperatures ranging from 1 ° to 45 °C, has high tolerance for salts including NaCl and the ability to grow at quite low pH, hence difficult to control during both food processing and food preservation²³⁻²⁷. Di- and trivalent cations have been reported to affect the activity of antimicrobial compounds such as was observed for the nisin-resistant strain of *L. monocytogenes* Nis^f where divalent cations (Ca²⁺, Mg²⁺, Mn²⁺ and Ba²⁺) increased resistance to nisin, but had no effect on the wild type strain²⁸. Abee *et al.*²⁹ observed a decrease in the rate of K⁺ efflux caused by nisin Z from whole cells of *L. monocytogenes* Scott A in the presence of divalent cations (Mg²⁺, Ca²⁺) and the trivalent cation, Gd³⁺ with the lanthanide gadolinium

(Gd³⁺) completely inhibiting the effect at 0.2 mM. In contrast, an increase in activity was reported for daptomycin, a membranolytic cyclic lipopeptide, in the presence of Ca²⁺ ³⁰. The probable reasons for interference of antimicrobial peptide activity by cations is either because they induce increased peptide aggregation ^{26,31,32} thus decreasing the “active” peptide concentration ²⁷ or shield electrostatic interaction between the peptide and the target membrane ^{31,33–35}. It is possible that neutralisation of the phospholipids’ negative head group charges by cations leads to a more rigid membrane by inducing phospholipid condensation which would affect membrane insertion by AMPs ²⁹. The use of single or combined AMPs together with salts or salt-sequestering agents like EDTA can improve control of *Listeria* and inhibit the induction of bacteriocin resistant strains and species ²⁶.

Marques *et al.* ³ observed that increasing the concentration of Ca from 25 mg/L to 50 mg/L moderately antagonised the antibacterial activity for Trc A and analogues. Previous work by Spathelf ³⁵ showed that divalent cations especially Ca²⁺ had an influence on the self-assembly and mode of action (MOA) of the Trcs changing it from a lytic to non-lytic MOA. Spathelf ³⁵ found the optimal concentration for the apparent synergistic effect of CaCl₂ on Trc activity to be 7.5 mM. Higher concentrations up to 15 mM was also beneficial, but led to precipitation of some of the media constituents and at 30 mM there was loss of the synergistic activity. Further investigation of the influence of biological salts (MgCl₂, CaCl₂, KCl and NaCl) on the activity of Trcs is needed using diverse assays and will be discussed in this chapter.

In Chapter 3, we deduced that the identity of the aromatic amino acid at position 7 and of the aromatic dipeptide unit in the variable pentapeptide ArArNQAr unit (Ar = aromatic amino acid) as well as the presence of a trimethylated Orn in place of Orn as cationic residue in the conserved V+LFP pentapeptide unit (+ = cationic residue) are critical residues within the Trc structure for antilisterial activity. To verify if the mechanism by which cations influence Trc activity is related to interaction with the residues relevant for their activity which may cause the modification of the MOA previously observed, we evaluated the most active of Trc A analogues namely Trc A, sTrc AOMe₃, Phc A and Trc C analogues (Trc C, Trc B, Tpc C). The sequences, activity and biophysical characteristics of these peptides are detailed in Chapter 3. We also included the two most active RW-peptides (c-WFW and c-WWW) which are cyclic analogues of the synthetic hexapeptide (Ac)-RRWWRF-NH₂ (Ac-RW), sharing the predominant aromatic residue and cationic character with the Trcs (refer to Chapter 3). Previous studies by Junkes *et al.* ³⁶ showed that c-WFW and c-WWW have a primarily non-lytic MOA. The effect of the salts on these RW-peptides will allow us to determine if the synergistic effect of CaCl₂ on Trcs is a

peptide-specific phenomenon or target dependant. Furthermore, we shall analyse the role played by interactions with the lipid matrix of Gram-positive bacterial membranes in the biological effect of divalent cations on peptide activity by using model lipid vesicles.

4.2. Materials

Tyrothricin (extract from *Bacillus aneurinolyticus*), gramicidin S (from *Brevibacillus brevis* (Nagano)), Corning Incorporated[®] cell culture cluster non-pyrogenic polypropylene microtiter plates, propidium iodide, bis-benzimide, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, ethylenediaminetetraacetic acid tetrasodium salt dihydrate (Na_4EDTA or EDTA) and trifluoroacetic acid (TFA, >98%) were obtained from Sigma (St. Louis, USA). Culture dishes were obtained from Lasec (Cape Town, South Africa) and microtiter plates (Nunc[™]-Immuno Maxisorp) were from AEC Amersham (Johannesburg, South Africa). Saarchem (Krugersdorp, South Africa) supplied the NaCl, diethyl ether, acetone, butan-1-ol, acetic acid and propan-2-ol. Brain heart infusion broth (BHI) and BHI agar were supplied by Biolab Diagnostics (Midrand, South Africa). Capital Enterprises (Hillcrest, South Africa) provided the KCl. To obtain analytical grade water, water was filtered from a reverse osmosis plant via a Millipore Milli-Q water purification system (Milford, USA). Promega (Madison, USA) supplied the Cell Titer-Blue[™] Cell Viability Assay kit. Ethanol (>99.8%), Tris and CaCl_2 was supplied by Merck (Darmstadt, Germany). Falcon[®] tubes were from Becton Dickson Labware (Lincoln Park, USA). Dr Margitta Dathe from Leibniz Institute of Molecular Pharmacology (FMP), Berlin, Germany supplied the two pure RW-peptides, c-WFW and c-WRW. Liposome extrusion filters (polycarbonate membrane filters, 100 nm pore size) were from Avestin, Switzerland. Chloroform (99.8% A.C.S. spectrophotometric grade) was from Sigma-Aldrich (Germany). The other lipids were procured from Avanti Polar Lipids, Inc. (Alabaster, AL).

4.3. Methods

4.3.1. Bacteria culturing

Normal sterile techniques were used to culture the leucocin A sensitive wild-type meat-isolate (B73) *Listeria monocytogenes* strain³⁷ as described in Chapter 3. Following 24 hour culturing at 37 °C on BHI agar from freezer stocks selected colonies were grown overnight at 37 °C in BHI broth (37 g prepared with 1 L water) or BHI broth supplemented with 7.5 mM CaCl_2 to log phase. Subsequently the cultures were sub-cultured in the culture medium to mid-log phase (OD_{620} of 0.4) prior to use in assays.

4.3.2. Peptide preparation

Selected tyrocidine A analogues (Trc A, sTrc AOMe₃, and Phc A), C analogues (Trc B, Trc C, Tpc C), RW-peptides (c-WWW and c-WFW), as well as Trc mixture and GS were used. The natural Trcs were extracted from commercially available tyrothricin as well as from tyrothricin isolated from culture extracts of *Bacillus aneurinolyticus* ATCC 8185 strain as described in Chapter 2. Purification of the Trcs was done using semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) and analytical HPLC according to the methods of Rautenbach *et al.*³⁸ and Eyéghé-Bickong³⁹. Chemical purity and integrity was established using previously described ESMS and UPLC-MS methods^{4,38-40}.

Antilisterial activity of these peptides for IC₅₀ and IC_{max} determination is described in Chapter 3. The peptide stocks were prepared as previously described to 1 mM with 40% v/v ethanol in deionised sterile H₂O for the Trcs and with deionised sterile H₂O for the RW-peptides (to 2 mM) and GS (to 1 mM). The stock was further diluted to the appropriate concentrations relative to the IC₅₀ and IC_{max} separately with each of the salt solutions (NaCl, KCl, CaCl₂, and MgCl₂). Deionised sterile H₂O was used as control solvent. All solvents were previously filtered using 0.22 µm pore size filters.

4.3.3. Growth and metabolism inhibition assays

Growth and metabolism inhibition assays were carried out against the two bacterial targets according to previously described methods³⁵ with modifications. The mid-log phase cultures were diluted with growth media to OD₆₂₀ = 0.2. Ten µL of the peptide solutions was added to 90 µL of the cell suspensions in 96-well microtiter plates and incubated for 16 hours at 37 °C. The final concentration of the salts in the cell suspensions was 7.5 mM and 15 mM. After reading the light dispersion of the wells at 595 nm, 10 µL of CellTiter-BlueTM reagent was added to all wells and incubated for an additional hour. Resazurin (blue, absorption maximum at 605 nm) in the CellTiter-BlueTM is reduced to resofurin (pink, absorption maximum at 573 nm) in viable and respiring cells⁴¹. The plates were analysed spectrophotometrically at 570 nm and 600 nm using a Biorad microtiter plate reader and the percentage metabolism inhibition was computed using equation 2. The growth inhibition was computed as previously described in Chapter 3 using equation 1.

$$\% \text{ growth inhibition} = 100 - \frac{100 \times (A_{595} \text{ of well} - \text{Average } A_{595} \text{ of background})}{\text{Average } A_{595} \text{ of growth wells} - \text{Average } A_{595} \text{ of background}} \quad (1)$$

$$\% \text{ inhibition} = 100 - \frac{100 \times (A_{570}/A_{600} \text{ of well} - \text{Average } A_{570}/A_{600} \text{ of background})}{\text{Average } A_{570}/A_{600} \text{ of growth wells} - \text{Average } A_{570}/A_{600} \text{ of background}} \quad (2)$$

In another set of experiments, spectrofluorimetry was used to observe the effect of the divalent cation salt CaCl₂ on the metabolism inhibition pattern of Trc B and GS against *L. monocytogenes* B73 using the CellTiter-Blue™ reagent. The peptide and salt solutions were prepared as above to peptide concentrations representing < IC₅₀, IC₅₀ and IC_{max}. Peptide solutions prepared in deionised sterile water were used for control. The bacterial cultures were prepared as described above. After adding 10 µL of the peptide solutions to 90 µL of the cell suspensions in 96-well microtiter plates, 10 µL of CellTiter-Blue™ reagent was added to each well and the plates were immediately assessed for fluorescence (560 nm excitation and 590 nm emission) every 2 minutes over 30 minutes using a Varioskan™ spectrofluorimeter and SkanIt® software 2.4.1 for retrieving the data (ThermoElectron Corporation, Vantaa, Finland).

4.3.4. Membrane permeabilisation/lysis assays

The effect of the salts at 7.5 mM on the membrane activity towards *L. monocytogenes* B73 of the peptides was investigated using the membrane impermeable DNA chelator, propidium iodide. Following the sub-culturing of the bacterial cultures to mid-log phase, the cultures were separated from the culture medium by centrifugation at 200×g for 10 minutes, the supernatant was discarded followed by a wash in sterile NaCl (0.9% *m/v*) again by centrifugation at 200×g for 10 minutes. The cells were diluted to an OD₆₂₀ of 0.20 with sterile NaCl (0.9% *m/v*). Propidium iodide (1 mg/mL) was added to the cell suspensions in a 1:100 ratio. Ten µL of peptide solutions (final concentration = IC₅₀ and 2 × IC₅₀) as prepared above was added to 90 µL of the stained cells distributed in black 96-well sterile flat bottom LumiNunc/FluoroNunc microplates for spectrofluorimetry. The reaction mixtures were incubated at room temperature for ±5 minutes and assessed for propidium iodide fluorescence emission at 617 nm (excitation wavelength was set at 535 nm) every 2 mins for 30 mins using the Varioskan™ spectrofluorimeter and SkanIt® software 2.4.1 for retrieving the data. For positive control, some cells were lysed by freeze-thawing before staining with propidium iodide for use in assay. The average propidium iodide fluorescence was calculated over the 30 minutes for triplicate repeats

in the presence of the peptide dissolved in water or 7.5 mM chloride salt and 6 repeats in the absence of peptide.

4.3.5. Influence of EDTA on Trc activity

In order to further verify the effect of divalent metal ion on the activity and mode of antilisterial activity of the peptides, EDTA (ethylenediamine tetraacetic acid) tetrasodium salt dihydrate was used to sequester Ca^{2+} and Mg^{2+} ions at 7.5 mM.

In initial experiments to determine the minimum concentration of EDTA that affects Trc activity, peptide suspensions were made from diluting the Trc mixture (final concentration = 12.5 $\mu\text{g}/\text{mL}$) with the divalent cation salt solutions, deionised sterile H_2O or BHI broth. GS (final concentration of 10 μM) was used as reference peptide. EDTA was added to the peptide in salt or water solutions at final concentrations of 10, 5 and 2.5 mM and at 10, 5 and 2.5 μM to the peptide solutions in BHI broth. NaCl at concentrations four times higher than those of EDTA (40, 20 and 10 mM or μM) was used as control for the effect of the salt fraction of the EDTA formulation used. Ten μL of these solutions was separately added to 90 μL of the bacterial cultures prepared as described above for the previously described growth and metabolism inhibition assays and also to 90 μL of the bacterial cultures prepared as described above for the membrane permeabilisation assays. Following the results from these assays, the experiments in *Section 4.3.4* were repeated with the addition of 10 mM EDTA to the peptide suspensions with the divalent metal ion salts to verify that the observed effect on the membrane permeabilisation activity of the peptides was indeed due to the divalent metal cations.

In order to determine the nature of Trc-EDTA interaction in the presence and absence of divalent cation salts (CaCl_2 and MgCl_2), IC_{50} values were derived from dose-response curves and used in fixed ratios for fractional inhibition concentration (FIC) determination according to an adaptation of the methods by Chawira and Warhust⁴² and Fivelman *et al.*⁴³.

Twenty mM EDTA and 50 μM (0.05 mM) peptide were used to prepare the following concentration combination ratios: 20:0, 15:0.01, 10:0.03, 5:0.04, and 0:0.05 mM in solutions of CaCl_2 , MgCl_2 , or water which were then individually serially diluted in the respective solvents. Ten μL of the peptide preparations was added to 90 μL of the cell suspensions, incubated for 16 hours at 37 °C, and analyzed for growth and metabolism inhibition as previously described. The IC_{50} values and standard error of the mean for the various EDTA-peptide combinations in each solvent were determined from the dose-response curves plotted and analyzed using GraphPad Prism[®] 4.03 (GraphPad Software, San Diego, USA). A minimum of three technical repeats and

two biological repeats were carried out for each combination experiment. Two fractional inhibition concentration (FIC) values were calculated for each of the five EDTA-peptide combination ratios, one for EDTA and the other for the peptide according to equations 2 and 3 below adapted from Bell ⁴⁴ and Makowa ⁴⁵.

$$FIC_{EDTA} = IC_{50}^{EDTA + Peptide} \text{ (in combination)} / IC_{50}^{EDTA} \text{ (alone)} \dots\dots\dots(2)$$

$$FIC_{peptide} = IC_{50}^{EDTA + Peptide} \text{ (in combination)} / IC_{50}^{peptide} \text{ (alone)} \dots\dots\dots(3)$$

The FIC values were used to construct isobolograms on Graphpad Prism[®] 4.03 and to compute the fractional inhibition concentration index (FICI) which is the sum of FICs of EDTA and each peptide using equation 4 below ^{45,46}.

$$FICI = FIC_{EDTA} + FIC_{peptide} \dots\dots\dots(4)$$

The magnitude of the FICI determined the nature of the EDTA-peptide interaction as being either synergistic (FICI < 1.0), antagonistic (FICI > 1.0) or additive (FICI = 1.0) ^{44,46,47}. However, a more conservative interpretation requires that FICI ≤ 0.5 indicates absolute synergy, 1 > FICI > 0.5 shows slight synergy, FICI = 1 means additive activity, 1 < FICI < 4 is interpreted as non-interactive to slight/moderate antagonism, while FICI ≥ 4 indicates absolute antagonism ^{44-46,48}. The shape of the isobolograms also provided an indication of the nature of the interactive effect of EDTA and the peptides with a concave curve for synergy, a linear line for an additive to non-interactive effect or convex curve for antagonism with deviation of the curves from the additivity line indicating the strength of the interactive effect ⁴⁴.

4.3.6. Analyses of data

GraphPad Prism[®] 4.03 (GraphPad Software, San Diego, USA) was used to plot the dose-response curves as described in Chapter 3. IC_{max}, related to the maximum inhibitory concentration (MIC) was computed from the x-values at the intercept between the slope and the top plateau ⁴⁹. The log IC₅₀ is the x-value that denotes the response halfway between the top and bottom where IC₅₀ is defined as the peptide concentration leading to 50% growth inhibition ⁵⁰. For fixed concentration experiments the mean and standard error of the percentage inhibition, percentage haemolysis or fluorescence signals were computed using GraphPad Prism[®] 4.03 (GraphPad Software, San Diego, USA) and statistical analyses of the data was performed with the same software.

4.3.7. *Fluorescence microscopy*

Prior to cell imaging experiments, the listerial cultures at mid-log phase (OD = 0.4) were washed and diluted to OD = 0.2 with 0.9% *m/v* NaCl. Bis-benzimide (Hoechst) which is a blue, cell-permeable, DNA-binding fluorescent dye and propidium iodide (PI), a red DNA intercalating dye that is membrane impermeable were used to assess the membranolytic activity of the Trcs towards *L. monocytogenes* B73. The dyes were prepared in phosphate bovine saline (PBS) and added to the cell suspensions in 0.9% *m/v* NaCl (to final concentration of 20 µg/mL for bis-benzimide and 10 µg/mL for PI) followed by 15 minutes incubation in the dark at room temperature. One mM stock of Trc B prepared as described in *Section 4.3.2* was diluted in CaCl₂ or MgCl₂ solutions and 10 µL of the peptide suspension was added to 90 µL of stained cells (final salt concentration = 7.5 mM and final peptide concentration = 5 µM). Water was used as control solvent. Aliquots of stained cultures were mounted under cover slips on microscope slides and the images were acquired using an Olympus UPlanApo N 100×/1.40 Oil ∞/0.17/FN26.5 objective and Cell^R imaging software attached to an Olympus IX81 inverted fluorescent microscope. Excitation was at 360 nm and 572 nm for the blue and red staining respectively employing a Xenon-Arc burner (Olympus Biosystems GMBH) as light source. The emitted light was collected using a UBG triple-bandpass emission filter cube (Chroma) for capture by an F-view-II cooled CCD camera (Soft Imaging systems).

4.3.8. *Vesicle interaction studies*

Dynamic light scattering measurements (photon correlation spectroscopy) were done with the aim of investigating the effect of peptides and/or CaCl₂ on the size of lipid vesicles. The required combinations and molar lipid ratios at final concentrations between 5 and 20 mM (POPG:POPE, 3:1) were produced from stock solutions of POPG (1-palmitoyl-2-oleoylphosphatidyl-*sn*-glycerol) and POPE (1-palmitoyl-2-oleoylphosphatidyl-ethanolamine), with chloroform as solvent. The lipid films were subsequently dried under nitrogen (using vacuum drier alpha 2-4 LD plus Christ[®]) and suspended in the appropriate buffer by vortexing. The large unilamellar vesicles (LUVs) (prepared in buffer containing 10 mM Tris, 154 mM NaCl at pH 7.4) were produced by 35 times extrusion through two stacked 100 nm filters in a mini extruder (Avestin, Switzerland). The existence of a main population of vesicles (more than 95% mass content) with a mean diameter of 100 nm (polydispersity index 0.3) in 1 mM and 10 µM of LUVs was confirmed by dynamic light scattering measurements (N4 Plus, Coulter Corp.) prior to assays ^{36,51,52}.

Analytical stock solutions of peptides (1.00 mM) were prepared in appropriate solvent (40% v/v ethanol for Trc B and analytical quality water for other peptides) and diluted to desired concentrations using either buffer 1 (10 mM Tris, 154 mM NaCl, pH 7.4) or buffer 2 (10 mM Tris, 154 mM NaCl, 7.5 mM CaCl₂, pH 7.4). Samples for light scattering experiments were prepared by mixing of appropriate amounts of the vesicle suspensions (stock concentration 10 mM) and peptide solutions (stock concentration 250 μM). The least final peptide concentration in the 500 μL samples was 6.25 μM for Trc B, 4 μM for GS and 5 μM for c-WFW. The molar lipid to peptide ratio varied depending upon the peptide and lipid system. The samples were incubated for 10 mins prior to reading. A volume of 40 μL of each sample was transferred to a sterile cuvette for reading (for each sample 5 readings were recorded per measurement and 5 measurements per sample; each sample prepared in duplicate). The autocorrelation functions were measured, averaged and analyzed using a Zeta Sizer. The normalized unweighted particle size distributions, which correlate with the respective scattering intensity, were determined.

4.3.9. Fluorescence spectroscopy

The LUVs with or without the above peptide preparations were also assessed spectrofluorimetrically by determining the tryptophan fluorescence spectra. Measurements were made on a Perkin-Elmer LS 50B spectrofluorometer at 23 °C. Upon excitation at 280 nm, recording of the emission spectra was done between 300-500 nm. The emission was detected using a 290 nm red edge filter.

4.3.10. Light scattering assays

Light scattering of Trc B and GS in solution with or without divalent cation salts was measured to determine the size of peptide aggregates. 1 mM peptide stock was prepared with 50% acetonitrile for Trc B and water for GS, allowed to stand for 10 minutes in a warm water bath followed by sonication to obtain a clear solution. The solution was then diluted to 50 μM either with 7.5 mM CaCl₂, 7.5 mM MgCl₂ or analytical grade water in one or two steps. For the one step dilution, 5 μL of peptide stock was added to 95 μL of solvent, while in the 2 step dilution, 5 μL of peptide stock was added to 20 μL of solvent and then a further 75 μL of solvent was added. 40 μL of each sample was then read as described above for LUVs (5 readings made per measurement and 3 measurements per sample, 2 samples for each preparation).

4.4. Results and Discussion

Results from Chapter 3 agreed with previous studies by Spathelf and Rautenbach⁴ and showed the potency of the major tyrocidines (Trcs) from the natural tyrothricin complex (tyrocidines A, B, C, tryptocidine C and phenycidine A) against the leucocin A sensitive and resistant strains of *L. monocytogenes* B73 and B73-MR1 respectively. In addition, the synthetic Trc A analogue with a trimethylated Orn revealed activity comparable to the natural analogues. The IC₅₀ values ranged from 5-18 µM at high bacterial counts of $\sim 7 \times 10^8$ CFU/mL. In view of the potential use of Trcs as bio-preservatives against *Listeria* which is highly salt tolerant and due to the reported influence of salts on antimicrobial activity of AMPs, in this study we evaluated the influence of biological salts on the activity and biophysical properties of the most active Trc A (Trc A, sTrc AOMe₃, and Phc A) and Trc C (Trc B, Trc C, Tpc C) analogues as well as the Trc mixture isolated from commercially available tyrothricin complex. Gramicidin S (GS) was employed as a reference peptide. The most active RW-peptides c-WFW and c-WWW were also evaluated for comparison.

4.4.1. Influence of metal cations on antilisterial activity of the cyclic peptides

Testing the activity of the Trcs and RW-peptides at fixed concentrations of 10 µM and 40 µM respectively we observed that their growth and metabolism inhibitory activities against *L. monocytogenes* B73 were generally salt tolerant when the peptides were pre-incubated in 75 mM and 150 mM of chloride salts. Notably, the divalent cation salts (CaCl₂ and MgCl₂) at final concentration of 7.5 mM in the assay led to significantly increased growth inhibitory activity (defined as decrease in optical scattering of light at 595 nm) of Trc B and Trc C, (Fig. 4.1A). The sequence of growth inhibitory activity of Trc B and Trc C in the presence of chloride salt at 7.5 mM final concentration was Ca²⁺~Mg²⁺ > K⁺~Na⁺ in terms of metal cation. The activity, as determined at 595 nm, of 40 µM, c-WWW [*cyclo*(RRRW⁺WW)] was generally salt tolerant (Fig. 4.1B) while c-WFW [*cyclo*(RRRW⁺FW)] was sensitive to Na⁺, K⁺ and Ca²⁺ chloride salts (decrease in growth inhibitory activity) while Mg²⁺ led to an increase in activity (Fig. 4.1B). This could indicate that the aromatic residue Phe plays a unique role in modulating the influence of salts on antilisterial growth inhibition. The activity trend in terms of influence of solvent on c-WFW was: Mg²⁺ > K⁺ ~ Na⁺ > Ca²⁺.

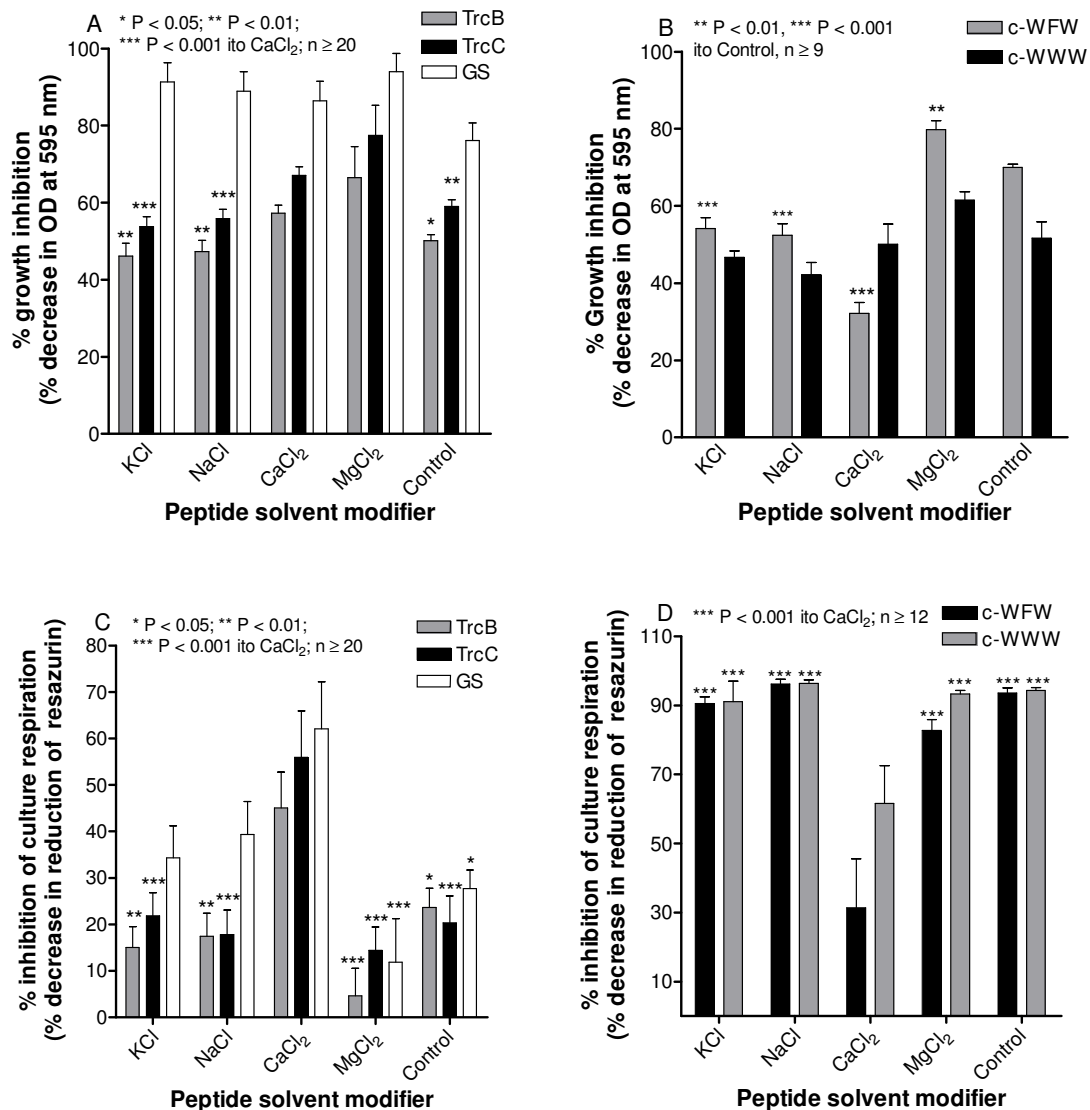


Figure 4.1 Comparison of the growth inhibitory activity of **A.** 10 μ M tyrocidine B, tyrocidine C and gramicidin S and **B.** 40 μ M c-WWW and c-WFW towards *Listeria monocytogenes* B73 in the presence of 7.5 mM chloride salts as measured by optical scattering of light at 595 nm. Comparison of anti-metabolic activity by **C.** 10 μ M tyrocidine B, tyrocidine C and gramicidin S and **D.** 40 μ M c-WWW and c-WFW towards *Listeria monocytogenes* B73 in the presence of 7.5 mM chloride salts as measured by decrease in resazurin reduction. Statistical analyses were done with two-way ANOVA using Bonferroni's post test.

The analysis of growth using optical methods is error prone if cells tend to clump or if biofilms form, so we reassessed the listerial growth and survival using the CellTiter-Blue™ assay which indicated metabolic activity. Antilisterial activity of the same preparations of Trcs in terms of the metabolism inhibitory activity as defined by decreased conversion of resazurin (blue) in the CellTiter-Blue™ reagent to resofurin (pink) by cells gave a very different result in that this sensitive method rather indicated that the Trcs were highly salt tolerant (Fig. 4.1C). The

influence of the cations also showed a different trend, namely $\text{Ca}^{2+} \gg \text{K}^+ \sim \text{Na}^+ > \text{Mg}^{2+}$. Thus, pre-incubation in MgCl_2 resulted in lower activity than expected while CaCl_2 significantly improved the metabolism inhibitory activity of the Trcs (Fig. 4.1C). Although we observed precipitation of components of the media and cell clumping at 15 mM final salt concentration, the high anti-metabolic activity by the Trcs in CaCl_2 solution was maintained at this concentration (results not shown). A significant increase in anti-metabolic activity in the presence of CaCl_2 above that in the control and MgCl_2 was also observed for GS, but this activity was not statistically different from that in the presence of Na^+ and K^+ (Fig. 4.1C).

In terms of the anti-metabolic activity, CaCl_2 was the only salt to significantly affect the activity of the RW-peptides. However, it led to a decrease in the activity of both c-WFW and c-WWW (Fig. 4.1D) which demonstrates that the calcium induced increase in anti-metabolic activity is unique to the peptides with the pentapeptide moiety VOKLFP (Trcs and GS).

Spathelf³⁵ also found, after testing preparations of Trcs over a range of CaCl_2 concentrations, that the increase in the antilisterial activity of Trcs provoked by calcium was optimal at 7.5 mM final concentration which is significantly high for *in vivo* systems. It is possible that this high calcium tolerance is related to the fact that the Trc producer *Bacillus aneurinolyticus*, may have evolved in soil where calcium content is 1-200 g/kg⁵³. Moreover, Welshimer⁵⁴ showed the survival of *L. monocytogenes* in soil for long periods. Therefore, Ca^{2+} might specifically modulate the antilisterial activity of Trcs. Inhibition of bacterial respiration (O_2 consumption) with an associated decrease in the redox status of the bacterial cells by Trcs was shown in early research by Dubos *et al.*¹. Dubos in another study proposed that Trcs caused cell death through the inactivation of bacterial glucose dehydrogenase⁵⁵. Therefore, it is possible that Ca^{2+} boosts the Trc anti-metabolic mode of action (MOA), contrary to Mg^{2+} which either antagonises Trc activity or enhances another MOA.

Over the 16 hour assay, the presence of CaCl_2 led to significantly higher inhibition of cell respiration by all the Trcs including the analogues Phc A and sTrc AOMe₃ compared to MgCl_2 and water (Fig. 4.2). However, Tpc C was not influenced at 15 μM , which may indicate that a small aromatic residue (Phe or Tyr) in position 7 is crucial for the calcium dependent increase in activity. This residue was previously shown to be specifically relevant to the MOA for antilisterial activity of Trcs in Chapter 3. Although calcium induced an opposite effect on the anti-metabolic activity of both RW-peptides, the relevance of the small aromatic residue Phe was

observed from the fact that c-WFW was more susceptible to the influence of CaCl_2 than c-WWW (Fig. 4.1B and 4.2).

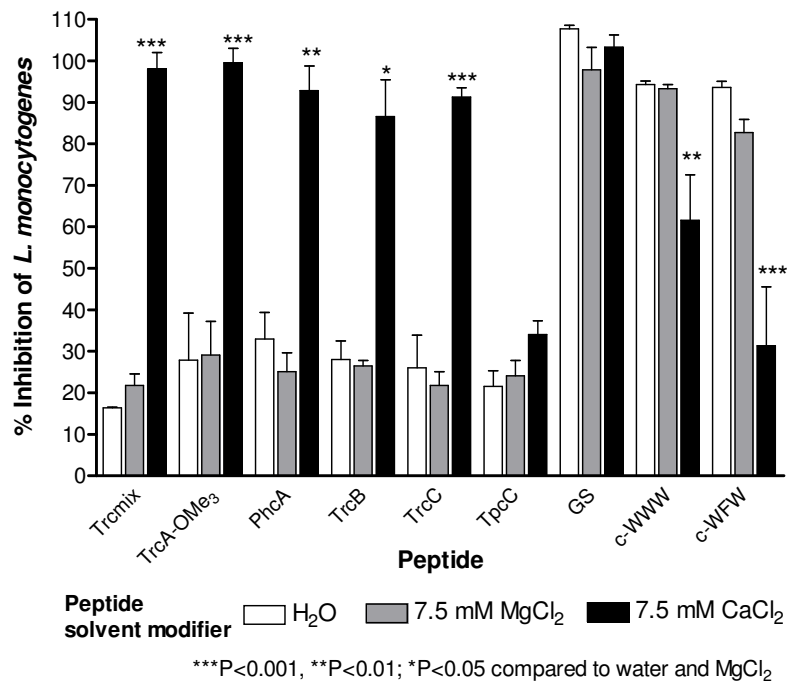


Figure 4.2 Comparison of the influence of 7.5 mM CaCl_2 or MgCl_2 on antilisterial activity by 12.5 $\mu\text{g}/\text{mL}$ of the tyrocidine complex, 10 μM of the different tyrocidine analogues and GS, and 40 μM of c-WWW and c-WFW. The percentage inhibition was determined after 17 hours using the resazurin reduction (CellTiter-Blue™ assay) to determine changes in redox potential and thus antilisterial activity. Means of 3-12 measurements in the presence of peptide \pm SEM are depicted. Statistical analysis was done with using Newman-Keuls multiple comparison test.

The influence of Ca^{2+} on activity of the lytic peptide GS and Trc B toward *Listeria* metabolism was monitored in kinetic assays over 30 minutes (Fig 4.3A and 4.3B). We observed that from 0-2 minutes, after peptide addition the resazurin fluorescence showed a sharp increase in the presence of CaCl_2 indicating increased metabolic activity (Fig. 4.3A and 4.3B). This increase was also found for Trc B alone, but not GS alone. The increase indicated that the peptides may have triggered a metabolic response, possibly an osmotic stress response. However, a sharp decrease in resazurin fluorescence after 2 minutes was observed for Trc B in the presence of Ca^{2+} , with the decrease leveling off between 88% and 67% in the presence and absence of CaCl_2 respectively indicating cell death or decrease in metabolism. For GS, there was an initial decrease after 2 minutes and a slow increase in metabolic activity close to the basal level. These

results indicated that there is a difference in the action of the lytic GS and Trc B on the cells if the metabolic response is considered.

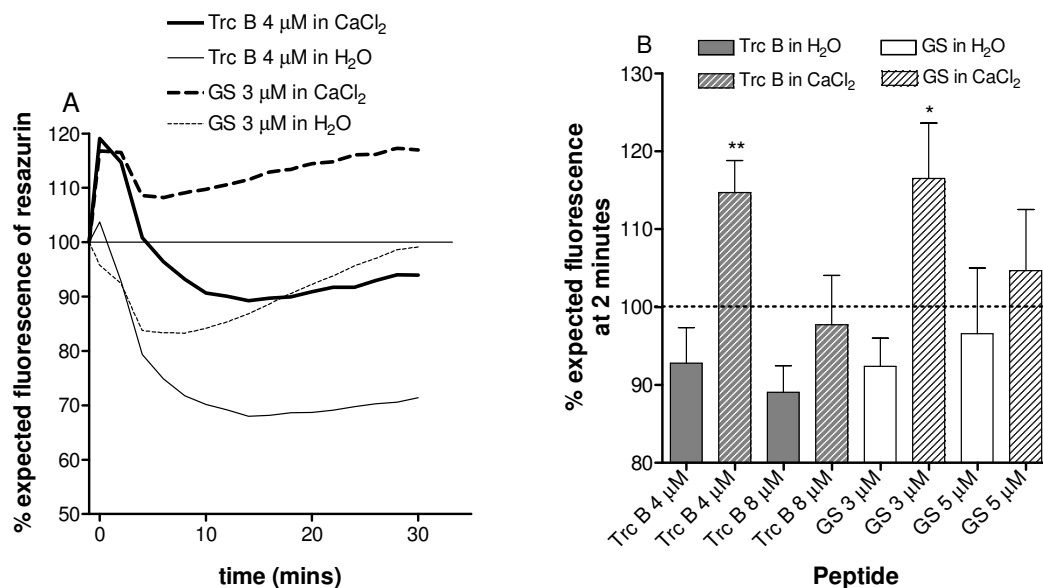


Figure 4.3 **A.** Kinetics of the influence of 4 μM Trc B and 3 μM GS in the presence of 7.5 mM CaCl₂ on the formation of resazurin as an indicator of energy metabolism over 30 minutes **B.** Comparison of the influence of 7.5 mM CaCl₂ on the fluorescence of resazurin after 2 minutes following treatment of cultures with varying concentrations (representing IC_{50} and 2 on 4 μM Trc B and 3 μM GS was significant (*

4.4.2. Influence of metal cations on membrane activity of the cyclic peptides

Given that the Trcs unlike the RW-peptides are known to have membrane permeabilising activity, it was necessary to ascertain the influence of the salts on this aspect of Trc antilisterial activity.

The membrane activity (permeability increase/lytic activity) of the Trc complex and GS as determined by propidium iodide leakage into the cells was not influenced by NaCl and KCl while, following pre-incubation in either MgCl₂ and CaCl₂ there was a decrease in the membrane activity of all peptides (Fig. 4.4A). The decrease in membrane activity was the result of slower permeabilisation in the presence of MgCl₂ and CaCl₂ (Fig. 4.4C). The permeabilisation rate by Trc B and Trc C was maintained in the presence of KCl and NaCl (Fig. 4.4B). Similar observations were made for the effect of alkaline earth metal chloride salts on the membrane permeabilisation activity of the purified Trc analogues (Fig. 4.5). The salts generally decreased the activity of the peptides including GS. However, the effect of CaCl₂ and MgCl₂ in comparison

to each other varied with peptide identity. Magnesium induced less lytic activity in GS and Trc C as opposed to a higher lytic activity in the Trc A analogues sTrc AOMe3 and Phc A as well as Tpc C (Fig. 4.5). The two salts had similar effects on Trc A and Trc B (Fig. 4.5).

As was expected the two RW-peptides were non-lytic at 40 μM to the bacterial cells following membrane permeabilisation/lysis assays using propidium iodide (results not shown). Previous experiments for membrane permeability have shown that the most active RW-peptides (c-WFW and c-WWW) do not rely primarily on membrane lysis for activity^{36,51,56}. Therefore, the primary MOA of the RW-peptides is different from that of the Trcs. Therefore, modulation of the activity of RW-peptide Ca^{2+} and Trcs is opposite, indicating differences in mode of action and possibly targets. Phe in the RW peptides sequence favoured a greater negative influence by salts and particularly CaCl_2 on activity, whereas the effect of CaCl_2 is lost in Tpc C containing Trp at position 7.

The presence of CaCl_2 results in both an apparent loss of membrane activity and increased anti-metabolic activity translating into gain in growth inhibitory activity of the Trcs. This indicates a change in the Trc MOA from a principally membranolytic MOA to one that influences the cell viability (ie energy metabolism) without overt membrane damage. In contrast, MgCl_2 induced apparent loss of membrane and anti-metabolic activities, though it gained apparent growth inhibitory activity as assessed at 595 nm. This could be due to overall loss of bactericidal activity or change in MOA/target that does not involve either membrane activity or the energy metabolism or alternatively results in the formation of biofilms which is not measured by the optical determination. The latter was indicated by flaky cell clumping in cultures. MgCl_2 was previously shown to reduce the lytic activity of another cyclic peptide of similar size containing β -turns, iturin A where at 1 mM MgCl_2 had a protective effect on lysis of *M. luteus* protoplasts and at 20 mM completely prevented lysis by up to 200 $\mu\text{g/mL}$ iturin A⁵⁷.

Calcium has been implicated to play a crucial role in drawing membrane bilayers closer together by partial dehydration of the membrane surface and cross-linking of opposing molecules of acidic phospholipids, a phenomenon not likely for magnesium⁵⁸. This is attributed to the non-uniform configuration of Ca with a coordination number of 7 or 8 contrary to the regular octahedral 6-coordination of Mg⁵⁸. Calcium is also more likely to lose part of its hydration shell, and to displace water upon complex formation⁵⁸. These differences on the specific membrane interaction by Ca and Mg could account for the differences in the mode of modulation of Trc activity by the two divalent metal ions.

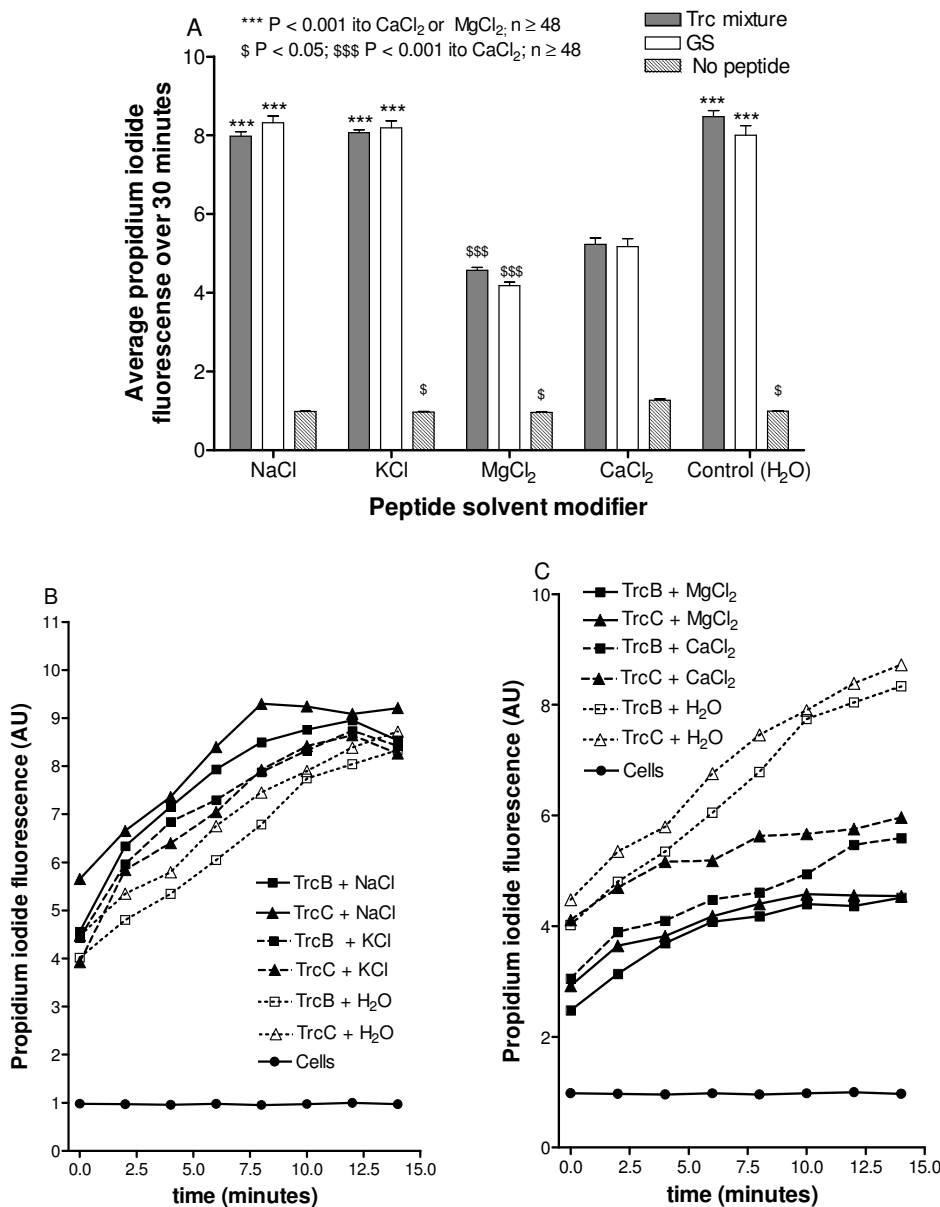


Figure 4.4 **A.** Comparison of the influence of chloride salts on listerial membrane permeabilisation by 12.5 µg/mL (~10 µM) Trc mixture and 10 µM GS using propidium iodide fluorescence over 30 minutes. Similar results were obtained for Trc B and Trc C. Statistical analyses were done with Two-way Anova using Bonferroni's post test (n ≥ 48 determinations). **B.** The kinetics of membrane permeabilisation by 10 µM mM Trc B or Trc C in the presence of 7.5 µM KCl or NaCl. **C.** The kinetics of membrane permeabilisation by 10 µM Trc B or Trc C in the presence of 7.5 mM CaCl₂ or MgCl₂. The data points are the means of triplicate measurements.

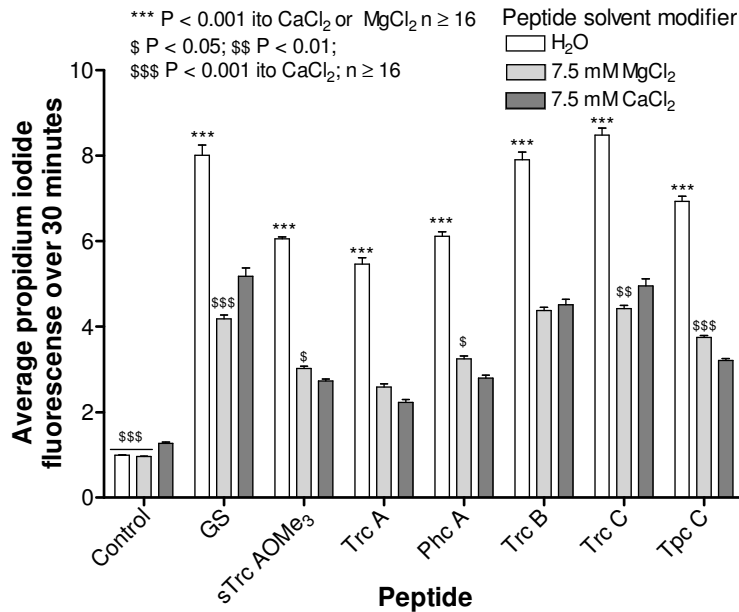


Figure 4.5 Comparison of the listerial membrane permeabilisation by 10 μ M of the purified Trcs in the presence of 7.5 mM chloride salt of alkali earth metals using propidium iodide fluorescence over 30 minutes. Means of ≥ 16 measurements in the presence of peptide \pm SEM are depicted. Statistical analysis was done with using Newman-Keuls multiple comparison test.

4.4.3. Antilisterial activity of tyrocidines in combination with EDTA and divalent cations

Addition of various concentrations of Na₄-EDTA to the tyrocidine complex (Trc mix) and GS in the presence or absence of either 7.5 mM MgCl₂ or CaCl₂ showed that EDTA activity was significant from 5 mM (Fig. 4.6A). EDTA restored the anti-metabolic activity of the Trcs in the presence of MgCl₂, albeit much higher concentrations of EDTA than for the Trcs in water were needed, indicating that MgCl₂ may have an antagonistic effect. In contrast, EDTA caused a significant loss ($P < 0.001$) of the anti-metabolic activity of the tyrocidines pre-incubated in CaCl₂ (Fig. 4.6B). The Trc activity in the presence of calcium was only re-established at 10 mM EDTA (Fig. 4.6A), however, EDTA is also antimicrobial at such a high concentration. These results indicate that Ca²⁺ and the Trcs may have synergistic antimicrobial activity, while Mg²⁺ and Trcs may be antagonistic with *L. monocytogenes* as target cell. EDTA addition also significantly ($P < 0.001$) increased the anti-metabolic activity of the tyrocidine complex in water (Fig. 4.6B) probably due to a synergistic activity between EDTA and the Trcs (refer Table 4.1 and the discussion below). EDTA could chelate Mg²⁺ and Ca²⁺ from the cell walls and weaken it for tyrocidine action. However, when EDTA was added to the culture medium it had minimal effect on the Trc activity and there was a significant difference ($P < 0.001$) from the activity of the Trcs dissolved in water modified with EDTA. It is therefore, more plausible that the EDTA is

chelating residual divalent cations such as Mg^{2+} that may be antagonistic towards the Trc action. Moreover, Na_4EDTA reversed the effect of $MgCl_2$ or $CaCl_2$ on membrane permeabilisation and restored the lytic effect of all Trc analogues (results not shown) and Trc mixture (Fig. 4.6B). EDTA has also been shown to improve the lytic activity of iturin A toward *M. luteus* protoplasts 57.

There was no significant effect of EDTA on the activity of GS in the presence or absence of the different cations (results not shown). Sodium at concentrations correlating to the concentration of Na^+ of the added EDTA also did not have any effect on the tyrocidine activity in the presence of the two earth metal chloride salts (results not shown).

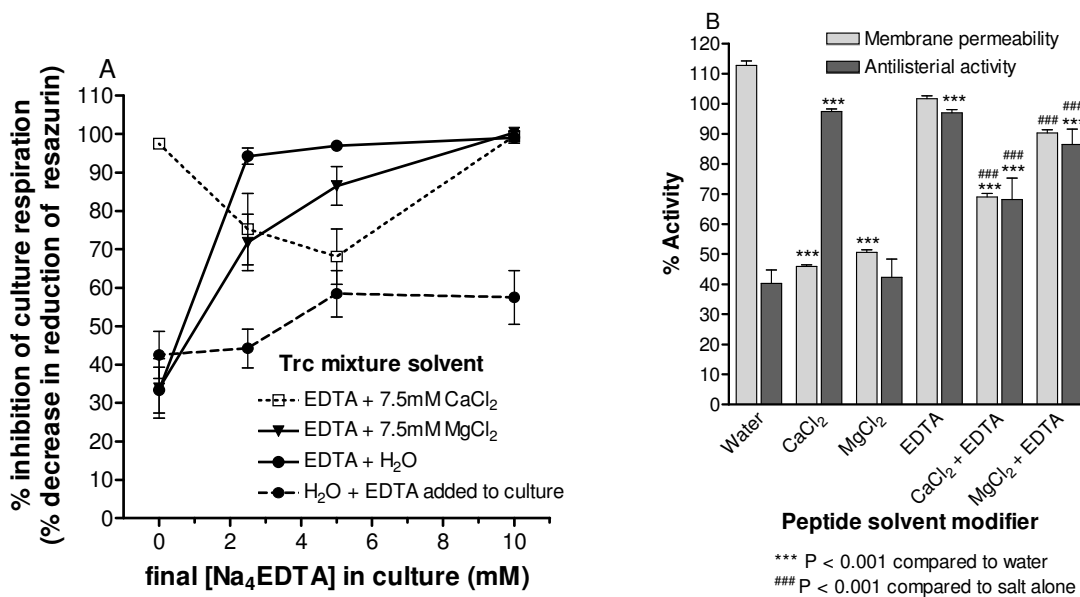


Figure 4.6 **A.** The antilisterial activity of 12.5 $\mu g/mL$ ($\sim 10 \mu M$) tyrocidine mixture in the presence of Na_4EDTA alone or Na_4EDTA and 7.5 mM $MgCl_2$ or $CaCl_2$ as measured by resazurin reduction. **B.** Comparison of the membrane and antilisterial activity of 12.5 $\mu g/mL$ ($\sim 10 \mu M$) tyrocidine mixture in the presence of 5 mM Na_4EDTA or Na_4EDTA and 7.5 mM $MgCl_2$ or $CaCl_2$. The antilisterial activity was measured by resazurin reduction ($n \geq 10$ determinations) and listerial membrane permeabilisation by propidium iodide fluorescence ($n = 48$ measurements). Statistical analyses were done with two-way ANOVA using Bonferroni's post test and Student t-test.

Due to the observed significant enhancement of the anti-metabolic activity of Trcs in water by EDTA we decided to test the hypothesis of a synergistic interaction between EDTA and Trcs towards *L. monocytogenes* B73 as well as the nature of the interaction in the presence of the divalent metal cation chloride salts. To assess the influence of EDTA on Trc activity in the presence and absence of divalent metal cations *in vitro* antilisterial activities of EDTA: Trc

mixture combinations were determined. GS was evaluated as reference peptide. Following determination of the IC₅₀ values of the combinations with dose-response assays as described in Chapter 3, FIC values were calculated and used to plot isobolograms (plots not shown) as well as determine the FIC indices (Table 4.1).

Table 4.1 Summary of the evaluation of the mode of *in vitro* interaction between EDTA and Trc complex or GS in different combinations in the presence and absence of the divalent metal chloride salts CaCl₂ and MgCl₂. FICs from activity in terms of anti-metabolism activity and calculated FIC indices were obtained from two biological repeats of experiments done in triplicate.

<i>Inhibition of culture respiration (conversion of resazurin to resofurin)</i>								
Combination ratio EDTA:peptide	EDTA FIC	GS FIC	EDTA:GS FIC index	Isobologram shape	EDTA FIC	Trc mix FIC	EDTA:Trc mix FIC index	Isobologram shape
Water								
15:0.01	0.59 ± 0.04	1.05 ± 0.11	1.60 ± 0.15	Convex	0.62 ± 0.01	0.26 ± 0.00	0.88 ± 0.01	Concave
10:0.03	0.23 ± 0.01	1.20 ± 0.12	1.40 ± 0.14		0.33 ± 0.08	0.43 ± 0.10	0.76 ± 0.18	
5:0.04	0.08 ± 0.02	1.30 ± 0.22	1.30 ± 0.24		0.14 ± 0.03	0.44 ± 0.02	0.58 ± 0.05	
CaCl₂								
15:0.01	0.32 ± 0.00	1.00 ± 0.12	1.30 ± 0.12	Convex	1.30 ± 0.24	1.50 ± 0.10	2.70 ± 0.34	Convex
10:0.03	0.16 ± 0.05	1.40 ± 0.31	1.60 ± 0.37		0.24 ± 0.02	1.06 ± 0.14	1.30 ± 0.16	
5:0.04	0.05 ± 0.00	1.38 ± 0.10	1.40 ± 0.10		0.08 ± 0.02	1.09 ± 0.23	1.20 ± 0.25	
MgCl₂								
15:0.01	0.30 ± 0.00	0.98 ± 0.37	1.30 ± 0.38	Convex	0.39 ± 0.02	0.26 ± 0.01	0.66 ± 0.01	Concave
10:0.03	0.21 ± 0.00	2.02 ± 0.77	2.20 ± 0.78		0.23 ± 0.01	0.48 ± 0.04	0.71 ± 0.04	
5:0.04	0.05 ± 0.00	1.51 ± 0.55	1.60 ± 0.55		0.11 ± 0.00	0.73 ± 0.02	0.84 ± 0.02	

The results from the shape of the isobolograms correlated well with the calculated FIC indices. The results confirmed slight synergy (concave shaped graph below the line of additivity) for growth inhibitory and anti-metabolic activity between EDTA and the Trc complex in water and MgCl₂ as indicated by the FIC indices ($1 > FICI > 0.5$). The anti-metabolic activity in the presence of CaCl₂ results indicated a non-interactive or slight/moderate antagonism (convex shaped graph above the line of additivity) according to the FIC indices ($1 < FICI < 4$) between EDTA and the Trc complex in CaCl₂. The results showed that EDTA was either non-interactive or slightly antagonistic with GS in all solvents.

4.4.4. *Fluorescent microscopy*

The increased Trc antilisterial activity caused by pre-incubation with CaCl₂ may be due to either complexation between calcium (refer to discussion under *Section 4.4.5*) and the Trcs which could lead to a more active conformation, formation of active complexes and/or antagonism of the Trc hydrogen-bonded structure(s) as indicated by circular dichroism analyses done by our group (personal communication, Dr. B. Battacharya, refer to *Section 4.4.5*). Another possible reason could be the increase in available calcium for integration in the bacterial cell wall, which may act as docking sites for the Trcs since most of the calcium content of *L. monocytogenes* is known to be in its cell wall⁵⁹. In order to evaluate the first hypothesis for the mode of modulation of Trc antilisterial activity by CaCl₂, Trc B was pre-incubated in 75 mM CaCl₂ (10× concentrate), allowing for pre-complexation before adding to *L. monocytogenes* B73 cultures in standard BHI growth medium. To test the second hypothesis, *L. monocytogenes* was cultured in BHI growth medium supplemented with 7.5 mM CaCl₂ allowing for prior calcium-bacteria interaction before evaluation of Trc activity.

Live-cell fluorescent microscopy showed that pre-incubation of 5 μM Trc B in water led to membrane lysis/leakage of *L. monocytogenes* as evidenced by the red fluorescence signal of propidium iodide (Fig. 4.7C), in contrast to the negligible membrane permeability observed for the cells previously grown in 7.5 mM CaCl₂-enriched BHI medium and/or treated with 5 μM Trc B pre-incubated in 7.5 mM CaCl₂ and MgCl₂ (Fig. 4.7D, E, F respectively). These results were in accordance with our previous results (refer to Figs. 4.4, 4.5 and 4.6B) and therefore, apparently pre-incubation of Trc B with CaCl₂ improves antilisterial activity by converting the mode of action from a lytic to a predominantly non-lytic mode of action. Lytic activity for antimicrobial peptides, such as the Trcs, involves self-assembly into active complexes probably within the membrane and upon reaching a certain significant peptide concentration this results in disruption of the membrane integrity⁶⁰. Therefore, putative tyrocidine-Ca²⁺ complexes could characterize another active form of the peptide, unlike putative complexes with Mg²⁺ which do not result in similar improved anti-metabolic activity. Ca²⁺ plays a significant role in the virulence of *L. monocytogenes* and its switch from a saprophyte to a predominantly anaerobic intracellular pathogen⁶¹. It is hence possible that the observed Ca²⁺-induced non-lytic MOA may result from both higher Ca²⁺ modulating the premature *L. monocytogenes* conversion from a saprophyte to an intracellular anaerobic pathogen and Trcs acting on sensitive non-membrane target(s) in the prematurely transforming pathogen.

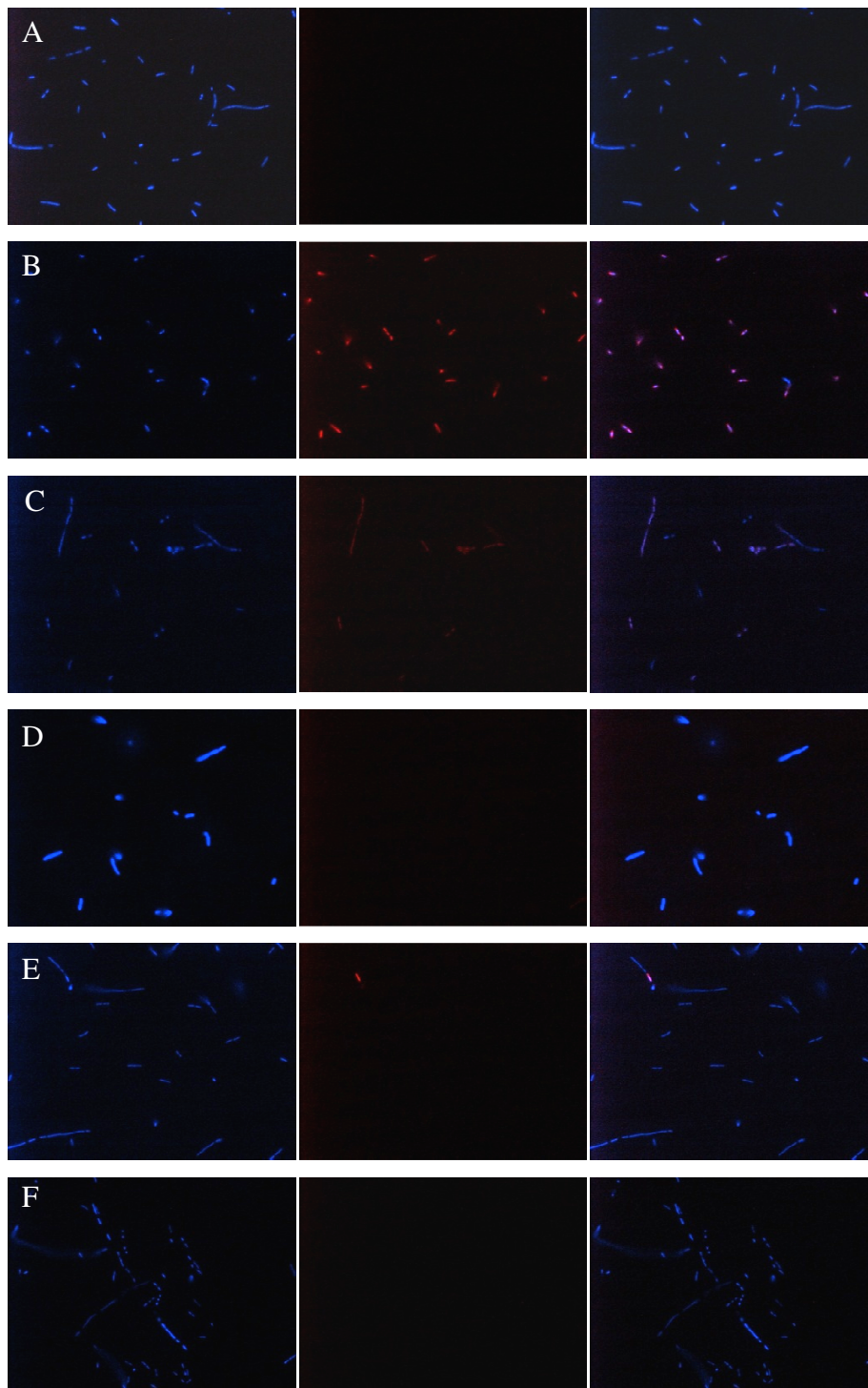


Figure 4.7 Live-cell fluorescent microscopy of *L. monocytogenes* B73 using bis-benzimide (left panels) and propidium iodide (middle panels) to evaluate the influence of Trc B on cell integrity in the presence and absence of CaCl_2 and MgCl_2 . **A.** Control **B.** Cells grown in BHI, treated with GS **C.** Cells grown in BHI, treated with Trc B + H_2O **D.** Cells grown in BHI + CaCl_2 , treated with Trc B **E.** Cells grown in BHI, treated with Trc B + CaCl_2 **F.** Cells grown in BHI, treated with Trc B + MgCl_2 . Bis-benzimide fluorescence (blue) indicates all cells, whereas propidium iodide fluorescence (red) indicates cells with compromised membrane integrity.

4.4.5. *Interaction of tyrocidines with metal cations*

The specific influence of selected metal cations on the antilisterial activity of the Trcs may be due to specific interaction of the tyrocidines with the metal ions which may influence their secondary structure and self-assembly/aggregation state. Alternatively, possible tight binding of peptide to a metal cation may yield a complex with larger positive charge and this could improve the binding interaction to negative cellular target(s). It seems that competition of the metal cations for negative target molecules in bacterial membrane/cell wall which will thereby compromise the binding of the peptides to the membrane/cell wall does not affect the Trc activity. Our results therefore, correlate with previous studies which demonstrated that complexation with Ca^{2+} is a determinant of Trc antilisterial activity and difference in sensitivity to CaCl_2 by the different Trc analogues could be due to distinct peptide-calcium interactions and/or aggregation tendencies which in turn are related to the structural differences among the Trcs³⁵.

Using photon correlation spectroscopy (PCS) we investigated the influence of 7.5 mM divalent metal cation chloride salts on the particle size of Trc B at 50 μM in aqueous solutions following either a one- or two-step dilution from 1 mM stock in 50% acetonitrile/water (which simulates the peptide preparation steps during the biological assays). The results showed that the one-step dilution did not yield any difference in the size of the Trc B particles (Fig. 4.8A) in all solvents (CaCl_2 , MgCl_2 and water). However, the two-step dilution gave particles in the following order in terms of solvent: $\text{MgCl}_2 < \text{CaCl}_2 < \text{water}$ (Fig. 4.8B). The particle sizes of 50 μM Trc B in diameter in water, CaCl_2 , and MgCl_2 were 59 nm; 51 nm and 38 nm respectively. The two step dilution allows more time for the solution for dissociation of the aggregates due to new interaction with the divalent cation interactions. Calcium and magnesium resulted in reduction of particle size of Trc B in aqueous solution following the two-step dilution indicating that these salts may act as chaotropic agents or lead to smaller defined oligomers/aggregates.

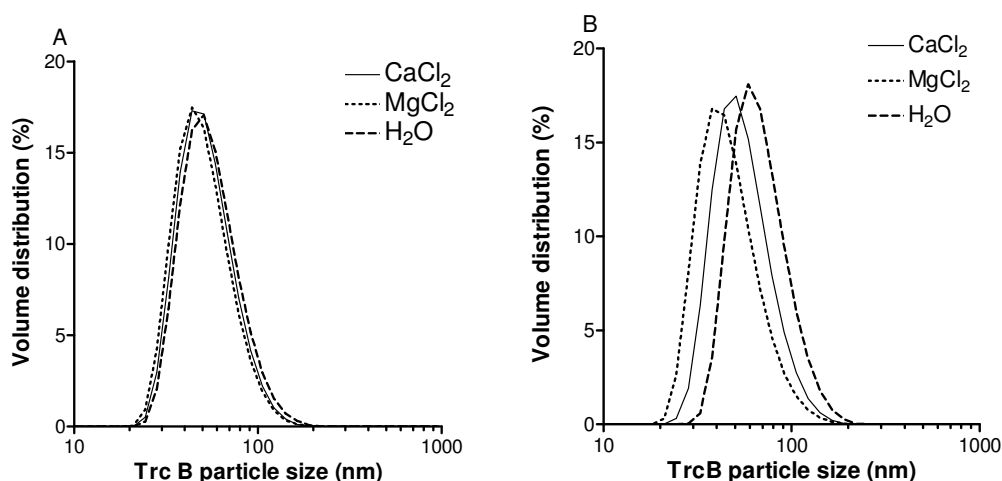


Figure 4.8 Effect of 7.5 mM calcium and magnesium chloride salts on the size of particles in 50 μ M Trc B diluted from 1 mM stock in 50% acetonitrile in either **A.** one step or **B.** two steps. The lines shown represent the average point to point line fit for triplicate measurements per solvent condition with 5 readings made per measurement and duplicate samples for each solvent condition.

We also investigated the influence of CaCl_2 on the intrinsic fluorescence signal of tryptophan in Trc B (at 6.25 μ M) both in aqueous solution (buffer containing 10 mM Tris, 154 mM NaCl at pH 7.4) and in 50 μ M of vesicles made of POPE and POPG (1:3 molar ratio respectively) representative of Gram-positive bacteria model membrane. The maximum wavelength (λ_{max}) of the fluorescence signal in buffer was 347.9 nm whereas in the liposome environment it decreased to 327.3 nm (Fig. 4.9), thus a significant red shift of 21 nm. The shift in fluorescence maxima indicates that the conformation of Trc B in aqueous solution, with particular emphasis on the Trp⁴ residue, differs from that in the liposome vesicles. Apart from the shift in λ_{max} , there was also quenching of the Trp fluorescence in the liposome environment from 33.84 to 25.06 (Fig. 4.9). Upon addition of CaCl_2 , we still observed a red shift of Trp fluorescence maxima between buffer and liposome environments by a magnitude of 23 nm, however, the fluorescence signal was comparable within both environments being 32.3 in buffer and 31.5 in liposome (Fig. 4.9). Therefore, calcium does not change the overall conformation of Trc B in terms of its Trp residue, but affects the binding of Trp with the membrane, changing its exposure within the membrane environment as indicated by the increased fluorescence intensity. Trp has been reported to be critical for membranolytic activity of AMPs⁶²⁻⁶⁴ hence; changing the membrane interaction of the unique Trp residue in Trc B could explain how calcium decreased the membrane permeabilising activity of the Trcs as observed in *Section 4.4.2*.

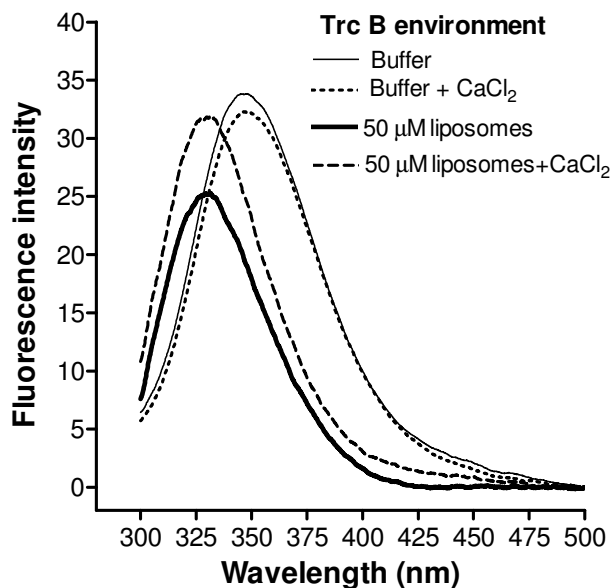


Figure 4.9 Effect of 7.5 mM calcium chloride salt on the Trp fluorescence spectra of 6.25 μM Trc B dissolved in either aqueous buffer with or without the chloride salt added as well as in the presence of 50 μM POPG:POPE (3:1) large unilamellar liposomes. The lines shown represent the average point to point line fit of three scans for each condition

A significant loss of ordered hydrogen bonded structures of Trcs was observed in the presence of the alkali and earth metal chloride salts in circular dichroism spectroscopy studies by Dr B. Bhattacharya from our group (personal communication). Spectra in chloride salts were compared and significant decreases in ellipticity over 200-220 nm (Fig. 4.10) and the $\theta_{206}/\theta_{217}$ ratio (structural change parameter) were observed after pre-incubation of the Trcs with either of the alkali or earth metal chloride salts. The intensity loss at both 206 nm and 217 nm minima for Trc B according to the type of cation was $\text{Mg}^{2+} \geq \text{Ca}^{2+} > \text{K}^+ > \text{Na}^+$. The chloride salts had very little influence on the GS structure according to the CD spectra (personal communication Dr B. Bhattacharya). It was generally found that Ca^{2+} caused the largest changes in the $\theta_{206}/\theta_{217}$ ratio decreasing it from 1.18 to 0.92, for Trc B indicating higher sensitivity of Trc hydrogen bonded structure to Ca^{2+} . These observations correlated with our PCS results that indicated the decrease in particle size when Trc B was diluted with either MgCl_2 or CaCl_2 (refer to Fig 4.8). However, the change of Trc structure did not compare with the calcium induced structural changes observed for daptomycin, a calcium dependent cyclic lipopeptide⁶⁵.

Previous studies on interaction of Trcs with metal cations by Spathelf³⁵ demonstrated differences in complexation between different Trcs and different metal cations. The influence of the metal cation chloride salts at 1 mM on the ESMS signal of singly charged free ions ($[\text{M}+\text{H}]^+$)

of the Trcs A, B, C and their Lys-containing analogues at $\sim 10 \mu\text{M}$ was evaluated. Spathelf³⁵ deduced from these studies that the Trc-divalent ions adducts were more stable under ESMS conditions because the Trc probably have a higher affinity for the divalent metal cation chloride salts.

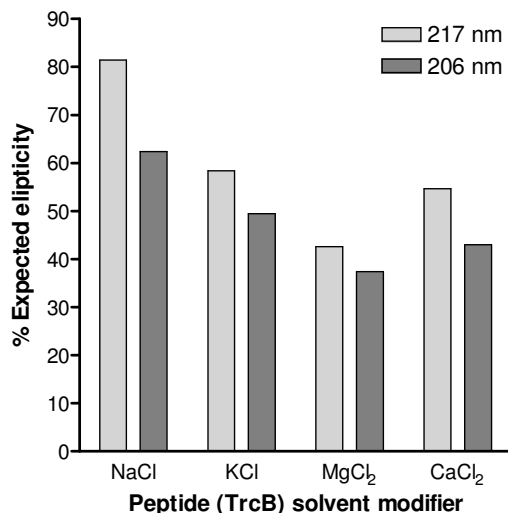


Figure 4.10 Effect of 100 mM chloride salt on the ellipticity of $0.19 \mu\text{M}$ Trc B dissolved in 12.5% *v/v* acetonitrile in water without chloride salt added at 217 nm and 206 nm. The % ellipticity (expected ellipticity) was calculated in terms of the ellipticity of the Trc B preparation without the added salt. The bars shown represent the average %ellipticity following point to point line fit of three scans for each solvent condition. Collection of scans was at 5000 data points per nm (Spectral data courtesy Dr. B. Bhattacharya)

An explanation of the calcium induced increase in antilisterial activity could be that a putative overall increase in positive charge of the Trc-calcium complex will not only increase the interaction with negative cell walls and membranes of target bacteria, but also result in electrostatic repulsion that would prevent unwanted formation of aggregates by self-association of Trcs (salting-in or chaotropic effect). Based on these observations it is proposed that any number of the seven carbonyl groups and one hydroxyl group in the variable pentapeptide moiety (X-D-X-Asn-Gln-Tyr, X=Phe or Trp) of the Trcs may form an electronegative cavity for metal cation interaction. Therefore, metal cations, especially Ca^{2+} and Mg^{2+} , may have a major influence on the Trc structure in aqueous solution, either acting as chaotropic agents or through specific interaction with the peptide in an electronegative cavity leading to disruption of secondary H-bonded structures. Our fluorescent studies (Fig. 4.9) showed that Ca^{2+} also affects the interaction of Trc B with model Gram-positive bacterial membranes. We then investigated the membrane interaction of the Trcs further using liposomes that model bacterial membranes.

4.4.6. Vesicle interaction studies

Interaction of selected peptides with model membrane systems was studied using dynamic light scattering measurements (photon correlation spectroscopy) with the aim of evaluating the effect of peptides and/or CaCl_2 on the size of lipid vesicles. The peptides studied were Trc B, GS and c-WFW based on the results from the *in vitro* antibacterial assays. The model membrane system under investigation was a combination of POPG and POPE in molar ratio 3:1 representing the phosphatidylglycerol-rich membrane of *L. monocytogenes* B73⁶⁶. The results were quantified in terms of the concentration lipid to peptide concentration ratio (cl/cp).

In the vesicle, the formation of larger particles up to several thousand nm in diameter was induced by Trc B at a lipid to peptide ratio of ≤ 5 . At cl/cp = 2, 100 nm particles no longer existed (destruction) (results not shown). Particle size was restored at cl/cp = 8. Ca^{2+} enhanced the peptide effect and the particle size was only restored at lipid to peptide ratio (cl/cp) five times higher than when Ca^{2+} was absent. GS disturbed the POPG:POPE 3:1 large unilamellar vesicle (LUV) integrity at cl/cp = 12.5. In the presence of Ca^{2+} , it was again at cl/cp = 12.5 that GS disturbed the LUV integrity although forming higher proportions of the larger particles. Thus Ca^{2+} also enhanced the effect of GS. In these type vesicles, Ca^{2+} had a greater effect on Trc B's activity than on the activity of GS which correlates well with *in vitro* antilisterial activity results above. This could be due to the fact that GS has a higher charge than Trc B. At cl/cp ≤ 10 , c-WFW disturbed POPG:POPE 3:1 LUVs. In the presence of Ca^{2+} , the effect of c-WFW was also enhanced. Therefore, the activity order in terms of the cl/cp required to disturb the LUV integrity was GS > c-WFW >> Trc B. This correlates well with the known high membrane lytic activity of GS⁵⁰ and the fact that membrane activity of c-WFW has been associated to lipid demixing⁶⁷⁻⁶⁹ which could cause packing defects allowing translocation of polar compounds into the cytoplasm⁷⁰. Therefore, in both lipid systems, Ca^{2+} enhanced the susceptibility of the particles to the effect of Trc B. The Gram + (POPG:POPE at 3:1 ratio) LUV resisted most the effect of Trc B (Table 4.2). The susceptibility of the POPG:POPE (3:1) liposomes to the activity of the peptides and also the effect of Ca^{2+} could be due to the fact that POPE has an unsubstituted quaternary ammonium group, which is protonated at neutral pH which has a small head group and is able to participate in hydrogen bonding interactions. The negatively charged POPG:POPE (3:1) liposomes will bind strongly to Ca^{2+} leading to reduction in the electrostatic charge of the head groups which will cause condensation of the bilayer which accounts for the observed increase in liposome size in the presence of CaCl_2 ⁵⁸.

Table 4.2 Summary of the influence of CaCl₂ on the activity of Trc B, GS and c-WFW in terms of the ratio of lipid concentration to peptide concentration (cl/cp) required to cause change in size of 100 nm POPG:POPE (3:1) large unilamellar vesicles *in vitro*.

Peptide	cl/cp of vesicle disturbance	
	POPG:POPE 3:1 (Gram + model)	POPG:POPE 3:1 (Gram + model) + Ca ²⁺
Trc B	< 5	< 8
GS	< 12.5	< 12.5
c-WFW	< 10	< 50

CaCl₂ distinctly enhanced the susceptibility of the POPG:POPE (3:1) liposomes, but this effect was peptide specific. This correlated with our *in vitro* studies that have shown that the different peptides respond differently to Ca²⁺. The activity of c-WFW towards the bilayers correlated with the good biological effect of c-WFW against bacteria (which is however not permeabilisation).

4.5. Conclusions

The antilisterial activity of the Trcs is tolerant to chloride salts of alkali (NaCl, KCl) and earth metals (MgCl₂, CaCl₂). However, Ca²⁺ might specifically modulate Trc activity as it was the only ion to cause an increase in overall activity while reducing their predominantly membranolytic action. This effect was specific to the Trcs as the antilisterial activity of other peptides tested (Tpc C, GS, c-WFW, and c-WWW) was either unaffected or decreased by the presence of CaCl₂. The results again indicated the relevance of a small aromatic residue (Phe or Tyr) in position 7 for the calcium dependent increase in antilisterial activity. This correlated with previous results showing that antilisterial activity of the Trcs rely on a Tyr or Phe in the NQAr unit (Chapter 3).

The influence of calcium could be partially due to a chaotropic effect or structural change as indicated by PSC and CD, respectively. Alternatively, it could be due to the calcium-induced change of conformation of Trcs in model Gram-positive bacteria membranes, as detected by fluorescence studies focussing on the Trp residues. D-Trp⁴ is probably directly involved in the membranolytic activity of the Trcs and calcium-induced change in the membrane interaction of this residue could account for observed decreased membranolytic activity of Trcs in the presence of Ca²⁺. However, the change in MOA of the Trcs could not be explained by their action on

model membrane in the presence of calcium. CaCl_2 in fact enhanced susceptibility of model Gram-positive bacteria liposomes to Trc B, GS and c-WFW, leading to a change the liposome size either through aggregation or disintegration. As model membrane targets are static, while the bacteria not only have cell wall barrier that have to be crossed but also a host of other stress and growth responses that must be overcome by the peptide, this result is not unexpected. Moreover, we observed a slight synergism against *L. monocytogenes* between EDTA and the Trc mixture in water and MgCl_2 , but a non-interactive or slight/moderate antagonism between EDTA and the Trc mixture in CaCl_2 was observed. The fact that Mg^{2+} and Ca^{2+} ions decreased the lytic activity of the Trcs and that EDTA, competing for the divalent ions, restored this Trc activity supports the hypothesis that Trcs probably interact with polar head groups of lipids in the cell membrane to exert their membranolytic effect ⁵⁷, but may have alternative target/MOA when associated with Ca^{2+} and the membrane interaction is inhibited.

In summary, the results indicated that the calcium influences the Trc solution/membrane bound structure, aggregation in solution, model membrane activity and MOA. Calcium complexation could change its cell membrane interaction, possibly targeting the Trc to the cell wall or allowing translocation over the cell membrane. Alternatively, an increased availability of Ca^{2+} released from the Trc-calcium complex could cause a premature *L. monocytogenes* transition from a saprophyte to intracellular anaerobic pathogen. The Trc- Ca^{2+} complexes may interact and inhibit key components that are essential to maintain redox potential and sustain metabolism, such as the membrane bound electron transport system or bacterial dehydrogenases, without leading to overt lysis of the target cell. The observed Ca-induced non-lytic mode of action may therefore, be the consequence of a combination of biophysical and biological effects.

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Chapter 5

Part II: Literature Review

Small cyclic antimicrobial peptides as anti-malarial compounds

5.1. Introduction

The seclusion of intracellular pathogens from host humoral and cellular immune response lead to infectious diseases linked with high mortality and morbidity that plague humans such as listeriosis and malaria ^{1,2}. The intracellular localisation of these pathogens also protects them from drugs, creating a reservoir for recurrence and re-infection and enhancing the development of drug resistant strains ³.

From immemorial times malaria has caused incredible human suffering including annihilation of human settlements, decline of civilisations, loss of wars and loss of millions of lives irrespective of social status which all slowed humanity's progress ^{4,5}. Although recent reports indicate a decrease in the number of malaria related cases worldwide, there were still about 219 million cases in 2010 leading to about 660 000 deaths ⁶. In 2011 an estimated 3.3 billion people were at risk to contract malaria worldwide ⁶. Most affected are immune deficient individuals like pregnant women (due to weakened ability to limit parasite replication during pregnancy) ⁶, children under five years of age and tourists (as immunity is established following repeated malaria infection) ⁶⁻⁸. The disease in humans is caused by parasites of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) which are transmitted by over 30 anopheline species through infected female mosquito bites ⁶⁻⁸. Malaria is predominantly overwhelming in sub-Saharan Africa, where about 80% of cases and 90% of deaths occur due to the prevalence of *P. falciparum* which is the most deadly form of the parasite ^{6,9,10}. Therefore malaria is still endemic especially in poor countries. Forty percent of the global total malaria deaths occurred in two African countries, the Democratic Republic of Congo and Nigeria ⁶. However, some countries like South Africa have about 90% of the population living in malaria-free regions ⁶ as a result of intervention policies and strategies some of which are detailed below. Nevertheless, *P. falciparum* is responsible for 99% of malaria cases in South Africa, the other 1% being due to infection with *P. vivax* ⁶. Other areas

affected by malaria include certain regions of South East Asia and South America⁷. Malignant malaria caused by *P. falciparum* can manifest as anaemia, cerebral complications (from coma to convulsions), hypoglycaemia and glomerulonephritis^{7,11,12}. Malaria is a major public health problem mainly due to the development of wide-spread resistance by *P. falciparum* to all available antimalarial drugs^{7,13-15}.

5.2. *Plasmodium falciparum*

P. falciparum is a unicellular eukaryotic microbe and thus has similar cell and molecular biology as other eukaryotes though unique by its intracellular lifestyle¹⁶. The protozoan parasite has both invertebrate (*Anopheles* mosquitoes) and vertebrate (human) hosts^{12,17}. The pathology of *falciparum* malaria is caused by a 48 hour dynamic asexual intra-erythrocytic life cycle involving many intermediate stages^{12,18-20}. *P. falciparum*, is different from the other human malaria species in that it is capable of unlimited replication in the human host achieving very high parasitemia levels, infecting >50% erythrocytes, of which most are immobilised by attachment to capillary walls, which generally correlate with clinical features and diagnosis^{8,21}. Four percent or higher parasitemia circulating is generally considered an indicator of a severe malarial threat in a low-transmission setting^{22,23}.

5.2.1. *Malaria pathogenesis*

The *Plasmodium* spp. sporozoites are transmitted *via* the saliva of infected female mosquitoes during a blood meal to the subcutaneous tissues of the human host²⁴. The sporozoites are then speedily transported to the liver where they invade hepatocytes and develop into tissue schizonts (exo-erythrocytic stage)²⁴ (Figure 5.1). The targeting of sporozoites to hepatocytes possibly involves interaction of the circumsporozoite protein (CSP) with heparin sulfate proteoglycans on the sporozoites and hepatocytes respectively¹⁷. By day 6-10 the tissue schizonts go through schizogony (Figure 5.1), during which they replicate via mitosis and burst infected hepatocytes releasing tens of thousands of merozoites in the form of membrane-bound merozoites into the blood stream^{25,26}. The development in the hepatocytes is asymptomatic¹². In *falciparum* malaria merozoite discharge from the liver is an incessant and asynchronous process²⁴. The intra-erythrocytic stage begins with invasion of red blood cells by merozoites, which occurs through localised disruption of the erythrocyte cytoskeleton induced by the merozoites¹⁶. Several merozoite surface proteins have been implicated in this invasion with the most characterised being MSP-1 which is uniformly distributed

around the merozoites and binds to the host receptor protein band 3^{27,28}. Moreover apical organelles of the merozoite stage namely: rhoptries, dense granules and micronemes are implicated in facilitating erythrocyte invasion and formation of the parasitophorous vacuole derived from invagination of the host cell membrane and components of the parasite^{16,29,30}. In the erythrocytes the merozoites lose their rhoptries, micronemes and pellicular membranes and develop into rings, immature trophozoites becoming mature trophozoites by ingesting erythrocyte cytoplasm^{12,30}.

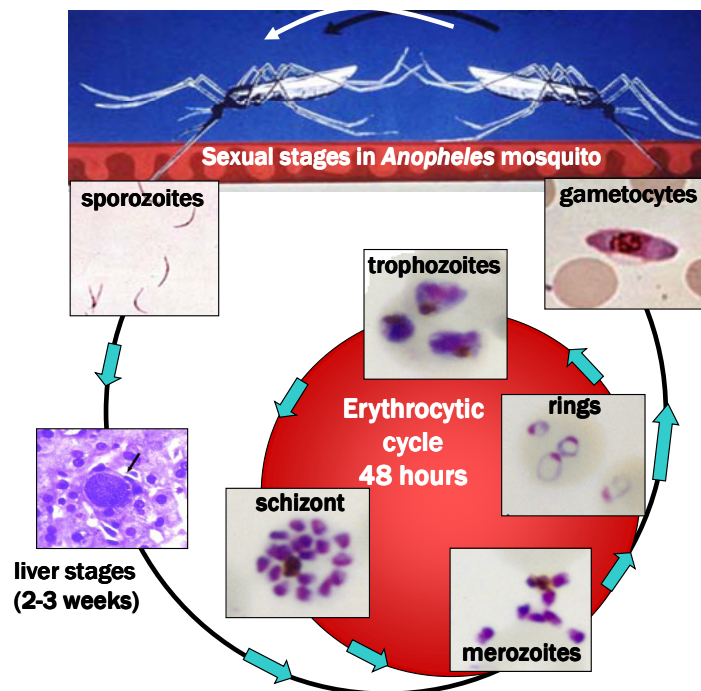


Figure 5.1 Life cycle of *Plasmodium falciparum* showing the detail in the human host of the different parasite stages (Picture courtesy of M Rautenbach and HC Hoppe).

The trophozoites mature to blood schizonts which each replicate to 8-32 daughter merozoites^{24,30}. During the growth and replication of the intracellular parasite the host red blood cell is remodelled into a stiff cell with decreased deformability which tends to stick to a variety of cell types^{21,29,31}. These changes are fundamental in the clinical complications of *P. falciparum* malaria such as anaemia, lactic acidosis, coma and death^{21,29,32}. Periodic fever is observed clinically following lysis of infected erythrocytes to release the merozoites due mainly to the stimulation of cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF)⁷. The released merozoites then invade new erythrocytes.

Some of the merozoites do not undergo asexual replication upon invasion of erythrocytes, but mature to a sexual stage called gametocytes which could be ingested by a mosquito during a blood

meal. Fusion of male and female gametocytes leads to formation of a zygote. It is during this diploid stage that sexual recombination of genetic material such as the chromosomal genes responsible for most drug resistance occurs²⁴. While in the mosquito's midgut the zygote develops into an oocyst, which in turn discharges sporozoites that travel to the mosquito salivary glands²⁴.

5.2.2. *Malaria related modifications of the red blood cell membrane*

Owing to the lack of an efficient fractionation procedure there is limited detailed knowledge available on the sub-cellular composition of the infected erythrocyte³³. For example no information is to our knowledge available on the lipid composition of the parasitophorous vacuolar membrane or the intraparasitic membranes as focus has been principally on the infected erythrocyte's plasma membrane³³. However, it is known that the fatty acid of the infected erythrocyte plasma membrane (IEPM) is very similar to that of the parasite, but cholesterol is almost absent in the parasite³³.

The membrane of the red blood cell or erythrocyte is composed of three layers: the carbohydrate-rich glycocalyx on the exterior, the lipid bilayer containing many transmembrane proteins in addition to its lipid constituents and the membrane skeleton on the interior which is a structural network of proteins³⁴. The flexibility and deformability of the erythrocyte, properties determined by proteins of the membrane skeleton, are important to allow it to deform and pass through capillaries whose diameters are less than half (3 μm) that of the erythrocyte (7 μm)^{34,35}. The mammalian membrane mass is equally distributed between lipids and proteins³⁶ while the total lipid bilayer mass is equally distributed between cholesterol and phospholipids³⁷. The major phospholipids are phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylserine (PS), and minor phosphoinositide (PI) components^{35,38,39}. While cholesterol is uniformly distributed between the two membrane leaflets, there is an asymmetric distribution of the major phospholipids with the neutral PC and SM in the outer leaflet while most PE, all PS and PI are found in the inner leaflet^{35,38,39}. Therefore, most anionic phospholipids are found in the intracytoplasmic layer⁴⁰. In addition to their other physiological roles PS and phosphatidylinositol-4,5-bisphosphate (PIP₂) interact with proteins in the membrane skeleton to control mechanical function of the membrane^{41,42}.

The changes that occur in the erythrocyte membrane during infection with *Plasmodium* spp. include increased fluidity⁴³⁻⁴⁶ possibly due to decreased SM⁴⁷ and cholesterol⁴⁸ levels, modification of phospholipid fatty acid^{30,47,49-53}, transbilayer distribution⁵⁴ and enhanced rate of transbilayer motion⁵⁵⁻⁵⁷, along with novel pores that boost membrane permeability⁵⁸⁻⁶². There is transfer of the

anionic PS from the inner leaflet to the outer leaflet of the bilayer which changes the membrane lipid asymmetry and the infected erythrocyte tends to be similarly anionic like bacterial cells^{63–65}. In addition, neoantigens are formed on the surface of the RBC through the alteration of resident integral erythrocyte membrane proteins^{66–68}.

It has been observed that modifications of the erythrocyte membrane structure and composition peak when the parasite is at the metabolically active trophozoite stage⁴⁷ and the membrane fluidity gradient between the bilayer leaflets is abolished at the schizont stage⁴⁶. Three membranes are involved in *Plasmodium*-infected erythrocytes: the IEPM, the parasitophorous vacuole membrane (PVM) which surrounds the parasite and the parasite's membrane^{30,47}. The erythrocyte membrane changes are directed by the parasite since mature human erythrocytes have limited lipid metabolism capacity⁶⁹ and the parasites are fully equipped for phospholipid biosynthesis⁵⁰ accounting for the rapid rise in erythrocyte lipid metabolism observed during infection^{30,47,49–51}. However, some authors have suggested that the parasite lacks the capacity to synthesise fatty acids and cholesterol *de novo*^{49,53} and must obtain these compounds from the serum or the erythrocyte membrane⁴⁷. It has been demonstrated that bidirectional exchange of lipids and associated proteins between the IEPM and parasite membrane or PVM^{47,69,70} possibly causes the IEPM from *P. falciparum*-infected erythrocytes (trophozoite stage) to be more similar to the parasite membrane or PVM and different from membranes of uninfected erythrocytes⁴⁷. This exchange involves delivery of parasite proteins to the erythrocyte membrane possibly via vesicular trafficking and fusion^{71,72} of novel membrane-bound organelles formed by the parasite in the erythrocyte cytoplasm⁷³. The PVM surrounding the parasite is a penetrable barrier through which the parasite actively alters the erythrocyte membrane to preserve an appropriate intracellular environment⁴⁷ such as when the erythrocyte's membrane permeability is increased for the parasite to obtain essential nutrients⁷⁴.

5.2.3. Anti-malarial strategies

The most effective tools for the eradication of malaria have been prevention and prophylactic and curative chemotherapy. Prevention of contact with the mosquito vector for control of transmission through house spraying with chemical insecticides, use of insecticide-treated bed nets and other materials, as well as other vector control strategies via nonchemical means including environmental and biological methods⁴. Chemotherapy to treat malaria is important for decreasing morbidity and mortality, but also to reduce transmission through decrease of parasite load in the human reservoir (Figure 5.1).

5.2.3.1. Vector control

The WHO considers vector control to be the most commonly effective means of malaria transmission prevention and it is one of the four fundamental technical components of the global malaria control strategies (GMCS) ⁷⁵. This is because successful vector control translates into less malaria patients to treat *via* chemotherapy ⁷⁵. Prior to targeting the anopheline vectors, efforts were dispensed towards eradication of the mosquito larvae which was not very successful ⁷⁵. Indoor residual spraying (IRS) with dichloro-diphenyl-trichloroethane (DDT), which is a residual insecticide and afterwards with others, took central stage in the malaria eradication campaign instigated in 1955 ⁷⁵. This led to the eradication of malaria in several parts of the world especially in temperate areas such as Western Europe and the United States as well as parts of South Africa ⁷⁵⁻⁷⁷. However, the logistic, human and financial resources suitable for malaria eradication through vector eradication were not available in all countries particularly in Africa ⁷⁵. With the resurgence of malaria, efforts are now directed towards control of transmission rather than vector eradication ⁷⁵.

The measures employed to control the malaria vector include ⁷⁵:

- 1) limiting contact between the mosquitoes and humans through the use of insecticide treated nets (ITNs), repellents and protective clothing for individuals as well as zooprophylaxis for communities;
- 2) IRS and space spraying to kill the adult mosquitoes;
- 3) destruction of the mosquito larvae domestically and at large scale in communities through sanitation and larviciding of water surfaces and
- 4) strategies for source reduction and social participation

However, ITNs and IRS remain the strategies with broad applicability both of which make use of insecticides to kill the adult mosquitoes as they get indoors to obtain a blood meal from humans ^{75,78}. The extensive use of DDT led to the development of resistant mosquitoes and prompted the use of other chemical groups to kill the adult mosquitoes. These other groups include organophosphates, carbamates and synthetic pyrethroids ⁷⁶. Nevertheless there has been the occurrence of multiple insecticide resistant mosquitoes making necessary the development of other measures for vector control ⁷⁶. Measures currently considered include the use of insect growth regulators, natural plant products, bio-control agents, bacterial and fungal pesticides, genetic manipulation and insect sterility techniques ⁷⁶. The need for efficient inter-sectoral coordination and community participation has been highlighted for the success of all strategies of vector control ⁷⁶. There is also a need to combine

the effectual tools including environmental management for an integrated approach towards vector control^{75,76}. The World Health Organisation Pesticide Evaluation Scheme (WHOPES) is involved in the development of new vector control tools and methods⁷⁶.

5.2.3.2. Antimalarial drugs and parasite resistance

According to Amina *et al.*⁷⁷ antimalarial drugs are currently classified under seven groups namely: 4-aminoquinolines, arylamino alcohols, antifolates, 8-aminoquinolines, inhibitors of the respiratory chain, antibiotics and artemisinin.

The activity of the 4-aminoquinolines (like chloroquine and amodiaquine) relies on complex formation with heme (ferriprotoporphyrine IX) which leads to inhibition of hemozoin formation in the parasite's food vacuole⁷⁷. The mechanism of action of chloroquine (CQ) involves its accumulation to millimolar levels inside the digestive vacuole of the intra-erythrocytic trophozoite where it inhibits heme polymerisation, leading to a accumulation of heme and/or a CQ-heme complex that is toxic to the parasite⁷⁹. Heme is a product of the parasite's digestion of haemoglobin for nutrients which is otherwise converted to hemozoin crystal(s) by the parasite to avoid toxicity^{11,79}. In chloroquine resistant strains, accumulation of chloroquine in the acidic digestive vacuole is altered due to point mutations in a gene *pfert* (*Plasmodium falciparum* chloroquine resistance transporter) which codes for a predicted transporter protein *PfCRT* situated in the parasite's digestive vacuole membrane⁸⁰. This leads to exclusion of the drug from the food vacuole⁷⁷. Due to widespread development of *P. falciparum* strains resistant to chloroquine, the use of this drug to treat *falciparum* malaria has been abandoned but it is still employed against malaria due to other species including *P. vivax*⁷⁷. Over 80% of *P. falciparum* strains are currently resistant to CQ^{77,81}. Emergence of CQ resistance was a disaster and halted elimination efforts leading to epidemic malaria resurgence^{15,77,82}. Amodiaquine is useful in cases of low CQ resistance although it has severe liver side effects following prolonged use^{77,83}. Moreover, resistance to amodiaquine has also been observed in Asia^{77,84}.

The arylaminoalcohols act by preventing access of haemoglobin to parasite's food vacuole⁷⁷. Examples include the widely used quinine and mefloquine and less currently used halofantrine and lumefantrine⁷⁷. Their detailed mechanism of action is not well understood, but seems to necessitate the presence of the racemate form⁷⁷. However, drugs in this class are generally associated with multiple albeit reversible side effects but halofantrine leading to severe cardiac side effects^{77,85}. *P. falciparum* strains resistant to the arylaminoalcohols possess a membrane-linked transport protein

called *Plasmodium falciparum* multidrug resistance-1 (*PfMDR-1*) which allows entry of the arylaminoalcohols into the food vacuole where they are inefficient⁷⁷. Resistance to quinine and mefloquine has also been observed in Asia⁸⁶.

The antifolate group consists of drugs that inhibit specific metabolic enzymes in the parasite such as dihydropteroate synthase (includes sulfadoxine) or dihydrofolate reductase (like pyrimethamine and cycloguanil)⁷⁷. The combination of sulfadoxine and pyrimethamine was previously used to replace chloroquine in the 1970s following widespread development of chloroquine resistance. *P. falciparum* has developed resistance to replacement first-line drug sulfadoxine/pyrimethamine (SP)^{77,87,88}. Multiple mutations in the genes that code for these enzymes are responsible for the resistance to antifolates that has been observed in 67% of isolates from South Eastern Asian countries⁸⁹.

The class of 8-aminoquinolines has one viable antimalarial drug, primaquine which is the recommended treatment of *P. vivax* malaria and can be used in chemoprevention against *P. falciparum*⁷⁷. It is efficient against both the sexual stage of parasites as well as their pre-erythrocytic or liver stages⁹⁰. Resistance to primaquine is rare, but has been observed in South East Asia and can be counteracted by increasing the dosage⁹¹.

The next group consists of respiratory chain inhibitors such as atovaquone. These drugs are used for prophylaxis and treatment of uncomplicated *P. falciparum* malaria⁷⁷. Atovaquone has been combined with proguanil as a means to fight development of resistance by parasites⁷⁷. It brings about inhibition of electron transport by targeting the binding site of ubiquinone in the cytochrome bc 1 complex^{77,92}. Resistance to atovaquone is spread across the globe and although combination with proguanil is efficient against certain strains, treatment failure of this combination therapy has been observed⁹³.

Antibiotics like doxycycline and clindamycin also have antimalarial activity⁷⁷. The function by targeting prokaryote-like proteins and prevent the development of the apicoplast during schizogony outside of the erythrocytes⁷⁷. However, they are only efficient following the second round of the intraerythrocytic parasite life cycle and so in the case of acute malaria they still need to be associated with faster acting drugs such as those from the arylaminoalcohols or artemisinin groups⁹⁴. Nevertheless, resistant strains to antibiotics have not yet been observed⁷⁷.

The artemisinin class is made up of several derivatives of artemisinin lactone which is the active principle of the *Artemisia annua* plant⁷⁷. Artemether and artesunate are the most common members of this group⁹⁵. This group consists of the most efficient and swift-acting antimalarial compounds

due to their ability to act against both early and late rings⁷⁷. The details of the mechanism of action of artemisinin are not well comprehended, but targets that have been proposed include food vacuole proteins or an endoplasmic reticulum adenosine-triphosphatase calcium pump (*Pf*ATPase6)⁷⁷. To delay resistance development, it is recommended to use the artemisinins in combination with other antimalarial drugs also known as artemisinin-based combination therapies (ACTs)⁹⁶. The last decade has seen over 90% of countries endemic for *P. falciparum* shifting their national treatment policies to ACTs⁶. However, there are recent reports of parasite resistance to artemisinins in four countries of the Greater Mekong sub region: Cambodia, Myanmar, Thailand and Viet Nam^{6,97}. Delayed-clearance phenotypes have been observed in South East Asia⁹⁸⁻¹⁰⁴ which emphasises the need for new drugs active against drug-resistant *P. falciparum*^{14,15,97,105,106}. The resistance is supposedly linked to a mutation in the adenosine-triphosphatase-6 gene of *P. falciparum*⁹⁷. It would catastrophic if artemisinin resistance spread to India or sub-Saharan Africa following the chloroquine resistance, because currently there is no other antimalarial drug to replace the ACTs in terms of efficacy and tolerability. Hence, there is urgent need for the development of new antimalarial drugs to which the parasite will not easily develop resistance⁷⁷. Unfortunately drugs with new targets are still lacking in the pipeline for new antimalarial drugs⁷⁷.

5.3. Potential of antimicrobial peptides as antimalarial agents

5.3.1. Antiplasmodial antimicrobial peptides

Several antimicrobial peptides (AMPs) from various sources have been investigated for antiplasmodial activity and promising results have been recorded. Three peptide antibiotics isolated from fungi: efrapeptins, zervamicins, and anti amoebicin have been shown to kill *P. falciparum* in culture with micromolar 50% inhibitory concentrations (IC₅₀)¹⁰⁷. Dermaseptins obtained from frog skin and their derivatives are selectively toxic to intraerythrocytic *P. falciparum* and/or infected red blood cells¹⁰⁸⁻¹¹¹. Gomesin isolated from the hemocytes of the spider *Acanthoscurria gomesiana* inhibited the growth of intraerythrocytic *P. falciparum* *in vitro* and compromised the maturity of the sexual and early sporogonic plasmodial stages *in vitro* and *in vivo*¹¹². Scorpin, a proposed cecropin-defensin hybrid from the venom of the African scorpion *Pandinus imperator* totally suppressed both fertilisation and formation of a fertilised zygote (ookinete) in *Plasmodium berghei*, a murine malaria species, at 50 and 3 µM, respectively¹¹³. Tryptophan-N-formylated gramicidin (NFG) was found to be active *in vivo* against *P. berghei* and was non-toxic to the host when administered in lipid

vesicles¹¹⁴. Δ Fd, a *de novo*-designed, cationic, lysine-branched amphipathic, helical peptide was observed to cause both the arrest of intra-erythrocytic *P. falciparum* stages without hemolysis, as well as prompted the premature release of the mature parasites from the red blood cells through membrane lysis⁶³. It was active against both chloroquin-sensitive (3D7) and CQ-resistant (INDO and Dd2) strains of the parasite. Other naturally occurring or modified AMPs with antiplasmodial activity include the phylloseptins isolated from the skin of *Phyllomedusa azurea*^{115,116}, isariins and isaridin, cyclic hexadepsipeptides from the fungus *Isaria*¹¹⁷, cecropin-mellitin^{118,119}, NK-2 a synthetic peptide derived from mammalian defensin NK-lysin⁶⁵, Shiva-1¹²⁰ and Shiva-3¹²¹ which are cecropin-like synthetic peptides, AddLp, a defensin-like protein (DLP) from the myxobacterium *Anaeromyxobacter dehalogenans*¹²², venturamides originating from the marine cyanobacterium *Oscillatoria sp.*¹²³, meucin-24 (cDNA clone from the venom gland of the scorpion *Mesobuthus eupeus*)¹²⁴, Psalmopeotoxin I and II (from the venom of the tarantula *Psalmopoeus cambridgei*)¹²⁵, jasplakinolide obtained from the marine sponge *Jaspis sp.*¹²⁶, apicidin which are cyclic tetrapeptides obtained from cultures of the fungus *Fusarium pallidoroseum*, gramicidin S^{127,128}, gramicidin D¹²⁸, and cyclosporine A¹²⁹. In 1944, Taliaferro *et al.*¹³⁰ discovered the antiplasmodial activity of the tyrothricin crude extract against *Plasmodium gallinaceum* in infected chicken. Previous work in our group revealed that this antiplasmodial activity could mainly be attributed to the six major tyrocidines which were shown to cause an inhibition of the development and life cycle progress of *P. falciparum*¹²⁷. A remarkable IC₅₀ of 580 pM was obtained for the most active tyrocidine preparation containing tyrocidine A, which equally had the highest selectivity index.

5.3.2. Possible mechanisms of antiplasmodial activity

The AMPs have two possible modes of antiplasmodial activity. They are either cytotoxic to both the host and parasite or they are selectively active against the parasite or infected erythrocyte¹²⁴.

Gomesin is a haemolytic peptide, but it is not evident if its antiplasmodial activity results from cytotoxicity to the host cell or if it directly interacts with the parasite¹¹². The dermaseptin S4 derivative K₄S4(1–13) (also known as “P”) effected its antiplasmodial activity through selective cytotoxicity to infected erythrocytes, but an aminoheptanoylated analogue (NC7-P) developed from peptide P showed decreased hemotoxicity and improved antiparasitic activity^{108–111}. The phylloseptins which are cationic peptides have very low haemolytic activity¹¹⁵ and toxicity in mice^{116,131}. Their helical structure induced by membrane environment (from a random coil conformation in aqueous solution) predisposes them for membrane activity thought to be the mode of action

against the malaria parasite ^{115,116,132}. However, their membrane interaction is less with mammalian cells due to differences in lipid composition and external membrane leaflet charge ^{116,133}. Notably, phylloseptin-1 has been observed to promote parasite growth and development at low concentration presumably as a result of homeostasis (an adaptive reaction of cells and organisms to modest and sporadic stress) ¹¹⁶. NK-2, a linear cationic and amphipathic helical peptide with a net charge of +10, was observed to cause selective lysis of IEPM mediated by the increased exposure of phosphatidylserine (PS) induced by the parasite infection ^{65,134}. NK-2 carried out its antiparasitocidal activity through both selective haemolysis and modification of the morphology of the parasite's membrane by forming temporary oligomers in the membrane ⁶⁵. Other possible binding partners of NK-2 include phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) whose membrane distribution is also affected by parasite infection of the red blood cells ^{65,135}. It was observed that labelled NK-2 localised preferentially onto the parasite's membrane which supports the hypothesis that PE is the peptide's binding partner as the membrane of trophozoites has low PS content ⁶⁵. The cationic dimeric peptide Δ Fd was suggested to cause selective lysis of erythrocytes infected with mature parasites (were more vulnerable) leading to premature release of trophozoites and schizonts following incubation of the cultures with the peptides at IC_{70} of 2.5 μ M (concentration leading to 70% parasite growth inhibition) for 24 hours resulting in parasite death ⁶³. The released trophozoites could not develop into schizonts and released schizonts containing knobs of amplified DNA and lacking the typical symmetric rosette appearance proliferated into merozoites that could not reinvade new host cells ⁶³. The peptide was selective for parasitized erythrocytes and up to $16 \times IC_{80}$ did not result in lysis of uninfected erythrocytes ⁶³. Fluorescence microscopy with the labelled peptide showed that it has the ability to cross the IEPM, the parasitophorous vacuolar membrane (PVM), the parasite's plasma and nuclear membranes to interact with DNA of the parasite ⁶³. The similarity of Δ Fd to poly-L-Lys led to the suggestion that their selective activity to infected erythrocytes relies on the differential exposure of anionic lipids on the surface of the infected red blood cells ⁶³. This initial binding then boosted the membrane anionic character reinforcing the peptide binding and speeding up the internalisation of peptides into the cells ⁶³.

Among the non-haemolytic peptides are the cecropins which cause pore formation in membranes predisposed for this by their helical amphipathic and hydrophobic conformation (with no detergent-like membrane lysis and do not require a specific membrane receptor as target) ^{136,137}. The difference in cholesterol content may account for selectivity between infected red blood cells (iRBCs) and uninfected RBCs since cholesterol affects membrane fluidity and dipole potential

which in turn affects the depth of insertion of the cecropins and their channel-forming ability¹³⁶. Cecropin-derivatives and analogues probably share this mechanism of action for example Shiva-1¹²⁰, Shiva-3¹²¹ (60% sequence identity to cecropin B and maintains its amphipathic and hydrophobic structure), and scorpion (cecropin-defensin hybrid)¹¹³. The fungal peptides zervamicins, antiamoebin and efraptins (at high concentration) form transmembrane helical bundles and the proposed mechanism for their antiplasmodial activity is that their ionophore channels cause dissipation of the parasite's mitochondria or plasma membrane potential¹⁰⁷. Although the mitochondrion is not essential for energy metabolism in the intraerythrocytic parasite stages of *P. falciparum* that rely mostly on glycolysis for ATP supply³⁰, the inhibition of mitochondrial activity is suggested to have other physiological implications on the parasite. Their activity at the plasma membrane level is also less likely to be overcome by a resistance mutation in the parasite¹⁰⁷. Selectivity of these peptides is due to the increased permeability of the infected erythrocytes to low molecular weight solutes¹⁰⁷.

It has been demonstrated that true ionophores (mobile ion carriers) that transport monovalent cations are the most active and selective in terms of inhibiting *P. falciparum* growth *in vitro* and cytotoxicity to mammalian cells¹²⁸. The quasi-ionophore (channel-forming) linear gramicidin D that is produced as part of the tyrothricin complex along with tyrocidines by *Bacillus aneurinolyticus*¹³⁸ and is specific to H⁺ had the highest *in vitro* antiplasmodial activity and lowest cytotoxicity¹²⁸. N-formylated gramicidin exert their antiplasmodial activity by inserting into the erythrocyte and form monovalent cation (K⁺) channels and do not act on the parasite itself^{114,139}. Rather it is the consequent loss of K⁺ ions that kills the parasites. Selectivity for the gramicidins is partly due to the fact that the life span of the channels relies on membrane surface pressure, which is decreased during plasmodial infection of the red blood cell leading to longer lasting channels^{140,141} and thus more ionophores are integrated in the membrane of the infected erythrocytes¹²⁸.

NC7-P was different from the predecessor dermaseptin S4 derivative in its ability to dissipate membrane potential and is thought to cause increased proton permeability in the parasite and thus shifting decreasing the intracellular pH without causing haemolysis¹¹⁰. It was suggested that the membrane-tropic and lipophilic character of NC7-P allows it to cross the erythrocyte membrane and result in nonspecific permeabilisation of the parasite's membrane¹¹⁰. Differences in the transmembrane potential between host and parasite membranes account for selectivity¹¹⁰. This mechanism of action was confirmed by the absence of parasite stage selectivity by the peptide contrary to NC7-P which was more active towards trophozoite- than ring-infected erythrocytes

relying on the increased change in host cell membrane structure caused by the more matured parasites ¹¹⁰. The rhodaminated-derivative (fluorescent label) of the peptide was only observed within infected erythrocytes associated specifically with the parasites and the host compartment of inclusions (turbulovesicular structures) and Maurer's clefts ¹¹⁰. In normal erythrocytes the labelled peptide was localised on the host cell membrane.

Psalmopeotoxin I and II ¹²⁵, and meucin-24 ¹²⁴ are non-haemolytic and non-toxic to bacteria and fungi but have antiplasmodial activity. Meucin-24 is a linear cationic peptide thought to act through initial selective adhesion to the membrane of infected erythrocytes followed by an affinity-driven uptake across the erythrocyte membrane to the parasite ¹²⁴. The detailed molecular mechanism of this uptake process is not fully understood especially as they possess an acidic molecular face that prevents antibacterial and antifungal activity but preserves antiplasmodial activity ¹²⁴. Jasplakinolide obtained from the marine sponge *Jaspis sp.* was found to interfere with red blood cell invasion by merozoites via their actin polymerising and filament-stabilising properties, thus reducing the parasitaemia of *P. falciparum* ¹²⁶.

Among the peptides with non-haemolytic antiplasmodial activity are small cyclic AMPs such as apicidin which has an inhibitory activity at low nanomolar scale towards protozoal histone deacetylase (HDA). It was found to be active against *P. berghei* in mice when administered orally. HDA is a vital nuclear enzyme involved in regulation of transcription. The enzyme controls the continuous acetylation/deacetylation of the ϵ -amino group of particular histone Lys residues which is necessary transcription. Apicidin's 2-amino-8-oxo-decanoic moiety seemingly imitates the ϵ -amino acetylated Lys residues of histone substrates, leading to effective reversible inhibition of HDA. The inhibition of HDA impedes cell proliferation ^{7,142}.

Another group of small cyclic peptides with significant antimalarial activities is the tyrocidines (Trcs) produced by the Gram-positive bacteria *Bacillus aneurinolyticus*. The activity of the peptides was linked to hydrophobicity with the most hydrophobic peptides Trc A and A₁ ¹²⁷ as well as tryptocidines showing the highest antiplasmodial activity ¹⁴³. Two hypotheses were proposed for the exceptional nanomolar antiplasmodial activity of the tyrocidines. First, the permeability of infected erythrocyte membranes may be disturbed by the tyrocidines thus affecting the ability of the parasite to regulate small molecule homeostasis in its host cell, leading to a progressive inhibition of the development of the parasite ¹²⁷. Second, the infected cell or parasite itself may possess a tyrocidine sensitive and specific molecular target causing a delay in maturation ¹²⁷. The latter hypothesis is

supported by the low values of anti-plasmodial IC₅₀s and relatively high cytotoxic concentrations obtained¹²⁷. The later parasite stages were found to be the most sensitive to the Trc activity interrupting re-invasion of new erythrocytes, by halting the development of schizonts to merozoites¹⁴³. The non-lytic MOA of the Trcs involves a disruption of parasite morphology leading to arrest of the parasite in the schizont stage which was related to a significant rise in parasite dynamin levels, a protein that is related to endocytosis mediators in mammalian cells. It was also suggested that the Trcs also affected haemoglobin digestion in both rings and trophozoites as the levels of undigested haemoglobin in the treated parasites was higher than in the controls¹⁴³. This was associated with the observed decrease in plasmepsin levels, which is a protein recognised for its role in the food vacuole and haemoglobin digestion in the *Plasmodium*¹⁴³. Unlike the Trcs, a related cyclic decapeptide with 50% sequence identity to the Trcs, gramicidin S (GS) which is a quasi-ionophore, has much reduced antiplasmodial activity and selectivity^{127,128}. Contrary to the Trcs, the antiplasmodial activity of gramicidin S is mediated merely by selective lysis of the infected erythrocyte¹². For this reason the membranolytic MOA of the Trcs has been linked to the VOLfP pentapeptide unit which it shares with GS, while its non-lytic MOA is associated with the ArArNQAr unit which contains the variable dipeptide unit (Ar = aromatic amino acid)¹²⁷.

5.4. Concluding remarks

As elaborated using the above examples, AMPs are able to cross the multiple membrane barriers in infected erythrocytes to exert their effects on the intracellular parasite and their activity is primarily at the membrane level, a mechanism of action less prone to parasite resistance strategies^{107,108,143}. Furthermore, most AMPs with antimalarial activity contain unusual amino acids and are constrained in a ring structure which renders them less vulnerable to proteases and thus increasing their bioavailability¹⁰⁷. Due to these attributes, the purpose of this part of the present project is to investigate the probable development of small cyclic AMPs (Trcs and RW-peptide analogues) as viable antimalarial drug leads (Chapter 6). The detailed elucidation of the mechanism of action(s) and identification of the target(s) of the Trcs will also facilitate the search for alternate therapeutic targets and approaches, as well as the design of Trc derived lead compounds (Chapter 7).

5.5. References

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Chapter 6

Role of antimicrobial peptide structure on antimalarial activity: tyrocidine and *cyclo*(RRRWF_n) analogues

6.1. Introduction

Among the diseases that occur in tropical countries, malaria is one of the most frequent causing approximately 225 million new infections and about a million deaths annually world-wide¹. The majority of malaria cases are caused by the unicellular protozoan and obligate intracellular parasite *Plasmodium falciparum*. Therefore, *falciparum* malaria is presently a public health problem especially in developing countries. Malaria control is part of the sixth Millennium Development Goal (MDG), Target 6.C – “to have halted by 2015 and begun to reverse the incidence of malaria and other major diseases”². Resistance by the parasites to antimalarial drugs has been the major drawback to the control of malaria and is also a current public health concern. For example chloroquine (CQ) which used to be the first line antimalarial drug because of its unprecedented benefits of low cost, low occurrence³ of side-effects and high efficacy³ has been abandoned due to an upsurge of resistant *P. falciparum*. The lurking threat of resistance by the parasites to the artemisinins which are the major components of the currently recommended antimalarial drugs (artemisinin combination therapies or ACTs) makes urgent the need to develop new drugs that have novel targets in *Plasmodium falciparum*⁴⁻⁸.

Among the current approaches to develop new antimalarial drugs, the modified erythrocyte membrane following infection by the malaria parasite is exploited as a target⁹⁻¹³. Some of the changes that occur in the infected erythrocyte plasma membrane (IEPM) include increased fluidity^{12,14-16}, decreased surface pressure¹³, novel pores that boost membrane permeability¹⁷⁻²¹, transfer of the anionic phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the bilayer which changes the membrane lipid asymmetry and the infected erythrocyte tends to be similarly anionic like bacterial cells^{9,22,23}. These erythrocyte membrane changes increase the likelihood that membrane active compounds like cationic antimicrobial peptides (AMPs) may have selective activity towards the infected RBC membrane^{24,25}.

Indeed AMPs have been investigated as a probable source of future antimalarial drugs due to some valuable properties including those considered for their use as new antimicrobials²⁶⁻²⁹. Though originating from various sources, AMPs exist as families of isoforms which has allowed for the fine-tuning of their physicochemical characteristics to improve their antimicrobial

properties using structure-activity relationship (SAR) studies and display the role of parameters such as amphipathicity, charge, hydrophobicity supramolecular organization, and conformational flexibility on the interaction with microbial targets ²⁶.

One of the first studies using peptides as antimalarials was on the tyrothricin complex which showed comparable activity to quinine towards *Plasmodium gallinaceum* in infected chicken by Taliaferro *et al.* ³⁰. The linear peptide gramicidin D that is produced as part of the tyrothricin complex along with tyrocidines by *Bacillus aneurinolyticus* ³¹ is a quasi-ionophore (channel-forming) specific to H⁺ ions that has shown high *in vitro* antiplasmodial activity and low cytotoxicity ³². It has also been shown that linear gramicidin A and its analogues inhibit growth of all life cycle stages of the parasite *in vitro*, and this activity tends to increase with the age of the parasite ³³. Tryptophan-N-formylated gramicidin A (NFG) was found to be active *in vivo* against *P. berghei* and was non-toxic to the host when administered in lipid vesicles ³⁴. N-formylated gramicidin exert their antiplasmodial activity by inserting into the erythrocyte and form monovalent cation (K⁺) channels but do not act on the parasite itself ^{34,35}. Rather it is the consequent loss of K⁺ ions that kills the parasites. Selectivity for the gramicidins is partly due to the fact that the life span of the channels relies on membrane surface potential, which is decreased during plasmodial infection of the red blood cell leading to longer lasting channels ^{36,37} and thus more ionophores are integrated in the membrane of the infected erythrocytes ³². Among the AMPs evaluated for antimalarial activity, small cyclic peptides have shown promising ability and have the added advantage of being better potential drugs than their linear analogues. This advantage is in terms of improved antimicrobial activity ^{38,39}, enhanced stability, resistance of proteolytic degradation, improved receptor selectivity, better bioavailability and available conformational proximity for receptor binding ^{40,41}. Some cyclic AMPs that have been shown to have antiplasmodial activity include the fungal metabolites isariins and isaridin which are cyclic hexadepsipeptides from the fungus *Isaria* ⁴² as well as apicidins which are cyclic tetrapeptides obtained from cultures of the fungus *Fusarium pallidoroseum* ⁴³. The cyclic peptide gramicidin S which has 50% sequence homology to the tyrocidines produced by *Aneurinibacillus migulanus* (previously classified as *Brevibacillus brevis*) ⁴⁴ and is another quasi-ionophore was also antiplasmodial although its activity was lower in comparison to that of gramicidin D and the tyrocidines ^{29,32}. Previous work in our group revealed the antiplasmodial activity of the six major tyrocidines which were shown to cause an inhibition of the development and life cycle progress of *P. falciparum* ²⁹. A remarkable IC₅₀ of 580 pM was obtained for the most active tyrocidine preparation containing tyrocidine A, which equally had the highest apparent selectivity index. The SAR analyses from this study deduced that anti-*P. falciparum*

activity of Trcs and selectivity for the infected erythrocyte or parasite was determined by overall hydrophobicity (measured as HPLC retention time) and steric factors (in terms of the side chain surface area)²⁹. Substitution of Orn in the conserved VOLfP pentapeptide by Lys led to a significant decrease in antiplasmodial activity, as well as decrease in haemolytic activity and toxicity. This suggested that antiplasmodial activity of Trcs may depend on their membrane activity which also relies on the chemical character of charged group in the cyclic peptide structure²⁹. Another relevant residue for Trc antiplasmodial activity indicated by the investigators was Phe in the first position of the FfNQY pentapeptide unit²⁹. Nevertheless, this study only evaluated Trc activity towards a single *P. falciparum* strain and considered only the six major Trcs in commercial tyrothricin. The findings from this study need to be refined by increasing the number of analogues as well as testing activity towards drug resistant strains of *P. falciparum* to determine parameters required for selective activity towards these drug resistant strains.

The promising activity of small cyclic AMPs warrants a further understanding of the structural parameters that determine their high antimalarial activity and improves their selectivity so that future drug development can involve a lead peptide from these libraries. We therefore aimed to assess the antimalarial activity of selected cyclic antimicrobial peptides. A library of small synthetic cyclic peptides, *cyclo*(RRRWWFV) and its analogues of (RW-peptides), will be tested in order to determine their potential and structural determinants for antiplasmodial activity and selectivity. The RW-peptides are a library of synthetic cyclic derivatives of the hexapeptide (Ac)-RRWWRF-NH₂ (R-, W-rich or RW-peptides), carrying substitutions of Trp and/or Arg (Chapter 3, Table 3.4). The RW-peptides and Trcs have common characteristics such as cyclic, aromatic and cationic character, as well as their active conformation which consists of two β turns, which is the smallest possible β -sheet^{39,45,46}. However, unlike Trcs the RW-peptides have been found to have low haemolytic activity⁴⁶⁻⁴⁸. By evaluating and comparing sequence and physicochemical property modifications among the RW-peptides we would gain valuable insight into their potential and structural motifs important for antimalarial activity. In addition, an extended library of the Trcs, grouped as Trc A and Trc C analogues, were tested against different *P. falciparum* strains namely chloroquine sensitive (CQS) strains *P. falciparum* D10 and 3D7 and against the chloroquine resistant (CQR) strain *P. falciparum* Dd2 to further elucidate structural determinants for antiplasmodial activity and selectivity. The Trc A [*cyclo*(fPFfNQYVOL)] analogues include Trc A₁ (ornithine (Orn) to Lys substitution), Trc B (Phe to Trp substitution), Phc A (Tyr to Phe substitution), sTrc A(Q-O) with a Gln to Orn substitution, sTrc AOMe₃ with an Orn to trimethylated-Orn substitution, and Tpc A (Tyr to Trp

substitution). The Trc C [*cyclo*(fPWwNQYVOL)] analogues investigated include Trc C₁ (Orn to Lys substitution), Trc B₁ (Orn to Lys substitution and D-Trp to D-Phe), Trc B (D-Trp to D-Phe substitution), and Tpc C (Tyr to Trp substitution); all natural analogues. It should be noted that Trc B doubles as both a Trc A and a Trc C analogue.

6.2. Materials

Tyrothricin (extracted from *Bacillus aneurinolyticus*), gramicidin S (from *Brevibacillus brevis* (Nagano)), and Corning Incorporated[®] cell culture cluster non-pyrogenic polypropylene microtiter plates, bis-benzamide trihydrochloride (Hoechst stain) and trifluoroacetic acid (TFA, >98%) were obtained from Sigma (St. Louis, USA). All the chemicals used to prepare the RPMI-1640 culture media (RPMI 1640 medium, glucose, HEPES, albumax II, hypoxanthine, NaOH, gentamycin, and sodium bicarbonate), sodium lactate, potassium chloride, NaCl, L-lactic acid, nitro blue tetrazolium (NBT), phenazine ethosulfate (PES), 3-acetylpyridine adenine dinucleotide (APAD), D-sorbitol, Dulbecco's modified Eagle's Medium (DMEM), 0.4% trypan blue solution, and DNA interchelator Giemsa stain mixture were obtained from Sigma-Aldrich (St. Louis, MA, USA). The synthetic tyrocidines were supplied by GL Biochem (Shanghai) Ltd, China. Resazurin reagent (CellTiter Blue[™]) was from Promega (Madison, WI). Sterile red standard cap 250 mL Cellstar tissue culture flasks, sterile Cryo.s PP tubes and sodium hydrogen phosphate were from Greiner Bio-One GmbH, Germany. Glycerol (AnalaR grade) was obtained from BDH Chemicals Ltd. Triton X-100 came from BDH Laboratory Supplies, Poole, England. Tris-HCl buffer was obtained from Boehringer Mannheim or Roche. Acetonitrile (ACN) (HPLC-grade, far UV cut-off) came from Romill Ltd. (Cambridge, UK). To obtain analytical grade water, water was filtered from a reverse osmosis plant via a Millipore Milli-Q water purification system (Milford, USA). Ethanol (>99.8%) was supplied by Merck (Darmstadt, Germany). Culture dishes and 0.2 µm-25 mm sterile cellulose acetate membrane syringe filters were obtained from Lasec (Cape Town, South Africa) and microtiter plates (Nunc[™]-Immuno Maxisorp) were from AEC Amersham (Johannesburg, South Africa). Falcon[®] tubes were from Becton Dickson Labware (Lincoln Park, USA). Foetal calf serum and penicillin–streptomycin were from Gibco BRL (Gaithersburg, MD, USA). Sterile VacuCap[®] 90PF filter unit w/0.8/0.2 µm Supor[®] membrane was obtained from Pall Corporation (Pall Europe Ltd, UK). SYTO[®] 9 green-fluorescent nucleic acid stain and HCS LipidTOX[™] neutral lipid stain were obtained from Invitrogen (Carlsbad, USA). Whole A⁺ blood stored in anticoagulant (citrate phosphate dextrose) containing enriched erythrocyte fraction in saline adenine-glucose-mannitol red blood cell preservation solution was donated by the Western Cape Blood services (or National Health Laboratory Services in South Africa). Asexual erythrocytic stage chloroquine sensitive (CQS)

Plasmodium falciparum D10 and 3D7 as well as chloroquine resistant (CQR) *P. falciparum* Dd2 (Asian/African) were benevolently supplied by Prof. Peter Smith from the University of Cape Town, Division of Pharmacology. COS-1 cells were provided by Prof. Pieter Swart from the University of Stellenbosch, Department of Biochemistry.

6.3. Methods

6.3.1. Parasite culturing

6.3.1.1. Culture media preparation

The composition of the media used to culture *P. falciparum* was RPMI-1640 (10.4 g/L) supplemented with glucose (4 g/L), HEPES (6 g/L), albumax II (5 g/L), hypoxanthine (0.4 g/L, dissolved previously in 1 mL of 1 N NaOH), gentamycin (50 mg/L), and sodium bicarbonate (2.1 g/L). The media was made up in analytical quality water; the pH was adjusted to 7.2 – 7.3 and sterility was achieved by filtering through a 0.2 µM filter^{49,50}.

6.3.1.2. Preparation of blood

Anonymous A⁺ donor blood (300 mL enriched erythrocyte fraction containing 63.0 mL citrate phosphate dextrose anticoagulant and 100 mL saline-adenine-glucose-mannitol red blood cell preservation solution) from the Western Cape Blood services (or National Health Laboratory Services in South Africa) conforming to relevant legislation and ethics were utilised during all experiments of this study.

Routinely A⁺ erythrocyte enriched blood of not older than two weeks was used since the *P. falciparum* cultures did not develop well on older erythrocytes⁵¹. Prior to use, the blood was washed twice in parasite culture media by centrifugation at 1300×g for 5 minutes per wash followed by decantation of supernatant (containing plasma and buffy coats if present).

6.3.1.3. *P. falciparum* culturing procedure

Culturing was carried out using normal sterile techniques according the methods of Trager and Jensen⁵⁰ and Lambros and Vanderberg⁵². Freezer stock cultures of CQS (D10 and 3D7) and CQR (Dd2) *P. falciparum* were thawed in a water bath at 37 °C and the cultures were transferred to 50 mL falcon tubes. The osmotic potential of the thawed parasite freezer stocks in glycerol was progressively reduced by stepwise dilution in three saline solutions prepared in analytical quality water namely solution A (12% NaCl), solution B (1.8% NaCl), and solution C (0.9% NaCl and 0.2% glucose)⁵³. Five volumes of solution A was first added drop wise with swirling to 1 volume of thawed cultures and allowed to stand for 5 minutes followed by slowly adding 10

mL of solution B. Subsequently the combination was centrifuged at 400×g for 5 minutes followed by aspiration of the supernatant. Ten mL of solution C was gradually added to the residual pellet with moderate swirling. The mixture was centrifuged at 400×g for 5 minutes followed by aspiration of the supernatant. The residual culture was washed with 20 mL of culture media by centrifuging at 400×g for 5 minutes and then returned to culture media for culturing.

Culturing involved incubating the parasitized blood (at 3-4% haematocrit, i.e. 3-4 mL blood/100 mL media) in sterile red standard cap 250 mL Cellstar tissue culture flasks at 37 °C under a gas mixture of 3% O₂, 4% CO₂, and 93% N₂ without shaking. The initial culture from freezer stocks was left to stand for 3-4 days. Culturing was carried out continuously for not longer than three months from one stock culture to avoid genetic modification⁵¹. The parasite development and parasitemia (number of parasites in the infected erythrocytes expressed as a percentage of the normal erythrocytes) were followed by taking samples of the cultures to prepare thin blood smears on microscope slides for staining with Giemsa and viewing under oil immersion at the 100× objective lens of a light microscope according to the methods of Reilly *et al.*⁵⁴. From 0.5% parasitemia, the media was replaced daily by centrifugation at 750×g for 3 minutes, aspiration of the supernatant and addition of fresh media.

It was important to maintain the cultures at the same developmental asexual stage and this was achieved by addition of 5 volumes of 5% D-sorbitol to 1 volume of pelleted parasite infected erythrocytes. *In vitro* synchronisation was carried out only when most of the parasites were at the ring stage (because the membranes of erythrocytes containing ring stage parasites are less permeable to solutes⁵⁵) according to the methods of Lambros and Vanderberg⁵². Following addition of sterile 5% D-sorbitol to the pelleted erythrocytes, the mixture was gently swirled and allowed to stand for 5 minutes in a water bath at 37 °C. Next, the mixture was centrifuged at 750×g for 3 minutes, the supernatant was aspirated and the pellet was washed once with culture media by centrifugation at 750×g for 3 minutes and aspiration of supernatant. The pellet was then returned to culture media and maintained according to the culturing procedures mentioned above. Synchronisation was limited to once a week due to the finding by Makowa⁵¹ that D-sorbitol favours the development of resistance to chloroquine.

Once a high parasitemia (>10%) was achieved, the cultures were either frozen away in glycerol at -80 °C for culture preservation or used for dose response assays. Freezing involved ring stage parasites only as their membranes are more robust according to the method of Diggs *et al.*⁵³. The glycerolyte medium in which the parasites were stored at -80 °C was made of 1.6% sodium lactate, 0.03% KCl, 1.38% sodium dihydrogen phosphate, 57% glycerol adjusted to pH of 6.8.

The solution was sterile filtered through 0.22 µm filters. One volume of glycerolyte medium was added to 3 volumes of pelleted infected erythrocytes in a drop wise manner with constant swirling. The mixture was allowed to stand for 5 minutes followed by addition of 2 volumes of glycerolyte medium. The cultures were stored in Cryo.s PP tubes by storage at -80 °C until required for use.

6.3.2. Peptide preparation

The major Trcs from commercial tyrothricin were purified and characterised as previously described in Chapter 2 according to the methods of Rautenbach *et al.*²⁹ and Eyéghé-Bickong⁵⁶. The other natural analogues (Tpc C, Trc A and Phc A) were extracted from cultures of *Bacillus aneurinolyticus* ATCC 8185 under specific nitrogen supplementation conditions, purified and characterised as described in Chapter 2. The synthetic Trcs were verified for purity and characterised as described previously in Chapter 2.

The analytically weighed purified peptides were subsequently used to analytically prepare stock solutions of 2.00 mM (purified peptides) or 2.00 mg/mL (Trc mixture) with 40% v/v ethanol in analytical quality water (Trcs) or with analytical quality water (gramicidin S and RW-peptides). Subsequently the stock solutions were used to construct quadrupling dilution series in polypropylene 96 multi-well plates containing the supplemented RPMI-1640 culture medium used for culturing the *P. falciparum*, but lacking albumax II. Preparation of stock solutions and subsequent dilution was performed about 30 minutes prior to each assay.

6.3.3. Determination of antimalarial and haemolytic activities of peptides

The antimalarial assays were carried out according to the method of Nkhoma *et al.*⁵⁷. Once the cultures reached a parasitemia of 5-15%, they were synchronised four days prior to the day of the assay as described above (*Section 6.3.1.3*) which allowed the cultures to recover from the stress caused by the D-sorbitol. On the day of the assay, the parasitemia was determined following observation by light microscopy after Giemsa staining of a thin blood smear from the culture (*Section 6.3.1.3*). The cultures at young trophozoite stage were diluted to 2% parasitemia and 2% haematocrit by addition of fresh RPMI medium and uninfected erythrocytes. This culture suspension was distributed in 96-well culture plates at 90 µL/well. The diluted peptides (see *Section 6.3.2*) were added to the cultures in triplicate at 10 µL/well to give a total volume of 100 µL/well. Total growth and total haemolysis were obtained by adding 10 µL of mixture of culture medium and solvent used in peptide preparation and 10 µL 1 mM gramicidin S (GS) respectively to the parasite suspension. The plates were incubated at 37 °C for 48 hours under an atmosphere

of 3% CO₂, 4% O₂ and 93% N₂. Assays were carried out in at least three biological repeats each consisting of three technical repeats to ascertain reproducibility.

To determine the haemolytic activity of the peptides, the plates were centrifuged using a swing-out rotor at 200×g for 3 minutes. The supernatant was diluted 1:8 in analytical quality water in separate 96-well plates and after mixing, the absorbance of the suspension was determined at 405 nm. The rest of the culture suspension was frozen away at –20 °C until determination of the residual lactate dehydrogenase activity of the parasites by the Malstat assay as adapted from Gomez *et al.*⁵⁸.

For the Malstat assay, the plates were transferred to –80 °C for 1 hour following overnight storage at –20 °C to ensure that all the suspensions in each well of the 96-well plate were frozen. The plates were then thawed at room temperature. The Malstat reagent consisted of 200 µL Triton X-100, 2 g L-lactic acid (substrate), 0.66 g Tris-HCl buffer and 0.011 g of 3-acetylpyridine adenine dinucleotide (APAD) (coenzyme for parasite lactate dehydrogenase) in 100 mL analytical quality water set to a pH of 9.0⁵⁷. The lactate dehydrogenase reaction was initiated by addition of a second solution, NBT/PES solution composed of 1.96 mM nitro blue tetrazolium (NBT) and 0.24 mM phenazine ethosulfate (PES) which was always protected from light. A 15 µL aliquot of the thawed and properly mixed suspension from the assay plates was added to 100 µL of the Malstat reagent, followed by addition of 25 µL of the NBT/PES solution. The reaction mixtures were then incubated at room temperature in the dark for 30 minutes followed by spectrophotometric measurement of the absorbance of the reduced APAD at 620 nm using a Model 680 Microplate reader from BioRad.

6.3.4. Determination of toxicity

COS-1 cells were cultured in DMEM containing 0.9 g/L glucose, 0.12% NaHCO₃, 10% foetal calf serum and 1% penicillin–streptomycin at 37 °C and under an atmosphere of 5% CO₂⁵⁹. Twenty hours before the assay, the cells were transferred to 96-well plates at a density of 1.5×10⁴ cells/well. The peptides from the dilution series were added to each well and incubated for 24 hours. The cell viability was measured by adding 20 µL/well resazurin reagent (CellTiter Blue™), followed by 4 hour incubation at room temperature in the dark and measurement of the absorbance at 560 nm and 600 nm in the microplate reader²⁹.

6.3.5. Assessment of dose-response data

The data obtained from spectrophotometric measurement of the plates following the lactate dehydrogenase (Malstat) assay were converted to percentage growth inhibition using equation 1

below as described by Rautenbach *et al.*⁶¹. The background was obtained from control wells in which the parasites were killed with 100 μ M GS while total growth was from the wells that were not submitted to peptides.

$$\% \text{ growth inhibition} = 100 - \frac{100 \times (A_{620} \text{ of well} - \text{Average } A_{620} \text{ of background})}{A_{620} \text{ of growth wells} - \text{Average } A_{620} \text{ of background}} \quad (1)$$

The background for the haemolysis evaluation (equation 2) was from wells in which the uninfected erythrocytes were lysed using 100 μ M GS while growth control was from the wells with uninfected erythrocytes that received no peptides²⁹.

$$\% \text{ haemolysis} = 100 - \frac{100 \times (A_{405} \text{ of well} - \text{Average } A_{405} \text{ of background})}{A_{405} \text{ of growth wells} - \text{Average } A_{405} \text{ of background}} \quad (2)$$

The percentage COS-1 cell death (CellTiter Blue™ assay) was similarly calculated using equation 3 from absorbance values.

$$\% \text{ COS-1 inhibition} = 100 - \frac{100 \times (A_{570}/A_{600} \text{ of well} - \text{Average } A_{570}/A_{600} \text{ of background})}{\text{Average } A_{570}/A_{600} \text{ of growth wells} - \text{Average } A_{570}/A_{600} \text{ of background}} \quad (3)$$

All the dose–response assay data were evaluated using GraphPad Prism 4.03 (GraphPad Software, San Diego, USA) followed by non-linear regression and sigmoidal curves were fitted (having variable slope and a constant difference of 100 between the top and bottom plateau). Equation 4 was used to fit the dose-response curves.

$$Y = \text{bottom} - (\text{top} - \text{bottom}) / (1 + 10^{\log \text{IC}_{50} \times \text{Activity slope}}) \quad (4)$$

Following calculation of the 50% *P. falciparum* inhibitory concentration (IC₅₀), 50% haemolytic concentration (HC₅₀) and 50% lethal concentration (LC₅₀) for COS-1 cells⁶¹, the selectivity indices were determined as the ratios HC₅₀/IC₅₀ and LC₅₀/IC₅₀²⁹.

6.3.6. Interaction between chloroquine and selected tyrocidine analogues

In order to determine the nature of Trc-CQ interaction, IC₅₀ values were derived from dose-response curves of fixed ratios of Trc and CQ for fractional inhibition concentration (FIC) determination according to an adaptation of the methods by Chawira and Warhurst⁶² and Fivelman *et al.*⁶³.

CQ at 400 ng/mL (0.2 μ M) and 50 μ M peptide were used to prepare the following concentration combination ratios: 0.2:0, 0.15:12.5, 0.1:25, 0.05:37.5 and 0:50 in supplemented RPMI-1640 culture medium used for culturing the *P. falciparum*, but lacking albumax II. Each drug:peptide combination was then serially diluted in the culture medium. The CQ, peptide or drug:peptide combination (10 μ L) was added to 90 μ L of the cell suspensions, incubated for 48 hours at 37 °C under an atmosphere of 3% CO₂, 4% O₂ and 93% N₂ and analyzed for growth inhibition as previously described using the Malstat assay. The IC₅₀ values and standard error of the mean for the various CQ-peptide combinations were determined from the dose-response curves plotted and analysed using GraphPad Prism[®] 4.03 (GraphPad Software, San Diego, USA). A minimum of three technical repeats and two biological repeats were carried out for each combination experiment.

Two fractional inhibition concentration (FIC) values were calculated for each of the five CQ-peptide combination ratios, one for CQ and the other for the peptide according to equations 2 and 3 below from Bell⁶⁴ and Makowa⁵¹.

$$\text{FIC}_{\text{CQ}} = \text{IC}_{50}^{\text{CQ + Peptide (in combination)}} / \text{IC}_{50}^{\text{CQ (alone)}} \dots \dots \dots (2)$$

$$\text{FIC}_{\text{peptide}} = \text{IC}_{50}^{\text{CQ + Peptide (in combination)}} / \text{IC}_{50}^{\text{peptide (alone)}} \dots \dots \dots (3)$$

The FIC values were used to construct isobolograms on Graphpad Prism[®] 4.03 and to compute the fractional inhibition concentration index (FICI) which is the sum of FICs of CQ and each peptide using equation 4 below^{51,65}.

$$\text{FICI} = \text{FIC}_{\text{CQ}} + \text{FIC}_{\text{peptide}} \dots \dots \dots (4)$$

The magnitude of the FICI determined the nature of the CQ-peptide interaction as being either synergistic (FICI < 1.0), antagonistic (FICI > 1.0) or additive (FICI = 1.0)⁶⁴⁻⁶⁶. However, a more conservative interpretation requires that FICI \leq 0.5 indicates absolute synergy, 1 > FICI > 0.5 shows slight synergy, FICI = 1 means additive activity, 1 < FICI < 4 is interpreted as non-interactive to slight/moderate antagonism, while FICI \geq 4 indicates absolute antagonism^{51,64,65,67}. The shape of the isobolograms also provided an indication of the nature of the interactive effect of CQ and the peptides with a concave curve for synergy, a linear line for an additive to non-interactive effect or convex curve for antagonism with deviation of the curves from the additivity line indicating the strength of the interactive effect⁶⁴.

6.4. Results and Discussion: Part I – *cyclo*(RRRWFW) analogues

6.4.1. Antimalarial and haemolytic activities

A total of 14 analogues of the hexapeptide *cyclo*(RRRWFW) (Table 6.1) were evaluated for both their growth inhibitory activity towards the chloroquine sensitive *P. falciparum* D10 and their haemolytic activity towards human erythrocytes. As described in Chapter 3 these peptides were grouped according to modifications in the hydrophobic and/or polar domains of the parent peptide.

Table 6.1 Analogues of *cyclo*(RRRWFW) evaluated for antiplasmodial and haemolytic activities

Group Number	Domain altered	Analogue Sequence	Code	Amino acid substitution
1	Hydrophobic	c(RRRWFW)	c-WFW	Parent compound
		c[RRR(b3-hW)F(b3-hW)]	c-b3hW	W to b3-hW
		c(RRRWWW)	c-WWW	F to W
		c(RRRWIW)	c-WIW	F to Igl
		c[RRR(1MeW)F(1MeW)]	c-1MeW	W to 1MeW
		c[RRR(5MeW)F(5MeW)]	c-5MeW	W to 5MeW
		c[RRR(Bal)F(Bal)]	c-Bal	W to Bal
2	Polar	c(RRRWFW)	c-WFW	Parent sequence
		c(KRKWWW)	c-KRK	R to K
		c(KKWWKF)	c-KW	R to K
		c(RRRWWW)	c-WWW	Parent sequence
		c(KKKWWW)	c-KWW	R to K
3	Polar and hydrophobic (ring size)	c(RRRWFW)	c-WFW	Parent sequence
		c(RRRRWFWF)	c-WFW 8	Addition of R and F
		c(RRRRRWFWFW)	c-WFW 10	Addition of RR and FW
		c(RRRRRRWFWFWF)	c-WFW 12	Addition of RRR and FWF
4	Polar and hydrophobic (destruction of clusters)	c(KKKWWW)	c-KWW	Parent sequence
		c(KWKWKW)	c-WKW	Altered sequence
		c(RRRWWW)	c-WWW	Parent sequence
		c(RWRWRW)	c-WRW	Altered sequence

In general, all of the hexapeptides had low haemolytic activities (< 20% erythrocyte lysis) at up to 200 μ M (Fig. 6.1). Increasing the size of the peptides from eight through to twelve residues with concomitant increase in hydrophobicity (see Chapter 3, Table 3.6) resulted in increased haemolytic activity (40-70% erythrocyte lysis) at the same concentration. Nevertheless, the haemolytic activity observed was significantly less than that of the Trcs (refer to discussion in Part II of this chapter). This observation is similar to that made in previous studies⁶⁸ which underlined the fact that activity towards the neutral membranes of human RBCs will rely more

on hydrophobicity and amphipathic character of the peptides than on electrostatic interactions. Hence, increase in peptide hydrophobicity favours interaction with and lytic activity towards erythrocytes.

The RW-peptides displayed much lower antiplasmodial activity to that of the tyrocidines (study of Rautenbach *et al.*²⁹ and this study, Part II) (Tables 6.2 and 6.3). Most of the peptides gave only 20-40% growth inhibitory activity towards *Plasmodium falciparum* D10 cultures at 12.5 μM (Fig. 6.1). However, we observed a substantial increase in antimalarial activity with increase in hydrophobicity (refer to Table 3.6, Chapter 3) within the groups of analogues. In group 1 with hydrophobic domain modification through the use of Trp and Trp unnatural analogues without changing the number of residues, the activity trend was (c-WFW, c-b3hW, c-WWW, c-WIW) < (c-1MeW, c-5MeW, c-Bal). Only c-Bal in group 1 gave a measurable IC_{50} value (38 μM) (Table 6.2).

Table 6.2 Summary of the growth inhibitory activity parameters toward the chloroquine sensitive strain of *Plasmodium falciparum* (D10) and the haemolytic activity parameters towards human erythrocytes of the *cyclo*(RRRWW) analogues. Concentrations are given in μM . Every value denotes the average of **n** biological repeats (number of repeats given in brackets), with 3 technical repeats per assay \pm SEM given in 2 significant figures.

Group	Peptide	Parasite infected erythrocytes $\text{IC}_{50} \pm \text{SEM}$ (n)	Uninfected human erythrocytes $\text{HC}_{50} \pm \text{SEM}$ (n)
1	Parent sequence c-WFW	> 100 (3)	> 100 (3)
	c-b3hW	> 100 (2)	> 100 (2)
	c-WWW	> 100 (3)	> 100 (3)
	c-WIW	> 100 (3)	> 100 (3)
	c-1MeW	> 100 (2)	> 100 (2)
	c-5MeW	> 100 (2)	> 100 (2)
	c-Bal	37.9 ± 1.10 (2)	> 100 (2)
2	c-KW	> 100 (2)	> 100 (2)
	c-KWW	> 100 (3)	> 100 (3)
	c-KRK	> 100 (3)	> 100 (3)
3	c-WFW8	13.4 ± 0.67 (3)	> 100 (3)
	c-WFW10	9.0 ± 0.19 (2)	> 100 (3)
	c-WFW12	7.0 ± 0.48 (2)	> 100 (3)
4	c-WKW	> 100 (3)	> 100 (3)
	c-WRW	> 100 (3)	> 100 (3)

The one physicochemical property that groups the residues 1MeW, 5MeW and Bal is the fact that they have higher hydrophobicity than W which explains the increase in the activity of the peptides containing these residues over c-WFW. However, when compared to activity of c-WWW, only that of c-Bal was significantly higher. Although b3hW also has higher

hydrophobicity than W, there was no significant increase in the activity of c-b3hW over c-WWW probably indicating that increase in size and flexibility of the backbone ring in b3hW⁶⁹ diminishes the propensity of hydrophobicity to increase antiplasmodial activity. The modification of the cationic domain in group 2 by changing R to K did not affect the activity. In group 3, the increase in peptide size by the addition of residues to both the hydrophobic and cationic domains yielded higher activity. The activity trend in this group was c-WFW < c-WFW 8 < c-WFW 10 < c-WFW 12 which corresponds to the order of increasing hydrophobicity (refer to Addendum Table 6.7 for more details). This is consistent with the fact that the greater the interaction of the peptides with the erythrocyte membrane the better their translocation into the cell and the greater their access to the intra-erythrocytic parasites. Scrambling of residues to offset the aromatic and cationic clusters in group 4 did not significantly affect the antimalarial activity (Addendum Table 6.8).

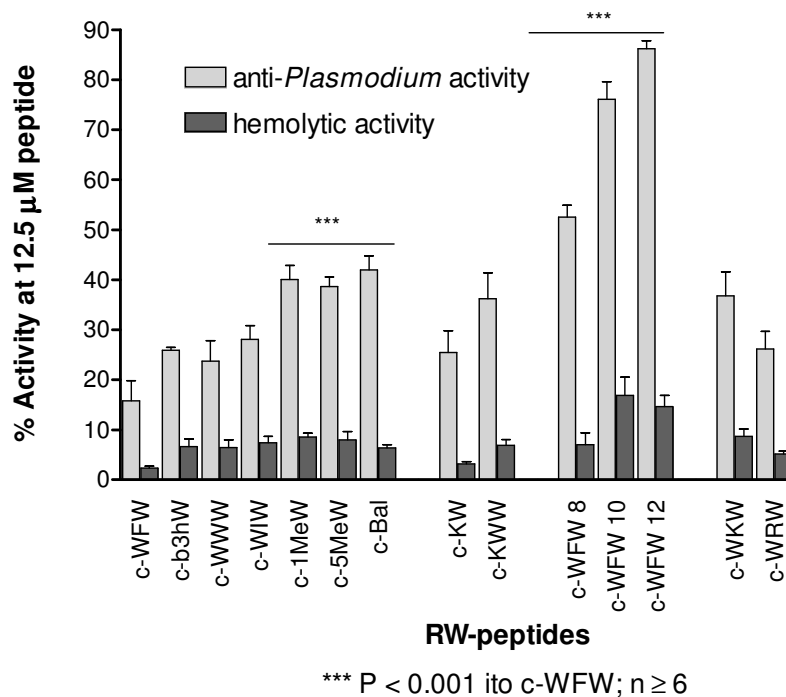


Figure 6.1 Comparison of the activity of RW-peptides towards *Plasmodium falciparum* D10 and human erythrocytes at 2% (v/v) parasitemia and 2% v/v haematocrit. Antiplasmodial activities of the peptides at 12.5 μM were compared within their specific subgroups using the Newman-Keuls multiple comparison test (Addendum Tables 6.5, 6.6, 6.7, and 6.8).

From these studies it is clear that there is a definitive prerequisite for hydrophobic character and good interaction with the erythrocyte (correlating with increased haemolytic activity) to ensure antimalarial activity. However, these peptides were designed to have low haemolytic activity and high specificity for bacteria. It is therefore, not unexpected that these peptides did not show

potent antimalarial activity and we hence decided to focus the rest of study on the tyrocidines and analogues (Part II).

6.5. Results and Discussion: Part II – tyrocidine A and C analogues

6.5.1. Antimalarial activity and cytotoxicity

Eleven Trc analogues, the Trc complex (called Trc mix) and GS were tested for their antiplasmodial activity against the chloroquine (CQ) sensitive *P. falciparum* D10. They were also concomitantly evaluated for their toxicity to human erythrocytes by employing a 48-hour dose-response assay. The activity was evaluated according to the IC₅₀ values (concentration that gives 50% parasite growth inhibition⁶⁰) for antiplasmodial activity and HC₅₀ values (concentration that gives 50% haemolysis^{29,60}).

As previously observed, the natural Trc analogues had significantly higher antiplasmodial activity than GS^{29,70}, ranging between 48 and 600 nM. The haemolytic activity of the natural Trc analogues towards infected erythrocytes was in the range of 5-9 μM comparable to that of GS at 6.2 μM (Fig. 6.2). If only the lowest range of the IC₅₀ values were considered for each of the tyrocidines, the antiplasmodial activities were comparable to previous antimalarial activity studies done by our group^{29,70}. The activity of the re-evaluated Trc C analogues and GS were comparable to results from Spathelf⁷⁰ and Rautenbach *et al.*²⁹ but there was about a 10-50 fold increase in the IC₅₀ values for Trc A and TrcA₁. One reason for this difference could be differences in the assay conditions which may influence the self-assembly/aggregation properties of these more hydrophobic peptides which in turn affected their antiplasmodial activity. Studies by Rautenbach *et al.*²⁹ involved peptides in the aggregated form as a single stock solution was used and was repeatedly dried by blowing off the ethanol solvent without freeze-drying which may have trapped the peptides in the aggregated conformation (M. Rautenbach, personal communication). Moreover, dilution and application of peptides was done immediately before assay which may have limited the disaggregation of the peptides. It was also observed that if the same peptide was dissolved in media and left to stand for 24 hours, it gave a >10× lower IC₅₀ value against *P. falciparum* D10 than when used immediately after preparation in the same solvent (M. Rautenbach, personal communication). In the previous studies it was found that the IC₅₀ values had a significant correlation with retention time on a C₁₈ RP-HPLC column which represents peptide hydrophobicity, with the activity increasing with increase in hydrophobicity²⁹. Alternatively, the Trc A and TrcA₁ sample preparations in previous studies^{29,70} may have contained minute amounts of the highly active gramicidin A and/or Tpc A which may have eluded detection with the less sensitive ESMS and HPLC analyses used in these studies^{29,70}.

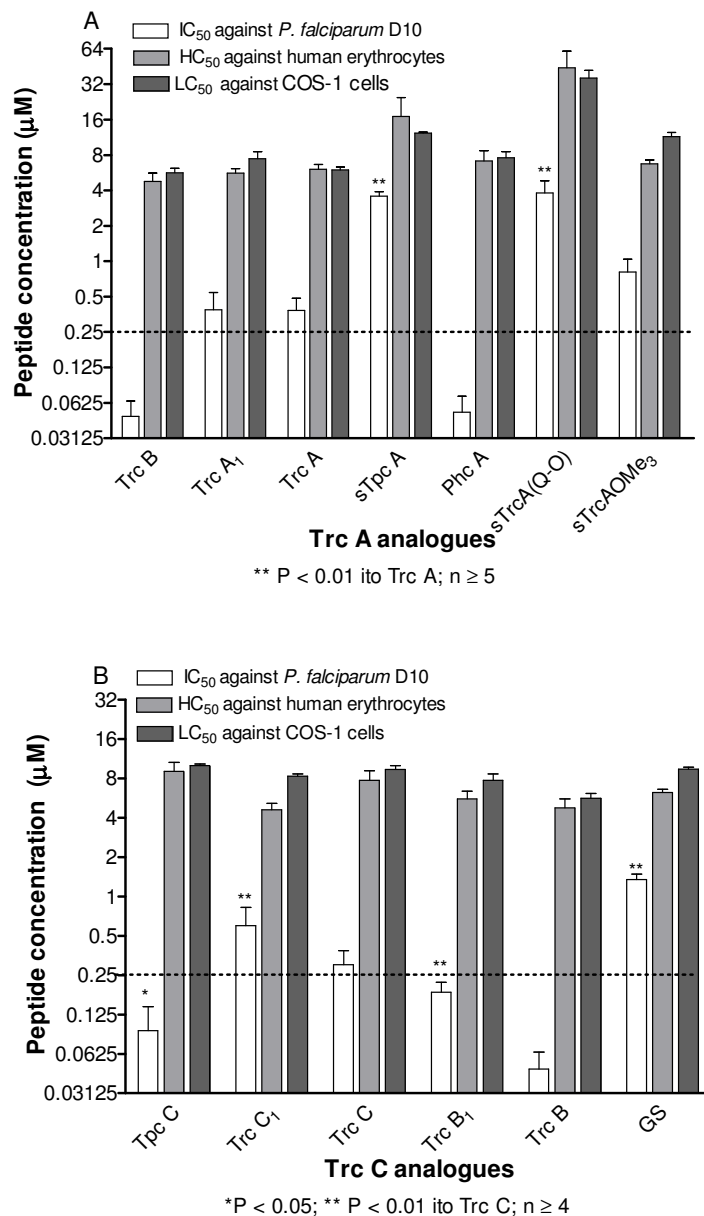


Figure 6.2 Comparison of the activity towards the chloroquine sensitive *P. falciparum* D10, and toxicity towards infected human erythrocytes and COS-1 cells **A.** of Trc A analogues **B.** of Trc C analogues. GS was included as a reference peptide. Antiplasmodial activity parameters of the peptides were compared to that of Trc A or Trc C using the Bartlett's test for equal variances (Parameter data are given in Addendum Table 6.9).

Gramicidin A has picomolar activity³³ against *P. falciparum* and the late eluting tyrocidines, Trc A and TrcA₁ tend to co-elute with gramicidin A during C₁₈-HPLC (this study and study by Eyéghé-Bickong⁵⁶). In this study the quality control of the peptides was done with one of the most sensitive high resolution mass spectrometers linked to state-of-the-art UPLC, which ensured that none of the peptides in the study contained any trace of gramicidins (refer to Chapter 2).

According to the overall results, the lowest average IC₅₀ values at < 250 nM were recorded for Trc B, Trc B₁, Tpc C, and Phc A. Spathelf⁷⁰ also noted high activity of partially purified Tpc B and Tpc C. Trc B, Tpc C and Phc A also had the highest selectivity (>100 fold) for activity towards the parasite (Fig. 6.2).

6.5.1.1. Trc A analogues

Only two of the Trc A analogues, Trc B and Phc A exhibited an average IC₅₀ < 250 nM against *P. falciparum* D10 (Fig. 6.2A). Comparing the IC₅₀ values for activity against *P. falciparum* D10 using the Bartlett's test for equal variances, the Trc A analogues showed the following sequence: (Phc A, Trc A, Trc B, Trc A₁, sTrc AOMe₃) > (sTpc A, sTrc A(Q-O)).

The HC₅₀ of the doubly charged sTrc A(Q-O) was significantly higher than that of the other analogues. Previous studies by Kohli *et al.*³⁸ also found that a doubly charged Trc A analogue in which D-Phe⁴ was substituted by D-Orn residue had a minimum haemolytic concentration of 60 μM in comparison to 4 μM for the parent Trc A. Therefore, evidently increasing charge reduces haemolytic activity of the peptide due to the reduced affinity between the more charged peptide and the zwitterionic phospholipids in the membrane of human erythrocytes³⁸. Synthetic Trc A with a trimethylated Orn was similarly active as the parent sequence Trc A (*cyclo*[fPFfNQQYVOL]) and the other active natural analogues. Therefore, the N^ε-trimethyl Orn groups that are involved with creation of electrostatic interactions with anionic phospholipids leading to tighter membrane interactions, as well as the increase in bulkiness and hydrophobicity of the charged residue with loss of hydrogen bonding character⁷¹ did not translate into higher antimalarial activity in this case. Unlike Lys N^ε-trimethylation at certain positions of the antimicrobial cecropin A-melittin hybrid peptide⁷¹ Orn N^ε-trimethylation did not result in improvement of Trc A selectivity. There was nearly 10-fold decrease in parasite growth inhibition activity following substitution of the Tyr in Trc A with a Trp in the synthetic Tpc A which was also three-fold less toxic to the red blood cells. This could indicate a preference for either a Tyr or Phe in position 7 with a bulky Trp leading to steric interference with membrane/target interaction. However, the haemolytic activity of sTpc A is significantly lower than expected for a tryptocidine if compared with Tpc C, which could indicate that this synthetic peptide preparation may not be a true mimic of the natural Tpc A, possibly due to residual trifluoroacetic acid in the preparation.

When Gln in Trc A was replaced by an Orn in the synthetic Trc A(Q-O) with an increase in charge to +2, this resulted in a 10-fold decrease in antiplasmodial activity. Thus, increasing charge did not favour increase in activity, similar to what we observed for its antilisterial activity

(Chapter 3). GS with a similar charge also showed lower antiplasmodial activity. This could be because the transfer of the anionic PS (phosphatidyl serine) from the inner leaflet to the outer leaflet of the bilayer of the red blood cell membrane modifies the membrane lipid asymmetry rendering the infected erythrocyte to be similarly anionic like bacterial cells^{9,22}. Thus the peptides that are able to interact better with the anionic infected erythrocyte plasma membrane (IEPM), the parasitophorous vacuole membrane (PVM) which surrounds the parasite and the parasite's membrane^{24,72} will be trapped and have less ability to reach the purported internal target²⁹. Although not statistically significant, the presence of Phe at position 7 in place of Tyr in Phc A improved the antimalarial activity up to 7 fold while that of Trc B (Phe³ to Trp³ substitution) was 8 fold higher than that of Trc A. Thus, the aromatic amino acids (Trp, Phe and Tyr) in the Trc analogues are pivotal in determining antimicrobial activity as previously seen with *Listeria* in Chapter 3. Phe has a greater lipophilicity compared to Trp and has also been found to integrate the membrane deeper while the indole and phenol analogues (Trp and Tyr) are shallower^{73,74}. On the other hand, Trp which is the largest of the aromatic amino acids should display better anchoring also as a result of the formation of hydrogen bonds between its NH-group and lipid carbonyl groups⁷⁵. However, a very tight membrane association does not always translate into better activity and there is need for an optimal amphipathicity which still allows for efficient membrane integration and self-assembly into active lytic complexes and/or translocation to an internal molecular target^{76,77}.

Activity of Trc A analogues against *P. falciparum* D10 is therefore sensitive to the amphipathic balance, with hydrophobic interactions being essential while an increase of electrostatic interactions leads to a decrease in Trc A activity. Trapping of the Trc A(Q-O) in the membrane could counteract their mode of action. A further prerequisite seems to be size and hydrogen bonding ability of residue 7 with Tyr and Phe preferred over Trp.

6.5.1.2. *Trc C analogues*

Three of the Trc C analogues, Tpc C, Trc B and Trc B₁ exhibited an average IC₅₀ < 250 nM against *P. falciparum* D10 as target (Fig. 6.2B). Using the Bartlett's test for equal variances to compare IC₅₀ values, the average IC₅₀ values against *P. falciparum* D10 of the Trc C analogues decreased in the following sequence: (Tpc C, Trc B, Trc B₁) > Trc C > Trc C₁. All Trc C analogues had comparable haemolytic activity (Fig. 6.2B) therefore modifying activity of this group led to a modification of the selectivity index.

Replacing Orn with Lys in the conserved VOLFP pentapeptide significantly decreased the activity of Trc C₁ (*cyclo*[fPWwNQYVKL]), indicating that there is a preference for an Orn

above Lys in the sequence. This result correlated with the observations of Rautenbach *et al.*²⁹. Trc C and TrcC₁ have Trp-Trp in the variable aromatic dipeptide unit and the replacement of D-Trp⁴ with D-Phe⁴ to Trc B and B₁ which have a Trp-Phe aromatic unit led to an increase in the activity. Phe and/or Lys contribute to tighter membrane binding and Trp and/or Orn amend this binding and limit peptide trapping in the membrane⁷⁷. Lys with a butylene moiety in its side chain, has been shown to carry out “snorkeling” into membranes^{29,77,78}. The tighter membrane interaction will in this case lead to lower antiplasmodial activity. Substituting the larger and more rigid D-Trp residue with a greater hydrogen-bonding ability at position 4 by the relatively smaller and non-polar D-Phe resulted in an increase in activity. It indicates that activity in this series is determined by membrane interaction since Phe has been found to integrate the membrane deeper than Trp and also Phe has a greater lipophilicity compared to Trp^{73,74}. Peptides containing Phe will have a preference for the water-phospholipid interface and should be more active^{29,74,75,79}. As mentioned earlier a very tight membrane association does not always translate into better activity and there is need for an optimal amphipathicity which still allows for efficient membrane integration and self-assembly into active lytic complexes and/or translocation to an internal molecular target^{76,77}. However, the mutation of the Tyr⁷ in Trc C to Trp⁷ decreased the IC₅₀ > 3-fold which indicates that Trp⁷ could be important in modulating antiplasmodial activity. This could also be more likely a sequence specific effect as the substitution of Tyr by Trp in Trc A to obtain Tpc A rather led to a loss of activity.

The LC₅₀ values towards COS-1 cells ranged from 6 to 42 μM, with the least toxic being synthetic Trc A having a Gln to Orn substitution (Fig. 6.2). The profile is quite similar to that obtained for haemolytic activity towards the infected human erythrocytes.

6.5.1.3. Strain susceptibility

The variability in terms of peptide preparation was eliminated at the onset of this study as stock peptide preparations were carefully prepared for each peptide and aliquoted to ensure repeatability. However, we observed variability in the IC₅₀ values obtained on different assay dates to some of the Trc analogues (Fig 6.3), while the HC₅₀ values, as well as the inhibition parameter data for the control peptide, GS were highly repeatable (Fig. 6.3). The results for GS antiplasmodial activity towards the CQ sensitive *P. falciparum* D10 strain was similar to that obtained by Rautenbach *et al.*²⁹ towards the more CQ sensitive *P. falciparum* 3D7 strain. In order to eliminate any errors that may have occurred in parasitemia determination, the IC₅₀ values of all the peptides were normalised to that of GS as an internal standard. After normalising the data, similar variability was found (results not shown). Specifically, the more

active Trc A analogues: Trc A, TrcA₁ and Phc A and the highly active Tpc C showed much more variability than the other Trc C analogues (Fig 6.3A).

We observed that the IC₅₀ values became higher as we persisted with the same parasite culture over time or used different starter cultures (Fig. 6.3B). From Figure 6.3B it can be observed that unlike Tpc C that demonstrated a steady decrease in activity over time towards the parasites, Trc A and Phc A had a reciprocal trend to each other while GS activity remained constant. It was assumed therefore, that such variability in the activity was specific to the Trc analogues and was culture derived, which correlated to observations made from previous studies in our group for chloroquine activity towards the chloroquine sensitive (CQS) *P. falciparum* D10 strain⁵¹. Makowa⁵¹ determined that the synchronisation process which involves treating the cultures with 5% v/v D-sorbitol solution induced the development of a CQ resistant (CQR) strain. It was hypothesised that a similar reason may account for the increased and variability of IC₅₀ values observed for the Trc analogues. To test this hypothesis, we tested the activity of Trc A and the most active peptides (Trc B, Trc C, Tpc C and Phc A; IC₅₀ < 250 nM) against the CQR strain *P. falciparum* Dd2 (Indochina).

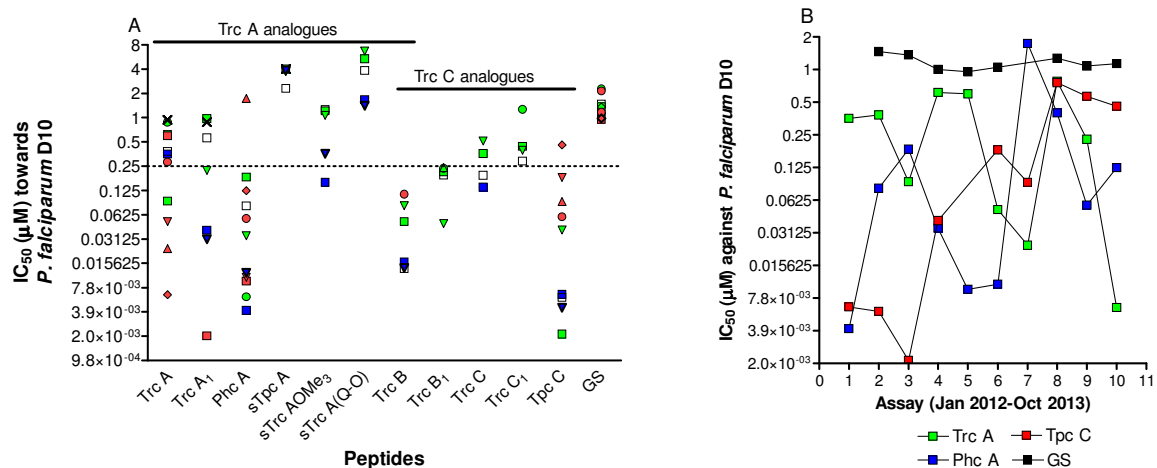


Figure 6.3 Variability of IC₅₀ values of Trc analogues obtained at different dates. **A.** Each shape represents a specific assay date. Blue symbols represent assays done in 2010, unfilled squares: 2011, green symbols: 2012, black and red symbols 2013. Dotted line shows 250 nM **B.** Activity variability trends for peptides Trc A, Tpc C and Phc A. GS was included as reference compound.

There was a significant decrease (2-18 fold) in activity against the CQR strain in comparison to the CQS strain for all the peptides, with degree of the decrease in activity as follows: Trc B > Phc A > Trc A > Tpc C > Trc C >> GS (Fig. 6.4A and 6.4B, Table 6.3). Therefore, the analogues with D-Phe at position 4 are more susceptible to the effect of CQ resistance in the Dd2 strain than the analogues possessing D-Trp⁴. It is possible that the aromatic residue at position 4 is relevant to the process of target interaction in the food vacuole and that the larger size and

lower lipophilicity of Trp compared to Phe^{73,74} makes it less susceptible to mutations linked with CQ resistance in the Dd2 strain. GS, as expected had the least resistance index of one (Table 6.3) since its activity is linked to lysis of the erythrocyte membrane. The slight difference noticed could be due to different effects on the IEPM by the different parasite strains. GS, which has a predominantly lytic activity^{80,81}, was slightly less active against the Dd2 strain which could indicate that this strain may have a different effect on the erythrocyte membrane leading to the loss of the selective lytic activity of GS. This can explain some of the loss in activity of the Trcs and analogues. If a secondary target, other than the primary action of the Trcs on the erythrocyte membrane which is the first point of interaction, is also changed or the interaction with the peptides is limited this probably would lead to such a major loss in activity.

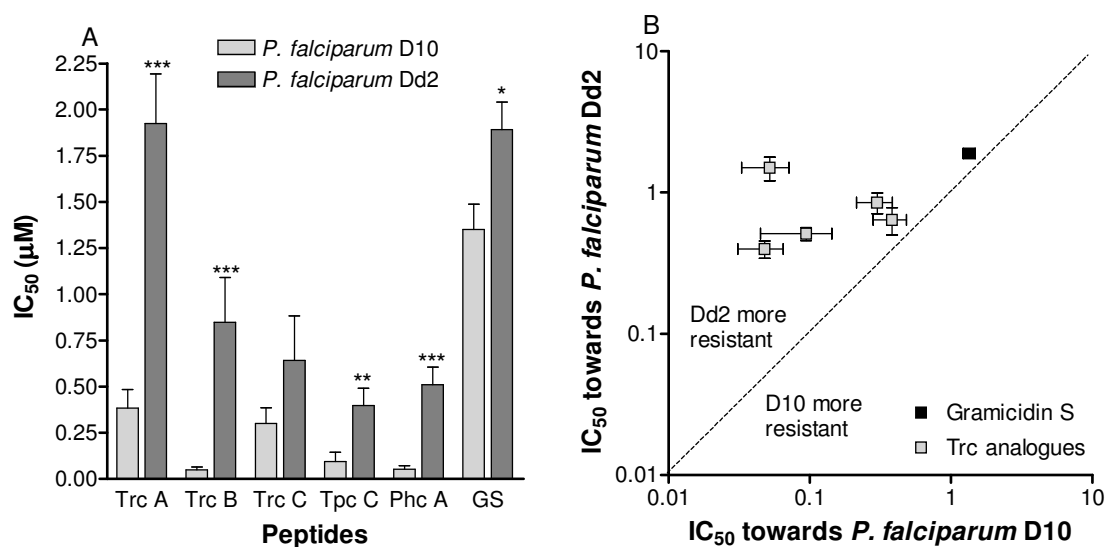


Figure 6.4 Comparison of the activity of selected Trc analogues towards the chloroquine sensitive strain *P. falciparum* D10 and chloroquine resistant strain *P. falciparum* Dd2. **A.** Bars represent the average of 3-11 biological repeats (each consisting of triplicate technical repeats) and standard error of the mean. Parameter data are given in Addendum Table 6.9. Statistical comparison of activity towards the two strains was done with the student t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). **B.** Direct comparison of IC₅₀ against *P. falciparum* D10 and *P. falciparum* Dd2.

Rautenbach *et al.*²⁹ suggested that such a putative target relies on the D-Phe⁴ of the aromatic dipeptide unit and Orn in the conserved pentapeptide. The 18-fold loss in activity of Trc B and 10-fold loss in activity of Phc A is consistent with this hypothesis. The least affected peptide was Trc C with Trp⁴ in the aromatic dipeptide unit. However, Tpc C was also found to be about 4-fold less active, similar to the 5-fold loss of Trc A activity. This similarity could be that the changes in the membrane target important for interaction and the putative internal target(s) has a differential influence on these analogues. These results further support our hypothesis (refer to Chapter 7 for more information) that at least for the most active Trcs there could be a parasite

target related to the digestive vacuole since the phenomena in the CQR strain that result in reduced accumulation of CQ⁸² in the vacuole also affects the Trcs leading to loss in activity.

According to Fidock *et al.*⁸³ CQ resistance arises due to:

- 1) Change in CQ influx or efflux at the plasma membrane of the intra-erythrocytic parasite⁸³
- 2) Change of H⁺ flux or CQ influx at the parasite's food vacuole membrane^{84,85}
- 3) Decreased access of CQ to its target, haematin which is produced in the food vacuole following haemoglobin digestion^{86,87}
- 4) Enhanced detoxification of CQ-haematin complexes mediated by glutathione⁸⁸

The molecules that have been suggested to be implicated in CQ resistance include P-glycoproteins, a Cl⁻ channel regulator, a Na⁺/H⁺ cation exchanger, a food vacuole H⁺ pump, a trait that lessens CQ access to haematin, and glutathione S-transferase or a similar molecule implicated in heme detoxification⁸³. The main mutation that brings about CQ resistance is a Lys⁷⁶ to Thr⁷⁶ mutation in the PfCRT which is suggested to lead to a loss of a positive charge in a putative pore-forming transmembrane domain (in the food vacuole membrane) facilitating the escape of diprotonated CQ from the parasite's food vacuole^{3,89}. It is known that some of the compounds that rely on the drug/metabolite transporter family, of which PfCRT is a member, are amino acids, weak bases and positively charged organic ions⁸⁹ as well as small peptides which are probably produced following degradation of haemoglobin in the food vacuole⁹⁰. Mutated PfCRT has been shown to directly transport the radio-labelled peptide YPWF-NH₂ rich in aromatic amino acids⁹¹. It is currently evident that PfCRT is also implicated in the resistance of *P. falciparum* to several antimalarial drugs such as quinine and quinidine⁹² as well as changed susceptibility to amodiaquine⁹³, halofantrine and may be mefloquine⁹⁴.

Another transporter protein found in the food vacuole membrane and known to contribute to CQ resistance is PfMDR1 (*Plasmodium falciparum* multi-drug resistance transporter 1). PfMDR1 also known as Pgh-1 is the P-glycoprotein aforementioned^{83,95} that is implicated in CQ resistant phenotypes^{90,93} such as the CQR strain Dd2, a clone obtained from the CQR W2 strain first isolated from Southeast Asia^{96,97}. An Asn to Tyr substitution at position 86 in the PfMDR1 has been suggested to contribute to elevated CQ resistance in the Dd2 parasite as this was observed to completely change the substrate affinity of PfMDR1 from a quinine and CQ transporting ability to a halofantrine transporting function following expression of the gene in *Xenopus laevis* oocytes⁹⁸. The role of PfMDR1 in CQ resistance is suggested to involve diminished uptake of the drug into food vacuole due to mutations in the transporter⁹⁰. It is not certain what the physiological role of PfMDR1 is but speculations are taken from homologues of the protein in mammals and plants where they function in transport of lipids, ions and glucosides^{99,100}. There

is also evidence that *PfMDR1* functions in transport of small peptides which arise from incomplete catabolism of haemoglobin from the food vacuole to the cytoplasm¹⁰¹. The presence of an analogous MDR1 in mammalian cells led to cross-resistance to a synthetic tripeptide (N-acetyl-leucyl-leucyl-norleucinal)¹⁰², gramicidin D (15 residue linear peptide)^{103,104} and valinomycin (a cyclic decapeptide)¹⁰³. These facts suggest that the Trcs may be among the substrates for the CQ transporters and may have an intracellular molecular target in the digestive vacuole. In a subsequent experiment, we also evaluated the activity of our Trc A preparation and the two newly evaluated and among the most active analogues towards *P. falciparum* D10 (Phc A and Tpc C) against the most CQ sensitive strain *P. falciparum* 3D7. The increased susceptibility to CQ of this strain was verified with an $IC_{50} = 15$ nM (in this study) as opposed to $IC_{50} = 56$ nM in strain D10 and $IC_{50} = 280$ nM in strain Dd2 (established by Makowa⁵¹) (Table 6.3). We observed that strain 3D7 showed higher susceptibility to Trc A, but similar susceptibility to Phc A and Tpc C relative to the D10 strain. Phc A had the lowest activity which was comparable to that of chloroquine (Table 6.3).

Table 6.3 Differences in susceptibility of three strains of *Plasmodium falciparum* to Trc analogues with GS as reference peptide. All IC_{50} values are the means \pm SEM in nM and numbers in brackets indicate the biological repeats, each with at least triplicate technical repeats. The resistance index is given as a ratio of activity against CQ resistant strain Dd2 to activity against CQ sensitive strain D10.

Compounds tested	Parasite strains			Resistance index ²² $IC_{50}Dd2/IC_{50}D10$
	<i>P. falciparum</i> 3D7	<i>P. falciparum</i> D10	<i>P. falciparum</i> Dd2	
Trc A	178 \pm 9 (6)	383 \pm 101 (11)	1920 \pm 270 (3)	5
Trc B	nd	48 \pm 17 (6)	848 \pm 243 (3)	18
Trc C	nd	300 \pm 85 (4)	642 \pm 242 (3)	2
Phc A	15 \pm 4 (2)	52 \pm 19 (10)	511 \pm 96 (3)	10
Tpc C	160 \pm 31 (4)	94 \pm 49 (9)	398 \pm 94 (3)	4
GS	1500 \pm 160 (2)	1350 \pm 137 (11)	1890 \pm 149 (3)	1
CQ	15 \pm 0.11 (2)	56 \pm 10 (2)	280 \pm 18 (6) *	5

* Value from Makowa⁵¹

The IC_{50} of Trc A at 178 nM was much higher than 0.58 nM obtained by Rautenbach *et al.*²⁹. As previously mentioned, this difference could be due to peptide preparation disparities. The activity of GS was quite consistent in all strains tested, which corroborates the hypothesis that the Trcs have an internal target(s) in *P. falciparum*.

6.5.2. Evaluation of antimalarial activity of tyrocidines in combination with chloroquine

There is possibly at least one common mechanism of action between some of the Trcs and chloroquine as observed from the significant differences in the antiplasmodial activities of the most active Trc analogues against different strains of *P. falciparum* characterised by their susceptibility to CQ. We carried out similar 48-hour dose response assays using different combinations of Trc A, Phc A or Tpc C with chloroquine to evaluate for interaction between the compounds (Table 6.4) and the possible role of the peptide sequence in case of interaction.

Table 6.4 Summary of the evaluation of the mode of *in vitro* interaction between chloroquine and Trc A, Phc A and Tpc C in different combinations towards CQ sensitive *P. falciparum* D10. FICs from activity in terms of growth inhibition as determined by Malstat assay and calculated FIC indices were obtained from three biological repeats of experiments done in triplicate.

Peptide	Combination ratio CQ:peptide	CQ FIC	Peptide FIC	CQ:peptide FIC index	Isobologram shape
Tyrocidine A	0.15:12.50	1.77 ± 0.29	1.99 ± 0.36	3.76 ± 0.65	Convex (antagonism)
	0.10:25.00	0.66 ± 0.02	0.95 ± 0.18	1.60 ± 0.20	
	0.05:37.50	0.13 ± 0.10	1.21 ± 0.29	1.35 ± 0.37	
Phencylidine A	0.15:12.50	1.09 ± 0.03	2.44 ± 0.20	3.53 ± 0.22	Convex (antagonism)
	0.10:25.00	0.33 ± 0.15	2.82 ± 0.48	3.15 ± 0.33	
	0.05:37.50	0.05 ± 0.02	0.49 ± 0.22	0.54 ± 0.23	
Tryptocidine C	0.15:12.50	0.36 ± 0.07	0.90 ± 0.40	1.26 ± 0.36	Convex (antagonism)
	0.10:25.00	0.23 ± 0.08	1.24 ± 0.26	1.47 ± 0.28	
	0.05:37.50	0.08 ± 0.03	0.93 ± 0.16	1.02 ± 0.17	

All three peptides tested were mostly only slightly antagonistic with chloroquine according to the shape of the isobolograms and the FIC indices i.e. $1 < FICI < 4$ (Table 6.4). Tpc C had the least antagonistic interaction. However, the two Phe-Phe containing peptides Trc A and Phc A showed overt antagonism ($FICI \sim 4$) at 0.15:12.5 CQ:peptide ratio. One of the ways in which these peptides could antagonise CQ action is by interfering with the food vacuole membrane integrity and leading to leakage of CQ from the food vacuole; thereby limiting the accumulation of CQ that is essential to its antimalarial activity. It is also possible that the aromatic residues may play

a role in obstruction of *Pf*CRT-mediated CQ uptake as previously observed for the transporter protein functionally expressed in oocytes of *Xenopus laevis* in the case of the radio-labelled linear tetrapeptide YPWF-NH₂⁹¹.

These results support the hypothesis that the Trcs and CQ may share at least one of their targets, such as the parasite food vacuole, and when both compounds are present in this target there is an antagonistic effect.

6.6. Conclusion

The RW-peptides had very weak antiplasmodial activity in general which was primarily non-lytic. Optimal peptide hydrophobicity and membrane interaction favoured improved activity without increase in haemolysis particularly observed in the hexapeptide analogue *cyclo*[RRR(Bal)F(Bal)] in which the unnatural Trp analogue Bal (β -(benzothien-3-yl)-alanine) replaces Trp in the parent sequence *cyclo*(RRRWFW). However, increase in size and flexibility of the backbone ring in b3hW⁶⁹ diminishes the propensity of hydrophobicity to increase antiplasmodial activity. Their activity was however, less than that of the Trcs and they were less toxic to human erythrocytes than the Trcs.

Generally, the Trcs showed potent antimalarial activity that was sequence specific and primarily non-lytic. The results confirm previous findings that natural Trc analogues had significantly higher antiplasmodial activity than GS^{29,70}. The activity of the Trcs in terms of the IC₅₀ (concentration that yields 50% growth inhibition as determined by the Malstat assay) ranged from 48 and 600 nM. A further prerequisite seems to be size and hydrogen bonding ability of residue 7 with Tyr and Phe preferred over Trp. Improved peptide selectivity was achieved in the Trc C library by the replacement of D-Trp⁴ with D-Phe⁴ in Trc C and TrcC₁ that have Trp-Trp in the variable aromatic dipeptide unit. There was also a requirement for a Trp at position 7 for improved activity and selectivity.

We observed a culture derived loss of susceptibility of the chloroquine sensitive strain *Plasmodium falciparum* D10 parasites that was sequence specific. This observation along with the correlation of CQ resistance in the Dd2 parasites with resistance to the Trcs as well as the antagonism between Trcs and CQ when present together especially at higher CQ concentration, supposed that the Trcs and CQ share a common intracellular target that is probably located in the food vacuole of the parasite. The aromatic residue D-Phe at position 4 and Orn in the conserved pentapeptide are probably involved in target interaction as previously proposed by Rautenbach *et al.*²⁹.

From the structure to activity relationship in all libraries we propose the following sequence as lead peptide for future antimalarial libraries: *cyclo*[VOLfP(Bal)fNQ(Bal)]. We suggest that including the Trp analogue Bal in the Trc structure could potentially improve the antimalarial activity, without increasing haemolytic activity as observed with the Arg and Trp-rich peptides.

6.7. References

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6.8. Addendum

Table 6.5 Summary of the P-values from the Newman-Keuls multiple comparison test percentage growth inhibition of *Plasmodium falciparum* D10 at 12.5 μ M for Group 1 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05

	c-b3hW	c-WWW	c-WIW	c-1MeW	c-5MeW	c-Bal
c-WFW	ns	ns	ns	0.001	0.001	0.001
c-b3hW		ns	ns	ns	ns	ns
c-WWW			ns	ns	ns	0.05
c-WIW				ns	ns	ns
c-1MeW					ns	ns
c-5MeW						ns

Table 6.6 Summary of the P-values from the Newman-Keuls multiple comparison test percentage growth inhibition of *Plasmodium falciparum* D10 at 12.5 μ M for Group 2 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05

	c-WFW	c-KW	c-KWW
c-WWW	ns	ns	ns
c-WFW		ns	0.01
c-KW			ns

Table 6.7 Summary of the P-values from the Newman-Keuls multiple comparison test percentage growth inhibition of *Plasmodium falciparum* D10 at 12.5 μ M for Group 3 RW-peptides. The precise P-values are less than the limit value shown in table.

	c-WFW8	c-WFW10	c-WFW12
c-WFW	0.001	0.001	0.001
c-WFW8		0.001	0.001
c-WFW10			0.05

Table 6.8 Summary of the P-values from the Newman-Keuls multiple comparison test percentage growth inhibition of *Plasmodium falciparum* D10 at 12.5 μ M for Group 4 RW-peptides. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05

	c-WRW	c-WFW	c-WWW
c-WKW	ns	ns	ns
c-WRW		ns	ns
c-WFW			ns

Table 6.9 Summary of the growth inhibitory activity (IC₅₀) of the purified tyrocidine analogues, Trc complex and GS toward the chloroquine sensitive (D10) and resistant (Dd2) strains of *Plasmodium falciparum* given in nM, the haemolytic activity (HC₅₀) towards human erythrocytes and the cytotoxicity towards COS-1 cells (LC₅₀) are given in μM. Every value denotes the average of n biological repeats (number of repeats given in brackets), with 3 technical repeats per assay ± SEM given in 2 significant figures.

Peptides	<i>P. falciparum</i> D10	<i>P. falciparum</i> Dd2	Human erythrocytes	COS-1 cells
	IC ₅₀ ± SEM (n)	IC ₅₀ ± SEM (n)	HC ₅₀ ± SEM (n)	LC ₅₀ ± SEM (n)
Trc mix	80 ± 18 (5)	350 ± 96.0 (3)	5.8 ± 0.48 (6)	8.03 ± 1.8 (2)
sTrc A(Q-O)	3800 ± 1030 (5)	nd	44 ± 17.0 (3)	36.0 ± 5.9 (2)
Trc C ₁	600 ± 23 (4)	1200 ± 280 (3)	4.6 ± 0.54 (5)	8.4 ± 0.24 (2)
Trc C	300 ± 85 (4)	640 ± 240 (3)	7.8 ± 1.4 (3)	9.4 ± 0.62 (2)
Trc B ₁	186 ± 35 (5)	560 ± 103 (3)	5.6 ± 0.79 (5)	7.7 ± 0.91 (2)
Tpc C	94 ± 49 (9)	400 ± 94 (3)	9.1 ± 1.5 (4)	10.02 ± 0.29 (2)
Trc B	48 ± 17 (6)	850 ± 240 (3)	4.8 ± 0.84 (4)	5.7 ± 0.51 (2)
sTrc AOMe ₃	1200 ± 58 (3)	nd	6.7 ± 0.56 (3)	11.0 ± 1.02 (2)
Trc A ₁	390 ± 160 (7)	nd	5.6 ± 0.52 (4)	7.4 ± 1.1 (2)
Trc A	380 ± 101 (11)	1900 ± 270 (3)	6.1 ± 0.59 (5)	6.0 ± 0.32 (2)
GS	1400 ± 140 (11)	1900 ± 150 (3)	6.2 ± 0.38 (9)	9.4 ± 0.32 (2)
sTpc A	3600 ± 320 (5)	nd	17.0 ± 7.5 (3)	12.00 ± 0.23 (2)
Phc A	52 ± 19 (10)	510 ± 96 (3)	6.7 ± 1.9 (4)	7.6 ± 0.94 (2)

Table 6.10 Summary of the P-values from the Newman-Keuls multiple comparison test on IC₅₀ of Trc A analogues towards *Plasmodium falciparum* D10. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05

	sTpcA	Trc A	Trc A ₁	sTrc AOMe ₃	Trc B	sTrc A(Q-O)
Phc A	0.001	ns	ns	0.01	ns	0.001
sTpc A		0.001	0.001	0.001	0.001	ns
Trc A			ns	ns	ns	0.001
Trc A ₁				ns	ns	0.001
sTrcA OMe ₃					0.01	0.001
Trc B						0.001

Table 6.11 Summary of the P-values from Newman-Keuls multiple comparison test on IC₅₀ of Trc C analogues towards *Plasmodium falciparum* D10. The precise P-values are less than the limit value shown in table. “ns” denotes a P-value > 0.05

	Tpc C	Trc C ₁	Trc C	Trc B ₁
Trc B	ns	0.001	0.01	ns
Tpc C		0.001	0.01	ns
Trc C ₁			0.001	0.001
Trc C				ns

Chapter 7

Investigation of tyrocidine and tryptocidine antiplasmodial mechanism of action using light and fluorescence microscopy

7.1. Introduction

The tyrocidines (Trcs) are cyclic amphipathic decapeptides produced through non-ribosomal synthesis as part of the tyrothricin complex by *Bacillus aneurinolyticus*¹⁻³. From the natural library of Trc isoforms, 28 cyclic decapeptide analogues have been identified and characterized⁴⁻⁹. Previous work in our group revealed the antiplasmodial activity of the six major tyrocidines, Trc A/A₁, B/B₁, C/C₁, which were shown to cause an inhibition of the development and life cycle progress of *Plasmodium falciparum* and to have nanomolar range IC₅₀ values (concentration that causes 50% growth inhibition¹⁰) against the chloroquine (CQ) sensitive strains of *P. falciparum* 3D7 and D10^{11,12}. Successful manipulation of the Trc producer enabled us to obtain two more natural analogues in high purity namely phenycidine A (Phc A), thus named by our group, and tryptocidine C (Tpc C)¹² in which the Tyr residue at position 7 is substituted by Phe or Trp respectively (see Chapter 2 for details). These two peptides were shown to have comparable antiplasmodial activity and selectivity as the best of the previously evaluated natural analogues (refer to Chapter 6).

Through structure-activity relationship (SAR) studies we identified structural prerequisites including hydrophobicity and size parameters that are most relevant in the antimalarial activity and selectivity of the Trcs with higher activity/selectivity recorded for smaller and more hydrophobic Trcs (this study and previous studies^{11,12}) except for Tpc C which seemed to have a particular sequence relevant for activity (this study). We aim to investigate the possible mode of action of this peptide to assess if it acts by a different mechanism as that suggested for the previously studied Trc A¹¹.

Based on the relevant physicochemical properties relevant to activity it is possible to design peptide mimetics on the basis of the natural Trc peptide scaffolds as drug leads against malaria. In order for these mimics to specifically target the non-lytic antimalarial mode of action of the Trcs, as well as avoid the chloroquine cross-resistance (refer to Chapter 6) it is crucial to identify the possible targets of the Trcs, particularly as it could include a novel selective drug target. There is urgent need to

develop drugs with new targets that are less prone to resistance by *P. falciparum* as the imminent widespread resistance to artemisinin will be devastating since there is no successor drug in sight¹³⁻¹⁶. Artemisinin is currently approved as the first line drug for the treatment of uncomplicated falciparum malaria by almost all malaria control programs all over the malaria-endemic world¹³.

The possible non-lytic modes of antimicrobial action of the Trcs include inactivation of the glucose dehydrogenase system which consequently affects metabolic activity as was observed in bacteria^{1,17}. Tyrocidine has been observed to specifically and reversibly interact with and inhibit acetylcholinesterase found in excitable membranes¹⁸. A similar Trc inhibition was found with β -galactosidase, a soluble cytoplasmic enzyme¹⁸. Trcs are able to bind to DNA in the producer strains as non-specific repressors leading to inhibition of transcription¹⁹⁻²¹, and their antimicrobial action could also involve a similar mechanism¹². The results from Chapter 6 demonstrated that resistance to CQ correlated with loss of Trc antiplasmodial activity and when both compounds were present there was an antagonistic effect. These observations supported the hypothesis that the Trcs and CQ may share at least one of their targets, such as the parasite food vacuole, as well as possibly a similar resistance mechanism.

7.2. Material and methods

7.2.1. Materials

Tyrothricin (extracted from *Bacillus aneurinolyticus*), gramicidin S (from *Brevibacillus brevis* Nagano), and Corning Incorporated[®] cell culture cluster non-pyrogenic polypropylene microtiter plates, bis-benzamide trihydrochloride (Hoechst stain) and trifluoroacetic acid (TFA, >98%) were obtained from Sigma (St. Louis, USA). All the chemicals used to prepare the RPMI-1640 culture media (RPMI 1640 medium, glucose, HEPES, albumax II, hypoxanthine, NaOH, gentamycin, and sodium bicarbonate), sodium lactate, potassium chloride, NaCl, L-lactic acid, nitro blue tetrazolium (NBT), phenazine ethosulfate (PES), 3-acetylpyridine adenine dinucleotide (APAD), D-sorbitol, Dulbecco's modified Eagle's Medium (DMEM), 0.4% trypan blue solution, and DNA interchelator Giemsa stain mixture were obtained from Sigma-Aldrich (St. Louis, MA, USA). Sterile red standard cap 250 mL Cellstar tissue culture flasks, sterile Cryo.s PP tubes and sodium hydrogen phosphate were from Greiner Bio-One GmbH, Germany. Glycerol (AnalaR grade) was obtained from BDH Chemicals Ltd. Acetonitrile (ACN) (HPLC-grade, far UV cut-off) came from Romil Ltd. (Cambridge, UK). To obtain analytical grade water, water was filtered from a reverse osmosis plant

via a Millipore Milli-Q water purification system (Milford, USA). Ethanol (>99.8%) was supplied by Merck (Darmstadt, Germany). Culture dishes and 0.2 µm - 25 mm sterile cellulose acetate membrane syringe filters were obtained from Lasec (Cape Town, South Africa) and microtiter plates (NuncTM-Immuno Maxisorp) were from AEC Amersham (Johannesburg, South Africa). Falcon[®] tubes were from Becton Dickson Labware (Lincoln Park, USA). Foetal calf serum and penicillin-streptomycin were from Gibco BRL (Gaithersburg, MD, USA). Sterile VacuCap[®] 90PF filter unit w/0.8/0.2 µm Supor[®] membrane was obtained from Pall Corporation (Pall Europe Ltd, UK). SYTO[®] 9 green-fluorescent nucleic acid stain and HCS LipidTOXTM neutral lipid stain were obtained from Invitrogen (Carlsbad, USA). Whole A⁺ blood stored in anticoagulant (citrate phosphate dextrose) containing enriched erythrocyte fraction in saline adenine-glucose-mannitol red blood cell preservation solution was donated by the Western Cape Blood services (or National Health Laboratory Services in South Africa). Asexual erythrocytic stage chloroquine sensitive (CQS) *Plasmodium falciparum* D10 cultures were benevolently supplied by Prof. Peter Smith from the University of Cape Town, Division of Pharmacology.

7.2.2. *Methods*

7.2.2.1. *P. falciparum* culturing procedure

Culturing was carried out using normal sterile techniques according the methods of Trager and Jensen²² and Lambros and Vanderberg²³. Refer to Chapter 6 for details.

7.2.2.2. *Evaluation of tyrocidine activity using light microscopy*

To determine the effect of the most active Trc analogue (according to IC₅₀ from the antiplasmodial assay) on cultured *P. falciparum* and to assess its mode of action, the synchronised chloroquine sensitive cultures (D10 strain) at trophozoite stage (2% parasitemia, 1% haematocrit) were incubated with the drug at non-haemolytic concentrations 2-fold above its IC₅₀ and evaluated using both light and fluorescence microscopy according to methods described by Rautenbach *et al.*¹¹ and Wiehart *et al.*²⁴ with modifications as described below. The mixture of Trcs (Trc mix) was also evaluated for comparison at a non-haemolytic concentration.

For light microscopy, following addition of the peptides, aliquots were collected from the cultures at different time-points for preparation of Giemsa-stained blood smears. The assay was done in duplicate and lactate dehydrogenase activity of the residual cultures was determined after 48 hours.

Prior to staining for fluorescence microscopy, the cultures were centrifuged at $300\times g$ for 4 minutes, then the supernatant was discarded and cells were suspended to the same percentage haematocrit in a solution made of one part analytical quality water and three parts sterile PBS (pH 7.2-7.3) in order to reduce solute concentration. Bis-benzimide (Hoechst) (blue fluorescent membrane permeable DNA chelator) and trypan blue (red fluorescent membrane impermeable protein binding dye) in PBS (to final concentrations $1\ \mu\text{g}/\text{mL}$ and $0.01\% v/v$, respectively) were added to the samples collected from the cultures at 1 hour and 5 hours post incubation with peptides for a trypan blue exclusion assay analysed by fluorescence microscopy. Aliquots ($30\ \mu\text{L}$) of stained cultures were applied to wells of an 8 multi-well microscope dish and the images were acquired using an alpha Plan-Apochromat $100\times/1.46$ Oil DIC M27 Elyra objective and ZEN 2011 imaging software attached to a Carl Zeiss LSM 780 confocal microscope with Elyra S.1 super-resolution platform. Excitation was at 561 nm and 405 nm for the red fluorescent and blue fluorescent stains respectively using MBS-488/561/633 beam splitter for red and MBS-405 for blue. The emitted light was collected using a GaAsP detector.

7.2.2.3. Evaluation of tyrocidine activity using fluorescence microscopy

A second study using fluorescence microscopy involved staining suspended cultures prepared as described above (except for not being synchronised within the last 48 hours of culturing) with combinations of trypan blue (red fluorescence) and either SYTO[®] 9 green-fluorescent nucleic acid stain or LipidTOX[™] neutral lipid stain (green fluorescence). To view the neutral lipids, the cells were stained with LipidTOX[™] (stock solution dilution at 1:200), incubated for 30 minutes at room temperature before staining with trypan blue at final concentration of $0.002\% v/v$. For nucleic acid staining, the cells were stained with SYTO[®] 9 (stock solution dilution at 1:1000) and trypan blue at final concentration of $0.002\% v/v$ and incubated at room temperature for 15 minutes. Super-resolution structured illumination (SR-SIM) fluorescence microscopy was carried out as follows: thin ($0.1\ \text{mm}$) z-stacks of high-resolution image frames were accumulated in 5 rotations using an alpha Plan-Apochromat $100\times/1.46$ oil DIC M27 ELYRA objective, employing an ELYRA S.1 (Carl Zeiss Microimaging) microscope equipped with a 488 nm laser (100 mW), 561 nm laser (100 mW) and Andor EM-CCD camera (iXon DU 885). Images were re-enacted using ZEN software (black edition, 2011, version 7.04.287) based on a structured illumination algorithm²⁵. Image projections and animations were carried out on reconstructed super-resolution images in ZEN.

7.3. Results and discussion

Our antimalarial activity results revealed that Tpc C, which was investigated in high purity for the first time in this study, was among the most active and most selective analogues towards *P. falciparum* D10. Rautenbach *et al.*¹¹ had shown that the Trcs (notably Trc A) have a non-lytic mechanism of action which influenced parasite progress through the life cycle. In order to find out if this observation applied to Tpc C, we employed the peptide at 200 nM which is about $2\times IC_{50}$ but still sub-haemolytic (Fig. 7.1A) to observe the influence on parasite life cycle progress using light microscopy (Fig. 7.2C). This concentration will lead to overt antimalarial activity within the parasite population without haemolytic activity to allow visualisation through microscopy. The concentration initially corresponded to about $10\times IC_{50}$ for the more susceptible parasite cultures (see Fig. 6.3, Chapter 6) and was maintained even when we observed more resistant cultures. The Trc mix, which showed a similar dose-response and selectivity to Tpc C (Figs. 7.1B), was included for comparison at sub-haemolytic concentrations of 200 ng/mL ($2\times IC_{50}$) in assessing the influence of peptide treatment on the parasite stage (Fig. 7.2B).

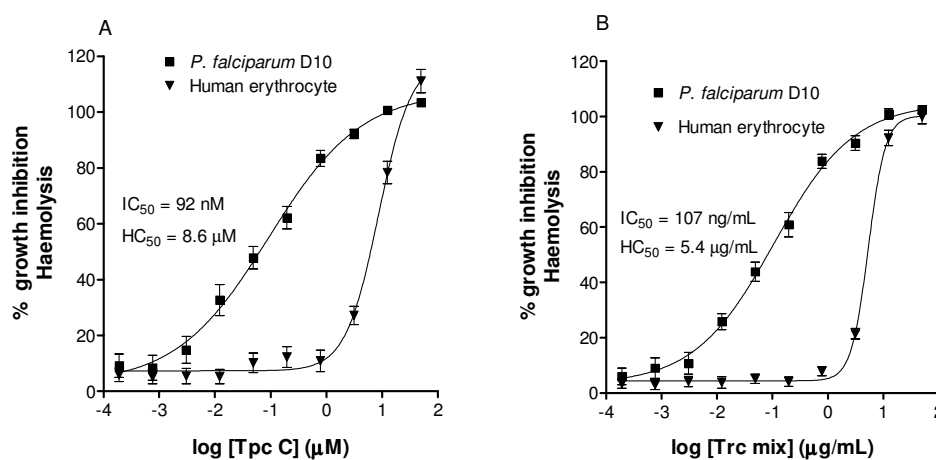


Figure 7.1 Representative dose-response curves for antiplasmodial activity against *P. falciparum* D10 (using the Malstat or lactate dehydrogenase activity assay) and haemolytic activity to infected human erythrocytes of **A.** Tpc C and **B.** Trc mix. Each data point represents the average \pm SEM of 3-6 biological repeats for each assay done in triplicate. The activity parameters IC_{50} (concentration that leads to 50% growth inhibition) and HC_{50} (concentration that leads to 50% erythrocyte lysis) for each peptide are indicated on each graph.

7.3.1. *Effect of tyrocidine and tryptocidine on intra-erythrocytic life cycle parasite stages*

7.3.1.1. *Light microscopy observation of life cycle progression*

Following the determination of the parasitemia through Giemsa staining and light microscopy, we observed that there was a delay in the occurrence of the schizont stage for the peptide-treated cultures. Whereas in the untreated cultures (Fig. 7.2A) the schizonts were observed from 12 hours post incubation, they were only seen after 24 hours in the treated cultures (Fig. 7.2B and C).

It is, however, possible that schizont only occurred between 12 to 24 hours in the treated cultures because ring stage parasites appeared at 24 hours post incubation for all cultures though at lower parasitemia for the treated cultures compared to the untreated ones (Figs. 7.2B and 7.2C). This might have affected the turnover into trophozoites at the end of the 48 hour incubation resulting in the pronounced difference in parasitemia observed between treated cultures and untreated cultures. The Malstat assay performed on the residual cultures indicated that 200 nM corresponds to 60-70% inhibition of parasite growth for the treated cultures (not shown). Rautenbach *et al.*¹¹ observed a high difference in parasitemia between cultures treated with either Trc A or C₁ compared to untreated cultures after 21 hours of incubation. In their work, they indicated that the Trcs did not affect progression from starter ring cultures to trophozoite stage although the morphology of the resulting trophozoites seemed abnormal. In accordance with this, in the present study the parasites progressed from rings to trophozoite after 24 hours but the parasitemia gradually declined suggesting a slow cytotoxic rather than a static effect of the peptides on the parasites¹¹.

7.3.1.2. *Confocal fluorescence live-cell imaging for membrane integrity and DNA packing*

In a bid to examine if the peptides affected the nuclear material of the parasites as was previously suggested for Trc A¹¹, cultures at trophozoite stage treated with Tpc C and Trc mix at $2 \times IC_{50}$ were observed using fluorescence microscopy 1 to 6 hours post incubation. Trypan blue, a membrane impermeable dye, was used as a marker for cell leakage^{11,24} while bis-benzimide, a membrane permeable DNA marker, was used to stain the parasite's nuclear material¹². In agreement with previous studies¹¹, there was no difference in the permeability of erythrocytes' and parasites' membranes to trypan blue between the treated (with either Tpc C or Trc mix) and the untreated cultures after up to 6 hours incubation. This indicates that at this concentration neither the erythrocyte nor parasite membranes were compromised.

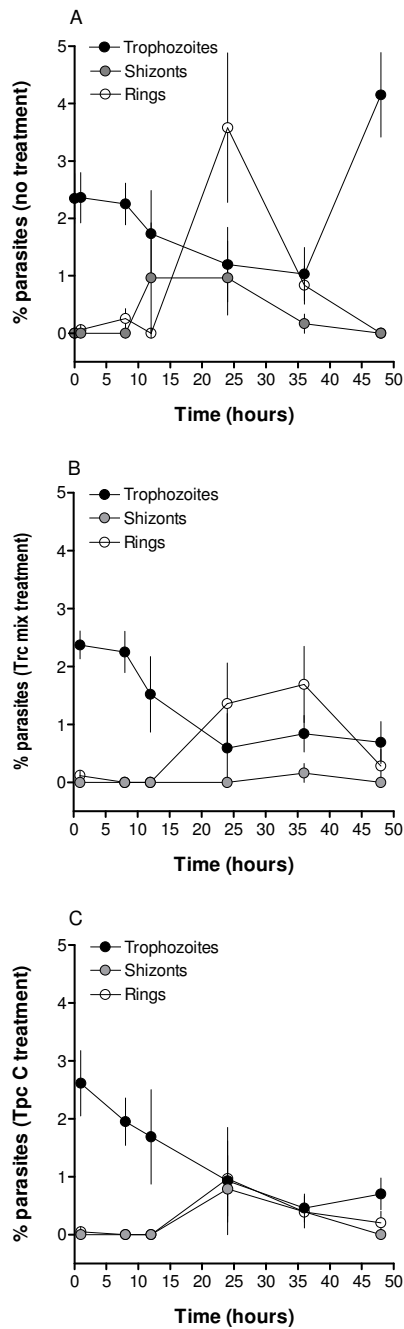


Figure 7.2 Distribution of trophozoite, schizont and ring parasite stages over time from 0 to 48 hours following treatment of synchronised *P. falciparum* D10 cultures at trophozoite stage incubated without (control) (A) or with 200 nM of tryptocidine C (Tpc C) (B) or 200 ng/mL tyrocidine mixture (Trc mix) (C). Each data point represents the mean \pm SEM of parasite counts made within 8-13 regions from 2 biological repeats.

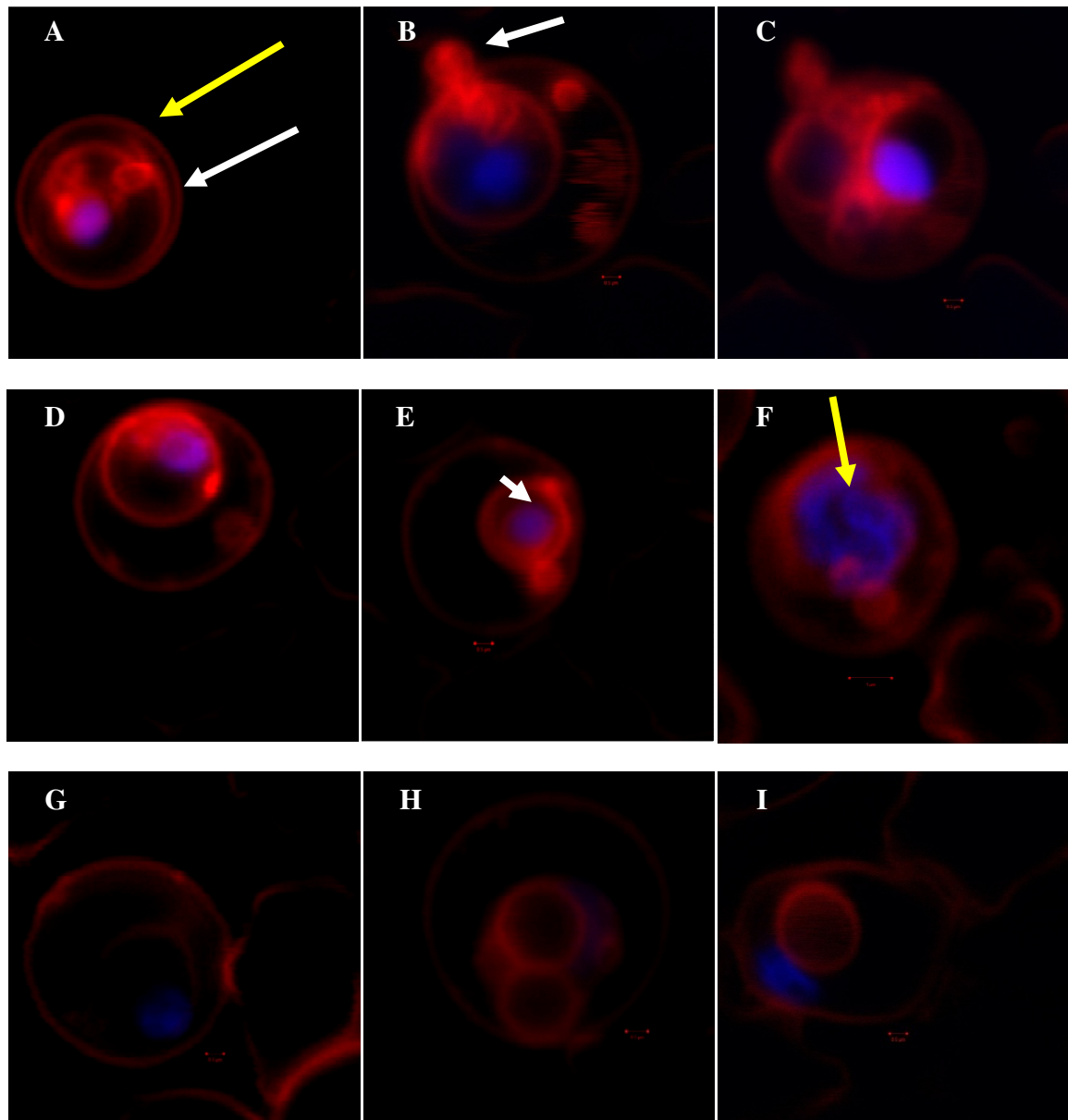


Figure 7.3 Fluorescence microscopy images of unsynchronised late intra-erythrocytic stages of *P. falciparum* D10 stained with protein binding and membrane impermeable fluorescent dye trypan blue (red) and the nucleic acid fluorescent stain bis-benzimide (blue). **A**, **B** and **C** are images of control culture with no peptide added; **D** shows tryptocidine C-treated cultures after 3 hours incubation; **E** and **F** tryptocidine C-treated cultures after 6 hours incubation; **G**, **H**, and **I** show Trc mix-treated cultures after 6 hours of incubation. The yellow arrow in **A** shows the infected erythrocyte plasma membrane (IEPM) and white arrow in **A** indicates the parasite's membrane. The white arrow in **B** shows vacuolar membranous structures. White arrow head in **E** delineates the nuclear membrane in the parasite. The yellow arrow in **F** shows the stained nuclear material.

We also observed that the nuclear membrane of the parasite was not damaged as confirmed by the confinement of the parasites' nuclear material within the nucleus as stained by bis-benzimide. Therefore, Tpc C similar to Trc A, as suggested by Rautenbach *et al.*¹¹, also acts by a non-lytic mechanism of action which slowly inhibits maturation of *P. falciparum*. In some of the images taken for the treated samples, we observed some abnormality in the packing or compactness of the chromatin (Fig. 7.3F and 7.3G) which could suggest that this is the consequence of the Tpc C activity. Tpc C is rich in Trp residues and it is known that Trp can interact with DNA²⁶. We decided to use a more sophisticated technique of super-resolution structured illumination microscopy (SR-SIM) to view the nuclear material at higher resolution.

7.3.1.3. Super-resolution structured illumination (SR-SIM) fluorescence microscopy

For the SR-SIM we only investigated the effect of the pure peptide Tpc C on the parasite's nuclear material using trypan blue as membrane impermeable dye (red) and SYTO[®] 9 (green) as membrane permeable nuclear stain. The results confirmed those in *Section 7.3.1.2*, but improved on the image quality and details observed. There was a deformation of the parasite/nuclear membrane (Fig. 7.4F) with change in the compact nature of the nuclear material (Fig. 7.4F-H). In addition we also observed that unlike the discrete segregated distribution of chromatin seen in the untreated schizont (Fig. 7.4E), in the treated samples there seemed to be a loss of the uniform segregation and morphology of the chromatin (Fig. 7.4J). This could mean that some of the merozoites derived from this schizont will not be viable and could explain the gradual decrease in parasitemia that was observed in *Section 7.3.1.1*. A similar effect on schizont chromatin morphology has been observed with GS¹¹ and Trc A²⁷ treatment in previous studies by our group. Fluorescence microscopy of a labelled form of the cationic dimeric peptide Δ Fd allowed for the observation of its ability to cross the IEPM, the parasitophorous vacuolar membrane (PVM), the parasite's plasma and nuclear membranes to interact with DNA of the parasite²⁸. Pre-maturely released schizonts following treatment with Δ Fd contained knobs of amplified DNA lacking the typical symmetric rosette appearance which could not be able to proliferate into merozoites to invade new host cells²⁸. These results and previous results from our group^{11,27} suggested that the Trcs and Tpc C could act in a similar manner.

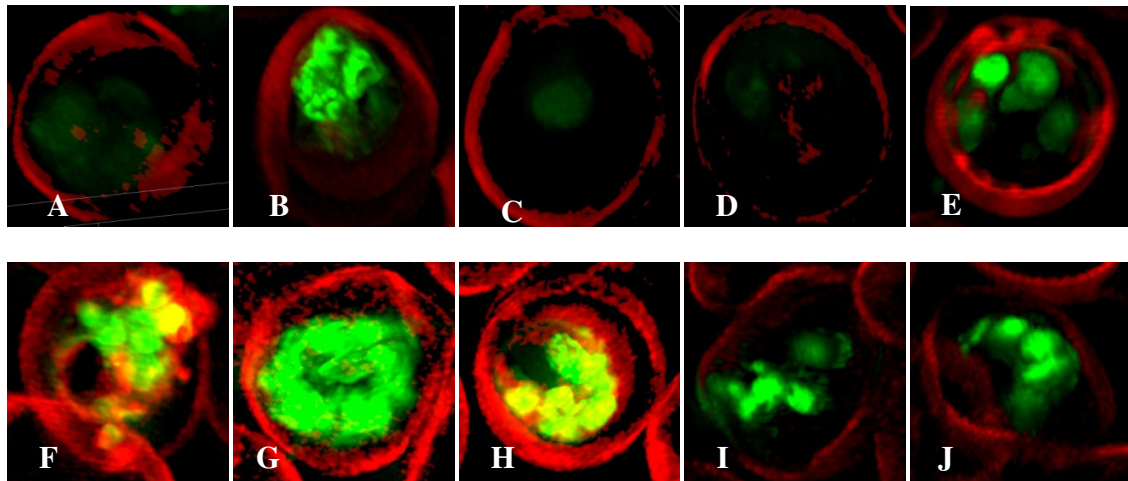


Figure 7.4 Super-resolution structured illumination fluorescence microscopy images of late intra-erythrocytic stages of *P. falciparum* D10 stained with the membrane impermeable fluorescent dye trypan blue (red) and the permeable nucleic acid fluorescent dye SYTO[®] 9 (green). Images **A**, **B**, **C**, and **D** show untreated normal trophozoites and **E** a normal schizont. Images **F**, **G** and **H** show Tpc C-treated trophozoites (post 6 hours treatment) and **I** and **J** show Tpc C-treated schizonts (post 6 hours treatment).

Apart from overt changes in parasite morphology, we also observed some abnormality in the shape and compactness of a dark structure thought to be the haemozoin crystal in the treated samples (Fig. 7.5). Unlike in the untreated sample, in some instances only observed in the treated samples there were dense dark elongated structures (Fig. 7.5) rather than round and uniform dark structures observed in the control samples (results not shown). Spathelf¹² noticed that the size of the haemozoin crystal was smaller in Trc A-treated parasites after 24 hours incubation compared to normal cultures. This suggested a compromise in the parasite's ability to form haemozoin which could favour toxicity by heme following the parasite's digestion of haemoglobin¹². Chloroquin has also been observed to cause the clumping of haemozoin crystals²⁹⁻³¹.

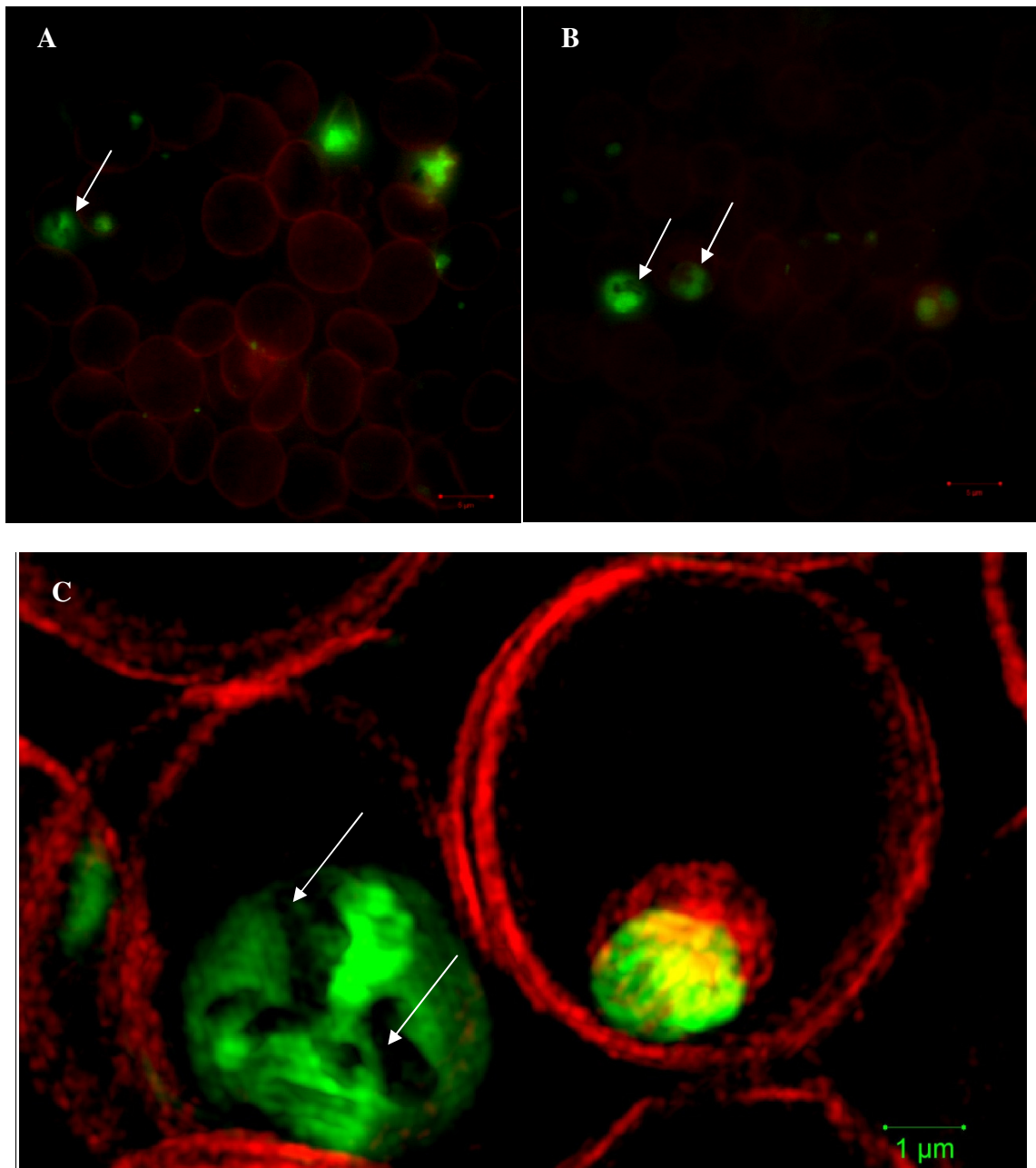


Figure 7.5 Super-resolution structured illumination fluorescence microscopy images of late intra-erythrocytic stages of *P. falciparum* D10 stained with the membrane impermeable fluorescent dye trypan blue (red) and the permeable nucleic acid fluorescent dye SYTO[®] 9 (green). **A** and **B** show wide-field images with arrows indicating changes in parasite morphology after addition of Tpc C to cultures. **C**. Processed image of **A** showing close up view of dark elongated particles, which could indicate haemozoin crystals, within or close to the nuclear material mass.

7.3.2. Visualization of the effect of tryptocidine C on parasite neutral lipids

Due to the possibility that the molecular target for the Trcs is located in the food vacuole as suggested by increased resistance of CQ resistant *P. falciparum* to the Trcs and the antagonism observed when both compounds were present, it can be suggested that this non-lytic mode of action involves heme toxicity as is the case for chloroquine. It has been suggested that haemozoin crystals in the malaria parasite are associated with neutral lipid droplet-like structures³² within the food vacuole and that these lipid droplets along with phospholipid membranes are involved in the process of heme crystallisation both *in vivo* and *in vitro*³²⁻³⁸. In an attempt to have a closer look at the food vacuole we used a SR-SIM method to visualize the effect of Tpc C on the neutral lipid accumulation using the green fluorescent LipidTOX™ neutral lipid stain.

We observed that most of the neutral lipids accumulated in the intra-erythrocytic cellular space possibly part of the parasites' membranes. The morphological details observed were unlike any yet described to the best of our knowledge and interpreting them is not within the scope of this project. Notably, the regular and symmetric shape of the lipid structures (Fig. 7.6A-I) was distorted after treatment with Tpc C (Fig. 7.6J-R).

A striking increase in phospholipid and neutral lipid content has been reported as one of the main changes that occur following malaria infection of human erythrocytes³⁹⁻⁴³. This increase in lipid content is geared towards synthesising the complex membranous system of the *Plasmodium*-infected erythrocyte³⁹. Our results agree with the observation that neutral lipids are previously not detectable in uninfected red blood cells^{39,40,43} as we only saw the stained lipids within infected erythrocytes. The neutral lipids are said to include fatty acids, diacylglycerol and triacylglycerol which are closely associated with the food vacuole originating from the digestion of phospholipids of transport vesicles used for haemoglobin ingestion³⁸. The formation of the neutral lipid structures depends on the stage of the parasite and peak during the mid- and late-trophozoite stages⁴² which will justify the stage selective activity of the Trcs which is more active towards trophozoites and schizont stages^{11,27}. The lipid structures observed in this study seemed to extend from the parasite to the erythrocyte membrane and may thus not be limited to neutral lipid bodies within the food vacuole.

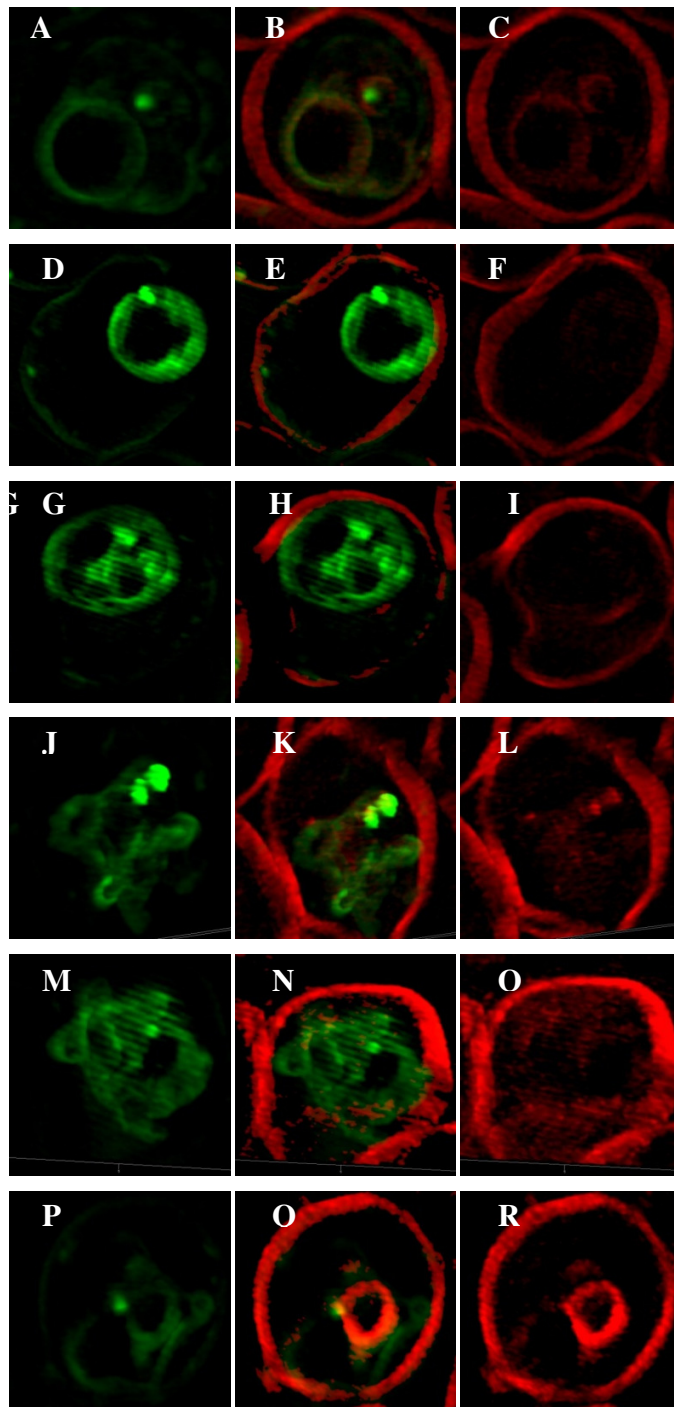


Figure 7.6 Super-resolution structured illumination fluorescence microscopy images of late intra-erythrocytic stages of *P. falciparum* D10 stained with trypan blue (red) and neutral lipid binding fluorescent dye LipidTOX (green). Right panel shows images with trypan blue stain alone while left panel shows images with LipidTOX alone. Middle panel are resulting images from super position of both. Images A-I show control cultures (no peptide added) and images J-R show cultures treated with Tpc C after 5 hours.

The parasite is also known to induce the formation of an interconnected network of turbovesicular membranes within 33 hours post-infection which indeed runs from the parasites vacuolar membrane to the erythrocyte membrane and are involved with transport of nutrients to the parasite such as nucleosides and amino acids ⁴⁴. All these vital roles played by the neutral lipid membranous structures could be interfered with as a result of change in their morphology that was observed following treatment with the membrane active Tpc C. It has been suggested that the mode of antimalarial action of CQ and other quinolines involves entry into the neutral lipid microenvironment which interferes with the heme crystallisation process that is facilitated by the complex neutral lipid mixture ³². This could explain the observed antagonism between the Trcs and CQ if they compete for interaction with the neutral lipid structures. Otherwise the Trcs could interfere with haemoglobin ingestion and or heme crystallisation by disturbing the network of membranes made of neutral lipids. This can also interfere with the transport of other vital nutrients to the parasite and indirectly be fatal to the parasite over time. A link can be made between the observed halt in the life cycle caused by the Trcs and observed damage of the turbovesicular network as the same link was purported to account for the cell-cycle arrest caused by monoclonal antibodies to the cell surface protein *Plasmodium falciparum* 60S stalk ribosomal acidic protein P2 (PfP2) (PFC0400w) ⁴⁵.

7.4. Conclusion

Following light and fluorescence microscopic observation of tyrocidine and tryptocidine treated *Plasmodium falciparum* D10 cultures we were able to observe and propose as possible tyrocidine/tryptocidine targets: the disorganisation of chromatin that could account for halted growth in late trophozoite/early schizont stage, as well as disorganisation of neutral lipid structures that could account for change in the uniform appearance of the haemozoin crystal. The latter supports the previous hypothesis that the Trcs and chloroquin have a common target in the malaria parasite.

7.5. References

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Chapter 8

Summary, conclusions and outlook

8.1. Introduction

In this study we set out to characterise small cyclic peptides with antilisterial and antimalarial activity. Focus on the two microbial targets *Listeria monocytogenes* and *Plasmodium falciparum* stems from the fact these are both intracellular pathogens which are the causative agents of listeriosis and malaria respectively, each a threat to public health ¹⁻⁵. The burden due to these two diseases is exacerbated with the development of resistance by the pathogens to all currently available drugs, which is probably favoured by their intracellular localisation. Antimicrobial peptides (AMPs) have been found to have the propensity to interact with cell membranes and reach intracellular targets and their positive charge also makes them quite selective for the negatively charged microbial surfaces in contrast to the zwitterionic mammalian cell surfaces. Hence AMPs are a possible source of a new class of drugs against drug resistant microbes. The interest in small cyclic peptides over their linear counterparts is due to improved stability, resistance to proteolytic degradation, receptor selectivity, better bioavailability and available conformational proximity for receptor binding ^{6,7}. There is a need to extensively refine the relationship of the structure of these peptides to their antimicrobial activity which will allow for the development of drug leads based on the structural pre-requisites derived from these studies.

Two libraries of small cyclic peptides were considered in this qualitative structure to activity relationship (QSAR) and mode of action (MOA) studies namely the tyrocidines and Trp/Arg rich cyclic peptides (RW-peptides). Given that up to 28 natural tyrocidines ⁸ have been identified and that other investigators have successfully synthesized unnatural tyrocidine analogues with promising activity ⁹, we extended the library of tyrocidine analogues tested against *Listeria monocytogenes* and *Plasmodium falciparum* in order to establish the structural motifs and pre-requisites required for optimal activity and selectivity. We have for the first time determined the antimicrobial activity of three previously identified tyrocidine (Trc) analogues namely phenycidine A (Phc A), tryptocidine C (Tpc C) and tryptocidine A (Tpc A), with the former two obtained in high purity (>90%) through selective amino acid supplementation of *Bacillus aneurinolyticus* (formerly called *Bacillus brevis*), cultures while the latter was chemically synthesised. Two new synthetic Trc A analogues, one in which the charged ornithine residue was trimethylated and the other bearing two ornithines due to the substitution of glutamine by

ornithine, were also evaluated. The motivation for determining the antimicrobial activity of new Trc analogues was the fact that in previous studies the antimicrobial activity of the Trcs unlike their membranolytic activity had been linked to the identity of some variable residues within the structure of the related six major analogues isolated from commercially available tyrothricin notably three residue positions Trp^{3,4}/Phe^{3,4} and Lys⁹/Orn⁹ ¹⁰⁻¹². This study also sought to use new techniques to verify certain proposed theories on the mechanism of action of the Trcs towards *L. monocytogenes* and *P. falciparum* which could facilitate rational design of more active and selective drug leads.

Furthermore, the availability of a library of synthetic AMPs which share the cyclic, aromatic and cationic character of the Trcs, but lack their high membranolytic activity generated interest to also test these peptides so as to identify the structural pre-requisites that could be transferable to the Trc library to improve selectivity. We established for the first time the antilisterial and antimalarial activities of this library of short, cyclic peptides RW-peptides. These peptides could also serve as scaffold for the development of lead peptides against the two pathogens of interest.

8.2. Summary of findings and future prospects

8.2.1. Production of selected natural Trcs

We identified the need for efficient strategies to produce the rare and potentially useful natural Trc analogues in sufficient amounts and purity for bioactivity analyses and physicochemical characterisation so as to improve their inherent activity or bring about new activities. Past studies found Trc production by *B. aneurinolyticus* to be governed by nitrogen supplementation with urea ¹³ and amino acids ¹⁴⁻¹⁶ and established that *B. aneurinolyticus* ATCC 8185 is an ideal strain to principally produce Trcs with little or no gramicidins ¹⁷. Based on this knowledge coupled with the low specificity of the enzyme systems in charge of incorporating some structurally related amino acids ¹⁴, we were successful in obtaining high yields of three analogues namely Trc A, Phc A and Tpc C in high purity (Chapter 2). Using preparative HPLC, the natural Trcs were obtained at >90% purity as determined by analytical HPLC, UPLC-MS and ESMS either from the commercial extract or from the extracts of manipulated cultures. The high purity achieved was very important for the analysis of their structure (Chapter 2), biological activity and qualitative structure to activity relationships (QSAR) (Chapter 3 and 6).

There is potential in employing this strategy for the production of other rare peptide analogues although some factors will have to be closely examined that we found could potentially affect the output namely strain of bacterial culture, depth of culture flasks and aeration, concentration of

amino acids as well as interaction between certain amino acids. For the latter it will be of interest to use cell-free enzyme extracts in future studies to produce rare peptides with modified residues. For example we have identified a few unnatural analogues (Bal, 5MeW and 1MeW) of the relevant amino acid tryptophan which improved the antimicrobial activity of the RW-peptides and which could be incorporated into the structure of the most active Trcs in place of the aromatic residues. It will be cost effective and sustainable to produce these analogues using the natural enzyme synthetase system rather than through chemical synthesis^{18 19} during which other agents are added that could interfere with the critical self-assembly mechanism of the peptides and disturb their antimicrobial activity. Previously Fujikawa *et al.*²⁰ succeeded in using the partially purified Trc synthetase enzyme preparation of *B. aneurinolyticus* ATCC 8185 to introduce unnatural amino acids like 5MeW, *p*-fluorotryptophan, thienylalanine and *p*-fluorophenylalanine into the structure of tyrocidines.

8.2.2. QSAR of antilisterial activity of small cyclic peptides

After obtaining the libraries of small cyclic AMPs in high purity we determined certain physicochemical parameters related to the structure of the analogues. This was done experimentally (Chapter 2 for the Trcs) through analytical HPLC for the retention time on the C₁₈ column (hydrophobicity/amphipathicity parameter) and ESMS for the molecular mass (M_r) (steric parameter) or through *in silico* molecular modelling (Chapter 2) to determine the molecular volume (MV), solvent accessible surface area (SASA) and solvent accessible volume (SAV) (all size/steric parameters) as well as through computation of the side chain surface area (SCSA), MV, lipophilicity, hydrophobicity, hydrophathy and interphase properties of the analogues from theoretical values of the constituent amino acid residues. These parameters were then correlated to activity parameters from antilisterial assays namely the IC₅₀, IC_{max}, IC_F and the newly coined activity product (A_P) for the Trcs; A₂₅ (percentage inhibitory activity at 25 μM) and A₁₀₀ (percentage inhibitory activity at 100 μM) for the RW-peptides. We decided to include the composite parameters IC_F (IC₅₀/IC_{max}) and A_P (IC₅₀ × IC_{max}) in our analyses to improve selection of the most active Trc analogues, while still incorporating conventional inhibition parameters.

As was previously established¹¹ we also found that the Trcs have potent activity with drug/disinfectant potential, especially against leucocin A resistant *Listeria monocytogenes* (B73-MR1) (Chapter 3 Part I). The activity of these decapeptides was found to be highly dependent on an amphipathic balance. QSAR analysis combined with principal component analysis showed the best activity correlation with hydrophathy, hydrophobicity and interphase properties

(hydrophobicity parameters), SASA, SAV, M_r and molecular volume (MV) (steric/size parameters), coupled with rigid sequence and charge prerequisites. For potent activity against *Listeria monocytogenes* strains, there is a prerequisite for a Tyr or Phe in the (W/F)(w/f)NQ(Y/F/W) sequence of the variable pentapeptide and ornithine (Orn, O) as cationic residue in the conserved V(K/O)fP pentapeptide, particularly with Trp in the aromatic dipeptide moiety of the variable pentapeptide. The roles of Trp and Orn in the Trcs were confirmed with the most active peptide, tyrocidine B (Trc B) containing Orn and a Trp-D-Phe in the aromatic dipeptide moiety. However, a novel analogue with a trimethylated ornithine and Phe-D-Phe showed an activity rivalling that of Trc B. It was also found that an analogue with two positive charges (Gln to Orn) lost activity against *L. monocytogenes*, indicating that increase electrostatic interaction may impede activity or that Gln is an important residue in the tyrocidine structure for antilisterial activity. Our results emphasised that activity is dictated by interplay between the character of the aromatic residues in the variable pentapeptide and the cationic residue. Any residue change resulting in tighter membrane/cell wall interaction is likely to trap tyrocidines and impede their mechanism of action. With the results from the QSAR of Trcs we can predict the following lead peptide structure for future libraries *cyclo*[VOMe₃LfPWfNQ(Y/F)].

Our findings on the antilisterial activity of the RW-peptides closely agreed with previous studies on their activity towards other Gram-positive bacteria. We also found relevant to their activity the clustering of the aromatic and cationic residues, a ring size of six amino acids and Arg instead of Lys as charged residue (Chapter 3 Part II). As with the Trc library, we found that the hydrophobicity of aromatic residues and an amphipathic structure is critical for antilisterial activity of AMPs and that there exists an optimal balance between peptide size and hydrophobicity/amphipathicity for maximum activity and selectivity.

The two target strains of *L. monocytogenes* are different in their cell wall²¹ and membrane composition²², as well as metabolism²³ and their susceptibility or resistance to the class IIa bacteriocin leucocin A. Most of the cyclic peptide tested showed higher activity against the leucocin A resistant strain. We also demonstrated for the first time that for the peptides to specifically target *L. monocytogenes* B73-MR1 (resistant strain) a small hydrogen bonding aromatic residue is a pre-requisite in the peptide structure.

8.2.3. Salt sensitivity and tolerance of antilisterial activity of the cyclic peptides

Previous work by Spathelf¹² established that at 7.5 mM CaCl₂ induced a change in the MOA of the Trcs towards *L. monocytogenes* from a primarily lytic to a non-lytic mechanism. Analysis by our group indicated a change in Trc structure, possibly due to a chaotropic effect of metal

chloride salts especially MgCl_2 and CaCl_2 on the Trcs¹² (personal communication Dr B. Bhattacharya). Given that *L. monocytogenes* is a food-borne pathogen and that the antimicrobial activity of AMPs along with the currently recognised food preservative nisin is affected by the presence of salts^{24, 25}, it was important to verify this phenomenon and identify the parameters that contribute to it. This information would be useful for Trcs to be considered as a new source of bio-preservative to protect from *L. monocytogenes* which is a food safety threat²⁶. We investigated the relationship between peptide structures with the effect of CaCl_2 among other metal chloride salts. To verify the effect of peptide structure, we evaluated the most active Trc analogues towards *L. monocytogenes* in comparison with the known lytic GS and non-lytic RW-peptide analogues. We also confirmed the effect of divalent cations by including a metal chelator EDTA in the assays and investigated the mode of action using novel techniques. The novel techniques employed included high throughput spectrofluorimetry, fluorescence spectroscopy and photon correlation spectroscopy also making use of Gram-positive model membrane vesicles as well as CellTiter-BlueTM reagent which allowed us to determine the anti-metabolic activity of the peptides.

The Trcs were found to be salt tolerant as they maintained their activity towards *Listeria monocytogenes* in the presence of selected chloride salts of earth (MgCl_2 , CaCl_2) and alkali (NaCl , KCl) metals. The Trcs and gramicidin S lead to an increase in membrane permeability of the listerial cells, confirming their predominantly membranolytic mode of action. However, pre-incubation of the Trcs with CaCl_2 significantly decreased their ability to permeabilise listerial membranes, but conversely increased their inhibition of actively metabolising listerial cells. This indicated a calcium-dependent change to a non-lytic Trc MOA and target. The non-lytic MOA may be related to the inhibition of a key component in cell respiration by the tyrocidine- Ca^{2+} complex and/or a combination of increased Ca^{2+} availability modulating *L. monocytogenes* transition from a saprophyte to intracellular pathogen and tyrocidine acting on an sensitive target involved in metabolism. We determined that a small aromatic residue (Phe or Tyr) in position 7 is important for the calcium dependent increase in antilisterial activity. It was also found that calcium-induced change in the interaction of D-Trp⁴ that would be directly involved in the membranolytic activity of the Trcs could explain how Ca^{2+} brings about the observed decreased membranolytic activity of Trcs. Future studie will involve elucidation of the calcium-dependent MOA of the Trc and identification of the target(s).

In this study confirmed that additive agents for formulations, namely, EDTA or CaCl_2 , to would increase the antilisterial potency of the Trcs in their role as bio-preservatives. We also found salt tolerance and that use of the Trcs in a high salt environment such as during food processing will

not interfere significantly with their activity. Future studies in which these preparations of Trcs would be applied to infected food samples to determine the effect of other parameters such as food texture as well as use of Trcs in combination with other food preservation techniques (hurdles^{27,28}) are recommended.

8.2.4. *Antimalarial activity of small cyclic peptides*

Activity of our peptide libraries towards the second target intracellular pathogen chloroquine (CQ) sensitive *P. falciparum* D10 was investigated through growth inhibition assays (Chapter 6) to determine activity parameters for QSAR analyses. However, this goal was complicated by the discovery of culture derived decrease in susceptibility of the parasites towards our most active peptides, the Trcs (Chapter 6 Part II). Nevertheless, we once again observed that the Trcs were more active than the RW-peptides and that peptide hydrophobic membrane interaction propensity was relevant to activity. For this reason the aromatic residues were found to also determine antiplasmodial activity with preference found for smaller and more lipophilic residues (Tyr and Phe). However, we demonstrated for the first time potent antimalarial activity of a purified tryptocidine, namely Tpc C, which have a specific amino acid sequence rich in Trp residues. From evaluation of the RW-peptides, we once more identified the Trp analogue Bal as being positive in improving activity without increase in cytotoxicity. This residue can be incorporated into the Trc scaffold in future libraries for improved selectivity. We also confirmed that the RW-peptides do not act primarily through membrane lysis due to their very low cytotoxicity to mammalian cells (erythrocytes and COS-1 cells) (Chapter 6 Part I). Thus, a possible lead peptide for increased selectivity and antimalarial activity would be *cyclo*[VOLfP(Bal)fNQ(Bal)].

The culture derived loss of Trc susceptibility indicated a shared intracellular target between the Trcs and CQ for which research in our group had observed a similar phenomenon brought about by the use of D-sorbitol to synchronize cultures²⁹. Our hypothesis was confirmed by the loss of activity observed when the Trcs were tested against the CQ resistant *P. falciparum* Dd2, as well as by the antagonism of the two compounds when tested together (Chapter 3 Part II). Light and fluorescence microscopy were employed to further investigate the MOA of the Trcs towards *P. falciparum*. These studies revealed that the Trcs and Tpc interrupted life cycle progression of the parasites through the observed Trc induced change in chromatin packing or morphology of neutral lipid structures associated with the parasites. Given the central role played by neutral lipid vesicles in heme crystallisation³⁰⁻³⁶, we hypothesise that the Trcs/Tpc interfere with haemozoin crystal formation as a shared target with chloroquine explaining the correlation

between loss of activity of peptides and chloroquine resistance as well as antagonism when both are present.

Future studies to verify this hypothesis are needed such as biophysical characterisation of the effect of Trcs on *in vitro* heme crystallisation to haematin within neutral lipid environments^{30–32}, as well as identifying the Trc target(s).

8.3. Last word

By comparing libraries of peptides with related structures we were able to identify structural parameters and particular amino acid residues that play a pivotal role in antilisterial and antimalarial activity. These parameters would be useful in the design of lead peptides and peptide mimics for drug development towards resistant pathogens like *L. monocytogenes* and *P. falciparum*.

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