The design and Synthesis of peptide-inspired antileishmanial agents

CHADBOURNE, FRANCES,LAURA

The Design and Synthesis of peptide-inspired antileishmanials agents

PhD Thesis

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Abstract

Leishmaniasis is a tropical disease caused by protozoan parasite of the genus *Leishmania.*

The temporins are a class of antimicrobial peptides (AMPs) and have documented antibacterial and antileishmanial activity. Temporins A, B, C, F, L and 1Sa were synthesised. Fluorescein and tetramethylrhodamine, used as biological imaging agents, were attached to temporins A and B and used in biological testing to track the progress of the peptides through infected macrophage cells, and in an *in vitro* skin model.

Temporins A and L were found to be active against both promastigotes and amastigotes, and alanine and lysine scans of these peptides were performed to attempt to identify any residues causing activity. No residues to this effect were identified, however based on this work, the largest library of antimicrobial peptides to date was synthesised and tested gainst *Leishmania mexicana* promastigotes and axenic amastigotes. Data obtained was subsequently used in the first reported study of computational modelling to predict the sequences of antileishmanials peptides. Based on this work, peptide sequences were predicted that may show activity as antileishmanials agents.

The Ciliatamides consist of three lipopeptides named Ciliatamides A-C, of which Ciliatamide B was shown to possess high levels of antileishmanial activity. (*S,S*), (*R,S*), (*S,R*) *and* (*R,R*) forms of Ciliatamide B were synthesised and used in biological testing.

The activity of both temporins A and B was assayed against *L. mexicana* promastigote and axenic amastigotes, as well as murine macrophages: Allowing for an as yet undocumented comparison between both stages of the *Leishmania* spp. lifecyle. Differences were noted in the activity of the promastigotes and amastigotes lifecycle stages of the parasite, with promastigotes being significantly more responsive to AMPs than the amastigotes. As all previous studies had taken place on the promastigotes lifecycle stage, this finding must be taken into consideration in planning future studies.

Acknowledgements

I would like to thank my supervisor, Dr. Steven Cobb, for his supervision, guidance, support and patience. I would also like to thank Dr Paul Denny for his guidance, support and advice on biological testing; Dr Aileen Congreve for her time and patience in developing a purification method for peptides; and Dr. Patrick Steel and his group use of their lab space and the microwave equipment. I would also like to thank Dr. Jackie Mosely and the other members of the mass spectroscopy service for analysing peptides by MALDI-ToF and Dr Alan Kenwright for providing NMR analysis. For helping to set up and maintain cell lines used in biological testing I would like to thank Mr. Hayder Al-Samra'ay, and I would like to thank Catroina Raleigh for initiating the biological testing of peptides. Finally, thanks to the members of lab group CG169.

Declaration

The work in this report was carried out at the University of Durham in the Department of Chemistry between October 2009 and October 20013. It has not been submitted for any other degree, and is the author's own work, except where acknowledged by reference.

Copyright

Abbreviations

Amino Acids

Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic Acid
Cys, C	Cysteine
Glu, E	Glutamic Acid
Gln, Q	Glutammine
Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

Protecting groups

Boc	*tert*-butoxycarbonyl
Fmoc	9H-fluoren-9-ylmethoxycarbonyl

Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
^{t}Bu	*tert*-butyl
Trt	trityl

Reagents

DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
FAM	5-6-Carboxyfluorescein
HIFBS	Heat Inactivated Foetal Bovine Serum
IC_{50}	50% Inhibitory Concentration
P/S	Penicillin / Streptomycin
PBS	Phosphate Buffered Saline
PyBOP	Benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate
NMM	*N*-methymorpholine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

Peptides

AMP	Antimicrobial Peptide
FAM-TB	5-6-Carboxyfluorescein – Temporin B
His-5	Histatin-5

Other

Amp	Amphotericin B
eq.	equivalents
ES+	Electrospray positive mode
Fmoc-SPPS	Fmoc Solid Phase Peptide Synthesis
LPG	Lipophosphoglycan
MALDI-ToF	Matrix Assisted Laser Desorption/Ionisation – Time of Flight
MS	Mass Spectrometry
MTT	3(4,5-dimthyl-thiazol-20yl)-2,5-diphenyl tetrazolium bromide
PE	Phosphatidylethanolamine
PPG	Proteophosphoglycans
PS	Phosphatidylserine

Figures and Tables

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Publications

1. "Evaluation of a solid-supported Tagging Strategy for Mass Spectrometric Analysis of Peptides." Siân R. Hudson, Frances L. Chadbourne, Philip A. Helliwell, Elsa Pflimlin, Jane E. Thomas-Oates, and Anne Routledge, *ACS Comb. Sci.*, 2012, *14* (2), 97–100.

2. "Studies on the Antileishmanial Properties of the Antimicrobial Peptides Temporins A, B and 1Sa." F. L. Chadbourne, Catroina. L. Raleigh, Hayder. Z. Ali, Paul. W. Denny, Steven. L. Cobb, *J. Peptide. Sci.* 2011, 17 (11), 751–755.

3. "Two-Photon Induced Responsive f-f Emissive Detection for Cyclin A with Europium-Chelating Peptide." Hoi-Kwan Kong, Frances L Chadbourne, Ga-Lai Law, Hongguang Li, Hoi-Lam Tam, Steven L Cobb, Chi-Kong Lau, Chi-Sing Lee and Ka-Leung Wong, *Chem. Commun.*, 2011, 47, 8052-8054.

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Chapter I

Introduction

Chapter 1 **Introduction**

Tropical diseases are defined as those diseases that mainly occur in tropical or subtropical regions. A sub-group of tropical diseases, designated as neglected tropical diseases includes 17 diseases, according to the first World Health Organisation (WHO) report on the subject. To be regarded by the WHO as a neglected tropical disease, a disease must meet the following criteria: (1) it prominently affects poor countries, (2) it affects low-income and politically marginalized populations, (3) it does not spread widely as its distribution is restricted by climate and the effects of climate on the distribution of vectors and reservoir hosts, (4) it causes stigma and social discrimination, especially in women, (5) it has a relevant impact on morbidity and mortality, (6) it is relatively neglected by researchers[1].

The seventeen tropical diseases classified by the WHO as being neglected are: Buruli Ulcer (Mycobacterium ulcerans infection), Chagas disease, Dengue/Severe dengue, Dracunculiasis (guinea-worm disease), Echinococcosis, Foodborne trematodiases, Human African trypanosomiasis (Sleeping sickness), Leishmaniasis, Leprosy, Lymphatic filariasis, Onchocerciasis (River blindness), Rabies, Schistosomiasis, Soil transmitted helminthiases, Taeniasis/Cysticercosis, Trachoma, and Yaws (Endemic treponematoses); other 'neglected' conditions are: Podoconiosis, Snakebite and Strongyloidiasis.

Infectious diseases caused by parasites are a major threat to mankind, especially in the tropics. More than 1 billion people world-wide are directly exposed to tropical parasites such as the causative agents of trypanosomiasis, leishmaniasis, schistosomiasis, lymphatic filariasis and onchocerciasis, which represent a major health problem,

particularly in impecunious areas. The selection of antiparasitic drugs varies between different organisms. Some of the currently available drugs are chemically *de novo* synthesized, however, the majority of antimalarial drugs are derived from natural sources such as plants which have subsequently been chemically modified to warrant higher potency against these human pathogens[2].

1.1 Leishmaniasis

Tropical diseases such as leishmaniasis, malaria, and Chagas' disease affect millions of people in equatorial countries each year[3]. As per World Health Organization statistics, leishmaniasis currently threatens 350 million men, women, and children in 88 countries around the world. As the disease is vector borne, the transmission of leishmaniasis is most favourable in developing world countries due to poor living conditions and sanitation [4]. Over 90% of the cases of leishmaniasis occur in parts of India, Bangladesh, Nepal, Sudan, and Brazil [5]. Inhabitants of these developing-world tropical countries are at high risk from infection and are often those with the least socioeconomic ability to obtain proper treatments; therefore, they are the most likely to develop serious and often fatal illnesses[6]. An increase in the incidence of leishmaniasis can be associated with urban development, forest devastation, environmental changes and migrations of people to areas where the disease is endemic[7].

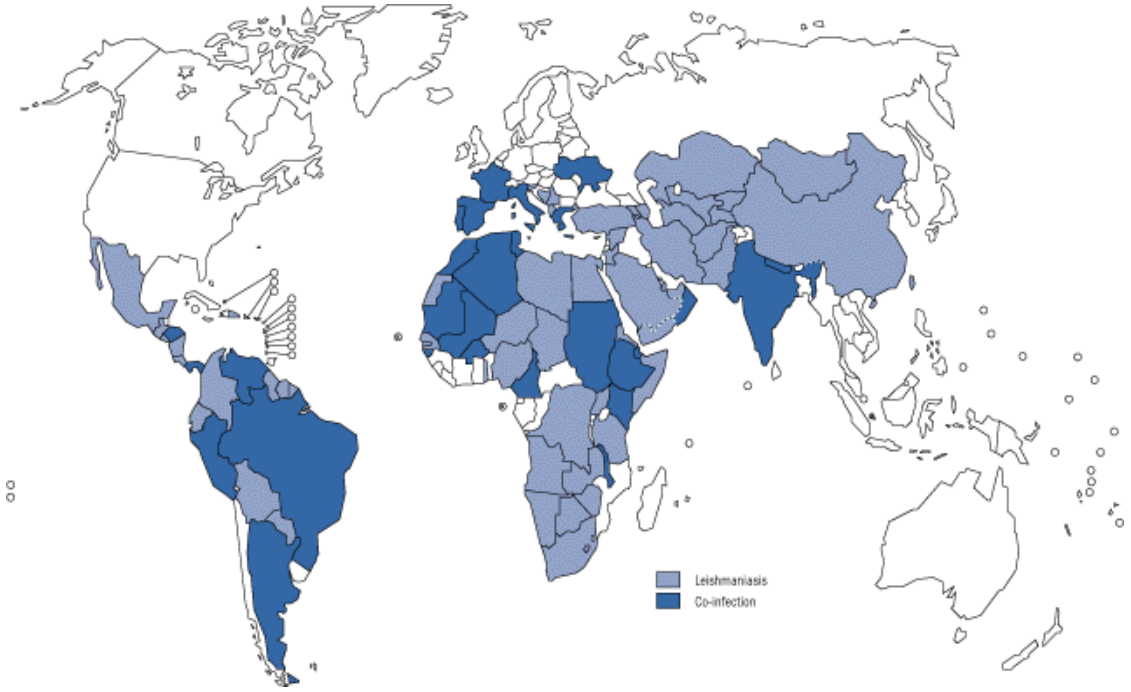


Figure 1.1 Regions of the world affected by leishmaniasis (dark blue) and co-infection with HIV (Light blue)

http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish/en/

Tropical diseases have been largely overlooked in drug discovery programs by major pharmaceutical companies due to the lack of significant financial return on this expensive and time-consuming process (only 13 of the 1300 new drugs introduced during the period 1975 through 1999 were for treating tropical diseases)[3].

Species of the genus *Leishmania*, a protozoan member of the hemoflagellate group, are the causative agents of human leishmaniasis, which has a reservoir in rodents, dogs, saguins, marsupials and others in the wild animal population[8]. Two genera of sandfly transmit *Leishmania* to humans: *Lutzomyia* in the New World and *Phlebotomous* in the Old World[9]. The disease is formerly known as Orient Boil, Baghdad Boil, black fever, sand fly disease, Dum-Dum fever, or espundia[9]. Leishmaniasis presents in several areas

including Africa, Central and South America, the Middle East, the Indian subcontinent and more recently southern Europe. The disease is caused by *Leishmania*, protozan parasites and presents in four forms in humans, as shown in Figure 1.2; cutaneous leishmanisis (CL), diffuse cutaneous leishmanisis (DCL), mucocutaneous leishmanisis (MC) and visceral leishmanisis (VL) which is also known as kala-azar[10]. The symptoms of these various leishmaniasis forms range in severity from skin lesions (CL) to serious disFigurement (MC) and in the worse cases fatal systemic infection (VL)[11]. Visceral leishmanisis carries a significantly higher mortality rate than other forms and if left untreated 100% of cases prove fatal. Cutaneous leishmanisis is the most prevalent form of the disease, with an incidence of 1.5 million cases each year concentrated mainly in central and South America[11].

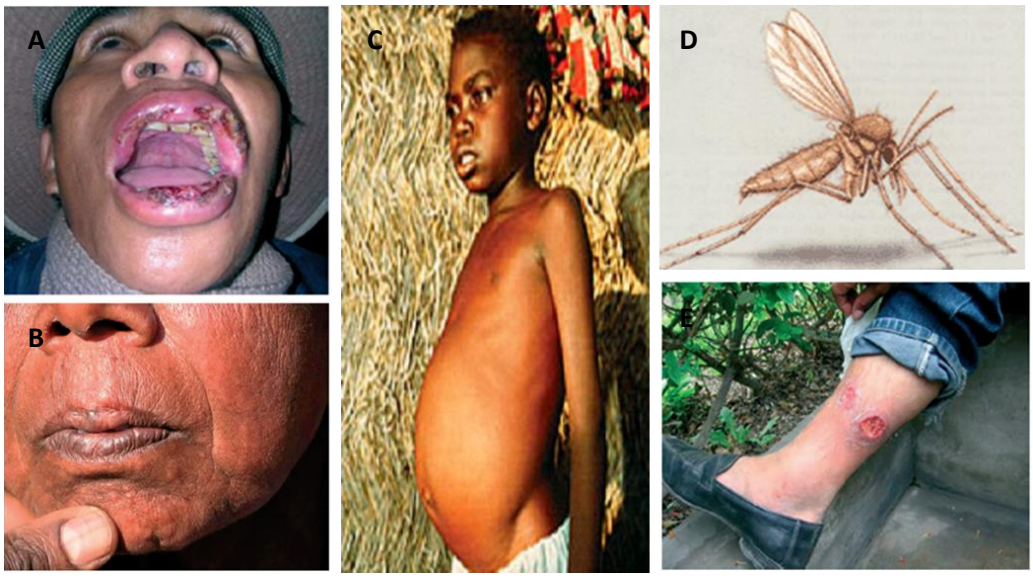


Figure 1.2 Typical Presentations of leishmaniasis (A) Mucocutaneous leishmaniasis; (B) Post kala azar dermal leishmaniasis (PKDL); (C) Visceral leishmaniasis; (D) Sandfly Vector; (E) Cutaneous leishmaniasis[10a,12].

The disease is initiated through the bite of female phlebotomine sandflies infected with the *Leishmania* protozoan. The parasite is internalised via macrophages in the liver,

spleen and bone marrow.[13] Following an incubation period of two to eight weeks, an erythematous papule arises at the site of the sandfly bite. The papule gradually develops into a nodule, increasing in size until ulceration occurs. Multiple lesions may occur if the patient has been bitten in several places. The lifecycle of the *leishmania* parasite is shown in Figure 1.3. The cutaneous form of the disease may also be further complicated by secondary bacterial infections at the site of the ulcers. The lesions formed as a symptom of cutaneous leishmaniasis may heal spontaneously, however a growing problem is increasing incidence of co-infection of the disease with patients previously infected with HIV. In these cases cutaneous leishmaniasis may not heal for many years[14]. Mucocutaneous leishmanisis occurs following a primary infection of cutaneous leishmanisis. Lesions arise in the tissues of the nose or palate and subsequently increase in size, destroying mucosa and cartilage. Obvious effects of this form of the disease are to cause serious disfigurement, with resulting social isolation and psychological impact upon the patient. In addition, if the lesions associated with this form of the disease obstruct the trachea or lungs, death can result rapidly.[15]

The problem of leishmaniasis has been worsened by the evolution of AIDS due to parallel infections in AIDS patients, as well as by the development of drug-resistance by parasites[16].

1.1.2 Leishmania lifecycle

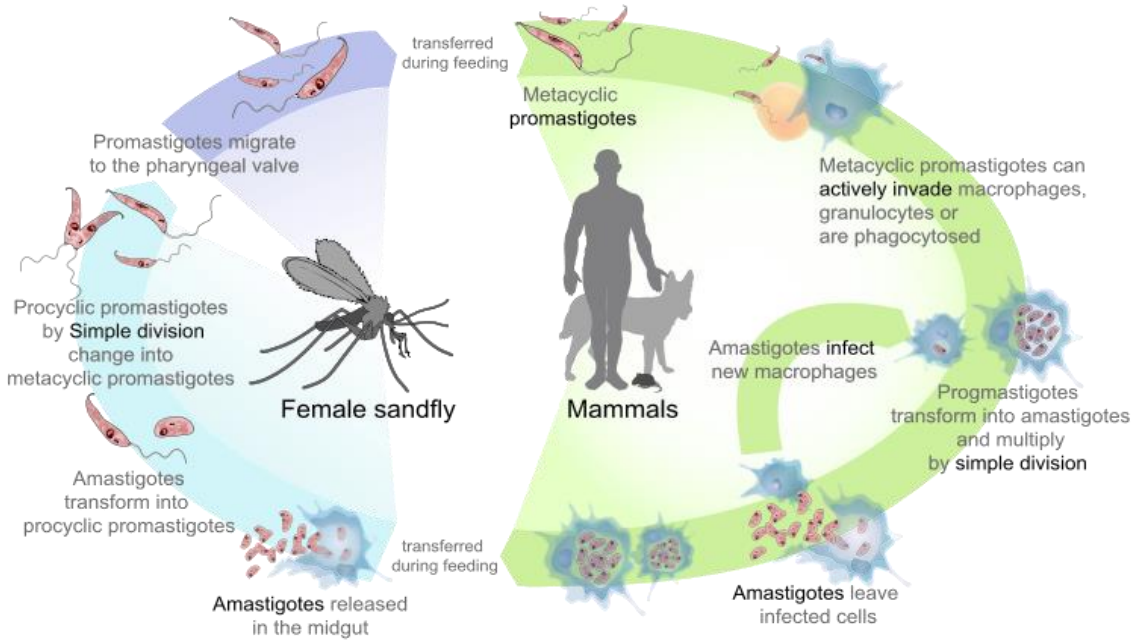


Figure 1.3 *Leishmania* lifecycle

http://www.dpd.cdc.gov/dpdx/HTML/leishmaniasis.htm

Leishmania have a digenic life cycle, Figure 1.3. In the mammalian host, the non-motile amastigote is an obligate intracellular parasite, multiplying within a parasitophorous vacuole. *Leishmania* amastigotes are adapted to thrive in the phagolysosome of mammalian macrophages. Host cells with a heavy burden of parasites are prone to lysis, releasing amastigotes which can infect further macrophages. Blood-feeding activity by a sandfly vector can result in ingestion of infected macrophages. Amastigotes are released into the insect gut as the host cell is digested, and the parasite undergoes a developmental switch to the promastigote stage. This transformation is exemplified by elaboration of a motile flagellum and can be reproduced under axenic conditions by changes in temperature and pH of *in vitro* culture media[17]. In the lumen of the insect

gut, promastigotes multiply and give rise to non-dividing metacyclic promastigotes which are able to establish infection in a mammalian host.

Until recently, *Leishmania* promastigotes have been the focus of much study. However, developments in techniques permitting the *in vitro* growth of amastigote-like organisms have allowed studies to take place in order to draw comparisons and highlight important differences between the *Leishmania* promastigotes and amastigotes[18].

Amastigotes are the cause of acute disease (ranging from self-healing cutaneous infections to severe disfiguring mucocutaneous and lethal visceral disease), as well as chronic or latent infections that can persist for the life time of the host. Despite the importance of amastigotes in perpetuating disease and as the target of antileishmanial drugs, comparatively little is known about either the metabolism of this stage *in vivo* or the biochemical composition of the phagolysosome.[18]

The cell surface of procyclic promastigotes (the insect stage of the parasite) is surrounded by a glycocalix layer, composed mainly by the lipophosphoglycan (LPG), a highly negative molecule encompassing phosphorylated disaccharide repeating units and bound to the membrane through a glycosylphosphoinositol (GPI) anchor [19]. LPG is absent or very low in amastigotes (the intracellular pathological form of the parasite for vertebrates) [20], and this might explain the weak activity of the majority of cationic AMPs towards this stage of the parasite. Also metacyclic promastigotes (the circulating form of *Leishmania* in the blood of an infected mammal for about 24 h, before being engulfed by macrophages and transformed into amastigotes) are less sensitive to the activity of AMPs. This could be related to the fact that the number of repetitive units of LPG is double on the metacyclic stage of the parasite, favouring electrostatic

interactions with AMPs, and thereby limiting the peptides' partitioning into the cytoplasmic membrane, Figure 1.4.[21]

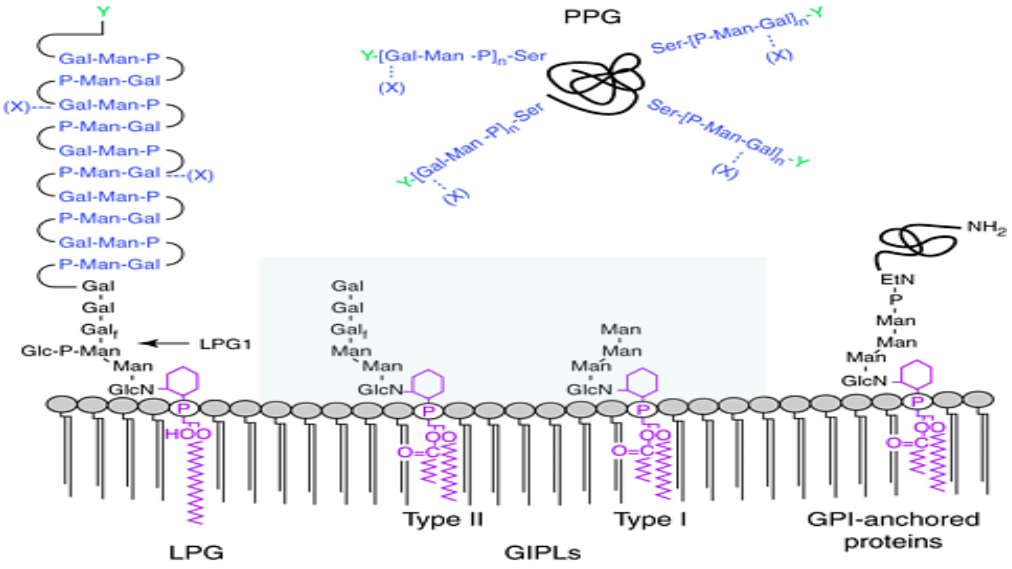


Figure 1.3 Structure of *Leishmania* parasite cell surface[18] lipophosphoglycan (LPG), glycoinositol phospholipids (GIPLs), glycosylphosphatidylinositol (GPI), proteophosphoglycan (PPG).

In order to fully understand the differences displayed between amastigote and promastigote forms of *Leishmania spp.*, genetically modified mutants have been designed to exploit the discrepancy between the two forms. Promastigotes particularly, have been developed lacking key surface coat constituents[18, 22]. The GPI anchored proteins, LPG and GIPL membrane constituents have been a focus of study [23, 24].

Research into *L. major* Lpg1 parasitic mutants reveals that deletion of genes used for LPG or GPI anchor synthesis result in a loss of virulence [25]. In *L. mexicana*, the inability to produce clones containing a deletion of GPI proteins suggests that these glycolipids may be essential for parasite growth [17]. In some cases modifications of the surface coat result in reduced growth rate, inability to transform, altered morphology

and, occasionally, no change at all [22, 23]. *Leishmania spp.* mutants remain an essential tool for understanding the mechanism of survival and transformation of the parasite. Mutants can also be beneficial to drug screens, where alterations to the target molecule or site can be utilized to fully understand the mechanism and site of drug action.

In vitro cultivated amastigotes (derived from *in vitro* differentiated promastigotes or lesion amastigotes) have been used to investigate some aspects of amastigote metabolism, these stages are typically grown in rich medium that is unlikely to mimic the biochemical milieu of the phagolysosome[18]. Analysis of amastigote metabolism *in vivo* is further complicated by the fact that different species of *Leishmania* reside within different populations of macrophages and can induce morphologically distinct phagolysosomes within the same population of macrophages [26].

For example, members of the *Leishmania mexicana* complex induce spacious communal vacuoles rather than the tight fitting individual phagolysosomes occupied by many other species (e.g. *Leishmania* major, *Leishmania donovani*). Moreover, the *L. mexicana*-occupied phagolysosomes become filled with electron-dense material over the course of several days, thus indicating temporal changes in the biochemical composition of the phagolysosome lumen [27].

The treatment of leishmaniasis is difficult because of the intramacrophagic location of the infectious form. Victims of this illness present an immune deficiency and are not able to eliminate the parasites through a natural mechanism of defence[8]. The problem of leishmaniasis has been worsened by the evolution of AIDS due to parallel infections in AIDS patients, as well as by the development of drug-resistance by parasites[16].

1.2 Current Chemotherapy of Leishmaniasis

Treatment of cutaneous leishmaniasis (CL) should be directed toward the eradication of the amastigotes and reduction of the size of the lesions with minimal scarring and toxicity. No single treatment modality to date has been shown to be indisputably superior to others[28]. The majority of current treatments are based on chemotherapy (although other treatments including heat therapy are also possible), relying on a handful of drugs with significant limitations, including high cost and toxicity, difficult route of administration and lack of efficacy in rural areas[11]. The most common syndrome is localised cutaneous leishmaniasis, which is frequently caused by *Leishmania major* and *Leishmania tropica* in the old world (Mediterranean basin, Middle East and Africa), and by *Leishmania braziliensis*, *Leishmania Mexicana*, and related species in the New World (Mexico, Central America and South America)[29].

Several uncontrolled trials, have demonstrated moderate to excellent treatment efficacy and cosmetic outcomes via cryotherapy or heat administration. The use of surgical excision and curettage is rarely recommended in New World disease because of high risk of relapse, lymphatic dissemination, and disfigurement. Several drugs such as allopurinol (**1**), nifurtimox (**2**), dapsone (**3**), chloroquine (**4**), and rifampicin (**5**) have been proposed as treatment alternatives for leishmaniasis[30] Figure 1.5. Their implementation, however, is not widely accepted as treatment efficacy is not convincing, and experience with their use is limited.[28]

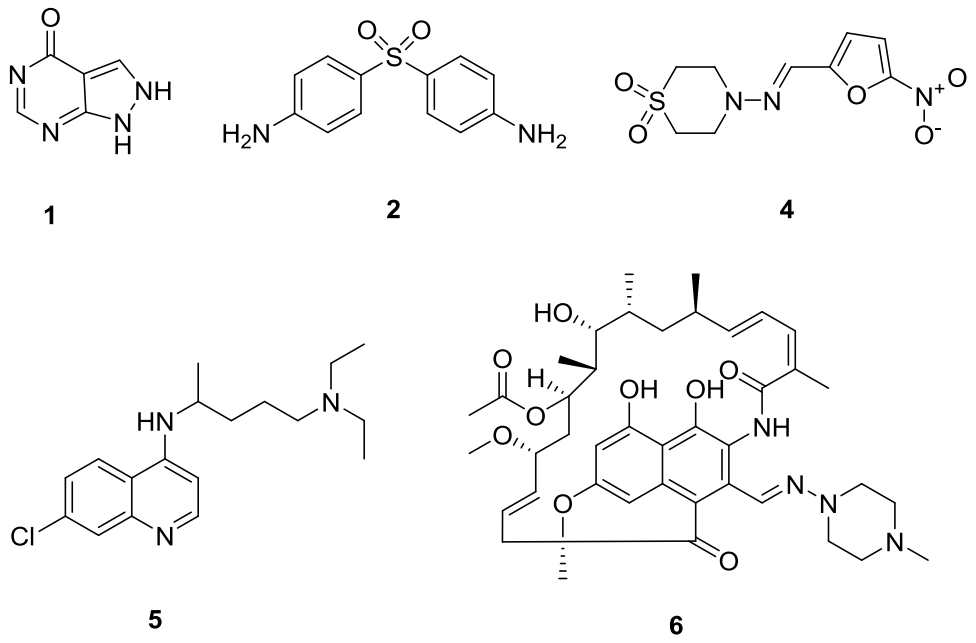


Figure 1.4 Drugs currently approved for the treatment of other diseases proposed as treatment alternatives for leishmaniasis

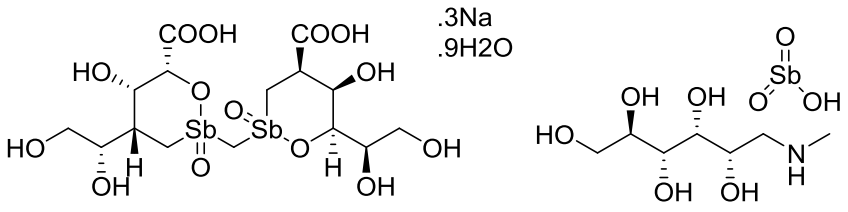
1.2.1 First line therapies: Pentavalent antimonials

The pentavalent antimonials such as sodium stibogluconate (Pentostam) (**7**) and meglumine antimoniate (Glucantime) (**8**) have been used for the treatment of leishmaniasis for over 70 years[31], unfortunately, resistance to the pentavalent antimonials is increasing, and in some areas, particularly Bihar, India, their use is becoming severely limited due to lack of efficacy[12]. This class of drugs are toxic with severe side effects including acute pancreatitis and cardiac arrthymia, and usually reversible muscle pains, renal failure, cardiotoxicity and hepatoxicity[31].

The poor accumulation of antimonial drugs in the skin has caused some posologic differences in their administration in patients affected with cutaneous leishmaniasis. In addition, little or no immune response at the lower temperature of the skin (33.0 °C) in

comparison to core body temperature (37.0 °C) can explain the longer regimes of antimonial medication against dermal leishmaniasis. Several randomised trials performed against New World cutaneous leishmaniasis demonstrated that, in following the WHO recommendations, the efficacy of sodium stibogluconate is far from being complete and relapses occur after healing. However, with increased doses or an extension of the length of time of treatment, cures and no relapses have been reported.[32] The formulation's active ingredient is the heavy metal antimony (Sb), and in order to obtain significant plasma levels, a large dose of drug must be injected. The spleen is one of the organs of accumulation, targeting *Leishmania*-infected macrophages.

As is the case with arsenic, pentavalent antimony is reduced to more toxic trivalent forms that may be responsible for the toxic side effects in the host. Antimonials are not safe drugs, and the WHO limitation can prevent use of toxic levels of the drug. In patients for whom medication must be increased because of relapse, no healing, or persisting symptoms in overweight patients – abdominal cramps, myalgais, artharlgias, alteration of hepatic transaminases, and changes (frequently reversible) in electrocardiographic patterns – are common.[32]



7 **8**

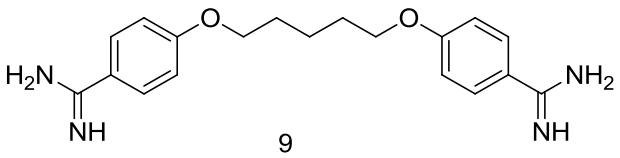
Sodium stibogluconate (**7**) and meglumine antimoniate (**8**)

1.2.2 Second-line therapies

Pentamidine isethionate

Pentamidine isethionate (**9**) has been shown to have clinical activity against a number of protozoa such as *Leishmania*, certain strains of *Trypanosoma*, and *Babesia*, as well as certain fungi, such as *Candida albicans*. The precise mode of its antiprotozoal action is not fully understood. This treatment may also be used in individuals intolerant to antimonial treatment, or in cases of antimonial resistance.[28]

The pentamidine regimen has the advantage of a short time course. Despite this, frequent adverse reactions with moderate morbidity have been associated with its use, to include an unusually high rate (50%) of hyperglycemia, most likely as a result of pancreatic damage, as well as hypotension, tachycardia, and electrocardiographic changes.[30a]



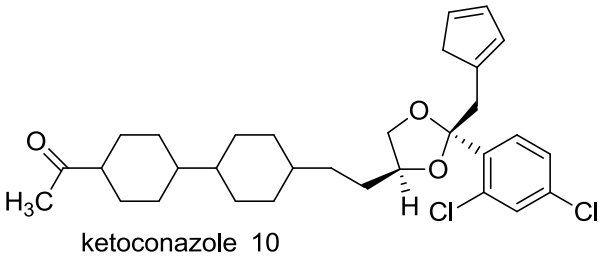
9

Pentamide isethionate (**9**)

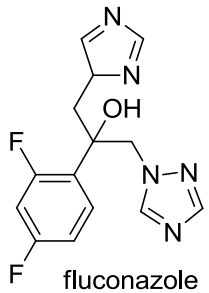
Inidazoles/triazoles (ketoconazole, fluconazole, itraconazole)

Imidazoles (eg ketoconazole (**10**)) and triazoles (eg fluconazole (**11**) and itraconazole (**12**)) are separate classes of antifungal compounds sharing the same antifungal

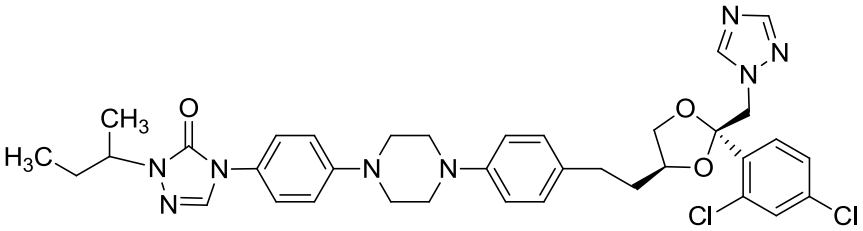
spectrum and mechanism of action, however triazoles are metabolised more slowly, interfere less with human sterol synthesis, and are therefore less toxic than imidazoles.[33]



ketoconazole 10



fluconazole 11



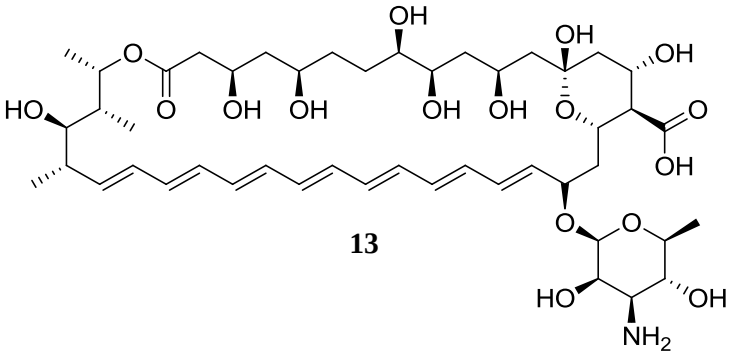
itraconazole 12

Studies on use of oral ketoconazole for the treatment of New World CL emphasize the importance of speciation because the efficacy of treatment has been shown to vary according to species. Moreover, in vitro studies have failed to produce consistent results on the susceptibility of each of the *Leishmania* species to the azole compounds.[28] The modest activity of oral ketoconazole against *L. mexicana*, and the substantially lower efficacy against *L. braziliensis* has, also, been demonstrated by other trials.[34] It appears that oral ketoconazole may be effective in the treatment of the more readily self-curing forms of CL (cutaneous disease caused by *L. mexicana* and *L. panamensis*) and, therefore, can reasonably but cautiously be recommended as initial treatment.[35] As of this date, no studies on the use of fluconazole or itraconazole for the treatment of New World CL were available.

Amphotericin B

Amphotericin B (**13**), a widely used antifungal compound, is also commonly used where antimonial failure occurs. Newer drugs, including the lipid formations of amphotericin B (AmBisome, Amphocil and Abelcet) have been shown to be effective in the treatment of visceral leishmaniasis[36]. However although these preparations are less toxic they are also more expensive. This is a prohibitive factor in the use of these formulations as treatments, a direct result of which, testing against cutaneous leishmaniasis has been severely limited, thus reducing treatment for the majority of patients presenting with visceral leishmaniasis[36]. Amphotericin B is generally not indicated for CL, except for mucosal lesions unresponsive to antimonial therapy.[37]

A limited number of studies have been conducted to determine the treatment efficacy of amphotericin B in New World CL. Solomon *et al* [38] reported complete clinical cure with liposomal amphotericin B in seven patients with cutaneous lesions caused by *L. braziliensis* infection. This study, however, was nonrandomized and included relatively few patients. Similarly, several case reports of successful treatment of New World CL (most caused by *L. braziliensis*) with amphotericin B exist.[38b, 39]

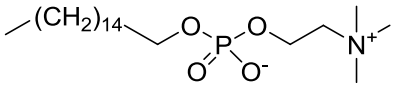


13

Amphotericin B (**13**)

Miltefosine

Miltefosine (**14**) was originally developed as an anticancer drug[40], and is the most recent drug to be introduced into clinical practice. Promisingly, this has been shown to be an effective oral treatment for visceral leishmaniasis in India[40] and has also been used for the treatment of cutaneous leishmaniasis caused by *L. vianna panamensis*, but not *L. V. braziliensis*[40]. At the date of data being published (2009), there were no reported cases of miltefosine resistant leishmaniasis, although it is expected that non-adherence to the recommended treatment regimen could lead to the emergence of parasite resistance[41], due largely to the relative ease with which miltefosine resistant parasites can be generated *in vivo*[42]. It is expected that miltefosine will be the major form of treatment in India and the surrounding regions in the foreseeable future[36].



14

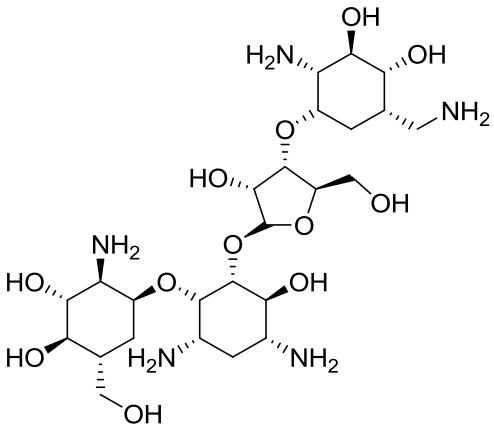
Structure of alkylphospholipid miltefosine (**14**)

Paromycin (aminosidine)

Paromomycin sulphate (**15**) is a broad-spectrum aminoglycoside antibiotic, shown to possess antileishmanial properties for both visceral[43] and cutaneous[44] leishmaniasis. The advantages of this treatment include a broad spectrum of activity, and low cost. The drug is marketed as an oral antiparasitic drug and a topical antileishmaniasis agent.[28] in addition, although resistance to aminoglycosides is well documented in bacteria, no clinical resistance has been reported for *Leishmania*.

Strains of *L. mexicana*, on the other hand, have been found to be highly susceptible to paromomycin sulfate treatment.[45] Currently, topical treatment of New World CL is not recommended except in cases of infection with *L. mexicana* where the risk of progression to mucosal leishmaniasis is unlikely.[37, 46] In cases of *L. mexicana* infection topical 15% paromomycin/12% methylbenzethonium ointment may be an acceptable, less costly alternative if first-line treatment with pentavalent antimony is not readily available.

A combination of 15% paromomycin and 0.5% gentamicin was used topically twice daily for 10 days to treat CL in mice. For ulcers caused by *L. mexicana, L. panamensis*, and *L. amazonensis*, the researchers reported 100% cure rate and no evidence of relapse.[47] However, a phase II trial in human beings that was conducted in Colombia failed to reproduce these findings. Treatment with this combination ointment resulted in nonstatistically significant difference in the cure rates between those individuals who were treated and those who were given a placebo.[48]



15

Paromomycin dihydrogen sulfate salt (**15**)

1.3 The development of antileishmanial vaccines

Visceral leishmaniasis is a deadly disease, however there are as yet no human vaccines licensed for use, and most vaccines studies have focused on the less severe cutaneous form of the disease[49] Despite the knowledge about various life stages of the parasite and the ongoing work, designing an effective vaccine against leishmaniasis is still a matter of research, there are a number of potent vaccine candidates, however issues regarding the cost, antigenic complexity along with the variability of organisms and the mixed type of responses produced in the host are limiting the progress in the relevant direction. Thus the technical challenges and the complexity in the immunity against the parasites clearly contribute to the absence of vaccines. The major hurdle in developing a potent vaccine is lack of more than one experimental model for studies which do not provide us all the facets of immune responses in humans and the safety issues further limits their development.[50]

1.4 Natural products as drug molecules

The increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived substances, an untapped source of antimicrobial chemotypes, which are used in traditional medicine in different countries.[51]

Nature has been a source of medicinal products for millennia, with many useful drugs developed from plant sources. Following discovery of the penicillins, drug discovery from microbial sources occurred and diving techniques in the 1970s led to more drug discovery from marine based compounds. Combinatorial chemistry (late 1980s), shifted

the focus of drug discovery efforts from Nature to the laboratory bench, however the move is now back to drugs derived from nature.[52]

The explosion of genetic information led not only to novel screens, but the genetic techniques permitted the implementation of combinatorial biosynthetic technology and genome mining. The knowledge gained has allowed unknown molecules to be identified. These novel bioactive structures can be optimized by using combinatorial chemistry generating new drug candidates for many diseases.[52]

1.5 Natural products as a new source of antileishmanials

Research is taking place to discover new antileishmanial agents derived from natural products, as these have been shown to be rich sources of active bioactive compounds for the treatment of *Leishmania*.[53]

1.5.1 Plant derived natural products

The potential antileishmanial properties of plant-derived compounds have also been investigated, with many being used as folk-remedies. Plant-derived products form a large group of compounds containing several products with leishmanicidal activity that includes quinines, alkaloids, termenes, saponins, phenolic derivatives and other metabolites[54]. The most promising antileishmanial compounds have been shown to be some alkaloids including benzoquinolizidine alkaloids[55], terpenes: diterpenoids[56], sesquiterpenes[57] and phenolics such as neolignans[58] or naphthoquinones[59]. Novel compounds isolated shown to possess antileishmanial activity include manzamine alkaloids[60], triterpenoids[61], compounds isolated from medicinal plants of ivory coast[62], ferns[63] and 2-substituted quinolinines[64]. However, most of these plant-derived compounds do not meet the required levels of *in vivo* activity or cytotoxicity for drug development, and would require additional modifications to their chemical structure in order to be considered further.

1.5.2 Antimicrobial Peptides

In recent years, the widespread overuse of conventional antibiotics has led to the emergence of multi-drug-resistant bacterial and fungal strains. This has become a serious concern in the developing world, increasing the urgency of the search for new antimicrobials with new modes of action[65]. In mammals, AMPs produced in response to infection and represent key components of the innate immune system[14]. They are expressed by a wide variety of cell types and predominate at portals of microbe entry such as the gastrointestinal, respiratory, urogenital tracts and in the skin,[65-66] and are also expressed in plants.

AMPs consist of short polypeptides of 10-40 residues produced in bacteria and fungi[66, 67,68], in addition to higher eukaryotes (plants, animals and humans) where they represent key components of the immune system[68, 69, 70, 71, 72]. Since their discovery in plants in 1972, over 800 AMPs have been isolated from members of almost every kingdom and phylam including humans[73, 74]. Due to the high cost for the commercial production of long peptides, particular interest has been given to small linear peptides that can be efficiently made by chemical synthesis at competitive costs and that have reduced or no immunogenicity[21].

Based on their secondary structures AMPs are classified into four major classes: α-helical, β-sheet, looped, and extended peptides [75]. Importantly, AMPs are active against pathogens resistant to traditional antibiotics[75-76], and thus offer the possibility to develop a new class of antibiotics. In addition some AMPs might be useful in treating cancer,

and there are already several peptides in preclinical and clinical trials[77]. Certain subsets of AMPs (defensins and some cathelicidins) have been found to be cysteine-rich, allowing extensive intra-disulfide bonding to occur, which is important for activity[78].

Antimicrobial peptides (AMPs) are promising novel antibiotics and antiprotozal agents, as they have been shown to exhibit broad antimicrobial spectra and do not easily induce resistance[79]. However, the use of AMPs as antiprotozal agents has yet to be fully investigated, largely attributed to parasitic infections being largely prevalent in developing countries, and therefore not being financially viable for pharmaceutical companies.

There is a consensus that, in general, cationic AMPs and some lipopeptides recognize and interact with the acidic phospholipids (e.g., phosphatidylglycerol, cardiolipin) exposed on the outer leaflet of the bacterial membrane. In contrast, in mammalian cells, the outer leaflet is zwitterionic, which should reduce the binding capacity of the cationic AMPs. This can account for the preferential activity of AMPs against bacteria and partially against fungi [80].

In addition to their large spectrum of antibacterial and antifungal activities, it has been shown that several amphibian AMPs can also inhibit cell-mediated HIV capture and infection,[81] a useful attribute as HIV co-infection with leishmaniasis is rapidly becoming a problem in the developing world[82].

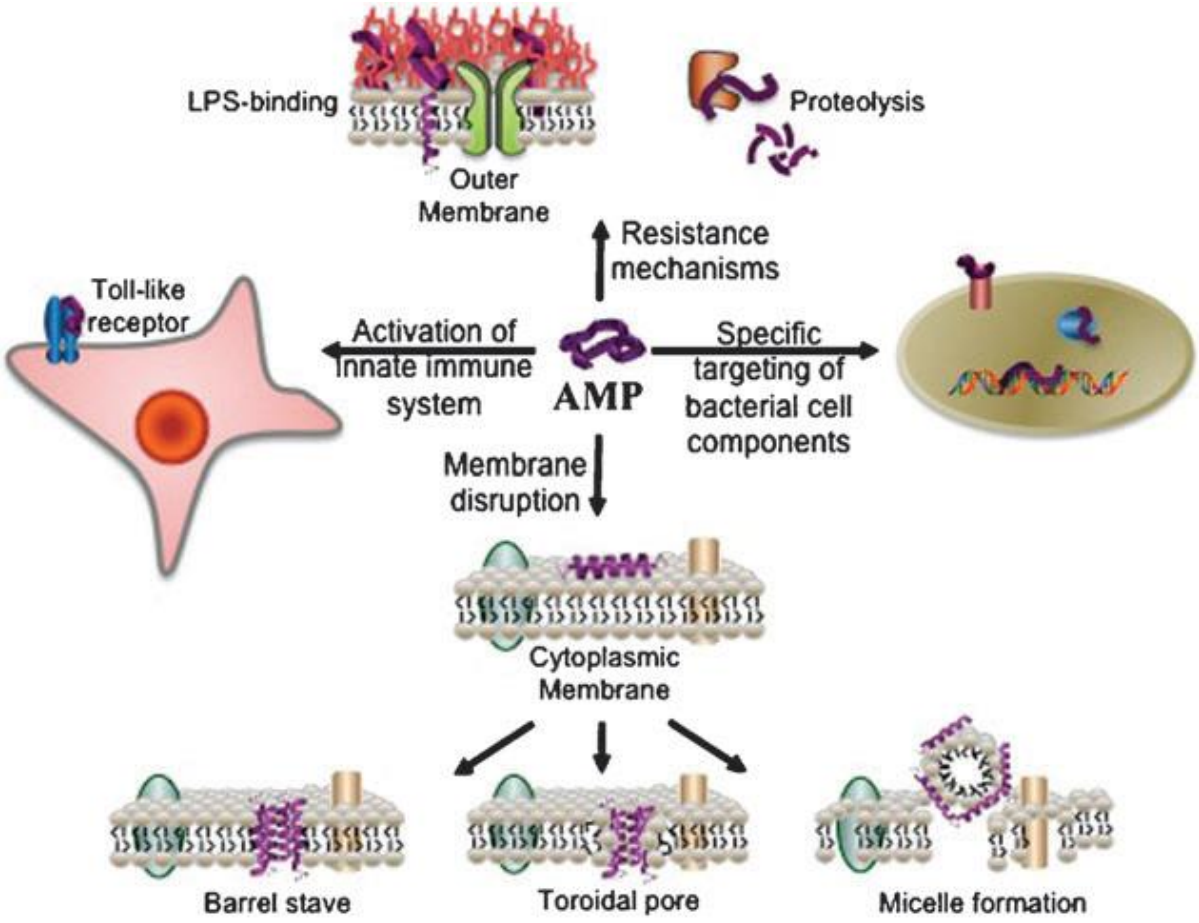


Figure. 1.9 Overview of the broad spectrum of cellular interactions associated with antimicrobial peptides. In addition to exerting antimicrobial activity by disrupting bacterial membranes, peptides may also bind to specific target proteins within microbial cells and activate the innate immune system. The binding of peptides to cell-surface LPS molecules and proteolysis contribute to bacterial resistance to AMPs.[83]

1.5.3 Antileishmanial activity of AMPS

AMPs are a promising target for development of antileishmanial agents for a number of reasons (see figure 1.6); they have been shown to exhibit a lack of toxicity towards mammalian cells at concentrations required to kill *Leishmania* parasites, and importantly, their mechanism of action has been shown to operate via disruption of biological membranes, a mechanism significantly different to those of current therapies

to which resistance has developed. There are however trials beginning to take place within countries where the prevalence of leishmaniasis is high, in order to test potential therapies within the country where the treatment is most needed.

In the case of other microorganisms, such as parasites of *Leishmania* genus, the cell surface of procyclic promastigotes (the insect stage of the parasite) is surrounded by a glycocalix layer, composed mainly by the lipophosphoglycan (LPG), a highly anionic molecule encompassing phosphorylated disaccharide repeating units and bound to the membrane through a glycosylphosphoinositol anchor[19]. LPG is absent in amastigotes (the intracellular pathological form of the parasite for vertebrates)[20], and this might explain the weak activity of the majority of cationic AMPs towards this stage of the parasite. Also metacyclic promastigotes (the circulating form of *Leishmania* in the blood of an infected mammal for about 24 h, before being engulfed by macrophages and transformed into amastigotes) are less sensitive to the activity of AMPs.[21]

AMPs do not easily induce resistance due to their proposed mechanism of action, (Figure 1.6) in which they physically permeate and destroy the cell membrane, causing damage that is difficult for the bacteria to repair, rather than acting via a receptor-mediated mechanism[84]. Conversely, commonly used drugs operate on specific intracellular targets and do not modify the bacterial morphology, making it easy for the microorganisms to become resistant to those drugs[84].

More than a dozen companies are developing antibiotic peptides and peptidomimetics, including the cationic peptide Melamine, as a coating, to help reduce the risk of contact-lense related infections, and numerous others of which are in clinical trials[85]. However

the trials are limited to topical applications, due the fact that peptides which have previously indicated limited toxicity towards mammalian cells *in vitro* are usually toxic when injected into the bloodstream, although this issue had not been well documented[79, 86]. The synergistic effects of AMPs, both as combinations of different AMPs[68] as well as in combination with other host defence mechanisms, are currently being investigated. In the area of the temporin peptides, these effects are in relation to the use of Temporin L in synergism with other Temporin peptides[84]. Temporins A and B, in combination with temporin L, have been shown to possess antibacterial activity against gram negative bacteria[87].

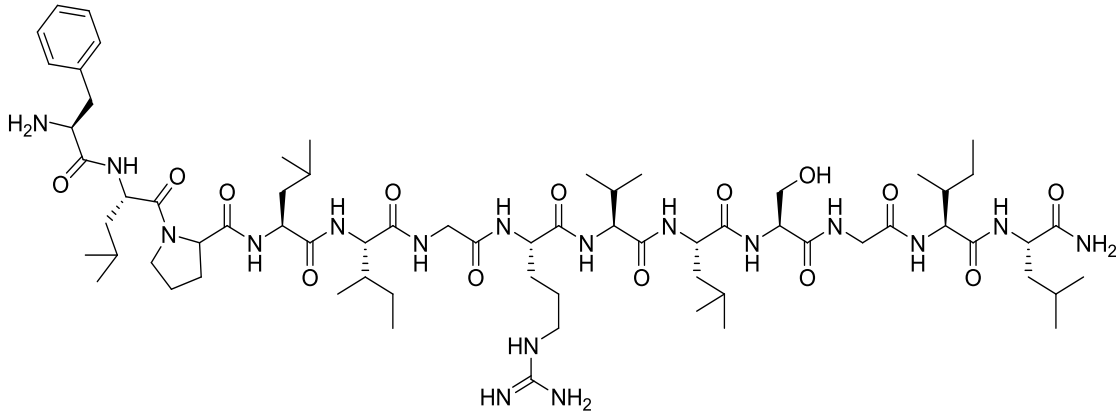
Amphibian skin secretions represent one of the richest natural sources of AMPs, with one such family of peptides, isolated from this source, the temporins being the smallest AMPs found in nature to date[65].

1.5.4 The Temporin peptides

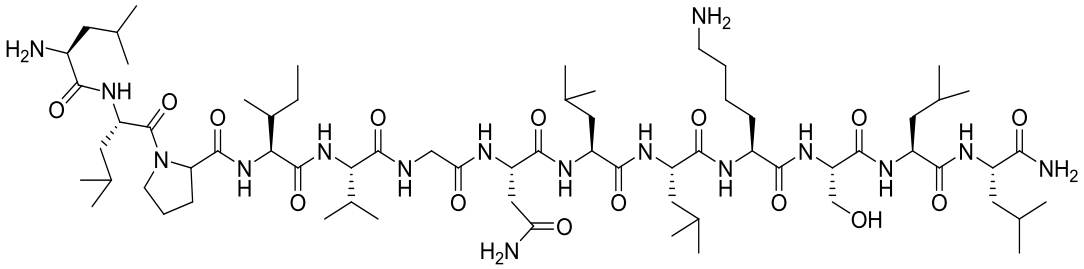
The temporin family of peptides includes more than forty members possessing properties to render them molecules of interest for further investigation of their biological function and mode of action. The properties leading to the temporins being of considerable interest are as follows: (i) temporins are among the smallest amphipathic α-helical AMPs found in nature (10-14 amino acids); (ii) their net positive charge at neutral pH is low, ranging from 0 to +3; (iii) some act efficiently and rapidly against a wide range of pathogens (bacteria, viruses, fungi, yeasts and protozoa) and have low toxicity to mammalian cells; (iv) their mode of action includes perturbation of the cytoplasmic membrane, but in a different way to that proposed for the majority of cationic α-helical AMPs[87]; (v) some temporins display immunomodulatory effects[87];

(vi) they preserve biological function in serum[87]; and (vii) they possess an in vivo efficacy in preventing prosthetic graft infections[87] and against sepsis[87].

Recent studies have revealed that temporins A (**16**) and B (**17**) have a strong antiparasitic action on promastigotes of the *Leishmania* genus *L. donovani* (temporin A 50 % growth inhibition, 8.4 μM, temporin B 50 % growth inhibition, 8.6 μM) and axenic amastigotes of the *Leishmania* genus *Leishmania pifanoi*. (temporin A 50 % growth inhibition, 14.6 μM, temporin B 50 % growth inhibition, 7.1 μM)[88]. These results are of significance, as the antileishmanial activity is present at concentrations not toxic to human red blood cells, and that in addition, in contrast to most AMPs, temporins preserve biological function in serum[89].



Temporin A, FLPLIGRVLSGIL (**16**)



Temporin B, LLPIVGWLLKSLL (**17**)

Temporins are mainly active on Gram-positive bacteria, including clinically isolated methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* and *E. faecalis*, with minimal inhibitory concentrations ranging from 2.5 to 20 MM[90, 91]. Some members are also lethal to Gram-negative bacteria and fungi, such as *Batrachochytrium dendrobatidis*, which is associated with a global amphibian decline[87], and *Candida albicans*. Furthermore, a very broad spectrum of activity was observed with temporin L (net charge +3), against both Gram-positive and Gram-negative bacteria, yeasts, human erythrocytes and cancer cells[92], Table 1.

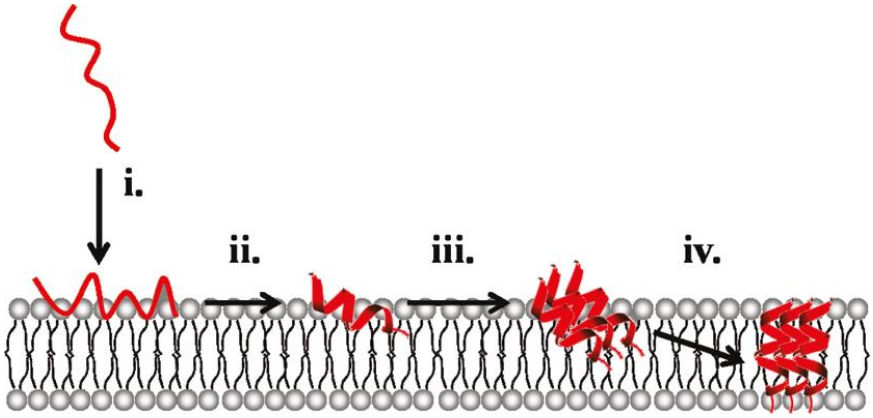
The mechanism of action of the temporin peptides is yet to be fully understood. It is thought by some groups that the presence of sphingomyelin and cholesterol in the plasma membranes of eukaryotes (a major difference from the plasma membrane composition of the bacterial cell membrane) acts to provide the most effective barrier against the insertion of AMPs into mammalian cells[93].

As previously mentioned, the temporins all possess a net positive charge at neutral pH, an attribute crucial to their proposed mechanism of action. It is thought that cationic AMPs interact preferably with the negatively charged bacterial membranes by altering the membrane permeability[94, 95].

Cationic side chains of the amphipathic α-helicalpeptides further interact electrostatically with negatively charged lipids, which neutralize the excess positive

charge of the surface associated peptides and reduce peptide–peptide repulsion[96]. The membrane bound AMP then associate to form aggregates, oligomers, which simultaneously cause membrane permeabilization. Membranes thus provide an environment where AMPs can and must adopt conformations and orientations, which promote peptide aggregation, all these processes being intimately coupled[97]. Monolayer experiments have revealed temporins B and L to be highly membrane active, effectively inserting into zwitterionic phosphatidylcholine (PC) monolayers. The lipid insertion was augmented by the negatively charged phosphatidylglycerol (PG), an abundant constituent of the bacterial target membranes[98].

Data reported on AMPs derived from a combinatorial library suggests that the antimicrobial activity of an AMP is related to the ability of the peptide to destabilise membranes by partitioning into membrane interfaces and disrupting the organisation of the lipids[99], Figure 1.7.



i) Electrostatically enhanced initial association of a random coil peptide to the membrane surface.

ii) The ensuing second step involves several simultaneous processes: intercalation into the bilayer, with the peptide long axis parallel to the membrane layer plane, a conformational change from a random coil to an amphipathic α-helix[100, 101], and ion-pairing of acidic phospholipids with positive residues of the peptide.

iii) Reorientation and membrane insertion of the peptide, its long axis becoming perpendicular to the monolayer surface[94, 102]. In this step some peptide associated acidic phospholipids can be removed from their initial location, so as to have their acyl chains oriented more or less parallel to the plane of the bilayer[103], as required for instance by the toroidal pore model[104].

Figure 1.11 Schematic illustration of the stages in the membrane association/folding/aggregation pathway. Reproduced from Carotenuto *et al.*[100]

The activity of temporins A (**16**) and B (**17**) has also been tested against wild-type parasites and mutants expressing diminished levels of the dense surface polyanionic glycoconjugate, lipophosphoglycan (LPG), suggesting that electrostatic forces between the peptide and the parasitic membrane are not an important deteriminant in their activity[104].

The temporin peptides are active against gram positive bacteria, including methicillin and vancomycin-resistant *Staphylococci* and *Enterococchi*, and are non-toxic towards mammalian cells. Temporin L, a temporin containing both an arginine and lysine residue in its sequence, has been shown to be the only member of the family to exhibit antibacterial activity against both gram positive and negative bacteria[105].

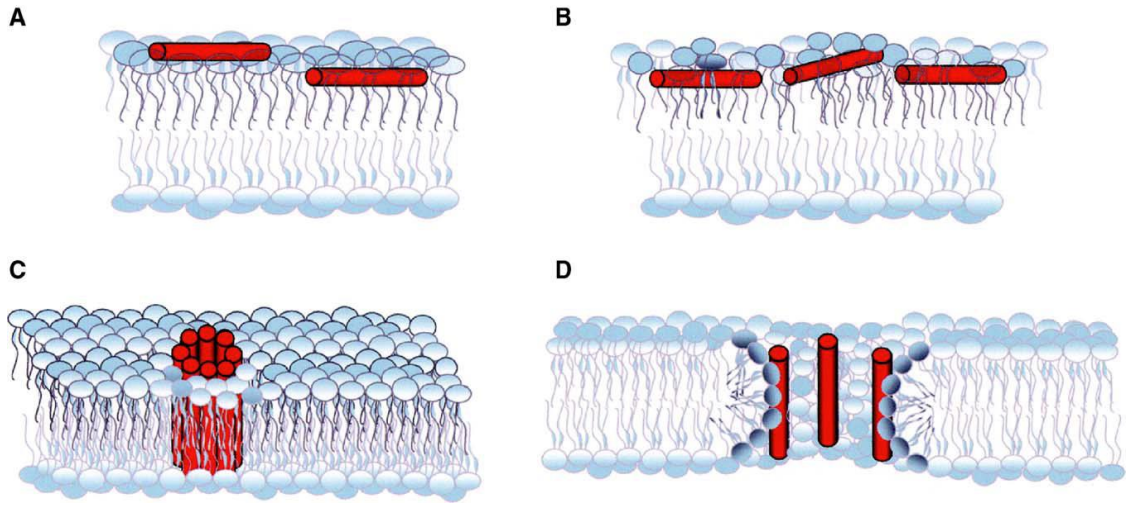


Figure 1.12: Models of transmembrane pore formations: (A) α-helix association with the membrane surface; (B) Peptide accumulation and disruption of membrane packing; (C) Peptide insertion into the membrane as a Barrel Stave pore; (D) Formation of a torroidal pore [75].

In vivo studies involving a large number of mice have been conducted, alongside in vitro experiments aimed at isolating resistant mutants, from which no resistant bacterial colonies have been recovered. The results of these studies suggest that the bacterial membrane not easily altered in order for drug resistance to emerge[106].

The small size and the low net positive charge of temporins could make it easier for them to cross the parasite's glycocalix and permeate the membrane compared with other AMPs that have a higher net positive charge and can stick easily to the negatively charged cell surface. Furthermore, unlike a few natural AMPs that exhibit antiparasitic properties, temporins preserve activity against the more resistant morphological stage of *Leishmania*, the amastigote[87]. This suggests that ionic interactions between the peptide and the parasite do not contribute significantly to the potency of temporins.[21, 87]

The length of temporins has also been shown to be important in determining antimicrobial activity. This is demonstrated by results showing that temporins H, LSPNLLKSLL-NH_2 and K, LLPNLLKSLL-NH_2, both with 10 amino acid residues, do not kill gram positive or negative bacteria (although temporin H has been shown to act synergistically against Gram negative strains when combined with classical antibiotics[(76)]. However, despite their shorter length and diminished charge when compared to other AMPs, temporins A (net charge +2, 13 amino acid residues) and B (net charge +2, 13 amino acid residues) have both been shown to be highly active against both promastigotes and amstigotes of *L.* d*onovani,* and *L. azurea* axenic amastigotes, wherein they act to disrupt the surface membrane and cause loss of intracellular ATP[76].

Temporins can also disintegrate the membrane of *Leishmania* parasites, causing a loss of intracellular material. The membrane of *Leishmania* protozoa is less anionic than that of bacteria and is devoid of phosphatidylglycerol. Temporins are among the smallest natural antiparasitic peptides reported so far that exert their activity by disrupting the parasitic membrane.[107]

Temporins are one of only a few AMPs have so far displayed anti-protozoa activity, and reports on their mode of action are scarce[106]. For *Leishmania,* these include the frog skin polypeptide YY[108], indolicidin isolated from granules of bovine neutrophils[108], gomesin, from the tarantula spider *Achantoscurria gomesiana* [109] and the cecropin-melittin hybrid peptides[87].

In contrast with temporins, which are highly active towards both the insect (promastigote) and the mammalian intracellular stage (amastigote) of the parasite, the

other mentioned AMPs exhibited considerably lower efficacy against amastigotes compared to promastigotes; (see table 1) however, the molecular basis for these differences is not yet clear. An appealing and therapeutically advantageous peculiarity of temporins is that they do not harm macrophages (the host cell for amastigotes) at doses that are lethal to the intracellular parasites[106].

Peptide	**Source**	**Sequence**	**Activity (% growth inhibition, concentration in µM)**		**Ref**
			promastigotes	axenic amastigotes	
Dermaseptin -S1 (DRS1)	amphibia	ALWKTMLKKLGTMALHAGKAALGAAADTISQ GTQ	*L. major* (50,4.5) *L. Mexicana* (50, 1.5)	n/a	[110]
Dermaseptin -S4 (DRS4)	amphibia	ALWMTLLKKVLKAAAKALNAVLGANA	*L. major* (50, 2.0)	n/a	[110a, 111]
Dermaseptin -O1) (DRS-01)	amphibia	GLWSTIKNVGKEAAIAAGKAALGAL-NH_2	*L. amazonensis* (100, 23.4)	n/a	[112]
Dermaseptin -H3) (DRS-H3)	amphibia	GLWISTIKNVGEAAIAAGKAALGAL-NH_2	*L. amazonensis* (78, 13.5)	n/a	[112]
Cecropin A	Insect	KWKLFKKIEKVGQNIRDGIIKAGPAVAWGQAT QIAK-NH_2	*L. donovani* (50, 0.3)	n/a	[113]
Melittin	Insect	GIGAVLKVLTTGLPALISWIKRKQQ-NH_2	*L. donovani* (50, 0.3)	n/a	[113]
Phylloseptin-1	amphibia	FLSLIPHAINAVSAIAKHN-NH_2	*L. amazonensis* (50,50)	n/a	[105]
Temporin A	amphibia	FLPLIGRVLSGIL-NH_2	*L. donovani* (50, 8.4)	*L. pifanoi* (50, 14.6)	[114]
Temporin B	amphibia	LLPIVGNLLKSLL-NH_2	*L. donovani* (50, 8.6)	*L. Pifanoi* (50, 7.1)	[114]
Temporin 1Sa	amphibia	FLSGIVGMLGKLF-NH_2	*L. donovani* (50, 18.1)	L. infantium (50, 22.8)	[115]
Bombin H2	amphibia	IIGPVLGLVGSALGGLL-NH_2	*L. donovani*	*L. pifanoi*	[116]

			(50, 7.3)	(50, 11)	
Bombanin H4	amphibia	[a]L*i*GPVLGLVGSALGGLLKKI-NH_2	*L. donovani* (50, 1.7)	*L. pifanoi* (50, 5.6)	[116]
Tachyplesin -1	crustacean	KWCFRVCYRGICYRRC	*L. braziliensis* (100, 12.5)	n/a	[87]
Skin polypeptide YY	mammal	YPPKPESPGEDASPEEMNKYLTALRHYINLVTR QRY-NH_2	*L. major* (100, 5.9)	*L. major*[d] (100, 6.2)	[117]
Decoralin	insect	SLLSLIRKLIT	*L. major* (50, 7.2)	n/a	[118]
Indolicidin	bovine	ILPWKWPWWPWRR	*L. donovali* (50, 35)	n/a	[119]
***P. duboscqui* defensin**	insect	ATCDLLSAFGVGHAACAAHCIGHGYRGGYCNS KAVCTCRR[c]	*L.major* (50, 68-85)	n/a	[119]
Gomesin	insect	Z**C**RRL**C**YKQR**C**VTY**C**RGR[b,c]	*L. amazonensis* (50, 2.5)	n/a	[106]
Defensin PTH1	plant	RNCKSLSHRFKGPCTRDSNC	*L. donovani* (50, 33.4)	n/a	[120]
Histatin-5	mammal	DSHAKRHHGYKRKFHEKHHSHRGY	*L. donovani* (50, 7.3)	*L. pifanoi* (50, 14.4)	[121]

[a] *i*, stands for D-allo-isoleucine,
[b]Z, stands for pyroglutamic acid
[c] single line indicates disulfide bridge between atoms
[d] testing was carried out using *ex vivo* amastigotes not axenic

Table 1.1: Antimicrobial peptides with activity against *Leishmania* spp.[122]

Histain-5 (His-5) (Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr-NH_2) is an AMP isolated from the saliva of higher primates and has been shown to possess defined antimicrobial, particularly candidacidal activity. As with many AMPs, His-5s activity is independent of enantiomeric composition, verified by a study on His-5 and analogues (the all-d-osomer is also active). However, unlike the majority of AMPs, His-5 has been shown to cross

the membrane of *L. donovani* and subsequently attack the mitochondria of the parasites, inhibiting the F1-F0 ATPase, resulting in disrupted oxidative phosphorylation. The observed higher accumulation of His-5 is accounted for by a naturally lower susceptibility to enzymatic degradation. The activity of His-5, particularly against amastigotes, is assumed to be enhanced due to the abundance of His residues in the primary structure; as the acidic pH of the phagolysosome increases the cationic character of the peptide. Whether His-5 has far reaching antileishmanial activity remains to be seen, however there is still merit in researching Histatin as a shuttle for other antileishmanial peptides or drugs[123].

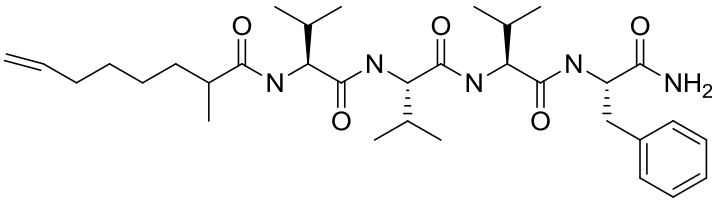
1.5.5 Lipopeptides

Antimicrobial lipopeptides (LiPs) are produced nonribosomally in bacteria and fungi during cultivation on various carbon sources. They are a class of antibiotics that are highly active against multidrug-resistant bacteria. Some also display antifungal activity.[124] Most native LiPs consist of a short (six to seven amino acids) linear or cyclic peptide sequence, with either a net positive or a negative charge, to which a fatty acid moiety is covalently attached to the N-terminus. [124b, 125]

1.3.3.1 Dragonamide E

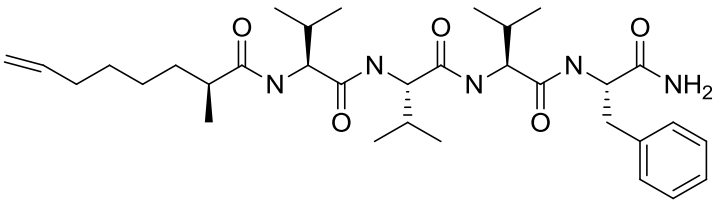
Research within The International Cooperative Biodiversity Group (ICBG) programme has been focused in Panama, searching among marine cyanobacteria and tropical plant endophytes for new, more efficacious, and less expensive treatments for tropical diseases[126, 127, 53]. During the course of this research, a new modified linear lipopeptide,

dragonamide E (**7**), as well as two related compounds previously reported in the literature, dragonamide A (**8**) and herbamide B (**9**), were isolated from a field-collected marine cyanobacterium and found to be active against leishmaniasis in an *in vitro* screening assay.



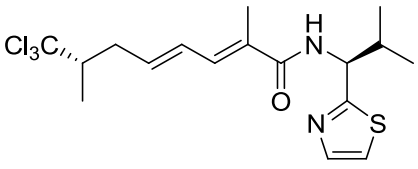
18

Dragonamide E (**18**)



19

Dragonamide A (**19**)



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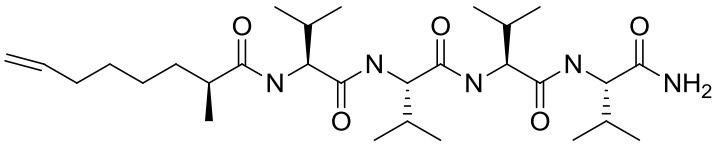
Herbamide B (**20**)



21

Dragonamide B (**21**)

Compounds **1**-**3** were tested for activity against three tropical disease parasites, *Plasmodium falciparum* (malaria), *Leishmania donovani* (*Leishmaniasis*), and *Trypanosoma cruzi* (Chagas' disease). Compound **4** was previously tested in these assays, and those results are also reported here to allow for direct comparison with the biological properties of dragonamides A (**19**), B (**21**), and E (**18**). Dragonamide E (**18**) exhibited moderately strong *in vitro* activity against *Leishmania promastigotes* (5.1 μM), with dragonamide A (**19**) and herbamide B (**20**) also showing comparable activity against this parasite (6.5 and 5.9 μM, respectively). Dragonamide B (**21**) was inactive against all of the tested parasites (up to 100 μM), indicating that the aromatic ring-containing residue at the peptide terminus is necessary for activity. The *in vitro* activity of dragonamide E (**18**) and herbamide B (**20**) against the *Leishmania donovani promastigotes*, the causative agent *of* visceral leishmaniasis supports their further examination through *in vivo* evaluations[128].

1.3.3.2 Ciliatamides

The Ciliatamides are a family of peptide-based compounds isolated from the deep sea sponge *Aaptos ciliate*, isolated as a result of a drug discovery project from Japenese marine invertebrates, discovered by a Nako et al[129].

The Ciliatamides consist of three lipopeptides named Ciliatamides A-C, shown in below.

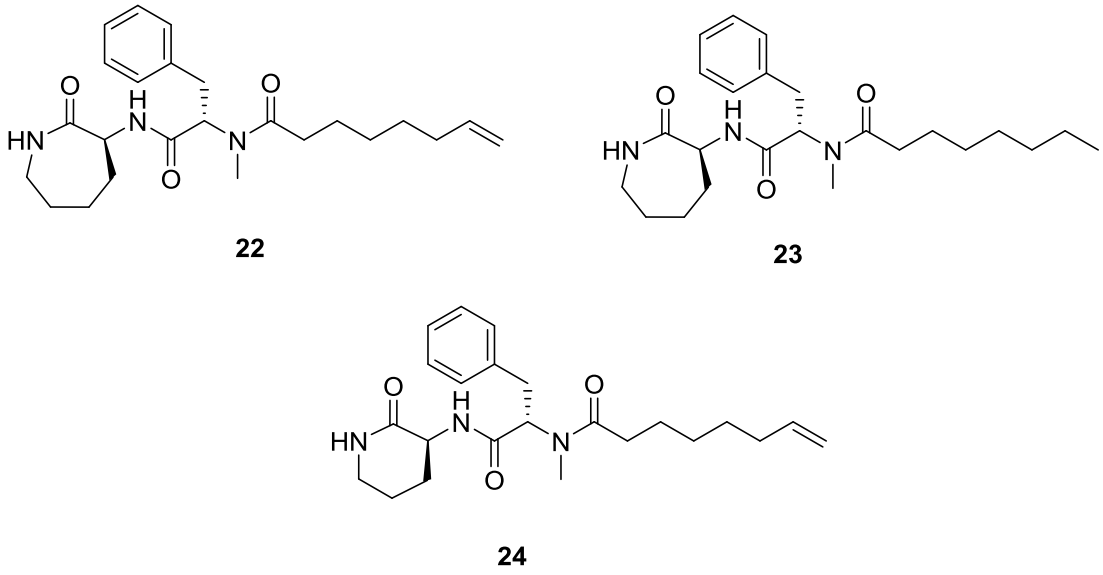


Figure 1.13 Ciliatamides A (**22**), B (**23**) and C (**24**), as reported by Nako *et al*.[129]

Ciliatamides A-C (**22-24**) have previously been tested for activity against promastigotes of *Leishmania* major. Ciliatamide C was found to be inactive, while Ciliatamide A and Ciliatamide B were shown to possess considerable activity. At concentrations of 10 µg/µl, Ciliatamides A and B showed 50 % and 45 % growth inhibition respectively, although the mechanism through which this occurs remains unknown. The organic extract of *Aaptos ciliate* exhibited 86 % growth inhibition at an identical concentration,

a result which suggests that there are other compounds present within the sea sponge *Aaptos ciliate,* which possess higher antileishmanial activities than the Ciliatamides. However at the present date, these compounds have not been isolated.

In August 2008, Lewis et al.[130] Published a total synthesis of Ciliatamides A-C, which included a revision of the stereochemistry proposed in the initial isolation paper. Synthesis of only *(S,S)* Ciliatamides was undertaken by Lewis *et al.*, as this was the stereochemistry reported by Nako et al. However due to disagreements in the suggested stereochemistry of the synthesised Ciliatamaides and those of the natural products, Lewis et al. undertook synthesis of *(S,S), (S,R), (R,S), and (R,R)* Ciliatamides A and B. Comparison of NMR data and optical rotations of the four enantiomers revealed the stereochemistry of the natural Ciliatamides to be *(R,R).* To date, work has yet to be undertaken to investigate the effect (if any) of stereochemistry on the antileishmanial activities of Ciliatamides A and B.

Ciliatamides A and B were selected as good synthetic targets as antileishmanial compounds as they have been shown to exhibit high activity against *Leishmania* major promastigotes, and the structures of these compounds are relatively simple when compared to those of current treatments. The Ciliatamides are therefore likely to be easier to synthesise prior to biological testing. Further biological testing against both promastigotes and amastigotes of a wider range of *Leishmania* species is required in order to determine the full therapeutic potential of these compounds. In addition, studies to determine the biological mode of action of Ciliatamide A would also be useful to determine their potential as antileishmanial agents.

1.4 Aims and Objectives

The aims of this research are to develop new peptide based antileishmanial agents. The development of antileishmanial agents will focus on the temporin family of antimicrobial peptides, and on the Ciliatamide family of lipopeptides. Initial testing will focus of the identification of active compounds against both promastigote and axenic asmastigote lifecycle stages of *Leishmania mexicana* parasites. Testing of a large scale library of temporin peptide will take place, in order to further develop structure activity relationships, and to develop compounds with increased activity in comparison to naturally occurring peptides. On generation of a large scale library of results, the data obtained will be used to attempt to predict active sequences of peptides through the use of computational modelling systems similar to those currently used in the generation of antibacterial compounds. This will be the first time this type of modelling will have been attempted in the prediction of antileishmanial agents.

Investigations in to the activity of the Ciliatamide family of compounds will take place; again with the intention of identifying structure activity relationships and synthesising and identifying related compounds with increased activity.

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Chapter VI

Initial Investigations into the potential application of antimicrobial peptides as new antileishmanials

2.1 Introduction and aims

2.1.1 Introduction

In the last 5 years antimicrobial peptides (AMPs) have emerged as a potential source of new and promising antiparasitic agents.[1] AMPs possess a mechanism of action through which bacterial resistance struggles to develop (i.e. cell membrane permeation) and it has been shown that a similar mode of action is in operation against parasite species for many AMPs[2]. This mode of action is one of the main reasons that researchers have begun to look at AMPs as a potentially new source of novel anti-parasitic agents. One of the parasitic infections that AMPs have been found to be active against is Leishmaniasis[3]. As previously discussed Leishmaniasis is a parasitic infection for which the development of new drugs is currently needed (Section 1, Introduction). The current first and second line therapies for the treatment of Leishmaniasis rely on a handful of drugs, to which resistance emerging, [4] and the use of which results in highly toxic side effects.[4-5] Although only six AMPs to have been tested against the amstigote forms of the parasite have currently been tested against *leishmania* species, they have shown activity against range of different species (i.e. *mexicana, donovani, infantum, pifanoi*).[1b] This range of activity coupled with a mode of action that differs from all current mean that AMPs are excellent candidates for further studies and the potential development of new antileishmanial agents.

2.1.2 Aims

The temporin peptides[6] were selected as a class of AMPs for further study as activity had previously been reported for temporins A, 1Sa and L against *leishmania mexicana,*

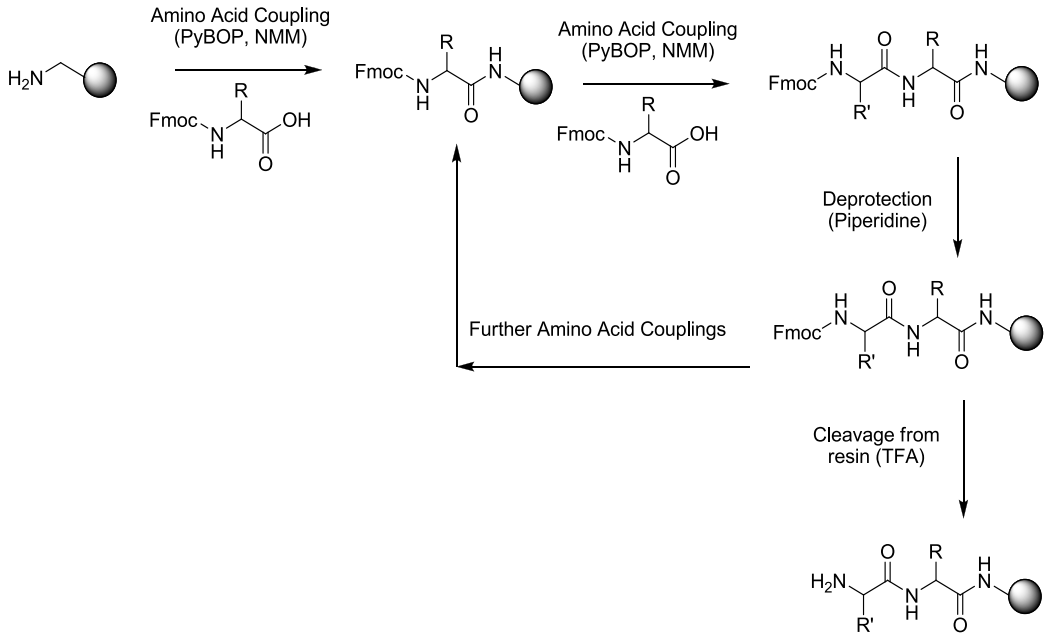
leishamania donovani, and *leishmania pifanoi* species[6]. It is worth noting that no temporin peptides had been screened against *L. mexicana*, the species of parasite of interest in our investigation. Additionaly the temporins appear to act via cell membrane permeation.[3] This mode of action is different to currently available treatments for leishmaniasis and given the complexity associated with altering cell membrane structure the chance of parasite resistance emerging to this class of peptides is relatively low. The initial aims of the investigation were therefore to synthesis of small number of temporin peptides using Solid-Phase Peptide Synthesis (SPPS) and screen these peptides for activity against *L. mexicana*. The results obtained from this initial screen would be used to inform the further selection of additional peptides for future studies.

2.2 Solid-Phase Peptide Synthesis

Solid-Phase Peptide Synthesis (SPPS) is the standard method used for the chemical synthesis of peptides. SPPS is a process by which chemical transformations are carried out in a step-wise fashion on a solid support. There are a number of benefits to the use of SPPS over conventional solution-phase peptide synthesis. These are namely that in the solution-phase synthesis of peptides, in particular longer sequences, the repetition of coupling and deprotection cycles are labour intensive, and often require purification of the intermediates. Conversely the use of a solid support in SPPS allows the individual peptide coupling reactions to be pushed to 'completion' through the use of excess reagents and multiple couplings.

SPPS proceeds via a repeating cycle of coupling and deprotection reactions, as shown in Scheme 2.1. A solution of the amino acid to be coupled, together with an activating agent is added to the resin and coupled under microwave conditions. Unreacted amino

acids were removed by draining the solution-phase from the microwave vessel. The coupled amino acids were deprotected by addition of base (piperidine) before coupling of the subsequent amino acid to the growing chain. Upon completion of the peptide chain, the peptide was cleaved typically using trofluoroacetic acid (TFA), which also served to remove side chain protecting groups present on amino acid side chains. A solid support was selected based on the requirements of the final peptide sequence. Considerations which are taken when choosing a solid support include; the length of the peptide to be synthesised, (low loading resins, 0.1mmol - 0.3 mmol were used in the synthesis of longer peptides) the solvent system used, (the reagents must be soluble in the same solvent system in which the resin swells) activating compounds used, and the presence of amino acids which may have presented difficulties coupling onto the growing peptide chain. Automated SPPS represents an important step forwards in the synthesis of peptides. The introduction of automated commercial peptide synthesisers has allowed chemists to synthesise peptides with minimal effort and in a reduce time frame when compared to previously utilised manual techniques.



Scheme 2.1 Outline of the general procedure used in Fmoc-SPPS.

2.3 Microwave peptide synthesis

Microwave irradiation has been around since the late 1940's, however it was not until 1986 that microwave energy was used in organic chemistry[7]. Microwave energy was an obvious source for completing chemical reactions in minutes that would otherwise take several hours to days[8]. The introduction of microwave heating technology into SPPS represented an important advancement in the area. It enabled the synthesis of medium to large sized peptides (15-40 amino acids) in a matter of hours, whereas previously this would have taken weeks or indeed months to complete. While conventional heating (e.g. water circulated jacket peptide vessels) had been applied successfully in SPPS, precise microwave irradiation to heat the reaction mixture during coupling and N_{α}-deprotection has become increasingly popular as it affords more control. Microwave heating can be used to overcome long standing difficulties that are associated with the coupling certain amino acid residues or sequences[7]. For example specific heating programmes can be used to introduce arginines and other bulky amino acid residues into

a peptide sequence. Microwave heating has been proven especially relevant for sequences which might form β-sheet type structures and for sterically difficult couplings. The beneficial effect of microwave heating appears so far to be due to the precise nature of this type of heating, rather than a peptide-specific microwave effect[7]. There is however no clear evidence that microwave is better than simple heating and some peptide laboratories regard microwave just as a convenient method for rapid heating of the peptidyl resin. In peptide synthesis, microwave irradiation has been used to complete long peptide sequences with high degrees of yield and low degrees of racemisation[8] It has been shown that heating to above 50-55 °C also prevents aggregation and accelerates the coupling[7].

Despite the main advantages of microwave irradiation of peptide synthesis, the main disadvantage is the racemisation which may occur with the coupling of cysteine and histidine residues[8]. It is thought that performing coupling of these amino acids at lower temperatures may overcome this[8]. Racemisation is a base-induced side reaction, and therefore occurs at the activation and coupling stages of synthesis. Deprotonation at the α-carbon of an α-amino acid residue results in racemisation as the carbanion intermediate can reprotonate on either side. Racemisation is fastest with strongly electron withdrawing groups (best leaving groups) and unhindered strong bases in dipolar aprotic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF). However, although strong bases will increase the rate of racemisation, the most important factor is the balance between the rate of racemisation, and the rate of coupling; the amount of racemisation taking place via this pathway is, in most cases insignificant[7].

Currently, there are two manufacturers of microwave-assisted peptide synthesizers, CEM and Biotage, both providing fully automated synthesizers. The major differences between the two systems are the liquid handling and the mixing of the reaction mixture, as the valve-based CEM instrument relies on nitrogen bubbling, while the Biotage instrument is a valve-free robot using vortexing to mix reactants. Most, if not all, microwave-assisted peptide syntheses reported in literature are on a scale below 0.2 mmol.[7]

2.4 The temporins

The temporin peptides are a family of AMPs isolated from the skin of the European red frog *Rana temporaria* and they are among some of the smallest AMPs found in nature (typically 13 amino acids long)[9]. The modes of action, along with their antimicrobial and reported antileishmanial properties make temporins good molecules for an in-depth understanding of host defence peptides in general. The temporin mechanism of action (against both bacteria and parasites[10]) has been shown to involve plasma membrane permeation based on the facts that; (i) they induce a rapid collapse of the plasma membrane potential, (ii) they induce the influx of the vital dye SYTOXTM Green, (iii) they reduce intracellular ATP levels and (iv) they severely damage the membrane of the parasite, as shown by transmission electron microscopy.[10]

2.4.1 Selection of temporins A, B and 1sa

AMPs isolated from a range of organisms have been screened against different *Leishmania* species however there are currently no reports of studies being carried out against the clinically relevant amastigote form of *Leishmania mexicana*. Temporin A FLPLIGRVLSGIL-NH_2 (**16**), temporin B LLPIVGNLLKSLL-NH_2 (**17**) and temporin 1Sa FLSGIVGMLGKLF-NH_2 (**25**) were selected as they had previously been shown to have activity at low micro-molar concentrations (promastigotes, temporin A 50 % growth inhibition *L. donovani,* 8.4 µM temporin B 50 % growth inhibition *L. donovani, 8.6* µM temporin 1Sa 50 % growth inhibition *L. infantium, 18.1* µM; axemic amistoges temporin A 50 % growth inhibition *L. pafanoi, 14.6 µM,* temporin B 50 % growth inhibition *L. pafanoi, 7.1 µM,* temporin 1Sa 50 % growth inhibition *L. infantum, 22.8 µM)* against *Leishmania* species [10-11]. Given the ease of cell culture, AMPs have most commonly been screened against insect stage, promastigote *Leishmania*[1b]. However, in order to fully assess the efficacy of any compound, it must be assayed against pathogenic, mammalian stage axenic amastigotes. Therefore to facilitate comparative analyses of the antileishmanial action of selected, synthesised temporins, it was chosen to utilise *L. mexicana*, where axenic culture of both lifecycle stages is long established[12].

The antimicrobial activities of temporins 1Sa (**25**), temporin 1Sb (**26**) and 1Sc (**27**) have previously been tested for biological activity by Ladram *et al*. The peptides were assayed against Gram-positive and Gram-negative bacteria, filamentous fungi, and yeasts (Table 2.1). It was found that their spectra of action differ considerably. Whereas temporin 1Sa (**25**) was active against most of the tested microorganisms at micromolar concentrations, temporin 1Sc was found to be inactive against Gram-negative bacteria

and temporin 1Sb was inactive or weakly active against all the tested microorganisms. The ability of the peptides to kill the *L. infantum* promastigotes and axenic axenic amastigotes was also investigated. Temporin 1Sa (**25**) exerted a significant leishmanicidal activity against both the promastigotes (insect stage) and axenic axenic amastigotes (mammalian stage) with an IC_{50} of 18.1 mM (log IC_{50} = - 4.741 ± 0.034) and 22.8 mM (log IC_{50} = -4.643 ± 0.036), respectively (Table 2.1). No activity was detected for either temporin1Sb or temporin 1Sc. It was for this reason that temporin 1Sa (**25**) was selected for investigation in our initial screening.

	Temporin 1Sa FLSGIVGMLGK LF-NH_2 (25)	**Temporin 1Sb FLPIVTNLLSGL L-NH_2 (26)**	**Temporin 1Sc FLSHIAGFLSNL F-NH_2 (27)**
MIC (MM)			
Gram-positive bacteria			
S. *aureus* ATCC 25923	3	58	10
E. *faecalis* ATCC 29212	10	>116	>80
B. *megaterium*	2	46	4
Gram-negative bacteria			
E. *coli* ATCC 25922	10	234	161
E. *coli* ATCC 35218	10	>116	>80
P. *aeruginosa* ATCC 27853	31	>231	>161
Fungus			
A. *flavus*	ND[a]	58	10
Yeasts			
C. *albicans* ATCC 90028	16	>116	20
C. *parapsilosis* ATCC 22019	31	>116	20
S. *cerevisiae*	8	>116	10
LC_{50} (mM)			
Erythrocytes	25	>116	>80
MICs and LC_{50} are expressed as average values from three independent experiments performed in triplicate. a not determined			

Table 2.1. Results obtained from previous testing of temporins 1Sa (**25**), 1Sb (**26**) and 1Sc(**27**)

2.4.2 Solid Phase Peptide Synthesis

Microwave-assisted Fmoc-SPPS was used for the synthesis of the target temporins, A (**16**), B (**17**) and 1Sa (**25**). The peptides were synthesised on Rink amide AM resin to ensure that an amidated C-terminus was provided upon cleavage from the resin (Figure 2.1) [7].

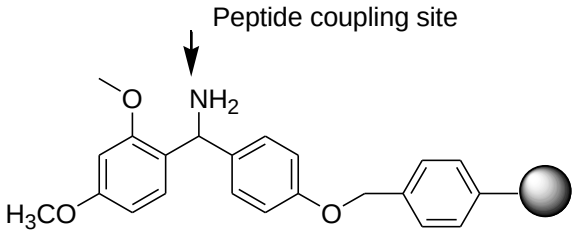


Figure 2.1 Rink Amide AM Resin

There are several common coupling agents that can be used in Fmoc-SPPS to enable amide bond formation. Common reagents include 1-ethyl-3-(3'-dimethylaminopropyl) carbodimide (**28**, EDCI), *N*,*N'*-Dicyclohexylcarbodiimide (**29**, DCC), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphateme (**30**, PyBOP™) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (**31**, HATU). Coupling agents are required in the process as there is a high activation energy to be overcome during amide bond formation. Amino acid derivatives with a strongly electron withdrawing group are formed with a range of activating agents used, making the carbonyl carbon more prone to nucleophilic attack and thereby achieving high reaction rates at room temperatures. Given its relative stability we selected PyBOP™ as a coupling reagent. PyBOP™ reacts to produce an activated benzotriazole ester (active ester) and avoids epimerisation taking place through formation of an oxazoline intermediate[7].

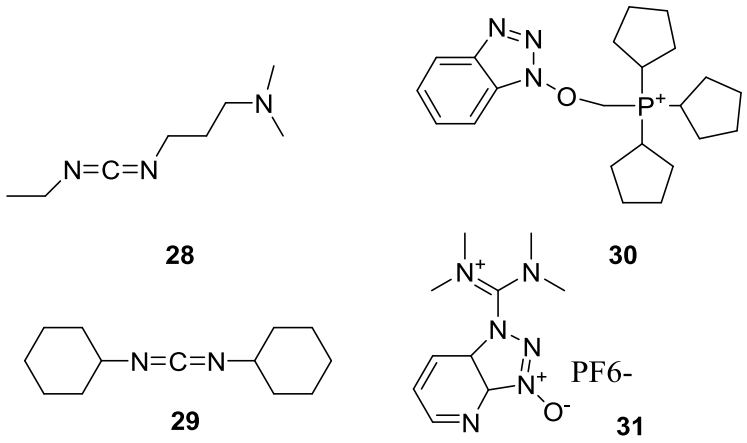
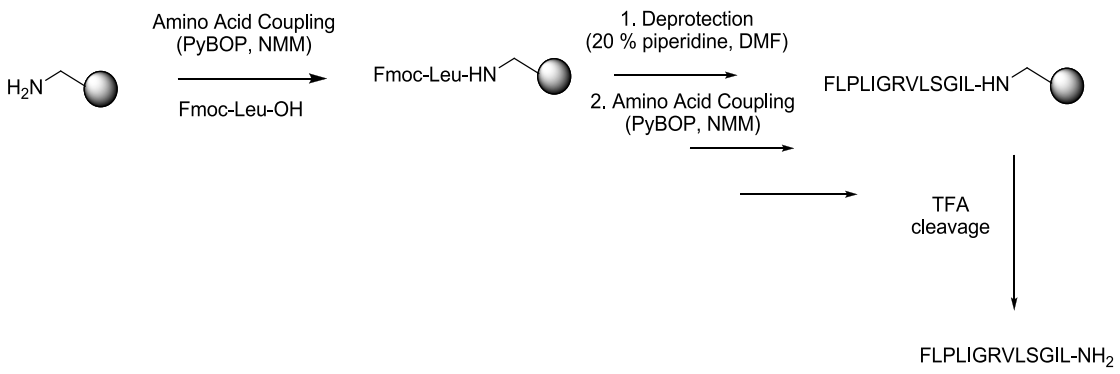


Figure 2.2 Common peptide coupling reagents utilised in Fmoc-SPPS

2.4.3 Synthesis of temporin A (16)

Temporin A (**16**), primary structure FLPLIGRVLSGIL-NH_2 was synthesised using the general Fmoc-SPPS procedure outlined in Scheme 2.2. The synthesis was carried out on 500mg (0.31 mmol) of Rink Amide AM resin and microwave assisted peptide couplings were used (See Experimental Section 7.2).



Scheme 2.2 General overview of the Fmoc-SPPS synthesis approached applied for the formation of temporin peptides **16**, **17** and **25** (sequence shown in reaction scheme is for temporin A).

During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. When all amino acids were coupled, a final Fmoc deprotection step was carried out using 20% piperidine/DMF, and the peptide was then cleaved from Rink Amid resin using TFA (90%), TIPS (5%), H_2O (5%) at room temperature. MALDI-TOF mass spectrometry analysis of the crude peptide was used to confirm the presence of the correct peptide. Temporin A (**16**) was purified using reverse phase HPLC (RP-HPLC). Fractions that were shown by mass spectrometry analysis to contain **16** were pooled and lyophilisation afforded the peptide as a white powder. The MALDI-TOF spectrum of the purified temporin A (**16**) is given in Figure 2.3. The purity of **16** was confirmed using reverse phase analytical HPLC prior to biological evaluation (Figure 2.4).

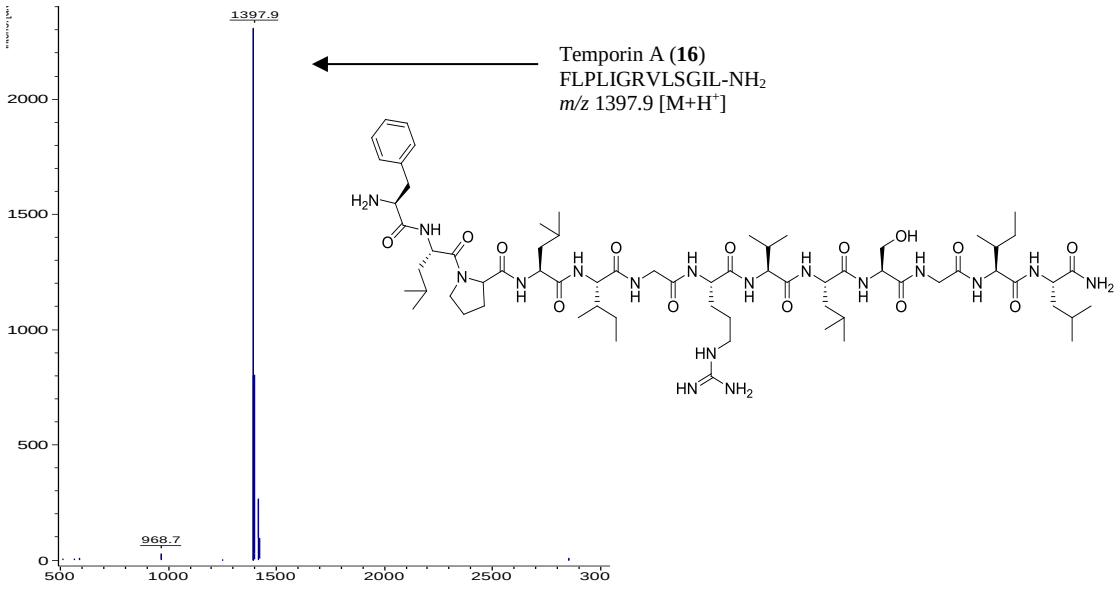


Figure 2.3 MALDI-TOF mass spectrum for purified temporin A (**16**)

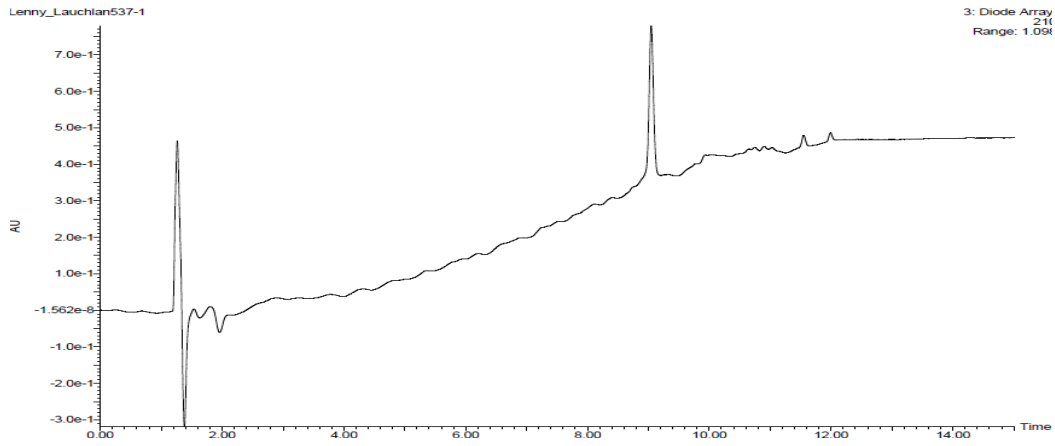


Figure 2.4 Analytical RP-HPLC analysis of purified temporin A (**16**)

2.4.4 Synthesis of temporin B (17)

Temporin B (**17**), primary structure LLPIVGNLLKSLL-NH_2 was synthesised using the general Fmoc-SPPS procedure outlined in Scheme 2.2. The synthesis was carried out on 500mg (0.31 mmol) of Rink Amide AM resin and microwave assisted peptide couplings were used (See Experimental Section 7.2).

During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. When all amino acids were coupled, a final Fmoc deprotection step was carried out using 20% piperidine/DMF, and the peptide was then cleaved from Rink Amid resin using TFA (90%), TIPS (5%), H_2O (5%) at room temperature. MALDI-TOF mass spectrometry analysis of the crude peptide was used to confirm the presence of the correct peptide. Temporin B (**17**) was purified using reverse phase HPLC (RP-HPLC). Fractions that were shown by mass spectrometry analysis to contain **17** were pooled and lyophilisation afforded the peptide as a white powder. The MALDI-TOF spectrum of the purified temporin B (**17**) is given in Figure 2.5. The purity of **17** was confirmed using reverse phase analytical HPLC prior to biological evaluation Figure 2.6.

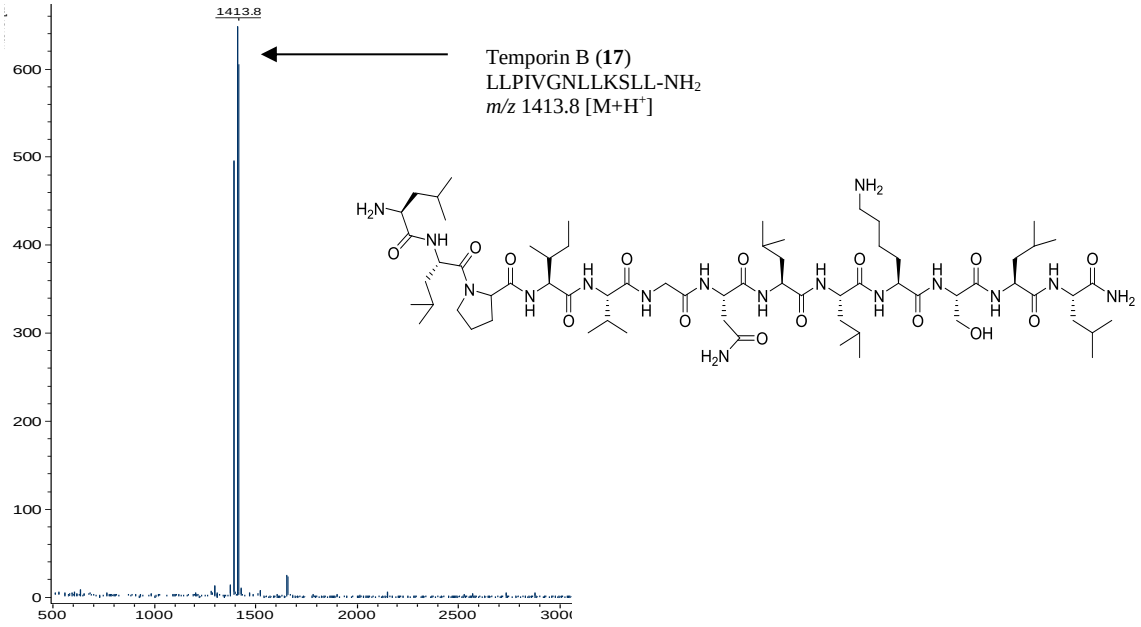


Figure 2.5 MALDI-TOF mass spectrum for purified temporin B (**17**)

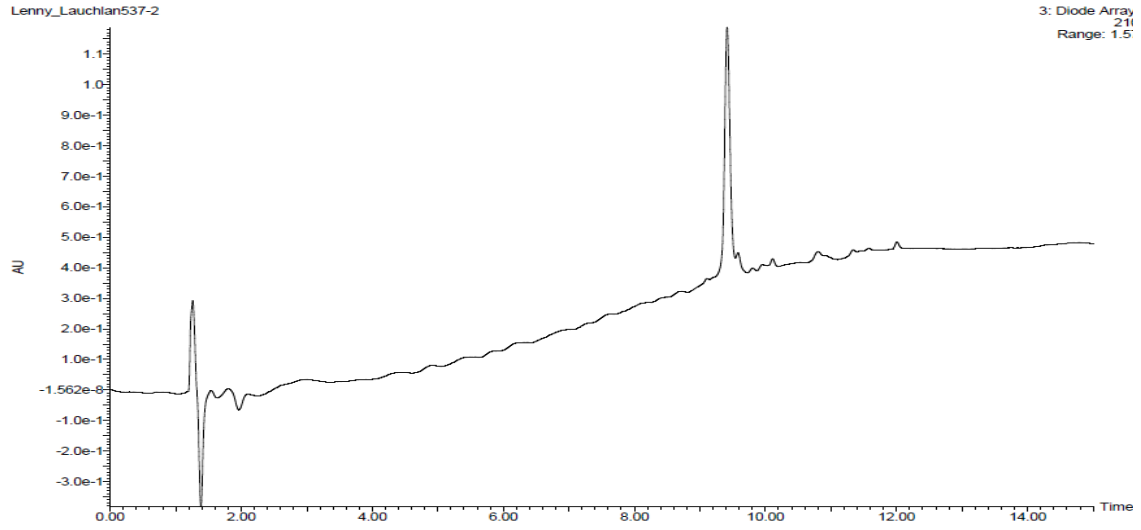


Figure 2.6 Analytical RP-HPLC analysis of purified temporin B (**17**)

2.4.5 Synthesis of temporin 1Sa (25)

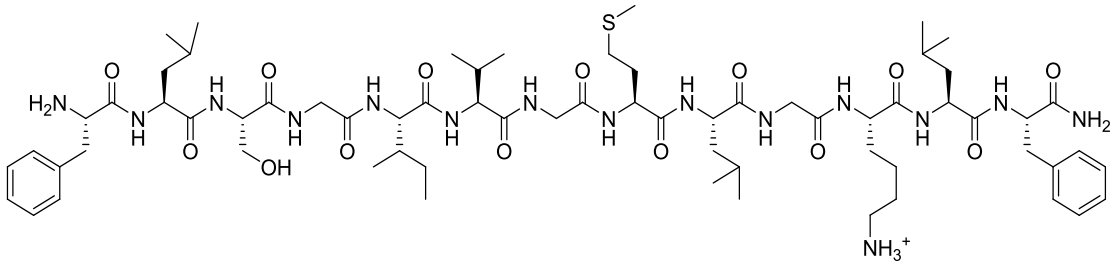


Figure 2.7 Chemical structure of temporin 1Sa (**25**)

Temporin 1Sa, primary structure FLSGIVGMLGKLF-NH_2 was synthesised using the general Fmoc-SPPS procedure outlined in Scheme 2.2. The synthesis was carried out on 500mg (0.31 mmol) of Rink Amide AM resin and microwave assisted peptide couplings were used (See Experimental Section 7.2).

During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. When all amino acids were coupled, a final Fmoc deprotection step was carried out using 20% piperidine/DMF, and the peptide was then cleaved from Rink Amid resin using TFA (90%), TIPS (5%), H_2O (5%) at room temperature. MALDI-TOF mass spectrometry analysis of the crude peptide was used to confirm the presence of the correct peptide. Temporin 1Sa (**25**) was purified using reverse phase HPLC (RP-HPLC). Fractions that were shown by mass spectrometry analysis to contain **25** were pooled and lyophilisation afforded the peptide as a white powder. The MALDI-TOF spectrum of the purified temporin 1Sa (**25**) is given in Figure 2.9. The purity of **25** was confirmed using reverse phase analytical HPLC prior to biological evaluation.

Figure 2.8 (left) Gel initially obtained on attempted synthesis of Temporin 1Sa (**25**), due to the presence of FLSGIVGMLGKL-NH_2 (middle) Partial gel obtained from synthesis of Temporin 1Sa analogue, ALSGIVGMLGKLF-NH_2, (right) Powder obtained on successful synthesis of Temporin 1Sa (**25**), FLSGIVGMLGKLF-NH_2.

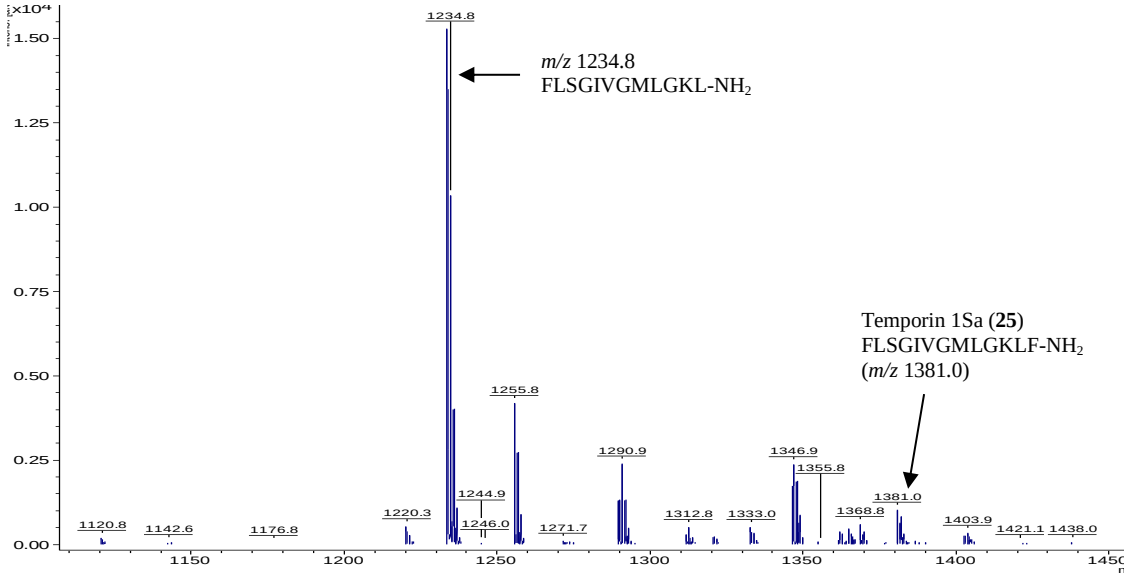


Figure 2.9 MALDI-TOF spectrum obtained upon initial synthesis of temporin 1Sa (**25**)

The problem of self-association is recognised in solid-phase synthesis, and arises suddenly, typically 6-12 residues into the synthesis[7]. It is thought that formation of the gel structure in Figure 2.8 may be due to alignment of peptide chains, either as β-sheets aligning through self-association, while attached onto the resin and once cleaved, aligning to form a gel. It is now accepted that a major contributing factor to the problem of aggregation originates in the intrinsic properties of the peptide sequence itself, and

that aggregation occurs primarily through the self-association of polymer-bound peptide sequences by intermolecular hydrogen bonding[7], as shown in Figure 2.10. The tendency for aggregation depends on the nature of the peptide and side-chain protecting groups, with sequences containing a high proportion of Ala, Val, Ile, Asn and Gln residues exhibiting a high predisposition to peptide aggregation. A fully solvated peptide-polymer is considered to contain a soluble peptide chain, whilst an aggregated matrix contains a partially soluble or insoluble chain[7]. In light of this, a possible solution to aggregation may be to use an alternative solvent such as DMSO or trifluoroethanol in order to aid solvation. In addition, Mendal et al has also described the use of PEGA gel resin to overcome aggregation in the synthesis of peptides other than 1Sa. Literature to date (September 2013) reports one chemical synthesis of temporin 1Sa[13], in which did not describe the occurrence of aggregation in Temporin 1Sa analogues.

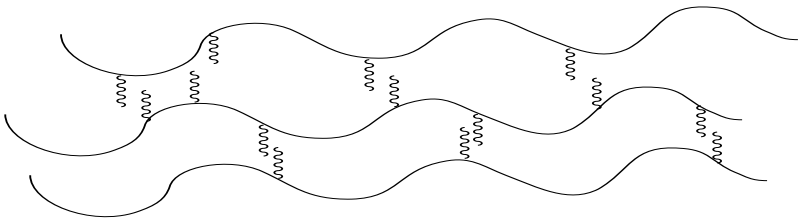


Figure 2.10 Intermolecular aggregation of polymer chains

Initial purification by RP-HPLC was attempted (on approximately ¼ of the crude material obtained) in order to separate Temporin 1Sa (**25**) from the unwanted analogues formed, peptides LSGIVGMLGKLF-NH_2 (**32**, temporin 1Sa analogue 1), and FLSGIVGMLGKLF-NH_2 (**33**, temporin 1Sa analogue 2). Fraction collection and subsequent mas spec. analysis confirmed that the peptide FLSGIVGMLGKL-NH_2 (temporin 1Sa analogue 2) was the major product of the synthesis. Unfortunately it was not possible to isolate any temporin 1Sa (**25**) during the purification which suggested

that although visible in MALDI-TOF analysis (Figure 2.9) **25** must have only been a very minor component of the crude reaction mixture. Given this it was decided not to attempt HPLC purification the remaining crude peptide mixture with but rather attempt a second synthesis of temporin 1Sa (**25**).

A subsequent synthesis of temporin 1Sa (**25**) was attempted on a smaller scale, using 250 mg (0.15 mmol) of Rink Amide resin, following peptide coupling procedure outlined in Scheme 2.2. The final coupling of amino acid residue 12 (lys) to 13 (phe) was carried out using a double coupling, and a fraction of the resin (50 mg) was removed and cleaved for analysis. The MALDI-TOF results obtained were very similar to those from the initial synthesis, indicating that FLSGIVGMLGKL-NH_2 (temporin 1Sa analogue 2) was again the major product produced.

A different approach was subsequently taken; LiCl can be used in peptide synthesis in order to disrupt aggregation, although this is not usually considered necessary to be used with peptides consisting of only 13 amino acids in length. An 8M solution of LiCl was used to wash the deprotected peptide FLSGIVGMLGKL-NH_2 prior to the final coupling of residues12 (lys) to 13 (phe) using standard peptide coupling conditions. The peptide was subsequently cleaved by use of microwave cleavage as outlined in procedure 4. MALDI-MS results obtained from this attempted synthesis showed no evidence of peaks corresponding to the presence of temporin 1Sa (**25**).

A synthesis of a temporin 1Sa analogue (**26**, ALSGIVGMLGKLF-NH_2) was attempted, substituting the alanine residue in place of the phenylalanine at residue 13. This used a ‘manual’ coupling of the initial phenylalanine (non-microwave conditions). All reagents used were identical to previous conditions, with the exception that the coupling took

place for two hours at room temperature, the reagents were removed by washing with DMF, and the process repeated a second time in an attempt to obtain successful coupling. This was attempted in order to determine if the problematic phenylalanine coupling was residue 1 or 13, and if coupling of a less bulky group onto the leucine residue 12 was possible, or if the physical properties of the 12-mer peptide following coupling of the leucine residue at the 12 position were preventing any further amino acid couplings taking place. Following a manual deprotection, a gel was obtained, and this coupling was shown to be successful, indicated by a MALDI-MS peak at *m/z* 1305.0 [$M+H^+$].

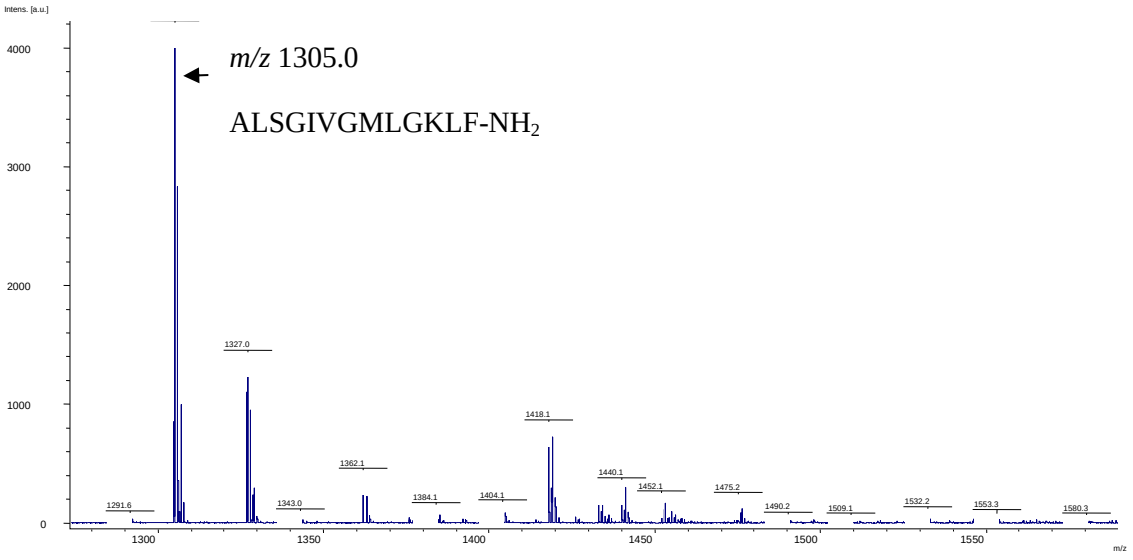


Figure 2.11 MALDI-TOF mass spectrum obtained upon synthesis of a temporin 1Sa analogue (**26**) (*m/z* 1305.0)

A repeated synthesis of temporin 1Sa (**25**) was attempted, following identical conditions to those used to successfully couple alanine as residue 13. Following cleavage from the

resin, a white powder was obtained, corresponding to the presence of temporin 1Sa, FLSGIVGMLGKLF-NH_2 indicated by a MALDI-MS peak at *m/z* 1380.5 $[M+H^+]$.

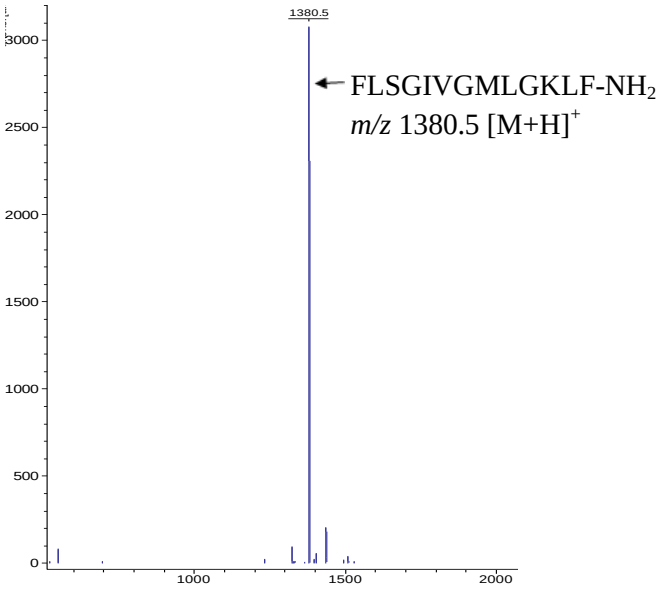


Figure 2.12 MALDI-TOF mass spectrum obtained for temporin 1Sa (25)

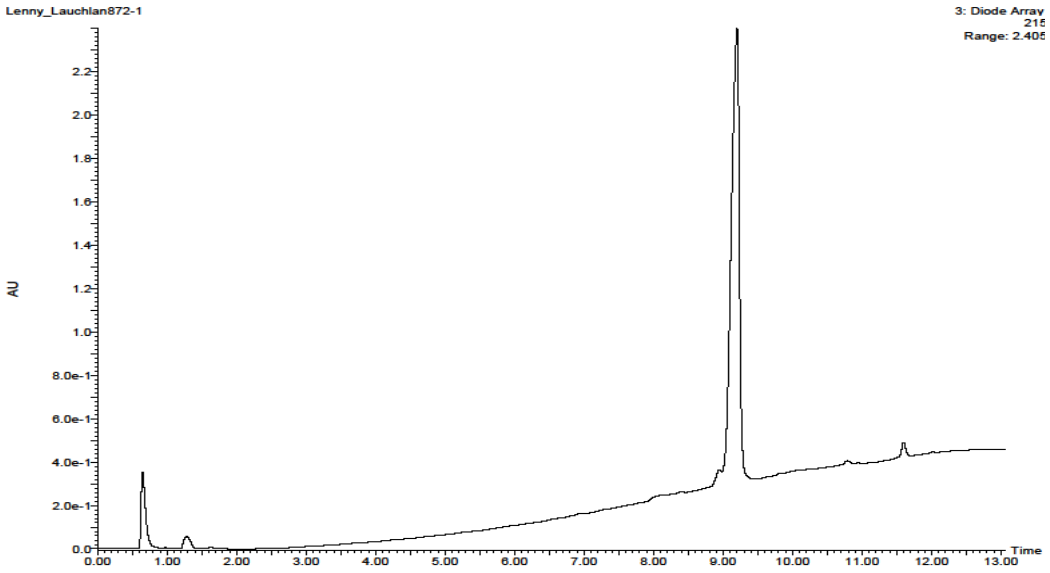


Figure 2.13 Analytical RP-HPLC analysis of purified temporin 1Sa (25)

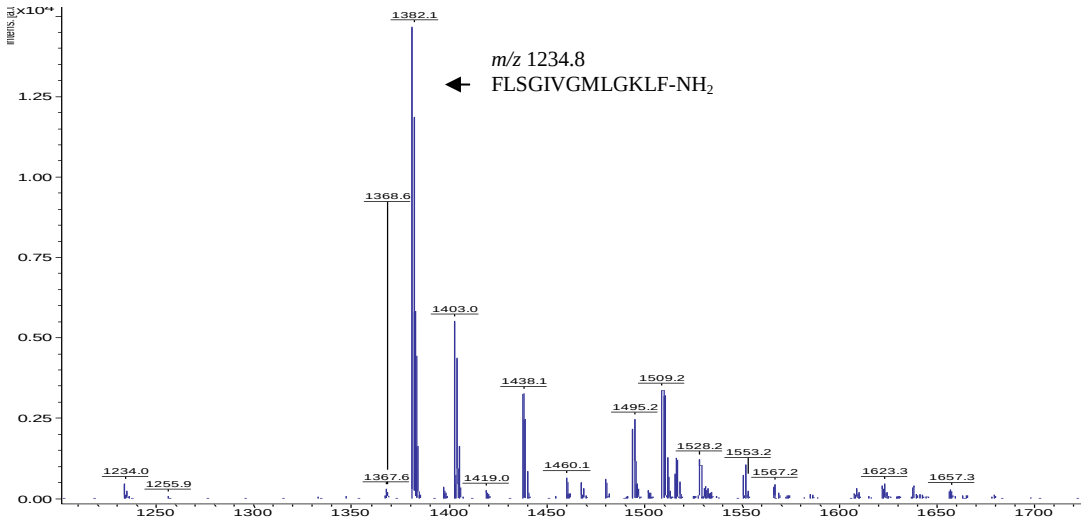


Figure 2.14 MALDI-TOF mass spectrum obtained upon synthesis of temporin 1Sa, using manual deprotection and coupling strategy, note the presence of *m/z* 1382.1 [M+H$^+$]

It is likely that temporin 1Sa analogues have aggregated to form β-sheets, not α-helices, due to the high levels of glycine present, which deters α-helical formation. A possible reason for the peptide aggregation of the temporin 1Sa analogues FLSGIVGMLGKL-NH_2 (**32**) and ALSGIVGMLGKLF-NH_2 (**33**) while temporin 1Sa FLSGIVGMLGKLF-NH_2 (**25**) is a powder may be that alignment of the β-sheets is able to occur without the presence of the two bulky phenylalanine groups at residues 1 and 13 (either end of the peptide) and when the final phenylalanine coupling has been successful, aggregation is disrupted and a powder is obtained in place of the gels obtained with temporin 1Sa analogues formed.

An alternative approach which may provide a partial solution to the problem of peptide aggregation could be to use a lower loading resin to lessen the degree of aggregation of the peptide chains as subsequent amino acids are coupled Although it is likely that in order for this approach to have any observable effect, the yield obtained would be significantly lowered and it is likely that there would still be a significant degree of

peptide aggregation observed. This would improve the likelihood of the initial phenylalanine coupling taking place, as the steric hindrance on the resin will be reduced, and as Temporin 1Sa did not aggregate once cleaved from the resin, will lessen the likelihood of an aggregated peptide being obtained on cleavage of the peptide from resin. There are also potential problems associated with methionine, with regards to the susceptibility of the thioether to alkylation and oxidation,[7] although these were not observed. It is also worth noting that the synthesis of other AMPs within the laboratory has resulted in a similar problem becoming apparent. It appears that an initial phenylalanine coupling is consistently problematic using the microwave assisted peptide synthesis conditions previously outlined. This may be due to the relatively bulky size of this particular amino acid, but this does not fit with the fact that non-microwave coupling conditions (i.e. rt coupling) works without any problems. At this time the exact reasons for the problems encountered with coupling a C-terminal phenylalanine residue on to the Rink Amide resin under microwave Fmoc-SPPS cannot be fully explained.

2.6 Antileishmanial testing Part I

Temporins A (**16**), B (**17**) and 1Sa (**25**) were synthesised and purified, experimental data is given in Table 2.2.

Peptide	R_t(HPLC)	Mr(Calc)[a]	Mr(Obs)[a,b]
Temporin A (**16**) FLPLIGRVLSGIL-NH_2	9.05	1395.9	1397.0
Temporin B (**17**) LLPIVGNLLKSLL-NH_2	9.41	1390.9	1413.8
Temporin 1Sa (**25**) **FLSGIVGMLGKLF-NH_2**	9.24	1379.8	1380.5

[a] The calculated (calc) and observed (obs) masses are monoisotopic.
[b] M_r (obs) are the observed protonated $[M + H]^+$ or sodiated $[M + Na]^+$ species as obtained by MALDI-TOF-MS.

Table 2.2 Sequence, chemical and physical data for temporins A (**16**), B (**17**) and 1Sa (**25**)

2.6.1. Optimisation of Assay

Temporins A (**16**), B (**17**) and 1Sa (**25**) were screened against both promastigote and axenic amastigote *L. mexicana* using the optimised Alamar Blue assay. To determine the most effective protocol for Alamar Blue® assay of MYNC/BZ/62/M379 *Leishmania mexicana* procyclic promastigotes two 96 well plates were set up using serial dilution to achieve a triplicate series of cell culture concentrations between 1.0 $x10^7$ cells/ml and 5.0 x 10^3 cells/ml. Each plate also contained a triplicate control of promastigote medium to indicate the minimum fluorescence that would be observed for cells incapable of metabolising Alamar Blue®. Following the recommended reagent protocol, 10% Alamar Blue® was added to each well. One plate was then incubated at 26^{o}C and the other at 37^{o}C, with absorbance measurements taken after the recommended 4 hour interval and the maximum recommended 24 hour interval. Temperatures were chosen to reflect the

recommended Alamar Blue® assay incubation temperature (37°C) and the temperature used to culture promastigotes (26°C).

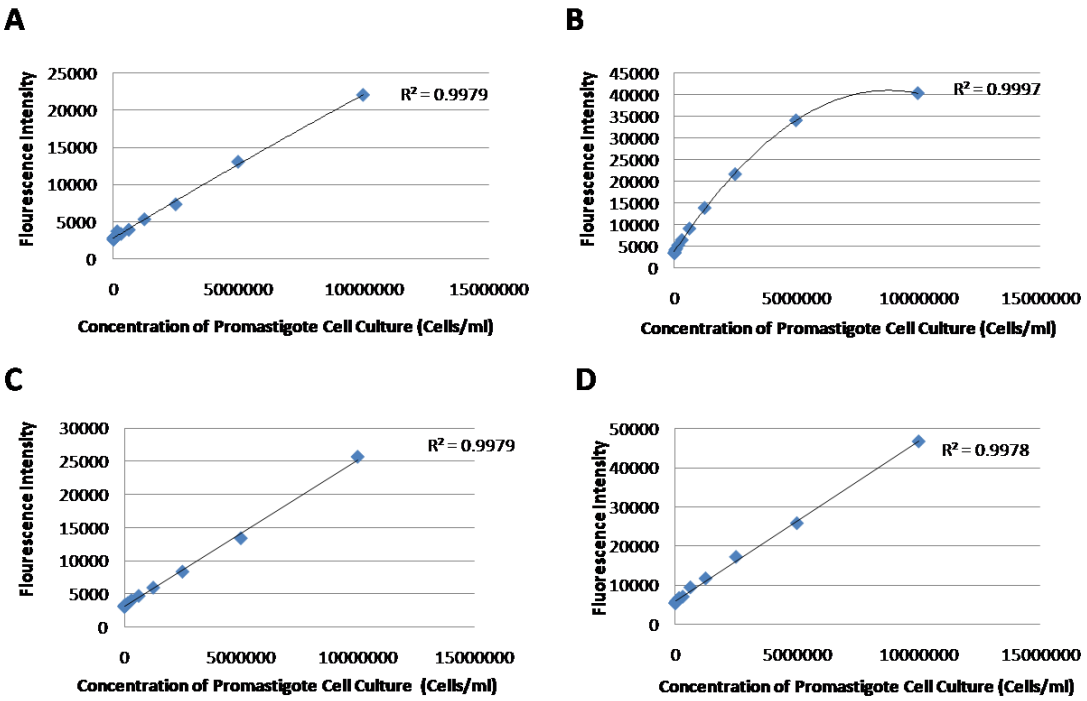


Figure 2.15 Alamar Blue® fluorescence assays for increasing concentrations of *L. mexicana* promastigote culture to determine the most effective conditions for Alamar Blue® usage. Conditions of assays: (A) 4 hours, 26°C; (B) 24 hours, 26°C; (C) 4 hours, 37°C; (D) 24 hours, 37°C.

Alamar Blue® was reduced by *L. mexicana* promastigotes in a time dependant and temperature dependant manner, seen in Figure 2.15. It was determined that 4 hour incubation is an inadequate time interval, as cell viability cannot be assessed effectively due to the small variance between the observed fluorescence for high concentrations of healthy promastigotes and those of the control (or low concentrations of healthy promastigotes). The poor metabolism of Alamar Blue® in the recommended assay timeframe may be a result of the thick glycocalyx, present on promastigotes, that is

absent in cell lines where a 4 hour incubation period is considered adequate (e.g. macrophage and *L. mexicana* amastigote). Physically, Alamar Blue® diffusion or transport through the glycocalyx and membrane into the promastigote cytosol may be slower than in cells lines lacking a thick glycocalyx. Temperature had been suggested as an explanation for the variation in efficacy of Alamar Blue results, and thus metabolism. However, the results observed at 4 hours, 37°C discount this theory: whilst there is a slight increase in observed maximal fluorescence, and therefore metabolism, it is still too low to be utilised effectively for a cytotoxicity assay; or to be considered comparable to amastigote or macrophage assay fluorescence results. Incubation at 37°C, whilst adequate at the 24 hour interval was not adopted as the protocol for *L. mexicana* promastigote Alamar Blue® assay. This is due to the fact that the 26°C, 24 hour incubation provided the widest range of observed fluorescence between high concentrations of healthy promastigotes and those of the control wells or low concentrations of healthy promastigotes. The smaller range of fluorescence discerned at 37°C, 24 hour can be accounted for by the level of fluorescence detected from the control wells: the 37°C assay showed higher baseline levels of fluorescence, suggesting that *L. mexicana* metabolism is increased at 37°C. Whilst this could be considered advantageous to cytotoxic assay results, as it is the recommended assay incubation temperature, the reduced fluorescence range precludes 37°C incubation as the most accurate measure of cell viability. The most effective assay, 26°C, 24 hour is run at the native culture temperature for *L. mexicana* promastigotes. Thus, administering the Alamar Blue® assay at 26°C not only provides the widest range of fluorescence results, allowing for the most accurate determination of cell viability; it also allows the promastigotes to proliferate and differentiate naturally. Consequently, any results for the

26°C, 24h assay would correlate more readily to other experimental *in vivo* and *in vivo* results. For these reasons the 26°C, 24h Alamar Blue® assay was adopted to determine cell viability for any cytotoxicity assay procedures described that utilise *L. mexicana* promastigotes.

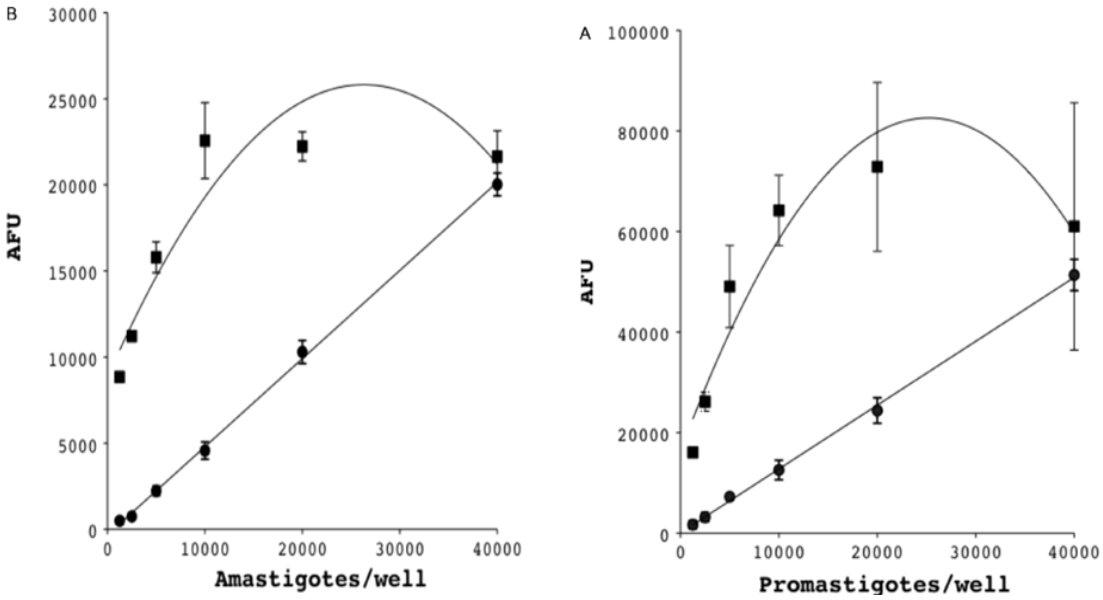


Figure 2.16 Twofold serial dilutions starting at 4×10^5 ml^{-1} of ***Leishmania mexicana*** promastigotes (A) and axenic axenic amastigotes (B) pre-incubated for 24 h before the addition of Alamar Blue and further incubation for 4 h (●) or 24 (▪) h. Note the linear correlation between cell number and fluorescent readout with a 4-h incubation (r^2 = 0.999 in A and B). The lower signal seen for axenic amastigotes (B) reflects their relatively slow rate of replication[14]. All points in triplicate with standard deviation indicated. AFU—arbitrary fluorescence units

In the presence of serum only temporin A (**16**) demonstrated any significant antileishmanial activity against *L. mexicana* promastigotes (Figure 2.16, 57% inhibition at 100 μM). None of the peptides tested showed significant activity at the concentrations tested against *L. mexicana* axenic amastigotes (Figure 2.18). Given that temporins A, B and 1Sa have previously been shown to be active other *Leishmania* species[1b], these

results were unexpected. However, by preincubating the parasites with the temporins studied in the absence of serum the peptide activity profiles were altered (Figure 2.17). In this assay, which reflects the conditions employed in previous studies [10], temporins A and B demonstrated improved efficacy against promastigote *L. mexicana* (Figure 2.17; 63% inhibition at 12.5 µM and 38% at 50 µM respectively). However, amastigote forms remained largely resistant to these compounds with only temporin A demonstrating any significant activity (Figure 2.19; only 23% inhibition at 12.5 µM but 98% at 100 µM). Notably, temporin 1Sa remained largely inactive against both lifecycle stages.

It is noTable that whilst the temporins A and B showed encouraging activity against insect stage promastigotes, a low level of activity was observed against the clinically relevant amastigote stage of *L. mexicana*. Many of the AMPs studied previously have focused on the *Leishmania* species promastigotes rather than the intra-macrophage amastigote form. Given the results obtained here it is evident that it is important to screen against axenic amastigotes, as well as promastigotes, if lead antileishmanial AMPs are to be identified and developed. Temporin peptides are believed to exert their antileishmanial activity through disruption of the parasite membrane[10]. Given this mode of action, it appears likely that the major differences seen in the surface structure of promastigote and amastigote *Leishmania* are responsible for the differential activity observed above[1b]. However, previous studies have reported significant activity of these peptides against both promastigotes and amastigiotes of *L. pifanoi* and *L. infantum*[10-11]. This may be because of more subtle differences in the surface structure between *Leishmania* species and indicates that it may be difficult to develop an AMP that has broad-spectrum antileishmanial activity.

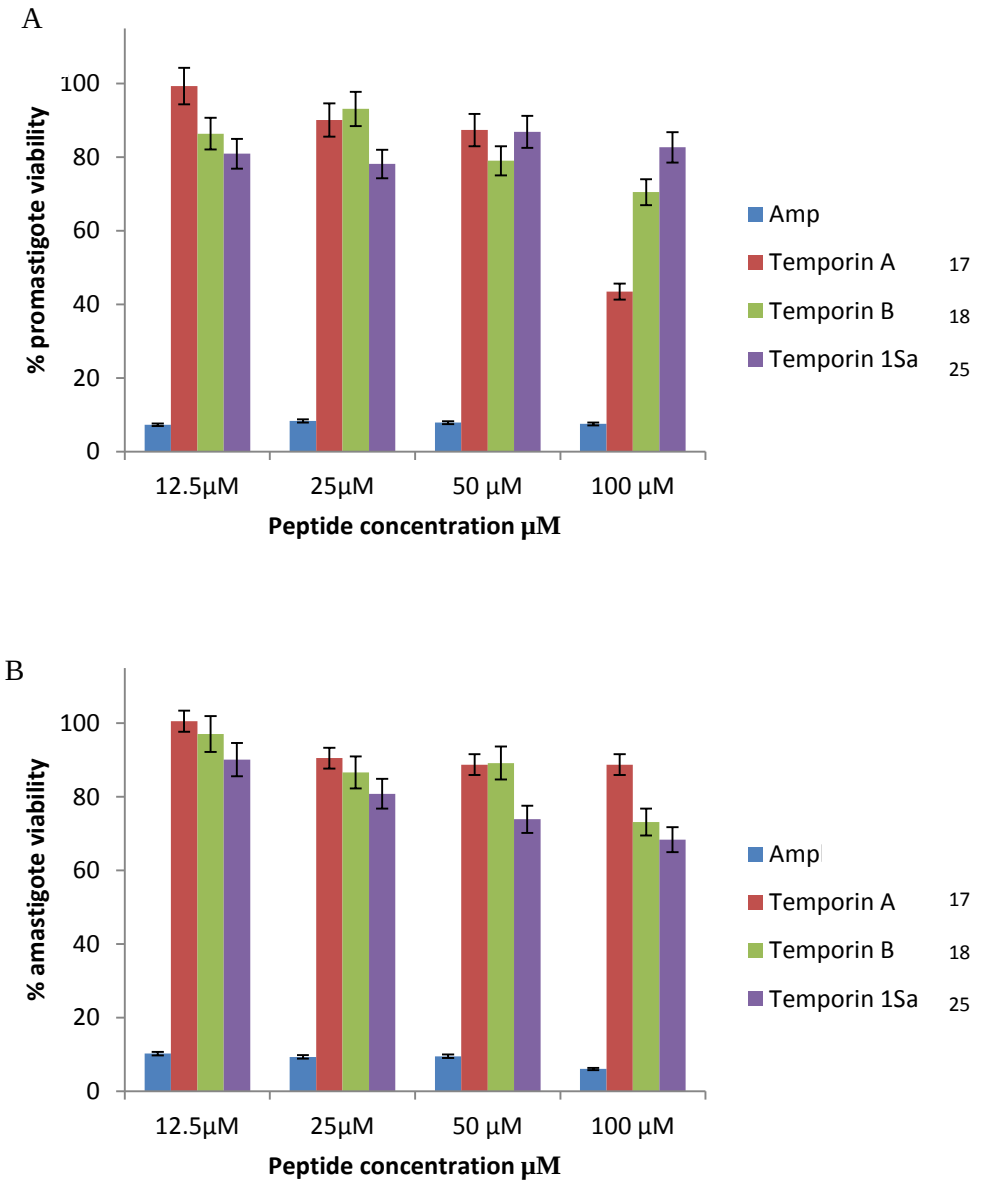


Figure 2.17 Using the Alamar Blue assay system, *L. mexicana* promastigote (A) and axenic amastigote (B) viability in the presence of various concentrations of temporins A (**16**), B (**17**) and 1Sa (**25**) was determined with respect to untreated, negative controls. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error indicated.

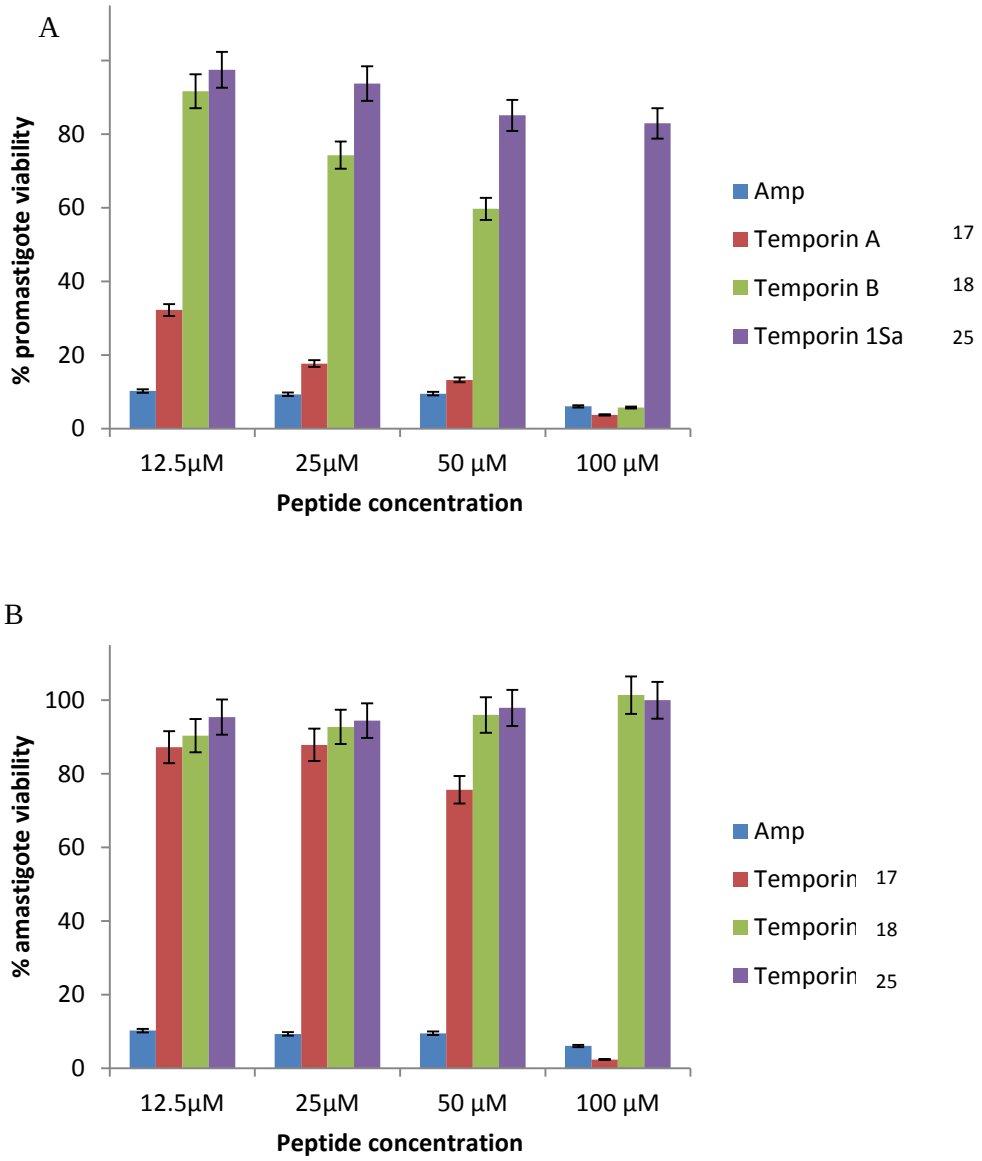


Figure 2.18 *L. mexicana* promastigote (A) and axenic amastigote (B) viability after exposure to various concentrations of temporins A (**16**), B (**17**) and 1Sa (**25**) in the absence of serum using the Alamar Blue assay system as above. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error is indicated.

2.7. Synthesis and testing of further temporins AMPs

Following the initial biological testing of temporins A (**16**), temporin B (**17**) and temporin 1Sa (**25**), additional temporins peptides were synthesised and tested for activity against *L. mexicana* promastigotes and axenic amastigotes. These peptides were selected based on previously reported activity against gram negative bacteria[15], and also structural similarities to temporins A (**16**), temporin B (**17**) and temporin 1Sa (**25**), with an initial aim to identify any structure activity relationships when minor alterations to the primary sequence occurred

2.7.1 Synthesis of temporin C

The synthesis of temporin C (**34**), primary structure LLPILGNLLNGLL-NH_2 was carried out following Fmoc-SPPS microwave coupling procedures in Scheme 2.2, starting with 500 mg (0.31 mmol) of resin. The synthesis proceeded without incident. During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of MALDI-MS. When all amino acids were coupled, a final Fmoc deprotection step was carried out following Fmoc-SPPS procedures in procedure 7.2, and the peptide cleaved from resin by use of TFA, TIPS and H_2O using microwave chemistry. On confirmation of the presence of the correct structure by use of MALDI-MS, analytical HPLC showed the product to elute at 7.80 minutes.

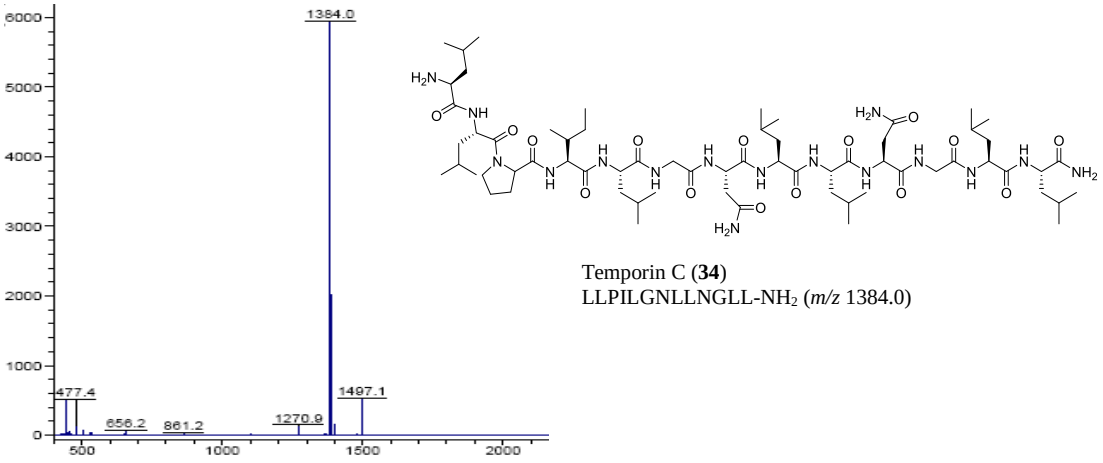
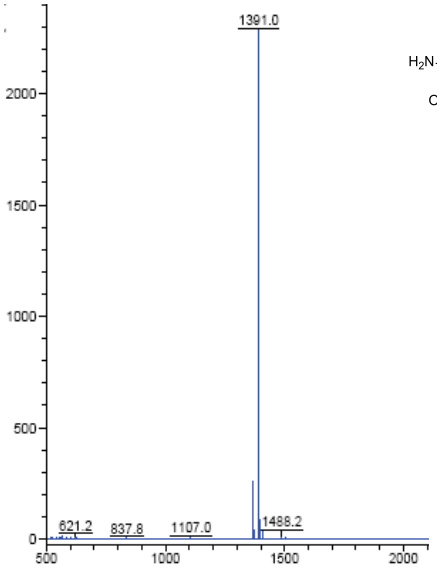
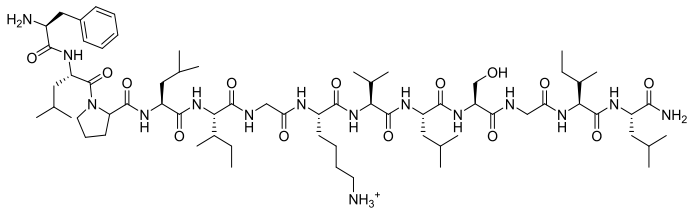


Figure 2.19 MALDI-TOF mass spectrum obtained upon synthesis of temporin C (**34**)

2.8.2 Synthesis of temporin F

The synthesis of temporin F (**35**), primary structure FLPLIGKVLSGIL-NH_2 was carried out following Fmoc-SPPS microwave coupling procedures in Scheme 2.2, starting with 500 mg (0.31 mmol) of resin. The synthesis proceeded without incident. During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of MALDI-MS. When all amino acids were coupled, a final Fmoc deprotection step was carried out following Fmoc-SPPS procedures in Scheme 2.2, and the peptide cleaved from resin by use of TFA, TIPS and H_2O using microwave chemistry. On confirmation of the presence of the correct structure by use of MALDI-MS, analytical HPLC showed the product to elute at 7.20 minutes.





Tenporin F (35)
FLPLIGKVLSGIL-NH_2
(*m/z* 1391.0)

Figure 2.20 MALDI-TOF mass spectrum purified temporin F (**35**)

2.8.3 Synthesis of temporin L

Literature results published have shown temporin L (**36**) to have a synergistic effect with temporins A and B when tested against Gram-negative bacteria in lipopolysaccharide detoxification.[9] However, these effects have yet to be tested in parasites. It was therefore decided to synthesise and purity temporin L in order to investigate the effects on the activity of these temporins used synergistically. Due to the nature of the primary structure of temporin L, this peptide presented a more challenging synthetic target than temporins A (**16**) and B (**17**) previously synthesised. The synthesis of temporin L, primary structure FVQWFSKFLGRIL-NH_2 was carried out following Fmoc-SPPS microwave coupling procedures in Scheme 2.2. starting with 500 mg (0.31 mmol) of rink amide resin. During the synthesis, the structure of the growing peptide chain was confirmed following coupling of phenylalanine and valine by use of MALDI-MS. When all amino acids were coupled, a final Fmoc deprotection step was carried out

following Fmoc-SPPS procedures in Scheme 2.2, and the peptide cleaved from resin by use of TFA, TIPS and H_2O using microwave chemistry. On confirmation of the presence of the correct structure by use of MALDI-MS, analytical HPLC showed the product to elute at 7.79 minutes. MALDI analysis showed a small peak for temporin L at *m/z* 1640.1 [$M+H^+$], but also that significant impurities were also present in the crude product cleaved from the resin.

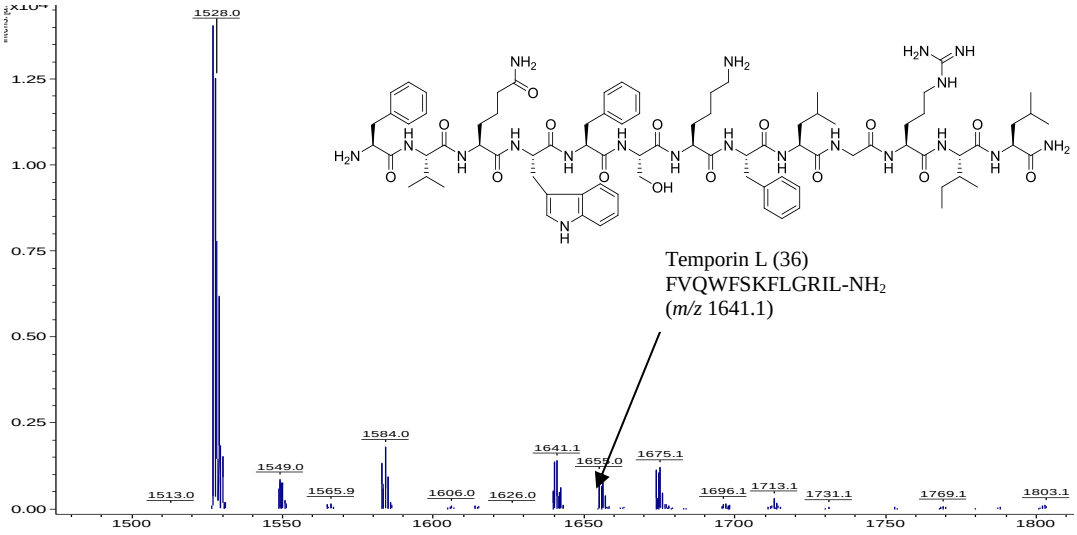


Figure 2.21 MALDI-TOF mass spectrum of purified Temporin L (**36**)

These fragments have been found to correspond to the presence of the fragments listed in Table 2.3 in MALDI-MS. As MALDI is a very mild ionisation technique, the presence of these fragments was not attributed to fragmentation in MALDI-MS. Instead, it was thought that there were problems when coupling amino acids in this particular sequence. In order to identify the structure of the fragment(s) corresponding to the large impurity present, MS/MS analysis could be performed, and resulting ions identified

based on the masses of fragments formed as isoleucine or leucine is present at different positions in each of the potential products identified.

***m/z* of impurity**	**Sequence of peptide fragment**	**Cause of impurity**
1527.0	FVQWFSKFGRIL-NH_2 or FVQWFSKFLGRL-NH_2 or FVQWFSKFLGRI-NH_2	Failed coupling of one residue of leucine or isoleucine (peak may be due to a mixture of these three peptide fragments).

Table 2.3 Assignment of peptide fragments responsible for the major peak given by the MALDI-TOF spectrum of Temporin L, and reasons for presence in spectrum.

Following the unsuccessful synthesis of Temporin L (**36**), it was decided that a subsequent synthesis will take place taking samples for analysis by MALDI-TOF following each coupling of leucine or isoleucine, in order to identify the coupling(s) which resulted in the presence of the impurities(s) shown in Table 2.3. A manual (non-microwave) coupling strategy from tryptophan onwards may also be used. Temporin L was subsequently purified by use of reverse phase HPLC.

2.9 Antileishmanial testing Part II

Temporins C (**34**), F (**35**) and L (**36**) were synthesised and purified, experimental data is given in Table 2.4.

Peptide	Mr(Calc)[a]	Mr(Obs)[a,b]
Temporin C (XX) LLPILGNLLNGLL-NH_2	1360.87	1384.0
Temporin F (XX) FLPLIGKVLSGIL-NH_2	1367.88	1391.0
Temporin L (XX) FVQWFSKFLGRIL-NH_2	**1638.93**	**1641.1**

[a] The calculated (calc) and observed (obs) masses are monoisotopic.
[b] M_r (obs) are the observed protonated $[M + H]^+$ or sodiated $[M + Na]^+$ species as obtained by MALDI-TOF-MS.

Table 2.4 Sequence, chemical and physical data for temporins C (**34**), F (**35**) and L (**36**).

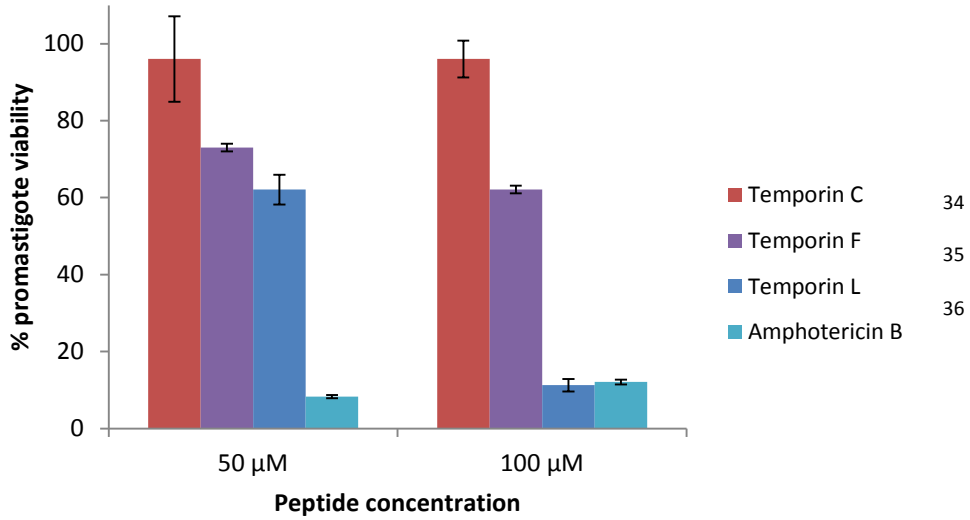


Figure 2.22 *L. mexicana* promastigote viability after exposure to 50 µM and 100 µM concentrations of temporins A (**16**), B (**17**), 1Sa (**25**), C (**34**), F (**35**), and L (**36**) in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error is indicated.

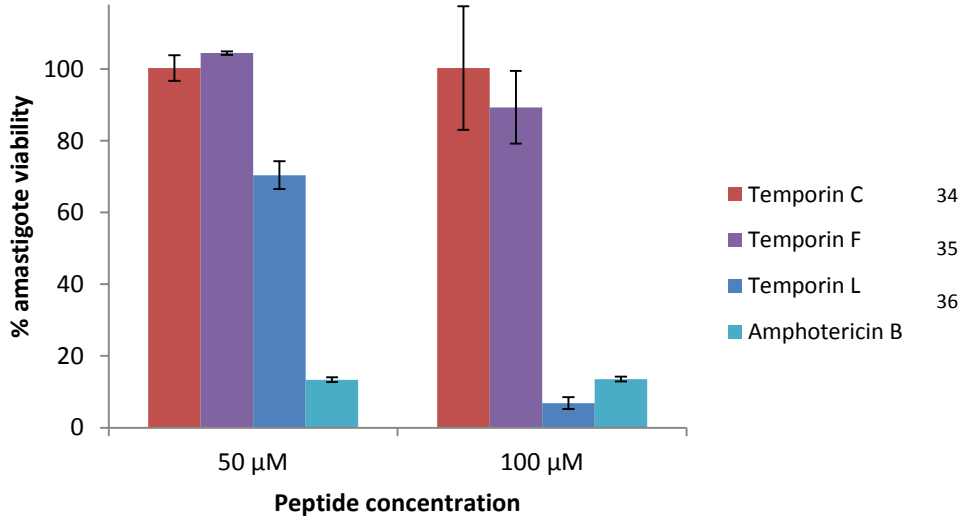


Figure 2.23 *L. mexicana* axenic amastigote viability after exposure to 50 µM and 100 µM concentrations of temporins A (**16**), B (**17**), 1Sa (**25**), C (**34**), F (**35**), and L (**36**) in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard deviation is indicated.

Temporins C (**34**), F (**35**) and L (**36**) were screened against both promastigote and amastigote *L. mexicana* using the optimised Alamar Blue assay decribed previously, involving incubation for 1 hour in the absence of serum. In this assay, which reflects the conditions employed in previous studies [10], temporin L (**36**) was the only peptide tested to show activity against *L. mexicana* axenic amastigotes (Figure 2.23; 60% cell viability at 50 µM and 10% at 100 µM respectively). Temporins C, and F remain largely inactive against both lifecycle stages.

A point of interest is that temporin L (**36**) exhibited greater activity when tested against *L. mexicana* axenic amastigotes, than the promastigote lifecylcle stage. This is in contrast to patterns of activity observed for other AMPs tested. It is possible that this

diference in acivity is due to temporin L being the only peptide tested to possess a +3 charge, which may affect the interactions with the parasite membrane, as this is known to differ between the two lifecycle stages of the *L. mexicana* parasites. It should however be noted that temporin L (35) has a significantly lower LD_{50} than other AMPs when tested against erythrocytes and macrophage cells[16]. Therefore although this peptide may possess higher activity against the *L. mexicana* amastigote lifecycle stage, it is likely that the peptide would prove to be toxic to other mamalian cells in clinically relevant concentrations.

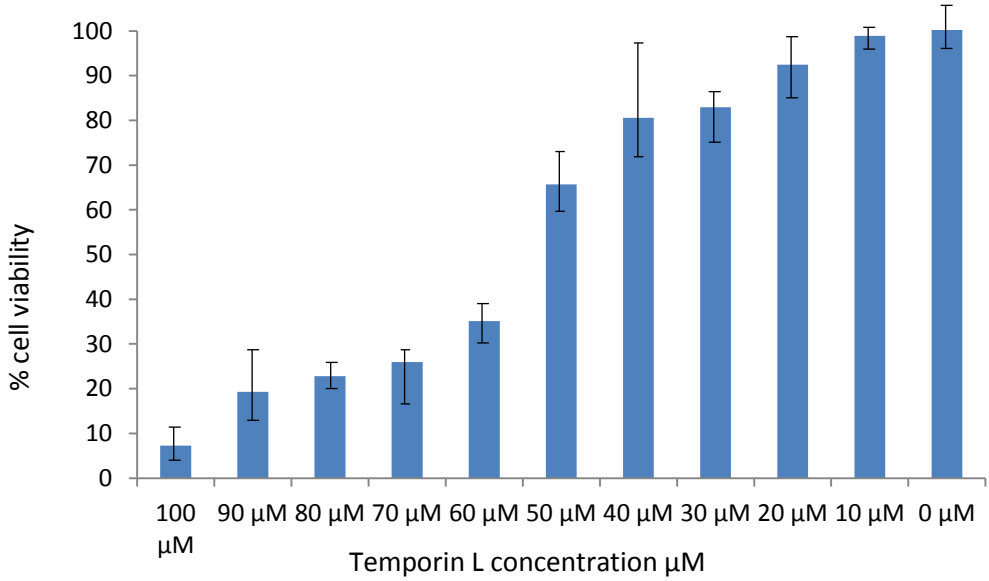


Figure 2.24 *L. mexicana* promastigote temporin L LD_{50} determined in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error indicated.

Previous studies have shown temporins A (**16**) and L (**36**) to possess synergistic activity when tested against bacteria. [17] However the results shown in figure 2.24 did not indicate this against *L. mexicana* promastigotes. Further investigations would be required to investigate these results, however it is likely that this occurred for similar

reasons to the other differences observed in activity when drawing comparisons between antileishmanials and antibacterial activity.

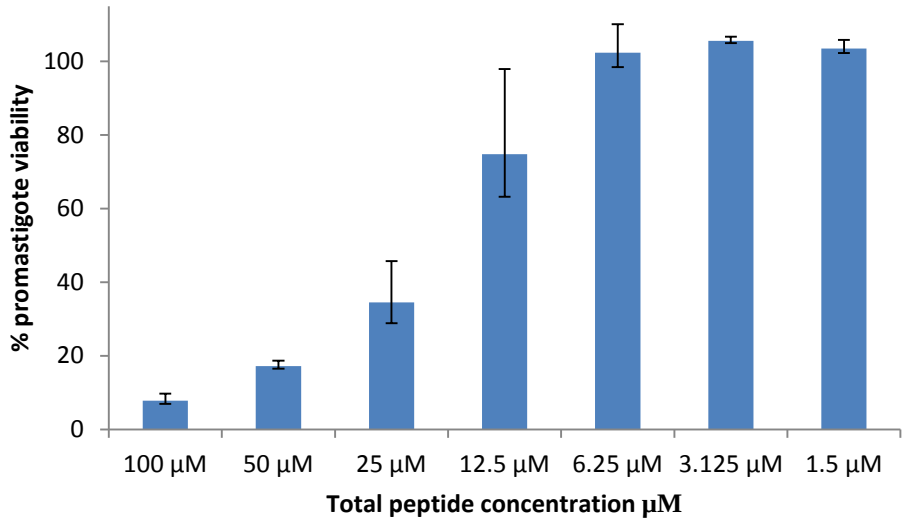


Figure 2.26 *L. mexicana* promastigote viability following exposure to combined concentrations of 100 µM to 1.5 µM (equal volunes of both peptides) of temporins A (**16**) and temporin L **(35**) in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error is indicated.

2.8.10 Peptide Toxicity

As temporins A (**16**) and L (**35**) were found to be the most active against the amastigote form of the parasite the toxicity of these peptide was investigated. It was important to investigate the toxicity of these peptides as peptides may be toxic to the host cells, in addition to *leishmania* parasites. Given the interest in **16** and **35** as antibacterial agents some toxicity data had previously been reported[16]. As summary of the published data is given in Table 2.6.

Temporin	Sequence	Testing against other axenic amastigotes	Murine macrophage testing IC_{50}	Haemolytic activity	Leathal concentration red blood cells	*L. mexicana* amastigote testing IC_{50}
Temporin L	FVQWFSKFLGR IL-NH_2	-	93 µM	2 µM[16]	55 µM[16]	50-100 µM (Cobb *et al.*, unpublished data)
Temporin A	FLPLIGRVLSGI L-NH_2	*L. pifanoi* (ama) 50% inhibition at 5.6 µM [10]	62.5 µM	1.25 µM[18]	>120 µM [19]	50-100 µM [11]

Table 2.6. A summary of the published toxicity data relating to temporin A (**16**) and temporin L (**36**).

Temporin L (35) hemolytic activity: beyond 2 µM, considerable haemolysis was observed, with 100% lysis at approx. 55 µM [16] Temporin A haemolytic activity: at 1.25 µM, 0.5 % lysis occurred, at 40.0 µM, 27.5 % lysis occurred. [18]

The toxicity of temporin A (**16**) against murine macrophages - 62.5 µM (C. Raleigh MSci report) Preliminary experiments have also shown that both temporins are devoid of cytotoxic

effects on murine macrophages (RAW 264,7 line)[10] Temporin A is chemotactic for human monocytes and neutrophils temporin A induced monocyte migration with a bell-shaped dose response curve. The peak response was observed at 250 nM.[20] Toxicity analysis of temporins A and L showed temporin A to be cytotoxic at 500 µM, and temporin L to be cytotoxic at 250 µM (shown in figure 2.26 below). These results are supported by results previously published[16], indicating that temporin L possesses a high toxicity against erythrocytes.

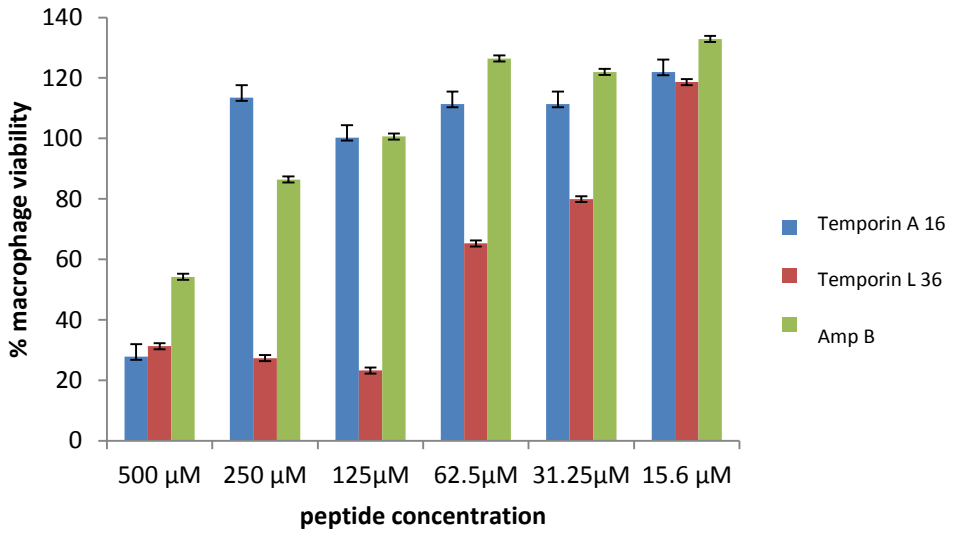


Figure 2.27 Percentage macrophage viability following 24 hours incubation with temporin A (**16**), temporin L (**36**) with combined concentrations as shown, in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 or 3 independent experiments performed in triplicate. Standard deviation is indicated. Viability is greater than 100 % in some cases due to statistical variation in controls used. Data points represent the mean of 2 independent experiments performed in triplicate. Standard error indicated.

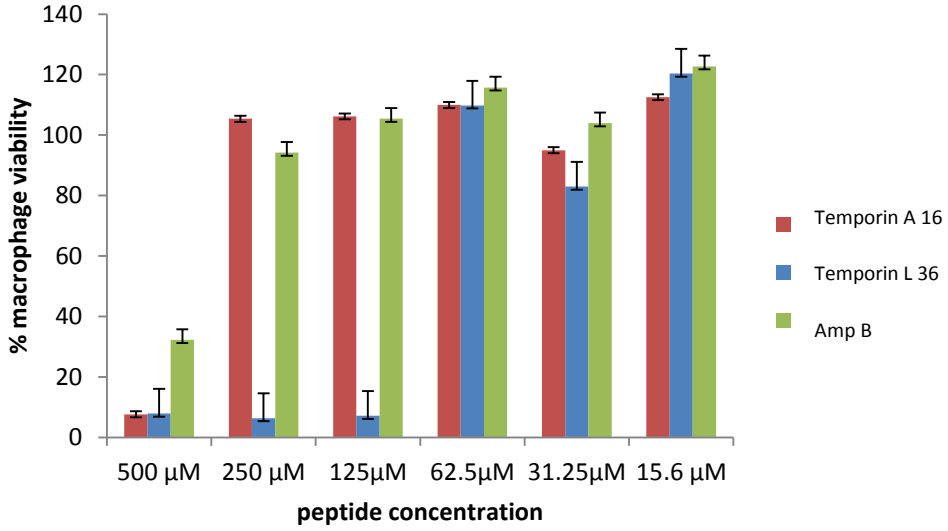


Figure 2.28 Percentage macrophage viability following 48 hours incubation with temporins A (**16**), temporin L (**36**) with combined concentrations as shown, in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard deviation is indicated.

2.9 Chapter Summary

Initially temporin peptides A (**16**), B (**17**) and 1Sa (**25**) were tested against *L. mexicana* axenic axenic amastigotes and promastigotes. Following on from this initial temporin peptides C (**34**), F (**35**) and L (**36**) were tested suing the same assay system. An overview of the anti-leishmanial results obtained from both these rounds of screening is presented in Figure 2.29 below.

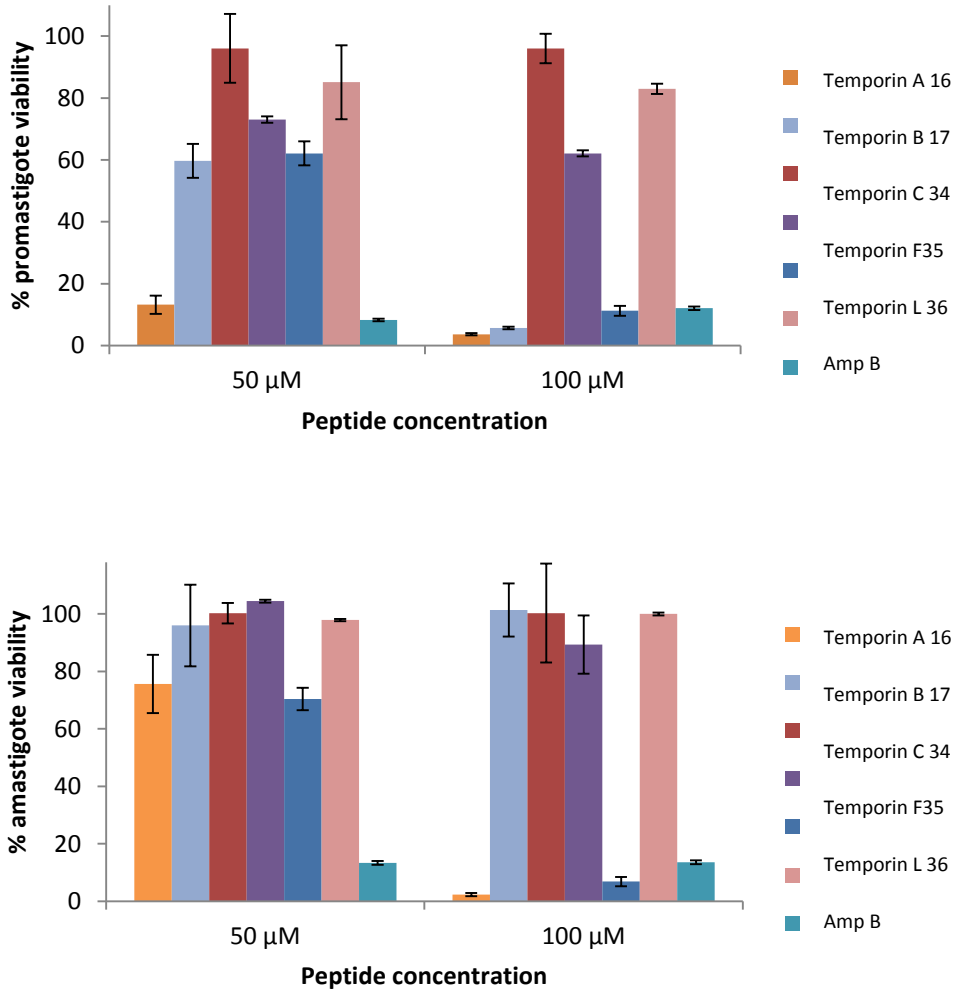


Figure 2.29 *L. mexicana* promastigote and amastigote viability following exposure to 50 µM and 100 µM concentrations of temporins A (**16**), B (**17**), C (**34**), F (**35**), and L (**36**) in the absence of serum using the Alamar Blue assay system as described previously. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 independent experiments performed in triplicate. Standard deviation is indicated.

However, as the data highlights temporins A (**16**) and L (**36**) were the only peptides tested that exhibited moderate to significant levels of activity against *L. mexicana* axenic axenic amastigotes, the clinically relevant form of *L. mexicana* published data has only reported activity on promastigotes. In contrast to insect stage promastigotes, pathogenic amastigote *L.*

mexicana are significantly more resistant to all of the temporins tested. This demonstrates the importance of screening against both forms of the parasite, particularly in light of available literature on the subject; in which reported testing on the amastigote lifecycle stage is scarce. Results obtained suggest that axenic amastigotes of different *Leishmania* species display varying susceptibility to peptides from the temporin family indicating that broad-spectrum antileishmanial AMPs may be challenging to develop. In addition, the ability of AMPs to translocate the host membrane and reach intra-macrophage axenic amastigotes has not been widely studied and remains unknown.

Given the size of the data set studied and the lack of any available data in the literature it is difficult to begin to draw useful conclusions about the properties that are required for biological activity. A summary of some of the physical and chemical properties for all the peptides analysed is given in Table 2.6.

Peptide	No. of residues	Known biological Activity	Charge	Average Hydrophobicity (Hopp & Woods)	pI	Ratio hydrophilic residues / total number of residues
Temporin A FLPLIGRVL SGIL-NH_2	13	Gram+, Virus, Chemotactic	+2	-0.9	14	15%
Temporin B LLPIVGNLL KSLL-NH2	13	Gram +	+2	-0.8	14	23%
Temporin C LLPILGNLL NGLL-NH_2	13	Gram +	+1	-1.1	14	15%
Temporin F FLPLIGKVL SGIL-NH_2	13	Gram+	+1	-0.9	14	15%
Temporin L FVQWFSKF LGRIL-NH_2	13	Gram+ & Gram-, Fungi, Mammalian cells, Cancer cells	+3	-0.9	14	30%
Temporin 1Sa FLSGIVGML GKLF-NH_2	13	Gram +	+2	-0.9	14	15%

Table 2.5 previously reported data associated with temporin peptides synthesised in this study.

Temporins A (**16**) and L (**36**) have been studied to a greater degree than any of the other temporin peptides. It is possible that the charge is important; as the parasite membrane is negatively charged a positively charged peptide may be attracted to the membrane through electrostatic attraction which may in turn facilitate the disruption of the parasite membrane, and account for a lower active concentration of drug needed to kill the parasites. Temporin L has the highest overall charge of the peptides studied in the table below (+3, compared to +1 or +2) which may facilitate the ability of the peptide to interact with the negatively charged parasite membranes. Temporin L also has a higher ration of hydrophobic residues /total number of residues (30, compared to 15-23 for other peptides), which may also account for the higher levels of antileishmanial activity observed with temporin L, this may be because the hydrophobic residues will more favourably interact with the lipid environment in the parasite membrane, and these interactions draw the peptide into the membrane more strongly than those peptides with proportionally fewer hydrophobic residues. The mean hydrophobicity is calculated using the Kyte-Doolittle scale. The Kyte-Doolittle scale is widely used for detecting hydrophobic regions in proteins. Regions with a positive value are hydrophobic. This scale can be used for identifying both surface-exposed regions as well as transmembrane regions, depending on the used window size. Short window sizes of 5-7 generally works well for predicting putative surface-exposed regions. Large window sizes of 19-21 is well suited for finding transmembrane domains if the values calculated are above 1.6[144]. These values should be used as a rule of thumb and deviations from the rule may occur. The values in the table for peptides synthesised in this work suggest a pattern of increasing mean hydrophobicity may indicate increasing antileishmanials activity. However the data set presented here is too small to form any further conclusions.

Temporins A and L the two most active peptides against axenic axenic amastigotes were then tested for cytotoxicity against uninfected murine macrophages, and against infected murine macrophages. Toxicity analysis of temporins A and L showed temporin A to be cytotoxic to 50 % of cells at 500 µM, and temporin L to be cytotoxic at 250 µM. These results are in alignment with previously published data[16], and show that temporin L possesses a high toxicity against erythrocytes. These results place the viability of temporin L as a potential therapeutic agent in question, as this peptide appears to be cytotoxic across a broad spectrum of biological tissues and would likely cause cell death in the host in addition to *leishmania* parasites. The eventual aim is to use AMPs in order to develop a topical leishmanicidal application; however it is likely that the levels of cytotoxicity reported here for temporin L would prevent the regrowth and healing of healthy tissues in the affected areas in addition to causing death of the *leishmania* parasites.

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Chapter VI

Overall Conclusions

Chapter 3: Evaluation of a second generation of antimicrobial peptides

3.1 Introduction

Following the synthesis and biological evaluation of a small number of the temporin peptides (Chapter II), it became apparent that a high throughput method of screening would be required in order to successfully identify AMPs that had promising antileishmanial activity. The initial testing carried out had been guided by previously reported activity for peptides against various *Leishmania sp.*[1] However, the results obtained from our initial study provide strong evidence that that antileishmanial activity against one *Leishmania sp*. cannot be used as an accurate indicator of potential antileishmanial activity against other *Leishmania sp*. In addition a clear difference in the biological activity of the temporin peptides against promastigotes and axenic amastigotes forms of the parasite showed that even within the same species there is a considerable difference in antileishmanial activity between the different life cycle stages of the parasite. It was also interesting to note that that antimicrobial activity cannot be used as a useful tool to accurately predict antileishmanial activity[2]. Given the considerable differences in the physical makeup of the parasite and bacterial membranes, which are the target for the temporin peptides this latter observation is not unexpected, but surprisingly it had not been well documented in the literature.

3.2 Computational modelling as a predictor of peptide biological activity

Antimicrobial peptides (AMPs) represent an important class of compounds in the search for new treatments against pathogens[3]. Membrane-active peptides (MAPs) represent a broad variety of molecules, and biological functions of most are directly associated with their ability to interact with membranes. Taking into account the effect of MAPs on living cells, they can be nominally divided into three major groups - fusion (FPs), antimicrobial/cytolytic (AMPs/CPs) and cell-penetrating (CPPs) peptides. Although spatial

structure of different MAPs varies to a great extent, linear alpha-helical peptides represent the most studied class. These peptides possess relatively simple structural organization and share a set of similar molecular features, which make them very attractive to both experimental and computational studies.[4]

Different molecular modelling methods are used depending on the purpose of the study, i.e. the application for which the AMPs studied are intended. The most sophisticated methods, such as molecular dynamics simulations, give information about molecular interactions driving peptide binding to the water-lipid interface, cooperative mechanisms of membrane destabilization and thermodynamics of these processes. Significant progress has been achieved in this field during the last few years, resulting in an increased interest in the identification of active peptides through these methodologies.[4]

As a result of the need to identify greater numbers of active AMPs, some *in silico* methods have been developed to find AMPs with potential therapeutic application.[5] Several algorithms take advantage of data mining and high-throughput screening techniques and apply vector-like analysis to scan protein and peptide sequences[6]. Other bioinformatics' strategies include supervised learning techniques, such as artificial neural networks (ANN) or support vector machines, in order to evaluate easily and reliably a great amount of complex data[7]. Although the majority of attempts have been centred in the prediction of highly active peptides using quantitative structure-activity relationships (QSAR) descriptors together with ANN[8] linear discriminant[9] or principal component analysis[10]. These systems use mainly 3D-QSAR descriptors to detail the antimicrobial properties of peptides. Recently, a QSAR-based ANN system was experimentally validated using SPOT high-throughput peptide synthesis, showing that this methodology can accomplish a reliable prediction by means of conventional and "inductive" QSAR descriptors[11]. However, the datasets used contained only peptides with fixed length and the leeds found

were only populated in few amino acids (W, R and K and, more limitedly, L, V and I). Although AMPs are actually enriched in these residues, a wide diversity in the amino acid content can be found in natural AMPs[5, 6b, 12].

Despite the inherent complexity in designing a prediction system only by means of computational chemistry and bioinformatics, the recent methods mentioned above have made remarkable advances. Hence, it is likely that in the future the combined use of bioinformatics and experimental screening techniques will be essential for the discovery and refinement of new anti-infective AMPs[5, 6b, 13].

Given the success that has been derived from the application of prediction systems in the development of antibacterial AMPs[14] we were keen to develop this approach to aid in the design of AMPs that had antileishmanial properties. However, when our work in the area was started there were no predictive systems in place that could be used to investigate antileishmanial, or even anti-parasitic properties of AMPs. In addition given that most AMPs disrupt the cell membrane of their targets, systems developed to predict antibacterial activity were unlikely to be of use in predicting antiprotozoal activity where the eukaryotic plasma membrane is the target. Furthermore, simply adapting the existing antibacterial prediction models is not straight forward as the experimental data sets available for peptides with, for example, antileishmanial activity are considerably smaller and gathered from across multiple, divergent species[15]. This makes them difficult to utilise in developing a predictive algorithm. Thus are vision was to generate a suitable data set of peptides that could then be used to develop a mathematical model to predict the activity of AMP sequences, and use that model to power a computational/rational design package aimed at producing new antileishmanial AMPs as lead compounds for CL (see Figure 3.1)

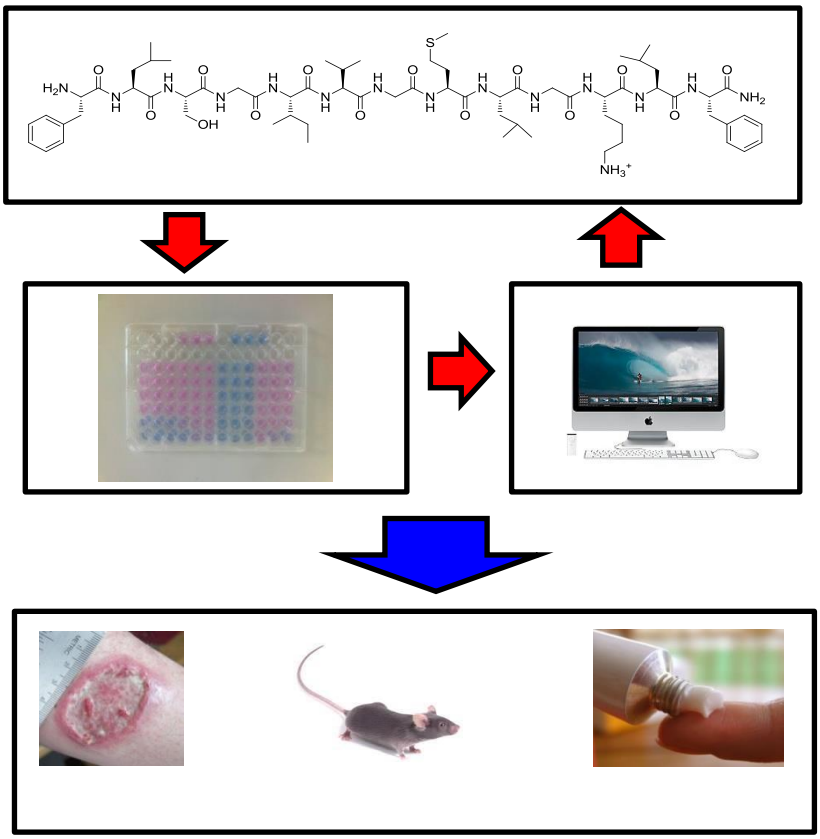


Figure 3.1 Schematic illustrating the process through which computational modelling will be used to predict active peptides. Step 1) synthesise and purify peptides, Step 2) test peptides for activity in assay, Step 3) enter testing data into computational modelling system and Step 4) preparation of a topical formulation and animal testing.

3.2 Evaluation of extended temporin peptide libraries

3.2.1 Peptide Selection

Our previous results, discussed in Chapter II had shown temporin A (**16**) and temporin L (**36**) to possess the greatest levels of antiparasitic activity against *Leishmania mexicana* promastigotes and axenic amastigotes.[2] In the presence of serum, only temporin A (**16**) demonstrated any significant antileishmanial activity against *L. mexicana* promastigotes (57% inhibition at 100 µM). Neither temporin A (**16**) or temporin L (**36**) peptides showed significant activity at the concentrations tested [temporin A: 98% at 100 µM; temporin L: 93% inhibition at 100 µM] against *L. mexicana* axenic amastigotes. Given that the temporins investigated in this study have previously been shown to be active against other

Leishmania species[16] these results were unexpected. Mangoni *et al*[16] have previously reported that temporins A (**16**) and B (**17**) are active on both the insect and the mammalian stages of *L. donovani* with complete inhibition of parasite viability at 15–25 μM.

However, by pre-incubating the parasites with the temporins studied in minimal serum the peptide activity profiles were altered. In this assay, which reflects the conditions employed in previous studies[18], temporins A (**16**) and B (**17**) demonstrated improved efficacy against promastigote *L. mexicana* (63% inhibition at 12.5 μM and 38% at 50 μM respectively). However, axenic amastigote *L. mexicana* remained largely resistant to these compounds with only temporin A (**16**) demonstrating any significant activity (only 23% inhibition at 12.5 μM but 98% at 100 μM).[2]

In order to identify potential residues within the sequences of temporin A (**16**) and temporin L (**36**) that could be modified to potentially enhance biological activity it was decided to carry out alanine scans of these peptides. An alanine scan is a commonly used technique in peptide chemistry in which every residue in the sequence is systemically replaced by an alanine[17]. As the α-carbon in alanine contains only a non-reactive methyl group, it can be viewed as essentially inert, in terms of contributing to factors such as target binding. This means that by sequentially substituting each amino acid residue with alanine, the effects of a particular residue can be observed. The aim would be to use this alanine scan approach to begin to develop a more detailed understanding of any structure activity relationships that might exist.

In the case where there is no defined molecular target, alanine scanning can be used to alter the secondary structure of the peptides, and thus the way in which the peptides are able to interact with each other, and with the parasite membrane.

In addition to the testing of alanine scans of temporins A (**16**) and temporin L (**36**), and a Lys scan of temporin L (**36**) would also be carried out (Section 3.2.2). Lysine scans can be used to evaluate the impact of charge interactions on the peptides' biological activity[18] .

Furthermore, a range of temporin peptides previously reported in the literature as possessing the greatest levels of activity against mammalian cells, gram positive bacteria, gram negative bacteria, fungi and viruses were also selected. These peptides were synthesised by Cambridge Research Biochemicals Ltd (CRB) as described in Section 3.2.2. Following testing of this library of peptides, a second library was synthesised and tested, to include those temporin peptides not previously reported as possessing no biological activity. Generally, activities of AMPs against bacteria, fungi and mammalian cells are determined by a complex interaction between cationicity, hydrophobicity, α-helicity and amphipathicity. The purpose of these syntheses is to develop analogs of temporins A (**16**) and temporin L (**36**) with a higher therapeutic index than the native peptides.

3.2.2 Preparation and screening of the Temporin A and Temporin L libraries

Both the alanine scan library of temporin A (**16**) and temporin L (**36**) were synthesised by CRB- using their in house PepArray™ system. The peptides were synthesised on a 3-5 µM scale, and were evaluated by CRB prior to providing them to us to have a purity of 70-90%. The evaluation of purity and quality control is determined by HPLC and MS analysis of a fraction (10%) of the peptides in the given library. The purity of the peptides supplied was deemed to be acceptable for initial screening applications, and with regard to cost and time it was the most reasonable method by which to screen larger libraries of peptides. It was anticipated that if a peptide was identified from the PepArray™ libraries as having antileishmanial activity, then it could be remade on a larger scale in house, purified, and

tested at a higher purity for more accurate analysis. Temporin A (**16**) was also synthesised by CRB as one of the peptides in the Peparray™, in order to provide another measure of the purity of the Peparray™ peptides produced. The activity of the "Peparray™" temporin A (**16**) would be compared to previous results obtained using a temporin A peptide that had been prepared and purified by HPLC previously at Durham. . The sequences for all the Temporin A peptides in the alanine scan library are shown in Table 3.1 below.

Peptide	Amino acid sequence
TA1	ALPLIGRVLSGIL-NH_2
TA2	FAPLIGRVLSGIL-NH_2
TA3	FLALIGRVLSGIL-NH_2
TA4	FLPAIGRVLSGIL-NH_2
TA5	FLPLAGRVLSGIL-NH_2
TA6	FLPLIARVLSGIL-NH_2
TA7	FLPLIGAVLSGIL-NH_2
TA8	FLPLIGRALSGIL-NH_2
TA9	FLPLIGRVASGIL-NH_2
TA10	FLPLIGRVLAGIL-NH_2
TA11	FLPLIGRVLSAIL-NH_2
TA12	FLPLIGRVLSGAL-NH_2
TA13	FLPLIGRVLSGIA-NH_2

Table 3.1 Alanine scan of temporin A (**16**)

All of the Tenporin A peptides (Table 3.1, TA1-TA13) were dissolved in DMSO to give 25 mM stock solutions and these were stored at -20°C until use. A solution of Amphotericin B, and pure DMSO were stored under identical conditions throughout the screening process. Temporin A peptides (Table 3.1, TA1-TA13) were initially tested for activity at a concentration of 200 µM. This concentration was selected so as to detect any active peptides. It also assumes that the concentrations of the peptides tested are likely to be less than predicted (i.e. below 200 µM) given the purity range of 70-90% provided by CRB the suppliers of the peptide library. All of the peptides were screened a minimum of two times, in triplicate. The result of the screening against *L. mexicana* promastigotes and *L. mexicana* axenic amastigotes are shown in Figures 3.2 and 3.3 respectively.

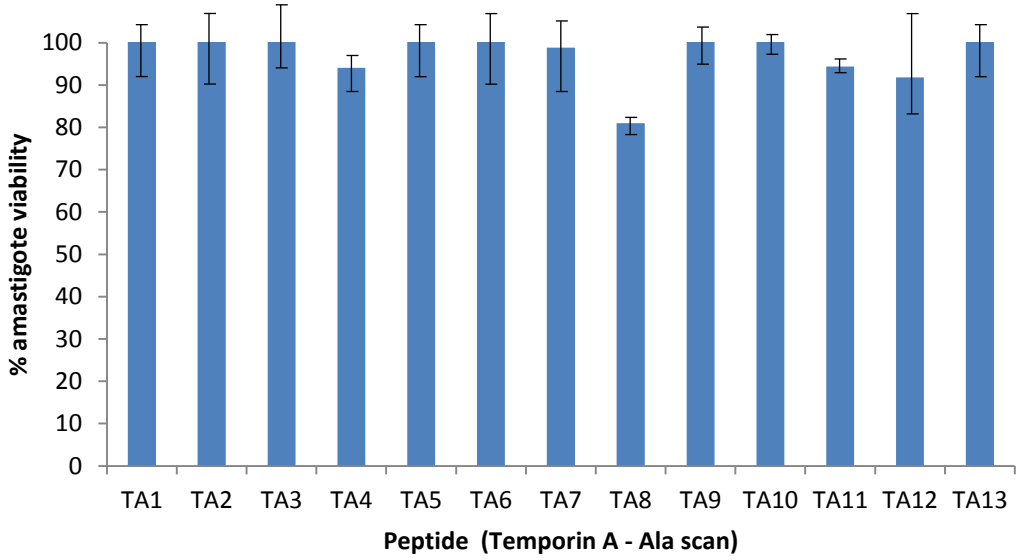


Figure 3.2 Alanine scan of temporin A (**16**), showing % amastigote viability following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

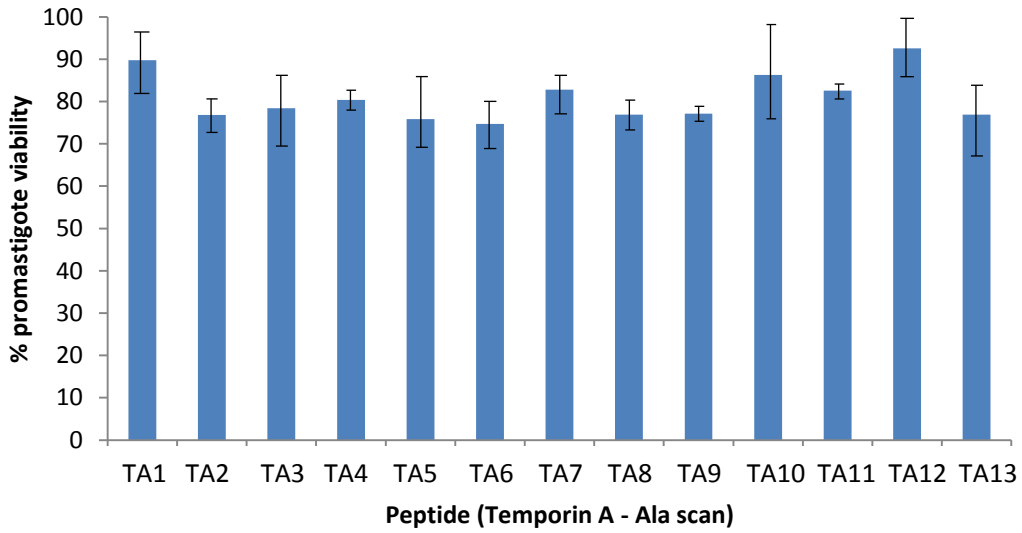


Figure 3.3 Alanine scan of temporin A (**16**), showing % promastigote viability following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Firstly, it should be noted that the temporin A (**16**) provided from CRB showed 5% promastigotes cell viability and 4% amastigotes cell viability at 200 µM (results not shown in Figures 3.2 and 3.3). These results correlate very well with those previously obtained within the group using HPLC purified temporin A (4% promastigotes cell viability and 3% amastigotes cell viability at 100 µM)[2]. This provided a level of confidence that the estimation of peptide purity of 70-90 % was likely to be accurate.

Upon initial evaluation of the data it was decided to set a threshold at which an 'active' peptide would be recorded as being gone which produced a percentage cell viability of 60 % or less. This activity threshold was set in order to prevent any 'hits' being missed in the initial screen. Also, as this was an initial large screening shotgun approach, any peptides producing a percentage cell viability of 60% or lower would be synthesised on a larger scale and purified in order to provide a more accurate evaluation of antileishmanial activity.

As the data in Figure 3.2 and Figure 3.3 highlight none of the peptides tested (Table 3.1, TA1-TA13) were found to show activity below the threshold against either promastigotes or axenic amastigotes. These results indicate that there are no residues within the Temporin A sequence that are suitable for further modification to enhance antileishmanial activity. It is worth while noting that peptides TA7 [FLPLIGAVLSGIL-NH_2] and Ta10 [FLPLIGRVLAGIL-NH_2] in which the overall charge of the peptide has been modified, little or no change in activity was observed. This is in contrast to structure-function relationship studies performed against a range of bacteria[19]. A previous study by Wade *et al*[19] reported that replacing isoleucine with leucine at amino acid positions 5 and 12 resulted in the greatest enhancement of antibacterial activity. In addition, this study also showed that there was little difference between the activities of temporin D (**16**) and its all-D enantiomer. This resulted indicate that the **16** probably exerts its biological effect on bacteria via non-chiral interactions with membrane lipids.

Previous structure-function relationship studies[17, 19] have been performed on temporin A (**16**), although to date, only one previous study has demonstrated the contribution of each individual amino acid residue to the antimicrobial and haemolytic activities of this peptide through alanine-positional scanning[17]. This previous study by Grieco *et al*[17] has reported an alanine scan of temporin A (termed temporin 1Ta in the paper). In this study, four analogs were identified with a higher hydrophobicity and higher percentage of α-helix compared to the natural peptide, in both DPC and SDS[17]. In the study by Grieco *et al*[17] the temporin A analogs (TA3, TA6, TA10, and TA11, sequences shown in Table 3.4) were tested for haemolytic activity, and also for activity against a range of gram-positive and gram-negative bacteria. All of the peptides tested displayed the same or enhanced antimicrobial activity compared to natural temporin A (**16**), but also a higher lytic effect on human erythrocytes[17]. Significantly, these substitutions reside at the hydrophilic face of the

peptide. However, substitution of Arg with Ala (TA7), also located at the polar face of the peptide, suppressed its antimicrobial effect against all of the microorganisms tested. This is in agreement with reports from other authors[20] and is probably due to the loss of the single positively charged residue (Arg) in the sequence, causing a weaker electrostatic interaction with the microbial membrane. In comparison, when alanine substitutions are introduced on to the hydrophobic side of the molecule (TA1, TA2, TA4, TA5, TA8, TA9, TA12, and TA13), a clear decrease or almost abolishment of the antimicrobial activity occurs, along with a significant decrease in the hemolytic activity. TA1 is an exception, because it exhibits two to threefold higher haemolytic activity compared to the parent peptide (Table 3.4).

							MIC (μM)					
					% Helix		Gram-negatives		Gram-positives			Yeasts
Peptides	Amino acid sequences	Charge	*H*	$^{\mu}H$	DPC	SDS	*E. coli*	*Y. pseudo-tuberculosis*	*S. aureus*	*B. megaterium*	*S. capitis*	*C. albicans*
1Ta	FLPLIGRVLSGIL-NH_2	+2	0.22	0.35	55	50	>40	20	5	2.5	5	5
TaA1	ALPLIGRVLSGIL-NH_2	+2	0.2	0.33	60	50	>40	>40	>40	20	>40	>40
TaA2	FAPLIGRVLSGIL-NH_2	+2	0.2	0.34	54	49	>40	40	20	5	20	10
TaA3	FLALIGRVLSGIL-NH_2	+2	0.25	0.33	69	57	>40	20	2.5	2.5	2.5	1.25
TaA4	FLPAIGRVLSGIL-NH_2	+2	0.2	0.36	53	49	>40	40	40	5	20	10
TaA5	FLPLAGRVLSGIL-NH_2	+2	0.19	0.31	50	46	>40	>40	40	20	40	20
TaA6	FLPLIARVLSGIL-NH_2	+2	0.23	0.35	71	65	>40	20	2.5	2.5	5	2.5
TaA7	FLPLIGAVLSGIL-NH_2	+2	0.38	0.21	63	52	>40	>40	>40	>40	>40	>40
TaA8	FLPLIGRALSGIL-NH_2	+2	0.2	0.34	54	51	>40	40	20	5	20	10
TaA9	FLPLIGRVASGIL-NH_2	+2	0.2	0.34	51	46	>40	>40	>40	>40	>40	>40
TaA10	FLPLIGRVLAGIL-NH_2	+2	0.26	0.32	56	50	>40	20	2.5	2.5	5	2.5
TaA11	FLPLIGRVLSAIL-NH_2	+2	0.23	0.35	71	61	>40	20	2.5	2.5	5	2.5
TaA12	FLPLIGRVLSGAL-NH_2	+2	0.19	0.32	55	49	>40	>40	40	20	>40	20
TaA13	FLPLIGRVLSGIA-NH_2	+2	0.2	0.35	55	50	>40	>40	>40	20	>40	20

Table 3.4. Temporin A Ala-scan comparison with respect to charge, *H*, $_{\mu H}$, % helicity and MIC on different microbial strains[3].

Screening of the large scale library against *L. mexicana* promastigotes and axenic amastigotes continued with the peptides synthesised in the Ala scan of temporin L. Results are shown in Figures 3.5 and 3.6.

Temporin L (**36**) was also synthesised by CRB, in order to provide a measure of purity of the Peparray^{TM} peptides. Temporin L (**36**) showed 3% promastigotes cell viability and 22% axenic amastigotes cell viability at 200 μM. These results correlate very well with those previously obtained by us when an HPLC purified 36 was utilised.[2] Again this result proved evidence that a purity of 70-90 % for the peptides used was accurate, and that peptides leading to less than 60 % cell viability are logically active at 100 μM or lower concentrations. The peptides synthesised in the alanine scan of temporin L (**36**) are shown below in Table 3.2.

Peptide	Amino acid sequence
TL1	AVQWFSKFLGRIL-NH_2
TL2	FAQWFSKFLGRIL-NH_2
TL3	FVAWFSKFLGRIL-NH_2
TL4	FVQAFSKFLGRIL-NH_2
TL5	FVQWASKFLGRIL-NH_2
TL6	FVQWFAKFLGRIL-NH_2
TL7	FVQWFSAFLGRIL-NH_2
TL8	FVQWASKALGRIL-NH_2
TL9	FVQWFAKFAGRIL-NH_2
TL10	FVQWFSKFLARIL-NH_2
TL11	FVQWFSKFLGAIL-NH_2
TL12	FVQWFSKFLGRAL-NH_2
TL13	FVQWFSKFLGRIA-NH_2
TL	FVQWFSKFLGRIL-NH_2

Table 3.2 Alanine scan of temporin L (**36**).

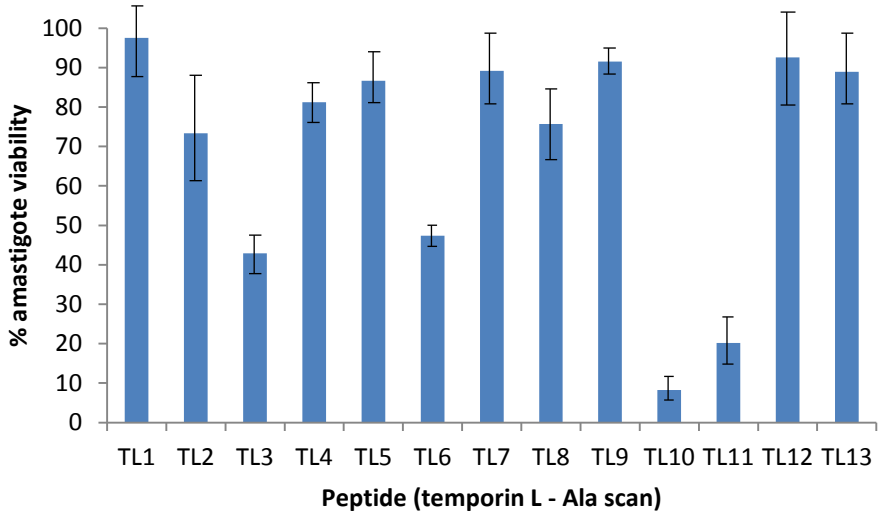


Figure 3.5 Alanine scan of temporin L (**36**), showing % axenic amastigote viability following a 24 hour incubation period with peptides at 200 μM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

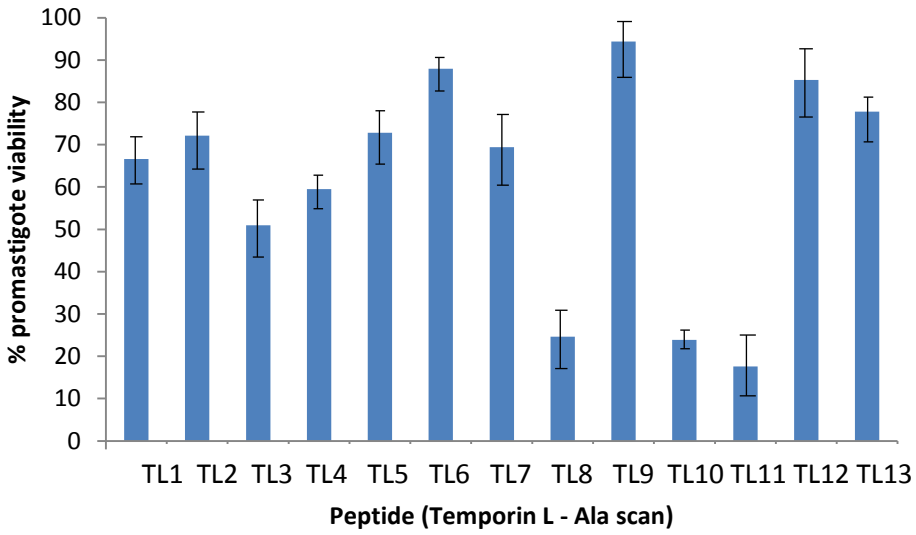


Figure 3.6 Alanine scan of temporin L (**36**), showing % promastigote viability following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Six peptides were found to be active against promastigotes, and three against amastigotes. The following sequences were active at 200 µM:

Peptide	Primary Sequence of Peptide	Promastigote cell viability	Axenic Amastigote cell viability
TL2	FAQWFSKFLGRIL-NH_2	43%	no activity
TL3	FVAWFSKFLGRIL-NH_2	52%	no activity
TL6	FVQWFAKFLGRIL-NH_2	48%	no activity
TL8	FVQWFSKALGRIL-NH_2	23%	8%
TL10	FVQWFSKFLARIL-NH_2	22%	20%
TL11	FVQWFSKFLGAIL-NH_2	20%	20%

Table 3.3 Biologically active peptides in alanine scan of temporin L (**36**)

Analogs of temporin L were shown to possess the greatest activity of all analogs tested against leishmnia mexicana promastigotes and axemic amastigotes; subsequently peptides were synthesised to produce a series of peptides with the amino acid lysine sequentially replacing each residue along the length of the chain. The lysine scan was conducted in order to determine the effect of insertion of a positive charge into the amino acid chain.

These peptides were synthesised on a 3-5 μM scale, and were tested at 70-90% purity. Peptides synthesised in the lysine scan of temporin L were as shown in Table 3.4.

Peptide	Amino acid sequence
TL14	KVQWFSKFLGRIL-NH_2
TL15	FKQWFSKFLGRIL-NH_2
TL16	FVKWFSKFLGRIL-NH_2
TL17	FVQKFSKFLGRIL-NH_2
TL18	FVQWKSKFLGRIL-NH_2
TL19	FVQWFKKFLGRIL-NH_2
TL20	FVQWFSGFLGRIL-NH_2
TL21	FVQWKSKALGRIL-NH_2
TL22	FVQWFSKFKGRIL-NH_2
TL23	FVQWFSKFLKRIL-NH_2
TL24	FVQWFSKFKGKIL-NH_2
TL25	FVQWFSKFLGRKL-NH_2
TL26	FVQWFSKFLGRIK-NH_2

Table 3.4 Lysine scan of temporin L (36)

Screening against *L. mexicana* promastigotes and axenic amastigotes continued with the peptides synthesised in the Lys scan of temporin L. Results are shown in Figures 3.7 and 3.8.

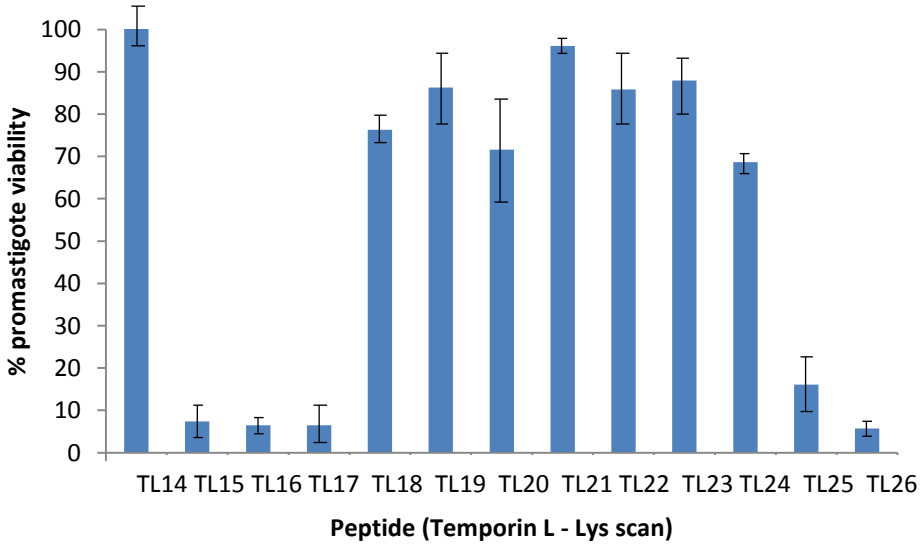


Figure 3.7 Lysine scan of temporin L, showing % promastigote viability following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

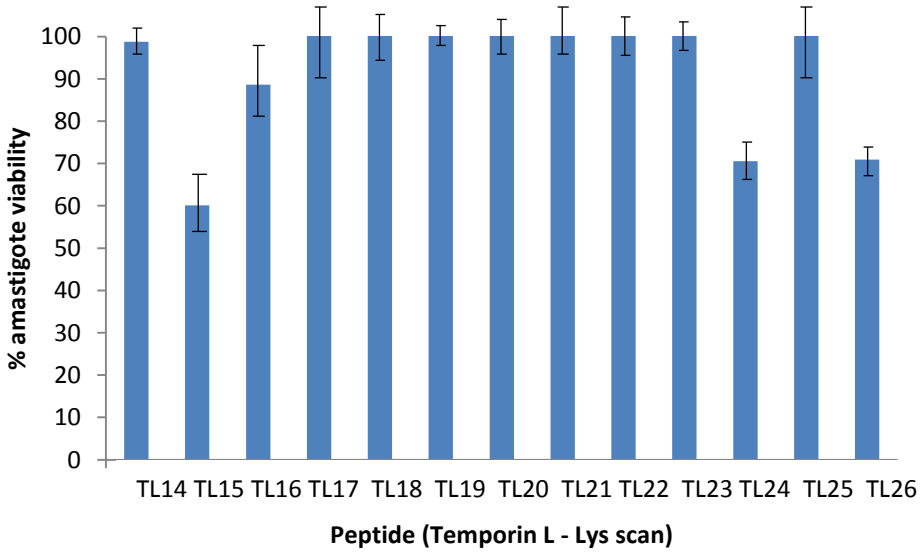


Figure 3.8 Lysine scan of temporin L, showing % amastigote viability following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Five peptides from the Lys scan were found to be active against *L. mexicana:* five peptides showed activity against promastigotes: TL15, TL16, TL17, TL25, TL26 and one sequence was active against both promastigotes and axenic amastigotes: TL15.

Peptide	Primary sequence	Promastigote cell viability	Axenic Amastigote cell viability
TL15	FKQWFSKFLGRIL-NH_2	7%	60%
TL16	FVKWFSKFLGRIL-NH_2	6%	no activity
TL17	FVQKFSKFLGRIL-NH_2	6%	no activity
TL25	FVQWFSKFLGRKL-NH_2	16%	no activity
TL26	FVQWFSKFLGRLK-NH_2	16%	no activity

Table 3.5 Active peptides from Lysine scan of temporin L. Peptides were tested at a concentration of 200 µM

3.2.3 Natural temporin peptide library

A library of temporin peptides were synthesised by Cambridge Research Biochemicals (CRB) using their PepArray™ technology. The peptides were synthesised on a 3-5 µM scale, and were evaluated by the suppliers to have a purity of 70-90%. This evaluation of purity is and quality control is determined by HPLC and MS analysis of a fraction (10%) of the peptides in the given library. The purity of the peptides supplied was deemed to be acceptable for initial screening applications, and with regard to cost and time it was the most reasonable method by which to screen larger libraries of peptides. It was anticipated that if a peptide was identified from the PepArray™ libraries as having antileishmanial activity, then it could be remade on a larger scale in house, purified, and tested at a higher purity for more accurate analysis. Temporin L was also synthesised by CRB, in order to provide another measure of the purity of the Peparray™ peptides produced. The activity of the "Peparray™" Temporin L would be compared to previous results obtained using a Temporin L peptide that had been prepared and HPLC purified in Durham. The full sequences for all the temporin peptides in the library synthesised are shown in Table 3.6.

Peptide	Sequence	Name
1	NFLGTLVNLAKKIL-NH_2	Temporin-1CSd
2	FLPILGKLLSGIL -NH_2	Temporin-1TGa
3	FLPLLASLFSRLL-NH_2	Temporin-1Oa
4	FLPLIGKILGTIL-NH_2	Temporin-1Ob
5	FLPIVGRLISGLL-NH_2	Temporin 1ARa
6	FLPIIAKVLSGLL-NH_2	Temporin 1Bya
7	ILPLVGNLLNDLL-NH_2	Temporin 1Ja
8	FLPIVGKLLSGLL-NH_2	Temporin 1M
9	ILPILGNLLNGLL-NH_2	Temporin 1Pra
10	FLPLVGKILSGLI-NH_2	Temporin 1VE
11	FLSSIGKILGNLL-NH_2	Temporin 1Va
12	FLSGIVGMLGKLF-NH_2	Temporin-SHa
13	FLSHIAGFLSNLF-NH_2	Temporin-SHc
14	ILPILGNLLNSLL-NH_2	Temporin-1PRb
15	HFLGGTLVNLAKKIL-NH_2	Temporin-1DRa
16	FLPVILPVIGKLLSGIL-NH_2	Temporin-1TGc
17	FFPLVLGALGSILPKIF-NH_2	Temporin-LTa
18	FFGSVLKLIPKIL-NH_2	Temporin-PTa
19	FLPGLIAGIAKML-NH_2	Temporin-LT1
20	FLPIALKALGSIFPKIL-NH_2	Temporin-LT2
21	FLPIITNLLGKLL-NH_2	Temporin-PRb
22	FVDLKKIANIINSIF-NH_2	Temporin-1CEa
23	FLPFLAKILTGVL-NH_2	Temporin-1Ca
24	FLPLFASLIGKLL-NH_2	Temporin-1Cb
25	FLPFLASLLTKVL-NH_2	Temporin-1Cc
26	FLPFLASLLSKV-NH_2	Temporin-1Cd
27	FLPFLATLLSKVL-NH_2	Temporin-1Ce
28	FLPIVGKLLSGLL-NH_2	Temporin-1P
29	FLPLLFGAISHLL-NH_2	Temporin-GH
30	FLPIVGKLLSGLL-NH_2	Temporin-1CSa
31	FLPIIGKLLSGLL-NH_2	Temporin-1CSb
32	FLPLVTGLLSGLL-NH_2	Temporin-1CSc
33	FLSAITSLLGKLL-NH_2	Temporin-1SPb
34	FIGPIISALASLFG-NH_2	Temporin-1DYa
35	FLPLVGKILSGLI-NH_2	Temporin-1PLa
36	FLPLLASLFSRLF-NH_2	Temporin-1Oc
37	FLPLLASLFSGLF-NH_2	Temporin-1Od

38	SILPTIVSFLSKVF-NH_2	Temporin-1Ga
39	SILPTIVSFLSKFL-NH_2	Temporin-1Gb
40	SILPTIVSFLTKFL-NH_2	Temporin-1Gc
41	FILPLIASFLSKFL-NH_2	Temporin-1Gd
42	FLPIVGKLLSGLSGLL-NH_2	Temporin-ALa
43	FLPIIGQLLSGLL-NH_2	Temporin 1AUa
44	FLPVIAGLLSKLF-NH_2	Temporin 1Ec
45	SIFPAIVSFLSKFL-NH_2	Temporin 1HKa
46	FLPFLKSILGKIL-NH_2	Temporin 1OLa
47	FLPFFASLLGKLL-NH_2	Temporin 1OLb
48	FLSIIAKVLGSLF-NH_2	Temporin 1Vb
49	FLPIVTNLLSGLL-NH_2	Temporin-SHb
50	FLPLVTMLLGKLF-NH_2	Temporin-1Vc
51	AVDLAKIANKVLSSLF-NH_2	Temporin-1TGb
52	FIITGLVRGLTKLF-NH_2	Temporin-LTb
53	SLSRFLSFLKIVYPPAF-NH_2	Temporin-LTc
54	FFGSVLKLIPKIL-NH_2	Temporin-PTa
55	FLSAITSILGKFF-NH_2	Temporin-1SPa
56	IPPFIKKVLTTVF-NH_2	Temporin-CPa
57	FLPIVGRLISGIL-NH_2	Temporin-CPb
58	FLPILGNLLSGLL-NH_2	Temporin-PRa
59	NFLDTLINLAKKFI-NH_2	Temporin-PRc

Table 3.6 List of the naturally occurring temporin peptides selected for the study.

Screening of the large scale library against *L. mexicana* promastigotes and amastigotes concluded with the remainder of the library of naturally occurring temporin peptides. Results are shown in Figures 3.9 and 3.10.

3.2.4 The Aureins and other antimicrobial peptides

The Aureins are a family of α-helical AMPs of a similar structure and size to the temporins[21]. They are also derived from frogs, in this case produced by the dorsal gland of the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*[22]. Aureins 2.1, 2.2, 2.3, 2.5,

2.6 and 3.1, were a gift from Dr Sarah Dennison (University of Lancaster, UK). Peptide sequences of aureins 2.1, 2.2, 2.3, 2.5, 2.6 and 3.1 are shown in Table 3.10

Peptide	Sequence	Chemical formula	MW
Aurein 2.1	GLLDIVKKVVGAFGSL-NH_2	$C_{76}H_{131}N_{19}O_{19}$	1614.98
Aurein 2.2	GLFDIVKKVVGALGSL-NH_2	$C_{76}H_{131}N_{19}O_{19}$	1614.98
Aurein 2.3	GLFDIVKKVVGAIGSL-NH_2	$C_{76}H_{131}N_{19}O_{19}$	1614.98
Aurein 2.5	GLFDIVKKVVGAFGSL-NH_2	$C_{79}H_{129}N_{19}O_{19}$	1648.99
Aurein 2.6	GLFDIAKKVIGVIGSL-NH_2	$C_{77}H_{133}N_{19}O_{19}$	1629.00
Aurein 3.1	GLFDIVKKIAGHIAGSI-NH_2	$C_{81}H_{136}N_{22}O_{20}$	1738.09

Table 3.10 Summary of the aurein peptides investigated.

All aurein peptides provided were tested as pure compounds (purified by RP-HPLC). Peptides were tested for activity against promastigote and axenic amastigote life cycle stages of *L. mexicana*. None of the aureins screened showed any activity against the axenic amastigotes. Of the aurein peptides that were investigated three showed activity against the promastigote form the parasite. Aureins 2.2, 2.3 and 2.5 (<50% parasite viability), but only at the higher concentration: aurein 2.2 (5% cell viability at 100 μM, 77% at 50 μM), aurein 2.3 (9% cell viability at 100 μM, 86% at 50 μM) and aurein 2.5 (38% cell viability at 100 μM, 84% at 50 μM). These results are shown in Figures 3.11 and 3.12.

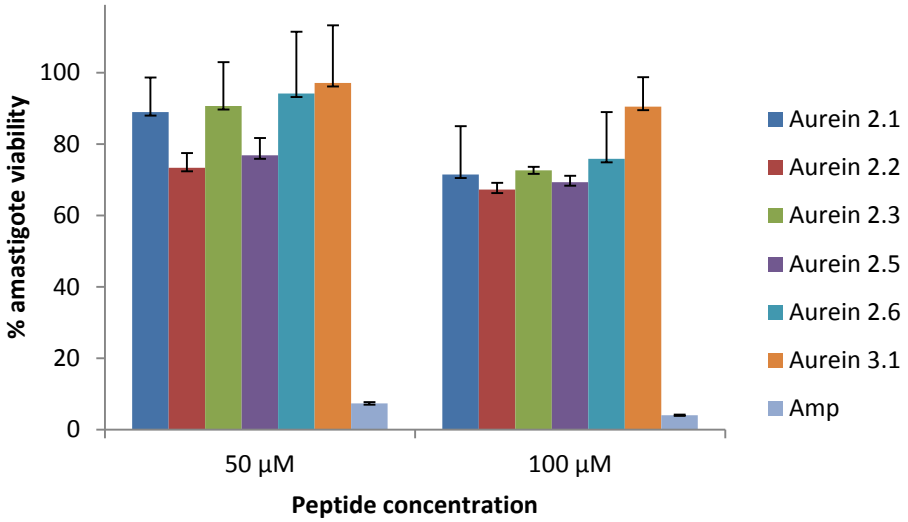


Figure 3.11 % viability (axenic amastigote) when incubated with aureins 2.1, 2.2, 2.3, 2.5, 2.6, 3.1. Amphotericin B was used as a control. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

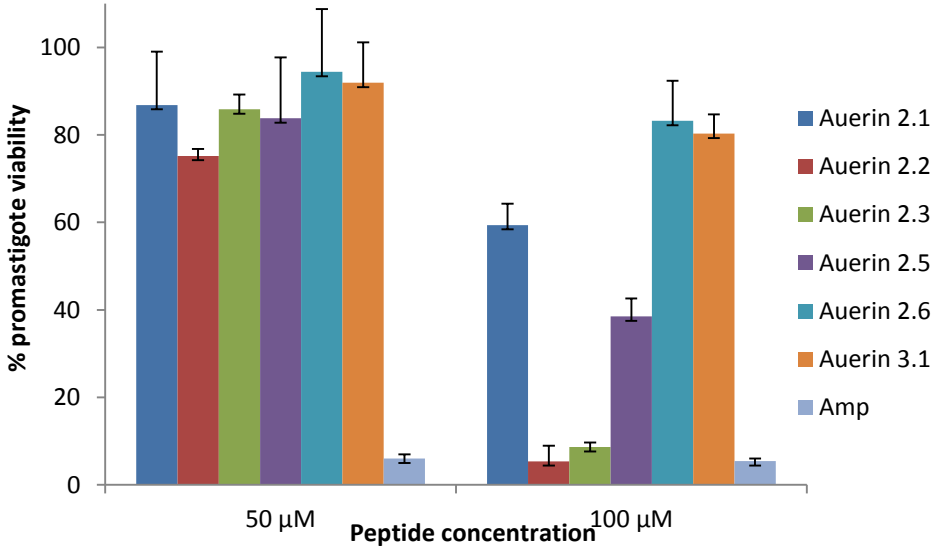


Figure 3.12 % viability (promastigotes) when incubated with aureins 2.1, 2.2, 2.3, 2.5, 2.6, 3.1 Amphotericin B was used as a control. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Results obtained from testing of the aureins support our previous findings[2] in which the promastigote lifecycle stage appears to be significantly more susceptible to the effects of the temporin AMPs. The aurein peptides are thought to act through bacterial membrane

disruption via the carpet mechanism[23], as has been reported for the temporin peptides[24]. The analogous reported mechanisms of action support findings reported here, in that both temporin and aurein peptides exhibit similar differences in activity in *L. mexicana* promastigote and axenic amastigote lifecycle stages.

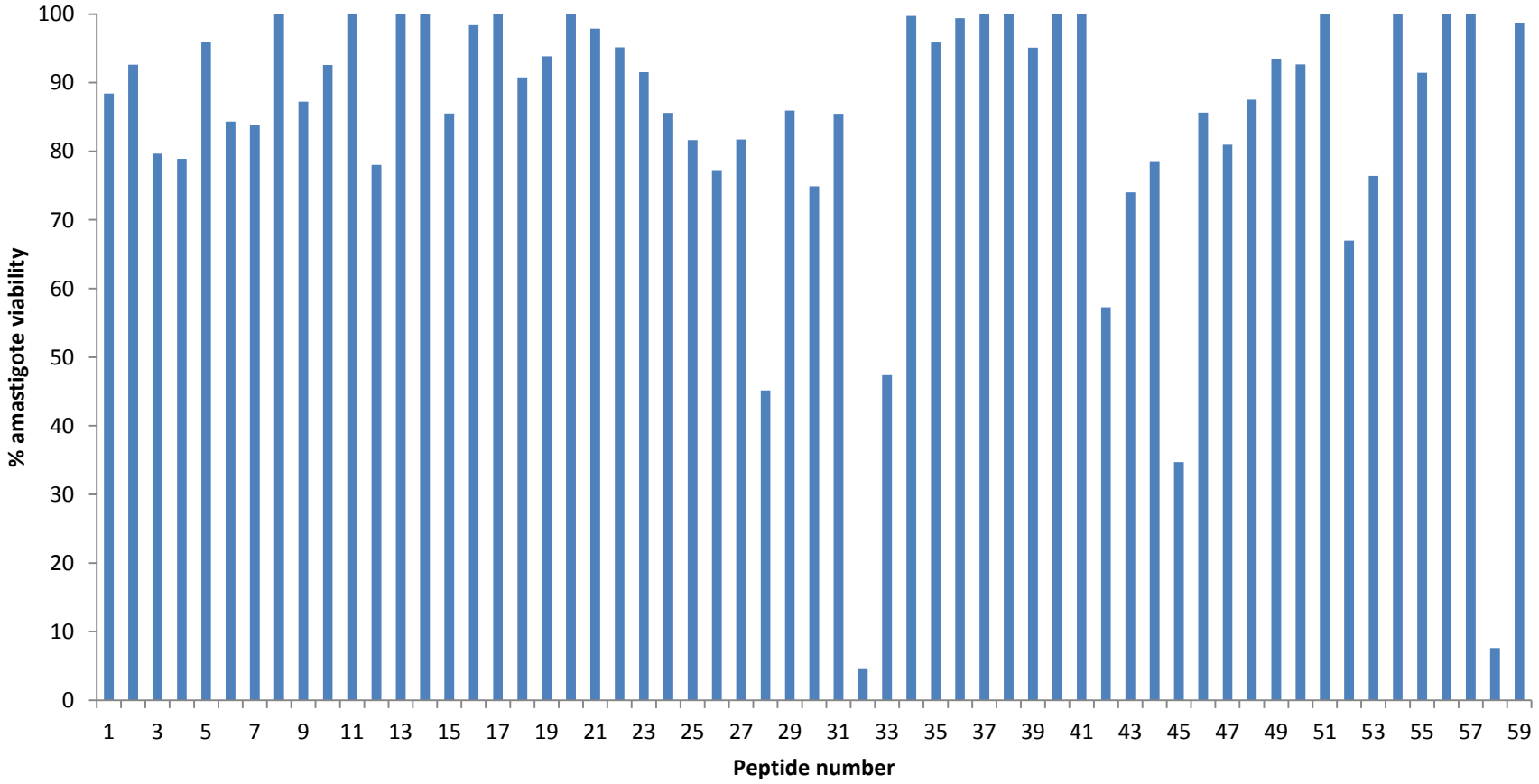


Figure 3.9 % viability (axenic amastigote) following a 24 hour incubation period with peptide library at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

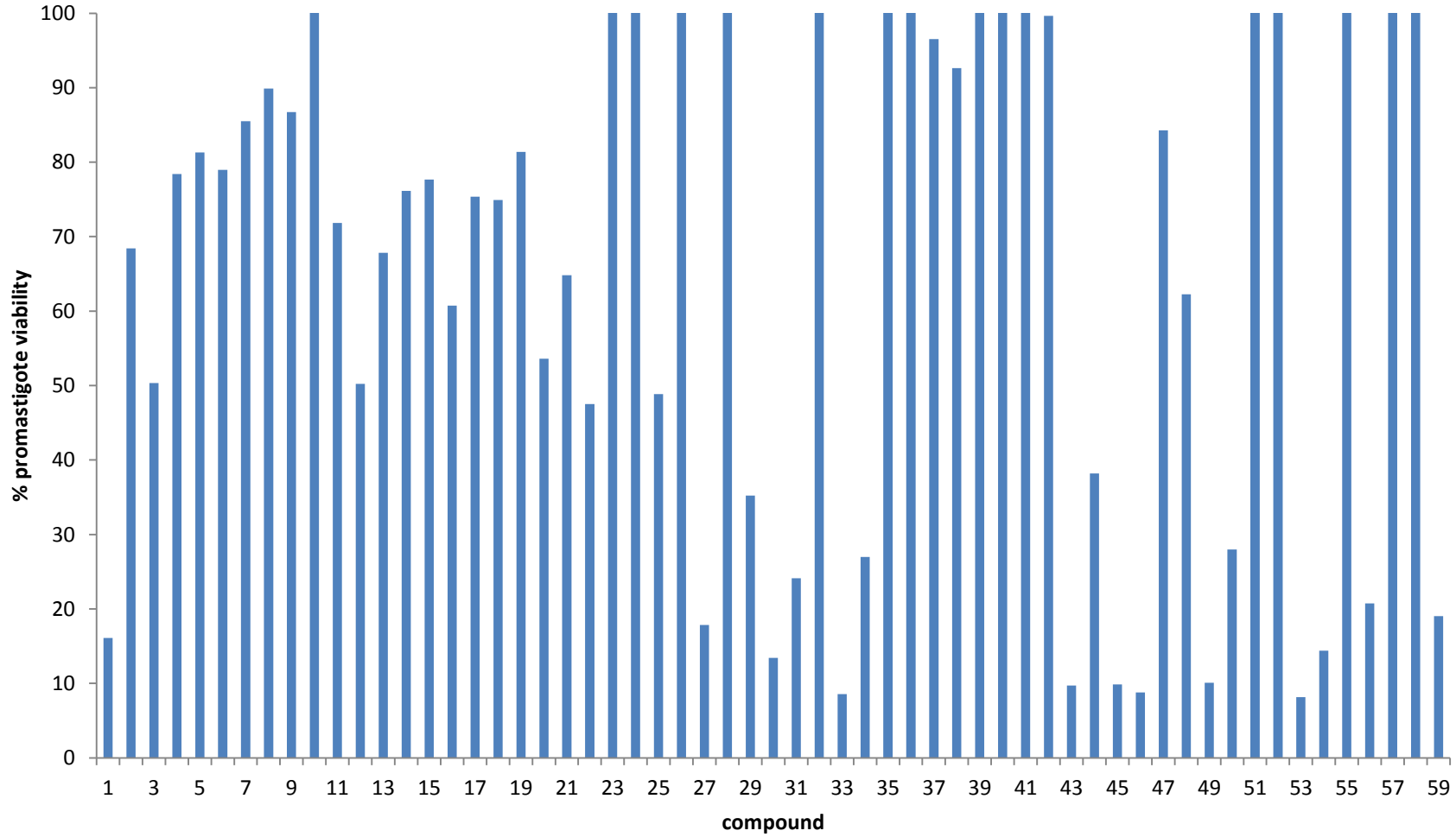


Figure 3.10 % viability (promastigote) following a 24 hour incubation period with peptides at 200 µM. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Peptides with the sequences shown in Table 3.7 displayed activity against promastigotes and peptides shown in Table 3.8 were found to possess activity against axenic amastigotes.

Peptide	Compound number	Active promastigotes only	Chemical formula	MW
Temporin-1Cc	25	FLPFLASLLTKVL-NH_2	$C_{74}H_{121}N_{15}O_{15}$	1460.85
Temporin-1Ce	27	FLPFLATLLSKVL-NH_2	$C_{74}H_{121}N_{15}O_{15}$	1460.85
Temporin-1CSa	30	FLPIVGKLLSGLL-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1368.76
Temporin-1CSb	31	FLPIIGKLLSGLL-NH_2	$C_{69}H_{119}N_{15}O_{14}$	1382.78
Temporin 1AUa	43	FLPIIGQLLSGLL-NH_2	$C_{68}H_{115}N_{15}O_{15}$	1382.74
Temporin 1Ec	44	FLPVIAGLLSKLF-NH_2	$C_{72}H_{117}N_{15}O_{14}$	1416.8
Temporin 1HKa	45	SIFPAIVSFLSKFL-NH_2	$C_{79}H_{122}N_{16}O_{17}$	1567.92
Temporin 1Vb	48	FLSIIAKVLGSLF-NH_2	$C_{70}H_{115}N_{15}O_{15}$	1406.76
Temporin-SHb	49	FLPIVTNLLSGLL-NH_2	$C_{68}H_{115}N_{15}O_{16}$	1398.74
Temporin-1Vc	50	FLPLVTMLLGKLF-NH_2	$C_{75}H_{123}N_{15}O_{14}S_1$	1490.94
Temporin-LTc	53	SLSRFLSFLKIVYPPAF-NH_2	$C_{99}H_{150}N_{22}O_{21}$	1984.4
Temporin-PTa	54	FFGSVLKLIPKIL-NH_2	$C_{75}H_{124}N_{16}O_{14}$	1473.89
Temporin-CPa	56	IPPFIKKVLTTVF-NH_2	$C_{76}H_{124}N_{16}O_{15}$	1501.9
Temporin L Lys scan	TL16	FVKWFSKFLGRIL-NH_2	$C_{84}H_{126}N_{20}O_{14}$	1640.03
	TL17	FVQKFSKFLGRIL-NH_2	$C_{78}H_{124}N_{20}O_{15}$	1581.95
	TL25	FVQWFSKFLGRKL-NH_2	$C_{83}H_{123}N_{21}O_{15}$	1655
	TL26	FVQWFSKFLGRLK-NH_2	$C_{83}H_{123}N_{21}O_{15}$	1655
TL ala scan	TL2	FAQWFSKFLGRIL-NH_2	$C_{81}H_{118}N_{20}O_{15}$	1611.94
	TL3	FVQWFAKFLGRIL-NH_2	$C_{83}H_{122}N_{20}O_{14}$	1623.99
	TL6	FVQWFSKALGRIL-NH_2	$C_{77}H_{118}N_{20}O_{15}$	1563.89
Temporin-1CSd	1	NFLGTLVNLAKKIL-NH_2	$C_{73}H_{127}N_{19}O_{17}$	1542.91
Temporin-1Oa	3	FLPLLASLFSRLL-NH_2	$C_{74}H_{121}N_{17}O_{15}$	1488.87
Temporin-SHa	12	FLSGIVGMLGKLF-NH_2	$C_{67}H_{109}N_{15}O_{14}S_1$	1380.75
Temporin-LT2	20	FLPIALKALGSIFPKIL-NH_2	$C_{93}H_{154}N_{20}O_{18}$	1840.35
Temporin-1CEa	22	FVDLKKIANIINSIF-NH_2	$C_{83}H_{136}N_{20}O_{20}$	1734.1

Table 3.7 Peptides that displayed activity against *L. mexicana* promastigotes.

Name	Compound number	Active promastigotes + amastigotes	Chemical formula	MW
Temporin-GH	29	FLPLLFGAISHLL-NH_2	$C_{73}H_{11}4N_{16}O_{14}$	1439.79
Temporin-1SPb	33	FLSAITSLLGKLL-NH_2	$C_{66}H_{115}N_{15}O_{16}$	1374.72
Temporin-1DYa	34	FIGPIISALASLFG-NH_2	$C_{69}H_{109}N_{15}O_{16}$	1404.7
Temporin 1OLa	46	FLPFLKSILGKIL-NH_2	$C_{76}H_{126}N_{16}O_{14}$	1487.92
Temporin-PRc	59	NFLDTLINLAKKFI-NH_2	$C_{79}H_{129}N_{19}O_{19}$	1648.99
Temporin L Lys scan	TL15	FKQWFSKFLGRIL-NH_2	$C_{84}H_{125}N_{21}O_{15}$	1669.03
TL ala scan	TL8	FVAWFSKFLGRIL-NH_2	$C_{81}H_{119}N_{19}O_{14}$	1582.94
TL ala scan	TL10	FVQWFSKFLARIL-NH_2	$C_{84}H_{124}N_{20}O_{15}$	1654.02
TL ala scan	TL11	FVQWFSKFLGAIL-NH_2	$C_{80}H_{115}N_{17}O_{15}$	1554.88
Temporin A		FLPLIGRVLSGIL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1396.77
Temporin L		FVQWFSKFLGRIL-NH_2	$C_{83}H_{122}N_{20}O_{15}$	1639.99

Table 3.8 Peptides showing activity against *L. mexicana* promastigotes and axenic amastigotes.

Name	Compound number	No activity against either form of the parasite	Chemical formula	MW
Temporin-1Ca	23	FLPFLAKILTGVL-NH_2	$C_{73}H_{11}9N_{15}O_{14}$	1430.83
Temporin-1Cb	**24**	FLPLFASLIGKLL-NH_2	$C_{73}H_{119}N_{15}O_{14}$	1430.83
Temporin-1Cd	**26**	FLPFLASLLSKV-NH_2	$C_{67}H_{108}N_{14}O_{14}$	1333.67
Temporin-1P	**28**	FLPIVGKLLSGLL-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1368.76
Temporin-1CSc	**32**	FLPLVTGLLSGLL-NH_2	$C_{66}H_{112}N_{14}O_{15}$	1341.69
Temporin-1PLa	**35**	FLPLVGKILSGLI-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1368.76
Temporin-1Oc	**36**	FLPLLASLFSRLF-NH_2	$C_{77}H_{119}N_{17}O_{15}$	1522.88
Temporin-1Od	**37**	FLPLLASLFSGLF-NH_2	$C_{73}H_{110}N_{14}O_{15}$	1423.75
Temporin-1Ga	**38**	SILPTIVSFLSKVF-NH_2	$C_{76}H_{124}N_{16}O_{18}$	1549.9
Temporin-1Gb	**39**	SILPTIVSFLSKFL-NH_2	$C_{77}H_{126}N_{16}O_{18}$	1563.93
Temporin-1Gc	**40**	SILPTIVSFLTKFL-NH_2	$C_{78}H_{128}N_{16}O_{18}$	1577.96
Temporin-1Gd	**41**	FILPLIASFLSKFL-NH_2	$C_{83}H_{130}N_{16}O_{16}$	1608.03
Temporin-ALa	**42**	FLPIVGKLLSGLSGLL-NH_2	$C_{79}H_{136}N_{18}O_{18}$	1626.04
Temporin 1OLb	**47**	FLPFFASLLGKLL-NH_2	$C_{76}H_{117}N_{15}O_{14}$	1464.84
Temporin-1TGb	**51**	AVDLAKIANKVLSSLF-NH_2	$C_{78}H_{134}N_{20}O_{21}$	1688.03
Temporin-LTb	**52**	FIITGLVRGLTKLF-NH_2	$C_{77}H_{129}N_{19}O_{16}$	1576.97
Temporin-1SPa	55	FLSAITSILGKFF-NH_2	$C_{72}H_{111}N_{15}O_{16}$	1442.75

Temporin-CPb	57	FLPIVGRLISGIL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1396.77
Temporin-PRa	58	FLPILGNLLSGLL-NH_2	$C_{67}H_{113}N_{15}O_{15}$	1368.71
Temporin L Lys scan	TL14	KVQWFSKFLGRIL-NH_2	$C_{80}H_{125}N_{21}O_{15}$	1620.99
	TL19	FVQWKSKFLGRIL-NH_2	$C_{80}H_{125}N_{21}O_{15}$	1620.99
	TL20	FVQWFKKFLGRIL-NH_2	$C_{86}H_{129}N_{21}O_{14}$	1681.09
	TL21	FVQWFSGFLGRIL-NH_2	$C_{79}H_{113}N_{19}O_{15}$	1568.87
	TL22	FVQWFSKKLGRIL-NH_2	$C_{80}H_{125}N_{21}O_{15}$	1620.99
	TL23	FVQWFSKFKGRIL-NH_2	$C_{83}H_{123}N_{21}O_{15}$	1655.00
	TL24	FVQWFSKFLKRIL-NH_2	$C_{87}H_{131}N_{21}O_{15}$	1711.11
		FVQWFSKFLGKIL-NH_2	$C_{83}H_{122}N_{18}O_{15}$	1611.98
Temporin B		LLPIVGNLLKSLL-NH_2	$C_{67}H_{122}N_{16}O_{15}$	1391.79
Temporin 1Sa		FLSGIVGMLGKLF-NH_2	$C_{67}H_{109}N_{15}O_{14}S_1$	1380.75
Temporin C		LLPILGNLLNGLL-NH_2	$C_{65}H_{116}N_{16}O_{15}$	1361.72
Temporin F		FLPLIGKVLSGIL-NH_2	$C_{62}H_{113}N_{17}O_{14}$	1320.67
TA ala scan	TA1	ALPLIGRVLSGIL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA2	FAPLIGRVLSGIL-NH_2	$C_{66}H_{115}N_{17}O_{14}$	1370.73
	TA3	FLALIGRVLSGIL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA4	FLPAIGRVLSGIL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA5	FLPLAGRVLSGIL-NH_2	$C_{69}H_{119}N_{17}O_{14}$	1410.80
	TA6	FLPLIARVLSGIL-NH_2	$C_{65}H_{110}N_{14}O_{14}$	1311.66
	TA7	FLPLIGAVLSGIL-NH_2	$C_{66}H_{113}N_{17}O_{14}$	1368.72
	TA8	FLPLIGRALSGIL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA9	FLPLIGRVASGIL-NH_2	$C_{68}H_{117}N_{17}O_{13}$	1380.77
	TA10	FLPLIGRVLAGIL-NH_2	$C_{69}H_{119}N_{17}O_{14}$	1410.80
	TA11	FLPLIGRVLSAIL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA12	FLPLIGRVLSGAL-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
	TA13	FLPLIGRVLSGIA-NH_2	$C_{65}H_{111}N_{17}O_{14}$	1354.69
TL ala scan	TL1	AVQWFSKFLGRIL-NH_2	$C_{77}H_{118}N_{20}O_{15}$	1563.89
	TL4	FVQWASKFLGRIL-NH_2	$C_{77}H_{118}N_{20}O_{15}$	1563.89
	TL5	FVQWFSAFLGRIL-NH_2	$C_{80}H_{115}N_{19}O_{15}$	1582.9
	TL7	FVQWFSKFAGRIL-NH_2	$C_{80}H_{116}N_{20}O_{15}$	1597.91
	TL9	FVQWFSKFLGRAL-NH_2	$C_{80}H_{116}N_{20}O_{15}$	1597.91
	TL12	FVQWFSKFLGRIA-NH_2	$C_{80}H_{116}N_{20}O_{15}$	1597.91
Temporin-1TGa	2	FLPILGKLLSGIL-NH_2	$C_{69}H_{119}N_{15}O_{14}$	1382.78
Temporin-1Ob	4	FLPLIGKILGTIL-NH_2	$C_{70}H_{121}N_{15}O_{14}$	1396.81
Temporin 1ARa	5	FLPIVGRLISGLL-NH_2	$C_{68}H_{117}N_{17}O_{14}$	1396.77
Temporin 1Bya	6	FLPIIAKVLSGLL-NH_2	$C_{69}H_{119}N_{15}O_{14}$	1382.78
Temporin 1Ja	7	ILPLVGNLLNDLL-NH_2	$C_{66}H_{116}N_{16}O_{17}$	1405.73
Temporin 1M	8	FLPIVGKLLSGLL-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1368.76
Temporin 1Pra	9	ILPILGNLLNGLL-NH_2	$C_{65}H_{116}N_{16}O_{15}$	1361.72
Temporin 1VE	10	FLPLVGKILSGLI-NH_2	$C_{68}H_{117}N_{15}O_{14}$	1368.76
Temporin 1Va	11	FLSSIGKILGNLL-NH_2	$C_{65}H_{112}N_{16}O_{16}$	1373.69
Temporin-SHc	13	FLSHIAGFLSNLF-NH_2	$C_{72}H_{105}N_{17}O_{16}$	1464.72

Temporin-1PRb	14	ILPILGNLLNSLL-NH_2	$C_{66}H_{118}N_{16}O_{16}$	1391.75
Temporin-1DRa	15	HFLGGTLVNLAKKIL-NH_2	$C_{77}H_{131}N_{21}O_{17}$	1623.00
Temporin-1TGc	16	FLPVILPVIGKLLSGIL-NH_2	$C_{90}H_{155}N_{19}O_{18}$	1791.32
Temporin-LTa	17	FFPLVLGALGSILPKIF-NH_2	$C_{94}H_{147}N_{19}O_{18}$	1831.30
Temporin-PTa	18	FFGSVLKLIPKIL-NH_2	$C_{75}H_{124}N_{16}O_{14}$	1473.89
Temporin-LT1	19	FLPGLIAGIAKML-NH_2	$C_{65}H_{111}N_{15}O_{13}S_1$	1342.74
Temporin-PRb	21	FLPIITNLLGKLL-NH_2	$C_{72}H_{124}N_{16}O_{15}$	1453.86

Table 3.9 Peptides that displayed no antileishmanial activity against either *L. mexicana* promastigotes or amastigotes

3.3 Retesting of selected temporin peptides

Peptides resulting in ≤ 60% cell viability were tested in assays to determine LD_{50} values. The values were determined using the peptides supplied by CRB (purity assumed to be 70-90%) with the exception of temporins A (**16**) and temporin L (**36**), which were determined using RP-HPLC purified peptides prepared in house. LD_{50} values were determined through testing peptides at concentration intervals of 10 µM three times in triplicate. These results were plotted on graphs and the values contained in the Table below obtained through a line of best fit.

Peptide	Compound number	Sequence	LD_{50} promastigotes (µM)	LD_{50} axenic amastigotes (µM)
Temporin-1Cc	25	FLPFLASLLTKVL-NH_2	150	>200
Temporin-1Ce	27	FLPFLATLLSKVL-NH_2	97	>200
Temporin-GH	29	FLPLLFGAISHLL-NH_2	105	147
Temporin-CSa	30	FLPIVGKLLSGLL-NH_2	103	>200
Temporin-CSb	31	FLPIIGKLLSGLL-NH_2	62	>200
Temporin-	33	FLSAITSLLGKLL-NH_2	86	74

1SPb				
Temporin-1DYa	34	FIGPIISALASLFG-NH_2	89	92
Temporin-1AUa	43	FLPIIGQLLSGLL-NH_2	72	98
Temporin-1Ec	44	FLPVIAGLLSKLF-NH_2	63	>200
Temporin-1HKa	45	SIFPAIVSFLSKFL-NH_2	41	>200
Temporin-1OLa	46	FLPFLKSILGKIL-NH_2	82	85
Temporin-1Vb	48	FLSIIAKVLGSLF-NH_2	147	>200
Temporin-SHb	49	FLPIVTNLLSGLL-NH_2	105	>200
Temporin-1Vc	50	FLPLVTMLLGKLF-NH_2	74	>200
Temporin-LTc	53	SLSRFLSFLKIVYPPAF-NH_2	49	>200
Temporin-PTa	54	FFGSVLKLIPKIL-NH_2	39	>200
Temporin-CPa	56	IPPFIKKVLTTVF-NH_2	66	>200
Temporin-PRc	59	NFLDTLINLAKKFI-NH_2	71	57
Temporin-1CSd	1	NFLGTLVNLAKKIL-NH_2	56	>200
Temporin-1Oa	3	FLPLLASLFSRLL-NH_2	174	>200
Temporin-SHa	12	FLSGIVGMLGKLF-NH_2	167	>200
Temporin-LT2	20	FLPIALKALGSIFPKIL-NH_2	154	>200
Temporin-1CEa	22	FVDLKKIANIINSIF-NH_2	147	>200
Temporin-A		FLPLIGRVLSGIL-NH_2	9	72
Temporin-L		FVQWFSKFLGRIL-NH_2	68	67
Temporin-L Ala Scan	TL2	FAQWFSKFLGRIL-NH_2		
		FVQWFAKFLGRIL-NH_2	147	>200
	TL3	FVQWFSKALGRIL-NH_2	123	>200
		FVAWFSKFLGRIL-NH_2	122	>200
	TL6	FVQWFSKFLARIL-NH_2	74	>200
	TL8	FVQWFSKFLGAIL-NH_2	67	>200
	TL10			
	TL11			
Temporin L Lys Scan	TL26	FVQWFSKFLGRLK-NH_2	170	>200
		FVKWFSKFLGRIL-NH_2	78	165
	TL16	FVQKFSKFLGRIL-NH_2	65	>200
	TL17	FVQWFSKFLGRKL-NH_2	43	>200
		FKQWFSKFLGRIL-NH_2	62	>200
	TL25			
	TL15			

Table 3.11 LD_{50} values for all peptides showing activity at less than 200 μM.

3.5 Synthesis and purification of active hits from library testing

Following more detailed analysis of the data obtained from screening the temporin peptide libraries, the peptides shown in Tables 3.12 and 3.13 were chosen to be synthesised, and the RP-HPLC purified peptides retested under identical conditions to those used in the initial library screening. As previously stated the purity of the commercial peptides was deemed to be greater be between 70-90% however in order to take any of the potential leads identified forward pure peptides that were synthesised in house were required. This work was carried out in collaboration with Miss Kathryn Sweeney (undergraduate MSci project 2013). The temporins that were chosen for re-synthesis were based on their activity profiles against promastigotes and axenic amastigotes (shown previously in Figures 3.9 and 3.10).

Peptide	Peptide Sequence	Charge at pH 7	Mean Kyte-Doolittle hydrophobicity	pI
Temporin 10a (3)	FLPLLASLFSRLL-NH_2	+2	1.73	14
Temporin 1CEa (22)	FVDLKKIANIINSIF-NH_2	+2	0.95	10.6
Temporin SHa (12)	FLSGIVGMLGKLF-NH_2	+2	1.67	14
Temporin 1CSd (1)	NFLGTLVNLAKKIL-NH_2	+3	0.90	14

Table 3.12 Peptides from the CRB library of natural temporins selected for resynthesis.

The temporin L analogs to be re-synthesized (TL 8, TL 10 and TL 11) were selected, based on their activity against promastigotes. TL10 and TL11 also displayed activity against axenic amastigotes. TA3 was selected as although it showed low activity against

Leishmania mexicana, it was active against gram negative bacteria including *S. aureus* and the yeast *C. albicans*[17].

Peptide	PeptideSequence	Charge at pH 7	Mean Kyte-Doolittle hydrophobicity	pI
Temporin L (36)	FVQWFSKFLGRIL-NH_2	+3	0.82	14
TL8	FVQWFSKALGRIL-NH_2	+3	0.75	14
TL10	FVQWFSKFLARIL-NH_2	+3	0.99	14
TL11	FVQWFSKFLGAIL-NH_2	+2	1.31	14
Temporin A (16)	FLPLIGRVLSGIL-NH_2	+2	1.81	14
TA3	FLALIGRVLSGIL-NH_2	+2	2.07	14

Table 3.13 Peptides from the alanine scans of temporin L (**36**) and temporin A (**16**) selected for re-synthesis and further investigation.

3.6 Biological Testing of temporin L and temporin A analogs

As described in Section 3.2.2, alanine scans of temporins L and A had previously been performed and the analogs tested against *L. mexicana* axenic amastigotes and promastigotes at 200 µM. Based on these results, the analogs in Table 3.13 were chosen to be resynthesised, purified and tested at varying concentrations using serial dilutions from 200µM.

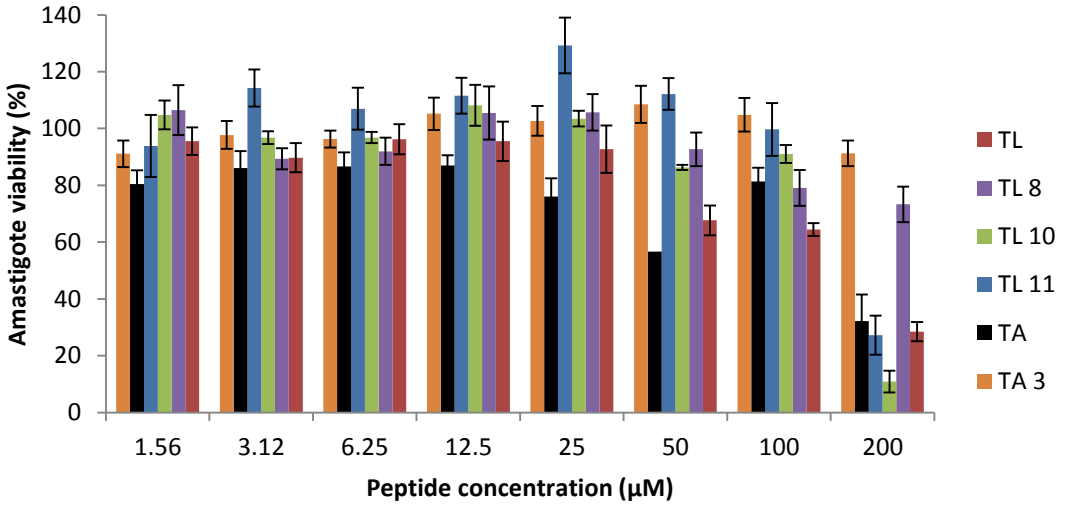
When treated with TL 8 [FVQWFSKALGRIL-NH_2], wild type promastigotes showed low viability at 200 µM and 100 µM, with an average of 5% and 15% respectively. At lower concentrations viability rapidly increased, with an average of 59% viability at 50 µM, 88% at 25 µM and 100% at lower concentrations (see Figure 3.13 (b)). When tested against axenic amastigotes, cell viability was, 73% at 200µM and even higher at lower concentrations (see Figure 3.3 (a)).

TL10 [FVQWFSKFLARIL-NH_2] resulted in promastigote viability of <10% at concentrations as low as 25µM. TL2 gave cell viability of around 10% at 200 µM when tested against axenic amastigotes but significantly higher cell viability at lower concentrations (see Figure 3.3 (a)).

Viability of promastigotes treated with TL11 [FVQWFSKFLGAIL-NH_2] was 8% at 200 µM and 43% at 100 µM and 50 µM. At concentrations lower than this there was little to no inhibition of growth, with viability being 87% at 25 µM and 100% at lower concentrations. Axenic amastigotes treated with TL11 showed 27% viability at 200 µM, and 100% at lower concentrations (see Figure 3.13 (b)).

Promastigotes and axenic amastigotes were also treated with the Temporin A analoge TA3 [FLALIGRVLSGIL-NH_2] however antileishmanial activity was low, as predicted based on

the results previously obtained (see figure 3.13). Viability of promastigotes was 71% when treated with this peptide at 200 μM and over 90% at lower concentrations. Viability of amastigotes was 91% at 200 μM and 100% at lower concentrations (Figure 3.13 (a)). The reduced activity of this peptide when compared to native temporin A may be due to a number of factors including possible alterations in the secondary structure of the peptide and in the possible removal of a residue affecting the antileishmanial properties of the peptide.



b)

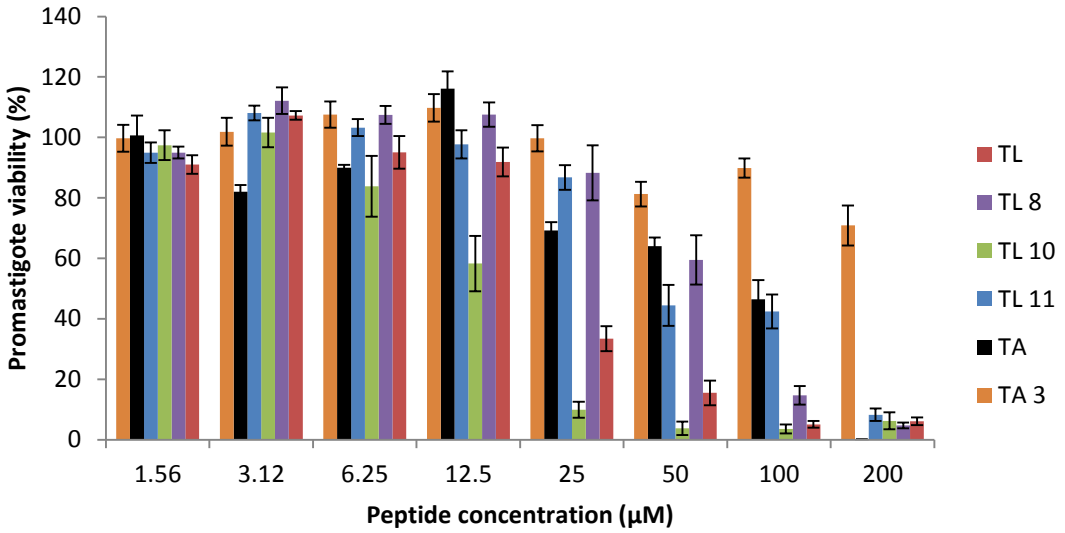


Figure 3.13 (a) axenic amastigote and (b) promastigote viability when treated with temporin L **(36)**, temporin A (**16**) and analogues TL 8, TL 10, TL 11 and TA 3. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

3.7 Testing against mutant *Leishmania mexicana*

As described previously in Chapter I, AMPs are thought to target cell membranes. Therefore, changes in the membrane composition of parasites are of interest in order to determine the effects of specific mutations on AMP activity.

Experimental work in this Section was carried out by Kathryn Sweeney (MSci 2013) and Dr Paul Denny. Peptides discussed in Sections 3.2.2 and their native equivalents were also tested against promastigote *lpg1* mutants. These parasites are unable to synthesise the glycan core of lipophosphoglycan (LPG). Given that axenic amastigotes lack LPG[25], this experimental work was carried out in order to attempt to determine the effect of the presence or absence of LPG to peptide activity.

Lpg1 mutant promastigotes treated with Temporin L (**36**) [FWQWFSKFLGRIL-NH_2] at 200µM were, on average, only 14% viable but at lower concentrations viability quickly rose to over 90% at concentrations up to 100µM, indicating temporin L (**36**) is less potent against these mutants than either the wild type promastigotes or axenic amastigotes. However, only one triplicate was successfully performed so this would have to be repeated to be confirmed (Figure 3.14).

Temporin L analogs tested all recorded efficacies against the mutants between those of the wild type promastigotes and axenic amastigotes. Mutants treated with TL8 gave around 48% viability at 200 µM. Viability at 100 µM was similar on average, increasing to 74% at 50 µM and over 90% at lower concentrations. Mutants treated with TL10 at 200 µM had 6% viability, increasing to 49% at 100 µM, 69% at 50 µM and over 80% at lower concentrations. Mutants treated with TL11 had 14% viability at 200 µM and over 90% at lower concentrations. Again, analogue TA3 showed no antileishmanial properties. The

native temporin A (**16**) was active at concentrations down to 50 µM and weak activity at 25µM (Figure 3.14).

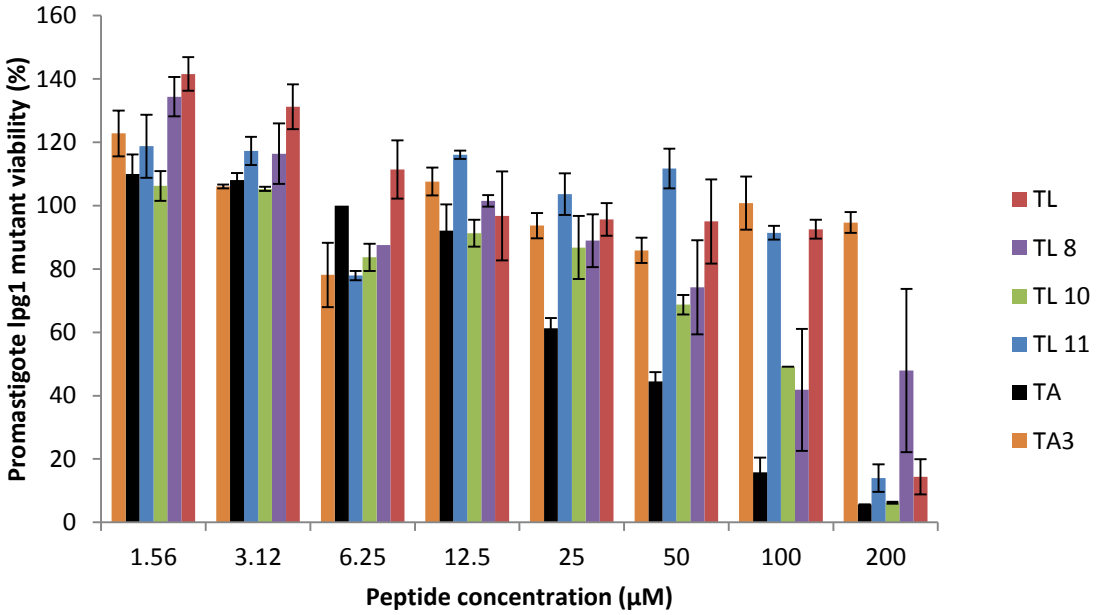


Figure 3.14 *lpg1* mutant viability when treated with **16**, **36** and analogues TL8, TL10, TL11 and TA 3. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

Based on previously discussed results, IC_{50} values for temporin L (**36**), TL8, TL10, TL11, temporin A (**16**) and TA3 were calculated for both *L. mexicana* promastigotes and axenic amastigotes. These values are shown in Table 3.14.

	TL	**TL 8**	**TL 10**	**TL 11**	**TA**	**TA 3**
Promastigote WT	24±3	55±6	12±1	70±11	60±7	>200
Axenic amastigote	>200	>200	>200	>200	191 ± 30	>200
Promastigote *lpg1* mutant	208±31	>200	69±4	>200	36±4	>200

Table 3.14 IC_{50} values for temporin A (16), temporin L (36) and analogues TL8, TL10, TL11 and TA3

The results for the Temporin L lysine scan analogs at 200 μM agree with those previously obtained (see Figures 3.7 and 3.8) and overall they show that of the 3 analogs TL10, [FVQWFSKFLARIL-NH_2], showed the greatest activity against both *L. mexicana* axenic amastigotes and promastigotes, with similar activity when compared to native Temporin L. This is unsurprising, given the only amino acid substitution is a glycine for an alanine, which is relatively minor given they have similar properties, both being small and neutral, despite their differences in hydrophobicity according to the Kyte-Doolittle model (native temporin L 0.82, TL8 0.75, TL10 0.99, TL11 1.31).

TL11, [FVQWFSKFLGAIL-NH_2], showed the least activity of all analogs tested against axenic amastigotes or promastigotes at lower concentrations. The removal of arginine leads to a peptide with a lower charge (+2 as opposed to +3) and higher hydrophobicity (69% as opposed to 62% hydrophobic residue), both of which could affect activity.

TL8, [FVQWFSKALGRIL-NH_2], also showed low levels of activity against both promastigotes and axenic amastigotes, which is perhaps surprising given the peptide has the same net charge (+3) and a very similar hydrophobicity (62% hydrophobic residue) as native Temporin L (**36**). However, the substitution of phenylalanine, a large, aromatic amino acid, for alanine, a small, aliphatic amino acid, will likely affect the integral structure of the peptide, including its ability to form helices, and hence its activity. Previously it has been shown that helicity can severely affect a peptides properties (study of temporin L analogues[18]). However, although it was shown that although helicity affected membrane permeability, it did not affect antimicrobial activity[17].

The initial alanine scan of temporin A showed that no analogues possessed antileishmanial activity against either axenic amastigotes or promastigotes. This suggests the

antileishmanial activity of native temporin A is very sensitive to even changes in its amino acid sequence.

The results obtained in this study (i.e. work carried out by K. Sweeney) for native Temporins A (**16**) and L (**36**) do not correspond exactly to previously obtained results (see Figures 2.20, 2.27 and 2.28). This discrepancy was attributed to an error in the weighing out of the peptide for the stock solution; given concentrated stocks were initially made up any small error in weighing could be significant. Temporin L (**36**) appears to be more active against promastigotes but less active against amastigotes than previously reported, whereas Temporin A (**16**) is less active against both forms of the parasite. Given the time-constrains of this study (carried out as an MSci project) there was not sufficient time to exams this further.

3. 8 Data Analysis of biological screening Part I: Analysis of known biological activity

Having screened over one hundred peptides analysis of the data to try and identify possible indicators that could be used to predict antileishmanial activity was carried out. This was done in two parts (Section 3.8 and Section 3.9). The following section explores finding possible links between known or published biological activity and that obtained in the screening against *L. mexicana*. Tables 3.16 and 3.17 provide summaries of the reported biological activity for peptides that had antileishmanial properties (promasitgotes only and both respectively). Table 3.18 lists the peptides that displayed no activity against the *L. mexicana* parasite. The net charge and the hydrophobic residue percentage for each peptide are also provided within the Tables.

At first glance it can be seen that the peptides that appear in all three tables display a range of reported biological activities and net positive charges (+1 to +4). There is no peptide that appears within the tables with reported activity against Gram-negative bacteria only. As this is a relatively small study, this does not necessarily indicate that AMPs that only display activity against Gram-negative bacteria are less likely to be antileishmanial peptides. Furthermore, the majority of AMPs isolated to date are activity against Gram-positive bacteria with far a fewer number displaying activity against both Gram-negative and Gram-positive bacteria. With no obvious pattern present and the low number of active hits identified it was not possible to draw any solid conclusions or useful information simply by looking at previously reported biological activities. In light of this a more rigorous computational analysis was undertaken (See Section 3.9).

Name	Primary Sequence	Reported Activity	Net charge	Hydrophobic residue %	Ref
Temporin-1Cc	FLPFLASLLTKVL-NH_2	Gram +ve	+2	69%	[26]
Temporin-1Ce	FLPFLATLLSKVL-NH_2	Gram +ve	+2	69%	[26]
Temporin-1CSa	FLPIVGKLLSGLL-NH_2	Gram +ve fungi mammalian cells	+2	61%	[27]
Temporin-1CSb	FLPIIGKLLSGLL-NH_2	Gram +ve	+2	61%	[28]
Temporin 1AUa	FLPIIGQLLSGLL-NH_2	Gram +ve	+1	61%	[29]
Temporin 1Ec	FLPVIAGLLSKLF-NH_2	Gram +ve	+2	69%	[30]
Temporin 1HKa	SIFPAIVSFLSKFL-NH_2	None reported	+2	64%	[31]
Temporin 1Vb	FLSIIAKVLGSLF-NH_2	Gram +ve	+2	69%	[31]
Temporin-SHb	FLPIVTNLLSGLL-NH_2	Gram +ve	+1	61%	[32]
Temporin-1Vc	FLPLVTMLLGKLF-NH_2	Gram +ve	+2	69%	[26]
Temporin-LTc	SLSRFLSFLKIVYPPAF-NH_2	Gram +ve Gram - ve HIV	+3	52%	[33]
Temporin-PTa	FFGSVLKLIPKIL-NH_2	Gram +ve Gram -ve HIV	+3	61%	[34]
Temporin-CPa	IPPFIKKVLTTVF-NH_2	Gram + ve Gram -ve, fungi	+3	53%	[35]
Temporin L Lys scan	FVKWFSKFLGRIL-NH_2	None reported	+3	62%	n/a
	FVQKFSKFLGRIL-NH_2	None reported	+3	54%	n/a
	FVQWFSKFLGRKL-NH_2	None reported	+3	54%	n/a
	FVQWFSKFLGRLK-NH_2	None reported	+3	54%	n/a
TL ala scan	FAQWFSKFLGRIL-NH_2	None reported	+2	62%	n/a
	FVQWFAKFLGRIL-NH_2	None reported	+2	69%	n/a
	FVQWFSKALGRIL-NH_2	None reported	+2	62%	n/a
Temporin-1CSd	NFLGTLVNLAKKIL-NH_2	Gram +ve Gram -ve fungi	+3	57%	[28]
Temporin-1Oa	FLPLLASLFSRLL-NH_2	Gram +ve Gram -ve	+2	69 %	[36]
Temporin-SHa	FLSGIVGMLGKLF-NH_2	Gram +ve Gram -ve fungi parasites	+2	61%	[32]
Temporin-LT2	FLPIALKALGSIFPKIL-NH_2	Gram +ve Gram - ve	+3	64%	[37]
Temporin-1CEa	FVDLKKIANIINSIF-NH_2	Gram +ve, cancer cells	+3	52%	[38]

Table 3.16 Summary of previously reported biological activity for peptides that displayed activity against *L. mexicana* promastigotes only.

Name	Primary Sequence	Reported Activity	Net charge	Hydrophobic residue %	Ref
Temporin-GH	FLPLLFGAISHLL-NH_2	Gram +ve	+1	69%	[39]
Temporin-1SPb	FLSAITSLLGKLL-NH_2	Gram +ve	+2	61%	[33]
Temporin-1DYa	FIGPIISALASLFG-NH_2	Gram +ve Gram -ve	+1	64%	[40]
Temporin-1OLa	FLPFLKSILGKIL-NH_2	None reported	+3	61%	n/a
Temporin-PRc	NFLDTLINLAKKFI-NH_2	Gram +ve	+2	57%	[41]
Temporin L Lys scan	FKQWFSKFLGRIL-NH_2	None reported	+3	54%	[42]
TL ala scan	FVAWFSKFLGRIL-NH_2	None reported	+2	69%	
TL ala scan	FVQWFSKFLARIL-NH_2	None reported	+2	69%	
TL ala scan	FVQWFSKFLGAIL-NH_2	None reported	+1	69%	
Temporin A	FLPLIGRVLSGIL-NH_2	Gram +ve Gram -ve HIV Parasites Chemotactic	+2	61%	
Temporin L	FVQWFSKFLGRIL-NH_2	Gram +ve Gram -ve Fungi Mammalian cells Cancer cells	+3	61%	

Table 3.17 Summary of previously reported biological activity for peptides that displayed activity against *L. mexicana* promastigotes and axenic amastigotes.

Name	Primary Sequence	Reported Activity	Net charge	Hydrophobic residue %	Ref
Temporin-1Spa	FLSAITSILGKFF-NH_2	None reported	+2	61%	[39]
Temporin-LTa	FFPLVLGALGSILPKIF-NH_2	Gram +ve Mammalian cells	+2	64%	[33]
Temporin-1TGc	FLPVILPVIGKLLSGIL-NH_2	Gram +ve Mammalian cells	+2	64%	[40]
Temporin-1DRa	HFLGTLVNLAKKIL-NH_2	Gram +ve Gram -ve Fungi Mammalian cells	+4	57%	[43]
Temporin-1VE	FLPLVGKILSGLI-NH_2	Gram +ve Gram -ve	+2	61%	[41]
Temporin-1PRb	ILPILGNLLNSLL-NH_2	Gram +ve Gram -ve Fungi	+1	61%	[42]

Table 3.18 Summary of previously reported biological activity for peptides that displayed no activity against *L. mexicana* promastigotes or axenic amastigotes.

3.9 Data analysis from biological screening Part II: Computational studies and development of predictive tools

The full data set that arose from the peptide screening was supplied to Dr Marc Torrent at the MRC, Cambridge. Dr Torrent has experience in the development of predictive tools and computational models that can be utilised to enhance the antibacterial properties of AMPs. A full copy of the report produced by Dr Torrent is provided in the Appendices and his initial conclusions are summarised below.

- The main overall conclusion is that a machine-learning approach has the potential to classify between active and inactive peptides.
- From the data we have at this stage we can conclude that the specificity is in general not good enough to accurately predict active peptide sequences.
- We have a high false positive rate, which decreases the specificity / accuracy of the predication. The reason for a high false positive rate is that we have a small number of active peptides compared with inactive peptides.
- We need to design several more peptides with high a probability of being active and screen them. The new peptides will provide us with more data to refine the model and, hopefully, complete our goal to design an algorithm able to predict antileishmanial compounds.

The key point was that additional experimental data that could be used to train the model further and enhance the reliability of the predications was required. The computational analysis also showed that there were significant differences between active and inactive peptides in terms of bulkiness, ß-sheet propensity and turn propensity. Peptides that could be used to probe these areas would be extremely useful in refining and improving the model. The peptides designed to probe bulkiness, ß-sheet propensity and turn propensity and that would have been synthesised had time permitted are outlined in the following section.

3.9.1 Refining the model via additional library screening (future work)

It is predicted that substitution of each residue in Aurein 2.1 (GLLDIVKKVVGAFGSL-NH_2) for Ile will give us information on bulkiness and ß-sheet propensity on a peptide that has been found to be active (at 200 μM) and relatively robust in the computational modelling. The information obtained could also help to dissect the importance of these two descriptors. An Ile scan of Aurein 2.1 would require the preparation of the 15 peptides shown in Table 3.19.

Peptide primary sequence (GLLDIVKKVVGAFGSL-NH_2)
ILLDIVKKVVGAFGSL-**NH_2**
G**I**LDIVKKVVGAFGSL-**NH_2**
GL**I**DIVKKVVGAFGSL-**NH_2**
GLL**I**VKKVVGAFGSL-**NH_2**
GLLD**I**KKVVGAFGSL-**NH_2**
GLLD**VI**VVGAFGSL-**NH_2**
GLLDIVK**I**VVGAFGSL-**NH_2**
GLLDIVKK**I**VGAFGSL-**NH_2**
GLLDIVKKV**I**GAFGSL-**NH_2**
GLLDIVKKVV**I**AFGSL-**NH_2**
GLLDIVKKVVG**I**FGSL-**NH_2**
GLLDIVKKVVGA**I**GSL-**NH_2**
GLLDIVKKVVGAF**I**SL-**NH_2**
GLLDIVKKVVGAFG**I**L-**NH_2**
GLLDIVKKVVGAFGS**I**-**NH_2**

Table 3.19 Isoleucine (**I**) scan of Aurein 2.1

Temporin A (**16**)(FLPLIGRVLSGIL-NH_2) which was found to be one of the most active peptides was highlighted for further investigation. The core of **16** is very sensitive to changes in terms of turn and ß-sheet propensity. As such variants of **16** with modifications in the core region would provide an insight on structural changes when i) a positive charge is added, ii) bulkiness is changed and iii) the structure is disrupted. Substitution of the Gly (G) residue in position 6 of temporin A by: K, R, L, I, P, W (6 peptides) would be carried out. Substitution of the Val (V) residue at position 8 in temporin A, by K, R, L, I, G, P, W (7 peptides) would also be carried out. As the original peptide (**16**) was active it is

predicted that a number of the new peptides (Table 3.20 and Table 3.21) may also be active, and this would help to improve model training by providing more active "hits".

Peptide primary sequence (FLPLI**G**RVLSGIL-NH_2)
FLPLI**K**RVLSGIL-**NH_2**
FLPLI**R**RVLSGIL-**NH_2**
FLPLI**L**RVLSGIL-**NH_2**
FLPLI**I**RVLSGIL-**NH_2**
FLPLI**P**RVLSGIL-**NH_2**
FLPLI**W**RVLSGIL-**NH_2**

Table 3.20 Substitution of the Gly (G) residue in position 6 of temporin A by: K, R, L, I, G, P, W

Peptide primary sequence (FLPLIGR**V**LSGIL-NH_2)
FLPLIKR**K**LSGIL-**NH_2**
FLPLIRR**R**LSGIL-**NH_2**
FLPLILR**L**LSGIL-**NH_2**
FLPLIIR**I**LSGIL-**NH_2**
FLPLIGR**G**LSGIL-**NH_2**
FLPLIPR**P**LSGIL-**NH_2**
FLPLIWR**W**LSGIL-**NH_2**

Table 3.21 Substitution of the Val (V) residue at position 8 in temporin A, by K, R, L, I, G, P, W

The peptides sequences below are all highly ranked (in terms of predicted activity) according to the computational models created and as such they would provide a good test of predictive power of the model at the current stage.

Peptide primary sequence
FLPLIKRKLSGIL-$\mathbf{NH_2}$
FLPLIRRRLSGIL-$\mathbf{NH_2}$
FLPLILRLLSGIL-$\mathbf{NH_2}$
FLPLIIRILSGIL-$\mathbf{NH_2}$
FLPLIGRGLSGIL-$\mathbf{NH_2}$
FLPLIPRPLSGIL-$\mathbf{NH_2}$
FLPLIWRWLSGIL-$\mathbf{NH_2}$

Table 3.22 Peptides designed to test the predictive power of the current model.

3.10 Conclusions

As the data in Figures 3.2 and 3.3 highlights, none of the peptides tested (Table 3.1, TA1-TA3) were found to show activity below the threshold against either promastigotes or axenic amastigotes. These results indicate that there are no residues within the natural temporin A (**16**) sequence that are suitable for further modification to enhance antileishmanials activity.

Lysine and alanine scans of temporin L (**36**) indicate that the nature of positively charged residues in the sequence of temporin L affect the antileishmanial activity of the peptide. Removal of a charged residue from temporin L (a peptide with reported antibacterial and antileishmanial activity) does appear to reduce antileishmanial activity. However, in this study there was not sufficient time to determine if this also affects the haemolytic activity; as a relatively high haemolytic activity of natural temporin L (**36**) currently prevents this peptide from being developed as a therapeutic agent.

The libraries of peptides synthesised commercially by Cambridge Research Biochemicals Ltd (CRB) were supplied with a suspected purity of between 70-90% (10% were analysed by HPLC and MS). Several peptides that showed activity were resynthesised in house at Durham. The biological activity obtained using the 'pure peptides' matched with a 10% error that obtained initially in the library screen. Temporin L (**36**) was also synthesised by CRB, in order to provide another measure of the purity of the Peparray^{TM} peptides produced. The activity of the peparray^{TM} temporin L (**36**) compared well to previous results obtained using a temporin L peptide that had been prepared and purified in Durham.

Computational analysis conducted by Dr Marc Torrent (Cambridge University) did not identify any patterns of activity when applying the algorithms applied as predictors of antibacterial activity. Conclusions reached through this analysis were that the parameters

applied to analyse peptide sequences for antibacterial activity are not suitable to be used in antiparasitic (antileishmanial) models of predictive activity. As this is the first study to use computational analysis to attempt to predict antileishmanial activity, a larger data set is required, as there were relatively few active peptides identified through this study, and as indicated in Tables 3.15 and 3.16, there appears to be no correlation between the physical parameters used in the algorithms and antileishmanial activity (or lack thereof). In conclusion, the data obtained through this library screening approach has not identified any sequences predictive of activity against *Leishmania mexicana*. However, as detailed in Section 3.9, the work carried out in our study has generated the largest data set in this field, and this will enable further studies in this area to be carried out. The ultimate aim is that the work carried out will lead to the first prediction systems being developed that can be used to rationally design peptides with anti-parasitic activities.

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Chapter VI

Temporin peptides as a means of reducing current drug toxicity

Chapter IV: Temporin peptides as a means of reducing current drug toxicity

4.1 Introduction and Aims

The main aim of the work present in this chapter was to investigate the ability of the temporin peptides to penetrate the host cell membrane (macrophage) and reach intracellular *Leishmania* amastigotes. In addition to probing the ability of these AMPs to act directly against the intracellular form of *L. mexicana*, any specific targeting properties of the temporins would be analysed. It was envisaged that if the temporins could enter *Leishmania* infected macrophages then perhaps they may be important delivery vehicles that could be used to increase the efficacy and reduce the toxicity of current treatments. Table 4.1 Summaries the AMPs that have previously been screened against the amastigote form of various *Leishmania sp*. Table 4.1 shows antimicrobial peptides reported as being tested against the amastigote forms of the parasite.

Peptide	Source	Sequence	Activity (% grow promastigotes	axenic amastigotes	Ref
Temporin A	amphibia	FLPLIGRVLSGIL-NH_2	*L. donovani* (50, 8.4)	*L. pifanoi* (50, 14.6)	[1]
Temporin B	amphibia	LLPIVGNLLKSLL-NH_2	*L. donovani* (50, 8.6)	*L. Pifanoi* (50, 7.1)	[1]
Temporin 1Sa	amphibia	FLSGIVGMLGKLF-NH_2	*L. donovani* (50, 18.1)	L. infantium (50, 22.8)	[2]
Bombin H2	amphibia	IIGPVLGLVGSALGGLL-NH_2	*L. donovani* (50, 7.3)	*L. pifanoi* (50, 11)	[3]
Bombanin H4	amphibia	[a]L*i*GPVLGLVGSALGGLLKKI-NH_2	*L. donovani* (50, 1.7)	*L. pifanoi* (50, 5.6)	[3]
Skin polypeptide YY	mammal	YPPKPESPGEDASPEEMNKYLTALRHY INLVTRQRY-NH_2	*L. major* (100, 5.9)	*L. major*[d] (100, 6.2)	[4]
Histatin-5	mammal	DSHAKRHHGYKRKFHEKHHSHRGY	*L. donovani* (50, 7.3)	*L. pifanoi* (50, 14.4)	[5]

[a] *i*, stands for D-allo-isoleucine, [b]Z, stands for pyroglutamic acid
[c] single line indicates disulfide bridge between atoms
[d] testing was carried out using *ex vivo* amastigotes not axenic
Table 4.1 Antimicrobial peptides with activity against *Leishmania* spp.[6]

4.2 Synthesis of Fluorescently labelled peptides

4.2.1 Selection of temporin peptides for investigation

Despite showing moderate activity against *L. mexicana* axenic amastigotes, temporin A (**16**) and temporin L (**36**) demonstrated no measurable efficacy against intra-macrophage *L. mexicana* parasites (see Chapter II, Figure 2.19). The next step would be to investigate whether this lack of efficacy against infected macrophages was due to problems penetrating the host cell and reaching the target amastigotes. However, it was decided that Temporin L would not investigated in this manner due to its generic toxicity, including against host cells. Temporins A (**16**) and B (**17**) demonstrated efficacy against *L. mexicana* promastigotes (63% inhibition at 12.5 µM and 38% at 50 µM respectively). However, the amastigote forms of the parasite remained largely resistant to these peptides with only temporin A (**16**) demonstrating any significant activity (23% inhibition at 12.5 µM but 98% at 100 µM). Temporin B was selected as it had been demonstrated that the peptide was not active against Leishmania Mexicana at the concentrations tested, and also that it was not toxic when tested against mammalian cells (see Chapter II).

4.2.1 Selection of Fluorescent dyes

In order to investigate the activity of the temporins against intracellular *L. mexicana* parasites, it was decided to investigate how they interact with both the un-infected murine macrophages and axenic amastigote infected murine

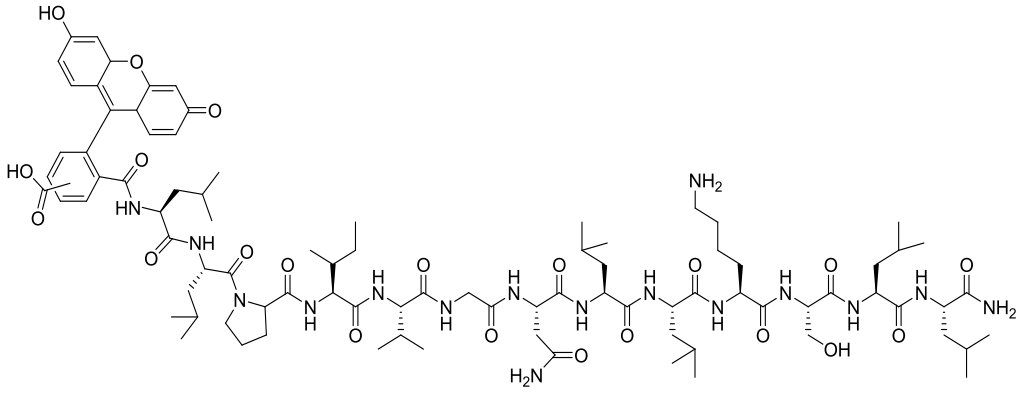
macrophages. To examine the mode of action (or toxicity to the host cell) we chose to synthesise a fluorescent analogue of two temporin peptides (temporin A and temporin B) so that fluorescence microscopy could be used to look at various aspects such as the cell penetration.

FAM was selected as one of the fluorescent tag as it is a cost effective peptide labelling dye. The 5-, 6- isomeric mixture of FAM was determined to be adequate for the initial synthesis as it was not clear whether labelling the peptide would be possible. For initial biological screening a single isomer of the dye was not deemed necessary as both the 5- and 6-isomers have identical fluorescent properties (460 nm) Importantly, FAM is membrane impermeable, implying that any cellular localization of FAM within the biological screening is due to membrane permeating properties of the attached peptide. FAM is particularly valuable to the biological screening of temporin B within infected and non-infected macrophage as it is a highly polar, water soluble dye that is frequently used to track liposomal and cellular interactions. In the context of this biological screen of the FAM tagged peptides would allow the movement and localization of the peptides within the macrophage to be probed. Fluorescein is a more pH-sensitive dye than tetramethylrhodamine. For optimum results, with Fluorescein the pH should be between 6 and 10. Fluorescence drops very sharply at pH values below 5.5, and it was speculated that this may cause a potential problem, given the acidic environment in the macrophage vacuoles where the parasite appears to reside (pH 5.5). It was for this reason that Rhodamine was also selected to be used in the tagging of the peptides.

4.2.2 Synthesis of Fluorescently labelled temporin B

The first step in the preparation of a fluorescently tagged temporin B required the synthesis of the un-tagged peptide. The synthesis of temporin B proceeded without incident on Rink Amide AM resin (0.31 mmol) using microwave Fmoc-SPPS. The successful synthesis of the peptide was verified with MALDI-TOF mass spectrometry. A third of the synthesised peptide (0.10 mmol) was then removed from the reaction vessel. The addition of the fluorescent tag, 5-6-Carboxyfluorescein (FAM) was achieved in the same manner as an amino acid coupling. First the *N*-terminal Fmoc was removed and the tagging reaction with FAM was carried out using a coupling agent (PyBOP) and a base (*N*-methylmorpholine). It is worth noting that the addition of FAM was carried out manual at rt due to the novel nature of the reaction and the potential sensitivity towards heating of the FAM group. The fluorescently peptide was then cleaved from the resin using standard reaction conditions. The crude fluorescently tagged peptide, FAM-TB (**37**) was then characterised by MALDI-TOF mass spectrometry. The MALDI-TOF mass spectrum obtained indicated that the coupling of FAM to temporin B (**17**) did not go to completion. The desired product, FAM-LLPIVGNLLKSLL-NH_2 (**37**) was detected as a minor peak with the major peak corresponding to unreacted temporin B (LLPIVGNLLKSLL-NH_2.) However, as MALDI-TOF analysis is largely dependent upon the peptide dissolving fully it was speculated that the differences in peak strength observed may simply be a result of the lower solubility of FAM-TB in the analysis solution (MeCN/ H_2O) compared to temporin B (**17**). This would correlate with the observation that often weaker MALDI-TOF signals for Fmoc protected peptides

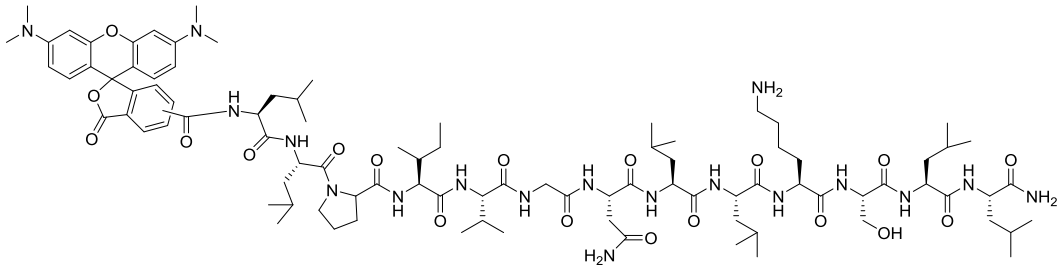
are obtained versus the free peptide. Given that both the Fmoc group and FAM are large aromatic systems as it is possible that the additional hydrophobicity of the FAM group prevented effective suspension of the peptide in the solvent required for MALDI-TOF analysis. The decision was therefore made to proceed with RP-HPLC purification of the crude reaction mixture. After purification MALDI-TOF analysis showed that a small amount of un-tagged temporin B (**17**) was still present in the sample. Given the sensitive nature of the FAM-TB (**37**) peptide it was deemed to be more appropriate to continue with biological screening rather than repeat the RP-HPLC purification and risk degrading the fluorescently tagged peptide.



Temporin B-fluorescein (**37**)

Temporin B, primary structure LLPIVGNLLKSLL-NH_2 was synthesised according to microwave coupling procedure illustrated in Scheme 2.2, starting with 500 mg (0.31 mmol) of rink amide resin. During the synthesis, the structure of the growing peptide chain was confirmed at approximately five amino acid intervals by use of MALDI-TOF mass spectrometry. 5,6-Carboxy-tetramethylrhodamine was coupled onto the resin bound peptide using 0.1 mmol

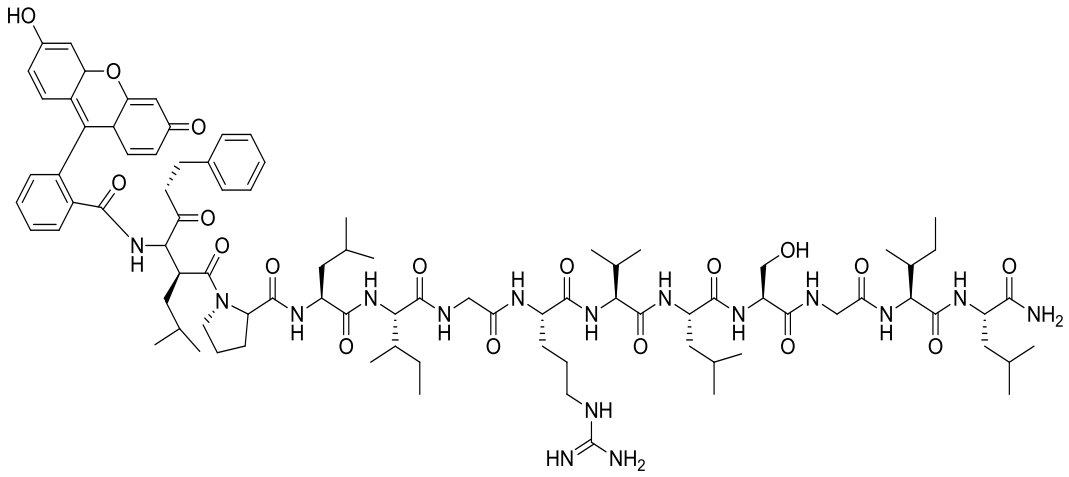
of 5,6-Carboxy-tetramethylrhodamine (TMR). The ratio of 1:3 was used as 5-Carboxy-tetramethylrhodamine is considerably more expensive than the amino acids used to form the growing peptide chain (a 1:5 ration used). When all amino acids were coupled, a final Fmoc deprotection step was carried out, and the peptide cleaved from resin by use of TFA, TIPS and H_2O using microwave chemistry. On confirmation of the presence of the correct structure, temporin A coupled to 5,6-Carboxy-tetramethylrhodamine was purified by use of HPLC and dried by lyophilisation. The purity of TMR-TB (**38**) was confirmed by use of analytical HPLC, and the compound subsequently used in biological testing.



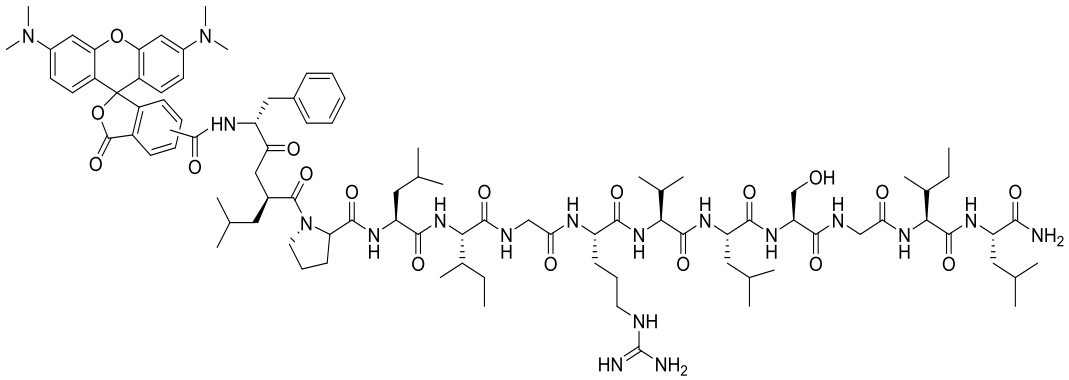
Temporin B-tetramethylrhodamine (**38**)

4.2.1 Synthesis of fluorescently labelled temporin A

Methods used for the synthesis of fluorescently labelled temporin A were as for the synthesis of fluorescently tagged temporin B. Full procedures are detailed in Chapter VII.



Temporin A-Fluorescein (**39**)

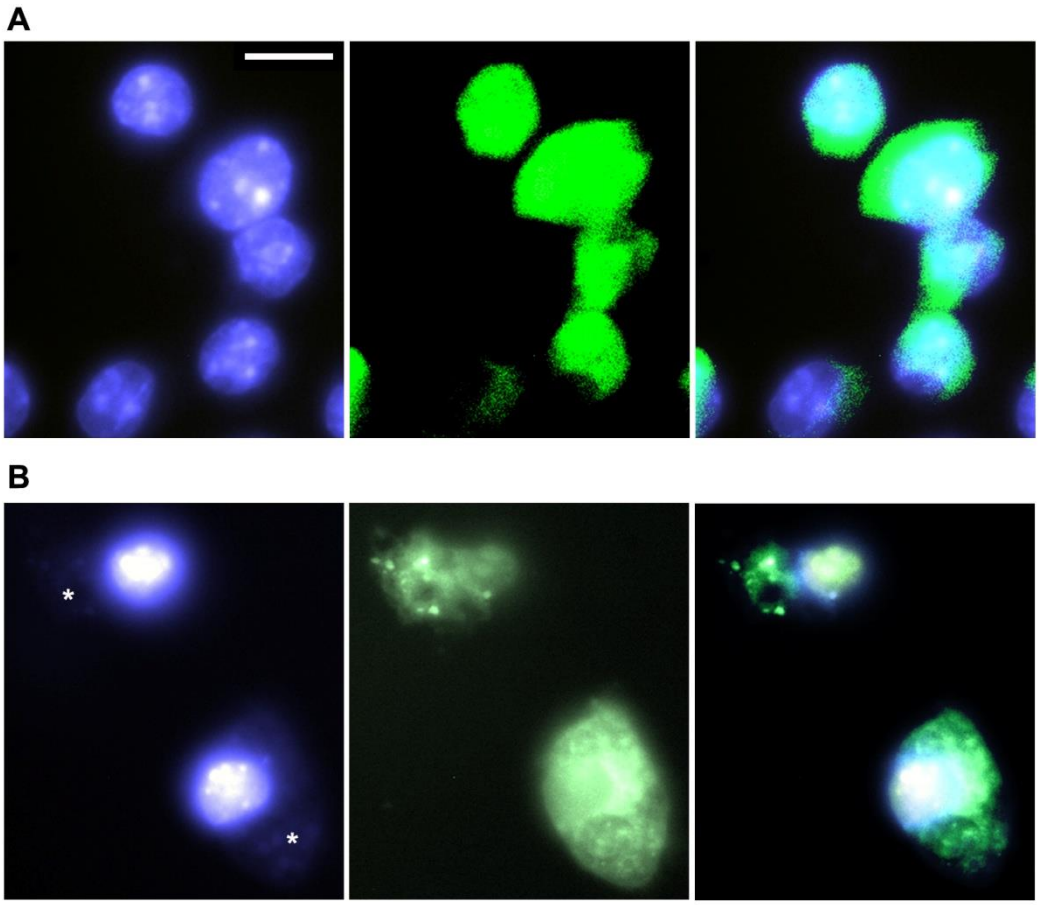


Temporin A-Tetramethylrhodamine (**40**)

4.3 Investigating the cell penetration and the targeting effects of the temporins

Control experiments showed that as expected fluorescein alone did not penetrate the host macrophage (data not shown). However, when conjugated to either

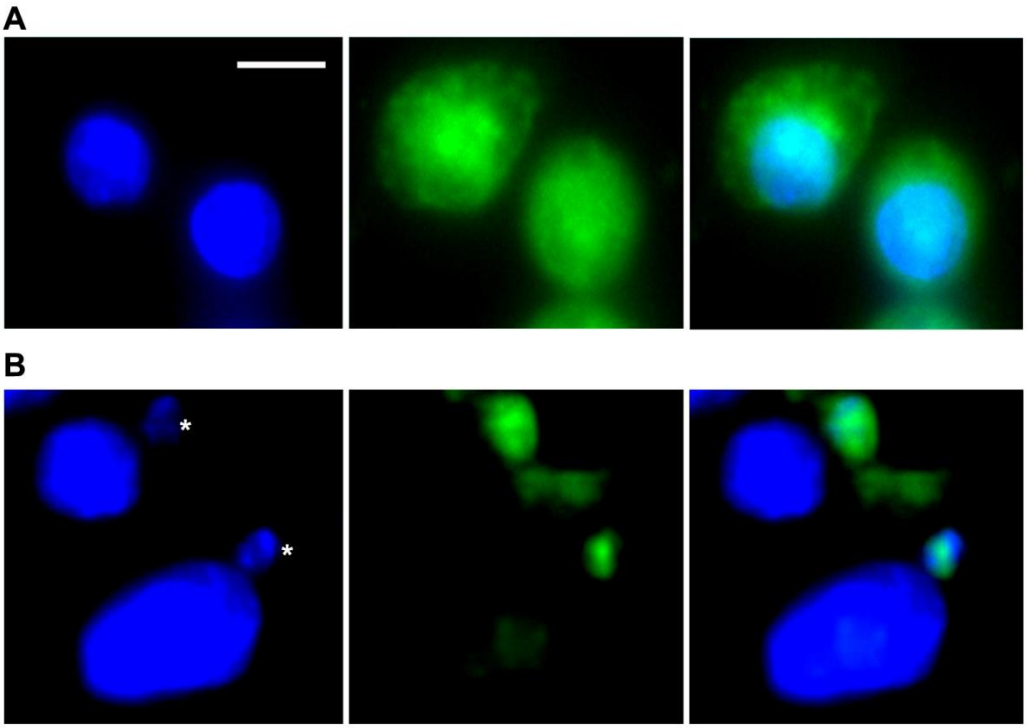
temporin A (FAB-TA, **40**) or B (FAB-TB, **37**) fluorescein was taken into uninfected macrophages when incubated with the cells in serum-free conditions (Figure 4.1 and 4.2). Notably this effect was temperature dependent with the uptake of the labelled peptides not being evident when incubated with the cells on ice (data not shown). In contrast to the diffuse staining observed in the uninfected macrophages, FAB-TB (**37**) clearly led to the accumulation of the fluorophore in the macrophage vacuole occupied by the amastigote parasites (Figure 4.2). A similar, although less distinct, effect was observed with FAB-TA (**39**) (Figure 4.1).



Panels from left: Blue, DAPI staining of DNA; Green, FAB-TA (**39**) localisation; Overlay of first two. Scale bar indicates 10 µm.

A. Uninfected RAW264.7 macrophage-like host cells. Nuclei stained blue. FAB-TA (**39**) conjugate demonstrated diffuse localisation.
B. *L. mexicana* infected RAW264.7 cells. Parasite nuclei and mitochondrial genomes (kinetoplasts) stained blue and indicated with *. FAB-TA (**39**) conjugate localised in a punctate pattern including the region occupied by parasites.

Figure 4.1 Experiments carried out using FAB-TA (**39**)



Panels from left: Blue, DAPI staining of DNA; Green, FAB-TB (**37**) localisation; Overlay of first two. Scale bar indicates 10 µm.

A. Uninfected RAW264.7 macrophage-like host cells. Nuclei stained blue. FAB-TB (**37**) demonstrated diffuse localisation.
B. *L. mexicana* infected RAW264.7 cells. Parasite nuclei and mitochondrial genomes (kinetoplasts) stained blue and indicated with*. FAB-TB (**37**) localised exclusively to the region occupied by parasites.

Figure 4.2 Experiments carried out using FAB-TB (**37**)

As data without confocal support does not confirm findings, this data suggests that the temporin-fluorophores conjugates (**37** and **39**) penetrate the host macrophage cell, and were also able to reach the phagolysosome in which the *leishmania* parasites reside. Whilst this may not lead to efficacy of the peptides studied here it indicated that temporins have the capacity and, furthermore, these short peptides offer the potential to target toxic, antiprotozoal compounds directly to their intended target. If develop further peptide such as temporin B may be useful delivery agents and help to reduce the undesirable cytotoxicity of current or

future antileishmanials. However, although a highly attractive prospect further experiments are required. Results obtained are not conclusive as to whether the fluorophore was taken into the lumen of the amastigote-containing phagolysosomes. Given the acidic nature of this compartment[7] the acid-labile fluorescein[8] would probably be significantly quenched. To aid in the investigation of this process the temporin conjugates were resynthesized with the acid stable fluorophore, tetramethylrhodamine[8] (to give TMR-TB (**38**) and TMR-TA (**40**)). This was important, as the rhodamine tags on the peptides would not be affected by the acidic environment in which the peptides concentrate. Both of the tetramethylrhodamine tagged temporin A and B conjugates also facilitated diffusion of the fluorophore into uninfected macropahges (data not shown as this experiment was performed once due to time constraints and difficulties working with the infected macrophages) and, although time constraints prevented the analyses of infected cells, these remain key tools for future studies.

4.4 The potential of temporins to penetrate the epidermis

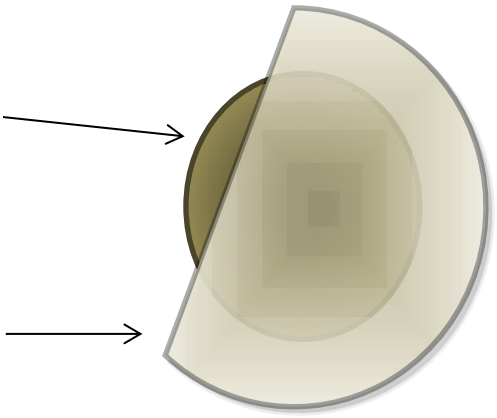
In considering the potential of temporins in the treatment of CL, either as direct treatments or as vehicles for other antileishmanials, it is important to investigate the permeability of skin to these agents. Microskin® is a sphere consisting of a central sphere of human dermal cells, surrounded by an outer sphere of epidermal cells (Figure 4.3). When grown together the two cell types produce proteins characteristic of the *in vivo* skin barrier. This testing was carried out in

conjunction with Mr Craig Manning, Department of Biological and Biomedical Sciences, Durham University.

Figure 4.3 Microskin® dermal model

Dermal sphere: cultured human Dermal cells form a central sphere

Epidermal shell: epidermal cells are supported by the dermal sphere



4 4.5 Results from intracellular testing

The images below were obtained following 18 Hr 37^{o}C treatment with FAB-TA (**39**) and TMR-TA (**40**) at 100μM. The Microskin® used was subsequently washed in excess PBS three times then imaged immediately.

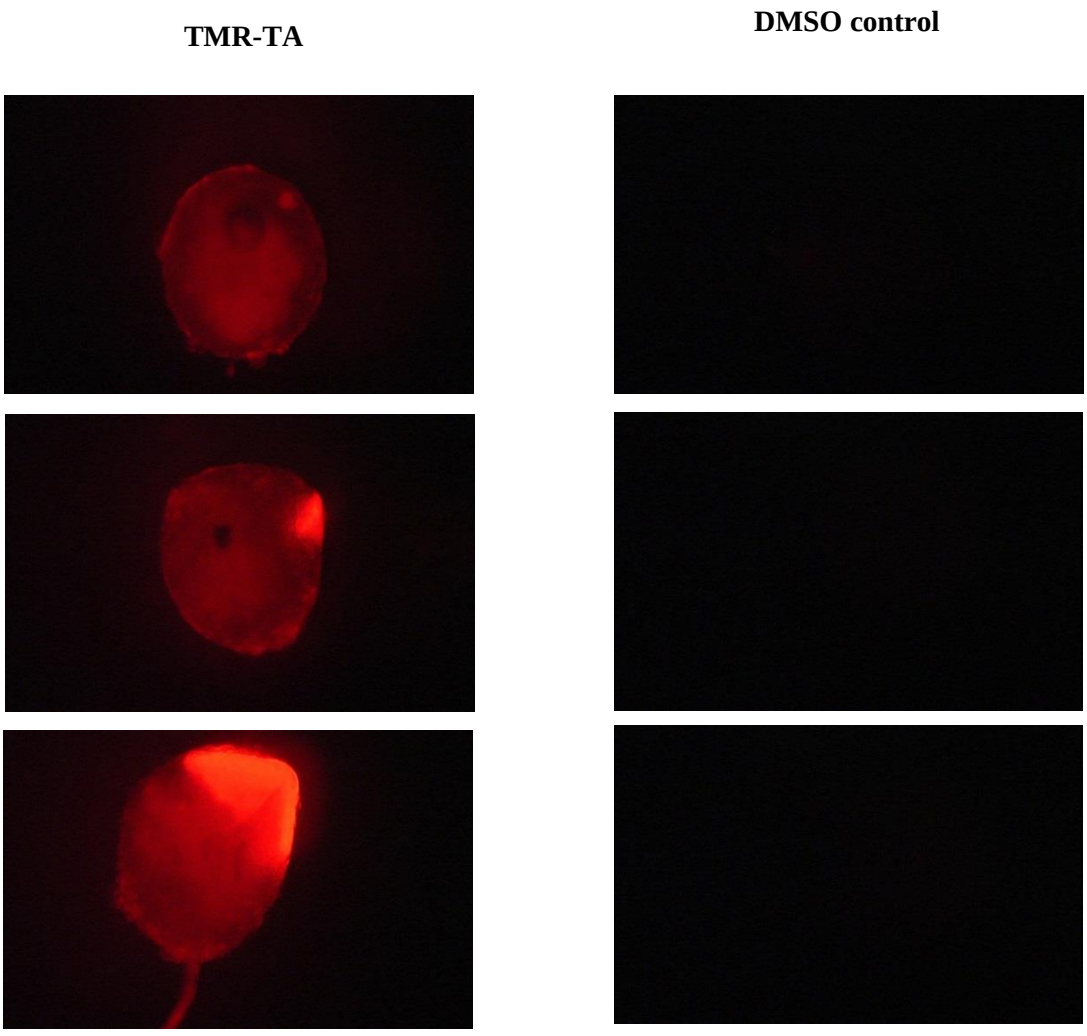


Figure 4.8 Microskin® treated with TMR-TA 100μM (left) and DMSO control (right). Top: 1hr peptide treatment (Immediate image) Middle: 1hr peptide treatment (18hrs incubation image) Bottom: 18hr peptide treatment (Immediate image).

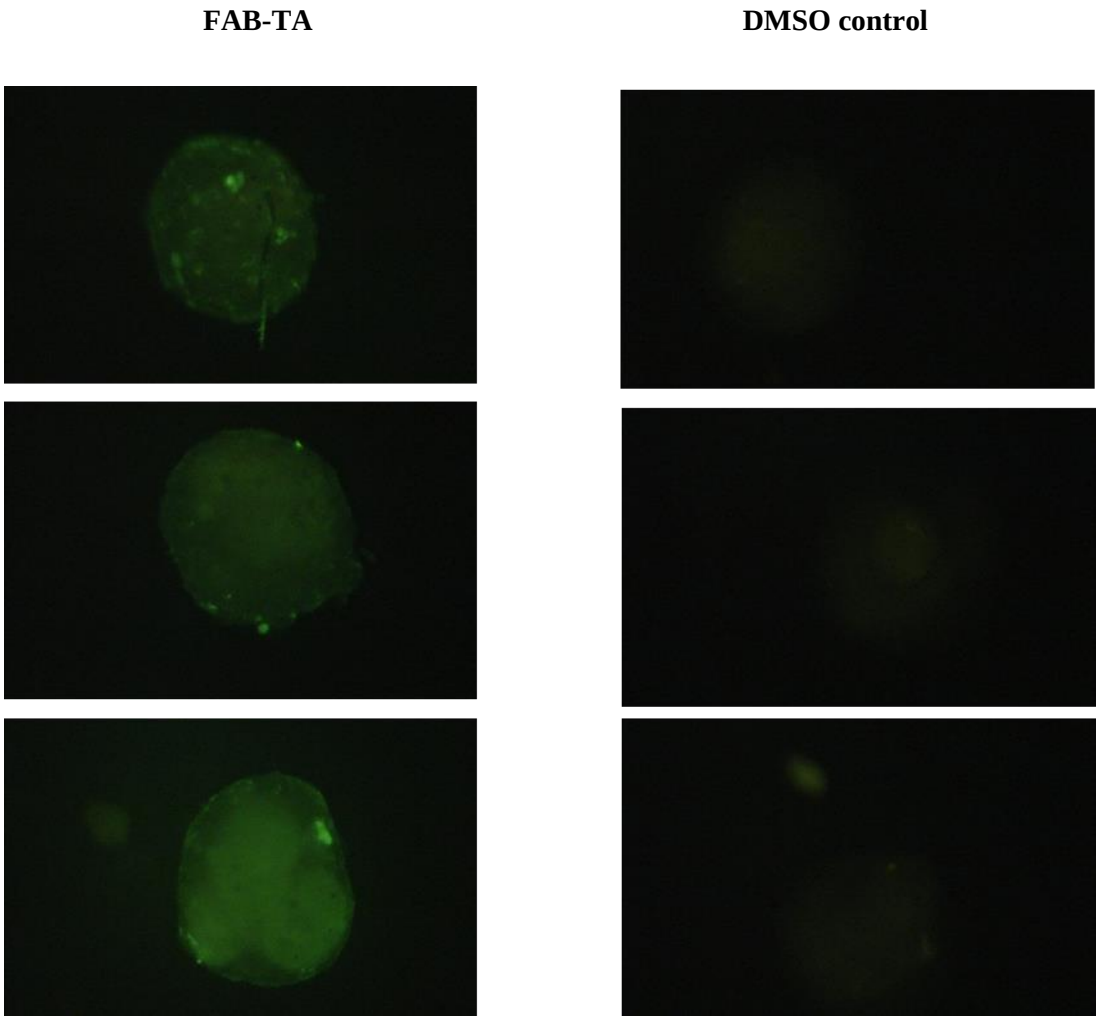


Figure 4.8 Microskin® treated with TA-TMR 100μM (left) and DMSO control (right). Top: 1hr peptide treatment (Immediate image) Middle: 1hr peptide treatment (18hrs incubation image) Bottom: 18hr peptide treatment (Immediate image).

It was observed that the spheres appeared to be alive (the spheres were intact, and have been shown to fall apart if not alive and healthy) 18hrs after a 1 hr peptide treatment. Therefore this shows that 100 μM peptide is not immediately fatal to the cells in the Microskin®. Peptides can penetrate the skin, and fluorescence is seen after 1 hr incubation for both flouroscein and tetramethylrhodamine labelled peptides. (A small amount of auto-flourecence were observed, however this is

clearly distinguishable from peptide treated samples). Therefore, longer treatment may result in deep dermal cell penetration. The fluorescence is gone 18hrs after the spheres are washed (1hr treatment). This could indicate that the flourophore has degraded however it is likely that the peptide remains in the Microskin®. An alternate explanation for this is that both the peptide and flourophore are degraded by cells in the Microskin®.

4.6 Conclusions

Temporins A and temporin B were both successfully synthesised and labelled with the dyes fluorescein (FAB) and tetramethylrhodamine (TMR). The labelled peptides (**37**-**40**) were subsequently used to investigate the cell penetration and toxicity of the temporin peptides through infected macrophage cells, and also through a Microskin® model.

The visualisation of FAM-TB (**37**) shows the distinct localisations observed in infected and non-infected macrophages (figures 4.1 and 4.2). Clearly FAM-TB (**37**) concentrates at the parasitophorous vacuole within the infected macrophage. This suggests that temporin activity is much more specific than originally thought; as non-specific membrane perturbation within an infected macrophage would affect cellular components distinct from the parasitophorous vacuole. Since this does not appear to be the case, a more complex, cell mediated, directed system must be considered. As AMP's are synthesised within the cell and are effectors of innate immunity, through both immunomodulatory and extracellular means, it is not unimaginable that AMPs have a lesser characterised intracellular role in addition to their extracellular role. As such, AMPs could be part of a complex cell

signalling response designed to degrade and destroy pathogens phagocytosed by the macrophage as part of the immune process. If this is the case then FAM-TB (**37**) could simply be incorporated into the natural immune response of the macrophage to the intracellular pathogens. This could explain the observed localisation around the intracellular amastigote *L. mexicana*. This is further supported by evidence that purports temporin B modulation of secretory phospholipase A_2 from bee venom[9]. This modification led to the enhancement, and subsequent lysis, of the anionic phosphatidylcholine liposomes studied. In this paper[10] it was suggested that the membrane perturbation enhance activity, however, given the evidence from this study it may be possible that Temporin B exerts an immunomodulatory effect, directing and enhancing the activity of the lytic enzymes.

The studies involving the microskin® model have shown the potential to use temporins A (**16**) and B (**17**) as cell penetrating peptides. As the biological activity of these peptides is not of the level required to be a potential therapeutic agent, it may be possible to use these peptides to attach onto a known drug treatment that does not easily cross the cell membrane. This could in turn decrease the toxicity of the known treatment if a lower dose is required following attachment to temporin A or B in order to facilitate crossing the macrophage cell membrane.

This is one of the first skin studies involving the temporin peptides, and as these peptides have been shown to have good activity against gram positive bacteria (see Chapters II and III), these have potential to be developed as topical antibacterials, in addition to the potential as new antileishmanials agents.

4.7 References

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Chapter VI

The development of lipopeptide antileishmanials

Chapter V: The development of lipopeptide antileishmanials

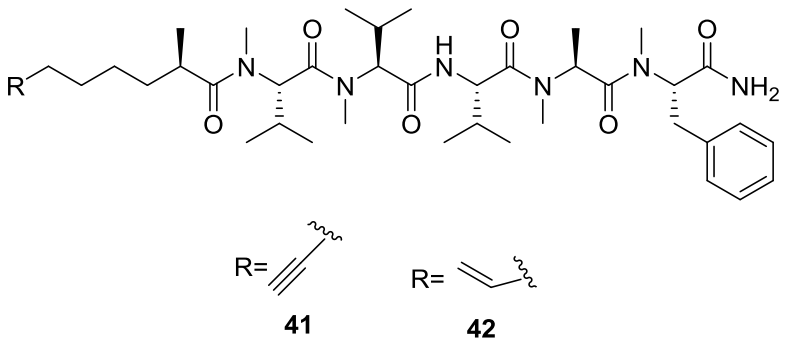
5.1 Introduction

First line treatments for Leishmaniasis now include liposomal Amphotericin B, which is highly effective and requires only a short course of treatment but is too expensive to be a viable treatment option in most developing nations.[1] Given the success of Amphotericin B we were keen to investigate if other simpler lipopeptides could be exploited as potential antileishmanial agents.

Lipopeptides are compounds that contain a cyclic or linear peptide linked to a lipid tail or other lipophilic moiety.[2] Lipopeptides are widely found in nature and as such they have become a wide investigated class of molecules.[3] Lipopeptides have been found to exhibit surfactant properties,[4] antimicrobial,[5] and cytotoxic activities.[2] In terms of their development as anti-infective agents lipopeptides are of particular interest because as like AMPs they typically target the plasma membrane of microorganisms. The considerable challenge presented to a microorganism to either repair or mutate their plasma cell membrane highlights that this structure is an ideal drug target. This mode of action also significantly decreases the likelihood of microorganisms developing resistance to lipopeptides which can occur with other molecules that target specific enzymes.[6] The number of reported cases of resistance towards lipopetides and AMPs is lower compared to other traditionally used antimicrobial agents.[7] This is, in part, due to the lower number of AMPs and lipopeptides use in animal husbandry vs. other antimicrobials, which has greatly reduced the opportunities available for microorganisms, such as *enterococci*, to develop mechanisms of resistance.[7]

Despite their attractive biological profiles, some lipopeptides have a high production cost (due to large size or complex structure), making them unsuitable for large scale production. In addition, there are inherent limitations on the *in vivo* stability of lipopeptides as their peptide component can often be readily degraded by peptidases. Thus, investments into the research and development of new lipopeptides, whether natural or synthetic, are extremely important, and the knowledge of the biological activities of different lipopeptides can be very useful to develop more biologically active compounds of this type.[2]

In relation to the development of new antileishmanials Sabchez *et al* have recently reported promising activity for the lipopeptides **41** and **42** against *L. donovani*. [1] Biological evaluation showed that **41** and **42** possessed strong *in vitro* antiparasitic activity against *L. donovani* axenic amastigotes (IC_{50} 2.4 and 1.9 μM, respectively. For reference, the two most widely used treatments against leishmaniasis (sodium stibogluconate and miltefosine) are active *in vitro* against *L. donovani* axenic amastigotes with IC_{50} values of 44.7 and 0.5 μM, respectively.[1]



41 **42**

Figure 5.1 Compounds **41** and **42** tested against *in vitro* against *L. donovani* axenic amastigotes [1].

5.2 The Ciliatamides

The Ciliatamides are a family of lipopeptides discovered by a group in Tokyo as a result of a drug discovery project from Japanese marine invertebrates. They are isolated from the deep sea sponge *Aaptos ciliate*.[5] The Ciliatamides consist of three Ciliatamides A-C (**22**-**24**), shown in Figure 5.2.

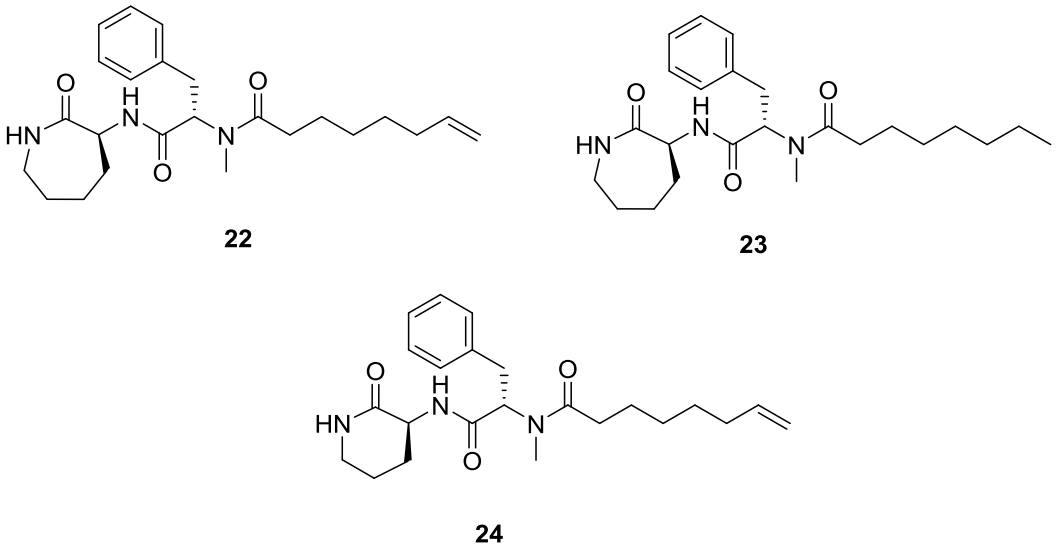


Figure 5.2 Structures of Ciliatamides A (**22**), B (**23**) and C (**24**), as reported by Nako *et al*.[5]

Ciliatamides A-C (**22-24**) were screened for activity against HeLa human uterine cervix carcinoma cells. Ciliatamide B (**23**) was found to exhibit marginal toxicity towards these cells (IC_{50} 4.5 μg/mL), Ciliatamides A (**22**), and C (**24**) also inhibited growth of the HeLa cells but to a lesser extent with IC_{50} values of 50 and 50 μg/mL, respectively[5]. The Ciliatamides were also tested for activity against promastigotes of *Leishmania* major[5]. Ciliatamide C (**24**) was found to be inactive at concentrations of 10 µg/ml, while Ciliatamides A (**22**) and B (**23**) were shown to possess moderate activity. At concentrations of 10µg/ml, Ciliatamides A (**22**)

and B (**23**) showed 50% and 45% growth inhibition respectively. The mode of action of the Ciliatamides is likely to proceed in a similar manner to that of other lipopeptides; through disruption of the cell membrane[2], although this was not investigated in the original isolation paper. It is interesting to note that the original organic extract from *Aaptos ciliate* exhibited 86% growth inhibition.[5] This result suggests that there are other compounds produced by *Aaptos ciliate*, which possess higher antileishmanial activities than the Ciliatamides, or that there is a synergistic activity at work.

In August 2008, Lewis *et al*.[8] published a total chemical synthesis of Ciliatamides A-C (**22**-**24**), which included a revision of the stereochemistry proposed in the initial isolation paper. Initially, Lewis undertook the synthesis of the *(S,S)* isomers of Ciliatamides A-C as this was the stereochemistry reported by Nakao et al.[5] However, discrepancies between the optical rotations of synthesised Ciliatamides and those obtained for the isolated compounds were obtained. To clarify the stereochemistry Lewis *et al*. undertook synthesis of (*S,S*), (*R,R*) (*S,R*) and (*R,S*), and isomers of Ciliatamides A and B. On comparison of the NMR data and the optical rotations of the four isomers the stereochemistry of the natural Ciliatamides A and B was determined to be (*R,R*).

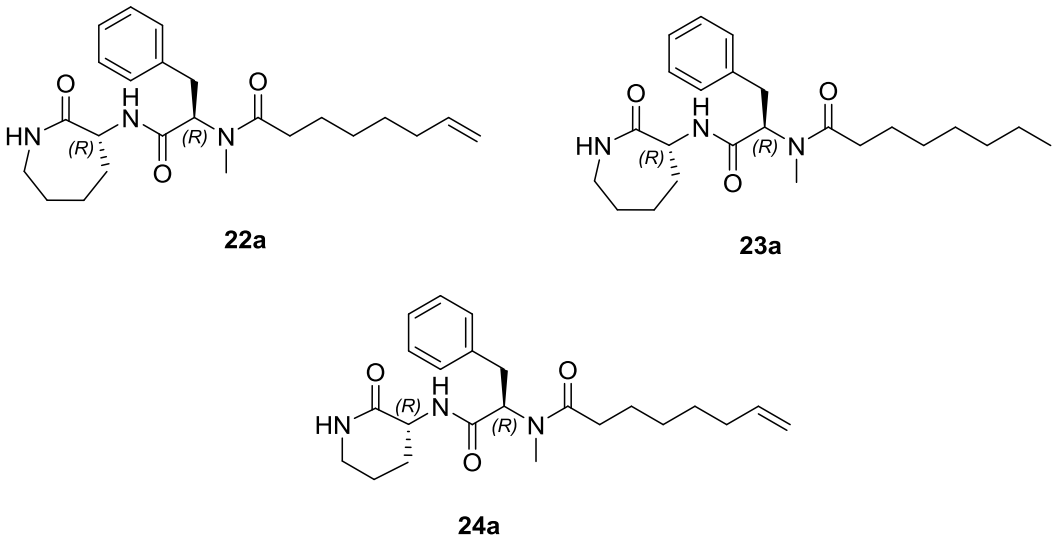


Figure 5.3 Revised structures for Ciliatamides A (**22a**), B (**23a**) and C (**24a**), as reported by Lewis *et al*[8].

5.3 Aims

Figure 5.5 shows the three Ciliatamides reported previously[8]. It was decided to initially focus on one of the Ciliatamides in this study.

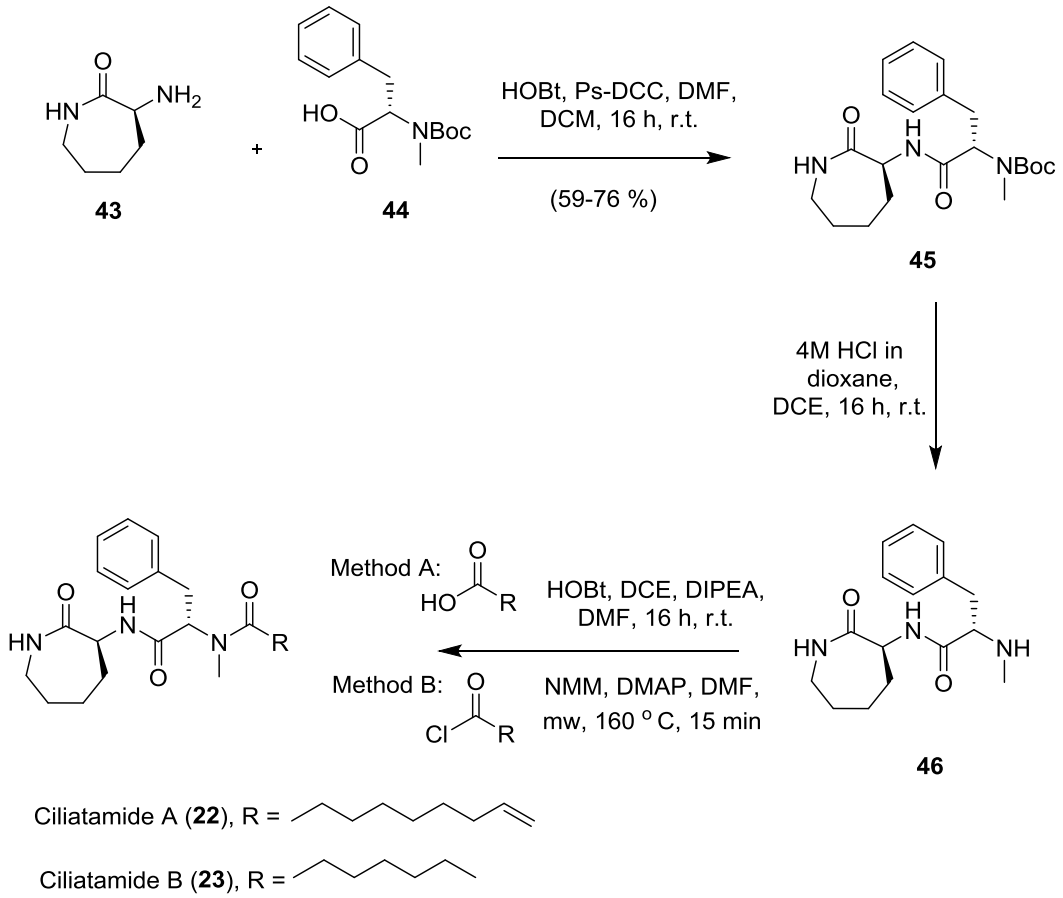
Ciliatamides B (**23**) was selected as its lipid tail could be assessed via commercial building blocks whereas this was not possible with the tail on Ciliatamide A (**22**). Ciliatamide B was also selected for further study in the development of new antileishmanial compounds as they had been shown to have greatest activity against *Leishmania major* promastigotes[5]. Further biological testing against both promastigotes and axenic amastigotes of *Leishmania mexicana* will be used to start to determine in more detail the therapeutic potential of these compounds in the treatment of CL In addition, we will look determine the biological mode of action, and the effect of stereochemistry if any, on the anti-infective activity of Ciliatamide B.

5.4 The total synthesis of *(S,S)* Ciliatamide B and isomers

5.4.1 Previous synthetic efforts from within the Cobb group

The synthesis of Ciliatamides A (**22**) and B (**23**) reported by Lewis *et al*. in 2008,[8] achieved following the route outlined in Scheme 5.1[8]. The synthesis used the starting materials L-α-amino-ε-caprolactam **43** (prepared from lysine), *N*-methyl Boc-protected phenylalanine (**44**), and decenoic acid (Ciliatamide A, **22**) or octanoyl chloride (Ciliatamide B, **23**). Initially, the additive Hydroxybenzoltriazole (HOBt)(**31**) was used together with resin-bound coupling reagent polymer-supported-Dicyclohexylcarbodiimide (PS-DCC), in order to couple the caprolactam ring onto a Boc-protected *N*-methyl phenylalanine. The initial step was followed by removal of the protecting group using of hydrochloric acid in dioxane. The final step was addition of either deconic or octanoic acid to the free nitrogen under the conditions detailed in Scheme 5.1**.** An alternative route was addition of deconoyl or octonyl chloride under microwave conditions (Method B, Scheme 5.1). This strategy was used with success in the first total synthesis of Ciliatamides A (**22**) and B (**23**). The same approach was also used to synthesise a further library of 50 (*S,S*) analogues via substitution of the lipid chains[8].

An email was sent to the authors of the original study[8], requesting samples of the compounds to test, however no reply was received. Work is continuing in the Cobb group to answer questions relating to the original study.

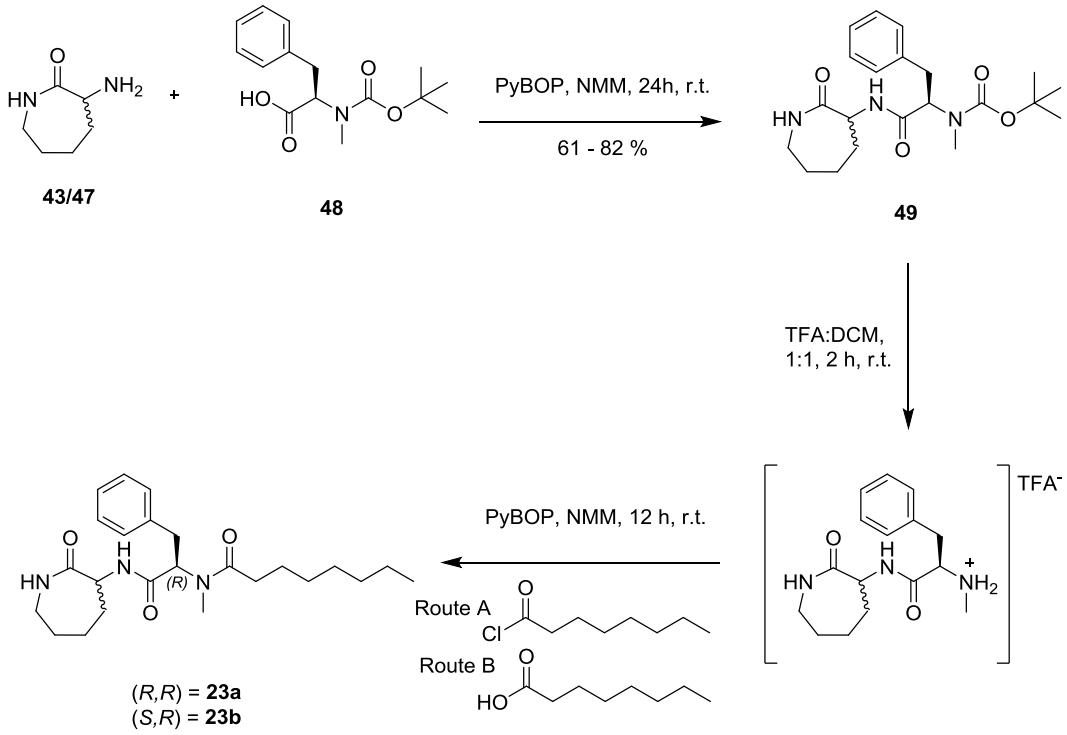


Scheme 5.1 Synthesis of Ciliatamides A (**22**) and B (**23**) as carried out by Lewis et al[8].

In order to perform biological testing to examine the effect of stereochemistry of Ciliatamide A on biological activity, it was necessary to synthesise both the (*R*,*R*)(compound **23a**), and (*S*,*S*)(compound **23**) enantiomers of the natural product. To produce a cost effective synthesis, it was hoped that the (*S*,*S*) enantiomer would possess equal or greater activity than the (*R*,*R*) enantiomer, as D-phenylalanine and the D-α-amino-ε-caprolactam (**47**) are considerably more expensive than L-phenylalanine and the L-α-amino-ε-caprolactam (**43**), thus the (*S*,*S*) enantiomer of Cilatamide B (**23**) would be more financially viable as a lead compound.

Given that all of the necessary building blocks were commercially available, an initial racemic approach to prepare the (*R,R)* isomer of Ciliatamide B (**23a**) had been previously attempted in the Cobb group (by Stephanie Maddrell, MChem student 2009, Scheme 5.2). This strategy was driven by the fact that the only commercially available forms of the caprolactam moiety are the pure L-form (**43),** and the racemic D/L-mixture (**43/47**), the initial strategy used within the group was to prepare dipeptides with D-stereochemistry at the caprolactam centre from a commercially available D/L-α-amino-ε-caprolactam mixture.

To obtain the required *R,R* lipopeptide the D/L-α-amino-ε-aprolactam (**43/47**) was used in the preparation of distereomeric dipeptide (**49**). Attempts to subsequently separate the resulting mixture of diastereomers by column chromatography on silica gel proved not to be possible. The decision was made to take the distereomeric dipeptide (**49**) through to a mixture of (*R,R*) **23a** and (*R,S*) **23b** diastereomers of Ciliatamide B. This was achieved as outlined in Scheme 5.2 below but again separation of the diastereoisomers was not achieved. In light of the problems associated with separating the stereoisomers at both the dipeptide and the final lipopeptide stage of the synthesis an alternative strategy was required.

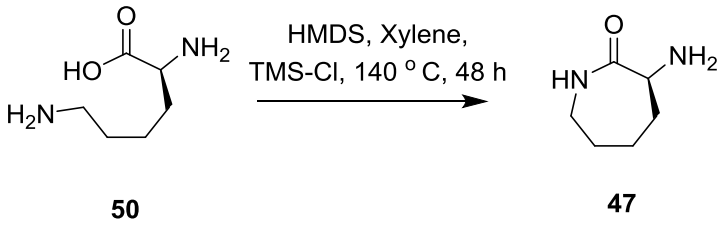


Scheme 5.2 Attempted Synthesis of the (*R,R*) and (*S,R*) isomers Ciliatamide B. (S. Maddrell, 2009).

5.4.2 Synthesis of the four possible stereoisomers of Ciliatamides B

Given that a racemic synthetic approach proved to be unsuccessful (Section 5.4.1) an alternative approach that would not require the separation of diasetreomers was sought. The alternative enatiomerically pure synthesis of the (*R,R*) isomer of Ciliatamide B would require the preparation of the D-α-amino-ε-caprolactam (**47**) as it is not commercially available. A method published in 1987 by Pellegata *et al.*[131] describes a method for the cyclisation of lysine (**51**) to yield L-α-amino-ε-caprolactam (**43**) using hexamethyldisilazane and catalytic trimethylsilylchloride (Scheme 5.3). Importantly, this reaction was reported not to racemise, the α-proton, and it was therefore thought that it would be possible to synthesise the D-

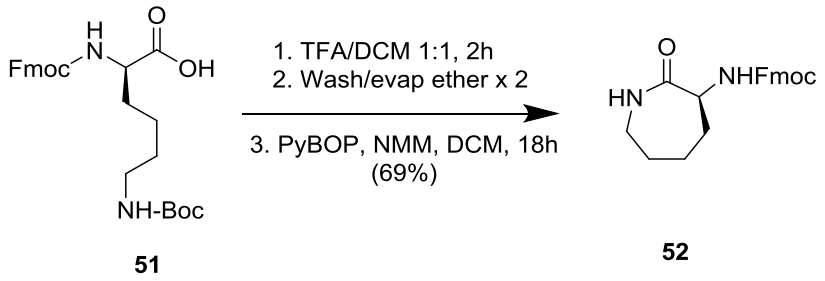
α-amino-ε-aprolactam (**47**) from D-lysine (**50**), a readily available and inexpensive starting material. Unfortunately the method outlined in Scheme 5.3 only gave D-α-amino-ε-aprolactam (**47**) in very low yield (<7%). Several attempts were made to vary the reaction conditions in which only unreacted starting material was obtained. Hence an alternative strategy was required in order to obtain D-α-amino-ε-caprolactam (**47**).



50 **47**

Scheme 5.3 Non-racemising cyclisation of D-lysine (**50**) to form D-α-amino-ε-caprolactam (**47**) (Attempted previously in Cobb group by S. Maddrell, 2009.)

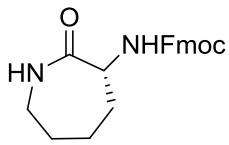
In order to produce the D-α-amino-ε-caprolactam (**47**) it was decided to remove the Boc-protecting group from Fmoc-D-Lys(Boc)-OH (**51**) by use of TFA, and then use an intramolecular ring closure to produce a protected lactam (**52**)[8] simply removal of the Fmoc group from **52** would generated the desired building block **47** as required for dipeptide formation. Following removal of the Boc group, (intermediate was not isolated) coupling of the free nitrogen to the acid is achieved by use of PyBOP. Using these conditions the formation of **52** occurred in a 69% yield, and the NMR data and physical data matching that provided in literature[8].



51 **52**

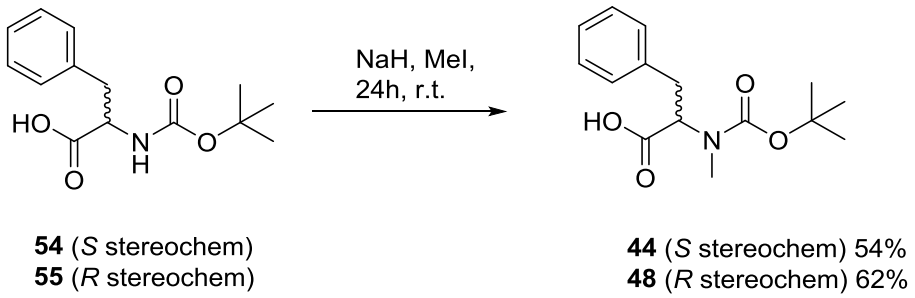
Scheme 5.4 Synthesis of an Fmoc protected D-α-amino-ε-caprolactam (**52**)**.**

The method used in Scheme 5.4 was also used to synthesise Fmoc protected *L*-α-amino-ε-caprolactam (**53**) in a 74 % yield.



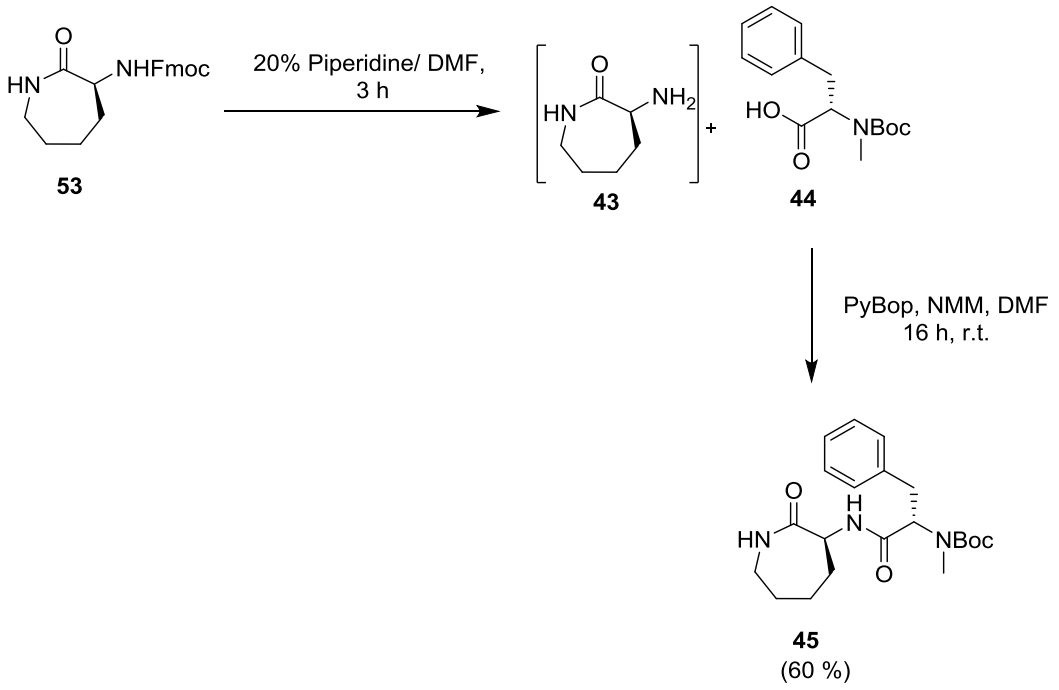
53

In order to prepare each of the four stereoisomers, enantiomerically pure Boc-Me-Phe-OH in both L- and D- forms are required. A similar problem arose to that encountered with the D-α-amino-ε-caprolactam (**47**), in that the L-Boc-*N*-Me-Phe-OH (**44**) is inexpensive and readily available, however although the D-Boc-*N*-Me-Phe-OH (**48**) is commercially available, it is considerably more expensive. Therefore, a simple procedure to synthesise **48** was sought. The literature showed that the reaction condition outlined in Scheme 5.5 to be the most suitable[8]. Synthesis initially proceeded via methylation of Boc-protected L-phenylalanine (**54**), as reported by Coggins *et al*. Synthesis of Boc-protected D-phenylalanine (**55**) proceeded by the same synthesis as shown in Scheme 5.5.



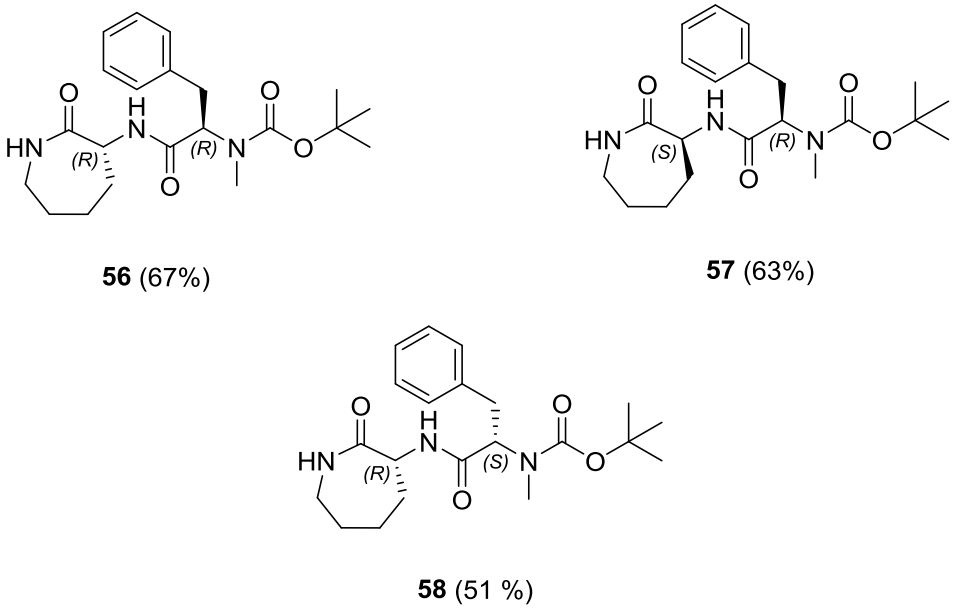
Scheme 5.5 Synthesis of N-methylated Boc-protected phenylalanines **44** and **48**.

Following formation of the Fmoc-protected L-α-amino-ε-caprolactam (**53**), the Fmoc group was removed using piperidine to give the L-α-amino-ε-caprolactam **43.** This building block was subsequently coupled immediately without further purification to the L-Boc-protected N-methyl phenylalanine **44** using PyBOP and NMM to generate the (*S*,*S*) dipeptide isomer (**45**), as shown in Scheme 5.6.



Scheme 5.6 Synthesis of (*S*,*S*)-dipeptide (**45**)

The ^{1}H NMR data of **45** indicated that rotamers were presents (doubling of peaks at 4.78 - 4.49 ppm and 2.75-2.65 ppm). Rotamers commonly occur for peptides that contain a a methyl group on the Boc-protected amine. The reaction sequence outlined in Scheme 5.6 was repeated with each α-amino-ε-caprolactam and methylated phenylalanine combination in order to obtain the remaining (*R*,*R*)(**56**), (*R*,*S*)(**57**), and (*S*,*R*)(**58**), isomers of the Boc-protected dipeptides (Figure 5.**.** The ^{1}H and ^{13}C NMR data obtained for **56-58** matched that data previously reported[8].



56 (67%)

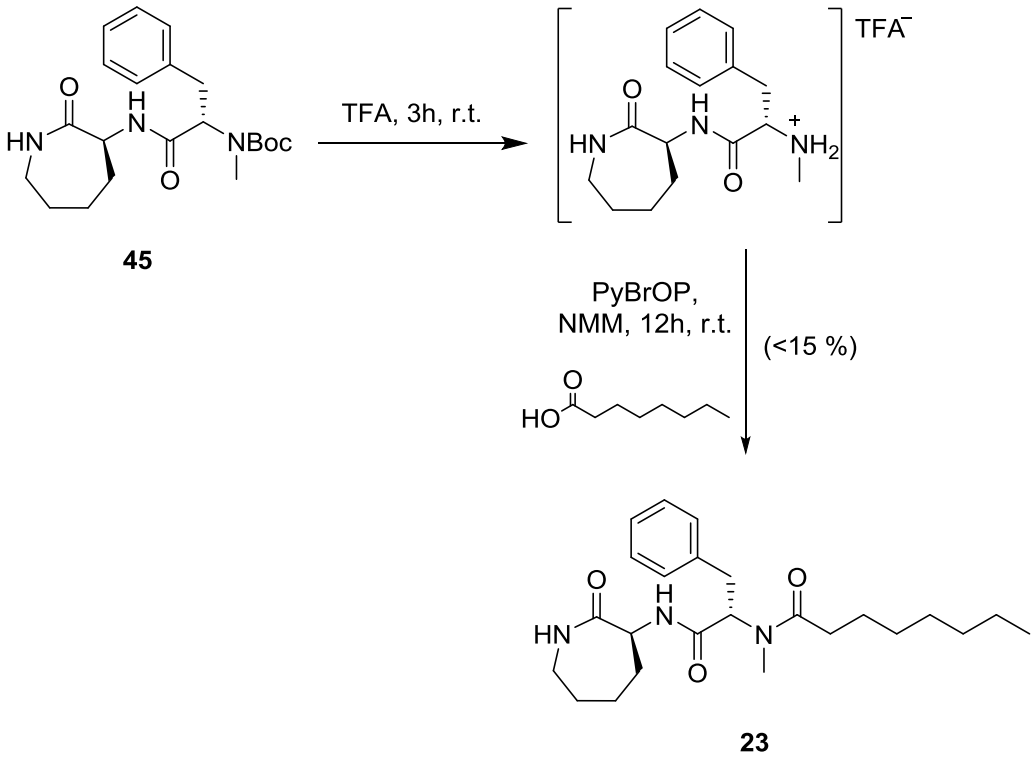
57 (63%)

58 (51 %)

Figure 5.4 N-Methylated dipeptides **56-58**

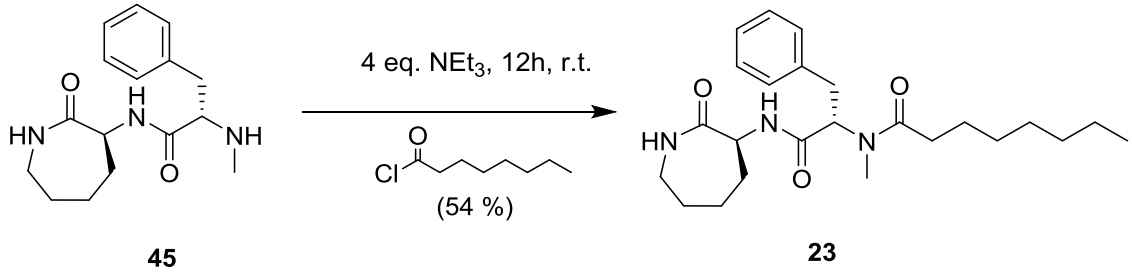
Previously within the Cobb group [Steph Maddrell, MChem 2009] removal of the Boc protecting group from the (*S*,*S*) di-peptide (**45**) using of TFA/ DCM, followed by coupling to octanoic acid using Bromotripyrrolidinophosphonium hexafluorophosphate (PyBrOP®) and NMM gave the desired lipopeptide (**23**). PyBrOP® was chosen over of PyBOP® as it is widely recognised to give greater yields for couplings onto methylated amino acids. However, even with the more

reactive PyBrOP® coupling reagent the reaction was found to proceed in very low yield (<15 %). In part this is due to purification problems (described later) which were also reported by Lindsley *et al*[13].



Scheme 5.7 Removal of Boc-group prior to coupling acylation and amide coupling of octanoic acid to a dipeptide using PyBrOP® as the coupling agent to give (*S,S*) Ciliatamide B (**23**).

In attempt to improve the reaction yield coupling using octanoyl chloride and TEA as shown in Scheme 5.8 as this method was found to give an improved yield of 54%[130].



Scheme 5.8 Reaction of octanoyl chloride with dipeptide to give (*S*,*S*) Ciliatamide B (**23**)

The method shown in Scheme 5.8 was then used to acylate all the Ciliatamide B dipeptide isomers **45** (*S*,*S*), **56** (*R*,*R*) **57** (*S*,*R*) and **58** (*R*,*S*) that had been prepared. Acylation resulted in the successfully synthesise of **23** (*S*,*S*), **23a** (*R*,*R*), **23b** (*S*,*R*) and **23c** (*R*,*R*) isomers of Ciliatamide B (Figure 5.5) Purity was confirmed by use of ^{1}H NMR and TLC. The biological evaluation of **23, 23a, 23b** and **23c** is discussed in Section 5.2.

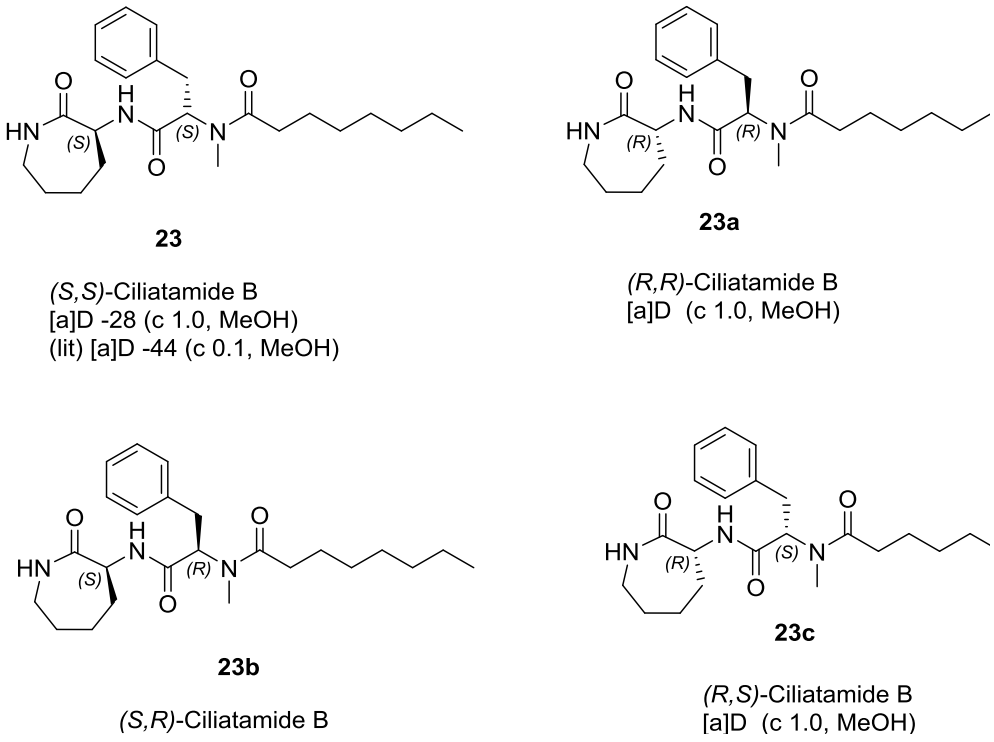


Figure 5.5 The four possible stereo-isomers of Ciliatamide B

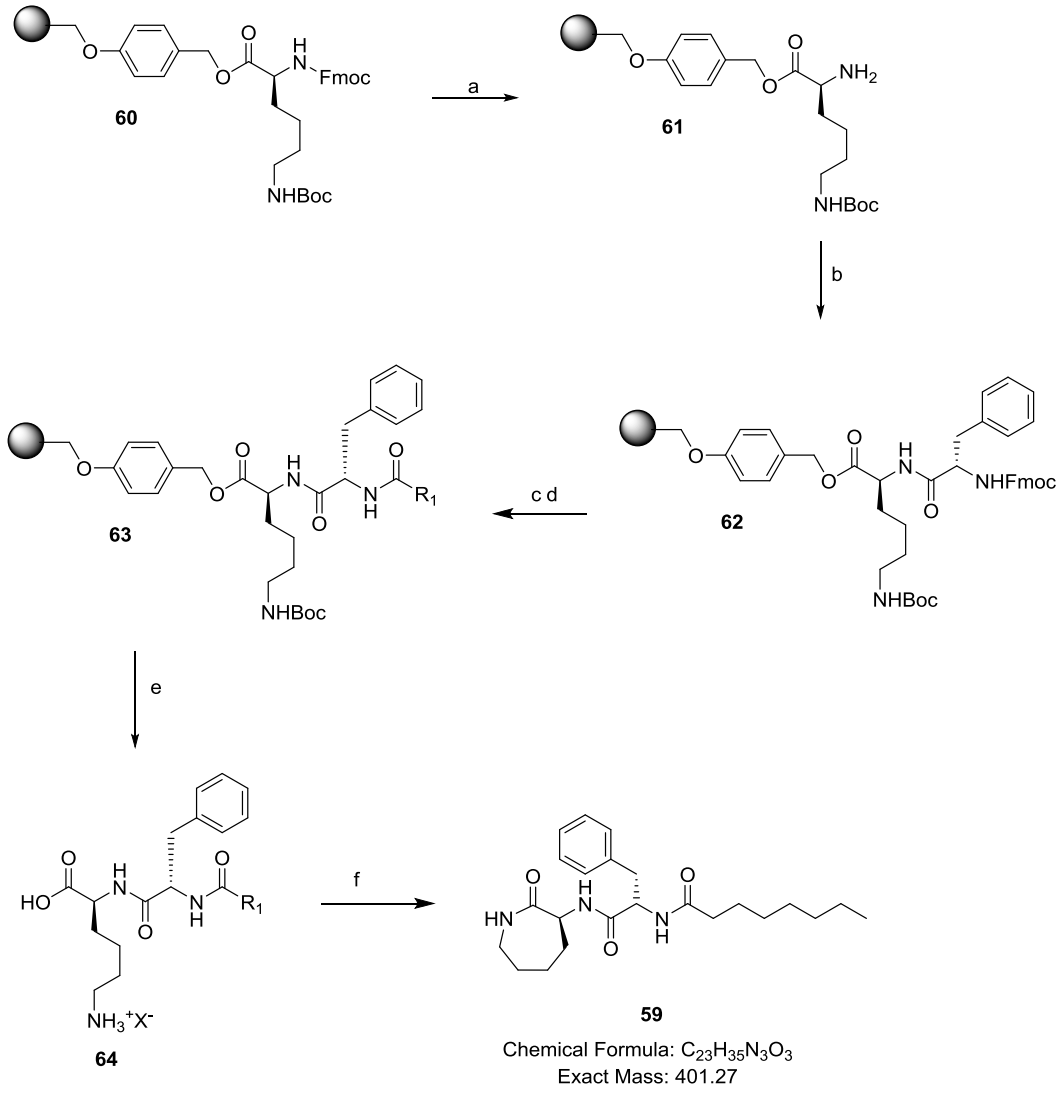
Regarding the synthesis of Ciliatamide B (**23**) and it's isomers, there are several points that should be noted regarding the technicalities of performing the reactions. It was found that complete removal of the residual TFA prior to acylation of the dipeptide (required for Boc deprotection) required repeated co-evaporation with diethyl ether steps under vacuum. It is also worth noting that as previously reported[8] the Ciliatamide lipopeptides proved to be very difficult compounds to purify using silica gel column chromatography. In particular, separation of tri-ethylamine (TEA) and PyBOP® from the Ciliatamides proved to be very problematic. Initially the crude reaction mixture was added directly to the column and separation using standard column chromatography (silica gel in a

range of solvent systems) was attempted. However, despite running the compounds through multiple columns, residual TEA and PyBOP® was still shown to be present by use of ^{1}H NMR. A second strategy was then employed, involving the use of 5% sodium bicarbonate to wash the reaction mixture in order to separate TEA and PyBOP® from the Ciliatamides prior to column purification. Following washing, the Ciliatamides were further purified by use of column chromatography in EtOAc/hexane 1:1, and then subsequently run through another column in DCM/MeOH 9:1, in order to obtain the purified Ciliatamides.

5.4.3 Attempted Solid Phase Peptide Synthesis (SPPS) of a Ciliatamide B analog (with Miriam Edwards, MSci 2012)

As previously discussed (Section 5.3.2), purification of the final Ciliatamide compounds proved to be problematic, producing unfavourably low yields of the compounds. It was decided to attempt a solid-phase approach to the synthesis as an alternative method for producing the natural Ciliatamides and Ciliatamide-like structures. Solid phase peptide synthesis (SPPS) presents notable advantages over solution phase synthesis as the use of a solid support allows remaining reactants and bi-products from the reaction to be washed away following each coupling/deprotect cycle. It was hoped that this approach would allow removal of octanoyl chloride prior to cleavage of the peptide, as this was the predominant problematic compound requiring separation from the peptide in solution-phase synthesis.

SPPS of a Ciliatamide B analog (**59**) was carried out using Wang resin preloaded with Boc-protected Lysine (**60**). As outlined in Scheme 5.9 below, preloaded resin was used in order to increase the efficiency of synthesis, removing the need to couple the first amino acid to the resin linker. An intramolecular cyclisation was carried out, using the nitrogen of the secondary amine on the lysine side chain as a nucleophile to attack the carbonyl centre and form a seven membered ring.



Scheme 5.9 SPPS of Ciliatamide-like Lipopeptide **59** on Preloaded Wang Resin. Reagents and Conditions: a) 20% piperidine in DMF, rt, 5 min, followed by DMF wash x 3, b) Fmoc-Phe-OH, TBTU, DIPEA, DMF, 75°C, 20 W, 10 min, c) 20% piperidine in DMF, rt, 5 min, followed by DMF wash x 3, d) octanoic acid, TBTU, DIPEA, DMF,

75°C, 20 W, 10 min, e) TFA, H_2O, TIPS, rt, 2 h, f) DCM, DIPEA, DIC, rt, 18 h, followed by wash with H_2O, extract with DCM x 3.

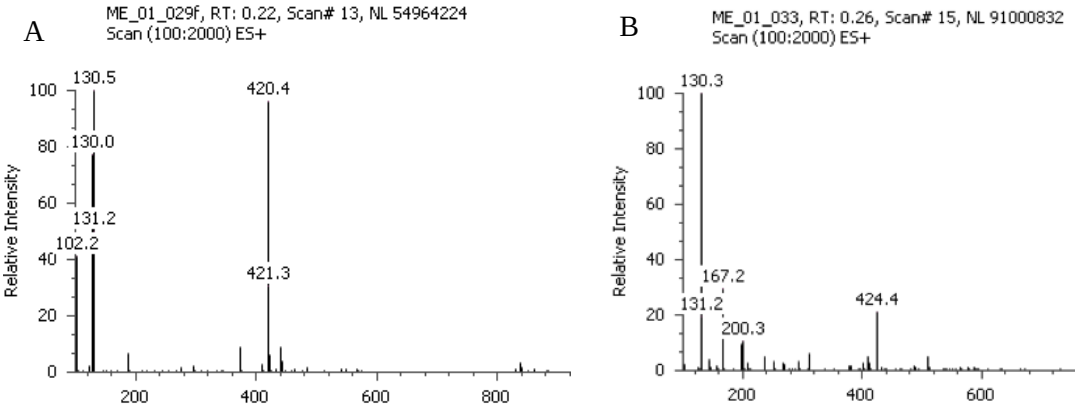


Figure 5.6 ESI spectra for: (a) the linear lipopeptide **64** prepared in Scheme 5.9 and (b) cyclised product **59,** *m/z* 424 $[M+Na]^+$.

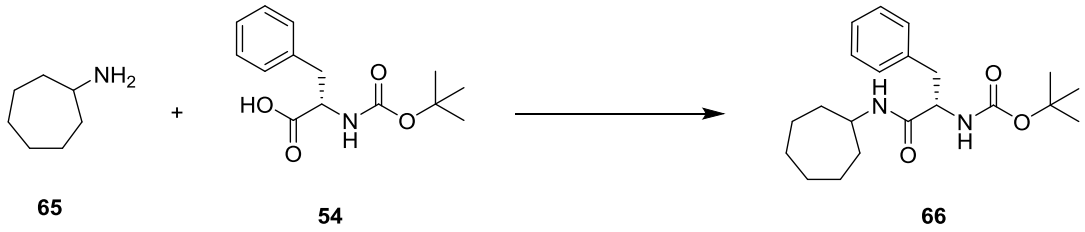
ESI analysis of the cleavage product obtained in Step e (Scheme 5.9) confirmed that the linear lipopeptide (**64**) had been made as indicated by the peak present at *m/z* 420.4 $[M+H]^+$ (Figure 5.6, Spectrum a). This suggested that solid phase synthesis of the linear lipopeptide **64** on resin was successful. Intramolecular cyclisation of the free lysine side chain to form a seven-membered ring and the target lipopeptide **59** was attempted (Scheme 5.9, Step f). ESI analysis of the crude reaction mixture after cyclisation is shown in Figure 5.6 (Spectrum b). The peak at *m/z* = 424.4 was assigned to be the Na adduct of the desired product **59**, indicating the ring closure had been successful. Purification of the crude reaction mixture was attempted, however due to time limitations, this was not pursued further. However, the SPPS approach does appear to be a potentially useful method by which to access analogs of Ciliatamide B more rapidly.

5

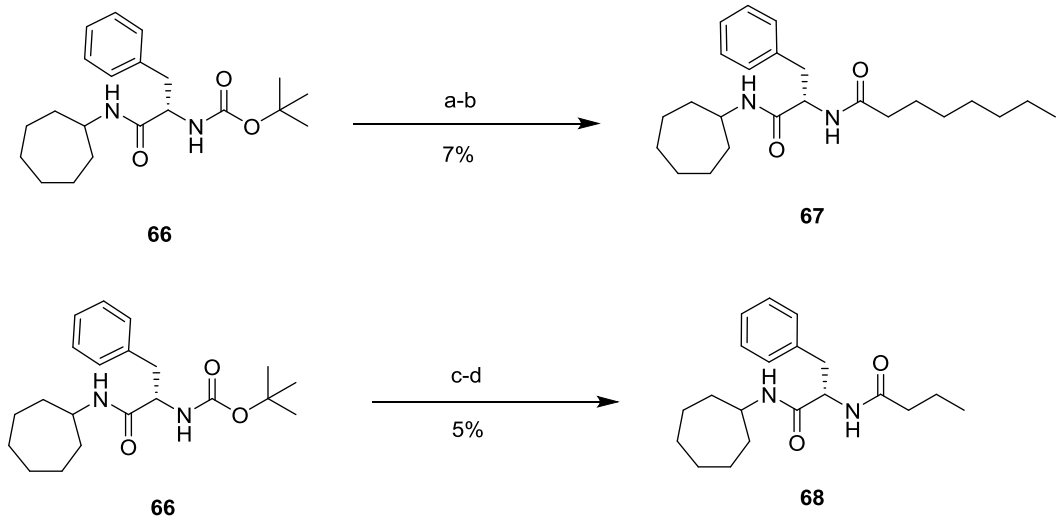
5.5 The synthesis of simplified Ciliatamide B analogs (carried out with Miriam Edwards, MSci 2012.)

As a result of the need to reduce the cost of the synthesis, and due to difficulties in preparing the lactams, it was decided to synthesise simplified Ciliatamide B analogs, containing a cycloheptane ring in place of the lactam ring.

Synthesis of dipeptide 66 was easily achieved as outlined in scheme 5.11 below.

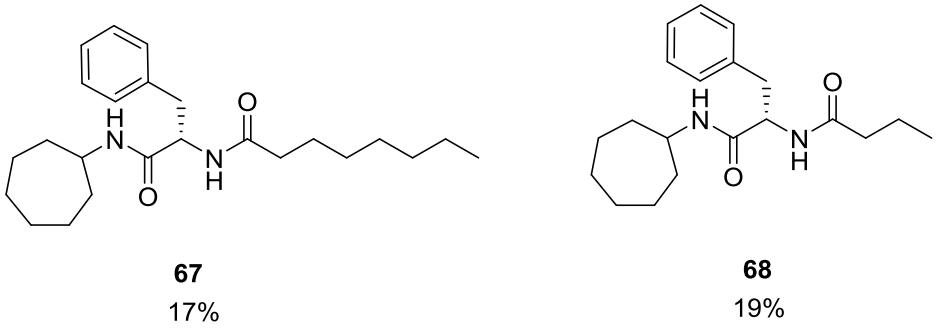


Scheme 5.11 Synthesis of pseudo dipeptide **66**: Reagents and Conditions: a) PyBOP / DCM, DIPEA followed by cooling to 0°C, addition cycloheptylamine, warm to rt, stirring 18 h



Scheme 5.12. Synthesis of lipopeptides **67** and **68**: Reagents and Conditions: a) TFA / DCM, rt, 2h b) DCM, octanoyl chloride, Et_3N, rt, 24 h c) TFA / DCM, rt, 2h d) DCM, butyryl chloride, Et_3N, rt, 24 h.

Following purification by column chromatography on a standard silica gel column, the targets lipopeptides **67** and **68** were recovered yields of 7% and 5% respectively. The reason for this is that the final peptides and the starting materials both have very similar polarities and as such they were extremely difficult to separate using standard silica gel column chromatography. In an attempt to increase the yields, lipopeptides **67** and **68** were resynthesized and purified using preparative TLC (Silica gel plates).



67
17%

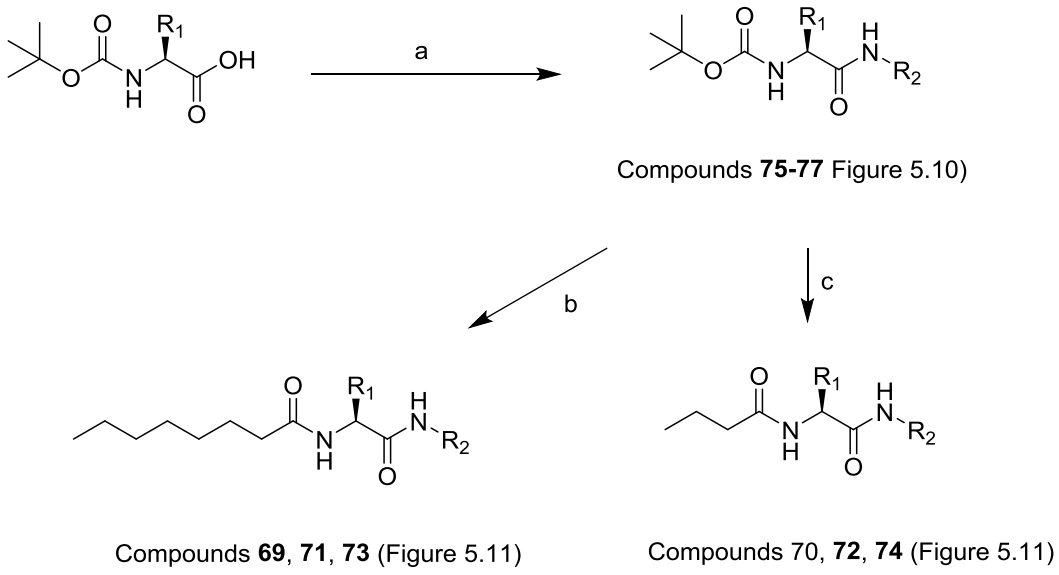
68
19%

Scheme 5.12 Synthesis of lipopeptides 67 and 68 from Boc-Phe-OH. Reagents and conditions: a) PyBOP / DCM, DIPEA followed by cooling to 0°C, addition cycloheptylamine, warm to rt, stirring 18 h. b) TFA / DCM, rt, 2 h followed by addition DCM, octanoyl chloride, Et_3N, rt, 24 h. c) TFA / DCM, rt, 2 h followed by addition DCM, butyryl chloride, Et_3N, rt, 24 h.

Due to the low yields of **67** and **68**, the reaction was attempted a second time, along with synthesis of the dipeptide precursor (Scheme 5.13). In this case the yields were increased when purification was achieved and the NMR spectra for the two compounds showed a greater removal of reactants through preparative TLC. This suggested that preparative TLC was a more successful purification method than column chromatography and so it was decided that all other lipopeptides synthesised utilising solution phase methods would be purified in this way. However, due to the presence of some contaminant, as discussed in section

5.4.3, yields are reported remain 'crude' yields and the pure yields could only be calculated once the contaminant had been removed.

Solution phase synthesis of the target molecules **69**-**74** was achieved with yields of 19, 18, 14, 11, 21 and 15% respectively, from their Boc-protected amino acid precursors (**75**-**77**) (Scheme 5.13). Structures of all final compounds and intermediates were confirmed by NMR and ESI analysis.



Scheme 5.13 General Solution Phase Synthesis of Ciliatamide-like Lipopeptides. Reagents and conditions: a) PyBOP / DCM, DIPEA followed by cooling to 0°C, addition cycloheptylamine, warm to rt, stirring 18 h. b) TFA / DCM, rt, 2 h followed by addition DCM, octanoyl chloride, Et_3N, rt, 24 h. c) TFA / DCM, rt, 2 h followed by addition DCM, butyryl chloride, Et_3N, rt, 24 h.

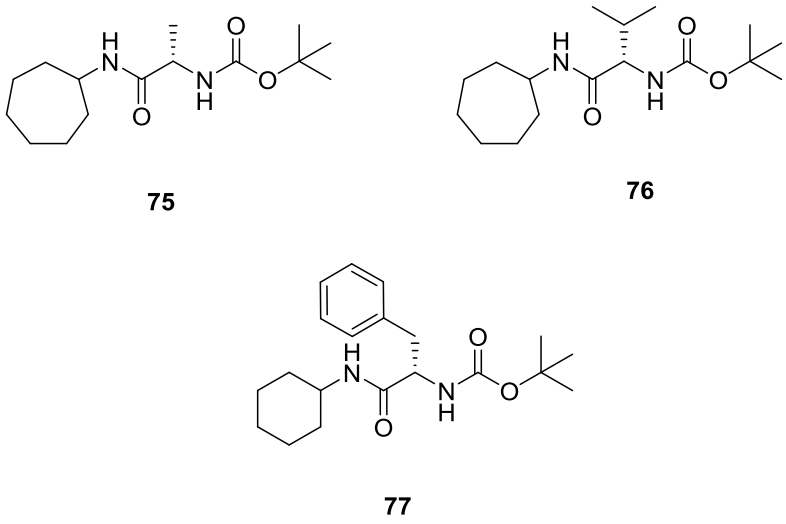


Figure 5.10 Intermediate pseudo-dipeptides **75**, 76 and **77** prepared.

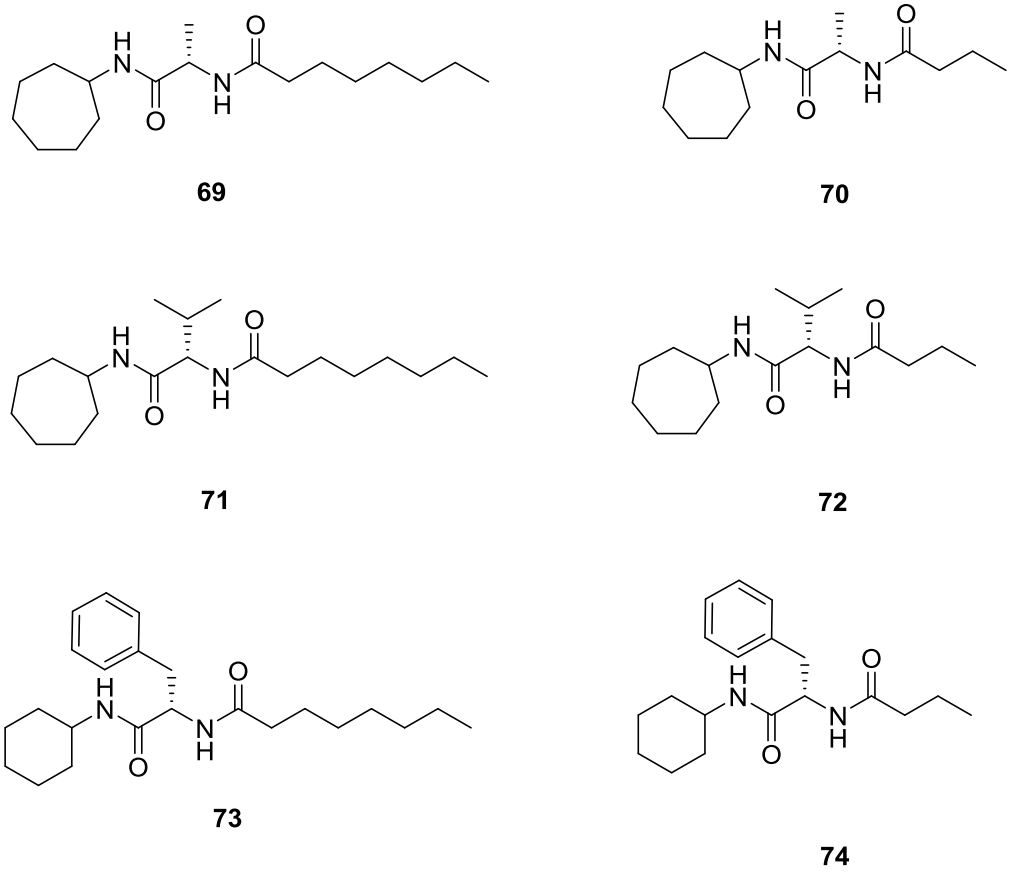


Figure 5.11 Chemical structures of the library of lipopeptides prepared (**69**-**74**).

5.6 Biological evaluation of lipopeptides

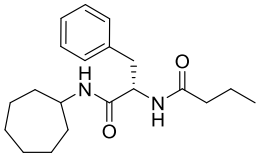
5.6.1 Initial testing of Ciliatamide B (and isomers)

Screening of Ciliatamides **23, 23a, 23b** and **23c** revealed no activity when tested against *Leishmania mexicana* axenic axenic amastigotes and promastigotes at concentrations of 200 µM. This is in contrast to previously reported testing against *Leishmania major* promastigotes[5]. Previously the Ciliatamide B (assumed to be *S*,*S*) **23** has previously been been reported to, at concentrations of 10µg/ml, give 45% growth inhibition[5]. The differences in results obtained in our study and that reported by Nako *et al*[5] are likely to be due to the differences in cell membrane structure between the different *Leishmania* species (major and mexicanna), as the lipopeptides are thought to target the cell membrane. Stark differences in the levels of activity that a given compound may show towards different species of *Leishmania* was also clearly highlighted in the previous Chapters II and III for the studies carried out on AMPs. It is likely that the Ciliatamides act via a similar mode of action to that proposed for the temporin AMPs, although there are differences in structural conformations in the two classes of compounds (i.e. in terms of secondary structure etc, which will be altered due to the presence of a lipid tail). Data has shown that pore formation in membranes occurs after lipopeptide oligomer binding, some of which are Ca^{2+} dependent multimers[9]. These pores may cause transmembrane ion influxes, including Na^{+} and K^{+}, which result in membrane disruption and cell death. The modes of action (if any) of the compounds tested in this chapter are difficult to propose, as my current results do not suggest any antileishmanial activity.

However, it is likely that the lipopeptide family of compounds disrupts cell membrane formation.[2]

5.6.3 Biological evaluation of simplified Cilliatamide analogs

Ciliatamide analogs **69-74** were screened for activity against *L. mexicana* axenic axenic amastigotes and the cell viability measured using the Alamar Blue fluorescence assay system (See Chapter VII, 7.5). The stock solutions of each compound and Amphotericin B (**13**) (used as a positive control) in DMSO were serially diluted to produce 100 and 50 μM concentrations of the compounds in the axenic amastigote cell culture. DMSO solutions of the same concentrations were used as a negative control for comparison and normalisation of the cell viability results. Compounds (**69**), (**70**), (**71**), (**68**) and (**73**) (see figure below) were screened.



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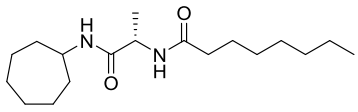

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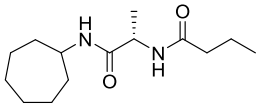

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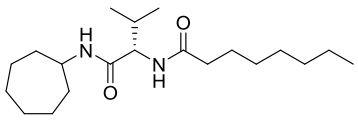

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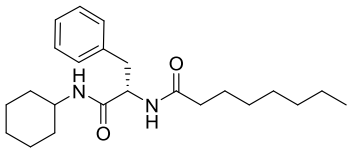

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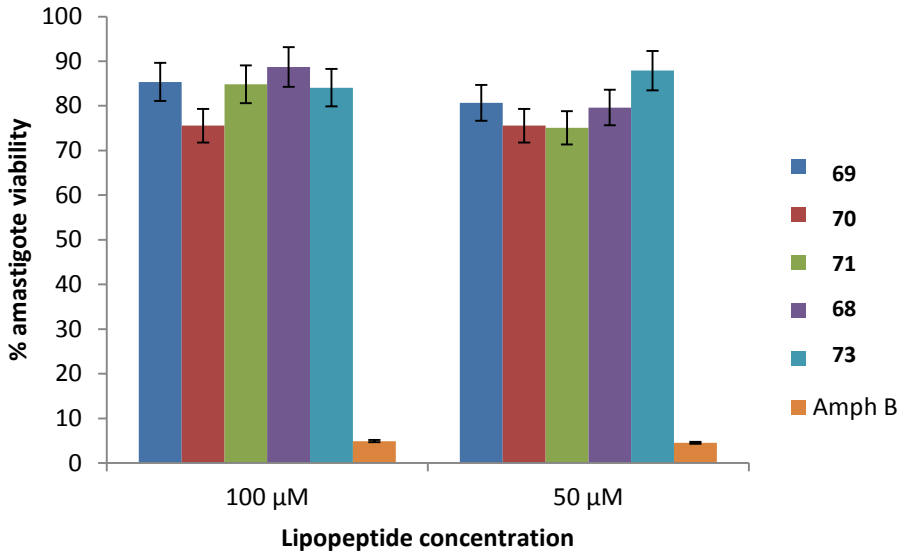

Figure 5.14 *L. mexicana* amastigote viability, as determined by Alamar Blue assay, when treated with different concentrations of targets **68**, **69**, **70**, **71**, **73** with Amphotericin B (Amph B) as a positive control. Viability was determined with respect to negative controls, which were untreated with drugs. Data points represent results of two independent experiments performed in triplicate. Standard error indicated.

The Alamar Blue viability assay results indicate that no of the compounds show significant (or any) activity against *L. mexicana* axenic axenic amastigotes at the concentrations tested (100 and 50 µM), with negligible difference between results at the different concentrations. It was therefore decided that further testing was unnecessary.

Compounds **67**, **68**, **69**, **70** and **74** (see Figure 5.15 below) were also screened against the bacterial species *E. coli*, *B. subtilis* and *S. epidermis* in order to determine the minimum inhibitory concentration of each compound. The degree of inhibition was determined from absorbance readings. Stock solutions of each compound and Ampicillin (used as a positive control) in DMSO were serially diluted to produce concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25

mg/L in nutrient broth. Serial dilutions of DMSO in nutrient broth were also prepared as a negative control for normalisation of the compound results. A further row of untreated broth was also run to represent a 0 mg/L concentration of each compound in order to confirm that any inhibitory activity is due to the compound screened. As none of the compounds screened resulted in complete growth inhibition of the bacteria species at the concentrations tested, minimum inhibitory concentrations could not be determined. Instead, results for % viability, calculated using untreated bacteria control absorbance values as 100% viability are shown for each species.

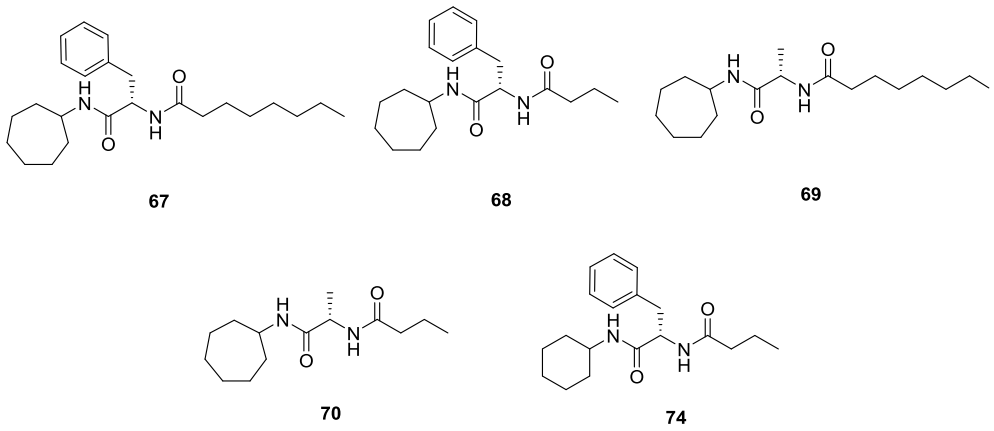

Figure 5.15 Compounds tested for antimicrobial activity, results shown in figure 5.16

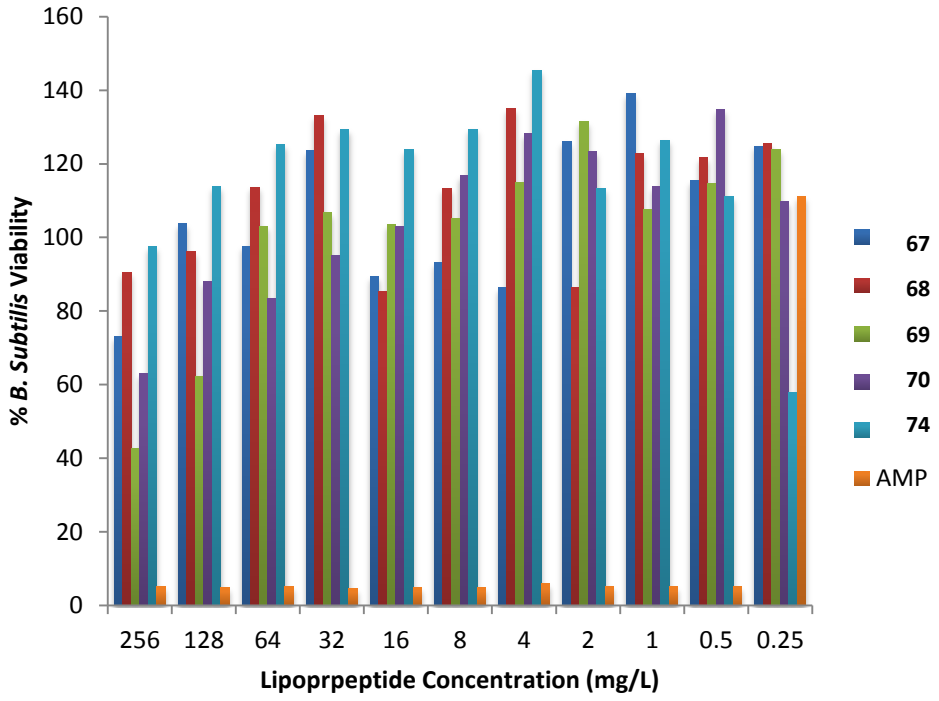

Figure 5.16 *B. subtilis* viability, treated with a range of concentrations of **67**, **68**, **69**, **70** and **74** with Ampicillin (Amp) as a positive control. Viability was determined with respect to the DMSO control untreated bacteria. Data points represent results of two independent experiments performed in triplicate. *Unfortunately the raw data for Figures 5.16, 5.17 and 5.18 was no longer available at the time of writing (as previously mentioned this data was obtained as part of a MChem project). It was however agreed to include the data in the printed state as these results form an important part of this chapter.

No real cytotoxicity against *B. subtilis* was demonstrated below concentrations of 128 mg/L (Figure 5.15) in any of the target compounds screened. Compounds **69** and **70** however, displayed reasonable activity at 256 mg/L concentrations with almost 60% growth inhibition demonstrated by **69**. As both **69** and **70** contain alanine units this suggests that the nature of the side chain may play a role in the molecules' antibacterial action. In order to further support these findings, the screening would need to be repeated in triplicate in order to show reproducibility of the results. Although the cytotoxicity is still overall, quite poor, especially when compared with the Ampicillin control so these target compounds do not

present particularly promising lead compounds for antibacterial agent development.

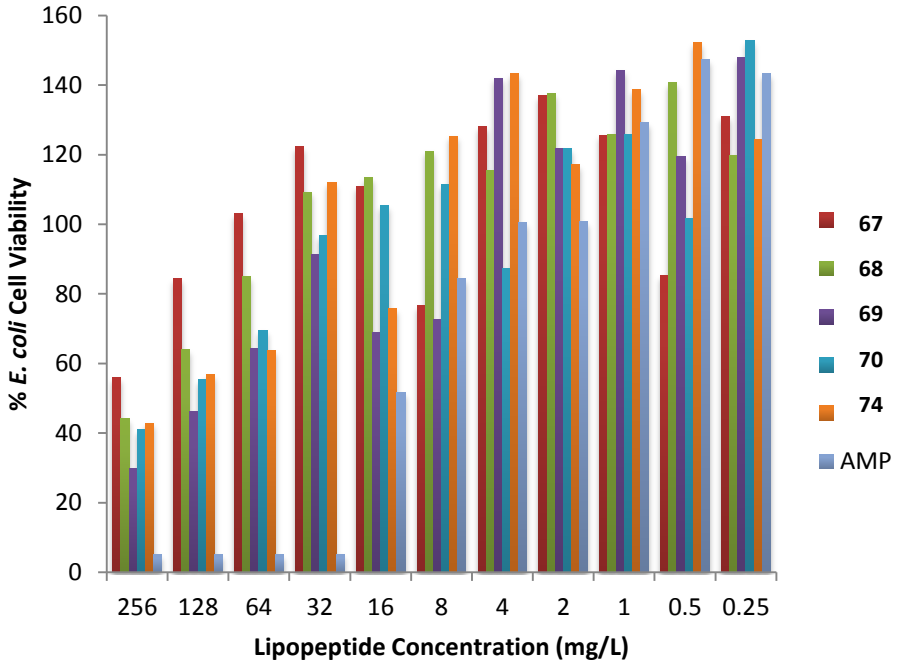

Figure 5.17 *E. coli* viability, treated with a range of concentrations of targets **67**, **68**, **69**, **70** and **74** with Ampicillin (Amp) as a positive control. Viability was determined with respect to the DMSO control untreated bacteria. Data points represent results of two independent experiments performed in triplicate.

Percentage viability results for the target compounds screened against *E. coli* and *S. epidermis* (Figure 5.16 are not particularly strong, however they do show a general increase in cytotoxicity with increased concentration. All compounds showed reasonable activity against *E.coli* and *S. epidermis* at 256 mg/L and interestingly, compound **69** showed the greatest cell growth inhibition, as was the case with *B. subtilis* screening; this pattern suggests that further development and modification of **69** could produce an antibacterial agent. However, further repeats of screening **69** would need to be carried out to determine the reliability of the

results. The compounds were also tested with the silica residue contaminant present and so the residue would need to be screened as a control to confirm whether it is the compound or the residue that possesses the biological activity.

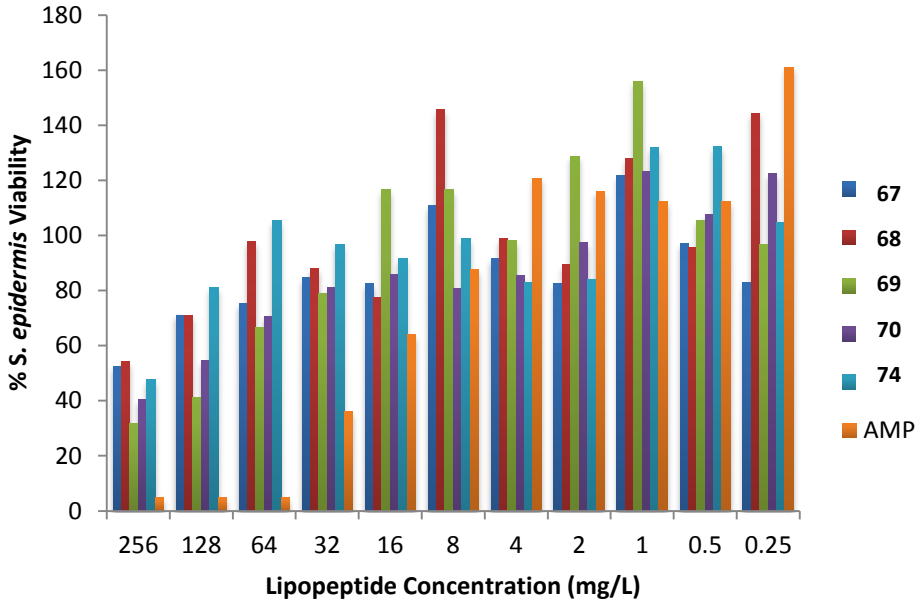

Figure 5.18 *S. epidermis* viability, treated with a range of concentrations of targets **67**, **68**, **69**, **70** and **74** with Ampicillin (Amp) as a positive control. Viability was determined with respect to the DMSO control untreated bacteria. Data points represent results of two independent experiments performed in triplicate.

The cytotoxicity results for the compounds screened against the bacterial species are not completely representative of the true antibacterial activity of the compounds in their pure form due to the presence of contaminant (as discussed above). These results do however give an indication of the potency of the compounds as antibacterial agents. Ideally, the compounds tested would be synthesised again retested as pure compounds.

5.7 Conclusions

There were several difficulties encountered with the purification and synthesis of the compounds discussed in this chapter. Namely, in obtaining a reasonable yield, and in the purification of the lipopeptides, as these compounds proved to be extremely difficult to separate from the starting materials.

The lipopeptide compounds tested did not show any antileishmanial activity against *Leishmania mexicana* when tested at concentrations of up to 200 μM. Testing the compounds at higher concentrations *in vitro* may result in activity against *Leishmania mexicana.* This may lead to the identification of analogs with activity at lower concentrations. It is unlikely that *in vitro* testing at concentrations higher than this would highlight a compound with *in vivo* activity at levels not cytotoxic to the host organism. The synthesis of the Ciliatamides using and SPPS approach may provide a solution to the difficulties encountered in the purification of these compounds, however additional work is required to further explore the viability of this option.

Considering the difficulties previously discussed in the synthesis and purification of these compounds, further investigations were not carried out to synthesise and test further analogs. It remains however an interesting question of the possible mode of action when tested against *Leishmania major*, and indeed if the Ciliatamides would prove to be effective antileishmanials agents when tested against other *Leishmania* species.

References

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Chapter VI

Overall Conclusions

6.1 Overall Conclusions

Temporins A (**16**) and L (**36**) were initially tested against axenic amastigotes, for cytotoxicity against uninfected murine macrophages, and against infected murine macrophages. Toxicity analysis (uninfected macrophages) showed temporin A (**16**) to be cytotoxic at 500 μM, and temporin L (**36**) to be cytotoxic at 250 μM. These results are in keeping with other published studies and they place the viability of temporin L (**36**) as a potential therapeutic agent in question, as this peptide appears to be cytotoxic across a broad spectrum of biological tissues and would likely cause cell death in the host in addition to *leishmania* parasites. The eventual aim is to use AMPs in order to develop a topical leishmanicidal application; however it is likely that the levels of cytotoxicity reported here for temporin L would prevent the regrowth and healing of healthy tissues in the affected areas in addition to causing death of the *leishmania* parasites.

Temporins A and L were initially found to be the only peptides tested to exhibit significant levels of activity against *L. mexicana* axenic amastigotes, the clinically relevant form of *L. mexicana* published data has only reported activity on promastigotes.

In contrast to insect stage promastigotes, pathogenic amastigote *L. mexicana* are significantly more resistant to all the temporins tested. This demonstrates the importance of screening against both forms of the parasite, particularly in light of available literature on the subject; in which reported testing on the amastigote lifecycle stage is scarce. Results obtained suggest that amastigotes of different *Leishmania* species display varying susceptibility to peptides from the temporin family indicating that broad-spectrum antileishmanial AMPs may be challenging to develop. Mass spectral analysis of the lipid content of the parasite cell walls from *L. mexicana* promastigotes and amastigotes was carried out in collaboration with Dr Jackie Mosely (MS, Durham University) and Dr Terry Smith (Chemistry Department,

University of St. Andrews). The analysis (data not reported) showed significantly higher levels of specific peptidoglycans in the promastigote cell walls, compared to the amastigote cell walls on comparison of two samples of equal volumes of cells in solutions analysed.

In addition, the ability of AMPs to translocate the host membrane and reach intra-macrophage amastigotes has not been widely studied and remains unknown. Temporins A and L have been studied to a greater degree than any of the other temporin peptides. It is possible that the charge is important; as the parasite membrane is negatively charged a positively charged peptide may be attracted to the membrane through electrostatic attraction which may in turn facilitate the disruption of the parasite membrane, and account for a lower active concentration of drug needed to kill the parasites. Temporin L has the highest overall charge of the peptides studied in the table below (+2, compared to 0 or +1) which may facilitate the ability of the peptide to interact with the negatively charged parasite membranes. Temporin L also has a higher ration of hydrophobic residues /total number of residues (30, compared to 15-23 for other peptides), which may also account for the higher levels of antileishmanial activity observed with temporin L, this may be because the hydrophobic residues will more favourably interact with the lipid environment in the parasite membrane, and these interactions draw the peptide into the membrane more strongly than those peptides with proportionally fewer hydrophobic residues.

Temporins A and B were both successfully labelled with fluorescein and tetramethylrhodamine. The labelled peptides were subsequently used to investigate the progress of the temporin peptides through infected macrophage cells, and also through a microskin® model.

The visualisation of FAM-TB (**37**) (Chapter IV) shows the distinct localisations observed in infected and non-infected macrophages. Clearly FAM-TB (**37**) concentrates at the parasite location within the infected macrophage. This suggests that temporin activity is much more specific than originally thought, and that a more complex, cell mediated, directed system must be considered.

The studies involving the microskin® model have shown the potential to use temporins A (**16**) and B (**17**) as cell penetrating peptides. As the biological activity of these peptides is not of the level required to be a potential therapeutic agent, it may be possible to use these peptides to attach onto a known drug treatment that does not easily cross the cell membrane.

The lipopeptide compounds tested (Chapter V) did not show any antileishmanial activity against *L. mexicana* when tested at concentrations of up to 200 μM. The synthesis of the Ciliatamides using solid phase synthesis may provide a solution to the difficulties encountered in the purification of these compounds, however additional work is required to further explore this option. Additional biological evaluation and toxicity studies of all the lipopeptides prepared is required to gain a more complete picture on whether or not these compounds are worth pursuing as anti-infective agents.

The large scale screening program, carried out generated a considerable amount of information and is the first real data set that could be used to design predictive models for the development of antiparasitic AMPs (Chapter V). The data obtained through this library screening approach did not identify any sequences predictive of activity against *Leishmania Mexicana*. However, it does represent the first step towards developing a computational model/prediction system that can be used to rationally design peptides with antiparasitic properties.

Chapter VII

Experimental Procedures

7.0 Experimental

7.1. General

7.1.1 Instrumentation

NMR spectra were recorded on a Brucker Avance-400 or Varian VNMRS-700 spectrometer. Mass spectra were recorded on a Micromass LCT ToF (LRMS) or Thermo-Finnigan LTQ FT (HRMS) electrospray ionisation instrument in positive (ES+) or negative (ES-) as specified. Optical rotations were measured on a Jasco P-1020 polarimeter (589 nm) at ambient temperature (20-22°C). RP-HPLC data was recorded on a Waters Mass Directed Prep System instrument, using a 3100 Mass Detector and Diode Array Detector. For analytical RP-HPLC, a 4.6 x 100 mm xbridge column was used, with a flow rate of 1 ml/min (run time 16.5 min). For Preparatory scale RP-HPLC, 1 19 x 100 mm xbridge column was used, with a flow rate of 17 ml/min (run time 16.5 min).

All the commercially available reagents and solvents were purchased and used without further purification. All reactions were carried out under an atmosphere unless otherwise stated. Microwave assisted reactions were performed on a Biotage Initiator™ Microwave. All fractions from column chromatography were monitored by thin layer chromatography using aluminium plates with a UV fluorescent indicator (Macherey-Nagel 60 SIL G/UV_{254}). One or more of the following methods were used for visualisation: UV absorption by fluorescence quenching, Ninhydrin in EtOH or PDMA in EtOH. Column chromatography was performed using Fluorochem type 60, 40-63 micron silica gel.

7.1.2 Characterisation

MALDI-TOF mass spectra were recorded on an Applied BiosystemsTM Voyager-DE STR instrument in positive ion mode using an α-cyano-4-hydroxycinnamic acid matrix. RP-HPLC

data was recorded on a Waters Mass Directed Prep System instrument, using a 3100 Mass Detector and Diode Array Detector. For analytical RP-HPLC, a 4.6 x 100 mm xbridge column was used, with a flow rate of 1 ml/min (run time 16.5 min). For Preparatory scale RP-HPLC, 1 19 x 100 mm xbridge column was used, with a flow rate of 17 ml/min (run time 16.5 min).

7.1.3 Materials

All commercially available reagents, solvents and Fmoc-protected amino acids were purchased and used without further purification.

7.2 Solid Phase Peptide Synthesis

7.2.1 General Procedures

All peptides were synthesised by use of microwave assisted couplings performed on a CEM Discovery microwave system unless otherwise indicated. In each of the reaction conditions listed, pressure is not included as peptide couplings take place in open vessels and therefore pressure cannot be altered from room pressure, 14.50 psi.

Procedure 1 Swelling of resin

All peptides were synthesised on Rink Amide AM resin with 0.62 mmol/g loading unless otherwise stated. The resin was swollen in DCM (7 ml) for 15 minutes and washed with DMF prior to use in subsequent steps.

Procedure 2 Fmoc deprotection conditions

Fmoc deprotection of peptides was achieved by addition of 5 ml of a 20 % piperidine in DMF solution to the resin in a SPPS microwave vessel, then reacted using the conditions outlined below:

Temperature 75 °C, Power 20 W, Time 3 minutes.

Following Fmoc deprotection, the resin was washed with DMF (5 x 5 ml) using a vacuum manifold.

Procedure 3 Peptide Coupling conditions (amide bond formation)

Coupling of peptides was achieved by addition of four equivalents of the amino acid, dissolved in DMF (2 ml) to which was added four equivalents of PyBOP™ in DMF (2 ml). The two solutions were mixed, and NMM (four equivalents) added. The solution was shaken and left four ten minutes before being added to deprotected resin in a SPPS microwave vessel, then reacted using the conditions outlined below:

Temperature 75 °C, Power 20 W, Time 10 minutes. Following coupling, the resin was washed with DMF (2 x 5 ml) using a vacuum manifold.

Procedure 4 Microwave Assisted Peptide Cleavage

To cleave a peptide from 500 mg of resin, TFA (9 ml), water (750 µl) and TIPS (750 µl) were added to the microwave vessel containing the resin.

Cleavage of peptides was achieved using the conditions outlined below:

Temperature 38 °C, Power 20 W, Time 18 minutes.

Following cleavage of the peptide, the solution-phase was filtered into a r.b.f. and the resin rinsed through with DCM to ensure complete removal of the peptide from the microwave vessel. Solvent was removed *en vacuo*, with addition of ether (5 x 10 ml) to ensure complete removal of TFA.

Procedure 5 Non-Microwave Assisted Peptide Cleavage

To cleave a peptide from 500 mg of resin, TFA (9 ml), water (750 µl) and TIPS (750 µl) were added to a microwave vessel containing the resin (in this instance the microwave vessel is used as a filter). The vessel was sealed and left to react at r.t for 3 h with stirring.

Following cleavage of the peptide, the solution-phase was filtered into a r.b.f. and the resin rinsed through with DCM to ensure complete removal of the peptide from the microwave vessel. Solvent was removed *en vacuo*, with addition of ether (5 x 10 ml) to ensure complete removal of TFA.

Procedure 6 Non-microwave-assisted Fmoc-deprotection

20% piperidine in DMF (5 ml) was added to the resin and shaken for five minutes. The resin was washed with DMF (5 x 5 ml) and 20% piperidine in DMF (5 ml) was added to the resin and shaken for five minutes. The resin was washed with DMF (5 x 5 ml) and 20% piperidine in DMF (5 ml) was added to the resin and shaken for five minutes. The resin was washed with DMF (5 x 5 ml) and used in the next step of the reaction.

Procedure 7 Peptide Purification

Following conformation of the presence of the desired peptide by MALDI-MS, peptides were purified by use of reverse phase RP-HPLC (RP-RP-HPLC). For analytical RP-HPLC, a 4.6 x

100 mm xbridge column was used, with a flow rate of 1 ml/min (run time 16.5 min). For Preparatory scale RP-HPLC, 1 19 x 100 mm xbridge column was used, with a flow rate of 17 ml/min (run time 16.5 min).

All peptides were purified gradually by RP-RP-HPLC, using the following method, with solution A, 95% Water 5% acetonitrile 0.1% TFA and solution B, 95% acetonitrile, 5% water and 0.1% TFA;

1) 100% solution A for 2 minutes (2ml/min)

2) 100% solution A to 100% solution B over 12 minutes (2ml/min)

3) 100% Solution B for 1 minute (2ml/min)

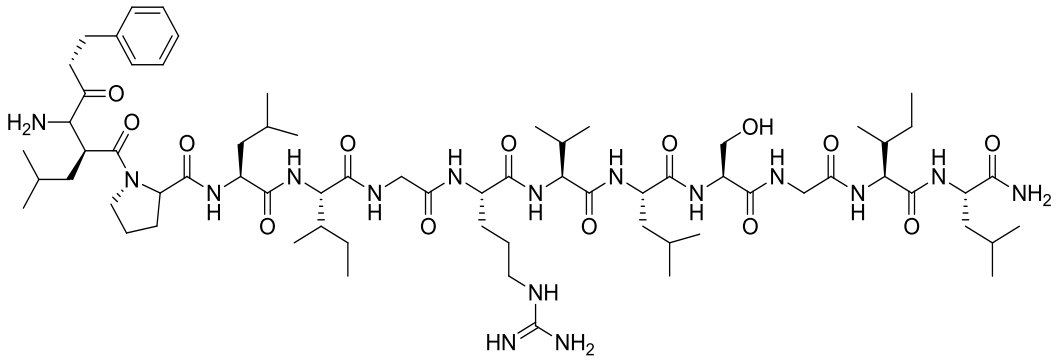
4) 100% solution B to 100% solution A over 1.5 minutes (2ml/min)

All peptides were dissolved in a mix of water and acetonitrile to a concentration of between 5 and 10mg/ml, with 1ml being injected for each run. A C18 reverse phase column was used. When between 3 and 6 runs for each peptide had been completed equivalent fractions were combined and freeze dried using procedure 16.

Analytical scale RP-HPLC was initially run for each peptide, in order to obtain an elution time for the individual peptides. On confirmation of the presence of the desired peptide, preparative scale RP-HPLC was subsequently run. UV-active fractions were collected and MS data was obtained for each fraction. Fractions containing the pure peptide were collected and solvent removed *en vacuo*. MALDI-MS was used to confirm the presence of the peptide and analytical-scale RP-HPLC run again to confirm the purity of the compound. The purified peptide was then lyophilised and stored under argon prior to use in biological testing.

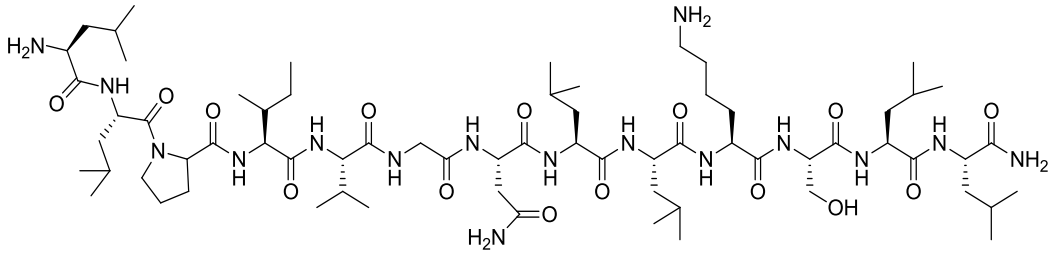
7.2.2 Synthesis of Temporins

7.2.2.1 Temporin A (**16**)



Synthesis of temporin A (**16**) with primary structure FLPLIGRVLSGIL-NH_2 was achieved following general procedures 1-4 (Section 7.2.1) suing 500 mg of Rink Amide resin (0.31 mmol). When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20% piperidine/DMF, and the peptide cleaved from resin by use of TFA (90%), TIPS (5%) and H_2O (5%) for four hours at room temperature. On confirmation of the presence of the correct structure, temporin A (**16**) was purified by use of RP-RP-HPLC (procedure 7) and dried by lyophilisation. The purity of temporin A (**16**) was confirmed by use of analytical RP-HPLC-MS (MALDI-TOF) *calcd* for $C_{68}H_{117}N_{17}O_{14}$ [M + H^+] 1396.8, found 1396.9. RP-HPLC: eluted at 9.05 minutes.

7.2.2.2 Temporin B (**17**)



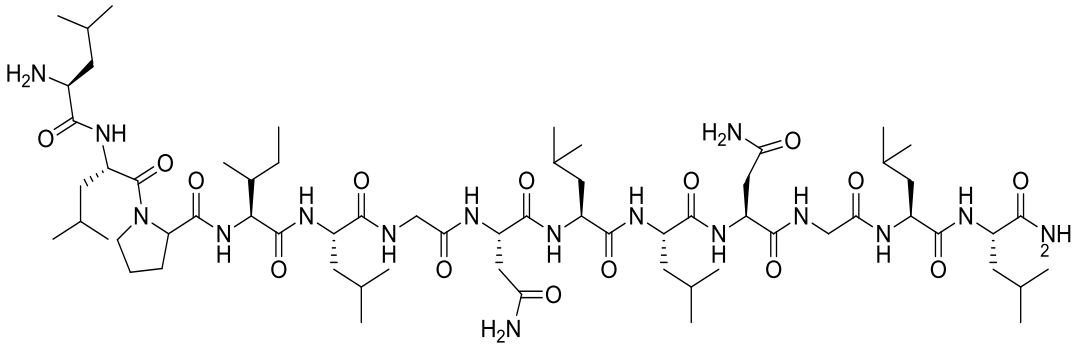
Synthesis of temporin B (**17**) with primary structure LLPIVGNLLKSLL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin.

When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20% piperidine/DMF, and the peptide cleaved from resin by use of TFA (90 %), TIPS (5 %) and H_2O (5 %) using microwave chemistry. On confirmation of the presence of the correct structure, temporin B was purified by use of RP-HPLC (procedure 7) and dried by lyophilisation. The purity of temporin B was confirmed by use of analytical RP-HPLC, and the compound subsequently used in biological testing.

MS (MALDI-TOF) *calcd* for $C_{67}H_{122}N_{16}O_{15}$ [M + Na^+] 1413.79, found 1413.8.

RP-HPLC: eluted at 9.41 minutes.

7.2.2.3 Temporin C (**29**)



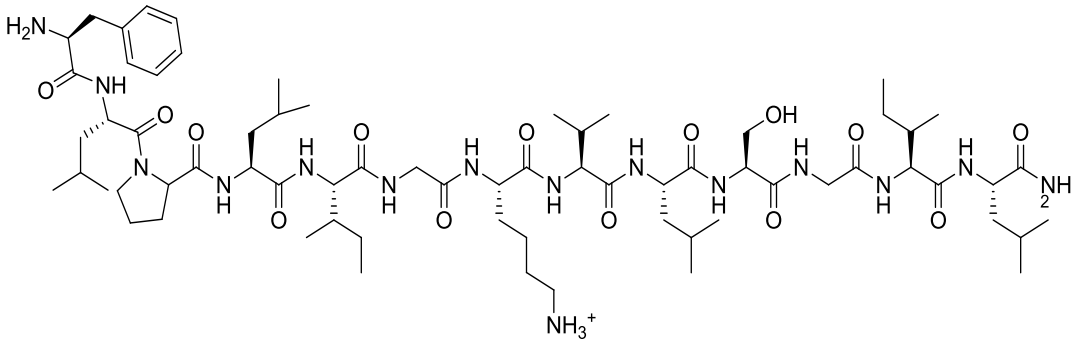
Synthesis of temporin C (**29**) with primary structure LLPILGNLLNGLL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin.

When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20 % piperidine/DMF, and the peptide cleaved from resin by use of TFA (90 %), TIPS (5 %) and H_2O (5 %) using microwave chemistry. On confirmation of the presence of the correct structure, Temporin C was purified by use of RP-HPLC and dried by lyophilisation. The purity of Temporin C was confirmed by use of analytical RP-HPLC, and the compound subsequently used in biological testing.

MS (MALDI-TOF) *calcd* for $C_{67}H_{122}N_{16}O_{15}$ [M + Na^+] 1384.0, found 1384.0.

RP-HPLC: eluted at 7.80 minutes.

7.2.2.4 Temporin F **(35)**



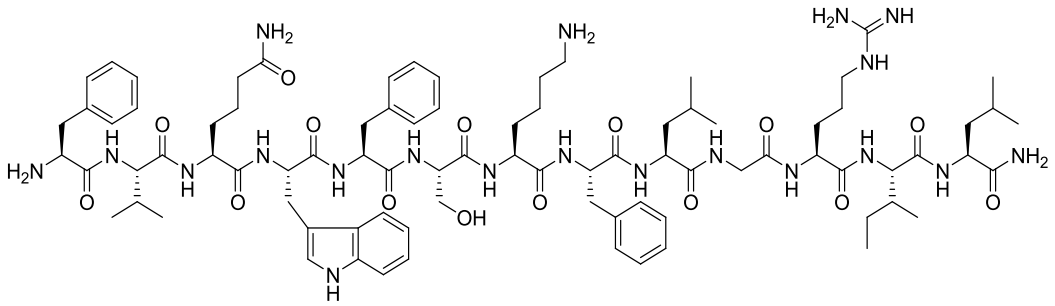
Synthesis of temporin F (**35**) with primary structure FLPLIGKVLSGIL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin.

When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20 % piperidine/DMF, and the peptide cleaved from resin by use of TFA (90 %), TIPS (5 %) and H_2O (5 %) using microwave chemistry. On confirmation of the presence of the correct structure, temporin F was purified by use of RP-HPLC (procedure 7) and dried by lyophilisation. The purity of temporin F was confirmed by use of analytical RP-HPLC, and the compound subsequently used in biological testing.

MS (MALDI-TOF) *calcd* for $C_{67}H_{125}N_{15}O_{15}$ [M + Na^+] 1391.0, found 1391.0.

RP-HPLC: eluted at 7.20 minutes.

7.2.2.5 Temporin L **(36)**

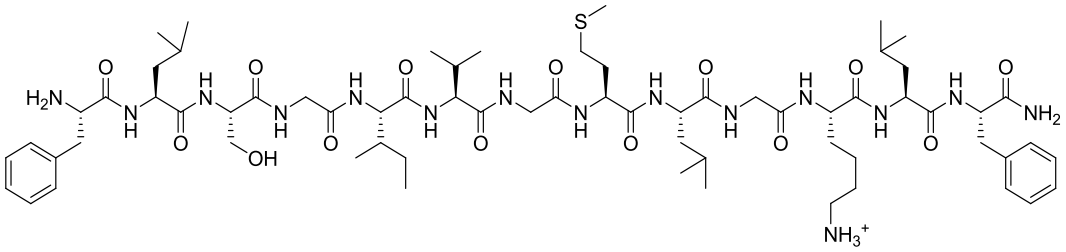


Synthesis of temporin L (**36**) with primary structure FVQWFSKFLGRIL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin. Following MALDI-MS analysis to confirm the correct structure of the peptide H_2N-WFSKFLGRIL-NH_2, the resin was split into two equal halves by weight and synthesis continued on one half to give the peptide FVQWFSKFLGRIL-NH_2. When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20 % piperidine/DMF, and the peptide cleaved from resin by use of TFA (90 %), TIPS (5 %) and H_2O (5 %) using microwave chemistry. On confirmation of the presence of the correct structure, temporin L was purified by use of RP-HPLC (procedure 7) and dried by lyophilisation. The purity of temporin L was confirmed by use of analytical RP-HPLC, and the compound subsequently used in biological testing.

MS (MALDI-TOF) *calcd.* for $C_{83}H_{122}N_{20}O_{15}$ [M + H^+] 1639.99, found 1640.1.

RP-HPLC: eluted at 7.79 minutes.

7.2.2.6 Temporin 1Sa **(25)**



25

Synthesis of temporin 1Sa (25) with primary structure FLSGIVGMLGKLF-NH_2 was achieved following SPPS procedures 1-4 with 250 mg of Rink Amide resin.

When all amino acids were coupled, a final Fmoc deprotection step was carried out by use of 20 % piperidine/DMF, and the peptide cleaved from resin by use of TFA (90 %), TIPS (5 %) and H_2O (5 %) using microwave chemistry. On confirmation of the presence of the correct structure, temporin B was purified by use of RP-HPLC (Procedure 7) and dried by lyophilisation. The purity of temporin B was confirmed by use of analytical RP-HPLC, and the compound subsequently used in biological testing.

MS (MALDI-TOF) *calcd* for $C_{67}H_{110}H_{15}O_{14}S$, 1381.1, found 1381.2 [$M+H^+$].

RP-HPLC: eluted at 8.92 minutes.

7.3 Synthesis of fluorescently labelled peptides

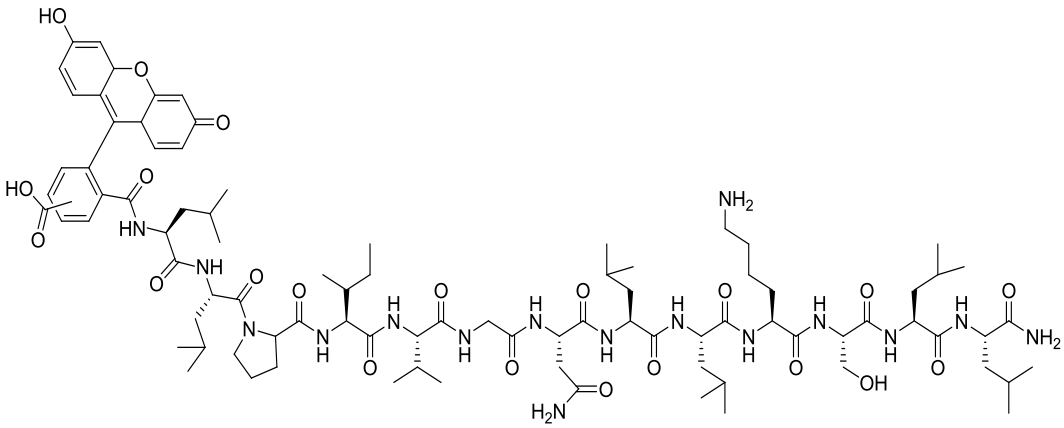
7.3.1 General information

Temporins A and B were synthesised by use of standard microwave SPPS, and labelled with fluorescein, and tetramethylrhodamine through peptide coupling procedure 3. Peptides were subsequently purified by use of RP-HPLC prior to use in biological testing

Synthesis of 5-6-carboxyfluorescein-Temporin B (FAM-TB) (**37**), structure FAM-LLPIVGNLLKSLL-NH_2, is achieved by coupling TB (**17**) with 5-6-carboxyfluorescein (FAM). One third of the Rink Amide resin (133 mg) + Fmoc-LLPIVGNLLKSLL-NH_2 peptide synthesised in the Temporin B (Microwave) section was deprotected following Fmoc-SPPS Procedure 2. The deprotected resin was then coupled with FAM following Fmoc-SPPS Procedure 3, with FAM used in place of the amino acid. Equivalents were scaled appropriately to the 133 mg of Rink Amide resin. The fluorescent peptide was then cleaved from resin following Fmoc-SPPS Procedure 5 and isolated as a yellow solid.

Synthesis was checked, where a small amount of LLPIVGNLLKSLL-NH_2 and FAM-LLPIVGNLLKSLL-NH_2 peptides + resin were cleaved from resin for MALDI-ToF analysis following Fmoc-SPPS Procedure 6. LLPIVGNLLKSLL-NH_2 spectrum MALDI-ToF (MS) *calcd* for $C_{67}H_{122}N_{16}O_{15}H^+$ [M + H^+] 1391.8 found: 1413.8 [M + Na^+]. FAM-LLPIVGNLLKSLL-NH_2 MALDI-ToF (MS) *calcd* for $C_{87}H_{132}N_{16}O_{19}H^+$ 1749.1 [M + H^+], found: 1750.4 [M + H^+] as a minor peak. Major peaks were observed corresponding to unreacted LLPIVGNLLKSLL-NH_2 [M + H^+] and [M + Na^+]. RP-HPLC analysis: LLPIVGNLLKSLL-NH_2 was observed with 9.41 min retention. FAM- LLPIVGNLLKSLL-NH_2 was observed with 11.63 min retention and ES+ (MS) for doubly charged fragments at 696.64.

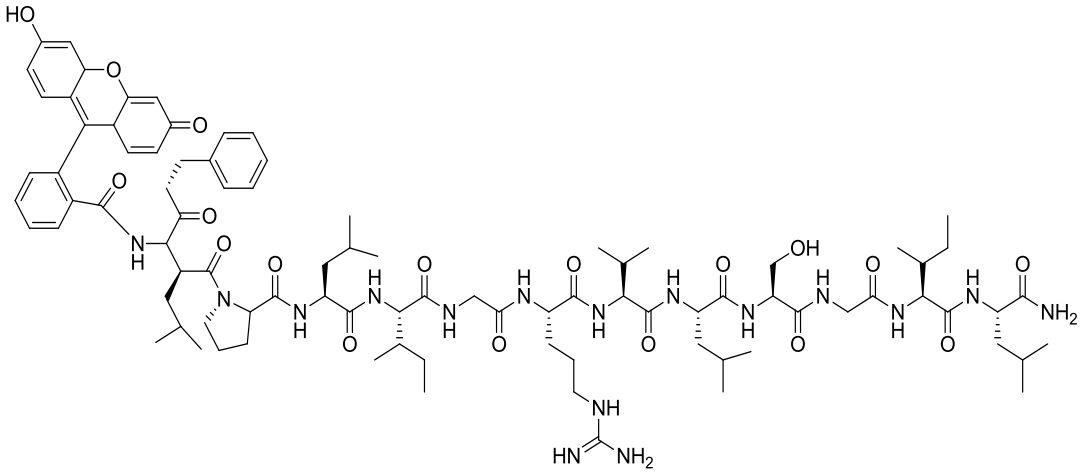
7.3.2 Synthesis of Temporin B – Fluorescein (**37**)



Synthesis of temporin B (17) with primary structure LLPIVGNLLKSLL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin as described I section 6.3.1 above. Synthesis of 5-6-carboxyfluorescein-Temporin B (FAM-TB) (34), structure FAM-LLPIVGNLLKSLL-NH_2, was achieved by coupling temporin B (18) with 5-6-carboxyfluorescein (FAM). One third of the Rink Amide resin (133 mg) + Fmoc-LLPIVGNLLKSLL-NH_2 peptide synthesised in the Temporin B (Microwave) section was deprotected following Fmoc-SPPS Procedure 2. The deprotected resin was then coupled with FAM following Fmoc-SPPS Procedure 3, with FAM in place of the amino acid. Equivalents were scaled appropriately to the 133 mg of Rink Amide resin. The fluorescent peptide was cleaved from resin following Fmoc-SPPS Procedure 5 and isolated as a yellow solid. The peptide was cleaved following microwave cleavage procedure 4. LLPIVGNLLKSLL-NH_2 spectrum MALDI-ToF (MS) *calcd* for $C_{67}H_{122}N_{16}O_{15}H^+$ [M + H^+] 1391.8 found: 1413.8 [M + Na^+]. FAM-LLPIVGNLLKSLL-NH_2 MALDI-ToF (MS) *calcd* for $C_{87}H_{132}N_{16}O_{19}H^+$ 1749.1 [M + H^+], found: 1750.4 [M + H^+] as a minor peak. Major peaks were observed corresponding to unreacted LLPIVGNLLKSLL-NH_2 [M + H^+] and [M + Na^+]. RP-HPLC analysis: LLPIVGNLLKSLL-NH_2 was observed with 9.41 min retention. FAM-LLPIVGNLLKSLL-NH_2 was observed with 11.63 min retention and ES+ (MS) for doubly

charged fragments at 696.64 MS (MALDI-TOF) *calcd* for $C_{67}H_{122}N_{16}O_{15}$ [M + H^+] 1391.79, found 1391.8. RP-HPLC: eluted at 11.63 minutes.

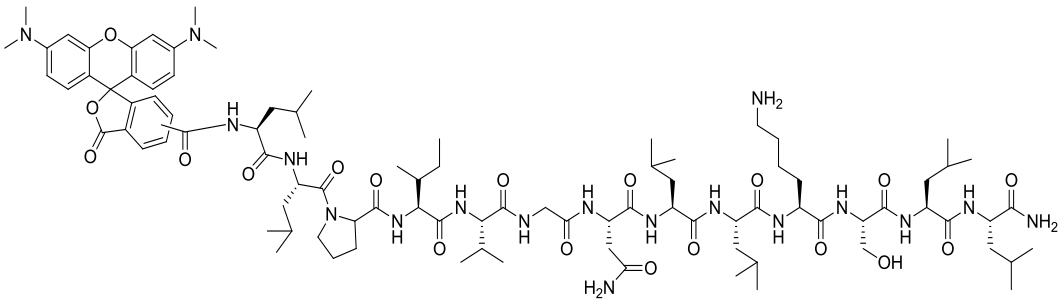
7.3.3 Temporin A-fluorescein (**39**)



Synthesis of temporin A (**16**) with primary structure FLPLIGRVLSGIL-NH_2 -NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin as described I section 6.3.1 above. Synthesis of 5-6-carboxyfluorescein-temporin A (FAM-TA) (**39**), structure FAM- FLPLIGRVLSGIL-NH_2, was achieved by coupling temporin A (**16**) with 5-6-carboxyfluorescein (FAM). One third of the Rink Amide resin (133 mg) + Fmoc-LLPIVGNLLKSLL-NH_2 peptide synthesised in the temporin A (Microwave) section was deprotected following Fmoc-SPPS Procedure 2. The deprotected resin was then coupled with FAM following Fmoc-SPPS Procedure 3, with FAM in place of the amino acid. Equivalents were scaled appropriately to the 133 mg of Rink Amide resin. The fluorescent peptide was cleaved from resin following Fmoc-SPPS Procedure 5 and isolated as a yellow solid. The peptide was cleaved following microwave cleavage procedure 4. FLPLIGRVLSGIL-NH_2 spectrum MALDI-ToF (MS) *calcd* for $C_{68}H_{117}N_{17}O_{14}$ H^+[M + H^+] 1396.7found: 1396.7 [M +

Na^+]. FAM- FLPLIGRVLSGIL -NH_2 MALDI-ToF (MS) *calcd* for $C_{88}H_{137}N_{17}O_{18}$ H^+ 1754.8 [M + H^+], found: 1754.8 [M + H^+] as a minor peak. Major peaks were observed corresponding to unreacted FLPLIGRVLSGIL-NH_2 [M + H^+] and [M + Na^+]. RP-HPLC analysis: FLPLIGRVLSGIL -NH_2 was observed with 9.05 min retention. FAM-FLPLIGRVLSGIL-NH_2 was observed with 11.19 min retention and ES+ (MS) for doubly charged fragments at 877.4 MS (MALDI-TOF) *calcd* for $C_{88}H_{137}N_{17}O_{18}$ [M + H^+] 1754.8, found 1754.8. RP-HPLC: eluted at 11.19 minutes.

7.3.4 Temporin B-Tetramethylrhodamine (**41**)

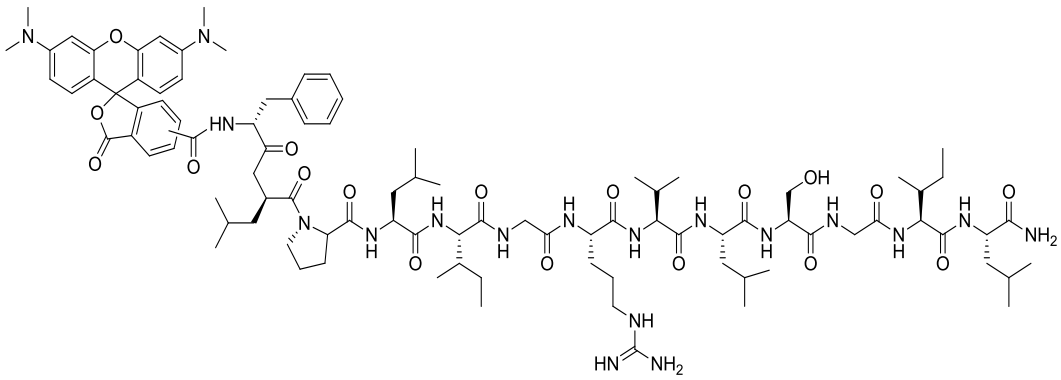


Synthesis of temporin B (**17**) with primary structure LLPIVGNLLKSLL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin as described I section 6.3.1 above. Synthesis of 5-6-carboxytetramethylrhodamine-temporin B (TMR-TB- (**41**), structure TMR-LLPIVGNLLKSLL-NH_2, was achieved by coupling temporin B (**17**) with 5-6-carboxytetrafluororhodamine (TMR). One third of the Rink Amide resin (133 mg) + Fmoc-LLPIVGNLLKSLL-NH_2 peptide synthesised in the temporin B (Microwave) section was deprotected following Fmoc-SPPS Procedure 2. The deprotected resin was then coupled with FAM following Fmoc-SPPS Procedure 3, with FAM in place of the amino acid. Equivalents were scaled appropriately to the 133 mg of Rink Amide resin. The fluorescent peptide was

cleaved from resin following Fmoc-SPPS Procedure 5 and isolated as a red solid. The peptide was cleaved following microwave cleavage procedure 4.

MALDI-ToF (MS) *calcd* for $C_{67}H_{122}N_{16}O_{15}H^+$ [M + H^+] 1391.8 found: 1413.8 [M + Na^+]. TMR-LLPIVGNLLKSLL-NH_2 MALDI-ToF (MS) *calcd* for $C_{92}H_{144}N_{18}O_{30}H^+$ 1821.4 [M + H^+], found: 1821.7 [M + H^+] as a minor peak. Major peaks were observed corresponding to unreacted LLPIVGNLLKSLL-NH_2 [M + H^+] and [M + Na^+]. RP-RP-HPLC analysis: LLPIVGNLLKSLL-NH_2 was observed with 9.41 min retention. FAM- LLPIVGNLLKSLL-NH_2 was observed with 11.13 min retention and ES+ (MS) for doubly charged fragments at 910.7, RP-HPLC: eluted at 10.32 minutes.

7.3.5 Temporin A-Tetramethylrhodamine (**40**)



Synthesis of temporin A (**16**) with primary structure FLPLIGRVLSGIL-NH_2 was achieved following SPPS procedures 1-4 with 500 mg of Rink Amide resin as described I section 6.3.1 above. Synthesis of 5-6-carboxytetramethylrhodamine-temporin A (TMR-TA) (**40**), structure TMR- FLPLIGRVLSGIL-NH_2, was achieved by coupling temporin A (**16**) with 5-6-carboxytetrafluororhodamine (TMR). One third of the Rink Amide resin (133 mg) + Fmoc-FLPLIGRVLSGIL-NH_2 peptide synthesised in the temporin A (Microwave) section was deprotected following Fmoc-SPPS Procedure 2. The deprotected resin was then coupled with TMR following Fmoc-SPPS Procedure 3, with TMR in place of the amino acid. Equivalents were scaled appropriately to the 133 mg of Rink Amide resin. The fluorescent peptide was cleaved from resin following Fmoc-SPPS Procedure 5 and isolated as a red solid. The peptide was cleaved following microwave cleavage procedure 4. FLPLIGRVLSGIL-NH_2 spectrum MALDI-ToF (MS) *calcd* for $C_{68}H_{117}N_{17}O_{14}$ H^+ [M + H^+] 1396.77 found: 1396.7 [M + H^+]. TMR- FLPLIGRVLSGIL-NH_2 MALDI-ToF (MS) *calcd* for $C_{93}H_{139}N_{19}O_{19}H^+$ 1827.15 [M + H^+], found: 1827.2 [M + H^+] as a minor peak. Major peaks were observed corresponding to unreacted FLPLIGRVLSGIL-NH_2 [M + H^+] and [M + Na^+]. RP-HPLC analysis: FLPLIGRVLSGIL-NH_2 was observed with 9.05 min retention. FAM-FLPLIGRVLSGIL -NH_2 was observed with 10.23 min retention and ES+ (MS) for doubly charged fragments at 913.5 MS RP-HPLC: eluted at 110.23 minutes.

7.4 Synthesis of Ciliatamides and Ciliatamide analogs[2]

7.4.1 General Procedures

Procedure 1: Preparation of Fmoc- α-amino Caprolactams

Fmoc-Lys-(Boc)-OH (**51**) (1.00 mmol) was added to TFA (10 ml) and DCM (10 ml) and reacted at r.t with stirring for 3 h. The solvent was removed *en vacuo* and co evaporated with ether (3 x 10 ml) to ensure complete removal of TFA. The resulting yellow oil was dissolved in DCM (10 ml) to which was added PyBOP (0.95 mmol, 454 mg) and NMM, (0.95 mmol, x mg). The reaction was left for 18 h at r.t. with stirring. Solvent was removed *en vacuo* to give the crude product. Purification took place by use of column chromatography.

Procedure 2: Preparation of *N*-methylated amino acids

Boc-Phe-OH (500 mg, 1.90 mmol) was dissolved in dry THF (~5 ml). The solution was cooled to 0°C. NaH (60 %) in mineral oil) (535 mg, 13.2 mmol) and methyliodide (940 µl, 15.20 mmol) were added slowly to the solution. The resulting mixture was stirred at 25°C for 24 h. On completion of the reaction the mixture was poured onto H_2O (~20 ml). THF was removed *en vacuo*. The mineral oil from the NaH was extracted by use of hexane (2 x 7 ml). The aqueous layer was acidified to pH 3 by use of 5 % citric acid. The product was extracted by use of EtOAc (3 x 25 ml), the EtOAc layer was then dried (Na_2SO_4) and filtered. Solvent was removed *en vacuo* to give the crude product. Purification took place by use of silica gel column chromatography.

Procedure 3: Preparation of di-peptides

A magnetically stirred solution of the Fmoc protected α-amino caprolactam (1.00 mmol) was dissolved in dry THF at 25 °C under Argon, to which was added piperidine (3 ml). After 3 h, the reaction mixture was concentrated *en vacuo* to afford the α-amino caprolactam, a pale yellow solid used without purification in the next step of the reaction sequence. A magnetically stirred solution of the *N*-methylated phenylalanine (1.0 mmol) was dissolved in DMF (5 ml), to which was added PyBOP (0.95 mmol) and NMM (2.0 mmol). After 30 minutes, the reaction mixture was cooled to 0°C and treated drop wise via cannula with a solution of the Fmoc deprotected α-amino caprolactam dissolved in DMF (8 ml). The reaction was left to react for 18 h at r.t. After 18 h, the mixture was diluted with HCl (5 ml of a 5 %v/v aq. Solution and extracted by use of diethyl ether (3 x 25 ml) and brine (1 x 10 ml) before being dried (MgSO4), filtered and concentrated *en vacuo* to give a light yellow oil.

Procedure 4: Attachment of lipid tails to Boc-protected di-peptides.

The Boc-protected dipeptide (1.00 mmol) was dissolved in DCM (2.5 ml). TFA (2.5 ml) was added and the resulting solution stirred for 2 h. On completion of the reaction, the solvent was removed *en vacuo*. Co-evaporation with ether was carried out three times to ensure complete removal of TFA. The resulting TFA salt was dissolved in DCM (5 ml). Octonylchloride (1.50 mmol) and TEA (3.00 mmol) were added to the solution. The resulting solution was stirred at 25 °C for 24 h. On completion of the reaction solvent was removed *en vacuo*.

Procedure 1a: Acetylation of dipeptides

The Boc-protected peptide (1.0 mmol, 1.0 eq.) was dissolved in DCM (2.5 mL) and TFA (3 mL) was then added and the resulting solution was stirred for 2 h at rt. The solvent was

removed *en vacuo* on completion of the reaction by co-evaporation with excess ether (x 5) The resulting crude TFA salt was dissolved in DCM (5 mL) and the acid chloride (1.5 mmol, 1.5 eq.) and Et_3N (3.0 mmol, 3 eq.) were then added to the solution. The resulting solution was stirred at 25 °C for 24 h. Solvent was removed *en vacuo* on completion of reaction to give the crude product which is purified by column chromatography on silica gel.

Procedure 2a: Coupling of peptide with cycloheptylamine

The Boc-protected peptide (1.0 mmol, 1.0 eq.) was dissolved in DCM (5 mL). PyBOP (1.0 mmol, 1.0 eq.) in DCM and DIPEA (2.0 mmol, 2.0 eq.) were added to the solution with stirring under inert conditions. After 30 minutes of stirring at room temperature, the mixture was cooled, using an ice bath, to 0 °C. Cycloheptylamine (1.2 mmol, 1.2 eq.) was added to the mixture via syringe and the resulting mixture was then stirred, whilst warming to room temperature, for 18 h. The solvent was removed *en vacuo*, to give the crude product, which was purified by column chromatography on silica gel.

Procedure 3a: Coupling of peptide with cyclohexylamine

The Boc-protected peptide (1.0 mmol, 1.0 eq.) was dissolved in DCM (5 mL). PyBOP (1.0 mmol, 1.0 eq.) in DCM and DIPEA (2.0 mmol, 2.0 eq.) were added to the solution with stirring under inert conditions. After 30 minutes of stirring at rt, the mixture was cooled, using an ice bath, to 0 °C. Cyclohexylamine (1.2 mmol, 1.2 eq.) was then added to the mixture via syringe. The resulting mixture was then stirred, whilst warming to room temperature, for 18 h. The solvent was removed *en vacuo* to give the crude product, which was purified by column chromatography on silica gel.

Procedure 5a: Coupling of acid with amino acid preloaded wang resin

DMF was added to the resin (1.0 mmol, 1.0 eq.) and left to stir at rt for 5 minutes to allow the resin to swell. The DMF was then removed *en vacuo*. The resin linker was then deprotected (Fmoc cleavage) by addition of 20% piperidine in DMF in excess with stirring for 5 minutes at rt. (x3) After each 5 minute stirring period, the piperidine was removed *en vacuo* and the resin was washed thoroughly with DMF.

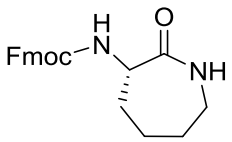
The acid (4.0 mmol, 4.0 eq.) was activated in a separate vessel by addition of TBTU (4.0 mmol, 4.0 eq.) and DIPEA (4.0 mmol, 4.0 eq.) with thorough mixing. The activated acid was then added to the deprotected, washed resin linker and just enough DMF was added to ensure all components were in solution. Coupling-3 programme was used in the manual microwave to couple the acid with the resin. The column was then washed through with DCM to remove any amine by-products.

Procedure 6a: Cleavage of peptide from resin

For 50 mg of resin, an 18:1:1 mixture of trifluoroacetic acid (TFA)(0.9 ml): water (0.05 mL): triisopropylsilane (TIPS) (0.05 mL) was added to the resin to cleave the peptide from the resin. Protecting groups that are acid sensitive are also removed from side chains in this step. The vessel was then left for 2 h, stoppered, with occasional agitation and the mixture filtered to remove the resin. The TFA solution was then partially removed by rotary evaporation (47 ^{o}C, 10 mbarr). The TFA was further removed by coevaporation with diethyl ether (6 ml), *en vacuo* (47 ^{o}C, 10 mbarr). Once a wet solid had formed, diethyl ether (6 ml) was added to precipitate out the peptide. To ensure complete removal of TFA and any remaining coupling agents, the excess diethyl ether was carefully pipetted off to leave the solid peptide in the bottom of the vessel. Diethyl ether (3 ml) was then added and removed *en vacuo* (47 ^{o}C, 10 mbarr) to give the peptide as a white solid.

7.4.2 Synthesis of Ciliatamide analogs and precursors

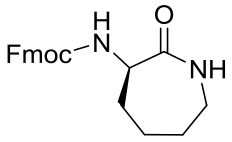
(*S*)-(9H-fluoren-9-yl)methyl 2-oxoazepan-3-ylcarbamate **(53)**



53

Fmoc protected α-amino caprolactam (**53**) was synthesised on a 1.07 mmol scale according to amide coupling Procedure 1. Purification via column chromatography (SiO_2 3:1 EtOAc /EtOAc, R_f 0.45 (EtOAc)) yielded **53** as a white powder (257 mg, 69%). δ_H (400 MHz; $CDCl_3$): 7.95-7.27 (10H, m, NH, Ar); 4.95 (2H, s, CH_2-O(C=O)N); 4.04-4.02 (1H, m, NH-C<u>H</u>) 4.15-4.05 (1H, m, CH2-C<u>H</u>); 3.35 – 3.20 (1H, m*,C<u>H</u>NHCH$_2$); 2.10-2.00 (2H, S, CH_2-O(C=O)); 1.31-1.22 (6H, m, CH_2, Lactam ring); δ_H ^{13}C (400 MHz; $CDCl_3$): 191.9 (C=O (Lactam)); 173.4 (C=O)O); 128.4-119.1 (C-Ar); 68.7 (<u>C</u>H$_2$O(C=O)); 52.5 (CH-NH); 47.1 (<u>C</u>H-CH$_2$O(C=O)); 40.1 (<u>C</u>H$_2$-NH(C=O)-NH); 32.5-22.4 (CH_2 (Lactam)); LRMS (ES+) *calcd* for $C_{21}H_{22}N_2O_3$ [M+H$^+$] 351.16, found 351.2. HRMS: *calcd.* 351.1709 (for $C_{21}H_{23}N_2O_3$), found 351.1710 [M+H$^+$]. Data obtained matches that in available literature[1].

(*R*)-(9H-fluoren-9-yl)methyl 2-oxoazepan-3-ylcarbamate **52**

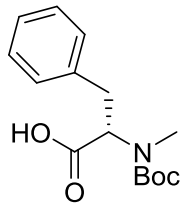


52

Fmoc protected α-amino caprolactam (**52**) was synthesised on a 1.07 mmol scale according to amide coupling procedure 1. The crude product was purified by column chromatography (SiO_2 3:1 EtOAc /EtOAc, R_f 0.45 (EtOAc))) gave **53** as a white powder (278 mg, 74 %). δ_H (400 MHz; $CDCl_3$): 7.95-7.27 (10H, m, NH, Ar)4.95 (2H, s, CH_2-O(C=O)N); 4.04-4.02 (1H,

m, NH-C<u>H</u>) 4.15-4.05 (1H, m, CH2-C<u>H</u>); 3.35 – 3.20 (1H, m,C<u>H</u>NHCH$_2$); 2.10-2.00 (2H, S, CH$_2$-O(C=O)); 1.31-1.22 (6H, m, CH$_2$, lactam ring); δ_H ^{13}C (400 MHz; CDCl$_3$): 191.9 (C=O (Lactam)); 173.4 (C=O)O); 128.4-119.1 (C-Ar); 68.7 (<u>C</u>H$_2$O(C=O)); 52.5 (CH-NH); 47.1 (<u>C</u>H-CH2O(C=O)); 40.1 (<u>C</u>H$_2$-NH(C=O)-NH); 32.5-22.4 (CH$_2$ (Lactam)); LRMS (ES+) *calcd* for $C_{21}H_{22}N_2O_3$ [M+H$^+$] 351.16, found 351.2. HRMS: *calcd.* 351.1709 (for $C_{21}H_{23}N_2O_3$), found 351.1709 [M+H$^+$]. Data obtained matched that in available literature[1].

(*S*)-2-(*tert*-butoxycarbonyl(methyl)amino)-3-phenylpropanoic acid (**44**)



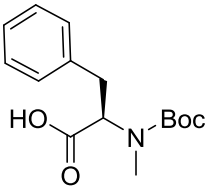
44

Boc-L-phenylalanine (500 mg, 1.90 mmol) was dissolved in dry THF (~5 ml). The solution was cooled to 0°C. NaH (60 %) in mineral oil) (535 mg, 13.2 mmol) and methyliodide (940 µl, 15.20 mmol) were added slowly to the solution. The resulting mixture was stirred at 25°C for 24 h. On completion of the reaction the mixture was poured onto H_2O (~20 ml). THF was removed *en vacuo*. The mineral oil from the NaH was extracted by use of hexane (2 x 7 ml). The aqueous layer was acidified to pH 3 by use of 5 % citric acid. **44** was extracted by use of EtOAc, (3 x 25 ml) the EtOAc layer was then dried (Na_2SO_4) and filtered. Solvent was removed *en vacuo* to give a dark orange oil. Column chromatography (SiO_2 1:1 hexane : EtOAc, R_f 0.46) afforded **44** as a yellow oil (321 mg, 61 %). δ_H (400 MHz; $CDCl_3$): 9.51 (1H, bs, OH); 7.28-7.10 (5H, m, 5 x Ar-H); 4.78 - 4.49 (1H, multiple signals*, H_α); 3.20 (1H, m, Ar-C<u>H$_a$</u>H$_b$); 2.93 (1H, m, Ar-CH$_a$<u>H$_b$</u>) 2.75-2.65 (3H, multiple signals*, HCH$_3$); 1.38-1.10 (9H, multiple signals*, C(CH$_3$)$_3$); δ_C (400 MHz; $CDCl_3$)*: 202.8, 232.4, 129.4, 129.0, 128.91, 128.6, 128.5, 126.8, 126.7, 107.6, 103.4, 90.9, 61.5, 60.5, 60.3, 45.4, 37.1, 35.3, 34.7,

32.7, 32.5, 28.3, 28.3, 21.1; LRMS (ES+) *calcd.* for $C_{15}H_{21}NO_4$ [M^-] 278.15, found 278.2. HRMS: *calcd.* 278.1392 (for $C_{15}H_{20}NO_4$), found 278.1401 [M^-].

*Doubling of peaks observed due to the presence of rotamers.

(*R*)-2-(*tert*-butoxycarbonyl(methyl)amino)-3-phenylpropanoic acid (**48**)

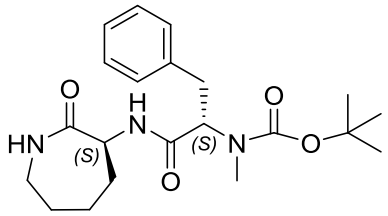


48

Boc-D-phenylalanine (500 mg, 1.90 mmol) was dissolved in dry THF (~5 ml). The solution was cooled to 0°C. NaH (60 %) in mineral oil) (535 mg, 13.2 mmol) and methyliodide (940 µl, 15.20 mmol) were added slowly to the solution. The resulting mixture was stirred at 25°C for 24 h. On completion of the reaction the mixture was poured onto H_2O (~20 ml). THF was removed *en vacuo*. The mineral oil from the NaH was extracted by use of hexane (2 x 7 ml). The aqueous layer was acidified to pH 3 by use of 5 % citric acid. **48** was extracted by use of EtOAc (3 x 25 ml), the EtOAc layer was then dried (Na_2SO_4) and filtered. Solvent was removed *en vacuo* to give the crude product **48** as a dark orange oil (427 mg, 81 %) The crude product was purified by column chromatography (SiO_2 1:1 hexane : EtOAc, R_f 0.46) gave **48** as a yellow oil (427 mg, 81 %). δ_H (400 MHz; $CDCl_3$): 10.37 (1H, bs, OH); 7.23-7.09 (5H, m, 5 x Ar-H); 4.79 - 4.52 (1H, multiple signals*, H_α); 3.34 (1H, m, Ar-C$\underline{H}_a$H$_b$); 3.05 (1H, m, Ar-CH$_a\underline{H}_b$) 2.82-2.64 (3H, multiple signals*, HCH_3); 1.38-1.16 (9H, multiple signals*, $C(CH_3)_3$); δ_C (400 MHz; $CDCl_3$)*: 128.9, 128.9, 128.6, 128.5, 126.7, 126.7, 61.5, 60.6, 60.4, 35.3, 34.7, 32.9, 32.5, 28.3, 28.2, 21.1; LRMS (ES+) *calcd* for $C_{15}H_{21}NO_4$ [M^-] 278.15, found 278.1. HRMS: *calcd.* 278.1392 (for $C_{15}H_{20}NO_4$), found 278.1405 [M^-]

*Doubling of peaks observed due to the presence of rotamers.

(S)-2-(tert-butoxycarbonyl(methyl)amino)-3-phenylpropanoic acid (**45**)

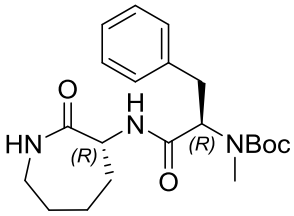


45

L-α-amino-ε-caprolactam **53** was coupled to methylated Boc-D-phenylalanine **44** on a 0.56 mmol scale following amide coupling procedure 3. The crude product was purified by column chromatography (SiO_2 3:1 hexane: EtOAc to 100% EtOAc, R_f EtOAc 0.45) gave **45** as a white solid (137 mg, 60 %). δ_H (400 MHz; $CDCl_3$): 7.71 (1H, s, NH); 7.45-7.10 (5H, m, Ar-H); 5.03-4.71 (1H, bs, multiple signals*, H_α (Phe)); 4.35-4.13 (1H, m, H_α (Lactam)); 3.41 (3H, s*, NCH_3); 2.9 (2H, m, CH_2); 2.80 (2H, m, CH_2); 1.98-1.80 (6H, m, CH_2 (caprolactam ring)); 1.22-1.10 (9H, d, $C(CH_3)_3$); δ_C (400 MHz; $CDCl_3$): 177.09 ((C=O) caprolactam); 169.99 (NH(C=O)); 156.85 ((C=O)O); 129.97-129.10 (C-Ar); 81.33 ($\underline{C}(CH_3)_3$); 62.50 ((C=O)$\underline{C}H_2N$); 60.67 (C_α (Lactam); 41.00 (CH_2NH); 35.07 (CH_2); 34.00-27.34 (CH_2 (Lactam), $C(\underline{C}H_3)_3$, NCH_3); LRMS (ES+) *calcd* for $C_{15}H_{21}NO_4$ [M + H^+] 389.23, found 390.1. HRMS: *calcd* for $C_{15}H_{21}NO_4Na^+$ [M + Na^+] 412.2701, found 412.2014 [M+Na^+].

*Doubling of peaks observed due to the presence of rotamers.

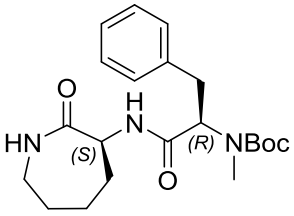
Tert-butyl-methyl((R)-azepan-2-one-3-ylamino-(S)-oxo-3-phenylpropan-2-yl)carbamate (**56**)



D-α-amino-ε-caprolactam **53** was coupled to methylated Boc-D-phenylalanine **48** on a 1.23 mmol scale following amide coupling procedure 3. The crude product was purified by column chromatography (SiO_2 3:1 hexane: EtOAc to 100% EtOAc, R_f EtOAc 0.45) gave **56** as a white powder (254 mg, 76 %). δ_H (400 MHz; $CDCl_3$): 7.89 (1H, s, NH); 7.31-7.13 (5H, m, Ar-H); 4.92-4.78 (1H, multiple signals*, H_α (Phe)); 4.21-4.09 (1H, m, H_α (Lactam)); 3.11 (3H, s*, NCH_3); 2.80 (2H, m, CH_2); 2.91 (2H, m, CH_2); 1.98-1.80 (6H, m, CH_2 (caprolactam ring)); 1.21-1.13 (9H, d, $C(CH_3)_3$); δ_C (400 MHz; $CDCl_3$): 177.09 ((C=O) caprolactam); 169.99 (NH(C=O)); 156.85 ((C=O)O); 129.97-129.10 (C-Ar); 81.33 ($\underline{C}(CH_3)_3$); 62.50 ((C=O)$\underline{C}H_2N$); 60.67 (C_α (Lactam); 41.00 (CH_2NH); 35.07 (CH_2); 34.00-27.34 (CH_2 (Lactam), C($\underline{C}$H3)3, NCH_3); LRMS (ES+) *calcd* for $C_{15}H_{21}NO_4$ [M + Na^+] 412.22, found 412.0. HRMS: *calcd* for $C_{15}H_{21}NO_4Na^+$ [M + Na^+] 412.2701, found 412.2933 [M+Na^+].

*Doubling of peaks observed due to the presence of rotamers.

Tert-butyl methyl((S)-azepan-2-one-3-ylamino-(R)-oxo-3-phenylpropan-2-yl)carbamate (**57**)

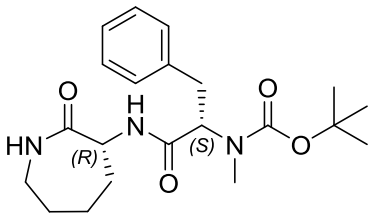


L-α-amino-ε-caprolactam **53** was coupled to methylated Boc-D-phenylalanine **48** on a 1.23 mmol scale following amide coupling procedure 3. The crude product was purified by

column chromatography (SiO_2 3:1 hexane: EtOAc to 100% EtOAc, R_f EtOAc 0.45) gave **57** as a white powder (214 g, 63 %). δ_H (400 MHz; $CDCl_3$): 7.70 (1H, s, NH); 7.28-7.05 (5H, m, Ar-H); 4.95-4.85 (1H, multiple signals*, H_α (Phe)); 4.23-4.11 (1H, m, H_α (Lactam)); 3.10 (3H, s*, NCH_3); 2.9 (2H, m, CH_2); 2.80 (2H, m, CH_2); 1.98-1.80 (6H, m, CH_2 (caprolactam ring)); 1.23-1.10 (9H, d, $C(CH_3)_3$); δ_C (400 MHz; $CDCl_3$): 177.09 ((C=O) caprolactam); 169.99 (NH(C=O)); 156.85 ((C=O)O); 129.97-129.10 (C-Ar); 81.33 ($\underline{C}(CH_3)_3$); 62.50 ((C=O)$\underline{C}H_2N$); 60.67 (C_α (Lactam); 41.00 (CH_2NH); 35.07 (CH_2); 34.00-27.34 (CH_2 (Lactam), $C(\underline{C}H3)_3$, NCH_3);LRMS (ES+) *calcd* for $C_{15}H_{21}NO_4$ [M + Na^+] 412.2, found 412.2. HRMS: HRMS: *calcd* for $C_{15}H_{21}NO_4Na^+$ [M + Na^+] 412.2701, found 412.3107 [$M+Na^+$].

*Doubling of peaks observed due to the presence of rotamers.

Tert-butyl methyl((R)-azepan-2-one-3-ylamino-(S)-oxo-3-phenylpropan-2-yl)carbamate (**58**)

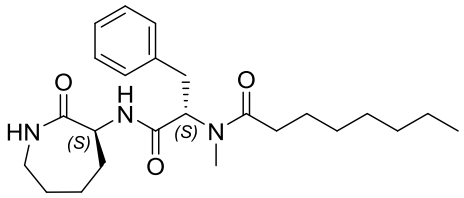


58

D-α-amino-ε-caprolactam **53** was coupled to methylated Boc-D-phenylalanine **44** on a 0.95 mmol scale following amide coupling procedure 3. The crude product was purified by column chromatography (SiO_2 3:1 hexane: EtOAc to 100% EtOAc, R_f EtOAc 0.45) gave **58** as a white powder (180 mg, 51%). δ_H (400 MHz; $CDCl_3$): 7.98 (1H, s, NH); 7.31-7.02 (5H, m, Ar-H); 4.96-4.84 (1H, multiple signals*, H_α (Phe)); 4.21-4.11 (1H, m, H_α (Lactam)); 3.10 (3H, s*, NCH_3); 2.91 (2H, m, CH_2); 2.80 (2H, m, CH_2); 1.99-1.80 (6H, m, CH_2 (caprolactam ring)); 1.24-1.10 (9H, d, $C(CH_3)_3$); δ_C (400 MHz; $CDCl_3$): 177.09 ((C=O) caprolactam); 169.99 (NH(C=O)); 156.85 ((C=O)O); 129.97-129.10 (C-Ar); 81.33 ($\underline{C}(CH_3)_3$); 62.50 ((C=O)$\underline{C}H_2N$); 60.67 (C_α (Lactam); 41.00 (CH_2NH); 35.07 (CH_2); 34.00-27.34 (CH_2 (Lactam), C($\underline{C}$H3)3, NCH_3);LRMS (ES+) *calcd* for $C_{15}H_{21}NO_4$ [M + H^+] 389.23, found 390.2. HRMS: *calcd* for $C_{15}H_{21}NO_4Na^+$ [M + Na^+] 412.2701, found 412.2711 [M+Na^+].

*Doubling of peaks observed due to the presence of rotamers.

N-methyl-N-((S)-1-oxo-1-((S)-2-oxoazepan-3-ylamino)-3-phenylpropan-2-yl)octanamide (**23**) *(S,S*-Ciliatamide B)

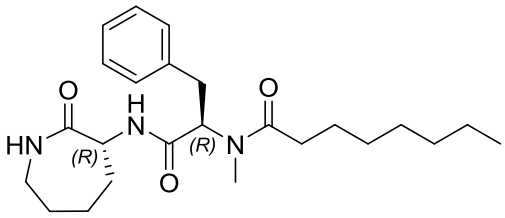


23

Dipeptide **45** was coupled to octanoyl chloride on a 0.257 mmol scale following amide coupling procedure 4. The crude product was purified by column chromatography (SiO_2 1:9 MeOH : DCM), R_f 0.45) gave **23** as an orange oil (57 mg, 54%). δ_H (400 MHz; $CDCl_3$): 7.58-6.84 (6H, m, Ar-H, NH); 5.51 (1H, bs, NH); 4.52 (1H, m, H_α (Lactam)); 3.46-3.11 (3H, m, CHNHC$\underline{H}_2$, Ar-C$\underline{H}_a$H$_b$); 3.06-2.76 (5H, m, Ar-CH$_a\underline{H}_b$, H_α(Phe), NCH_3); 2.31-0.54 (21H, m, 9x C$\underline{H}_2$, CC$\underline{H}_3$); δ_C (400 MHz; $CDCl_3$)*; 129.03, 128.90, 128.83, 128.42, 126.53, 57.43, 52.33, 52.21, 42.10, 34.62, 34.20, 34.02, 33.64, 31.74, 31.68, 29.22, 29.07, 29.03, 28.93, 28.81, 27.91, 24.92, 24.81, 22.62, 14.09; LRMS (ES+) *calcd* for $C_{24}H_{38}N_3O_3$ [M+H$^+$] 416.58, found 416.4. HRMS: *calcd.* 438.3071 (for $C_{24}H_{38}N_3O_3Na^+$), found 438.4140 [M+Na$^+$].

*Doubling of peaks observed due to the presence of rotamers.

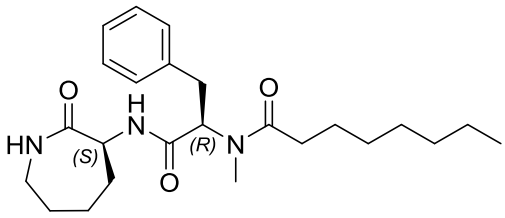
R,R-Ciliatamide B N-methyl-N-((R)-1-oxo-1-((R)-2-oxoazepan-3-ylamino)-3-phenylpropan-2-yl)octanamide (**23a**)



23a

Dipeptide **56** was coupled to octanoyl chloride on a 0.267 mmol scale following amide coupling procedure 4. The crude product was purified by column chromatography (SiO_2 1:9 MeOH : DCM), R_f 0.45) gave **23a** as a yellow oil (106 mg, 54 %). δ_H (400 MHz; $CDCl_3$): 8.02-7.94 (1H, m, NH); 7.49-7.17 (5H, m, Ar-H); 6.16 (1H, m, NH); 5.19 (1H, m, H_α(Lactam)); 3.58 (3H, s, NCH_3); 2.95-2.28 (4H, m, C$\underline{H}_2$Ar, CH_2N (lactam); 3.00-2.77 (2H, CH_2); 1.61-1.05 (18H, m, 9x CH_2) 0.83 (3H, m, CC$\underline{H}_3$); δ_C (400 MHz; $CDCl_3$)*; 177.20, 175.79, 172.60, 168.65, 136.25, 127.97, 129.90, 128.81, 128.45, 126.49, 107.55, 103.41, 42.82, 35.28, 34.06,31.68, 31.63, 31.58, 29.48, 29.23, 29.08, 29.01, 28.90, 28.85, 28.82, 24.68, 24.22, 23.90, 22.59, 14.05; LRMS (ES+) *calcd* for $C_{24}H_{38}N_3O_3$ [M+H^+] 416.58, found 416.4. HRMS: *calcd.* 438.3071 (for $C_{24}H_{38}N_3O_3Na^+$), found 438.3891 [M+Na^+].

S,R-Ciliatamide B N-methyl-N-((S)-1-oxo-1-((R)-2-oxoazepan-3-ylamino)-3-phenylpropan-2-yl)octanamide (**23b**)

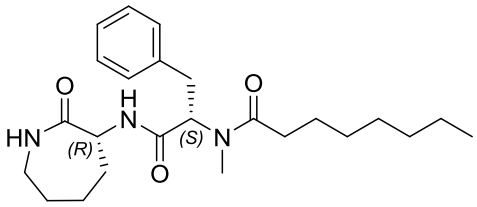


23b

Dipeptide **57** was coupled to octanoyl chloride on a .103 mmol scale following amide coupling procedure 4. The crude product was purified by column chromatography (SiO_2 1:9 MeOH : DCM) R_f 0.45) gave **23b** as a yellow oil (79 mg, 54 %). δ_H (400 MHz; $CDCl_3$): 7.34-7.14 (5H, m, Ar-H); 7.03 (1H, t, J_{HH} 6.10, NH); 5.56 (1H, dd, J_{HH} 6.44, J 9.82, NH); 4.54 (1H, m, H_α(Lactam)); 3.53-3.11 (3H, m, CHNHC$\underline{H}_2$, Ar-C$\underline{H}_a$$H_b$); 3.00-2.77 (5H, m, Ar-C$H_a$$\underline{H}_b$, H_α(Phe), NCH_3); 2.37-0.30 (21H, m, 9x C$\underline{H}_2$, CC$\underline{H}_3$); δ_C (400 MHz; $CDCl_3$)*; 178.20, 175.99, 174.60, 169.61, 137.29, 128.97, 128.91, 128.81, 128.36, 126.49, 77.35, 77.03, 76.71, 57.15, 52.03, 42.08, 34.13, 33.95, 33.57, 31.91, 31.66, 31.64, 31.49, 31.15, 29.68, 29.64, 29.34, 29.18, 29.00, 28.93, 28.71, 27.97, 24.91, 24.84, 24.82, 24.78, 22.70, 22.67, 22.66, 22.65, 22.64, 22.60, 22.59, 22.58, 22.52; LRMS (ES+) *calcd* for $C_{24}H_{38}N_3O_3$ [M+H^+] 416.58, found 416.4. HRMS: *calcd.* 438.3071 (for $C_{24}H_{38}N_3O_3Na^+$), found 438.3390 [M+Na^+].

*Doubling of peaks observed due to the presence of rotamers.

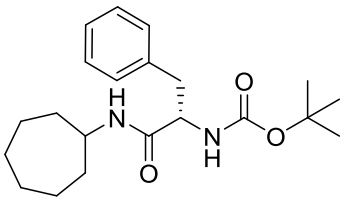
R,*S*-Ciliatamide B *N*-methyl-N-((R)-1-oxo-1-((S)-2-oxoazepan-3-ylamino)-3-phenylpropan-2-yl)octanamide (**23c**)



23c

Dipeptide **58** was coupled to octanoyl chloride on a 0.64 mmol scale following amide coupling procedure 4. Purification on SiO_2 (1:9 MeOH : DCM), R_f 0.45) gave **23c** as a yellow oil (117 mg, 44 %). δ_H (400 MHz; $CDCl_3$): 8.01-7.93 (1H, m, NH); 7.7 (1H, m, H_α(Lactam)); 7.20 (5H, m, Ar-H); 4.01 (3H, s, NCH_3); 3.34, (2H, m, $C\underline{H}_2Ar$) 3.00-2.77 (2H, CH_2); 1.60-1.05 (18H, m, 9x CH_2) 0.84 (3H, m, $CC\underline{H}_3$); δ_C (400 MHz; $CDCl_3$)*; 177.40, 172.76, 172.69, 171.15, 170.21, 169.55, 168.28, 143.45, 137.43, 133.01, 132.79, 132.56, 130.32, 129.44, 128.69, 128.49, 128.24, 126.72, 126.43, 126.06, 124.79, 124.30, 120.51, 120.09, 116.10, 115.49, 114.45, 108.16, 63.79, 60.38, 53.11, 48.05, 46.51,43.39, 42.80, 42.03, 39.98, 39.35, 38.54, 35.72, 35.51, 35.05, 33.94, 33.78, 33.43, 33.38,33.10,31.71, 31.67, 31.64, 31.60, 31.53, 31.50, 31.28, 30.75, 29.47, 29.26, 29.21; LRMS (ES+) *calcd* for $C_{24}H_{38}N_3O_3$ $[M+H^+]$ 416.58, found 416.4. HRMS: *calcd.* 438.3071 (for $C_{24}H_{38}N_3O_3Na^+$), found 438.0251 $[M+Na^+]$.

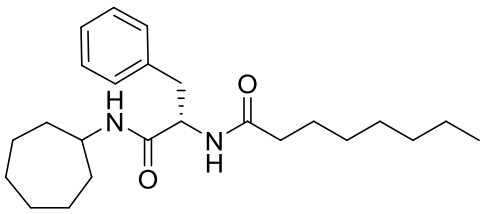
Synthesis of (**66**)



66

General procedure 2a was carried out on the Boc-protected peptide (Boc-Phe-OH) (715 mg, 2.69 mmol, 1.0 eq.) The crude product was then purified by column chromatography (solvent system of 3:1, hexane:ethyl acetate), giving **66** as a pale yellow solid (788 mg, 2.19 mmol, 81%). $[\alpha]_D^{25}$ = + 20.2 (c 1.0 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 1.45 (9H, s, tBu), 1.10 - 1.75 (12H, m, HN-CH($C\underline{H}_2$)$_6$), 1.75 – 1.85 (2H, m, 2 x CH), 2.95 (1H, dd, 16.0 and 24.0 Hz, $C\underline{H}_2$-Ar), 3.10 (1H, dd, *J* 12.0 and 24.0 Hz, $C\underline{H}_2$-Ar), 3.85 (1H, br s, NH-$C\underline{H}(CH_2)_6$), 4.25 (1H, q, Hα), 5.15 (1H, br s, NH), 5.50 (1H, br s, NH), 7.25 (5H, m, Ar); *m/z* (ESI) 383.4 (M^+ + Na), 743.7 ($2M^+$ + Na) Accurate mass: 383.2310 *m/z* = $[M+Na]^+$

Synthesis of (**67**)



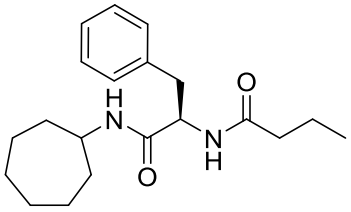
67

General procedure 1a was carried out on the Boc-protected peptide (**66**) (280 mg, 0.78 mmol). The crude product was then purified by column chromatography (solvent system of 3:1, hexane:ethyl acetate), leaving the product as a yellow solid (5%). δ_H (400 MHz, $CDCl_3$) 1.20 – 1.45 (12H, m, $CH_3(C\underline{H}_2)_6$), 1.47 – 1.75 (12H, m, HN-CH($C\underline{H}_2$)$_6$), 2.22 (2H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 2.40 (1H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 3.00 (1H, dd, *J* 8.0 and 12.0 Hz, $C\underline{H}_2$-Ar), 3.10 (1H, dd, *J* 4.0 and 8.0 Hz, $C\underline{H}_2$-Ar), 3.85 (1H, br s, NH-$C\underline{H}(CH_2)_6$), 4.70 (1H, q, *J* 8.0 Hz,

Hα), 6.1 (1H, s, NH), 6.84 (1H, br s, NH), 7.30 (5H, m, Ar); *m/z* (ESI) 409.5 (M^+ + Na), 795.8 ($2M^+$ + Na)

Synthesis of (**68**) General procedure 1a was carried out on 250 mg of the Boc-protected peptide (**66**) (0.94 mmol, 1 eq.) with octanoyl chloride (0.23 g, 1.41 mmol, 1.5 eq.). δ_H (400 MHz, $CDCl_3$) 1.20 – 1.45 (12H, m, $CH_3(C\underline{H}_2)_6$), 1.47 – 1.75 (12H, m, HN-CH($C\underline{H}_2)_6$), 2.22 (2H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 2.40 (1H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 3.00 (1H, dd, *J* 8.0 and 12.0 Hz, $C\underline{H}_2$-Ar), 3.10 (1H, dd, *J* 4.0 and 8.0 Hz, $C\underline{H}_2$-Ar), 3.85 (1H, br s, NH-$C\underline{H}(CH_2)_6$), 4.70 (1H, q, *J* 8.0 Hz, Hα), 6.1 (1H, s, NH), 6.84 (1H, br s, NH), 7.30 (5H, m, Ar); *m/z* (ESI) 387.5 (M^+ + H), 409.7 (M^+ + Na), 795.8 ($2M^+$ + Na).

Synthesis of (**72**)



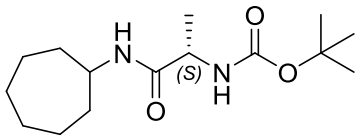
72

General procedure 1a was carried out on the Boc-protected peptide (**75**) (200 mg, 0.56 mmol, 1.0 eq.) with butyryl chloride (89 mg, 0.83 mmol, 1.5 eq.). The crude product was then purified by column chromatography (solvent system of 3:1, hexane:ethyl acetate), leaving the product as a yellow solid. (7 %) δ_H (400 MHz, $CDCl_3$) 1.20 – 1.35 (5H, m, $CH_3(C\underline{H}_2)_2$), 1.36 – 1.65 (5H, m, HN-CH($C\underline{H}_2)_6$), 2.15 (2H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 2.95 (1H, dd, *J* 12.0 and 16.0 Hz, $C\underline{H}_2$-Ar), 3.10 (1H, dd, *J* 8.0 and 16.0 Hz, $C\underline{H}_2$-Ar), 3.80 (1H, m, NH-$C\underline{H}(CH_2)_6$), 4.55 (1H, q, *J* 12.0 Hz, Hα), 5.7 (1H, s, NH), 6.44 (1H, br s, NH), 7.25 (5H, m, Ar)

Synthesis of (**70**)

General procedure 1a was carried out on 250 mg of the Boc-protected peptide (**74**) (0.94 mmol, 1 eq.) with butyryl chloride (150 mg, 1.41 mmol, 1.5 eq.). The crude product was purified by prep TLC using a solvent system of 3:1, DCM:MeOH. δ_H (400 MHz, $CDCl_3$) 0.70 – 0.90 (4H, m, $CH_3(C\underline{H}_2)_2$), 1.47 – 1.75 (12H, m, HN-CH($C\underline{H}_2$)$_6$), 2.22 (2H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 2.40 (1H, t, *J* 8.0 Hz, $C\underline{H}_3CH_2$), 3.00 (1H, dd, *J* 8.0 and 12.0 Hz, $C\underline{H}_2$-Ar), 3.10 (1H, dd, *J* 4.0 and 8.0 Hz, $C\underline{H}_2$-Ar), 3.85 (1H, br s, NH-C$\underline{H}$(CH_2)$_6$), 4.70 (1H, q, *J* 8.0 Hz, Hα), 6.1 (1H, s, NH), 6.84 (1H, br s, NH), 7.30 (5H, m, Ar); *m/z* (ESI) 353.3 (M^+ + Na), 683.6 ($2M^+$ + Na); δ_C

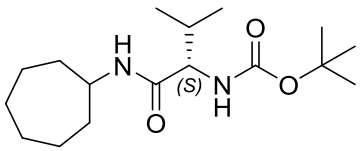
Synthesis of (**75**)



75

General procedure 2a was carried out on the Boc-protected peptide (Boc-Ala-OH) (500 mg, 2.64 mmol, 1.0 eq.). The crude product was then purified by column chromatography (solvent system of 3:1, hexane:ethyl acetate), leaving the product as a pale yellow solid (568 mg, 2.00 mmol, 76 %). $[\alpha]_D^{26}$ = -70.5 (c 1.0 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 1.33 (3H, d, *J* 8.0 Hz, CH_3), 1.37 – 1.65 (12H, m, HN-CH($C\underline{H}_2$)$_6$), 1.45 (9H, s, tBu), 1.85 – 1.93 (2H, m, 2 x C$\underline{H}$), 3.95 (1H, m, NH-C$\underline{H}$(CH_2)$_6$), 4.05 (1H, m, Hα), 4.95 (1H, br s, NH), 6.05 (1H, br s, NH); *m/z* (ESI) 307.4 (M^+ + Na), 591.6 ($2M^+$ + Na) Accurate mass: 307.2004 m/z = $[M+Na]^+$

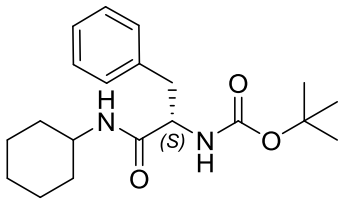
Synthesis of (**76**)



76

General procedure 2a was carried out on the Boc-protected peptide (Boc-Val-OH) (500 mg, 2.30 mmol, 1.0 eq.). The crude product was then purified by column chromatography (solvent system of 3:1, hexane:ethyl acetate), leaving the product as a pale yellow solid (430 mg, 1.38 mmol, 60 %). $[\alpha]_D^{26}$ = -18.9 (c 1.0 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 0.90 (3H, d, *J* 4.0 Hz, CH_3), 0.95 (3H, d, *J* 4.0 Hz, CH_3), 1.45 (9H, s, tBu), 2.10 (1H, m, $CH_2C\underline{H}CH_2$), 3.77 (1H, dd, *J* 8.0 and 4.0 Hz, Hα), 3.95 (1H, m, $CH_2C\underline{H}CH_2$), 5.05 (1H, br s, NH), 5.75 (1H, br s, NH); *m/z* (ESI) 335.4 (M^+ + Na), 647.7 ($2M^+$ + Na) Accurate mass: 335.2315 m/z = $[M+Na]^+$

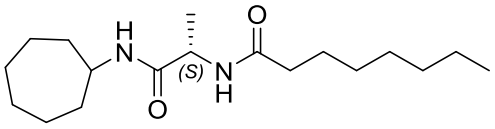
Synthesis of (**77**)



77

General procedure 3a was carried out on 1.00 g of the Boc-protected peptide (Boc-Phe-OH) (3.77 mmol, 1.0 eq). The crude product was purified by column chromatography using a solvent system of 3:1, hexane:ethyl acetate. To afford the product as a pale yellow solid (3.00 mmol, 80%) $[\alpha]_D^{26}$ = -13.2 (c 1.0 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 1.45 (9H, s, tBu), 1.10 - 1.75 (10H, m, HN-CH($C\underline{H}_2$)$_5$), 1.75 – 1.85 (2H, m, 2 x CH), 2.95 (1H, dd, 16.0 and 24.0 Hz, $C\underline{H}_2$-Ar), 3.15 (1H, dd, *J* 12.0 and 24.0 Hz, $C\underline{H}_2$-Ar), 3.70 (1H, br s, NH-$C\underline{H}(CH_2)_6$), 4.20 (1H, q, Hα), 5.10 (1H, br s, NH), 5.45 (1H, br s, NH), 7.25 (5H, m, Ar); *m/z* (ESI) 369.3 (M^+ + Na), 715.5 ($2M^+$ + Na); Accurate mass: 369.2173 m/z

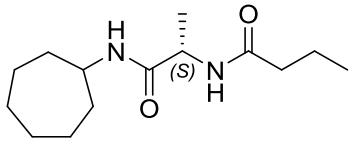
Synthesis of (**69**)



69

General procedure 1a was carried out on the Boc-protected peptide (**75**) (140 mg, 0.5 mmol, 1.0 eq.) with octanoyl chloride. The crude product was purified by prep TLC on silica gel. To afford the product as a pale yellow solid (19 %) δ_H (400 MHz, $CDCl_3$) 0.85 (8H, m, $C\underline{H}_3(C\underline{H}_2)_6$), 1.45 – 1.60 (12H, m, HN-CH($C\underline{H}_2)_6$), 1.85 – 1.93 (2H, m, 2 x CH), 3.90 (1H, m, NH-C$\underline{H}(CH_2)_6$), 4.50 (1H, m, Hα), 4.95 (1H, br s, NH), 6.65 (1H, br s, NH)

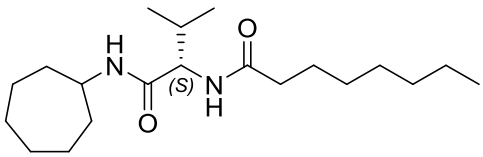
Synthesis of (**70**)



70

General procedure 1a was carried out on 140 mg of the Boc-protected peptide (**75**) (0.5 mmol, 1.0 eq.) with butyryl chloride (0.08 mL, 0.75 mmol, 1.5 eq.) and Et_3N (0.21 mL, 1.5 mmol, 3 eq.). The crude product was a pale yellow solid which was purified by prep TLC on silica gel to afford the product (18%) δ_H (400 MHz, $CDCl_3$) 0.90 (7H, m, $CH_3(C\underline{H}_2)_2$), 1.55 – 1.70 (12H, m, HN-CH($C\underline{H}_2)_6$), 2.37 (3H, t, *J* 8.0 Hz, CH_3), 3.90 (1H, m, NH-C$\underline{H}(CH_2)_6$), 4.40 (1H, q, *J* 8.0 Hz, Hα), 6.05 (1H, br s, NH); *m/z* (ESI) 255.4 (M^+ + H), 277.3 (M^+ + Na), 531.5 ($2M^+$ + Na);

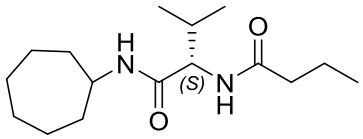
Synthesis of (**71**)



71

General procedure 1a was carried out on 120 mg of the Boc-protected peptide (**76**) (0.38 mmol, 1.0 eq.) with octanoyl chloride (0.09 g, 0.58 mmol, 1.5 eq.) and Et_3N (0.12 g, 1.15 mmol, 3 eq.). The crude product was a pale yellow solid, which was then purified by prep TLC on silica gel to give apale yello produc (14%). δ_H (400 MHz, $CDCl_3$) 0.80 – 0.90 (6H, m, 2 x CH_3), 1.20 – 1.35 (15H, m, $CH_3(C\underline{H}_2)_6$), 1.45 – 1.7 (12H, m, HN-CH($C\underline{H}_2)_6$), 2.30 (1H, m, $CH_2C\underline{H}CH_2$), 3.90 (1H, br s, Hα), 4.25 (1H, m, $CH_2C\underline{H}CH_2$), 7.23 (1H, br s, NH), 7.70 (1H, br s, NH); *m/z* (ESI) 361.4 (M^+ + Na), 699.7 ($2M^+$ + Na);

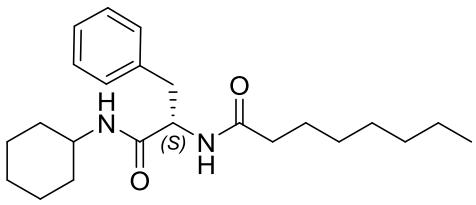
Synthesis of (**72**)



72

General procedure 1a was carried out on 120 mg of the Boc-protected peptide (**76**) (0.38 mmol, 1.0 eq.) with butyryl chloride (0.06 g, 0.58 mmol, 1.5 eq.). to give the crude product as a pale yellow solid, which was then purified by prep TLC on silica gel to afford the product as a pale yellow solid (11%) δ_H (400 MHz, $CDCl_3$) 0.90 – 1.0 (7H, m, $CH_3(C\underline{H}_2)_6$), 1.4 – 1.7 (12H, m, HN-CH$(C\underline{H}_2)_6$), 1.8 – 2.0 (3H, m, CH_3), 2.15 – 2.35 (3H, m, CH_3), 3.90 (1H, br s, Hα), 4.25 (1H, dt, *J* 8.0 and 24.0 Hz $CH_2C\underline{H}CH_2$), 6.55 (1H, br s, NH), 6.8 (1H, br s, NH); *m/z* (ESI) 305.4 (M^+ + Na), 587.6 ($2M^+$ + Na)

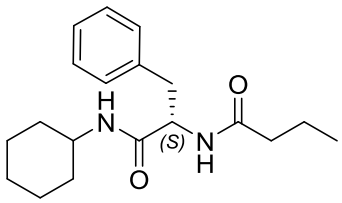
Synthesis of (**73**)



73

General procedure 3a was carried out on (0.95 mmol, 1 e.q.) **77** to afford **73** as a white solid, which was the purified by prep TLC on silica gel to afford the product (21 %). δ_H (400 MHz, $CDCl_3$) 0.08 – 0.09 (7H, m, $C\underline{H}_3(C\underline{H}_2)_6$), 1.20 - 1.35 (18H, m, HN-$C\underline{H}(C\underline{H}_2)_5$, $CH_3(C\underline{H}_2)_6$), 2.15 (2H, t, *J* 8.0 Hz $C\underline{H}_3(CH_2)_6$), 2.35 (1H, t, *J* 8 Hz, $C\underline{H}_3(CH_2)_6$), 2.95 (1H, dd, 12.0 and 16.0 Hz, $C\underline{H}_2$-Ar), 3.05 (1H, dd, *J* 8.0 and 16.0 Hz, $C\underline{H}_2$-Ar), 3.60 (1H, br s, NH-$C\underline{H}(CH_2)_6$), 4.55 (1H, q, *J* 12.0 Hz, Hα), 5.60 (1H, br s, NH), 6.45 (1H, br s, NH), 7.25 (5H, m, Ar); *m/z* (ESI) 373.4 (M^+ + Na), 767.7 ($2M^+$ + Na);

Synthesis of (**74**)



74

General procedure 3a was carried out on (0.95 mmol, 1 e.q.) **77** to afford **74** as a white solid, which was the purified by prep TLC on silica gel to afford the product (21 %). δ_H (400 MHz, $CDCl_3$) 0.07 – 0.09 (7H, m, $CH_3(C\underline{H}_2)_2$), 1.10 - 1.70 (10H, m, HN-C$\underline{H}$($C\underline{H}_2)_5$), 2.85 (1H, dd, 8.0 and 12.0 Hz, C$\underline{H}_2$-Ar), 3.05 (1H, dd, *J* 4.0 and 8.0 Hz, C$\underline{H}_2$-Ar), 3.60 (1H, br s, NH-C$\underline{H}$($CH_2)_6$), 4.50 (1H, m, Hα), 5.40 (1H, br s, NH), 6.2 (1H, br s, NH), 7.20 (5H, m, Ar); *m/z* (ESI) 339.4 (M^+ + Na), 655.6 ($2M^+$ + Na);

Accurate mass: 339.2 (M^+ + Na)

7.5 Assay

7.5.1 Maintenance of Cell Lines[3]

Promastigote: MYNC/BZ/62/M379 *Leishmania mexicana* (M379) procyclic promastigote parasites were cultured in sterile filtered Sigma-Aldrich® Schneider's Drosophilia medium (+) L-glutamine containing 15% Biosera heat inactivated (1h at 65°C) Foetal Bovine Serum (HIFBS), 1% GIBCO™ Penicillin and Streptomycin (P/S) (Promastigote medium) at pH 7.0, 26°C. Subcultures were seeded with two drops of procyclic promastigote parasite culture in 5 ml in a 50 ml flask and incubated at 26°C.

Amastigote: M379 amastigotes were cultured in sterile filtered Sigma-Aldrich® Schneider's Drosophilia medium (+) L-glutamine containing 20% Biosera HIFBS, 1% GIBCO™ P/S (Amastigote medium) at pH 5.5, 32°C. Subcultures were seeded with two drops of healthy culture in 5 ml in a 50 ml flask and incubated at 32°C.

Macrophage: RAW 264.3 mouse leukemic macrophages were cultured in sterile GIBCO™ Dulbecco's Modified Eagle Medium (+) glutamine (DMEM) containing 10% HIFBS, 1% P/S (Macrophage medium) at 37°C with 5% CO_2. Cultures were maintained in 50 ml flasks with vented caps. In order to facilitate subculture the medium was removed from 50-100% confluent cell cultures which were then exposed for 3 min to 1ml of a 1:10 GIBCO™ 0.5% Trypsin-EDTA 10X in phosphate buffered saline (PBS) solution at 37°C in with 5% CO_2 in a vented cap flask. The cells were then disturbed by shaking and 5 ml macrophage medium added to the flask. Subcultures were then seeded with 1ml of this culture in 5 ml of the macrophage medium in a 50 ml vented cap flask and incubated at 37°C with 5% CO_2.

Cell Culture from Frozen Stock: Promastigote cell cultures were continually brought up from frozen stocks stored at -180^{o}C. Cells were stored as 1 ml samples at 1 x 10^{5} cells/ml with 10% dimethyl sulphoxide (DMSO) in the appropriate medium. Promastigote frozen stocks were defrosted and added to 5 ml of the described promastigote medium. This was incubated at 26^{o}C for 24 h. In order to wash the cells of DMSO, the culture was centrifuged in a 15 ml falcon tube for 5 min at 3000rpm in a BOECO U-320R centrifuge. The media was then removed and the pellet re-suspended into fresh promastigote medium (5 ml). The culture was then treated as described in the **Promastigote** section described above.

7.5.2 Background and Analytical Procedures for Biological Screening[3]

AMP and Amphotericin-B Stock Solutions: Stock solutions of RP-HPLC Purified TA (**13**), RP-HPLC Purified TB (**9**), AmB (**2**) and FAM-TB (**10**) were prepared at 25 mM in DMSO. The stocks were then stored at -20^{o}C between uses. Before each use each stock was thoroughly defrosted and the peptide re-suspended using a Stuart Vortex mixer.

Cell Counting and Concentration (Procedure 1): Cell line culture concentrations were measured using a Neubauer improved bright-line haemocytometer. The required cell/ml concentration was calculated and the estimated amount of cell culture re-suspended in the appropriate fresh medium. This culture was then recounted to verify that the correct cell concentration was achieved.

Alamar Blue® Procedure 1 (Amastigote and Macrophage) (Procedure 2): The Invitrogen Alamar Blue® protocol [67] was followed for amastigote and macrophage cell cultures in order to monitor cell viability and proliferation. Cells were incubated at the appropriate conditions for each cell type for 4 h with 10 µl per 100 µl of cell culture. Fluorescence was detected

using an excitation wavelength of 540 nm in a BioTek FLx800 plate reader in conjunction with the Gen5 Reader Control Program.

Alamar Blue Procedure 2 (Promastigote) (Procedure 3): The Invitrogen Alamar Blue® protocol was insufficient to provide useful cell viability and proliferation results for the promastigote cultures. As such a procedure was developed as follows: Two Sarstedt 96 well plates were set up with wells containing 100 µl of promastigote cell culture at the following concentrations: 1.0 x 10^7 cells/ ml, 5.0 x 10^6 cells/ ml, 2.5 x 10^6 cells/ ml, 6.25 x 10^5 cells/ ml, 3.13 x 10^5 cells/ ml, 1.63 x 10^5 cells/ ml, 8.18 x 10^4 cells/ml, 4.09 x 10^4 cells/ml, 2.05 x 10^4 cells/ml, 1.02 x 10^4 cells/ml, 5.01 x 10^3 cells/ml. The above concentration series was achieved by serial dilution of the 1.0 x 10^7 cells/ ml concentration into wells containing fresh promastigote medium. In each plate the concentration series and a control well containing only promastigote medium were set up in triplicate. 10 µl of Alamar Blue® was then added to each well and the plates sealed with parafilm. One plate was then left to incubate at 26°C and the other at 37°C. Fluorescence was measured at 4 h and at 24 h using an excitation wavelength of 540 nm in a BioTek FLx800 plate reader in conjunction with the Gen5 Reader Control Program. The most effective protocol was that conducted at 26°C for 24 h. Thus the promastigote Alamar Blue® protocol was conducted with 10 µl of Alamar Blue® per 100 µl of cell culture. The plate was then sealed with parafilm and incubated at 26°C for 24 h. Fluorescence was then measured as above.

Cytotoxicity Screening

Macrophage Cytotoxicity Screen: Macrophage cell culture concentration was made up to 1 x 10^5 cells/ ml following **Background Procedure 1**. 200µl of this culture was added to the

starting wells of each row of a Starstedt 96 well plate. In the next 5 wells of each row 100 µl of culture was added. 4µl of the Pure TA (**13**) stock solution, Pure TB (**9**) stock solution, AmB (**2**) stock solution and DMSO were each added individually to three of the starting wells to give nine wells containing 500µM drugs solutions in cell culture and three negative control cell culture wells (DMSO). Each starting well was then thoroughly mixed using a pipette and serially diluted by progressively transferring and mixing 100 µl of cell culture containing the drug compound into the subsequent well to give wells containing the following drug concentrations: 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25µM. Positive control wells of only macrophage media and only macrophage cell culture were also set up in triplicate. The plates were then left to incubate at 37^{o}C with 5% CO_2 for 72 h. **Background Procedure 2** was then followed in order to correlate macrophage viability and proliferation to drug concentration. This plate was prepared in triplicate with separate macrophage cultures.

Promastigote Cytotoxicity Screen: Promastigote cell culture concentration was made up to 1 x 10^5 cells/ ml following **Background Procedure 1**. 4 µl of the Pure TA (**13**) stock solution, TB (**9**) stock solution, AmB (**2**) stock solution and DMSO were each added individually to 1 ml samples of this cell culture to give promastigote culture stock solutions containing 100 µM of each drug and a negative control cell culture stock solution (DMSO). 200 µl of each stock solution was added individually to three starting wells of a Starstedt 96 well plate to give 12 wells containing 200 µl of promastigote culture. 100 µl of the 1 x 10^5 cells/ ml culture without any drug compound was added to the next 5 wells of each row. Each starting well was then thoroughly mixed using a pipette and serially diluted by progressively transferring and mixing 100 µl of cell culture containing the drug compound into the subsequent well to give wells containing the following drug concentrations: 100 µM, 50 µM,

25 μM, 12.5 μM, 6.25 μM, 3.125 μM. Positive control wells of only promastigote media and only promastigote cell culture were also set up in triplicate. The plate was then sealed with parafilm and left to incubate at 26^{o}C for 24 h. **Background Procedure 3** was then followed in order to correlate promastigote viability and proliferation to drug concentration. The plate was prepared in triplicate with separate promastigote cultures.

Amastigote Cytotoxicity Screen: Amastigote cell culture concentration was made up to 1 x 10^5 cells/ ml following **Background Procedure 1**. 4μl of the Pure TA (**13**) stock solution, TB (**9**) stock solution, AmB (**2**) stock solution and DMSO were each added individually to 1 ml samples of this cell culture to give amastigote culture stock solutions containing 100 μM of each drug and a negative control amastigote culture stock solution (DMSO). 200 μl of each stock solution was added individually to three starting wells of a Sarstedt 96 well plate to give 12 wells containing 200 μl of amastigote culture. 100 μl of the 1 x 10^5 cells/ ml culture without any drug compound was added to the next 5 wells of each row. Each starting well was then thoroughly mixed using a pipette and serially diluted by progressively transferring and mixing 100 μl of cell culture containing the drug compound into the subsequent well to give wells containing the following drug concentrations: 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM. Positive control wells of only amastigote media and only amastigote cell culture were also set up in triplicate. The plate was then sealed with parafilm and left to incubate at 32^{o}C for 72 h. **Background Procedure 2** was then followed, ensuring that the plate was re-sealed with parafilm prior to incubation, in order to correlate promastigote viability and proliferation to drug concentration. The plate was prepared in triplicate with separate amastigote cultures.

7.5.3 Fluorescent peptide Screening[3]

Non-infected macrophage plate set up: Sterile cover slips were placed into two wells in two Sarstedt 24 well plates. Macrophage cell culture concentration was made up to 1 x 10^5 cells/ ml following **Background Procedure 1** and 500 µl of this culture was added to each of the four wells. Plates were then incubated at 37^{o}C, 5% CO_2 for 24 h to allow the macrophage to adhere to the cover slips and achieve 50-100% confluence. The plates were then ready to be used in the visualisation procedure.

Infected macrophage plate set up: Sterile cover slips were placed into two wells in two Sarstedt 24 well plates. Macrophage cell culture concentration was made up to 1 x 10^5 cells/ ml following **Background Procedure 1** and 500 µl of this culture was added to each of the four wells. Plates were then incubated at 37^{o}C, 5% CO_2 for 24 h to allow the macrophage to attach to the cover slips and achieve 50-100% confluence. After 24 h the media was removed from each of the wells and replaced with 500 µl of 1 x 10^6 cells/ml amastigotes in macrophage medium to allow for 1:10 macrophage to amastigote. The amastigote culture was prepared by counting and adjusting an amastigote culture in amastigote medium following **Background Procedure 1**, the culture was then centrifuged at 3000 rpm for 5 min. The amastigote medium was then removed and the amastigote pellet re-suspended in the same amount of macrophage medium. The amastigote culture was then recounted to ensure correct cell concentration. The Sarstedt 24 well plates were then incubated for 24 h at 37^{o}C, 5% CO_2 to allow for amastigote infection. After 24 h the amastigote culture was removed from each of the wells and replaced with 500 µl of macrophage medium. The plates were then incubated at 37^{o}C, 5% CO_2 for 24 h. The plates were then ready to be used in the visualisation procedure.

7.5.4 Visualisation of Fluorescent Peptides' Localisation in Infected and Non-infected Macrophage[3]: The plates described above were used throughout this procedure. Following the final 24 h incubation all plates had the media present removed and replaced with 500 µl 37^{o}C HIFBS free DMEM. All plates were then incubated at 37^{o}C with 5% CO_2 for 30 min in order to starve the cells. The media was then removed from all wells and replaced with 500 µl of 37^{o}C HIFBS free DMEM in the plates labelled HOT and 500 µl of 0^{o}C HIFBS free DMEM in the plates labelled COLD. All plates were then sealed with parafilm and incubated at 37^{o}C for 15 min. The media was then removed from all wells and the plates treated as follows: 500 µl of 37^{o}C 50 µM FAM-TB in HIFBS free DMEM was added to each well. The 50 µM FAM-TB solutions were prepared using the 4 µl of 25 mM stock solution to 1 ml of HIFBS free DMEM. Each plate was then re-sealed with parafilm and incubated at 37^{o}C for 1 h. The media was then removed from all wells. Each well was washed with 1ml of 0^{o}C HIFBS free DMEM thee times, with the final wash removed. The cells in each well were then fixed using 500 µl of 0^{o}C 3.7% formaldehyde in macrophage medium and left 10 min at room temperature. The formaldehyde solution was prepared using 37% formaldehyde in MeOH at 1:10 with macrophage medium. The formaldehyde solution was then removed and each well washed three times with 1 ml of 0^{o}C PBS. The final wash of PBS was removed from each well and replaced with 500 µl of 2.86 µM 4',6-diamidino-2-phenylindole (DAPI) in PBS and left at room temperature for 10 min. The DAPI solution was prepared using a 14.3 mM stock solution of DAPI at 1:5000 with PBS. The DAPI solution was then removed from each well and replaced with 500 µl of PBS and the wells examined using an Olympus 1X71 fluorescence microscope at 40X in conjunction with OpenLab 4.0.1.

Given ease of cell culture, AMPs have most commonly been screened against insect stage, promastigote *Leishmania*. However, in order to fully assess the efficacy of any compound it must be assayed against pathogenic, mammalian stage amastigotes. Therefore to facilitate comparative analyses of the anti-leishmanial action of selected, synthesized temporins it was chosen to utilise *Leishmania mexiciana,* were axenic culture of both lifecycle stages is long established.

The Alamar Blue viability assay has previously been validated for microtitre plate-based analyses of promastigote *L. major*; and *L. donovani*, *L. tropica* and *L. mexicana* promastigotes and amastigotes. In addition it has been utilised for screening *L. amazonensis, L. braziliensis* and *L. chagasi* promastigotes.[22] However, to facilitate comparison of the efficacy of the synthesized AMPs against both promastigote and amastigote axenic *L. mexicana*, the Alamar Blue assay was optimized to allow both lifecycle stages to be screened under equivalent conditions. To this end, and in light of previous studies, serial dilutions (starting at $4x10^4$ cells/well) of both lifecycle stages were incubated for 24 hours in 96-well plates at appropriate temperatures before the addition of Alamar Blue at 10% v/v for either 4 or 24 hours and the subsequent assessment of cell viability by fluorescent readout. The data clearly show that that a direct correlation of parasite numbers with readout was apparent in the case of both lifecycle stages incubated for 4 hours after the addition of Alamar Blue (r^2 = 0.999 for both promastigotes and amastigotes). However, deviation from this linear relationship was apparent at higher cell concentrations after 24 hours incubation with the indicator, particularly with respect to the promastigotes with the correlation breaking down at more than $5x10^3$ cells/well. A similar pattern has previously been noted with other *Leishmania* spp. To ensure a direct correlation between readout and cell number in the AMP screen of both lifecycle stages of *L. mexicana*, a starting concentration of $4x10^5$/ml ($4x10^4$ cells/well), followed by incubation with Alamar Blue for 4 hours, was employed in all

subsequent experiments.

Leishmania culture

Leishmania mexicana (MNYC/BZ/62/M379) parasites were maintained at 26°C in Schneider's Drosophila media (Sigma Aldrich) supplemented with heat inactivated foetal bovine sera (15% for promastigotes and 20% for amastigotes; Biosera). Promastigotes were transformed into axenic amastigotes by a pH and temperature shift as previously described. Cells were counted using a Neubauer Improved Haemocytometer.

Cytotoxicity assay

Cytotoxicity analyses were performed in 96-well plates (Nunc) using Alamar Blue (Invitrogen) with some modifications to the published protocol. [17]

Briefly, following optimization of the assay system, 100 µl of both promastigote and amastigote *L. mexicana* at $4x10^{5}ml^{-1}$ were incubated with compounds in triplicate (amphotericin B was used as a positive control, and untreated parasites as a negative control) for 24 hours before incubation with Alamar Blue (Invitrogen) for 4 hours prior to assessing cell viability using a fluorescent plate reader (Biotek; 560EX nm/600EM nm). The experiments described above were carried out on a minimum of two separate occasions to ensure a robust data set was collected.

7.6 References

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