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A bioactive peptide from the skin secretion of the bamboo leaf odorous frog, Odorrana versabilis

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A bioactive peptide from the skin secretion of the bamboo leaf odorous frog, *Odorrana versabilis*

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Contents

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Declaration

I declare that the research reported in this thesis was carried out by myself except where acknowledgement has been made. All work was performed in the Natural Drug Discovery Group, Faculty of Medicine, Health and Life Sciences, Queen's University, Belfast.

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Abstract

Due to the increased and inappropriate use of antibiotics, antibiotic resistance has emerged and become a global threat during the past decades. As the problem of antibiotic resistance gets worse, the need to search for novel antimicrobial molecules is high on the agenda. Antimicrobial peptides (AMPs) derived from amphibian defensive skin secretions have been found to possess remarkable bioactivities in some areas, such as antimicrobial activities, smooth muscle activities, anticancer activities, and so on. Therefore, AMPs are now recognised as one of the most promising groups of molecules for potential therapeutic applications in the future.

This thesis aimed to isolate and identify the structure of a bioactive peptide from the skin secretion of the Chinese bamboo leaf odorous frog, *Odorrana versabilis*, by applying 'shotgun' molecular cloning technology. After this was achieved, the synthetic peptide replicate obtained using solid phase peptide synthesis, was identified and purified by reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometry. Afterwards, an array of assays was carried out to assess the bioactivities of the peptide.

Indeed, a peptide, named QUB-2040, was isolated and found to possess antimicrobial activity against *S. aureus*, *E. coli and C. albicans* with minimum inhibitory concentrations (MICs) of 8 μM, 16 μM, and 8 μM, respectively. In addition, the peptide QUB-2040 was found to cause less than 30% lysis of the horse erythrocytes at effective concentrations. Nevertheless, QUB-2040 showed no inhibitory effects on the proliferation of the tested four cancer cells. Therefore, it has the potential to be developed as a promising antimicrobial drug in the future study.

Chapter1 Introduction

1.1 Background—Antibiotic Resistance

Antibiotics have brought great convenience to human life since Alexander Fleming, a Scottish scientist, discovered the first antibiotic—penicillin (Tan and Tatsumura, 2015). These drugs have resulted in an extraordinary decline in bacterial infections and numbers of deaths from these infections in the past 70 years. They are able to induce damage to bacterial cells, which causes either cell death or the inhibition of reproduction. However, with the increased use of antibiotics, antibiotic resistance, which is a growing global concern within the medical scientific world and the general public, has emerged. When antibiotic resistance emerges, it means that the antibiotic agent has lost its potency either to kill bacteria or to inhibit their growth (Morier, 2016).

1.1.1 The causes and effects of antibiotic resistance

Drug resistance is a natural phenomenon which can be explained by natural selection and it existed long before scientists discovered how to use antibiotics as medicines but has been facilitated by a number of human factors (Wright, 2010). The overuse and misuse in human beings or animals may be the chief factors in accelerating this process (Alanis, 2005). Since antibiotics produce their significant therapeutic effects on infections, they have been prescribed by physicians frequently to treat various bacterial infections with incorrect dosages or even for ineffectual circumstances such as for viral infections—seasonal colds and flu. Both patients and doctors became dependent on these ‘wonder drugs’. As a result, this overprescribing has led to the accumulation of antibiotic resistant genes in bacteria. Meanwhile, some patients have disregarded the treatment regimens of doctors and they stop using the drug as soon as they feel better which causes the remaining of some bacteria in the body, making the problem worse.

Antibiotics are also applied in agriculture often in much larger quantities than those used in humans (Ventola, 2015). People in both developing and developed countries use fodder mixed with antibiotics to feed livestock in order to prevent infections and to promote growth in which way a great number of antibiotics or even resistant organisms may accumulate *in vivo*. When antibiotics are used in this way in animals produced for the food chain, the antibiotics are without doubt ingested by humans (Ventola, 2015). The environment may be contaminated to some degree because of the antibiotics used in agriculture, promoting the spread of resistance.

There are some other social factors contributing to antibiotic resistance. When healthcare workers in hospitals do not follow infection control protocols strictly, resistance will be easily transferred through bacteria from person-to-person. This would be more serious among hospitalised patients with compromised health because they are vulnerable to bacterial infections and once they get infected, increased usage of different antibiotics is usually applied. This causes the rapid development of resistance. From another aspect, the lack of public knowledge about antibiotic agents increases the inappropriate use of antibiotics (Fair and Tor, 2014). Several antibiotics are available in pharmacies as OTC medicines, so they can be obtained without medical supervision. Apart from the above reasons, the difficulty in finding new antibiotics gives drug resistance the opportunity to develop.

This makes infectious diseases more serious since the bacteria display antibiotic resistance. More complicated diseases may spring up because of the metamorphic bacteria. It leads to more doctor visits, higher medical costs, longer hospital stays, and even increased mortality. In the USA, at least 2 million people get bacterial infections that are resistant to present antibiotics and more than 23 thousand infected people lose their life every year (Solomon and Oliver, 2014). More alarming, multi-drug

resistance (MDR) occurs when one kind of bacteria is resistant to more than one antibiotic agent which makes the problem more complicated.

1.1.2 The mechanism of antibiotic resistance

Inherent resistance, mutational resistance and acquired resistance are the three main types of antibiotic resistance and there are a few mechanisms of antibiotic resistance including inactivation of drugs by enzymes, inhibition of drug uptake, activation of drug efflux pumps, alteration of drug targets and so on (Figure 1.1).

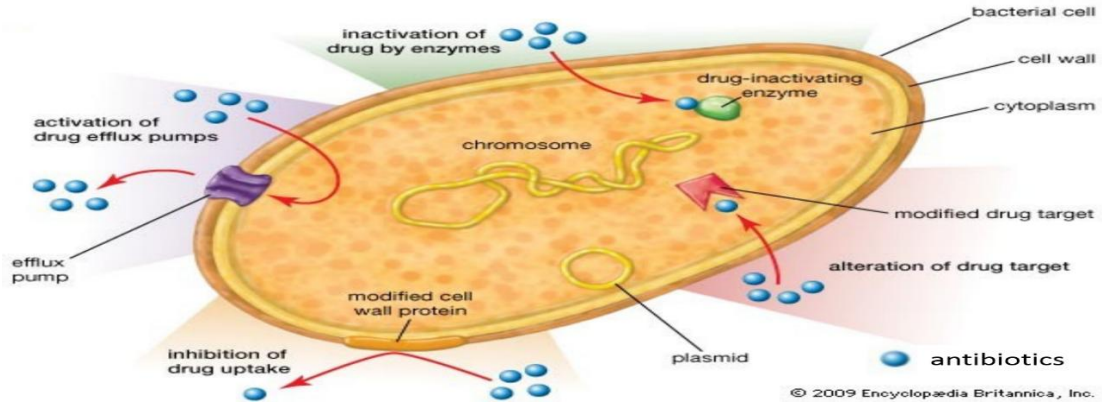

Figure 1.1 Examples of mechanisms of antibiotic resistance (Encyclopædia Britannica, Inc., 2009. *Antibiotic resistance: mechanisms of antibiotic resistance*. [image] Available at: https://www.britannica.com/science/antibiotic-resistance [Accessed 22 Nov. 2016])

Inactivation of drugs by enzymes

Bacteria are able to preserve themselves by producing enzymes that can destroy the active part of the antibiotic agent, rendering it inactive (Munita and Arias, 2016). The beta lactamase is a major inhibiting enzyme towards β-lactam antibiotics such as penicillin (Figure 1.2). It breaks the beta lactam ring of penicillin making the medicine ineffective in binding to PBPs (penicillin binding proteins), while PBPs are known as β-lactam targets which are enzymes found essential for the biosynthesis of the

bacterial cell wall (Zeng and Lin, 2013). Therefore, the agent does not have the ability to kill the bacteria since it cannot inhibit the role of PBPs in the cell wall synthesis of the bacteria (Tenover, 2006).

Inhibition of drug uptake

Almost all the antibiotics require certain access into the bacterial cell to reach the target point where they can display their intended effect. It is porin channels that provide a passageway through the outer membrane for some small hydrophilic antibiotics (Delcour, 2009), including aminoglycosides and β–lactams. However, a large number of bacteria have acquired resistance through a decrease in the number or change in the character of porin channels. As such, bacteria can defend themselves against the entry of drugs by modifying the porin channel number, size or selectivity (Džidić et al., 2008). In this case, aminoglycosides cannot reach their target site, the ribosomes, so they are unable to interfere with the normal function of the bacterial organism which leads to resistance.

Activation of drug efflux pumps

It is well established that efflux pumps are transport proteins responsible for exporting toxic substances such as metabolites and antibiotics to regulate the internal environment of microorganisms (Soto, 2013). When the pump encoded by bacteria is activated, it provides an exit path from within the cell into the external environment which drives antibiotics to be continuously pumped out through the channel (Figure 1.3). It results in low intracellular concentrations of antibiotics which means they are insufficient to fulfil the antibacterial function. This resistant strategy has been observed in many organisms including the efflux pump AcrB in *E. coli*, LmrS in *S.*

aureus, MexB in *P. aeruginosa* and MdeA in *Streptococcus mutans* and so on(Blair et al., 2015).

Alteration of drug target

Some bacteria evade antibiotics through changing or modifying the antibiotic target site. They may induce the mutation of the genes coding for target proteins to change the structure of targets. In doing so, the antibiotics then fail to recognise the site and no subsequent binding or inhibition will take place. Such resistance mechanisms can be found in *S. aureus* against methicillin (Blair et al., 2015). The changes in PBP or acquisition of different PBPs lead to the unsuccessful target recognition and binding of agents.

Other mechanisms are also analysed by researchers including modification of target ribosomes or reduced permeability of drugs by bacteria, whilst several mechanisms have been unclear so far. New resistance mechanisms are emerging and spreading without a break. The study on the mechanisms of antibiotic resistance need to be carried out continually since it is the key point of solving this problem.

1.1.3 How to solve antibiotic resistance

Many global or regional organisations, such as Centers for Disease Control and Prevention, USA (CDC), and the World Health Organisation (WHO) have made important efforts against antibiotic resistance, but it still remains a critical threat to human health and society. Although the problem becomes increasingly more intractable, there are a few potential solutions to lengthen the useful life of antibiotics.

First of all, societies need to be aware of the importance of combating antibiotic resistance as well as the rational use of antibiotics. To slow down the accelerating pace of antibiotic resistance and its spread, actions can be taken at different segments

of society. Insisting on some basic guidelines when using antibiotic agents is necessary for both healthcare workers and patients, including using the right antibiotic for corresponding infections, stopping unnecessary antibiotic prescriptions and finishing the full antibiotic treatment courses. Sticking to the approaches to infection control and creating a nice, clean environment for the public in order to prevent the spread of resistant organisms. Moreover, overuse of antibiotics in the agricultural sector needs to be banned and the usage of antibiotics to animals needs to be limited under veterinary supervision.

Another important method in the battle against drug resistance is to search for new antibacterial compounds. Since the rate of antibiotic resistance occurrence is faster than that of new effective antibiotic discovery, it is necessary for biotechnology and pharmaceutical companies to discover or develop new medicines to ensure the provision of effective medicines in the market. There have been some uplifting developments in the discovery of new antibiotic agents in recent years mostly belonging to conventional antibiotic classes, such as methicillin-resistant *Staphylococcus aureus* (MRSA) active cephalosporins among β–lactam agents (Kern, 2015). Scientists are also devoting themselves to studies on how to lengthen the life of existing antibiotics. As has been stated above, penicillin becomes ineffective against some bacteria because of penicillinase. Thus, a combination of penicillin with an inhibitor of penicillinase has been developed based on resistance mechanisms.

However, for a long-term solution, investing for novel antibacterial agents with different action modes is high on the agenda. Given that targeting the bacterial virulence and resistant mechanisms is a compelling method for the development of novel antimicrobials, several promising compounds such as small-molecule drugs and AMPs are included in the research. It is reported that the research on small molecule-

capped gold nanoparticles which induce bacterial resistance slowly may lead to a new direction in designing antibacterial agents (Zhao et al., 2010). In addition, AMPs have shown a broad range of biological activities in recent research. Their intrinsic properties, relative safety and efficacy in humans which are different from traditional antibiotics, makes them top candidates to broaden the limited therapeutic methods (Fosgerau and Hoffmann, 2015). In this thesis, the focus of attention is on these attractive compounds – the AMPs.

1.2 Antimicrobial peptides (AMPs)

An answer can always be found in Nature when searching for novel medicinal compounds. Antimicrobial peptides (AMPs), also known as host defence peptides (HDPs), are the key components of innate immunity found in most living creatures. They have been proposed as one of the primary alternatives to present drugs not only for their abundant distribution in Nature, but also for their broad-spectrum activity against a variety of microorganisms (e.g. virus, bacteria, fungi) and even cancer cells (Lakshmaiah Narayana and Chen, 2015). Scientists have attached so much importance to the study of AMPs that more than 4000 AMPs to date have been described based on the most recent AMP databases (Mai et al., 2016).

1.2.1 Diverse sources of AMPs

Ubiquitous in Nature, AMPs can be derived from a wide range of species, from prokaryotic organisms to eukaryotes. AMPs comprise a vital part in the defence system of hosts against the everyday exposure to a large number of potential pathogens.

AMPs from microbes

A number of papers show that AMPs exist in natural microbes like bacteria, fungi and others. It has been indicated that AMPs also take part in the fine-tuning interaction with commensal and symbiotic microbial populations (Maroti et al., 2011). For instance, a large proportion of bacteria and Archaea are able to produce a type of toxic peptides called bacteriocins. The bacteriocins cannot exert the antimicrobial function on the producers because of the inner co-expression of immunity proteins. They are actually active against other similar or closely related bacterial strains. Bacteriocins are aggressive molecules acting against rivals in the ecosphere rather than defenders. In the view of ecology, such toxic peptides promote and maintain the biodiversity of microbial communities (Maroti et al., 2011). Some representative bacteriocins can be seen in Table1.1. Several applications of bacteriocins have been carried out to assess their medical value as narrow-spectrum antibiotics (Cotter et al., 2013).

One of the big AMP families in fungi are the peptaibols which usually contain non-proteogenic amino acids, such as α–aminoisobutyric acid (Aib) (Ageitos et al., 2016). Acetylation of the N-terminal residues is common in peptaibols, and so is the amino alcohol at the C-terminus. Alamethicin is the most extensively studied peptaibol originating from the fungus *Trichoderma viride* which exhibits a broad-spectrum of biological activities, such as antibacterial and antifungal properties, especially against Gram-positive bacteria (Leitgeb et al., 2007). There are some other AMPs (plestasin, copsin, saturnisporins, and so on) found in fungi, some of which are shown in Table 1.1.

Table 1.1 Selected AMPs from bacteria and fungi (Ageitos et al., 2016)

Organism	AMP name	Activity	
		Gram+	Gram-
Bacillus brevis[1]	Gramicidin A	+	+
Bacillus subtilis[1]	Gageotetrin A	+	+
Escherichia coli[1]	Microcin J25		+
Trichoderma viride[2]	Alamethicin	+	
Pseudoplectania nigrella[2]	Plectasin	+	
Coprinopsis cinerea[2]	Copsin	+	

[1]Bacteria; [2]Fungi; Gram+: Gram positive bacteria; Gram-: Gram negative bacteria.

AMPs from animals

AMPs are widely distributed in the Animal Kingdom because they can be found not only in mammals, reptiles and amphibians, but also in fish and birds.

(1) AMPs from mammals. Mammals have a great number of AMPs in their innate immune systems, mainly including defensins and cathelicidins. There are also some AMPs from other families such as platelet antimicrobial proteins (PMPs), hepcidins and dermcidins. The original AMP from animals, defensin, was first reported from the rabbit in 1956 (Bahar and Ren, 2013). AMPs were proven to exist in human leukocytes as well during the same time (Zeya and Spitznagel, 1963). The AMPs in mammals can act *in vivo* just as antibiotics do, defeating infections by killing microbes but in a more moderated way.

(2) AMPs from reptiles. Due to their respectable antiquity and high ecological diversity, reptiles have ancient immune systems which attract great interest from scientists to discover useful AMPs. Similar to mammals, the prominent AMPs existing in reptiles belong to the cathelicidin and defensin families, as well as liver-expressed peptides, crotamine, and others (van Hoek, 2014). So far, AMPs have been discovered

mainly in the following three orders of reptiles - the testudines, crocodilians, and the squamates. Fifty cathelicidins and thirty-four β-defensins have been predicted in reptiles according to genomic analyses (Ageitos et al., 2016).

(3) AMPs from amphibians. A large number of amphibians researched over the past several years have been shown to have skins that are a rich source of AMPs. Amphibians display a diverse number of AMPs which are mostly present in the granular gland secretions of amphibian skin, with more than 500 different AMPs having been described to date (Ageitos et al., 2016). These active components are recognised as playing a crucial role either in the physiological regulation or in defence against various microorganisms or dangerous predators (Barra and Simmaco, 1995). In searching for AMPs from amphibians, one of the most promising target groups is the Ranidae family due to its great diversity and wide global distribution (Wang et al., 2016b).

(4) AMPs from fishes. It has been found that fishes are also an outstanding source of AMPs which exert the similar broad-spectrum antimicrobial activity as other species. Fish have almost all major classes of AMPs which are known as cathelicidins, defensins, hepcidins, histone-derived peptides, and there are also a special class of AMPs called piscidins, unique in fishes which are homologous to cecropins (Masso-Silva and Diamond, 2014). In addition to the common antimicrobial activities and immunomodulatory functions, fish peptides seem to possess some special properties, thus the fish peptides can be used as therapeutic agents against pathogenic microbes not only in human diseases but also in aquaculture.

(5) AMPs from birds. The two major families of AMPs existing in birds are cathelicidins and β-defensins (Zhang and Sunkara, 2014). Unlike the mammalian

peptides that usually adopt different spatial structures, α-helices are the most common conformation in the mature cathelicidins of birds. Meanwhile, the same situation can be seen in defensins of birds because there are only β-defensins, whereas mammals produce α- or θ-defensins additionally (Ageitos et al., 2016). Besides this, a type of β-defensin-related peptides, called ovodefensins, are unique in birds (Zhang and Sunkara, 2014).

AMPs from plants

Although plants do not protect themselves from the environment with specific immune systems like animals, they do produce several antimicrobial substances as part of a defence mechanism to relieve stress from drought, frost, pathogen attacks and other factors. At least 17 families of AMPs have been detected in the seeds, leaves, and roots of various plants, especially in the seeds (Tam et al., 2015). In general, they have abundant cysteines which may form one or more disulphide bridges in plant AMPs. Based on their structural characteristics, AMPs from plants can be classified into several families, with the major families including thionins, defensins, knottin-type peptides, hevein-like peptides, snakins, lipid transfer proteins, α-hairpinin families, and unclassified CRP-AMPs (Tam et al., 2015).

1.2.2 Structures and properties of AMPs

AMPs are mostly oligopeptides, with lengths ranging from five to over a hundred amino acids. In one online database for AMPs, about 97% of AMPs have less than 50 amino acid residues (Wang and Wang, 2004). There is a considerable diversity in the length, sequence and structure of natural AMPs, but the majority do share some common physical properties in general, including charge, hydrophobicity, and amphipathicity. Most of the available AMPs are proven to have a net positive charge

which are often called cationic antimicrobial peptides (CAMPs). Certainly, there are a few small anionic peptides described as having antimicrobial activities to some degree also, but they remain marginal compared with their cationic homologues and can only be found in a few animal species (Vargues, 2009). It has been indicated that around 90% are cationic peptides while only 10 percent of current AMPs are anionic peptides (Lakshmaiah Narayana and Chen, 2015).

Cationic antimicrobial peptides (CAMPs)

Cationic antimicrobial peptides (CAMPs) are the main subjects studied in current research. The positive charge ranging from +2 to +9 is imparted by the presence of basic amino acids such as lysine and arginine (Hemshekhar et al., 2016). Given the negative charge of most bacterial membranes, it is widely accepted that bacteria die because the bacterial membrane disrupts under the presence of some CAMPs. Apart from the direct antimicrobial activity against microorganisms, CAMPs are found to have an amazing modulatory capacity to influence the inherent and adaptive immune response (Brown and Hancock, 2006). These peptides are classified into four main sub-categories based on their secondary structures : (i) α-helical peptides, (ii) peptides composed of an array of β-sheets, sometimes with one or more disulphide bonds, (iii) peptides with unconventional structures like extended peptides and (iv) peptides that developed into loops, with the first two structures being the most common ones in Nature (Pushpanathan et al., 2013). For the sake of clarity, representative examples from each sub category are listed in Figure 1.4.

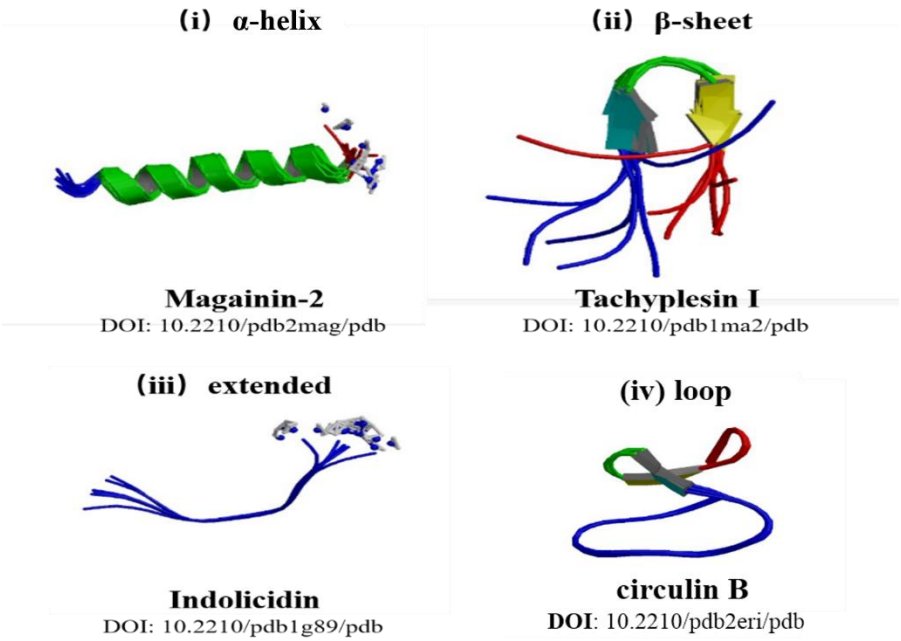

Figure 1.4 Representative peptide models indicating the structural differences of the four sub categories of CAMP. The above structures were obtained from the RCSB Protein Data Bank (PDB) with their Digital Object Identifier (DOI). For more details on each AMP, search the RCSB PDB and cross-reference the DOI.

Anionic antimicrobial peptides (AAMPs)

Contrary to CAMPs, AAMPs are negatively charged with the range from -1 to -7 and have been identified in invertebrates, vertebrates, and plants. With further study, the antimicrobial property of AAMPs is increasingly recognised although this property may be the secondary role for these anionic peptides, whilst for some AAMPs, post-translational modifications are necessary for their antimicrobial property. AAMPs have different structures as well as varying from α–helices of some amphibian peptides to the disulphide bridge structures found in some plant peptides. Their working mechanisms in some cases are not clear yet, so the research usually focus on the CAMPs. A few randomly selected AAMPs from human, amphibians, and cows are shown in Table 1.2.

Table 1.2 Selected list of anionic AMPs (Lakshmaiah Narayana and Chen, 2015, Harris et al., 2009)

Peptide	Source	Sequence
Dermcidin-1L	*Homo sapiens*	SSLLEKGLDGAKKAVGGLGKLGKDAVEDLES VGKGAVHDVKDVLDSVL
Temporin-1 Ja	Amphibians	ILPLVGNLLNDLL-NH_2
Maximin H5	Amphibians	ILGPVLGLVSDTLDDVLGIL-NH_2
Peptide B	Bovine	FAEPLPSEEEGESYSKEVPEMEKRYGGFMRF
Chromacin	Bovine	YPGPQAKEDSEGPSQGPASREK

1.2.3 Bioactivities of AMPs

Antimicrobial activities

A large number of AMPs possess a broad-spectrum antimicrobial activity against miscellaneous microbes, such as bacteria, fungi, viruses, and parasites in common, although the prominent activity conducive to host defence may show a rich diversity among disparate sites in a certain organism and between different kinds of organisms as well (Jenssen et al., 2006). Going into details, there are four main antimicrobial activities including antibacterial, antifungal, antiviral, and anti-parasitic activities.

(1) Antibacterial activity. Antibacterial activity is a common property among the natural AMPs and so antibacterial peptides are the most researched AMPs till now. CAMPs, which possess a net positive charge, make up a high percentage of the antibacterial peptides. Most of such AMPs are amphipathic due to the presence of both hydrophilic and hydrophobic residues. Considering their structural characteristics, the peptides can interact with the cytoplasmic membrane of bacteria by binding the hydrophobic region with lipid components and the hydrophilic region with

phospholipid groups (Bahar and Ren, 2013). The mutual effect on anionic lipids or other bacterial targets is often enhanced because of the net positive charge.

However, there are some AMPs which have been demonstrated to have a capability to kill bacteria at the minimal concentration without disrupting membrane integrity. For example, an antimicrobial peptide called buforin II from amphibians, is able to translocate through the membrane and accumulate intracellularly, binding directly to both RNA and DNA within the bacterial cytoplasm to exert its antibacterial function (Jenssen et al., 2006). In addition to inhibition of RNA and DNA synthesis, or interference with protein synthesis (Patrzykat et al., 2002), inhibition of cellular enzymatic activity (Kragol et al., 2001), disturbing the formation of cell wall (Kruszewska et al., 2004), other action modes have also been observed. It is likely that AMPs utilise more than one mechanism in killing various bacteria. Antibacterial peptides can often target other microbes such as fungi, viruses, and parasites, and therefore the term 'antimicrobial' is used appropriately.

(2) Antifungal activity. Many natural AMPs have potent antifungal activities which means they are capable of coping with fungal pathogens, solving the serious problem in both human health and food security. These peptides are varied in structures, ranging from large peptides, such as the histone H2A (13.6kDa) of the rainbow trout (*Oncorhynchus mykiss*), to much smaller molecules like the jelleines (8~9 amino acid residues) derived from the royal jelly of honeybees (*Apis melliferia*) (Fernandes et al., 2002) (Fontana et al., 2004). Indeed, they have polar and neutral amino acids in common. Peptides with antifungal activities usually act by either interfering with the cell wall or targeting the intracellular components (Jiang et al., 2008). The difference between antibacterial peptides is that most antifungal peptides are able to bind and target a substance called chitin which is a major component of the fungal cell wall.

Histatin 5, showing activity against some fungi, such as *Candida albicans*, is one of such antifungal peptides and has been relatively well-studied (Situ and Bobek, 2000).

Although a portion of these antifungal peptides are believed to hold promise in dealing with fungal infections, only a few have entered clinical trials because of their potential toxicity *in vivo.* For instance, XT-7, produced by the Western clawed frog, *Xenopus tropicalis,* shows a great antifungal activity against *C. albicans* when in its C-terminal amidated form (Ali et al., 2001). Nevertheless, evidence indicates that it has cytotoxicity and haemolytic activity. Fortunately, the toxicity can be reduced by proper modifications, such as the substitution of glycine at position 4 by L-lysine residue in XT-7 which can weaken the cytolytic activity without changing the anti-candidal potency (Conlon et al., 2008a).

(3) Antiviral activity. Antiviral peptides have been revealed to have a strong capacity in combating viruses and it is anticipated that this will cater for the current need to develop antiviral therapeutics. Antiviral peptides exert their functions by targeting various stages of the viral life cycle. This progression is probably associated with the viral invasion process, whilst it might also be a result of a direct attack on the envelope of virus. Antiviral peptides can inhibit the entry of viruses into host cells through interacting with specific cellular receptors. In previous research, it has been shown that antiviral peptides are able to target both enveloped RNA and DNA viruses (Horne et al., 2005). Besides, antiviral peptides have the ability to cross the membrane and accumulate inside the cell which may result in gene expressional changes, affecting the host cell antiviral mechanism (Jenssen et al., 2006).

So far, in order to identify the vital structural requirements for antiviral peptides, scientists have made several attempts through synthesising analogues of the natural

antiviral peptides. Considering the highly cationic and amphiphilic properties, some have focused their attention to the net charge and aromatic amino acids in antiviral peptides (Jenssen et al., 2004a). At the same time, other research has indicated that the net charge may be of less importance than the spatial position of the amino acids (Jenssen et al., 2004b). Moreover, cyclic lactoferricin with an internal disulphide bridge has shown a better inhibitory effect against HCMV IE/E antigen expression than the linear form (Andersen et al., 2001). The disulphide bridge may play a crucial role in the antiviral activity of such peptides. It is reported that, relationships between the structural features and the potency of antiviral peptides is not absolute. It is also concluded that the hydrophobic characteristic and positively charged amino acids are needed, but they are not sufficient conditions for antiviral activity (Giansanti et al., 2005). According to an online antiviral peptides database called AVPdb, 2683 peptides, including 624 modified peptides, have been verified experimentally for antiviral activity (Qureshi et al., 2014).

(4) Anti-parasitic activity. Compared to the above three activities, anti-parasitic activity is not as common in currently known AMPs. A number of natural and synthetic AMPs have been examined for anti-parasitic activity. One of the first AMPs discovered to act against parasites was magainin, which was reported to kill *Paramecium caudatum* (Zasloff, 1987). In some situations, anti-parasitic peptides exert the function in a similar way as the peptides with antibacterial, antifungal, or antiviral activities, by interacting with host cell membranes. Cathelicidin is one of such peptides which can form pores in cell membranes to strike down *Caernohabditis elegans* (Park et al., 2004). However, the anti-parasitic activity may be mediated by different mechanisms. There is also an online database of anti-parasitic peptides called

ParaPep, which contains 863 anti-parasitic peptides tested against 12 various kinds of parasites with 519 unique peptides (Mehta et al., 2014).

Other bioactivities

In addition to the direct antimicrobial function, AMPs encompass non-antimicrobial bioactivities related to wound healing, immune activation and regulation, inflammation and others. Additionally, a range of studies have claimed that the broad-spectrum antimicrobial activity against bacteria, fungi, viruses, and parasites is not the only bioactivity of several AMPs, they also possess similar activity against cancer cells. Both antimicrobial and anticancer activities probably share similar cell-killing mechanisms, for example, membranolysis (Kao et al., 2012). All of these bioactivities need to be explored further in order to develop more efficient agents for current needs.

1.2.4 Mechanisms of AMP action

The mechanisms of action are diverse and complex among various kinds of AMPs and are currently still under study. Independent of the proposed targets, interaction with the cytoplasmic membrane is the common first step in any mechanism of AMPs (da Cunha et al., 2016). This then results in two different possible action modes in accordance with specific AMPs and species of microorganisms: (i) membrane disruptive mechanisms, which cause cell lysis; and (ii) membrane non-disruptive mechanisms, which lead to the translocation across the cell membrane and targeting of intracellular sites (Maroti et al., 2011). The modes of action are directly influenced by the different compositions of cell walls in various types of microbes.

Membrane disruptive mechanisms

Following the initial interaction with the cytoplasmic membrane, AMPs, mostly with a net positive charge, attack the target cell membrane, forming transmembrane pores.

This results in the leaking of the endocellular contents, causing the eventual death of cells. The hypothetical modes of action for AMPs can be described by three classical models: (i) the Barrel-stave model; (ii) the Toroidal-pore model; and (iii) the Carpet model (Mai et al., 2016).

The Barrel-stave model (Figure 1.5.A) suggests that a number of individual peptides aggregate on the cell membrane surface. Then the aggregated AMPs perpendicularly insert themselves into the lipid bilayer of the membrane, forming transmembrane pores which cause the release of intracellular compounds and the death of cells in the end. In this model, hydrophobic regions are closely-packed together with the lipid core region of the bilayer, facing to the outer lipid environment, while the hydrophilic parts point inward to the newly formed transmembrane pore. Alamethicin is one of the most well-studied examples found to use the Barrel-stave model. Using X-Ray diffraction, the alamethicin-induced transmembrane pore is shown to consist of eight alemethicin helices belonging to the Barrel-stave type (Qian et al., 2008).

The Toroidal-pore model (Figure 1.5.B) has similarities with the Barrel-stave model to some degree. The difference between the two models is that the transmembrane pore in the Toroidal-pore type is composed of both the peptide and the phospholipid bilayer of the bacterial cell, while just the peptides themselves form the pores in the Barrel-stave model (Yeaman and Yount, 2003). The membrane is opened perpendicularly after the formation of a ‘flip-flop’ translocation channel (da Cunha et al., 2016). Throughout the whole process, the AMPs are closely associated with phospholipid head-groups which usually face towards the pore centre. In other words, the pores are formed together by the AMPs and lipid, with the membrane curving inward. The formation of the transmembrane pore and translocation of the peptide are dependent on a specific ratio of the peptide and the lipid (Giuliani et al., 2007).

In the Carpet model (Figure 1.5.C), AMPs exercise a great influence on the membrane architecture by covering some areas of the membrane surface, in a detergent-like mode (Rotem and Mor, 2009). It is actually a diffuse effect of the nonspecific membrane permeabilisation caused by AMPs (Yeaman and Yount, 2003). Similar to other models, peptides firstly connect to the membrane mainly with the help of electrostatic interactions (Shai and Oren, 2001). Small areas of membrane are coated with AMPs in a carpet-like fashion with hydrophobic sides facing inward. When the concentration of AMPs attached to the cell surface gets higher and reaches a threshold level, the AMP molecules form a number of micelles which can destroy the membrane. Then the AMPs penetrate the lipid bilayer to form the pore, disrupting the structure of membranes (Pushpanathan et al., 2013).

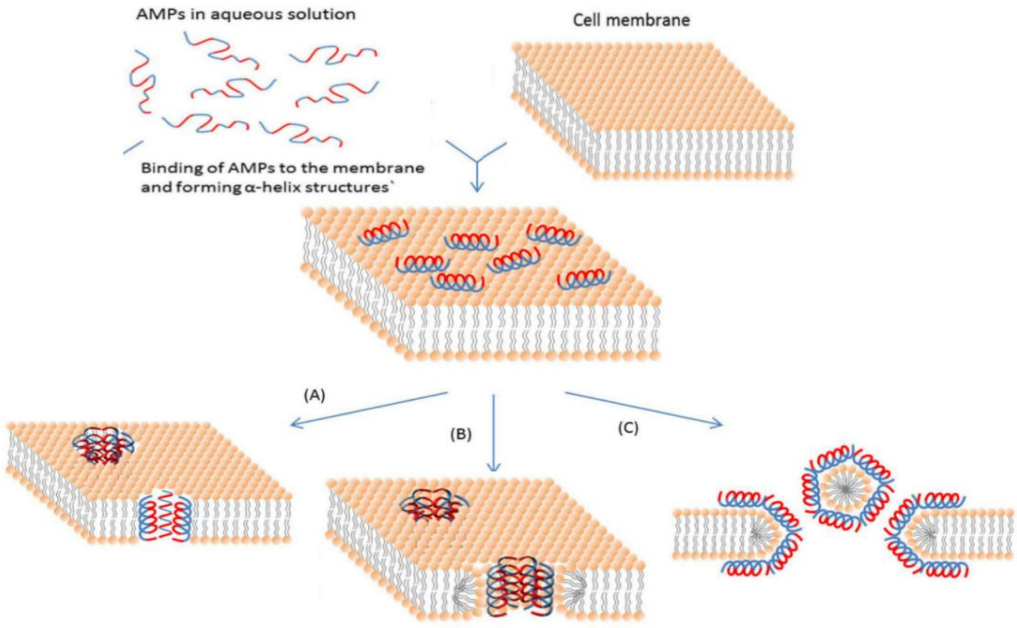

Figure 1.5 Schematic representation of the classical membrane disruptive mechanisms of AMPs (Bahar and Ren, 2013). (A) the Barrel-Stave model. (B) the Toroidal-pore model. (C) the Carpet model. In this figure, the blue colour represents the hydrophobic parts of AMPs, while the red colour indicates the hydrophilic portions in AMPs.

During recent years, a number of new disruptive models have been described as well as some models indirectly relating to membrane disruption, such as the Disordered toroidal model, the Charged lipid clustering model, the Membrane thinning/thickening model and so on (da Cunha et al., 2016).

Membrane nondisruptive mechanisms (Intracellular targets of AMPs)

In early AMP action mechanism studies, it was advised that the concentration of AMPs used should be high enough so that the microbes could be killed by lysis. However, it has been recently confirmed many times that AMPs are able to cause the death of microbes using other mechanisms without causing membrane permeabilisation. Many studies indicate that a number of AMPs induce cell damage at a low concentration through acting on one or more intracellular targets, including the inhibition of protein, DNA or RNA synthesis (Figure 1.6).

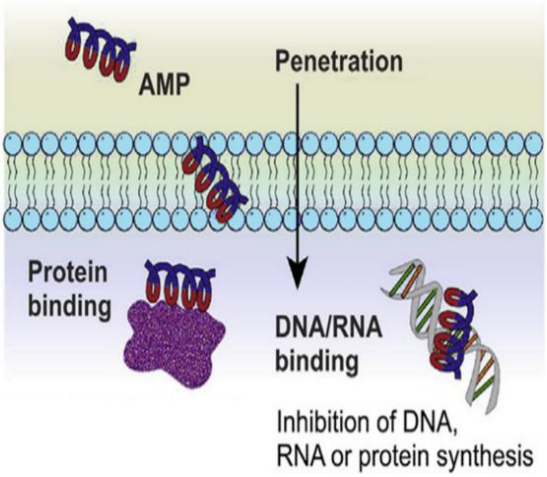

Figure 1.6 Proposed antimicrobial mechanisms of AMPs action based on intracellular targeting (Mai et al., 2016)

Some AMPs exert their functions via inhibiting the synthesis of DNA, RNA or protein. One example of this is indolicidin. Although indolicidin can form transient holes in the bacterial cytoplasmic membrane causing the membrane depolarisation, it neither

enters the cell nor leads to the cell wall lysis. This phenomenon suggests that it has more than one mechanism. The following study explains that the indolicidin has the ability to reduce the synthesis of DNA, leading to the filamentation of *E. coli* to express its antimicrobial activity (Nicolas, 2009). Similar to indolicidin, a peptide from pig intestine named PR-39, combats bacteria in a non-lytic method through stopping DNA and protein synthesis (Bahar and Ren, 2013). Another well-studied example is Buforin II which is derived from the Chinese toad, *Bufo gargarizans*. It has been found to inhibit the gene expression of DNA and mRNA during the transcription and translation processes (da Cunha et al., 2016).

Some peptides inhibit the synthesis of the cell wall. Without such essential cell structure, the cell would lyse because of the high cytoplasmic osmotic pressure (Guilhelmelli et al., 2013). Peptidoglycan is the main component in bacterial cell walls which is not found in eukaryotes. Therefore, if the synthesis of peptidoglycan is inhibited, the microbes must be destroyed. One AMP family known as Class I bacteriocins is capable of targeting a crucial precursor of peptidoglycan synthesis called lipid II in order to kill bacteria (Yount and Yeaman, 2013).

There are some other putative intracellular targets involving DNA transcription and RNA translation and some AMPs can trigger the cell death by induction of incorrect protein folding. Considering the obscure mechanism details, some action modes of AMPs need further research. Furthermore, some evidence compels us to think that there might be a multiple-hit strategy based on two or more co-instantaneous mechanisms in a single AMP since all the mechanisms are not exclusive. Meanwhile, it is necessary to be aware of the fact that the AMP action modes may change under different experimental conditions and that they can be affected by parameters such as temperature, osmolarity and media pH (Yeaman and Yount, 2003).

1.3 The frog investigated in this study

In this research, attention was focused on the AMPs from amphibian skin secretion. Amphibians have developed a remarkable diversity in life history by adapting to many different aquatic and terrestrial habitats (Clarke, 1997). To protect themselves against infections and the threat from predators, amphibians have developed an array of defence countermeasures. Their skin plays an important role in everyday life and helps to exploit widely disparate ecological niches. By further study, amphibian skin glands, especially granular glands, produce defensive skin secretions against infection and predators. The granular glands are expected to be the source of major effective compounds in amphibian skin through numerous investigations on amphibian skin gland secretions which contain a great deal of compounds with novel molecular structures and clinically useful functions. Bioactive peptides are the predominantly active components within the skin secretion. Thus, the granular gland secretions are considered to be an unequivocally intriguing source of raw materials of novel potential bioactive peptides. Since the clinical problems caused by bacterial and fungal infections become more serious, it is worth investigating the potentially useful AMPs derived from amphibian skin secretions.

1.3.1 *Odorrana versabilis*

Odorrana versabilis (Figure 1.7) is a species of frog belonging to the Ranidae family. Ranidae are also known as true frogs with a wide distribution and are one of the most diverse families in the order Anura. It contains several genera and more than 600 species are found worldwide (Wang et al., 2016b). Members of this family are mostly aquatic or semiaquatic, with a few being ground burrowers or arboreal. *Odorrana*, which are commonly known as the odorous frogs, comprise 58 species (Darrel R,

2016) and are a genus of the Ranidae family distributed in East Asia and surrounding regions. *Odorrana versabilis* is known from the Provinces of Guizhou, Jiangxi, Hunan, Guangdong, Guangxi and Hong Kong in China. Its natural habitats are subtropical or tropical moist lowland forests, mountainous forests and surrounding hill streams. It is one of the most sizeable and drably-coloured of the genus *Odorrana*. As its name suggests, it produces an offensive and pungent odour when it is under threat (Chen et al., 2006). It is a common species, listed as of least concern with regards to its relatively wide distribution (Liang and Cheng, 2004). Nevertheless, the species is in serious decline because of its loss of habitat.

Figure 1.7 *Odorrana versabilis* is commonly called the Chinese bamboo leaf odorous frog. (Chinese Academy of Sciences Kunming Animal Institute, (n.d.). *Odorrana versabilis*. [image] Available at: http://amuseum.cdstm.cn/AMuseum/dongwu/page/animal_detail_5152.html [Accessed 11 Dec. 2016])

1.3.2 AMPs from *Odorrana versabilis*

Several research papers on AMPs from *Odorrana versabilis* have been reported during the past years. A range of peptides derived from *Odorrana versabilis* have been discovered to possess pharmacological activities, whereas there are still more bioactive peptides waiting to be detected in future research.

The bradykinins and related peptides are widely considered as one of the most widespread families in amphibian defensive skin secretions. An avian bradykinin, ornithokinin, is reported to be found in the skin secretion of *Odorrana versabilis*. In the same study, the synthetic ornithokinin was proven to be active in both the rat ileum and the uterus (Lyu et al., 2014). Moreover, two novel non-canonical bradykinin antagonist peptides isolated from *Odorrana versabilis* are described by Wu, Yuxin. The two peptides contain a 'Rana box'-like structure which is a normal motif in AMPs at their C-terminals (Yuxin, 2013). Gk-22 amide is another example of an AMP derived from *Odorrana versabilis*. It is a 22-mer amidated peptide with an internal disulphide bond between Cysteine 11 and 20. A synthetic replicate of Gk-22 was reported to exert powerful and dose-dependent contraction on the smooth muscle of the rat urinary bladder (Tao et al., 2010). More examples of AMPs from *Odorrana versabilis* can be found in Table 1.3.

Table 1.3 Selected examples of AMPs from *Odorrana versabilis* with their corresponding entry IDs in UniProtKB (a protein knowledge database) and sequence (UniProtKB, 2016)

Peptide	Entry ID	Sequence
Ranatuerin-2Va	Q1JS92	GLLDTIKNTAKNLAVGLLDKIKCKMTGC
Ranatuerin-2Vb	Q1JS90	GIMDTVKGVAKTVAASLLDKLKCKITGC
Temporin-1V	Q1JS91	FLPLVGKILSGLI
Brevinin-1Vb	Q1JS93	FLPLIAGLAANFLPKIFCAITKKC
Esculentin-1Vb	Q1JS87	GIFTKINKKKAKTGVFNIIKTIGKEAGMDVIRAGIDTISCKIKGEC
Esculentin-2Vb	Q1JS89	GLFSILKGVGKIAIKGLGKNLGKMGLDLVSCKISKEC
Palustrin-3b	Q1JS88	GIFPKIIGKGIKTGIVNGIKSLVKGVGMKVFKAGLSNIGNTGCNEDEC
Palustrin-1c	Q1JS94	ALSILRGLEKLAKMGIALTNCKATKKC
Brevinin-1V	Q2UXV8	FLPLIASVAANLVPKIFCKITKKC

1.4 Trends and challenges

In recent years, the need for novel antimicrobial agents tends to be more and more urgent because of the increase of antibiotic resistance. In order to address the problems relating to antibiotic resistance, a great deal of work has been carried out to search for more efficient drugs with a remote possibility to develop resistance. The discovery of AMPs is a major stride in developing new therapeutic medicines. AMPs from amphibians have earned worldwide attention in their promise as potential antibiotics in human health. As a result, much research has steered in this direction. However, we are still faced with several challenges, such as the decline of amphibians, some restrictions of AMPs, and difficulties in developing AMPs.

Amphibians which contain more than 7,000 known species are now in decline with more than 70% of the species affected globally (Hayes et al., 2010). The population decline has been observed globally since the early 1990s (Wake, 1991). Currently, it is assessed that about 32% of amphibians are threatened, while almost 168 species are considered to have gone extinct and more than 2,469 species are in decline (AmphibiaWeb, 2016). Consequently, there is an increased tendency in the number of extinct and threatened species (Stuart et al., 2004). Such a global phenomenon is caused by the collaborative influence of several factors. Among the various factors, habitat destruction may be one of the major factors contributing to the decline, as well as environmental pollution and global climate change. It will be a great loss for the world if the amphibians undergo extinction.

As said above, AMPs have an array of advantages as potential antibiotics, such as a broad-spectrum of antimicrobial activity, low levels of induced resistance, and rapid onset of bacterial cell death (Leitgeb et al., 2007). Nevertheless, there are some

restrictions to the use of AMPs. On the one hand, AMPs actually exhibit potential toxicity to human cells which limits their systemic application, as well as potential susceptibility to proteolysis (Hancock and Sahl, 2006). On the other hand, AMPs may lose their activity in some ways when exposed to salt and serum conditions or under extreme pH environments because of their sensitivity to severe conditions (Leitgeb et al., 2007). AMPs usually have low oral availability so that injection is required in general (Vlieghe et al., 2010). Moreover, the confounding bioactivities of AMPs make difficulty in using AMPs as clinical medicines since some mechanisms of action modes have not been subjected to in-depth studies. Besides, AMPs are expected to withstand drug resistance, but it is unrealistic to claim that no microbial pathogens can obtain resistance to AMPs. Considering the already reported resistance against AMPs (Zhu et al., 2017), it is necessary to attach great importance to the appearance of resistant pathogens as well.

In addition to the intrinsic limitations of AMPs, another bothersome impediment in developing AMPs is the prohibitive cost of manufacture. The expensive price of screening and synthesising peptides which is often five to twenty times as high as that of traditional antibiotics has compelled many pharmaceutical companies to give up promoting the clinical use of peptides (Giuliani et al., 2007). From studies on synthesised and modified AMPs, it is obvious that the characteristics of a peptide can be changed to some extent even due to small modifications of the structure. Therefore, rational design of peptides is required to exert maximum function of AMPs with high specificity. However, the lack of robust guidelines for rational design has slowed down their development (Bahar and Ren, 2013).

1.5 Aims and objectives of this thesis

The aims and objectives of this study are to isolate, identify and determine the structure of a bioactive peptide from the defensive skin secretion of *Odorrana versabilis* through a series of experiments. A bioactive peptide is expected to be found through "shotgun" molecular cloning with its complete peptide sequence.

Once such a peptide has been unequivocally structurally-characterised, a chemically synthetic replicate of the natural peptide will be produced through solid phase peptide synthesis. The replicate will be identified and purified by employing reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometric analytical techniques to acquire a degree of purity appropriate for bioactivity assessments, including antimicrobial, haemolysis and cancer cell antiproliferation assays.

During this research, a number of biotechnology and chemical analysis methods will be learnt, including the "shotgun" molecular cloning, solid-phase peptide synthesis, RP-HPLC, MALDI-TOF-MS, antimicrobial assays, haemolysis assays and anti-cancer cell assays. All of these experimental technologies will prove to be of great use for our future studies and working knowledge.

Chapter2 Molecular cloning

2.1 Materials and methods

2.1.1 Frog skin secretion acquisition

The experimental amphibians acquired in China, *Odorrana versabilis*, were kept in a purpose-designed amphibian facility (in Medical Biotechnology Centre, Queen's University of Belfst) under simulated natural conditions with an adaptable temperature of 20-25 °C, a 12 h-light/12 h-dark cycle and vitamin/mineral-loaded crickets fed every 2-3 days for 4 months before secretion harvesting. The gentle electrical stimulation was used to obtain the amphibian skin secretion. The mild transdermal electrical stimulation (6 V DC; 50 Hz; 4 ms pules-width) was utilised for several periods of 20 s duration to induce the amphibian glands to secrete onto the dorsal surface. A bipolar electrode of 21G platinum was gently moved along the frog dorsal skin until the secretion was visible. Distilled deionised water was used to rinse off the resulting secretion, which was then collected into a chilled glass baker. Finally, the skin secretions were snap frozen in liquid nitrogen, lyophilised in a Hetosicc 2.5 freezer dryer (Heto, UK) and stored at -20 °C before analysis. All the procedures were subject to ethical approval and proceeded under appropriate UK animal research and project licenses.

2.1.2 mRNA isolation from the skin secretion

Intact polyadenylated mRNA was rapidly isolated from *Odorrana versabilis* skin secretion using a Dynabeads® mRNA Direct™ Kit (Invitrogen, Lithuania) following the protocol for mRNA isolation from solid plant/animal tissues based on the A-T base pairing.

Preparation of sample solution

Five mg of crude lyophilised skin secretion of *Odorrana versabilis* were dissolved in 1 ml Lysis/Binding Buffer in a tube. The tube was vortexed for 1 min and kept on ice for 1 min. The operation was repeated 10 times to ensure the complete dissolution, after which the tube was centrifuged at 18,000 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany).

Preparation of Dynabeads® Oligo $(dT)_{25}$ beads

The Dynabeads® Oligo $(dT)_{25}$ beads (5 mg/ml) were resuspended by mixing gently and thoroughly before 250 µl of the suspension were transferred to a 1.5 ml tube. The tube was then placed on a Dynal MPC-S magnet device to remove and discard the storage buffer. Afterwards, the beads were washed by mixing with 250 µl of Lysis/Binding Buffer gently. Finally, the beads were placed on the magnetic rack to remove the supernatant till the lysate was well-prepared.

Isolation of mRNA

When 1 ml supernatant of the sample solution were transferred into the 1.5 ml tube containing prepared beads, the mixture was blended by slowly and gently shaking for 15 min at room temperature. Then the tube was placed on the magnetic rack to remove the supernatant. The mRNA/beads complex was gently washed three times with 500 µl Washing Buffer A and twice with 500 µl Washing Buffer B at room temperature. The washing steps were operated with the help of the magnetic rack as well, discarding the buffer completely after each washing step. After washing, mRNA was eluted from beads using 18 µl of Elution Buffer (Tris-HCl, 10 mM) and was incubated at 80 °C for 2 min. Then the tube was placed onto the magnetic rack as soon as possible to

obtain the supernatant containing mRNA. Finally, the supernatant was pipetted into a PCR tube and placed on ice immediately.

2.1.3 cDNA library construction

A BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK) was applied to construct the cDNA library of *Odorrana versabilis* skin secretion.

For the preparation of 3'-RACE-Ready cDNA and 5'-RACE-Ready cDNA, two different kinds of reagent mixtures were prepared as described in Table 2.1. Following this, 3'-RACE-Ready cDNA in triplicate and 5'-RACE-Ready cDNA in duplicate were added into 5 individual PCR tubes.

Table 2.1 The components of 3'-RACE-Ready cDNA and 5'-RACE-Ready cDNA reactions

3'-RACE-Ready cDNA	5'-RACE-Ready cDNA
4 µl mRNA sample	3 µl mRNA sample
1 µl 3'-CDS primer	1 µl 5'-CDS primer
	1 µl SMART II A Oligo
BD SMART II	5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'
3'-CDS Primer A	5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30V N–3'
5'-CDS Primer	5'–(T)25V N–3' (N = A, C, G, or T; V = A, G, or C)

After being briefly centrifuged, all the five PCR tubes were incubated at 70 °C for 2 min and cooled on ice for another 2 min. Afterwards, 2 µl of 5×First-strand Buffer, 1 µl of DTT (20 mM), 1 µl of dNTP (1 mM) and 1 µl of reverse transcriptase were added into each tube (Table 2.2).

Table 2.2 Components of each tube for 3’ or 5’-RACE-Ready cDNA

Reagent	3’-RACE PCR	5’-RACE PCR
mRNA sample	4 µl	3 µl
3’-CDS primer (10 µM)	1 µl	-
5’-CDS primer (10 µM)	-	1 µl
SMART II (10 µM)	-	1 µl
5X First-Strand Buffer	2 µl	2 µl
DTT (20 mM)	1 µl	1 µl
dNTP Mix (10 mM)	1 µl	1 µl
BD RTase (100 Unit/µl)	1 µl	1 µl

All tubes were incubated at 42 °C for 90 min in a thermal cycler after being centrifuged briefly. When the programme was finished, 50 µl PCR water was added to the reaction mixture and then briefly centrifuged. The mixtures were kept at 72 °C for 7 min. When these were all completed, the reaction products were stored at -20 °C.

2.1.4 Rapid amplification of cDNA 3’ends

The 3’-RACE PCR reaction was carried out using the BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK).

The following components (Table 2.3) were combined and mixed completely by pipetting and an extra volume was calculated and added to ensure sufficient volume for the RACE-PCR reaction whilst PCR water was added to negative control instead of cDNA library. After brief centrifugation, the tubes were placed into a PCR machine with the following programme in Table 2.4. The final PCR products were stored at 4 °C.

Table 2.3 Components in each tube of sample group in RACE PCR

Reagent	Volume	Final concentration
PCR-Grade Water	3.1 µl	
10X BD Advantage 2 PCR Buffer	1.5 µl	1.5 X
dNTP Mix (10 mM)	0.2 µl	0.2 mM
NUP (20 µM)	0.5 µl	1 µM
Sense Primer (20 µM)/Anti-sense primer	0.5 µl	1 µM
50 X BD Advantage™ 2 Polymerase Mix	0.2 µl	1 X
cDNA library	5 µl	

Table 2.4 The programme setting for 3'/5'-RACE PCR

Stage	Parameter
Stage 1	Initial denaturation at 96 °C for 1min
Stage 2	40 cycles (denaturation at 96 °C for 20 s, primer annealing at 55 °C for 10 s, extension at 60 °C for 4 min)
Stage 3	Final extension at 72 °C for 10 min

2.1.5 Agarose gel analysis of 3'-RACE PCR products

The PCR products were analysed using a conventional gel electrophoresis which could separate different sized DNA fragments as well as detect whether the primer had annealed to cDNA template or not.

An agarose gel (≥ 1% w/v) was made up by adding 0.45 g agarose powder (Invitrogen, UK) to 35 ml freshly prepared 1 X Tris/Borate/EDTA (TBE) buffer (Invitrogen, UK) in a 200 ml flask. The mixture was heated in a microwave oven to dissolve agarose powder in TBE buffer completely. After being cooled down, 2.5 µl of 10 mg/ml Ethidium Bromide (EB) (Sigma-Aldrich, USA) was added into the flask and the flask

was gently swirled to mix fully. Then the molten gel was poured into a dry gel electrophoresis tank with combs inserted. After solidification of the gel for 30 min, the comb was vertically removed and sufficient running buffer (1 X TBE buffer) was added to submerge the solid gel. Afterwards, 2 µl 100 bp DNA ladder (BioLabs, UK) was injected into the outside well of a lane as a comparison, while 1.5 µl sample evenly mixed with 0.5 µl 6 X Loading Dye (Promega, USA) was added into the following wells. The electrophoresis was run at 90 V, and the process was finished when the yellow colour indicator reached two-third of the gel. The DNA bands on gel were observed under an electronic UV Transilluminator (Ultra-Lum Inc, USA). The size of the DNA fragments could be analysed through comparing with the ladder band.

2.1.6 Purification of 3'-RACE PCR products

The 3'-RACE PCR products were purified using a Cycle Pure Kit (Omega Bio-Tek, USA) to eliminate impurities such as buffer, polymerase and unreacted primers.

The 3'-RACE PCR products were mixed with 95 µl CP Buffer (quintuple CP buffer). After enough mixing, the mixture was loaded onto the centre of a filter cartridge with a sleeve. The cartridge was centrifuged at 15,000 × g for 1 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany) and the filtrate was discarded then. Afterwards, the cartridge was placed back into the sleeve and 700 µl DNA Washing Buffer was added into the cartridge. The centrifugation (15,000 × g, 1 min) was needed to discard the filtrate. Similarly, 500 µl DNA Washing Buffer was also added into the cartridge and centrifuged again at 5,000 × g for 1 min. Then, the empty cartridge with collection sleeve was centrifuged at 8,000 × g for 2 min to remove the remaining liquid completely. After that, the cartridge was transferred into a 1.5 ml tube and the former sleeve was discarded. 30 µl PCR water was added into the

cartridge membrane and the tube was incubated at room temperature for 2 min before being centrifuged at 8,000 × g for 1 min. Finally, the tube with products was placed into a concentrator for dying before being sealed and stored at -20 ºC.

2.1.7 Ligation of DNA products with pGEM-T Easy vector

The ligation step was performed using a pGEM®-T Easy Vector system (Promega, USA).

Eight microliters PCR water was added into the tube with 3'-RACE PCR purification products from section 2.3.5. The tube was vortexed for 1 min and then kept on ice for 30 s. This operation was repeated 5 times. After the complete dissolution, the tube was centrifuged briefly and kept on ice for the next step. Then the reaction reagents were transferred into a new PCR tube in the following order as well as the volumes shown in Table 2.5. When preparing the mixture, the reagents were added and mixed without producing bubbles. Finally, the mixture was incubated still at room temperature for 1 h to allow the ligation process before it was stored at 4 ºC.

Table 2.5 The reaction reagents used in ligation with the volume and final concentration

Reagent	Volume	Final concentration
2 X Rapid Ligation Buffer	2.5 µl	1 X
pGEM®-T Easy Vector (50 ng/µl)	0.5 µl	5 ng/µl
Diluted PCR products	1.5 µl	
T4 DNA Ligase (3 Unit/µl)	0.5 µl	0.3 Unit/µl

2.1.8 Transformation

The transformation was performed using JM109 High Efficiency Competent *E. coli* cells (>10^8 CFU/µg) (Promega, USA).

The *E. coli* cells were taken out of the freezer and thawed on ice for 4 min. 2.3 µl ligation product and 55 µl *E. coli* cells were transferred into a tube and mixed with each other by gently flicking. The tube was kept on ice for 20 min and then heat-shocked at 42 ºC for 47 s before being returned to ice for 2 min. Here 950 µl S.O.C medium (Invitrogen, USA) was added to the tube and the tube was incubated at 37 °C for 2.5 h with a shaking rate of 150 rpm. The transformation products were just obtained after the 2.5 h incubation.

LB Agar plates with 550 µl of Ampicillin (100 µg/ml) (Roche, USA) were prepared before the transformation. During the shaking incubation, 5 ready-to-use LB-ampicillin plates were taken out to prepare LB Agar plates with ampicillin/IPTG/X-Gal. 110 µl of 0.1 M IPTG (Promega, USA) were added into each dish and left to dry, after which another 20 µl X-Gal (50 mg/ml) (Promega, USA) was spread over each plate as well. Then the 5 plates were incubated at 37 °C for around 45 min.

One hundred microliters transformation product were pipetted into each plate respectively and spread symmetrically and gently with a spreader. All the Petri dishes were placed upside down and incubated at 37 °C for 18-22 h.

2.1.9 Blue-white screening

The spare LB-ampicillin plates were taken out of 4 °C storage and had IPTG and X-Gal added as per the method shown in section 2.3.7 to prepare LB Agar plates with ampicillin/IPTG/X-Gal after which the plates were incubated at 37 °C for 45 min.

The 5 plates with transformation product were carried out of the incubator and the blue-white screening was operated near the Bunsen burner. The white colonies (recombinants) differentiated from the blue colonies (non-recombinants) in appropriate areas were chosen and transferred to the new activated plate through drawing the continuous line resembling 'Z' with a sterilised inoculating loop. Three prepared LB Agar plates with ampicillin/IPTG/X-Gal were divided into numerous squares. One white colony was sampled to one square of a plate. When all inoculations had been finished, the three plates were placed upside down in a 37 °C incubator overnight.

2.1.10 Isolation of recombinant DNA from JM109 cells

Only pure white colonies were selected for the next cloning PCR step. Each white colony was carefully picked up using an autoclaved tip and resuspended in a corresponding 1.5 ml tube containing 20 µl PCR water. After this, all tubes were incubated in a heating block at 100 °C for 5 min and then were transferred onto ice immediately for another 5 min. Each tube was vortexed for 30 s and centrifuged at 8,000 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany) for 5 min. Therefore, the supernatant transferred into clean tubes was ready for next step.

2.1.11 Cloning PCR reaction

A Go-taq PCR kit was used for the cloning PCR reaction. The components of each PCR tube are shown in Table 2.6. Then the PCR tubes were centrifuged briefly before starting the PCR programme. All the PCR tubes were incubated in a PCR machine using the cloning PCR programme shown in Table 2.7. Finally, the cloning PCR products were stored at 4 °C.

Table 2.6 Components of each tube in cloning PCR

Reagent	Volume	Final concentration
5 X Cloning Buffer	10 µl	1 X
dNTP Mix (10 mM)	1 µl	0.2 mM
M_{13}F (20 µM)	2.5 µl	1 µM
M_{13}R (20 µM)	2.5 µl	1 µM
PCR-Grade water	31 µl	
Taq polymerase (5 Unit/µl)	0.25 µl	0.025 Unit/µl
DNA supernatant	2.5 µl	

Table 2.7 Cloning PCR programme

Stage	Parameter
Stage 1	initial denaturation at 94 °C for 1 min
Stage 2	31 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 3 min)
Stage 3	final extension at 72 °C for 3 min and storage at 4 °C prior to use

2.1.12 Gel analysis & purification of cloning PCR products

The cloning PCR products were subjected to gel electrophoresis as described in section 2.3.4. Then the selected cloning PCR products were purified and washed out with the same method mentioned in section 2.3.5.

2.1.13 Sequencing PCR reaction

The sequencing PCR reaction was carried out using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

For the sequencing reaction, the sequencing Master Mix was prepared in advance and 18.5 µl of the mixture was mixed with 2.5 µl of each purified DNA product (Table 2.8). The tubes were centrifuged briefly and then placed into the PCR machine and

incubated under the programme named ‘Sequencing’ (Table 2.9). When the programme was finished, all the PCR tubes containing the extension products were taken out and stored at 4 °C.

Table 2.8 Components in each sequencing reaction tube

Reagents	Volume
PCR-Grade water	12.4 µl
Diluted $M_{13}F$ or $M_{13}R$	1.14 µl
2.5 X Ready reaction mix	2.86 µl
5 X BigDye Sequencing Buffer	3.57 µl
Purified cloned PCR products	2.5 µl

Table 2.9 Sequencing reaction PCR programme

Stage	Parameter
Stage 1	initial denaturation at 96 °C for 1 min
Stage 2	26 cycles (denaturation at 96 °C for 20 s, annealing at 55 °C for 10 s, extension at 60 °C for 4 min)
Stage 3	preservation at 4 °C for 7 min

2.1.14 Purification of extension products

Each extension product was mixed well with 72 µl 95% Ethanol (Sigma-Aldrich, USA) before being transferred into a 1.5 ml tube containing 10 µl PCR water. All samples were vortexed for 30 s and incubated at room temperature for 20 min. Then, all tubes were centrifuged at 13,000 × g for 20 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany). After discarding the supernatant quickly using tissue paper, 260 µl 70% ethanol were added into each sample and vortexed for 30 s. The tubes were again centrifuged at 13,000 × g for 10 min, after which the supernatant was

quickly removed as mentioned before. Afterwards, the tubes were vacuum-concentrated until dried and the products were stored at room temperature.

2.1.15 Sequencing

All samples were dried in a concentrator for 45min to remove ethanol thoroughly before sequencing. 10 µl HiDi (highly deionised-formamide) was added into each tube and each tube was vortexed for 37 s. The tubes were centrifuged briefly before being heated at 95 °C for 4.5 min. After heating, the samples were immediately transferred onto ice and kept for 3 min.

All liquid in each tube was pipetted into a corresponding well of a 96-well sequencing plate without any bubbles. Afterwards, the plate was delivered to be sequenced using an ABI 3730 automated sequencer (Applied Biosystems, USA).

2.2 RESULTS

Through the 'shotgun' molecular cloning, a single precursor-encoding cDNA of a peptide was consistently cloned from the skin secretion-derived cDNA library of *Odorrana versabilis*. The open-reading frame (ORF) in the cDNA encodes a polypeptide which consists of 67 amino acids (Figure 2.1). The topological structure of the peptide precursor contains three distinct domains—a signal peptide sequence, an acidic spacer and a mature peptide sequence. The putative signal peptide with 22 amino acids is followed by the acidic spacer ending with two representative propeptide convertase processing sites (-KR-). The subsequent domain is the mature peptide sequence consisted of 21 amino acids.

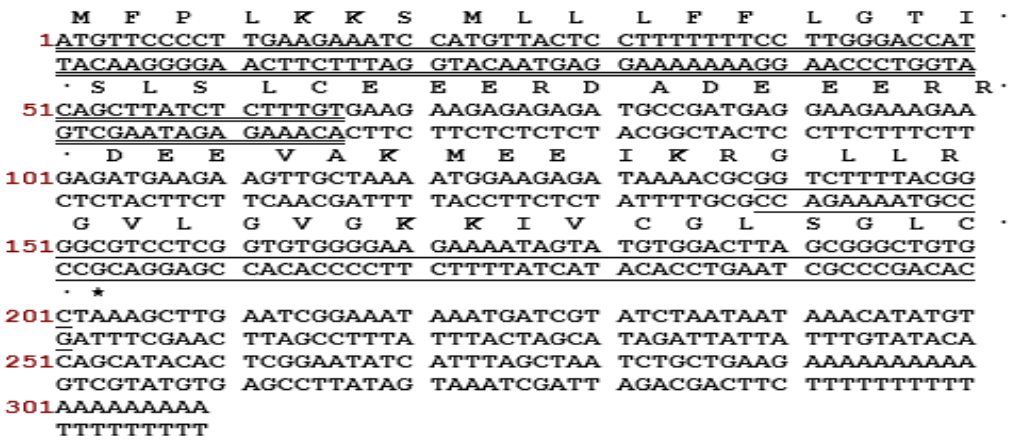

Figure 2.1 The open-reading frame (ORF) sequence of cDNA encoding the biosynthetic precursor of QUB-2040 from the *Odorrana versabilis* skin secretion-derived cDNA library. The putative signal peptide sequence (double-underlined), the mature peptide sequence (single-underlined) and the stop codon (asterisk) are indicated.

Both the mRNA and the ORF amino acid sequence were searched online using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). The alignments of several results are shown in Figure 2.2 (A) and (B). The sequence of QUB-2040 is similar to nigrocin-related peptide precursors isolated from other species of *Odorrana* frogs. Meanwhile, from the graphic summary in the ORF sequence analysis which was also shown in the BLAST results, the peptide QUB-2040 belongs to Antimicrobial 22 superfamily (Figure 2.3). It is almost identical to nigrosin-RA4 (Sequence ID in BLAST: ACZ71273.1) isolated from *Odorrana andersonii* according to the BLAST results of the mature peptide (shown in Figure 2.2 C). In addition, it is highly homologous with nigrosin-RA3 (Sequence ID in BLAST: ACZ71311.1) which is also derived from *Odorrana andersonii* and has a single amino acid difference in the fourth position.

(A)

```
QUB-2040     [Odorrana versabilis]  ATGTTCCCCTTGAAGAAATCCATGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133827.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133825.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133822.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133770.1   [Rana andersonii]      ATGTTCCCCTTGAAGAAATCCCTGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133833.1   [Rana andersonii]      ATGTTCCCCATGAAGAAATCCCTGTTACTCCTTTTCTTCCTTGGGACCATCAGCTTATCT
GU133838.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTTTTCCTTGGGACCATCAGCTTATCT
GU133830.1   [Rana andersonii]      ATGTTCACCTTGAAGAAACCCCTGTTACTCCTTTTCTTCCTTGGGACCATCAGCTTATCT
GU133824.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTCTTCCTTGGGACCATCAGCTTATCT
GU133829.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTCTTCCTTGGGACCATCAGCTTATCT
GU133826.1   [Rana andersonii]      ATGTTCACCTTGAAGAAATCCCTGTTACTCCTTTTCTTCCTTGGGACCATCAGCTTATCT
                                    ****** ** ******** ** ************** ************************

QUB-2040     [Odorrana versabilis]  CTTTGTGAAGAAGAGAGAGATGCCGATGAGGAAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133827.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133825.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133822.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133770.1   [Rana andersonii]      TTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133833.1   [Rana andersonii]      TTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133838.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133830.1   [Rana andersonii]      CTCTGTCAGGATGAGACAGATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133824.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133829.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
GU133826.1   [Rana andersonii]      CTCTGTCAGGATGAGACAAATGCCGAAG---AAGAAAGAAGAGATGAAGAAGTTGCTAAA
                                     * *** * ** **** * ******* *   ******************************

QUB-2040     [Odorrana versabilis]  ATGGAAGAGATAAAACGCGGTCTTTTACGGGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133827.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133825.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133822.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133770.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133833.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGGGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133838.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGGAGAAAATAGTA
GU133830.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133824.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133829.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
GU133826.1   [Rana andersonii]      ATGGAAGAGATAAAACGCGGTCTTTTAAGTGGCGTCCTCGGTGTGGGGAAGAAAATAGTA
                                    *************************** * ***************** ************


QUB-2040     [Odorrana versabilis]  TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATAAATGATCGTATCTAATAAT
GU133827.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATTTGATGTGGAATATCAT
GU133825.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGTGGAATATCAT
GU133822.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATTTGATGTGGAATATCAT
GU133770.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGGGGAATATCAT
GU133833.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATTTGATGTGGAATATCAT
GU133838.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATTTGATGTGGAATATCAT
GU133830.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGTGGAATATCAT
GU133824.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGTGGAATATCAT
GU133829.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGTGGAATATCAT
GU133826.1   [Rana andersonii]      TGTGGACTTAGCGGGCTGTGCTAAAGCTTGAATCGGAAATCATCTGATGTGGAATATCAT
                                    ************************************* *      *      ** **

QUB-2040     [Odorrana versabilis]  AAACATATGTCAGCATACACTCGGAA-TATCATTTAGCTAATCTGCTGAAG---------
GU133827.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACAGAAAAAAAAAA
GU133825.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACTGAAAAAAAAAA
GU133822.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACTGAAAAAAAAAA
GU133770.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTCGCATACAAAAAAAAAAAAAA
GU133833.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACTGAAAAAAAAAA
GU133838.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTTTTATAAATAATAAAAATGTTGCATACACTAAAAAAAAAAA
GU133830.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACTAAAAAAAAAAA
GU133824.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACTAAAAAAAAAAA
GU133829.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACCGAAAAAAAAAA
GU133826.1   [Rana andersonii]      TTAGCTAAATGCTAAATGTCTTATAAATAATAAAAATGTTGCATACACCCAAAAAAAAAA
                                     *  **  *    *     *   ** **  *   *  *    * *

QUB-2040     [Odorrana versabilis]  ----------------------
GU133827.1   [Rana andersonii]      AAAAAAAAAAAAAAAAAAA---
GU133825.1   [Rana andersonii]      AAAAAAAAAAAAAAA-------
GU133822.1   [Rana andersonii]      AAAAAAGAAAAAAAAAAAA--
GU133770.1   [Rana andersonii]      AAAAAAAAAAAA----------
GU133833.1   [Rana andersonii]      AAAAAAAAAAAAAAAAA-----
GU133838.1   [Rana andersonii]      AAAAAAAAAAAAAAAAA-----
GU133830.1   [Rana andersonii]      AAAAAAAAAAAAAAAA------
GU133824.1   [Rana andersonii]      AAAAAAAAAAAAAAAAAAAAAA
GU133829.1   [Rana andersonii]      AAAAAAAAAAAAAAAAAAA--
GU133826.1   [Rana andersonii]      AAAAAAAAAAAAAAAA------
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(B)

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QUB-2040     [O. versabilis]   MFPLKKSMLLLFFLGTISLSLCEEERDADEEERRDEEVAKMEEIKRGLLRGVLGVGKKIVCGLSGLC
ABG76329.1   [O. grahami]      MFTLKKSMLLLFFLGTISLSLCEQERNADEEERRDGEVAKMEEIKRGLLSGILGAGKNIVCGLSGLC
ABG76394.1   [O. grahami]      MFPLKKSPLLLFFLGTINLSFCQDETNAE-EERRDEEVTKMEEIKRGLLSGILGAGKHIVCGLSGLC
CAM35557.1   [O. schmackeri]   MFTLKKSILLLFFLGTINLSLCQDETNAE-EERRDEEVAKMEEIKRGILSGILGAGKSLVCGLSGLC
ADP05826.1   [O. andersonii]   MFTMKKSLLLLFFFGTINLSFCQDETNAE-EERRDEEVAKMEEIKRGLISGILGAGKKIVCGLSGLC
ABG76309.1   [O. grahami]      MFTLKKSLLLPFFLGTINLSLCQDETNAE-EERRDEEVAKMEEIKRGLLSGILGAGKHIVCGLSGLC
P0C2A5.1     [Rana grahami]    MFTLKKSQLLLFFPGTINLSLCQDETNAE-EERRDEEVAKMEEIKRGLLSGILGAGKHIVCGLSGLC
ABX58819.1   [O. grahami]      MFTMKKHMLLRFFLGTINLSLCREETNAE-EERRDEEVAKMEEIKRGLLSGVLGVGKKVPCGLSGLC
ABG76408.1   [O. grahami]      MFTMKKSLLPLFFLGTINLSLCQDETNAE-EERRDEEVAKMEEIKRGLLSGILGAGKHIVCGLSGLC
ABG76291.1   [O. grahami]      MFTLKKSLLLPFFLGTINLSFCQDETNAE-EERRDEEVAKMEEIKRGLLSGILGAGKHVVCGLSGLC
ABG76386.1   [O. grahami]      MFTLKKSPLLLFFLGTINLSLCQDETNAE-EERRDEEVAKMEEIKRGLLSGILGAGKHIVCGLSGL-
                               ** :**  *  ** ***.**:*.:* :*: ***** **:********:: *:**.** : ******
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(C)

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QUB-2040     [O. versabilis]      GLLRGVLGVGKKIVCGLSGLC
ACZ71273.1   [O. andersonii]      GLLRGVLGVGKKIVCGLSGLC
ADP05899.1   [O. andersonii]      GLLRGVLGVGKKIVCGLSGLC
ADP05898.1   [O. andersonii]      GLLRGVLGVGKKIVCGLSGLC
ACZ71311.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ACZ71312.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ACZ71293.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ACZ71279.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ACZ71263.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ADP05897.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
ADP05896.1   [O. andersonii]      GLLSGVLGVGKKIVCGLSGLC
                                  *** ******************
```

Figure 2.2 Alignmenst of QUB-2040 with the top 10 hits from the BLAST site of the NCBI using the online tool Multiple Sequence Alignment of Clustal Omega. (A) the alignment of the mRNA of QUB-2040; (B) the alignment of the ORF amino acid sequence; (C) the alignment of the mature peptide sequence. Each sequence ID in BLAST is displayed ahead of each sequence. The asterisks show identical nucleotides or amino acid residues. The ':' indicates high similarity, while '.' represents relatively low similarity.

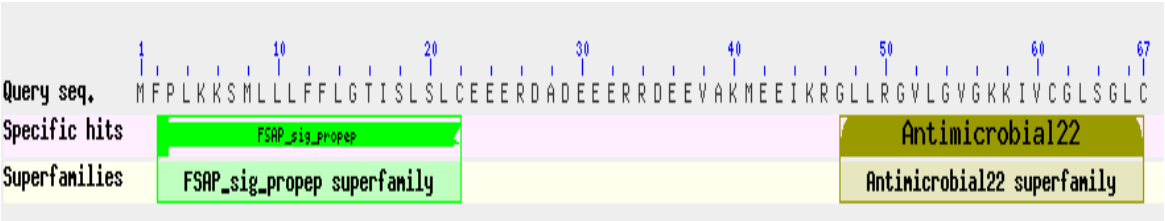

Figure 2.3 Graphic summary of the ORF amino acid sequence in BLAST results with putative conserved domains detected, indicating that the peptide QUB-2040 belongs to Antimicrobial 22 superfamily. The result is available by searching the full ORF amino acid sequence with the online tool BLAST.

Chapter3 Chemical synthesis of the peptide QUB-2040

3.1 Materials and methods

3.1.1 Preparation of the chemical synthesis of QUB-2040

The solid-phase peptide synthesis was used to chemically synthesise the peptide (GLLRGVLGVGKKIVCGLSGLC) derived from *Odorrana versabilis* skin secretion.

Calculations and weighings

To synthesise 0.3 mmol peptide, each amino acid contained in the peptide should be used in a 4 x molar excess. Thus 1.2 mmol for each amino acid were weighed into amino acid vials respectively, each of which also contained 1.2 mmol 2-(1H-Benzotriazol-1-YL)-1,1,3,3-Tetramethyluronium Hexafluorophosphate (HBTU) in order to catalyse each coupling. Each amino acid vial consists of a vial, a cap, a crimp cap and a septum. Here Wang resin combined with cysteine was utilised at 0.3 mmol and was weighed (0.5270 g [weight (g) = 0.3 mmol / loading capacity (0.57 mmol/g)]) into a clean 40 ml reaction vessel before the synthesis.

Reagents used in the synthesis

All the reagent bottles were checked and refilled with required reagents before the synthesis. The reagents used in the chemical synthesis are listed in Table 3.1.

Table 3.1 Reagents used in the synthesis

Bottle	Reagent	Effect
1 & 2	N,N-Dimethylformamide (DMF)	Washing reagent
3	Piperidine:DMF (1:4)	To release the Fmoc protecting group
4	4-Methylmorpholine (NMM):DMF (11:89)	To dissolve and activate amino acids
5	Dichloromethane (DCM)	Wash synthesised peptide and remove DMF

3.1.2 Solid-phase peptide synthesis

In this project, the chemical synthesis was performed using a Tribute^{TM} Peptide Synthesiser (Protein Technologies, USA). After all the amino acids and HBTU were weighed separately, the vials were loaded in the order from C-terminus to N-terminus. The reaction vessel with required resin was also loaded onto the synthesiser. The inline solvent filters and the source of nitrogen were checked before operation. The start and the end positions on the carousel as well as the appropriate coupling programme for each amino acid were selected and edited. Then, the peptide synthesiser was run under the major programme as shown below in Table 3.2. After automatically synthesising with the peptide synthesiser, the chemically synthesised peptide was still combined with resin and the amino acid side chain protecting groups remained as well. Thus, the following cleavage step was needed to obtain the target peptide.

Table 3.2 The major programme for peptide synthesis

Step	Action	Reagent Bottle	Effect
1’	RV_TOP	1	Wash resin
1	RV_TOP	3	Deprotection(N-terminus)
2	RV_TOP_Vent_Wash	1	Quick wash
3	RV_TOP	1	Generally wash
4	AA_Delivery	4	Activation
5	Mix	/	Coupling
6	RV_TOP_Vent_Wash	1	Quick wash
7	RV_TOP	1	Wash
	*Cycle: Step1~Step7, repeated until the last amino acid is attached		
8	RV_TOP	3	Final deprotection
9	RV_TOP_Vent_Wash	1	Quick wash
10	RV_TOP	1	Wash
11	RV_TOP	5	Eluting DMF with DCM
12	/	/	Dry-drain

3.1.3 Cleavage and deprotection of peptide

The purpose of this step was to separate the peptide from resin and to deprotect the side chain protecting groups at the same time. The amino acid vials and the reaction vessel were removed from the Tribute^{TM} Peptide Synthesiser when the synthesis was completed. The dried peptide’-resin was weighed on an electronic balance for calculating the total volume of cleavage cocktail (25 ml/g peptide’-resin). Then the peptide’-resin was transferred to a 50 ml round-bottomed flask. The cleavage cocktail was prepared as 94%TFA/2% H_2O/2%EDT/2%TIS (v/v/v/v) and added to the round-bottomed flask. The cleavage and deprotection reaction was operated at room temperature for at least 6 h with constant stirring on a magnetic stirrer. After this, the cleavage mixture was filtered into another 50 ml round-bottomed flask through a

Buchner funnel. Then the filtrate was concentrated to nearly 5 ml using a rotary evaporator at a temperature of no more than 40 °C. After that, about 45 ml Et_2O was added to the flask and then transferred to a 50 ml centrifuge tube. The product was labelled and kept at -20 °C overnight for peptide precipitation and to remove TFA from the peptide.

3.1.4 Washing and oxidation of peptide

The 50 ml centrifuge tube was centrifuged at 2500 × g for 5 min to collect the peptide pellet. The supernatant was discarded carefully in order to avoid the loss of peptide pellet at the bottom. Then, 45 ml ether was refilled and the washing step was repeated three times. After the washing, the tube was covered using tinfoil with holes and was placed in a fume cabinet at room temperature overnight for Et_2O volatilisation.

The following step was to oxidise the two cysteines in the synthesised peptide to form an intramolecular disulphide bond. When the peptide was dry, HPLC solution B (10-20 ml) was added to dissolve the peptide. Then the mixture was transferred to a round-bottomed flask. The oxidation process was accelerated in the presence of 40 µl H_2O_2 and operated at room temperature with stirring on a magnetic stirrer.

3.1.5 Lyophilisation of peptide

After the oxidation of peptide, the peptide solution was snap frozen in liquid nitrogen and lyophilised using an Alpha 1-2 freeze-dryer (SciQuip, UK) for around 60 h.

3.1.6 Identification and purification of the synthesised peptide

In this study, Reversed-phase High Performance Liquid Chromatography (RP-HPLC) and MALDI-TOF mass spectrometry were used to identify the purity of the chemically synthesised peptide and purify it if necessary.

Analysis of the synthesised peptide using RP-HPLC

One milligram of lyophilised synthesised peptide was dissolved in a mixture of 500 µl HPLC solution A and 500 µl solution B. Solution A consisted of trifluoroacetic acid (TFA) and distilled deionised water (0.05/99.5, v/v) while solution B was comprised of 80%ACN, 19.95%H_2O and 0.05%TFA. The sample was vortexed for 5 min and then was centrifuged at the maximum speed for 15 min. Before the sample injection, solution B was pumped to wash the column for 30 min and then equilibrated with solution A for another 30 min. After the equilibration, the clear supernatant of the sample was directly injected into the RP-HPLC using a Cecil Adept Binary HPLC system (Cambridge, UK) with a 1 cm×25 cm Jupiter 00G4025 semi-preparative C-5 reverse phase column (Phenomenex, UK). The column was eluted with a linear gradient formed from 100%A: 0%B to 0%A:100%B in 80 min at a flow rate of 1 ml/min. The wavelength of the detector was set at 214 nm. The fractions were collected respectively at every peak and the tubes were labelled with the corresponding time.

MALDI-TOF MS analysis

Two microliters of each RP-HPLC fraction were placed onto wells of a 100-well MALDI-TOF plate and then air-dried. A matrix solution, α-cyano-4-hydroxycinnamic acid (CHCA), was prepared in acetonitrile/water/TFA (50/49.95/0.05, v/v/v) to obtain a 10 mg/ml solution of CHCA. 1 µl matrix solution was added to each well and left to dry once again. Then the target plate was loaded into the instrument.

A MALDI-TOF Mass Spectrometer (Voyager DE, PerSeptive Biosystems, Framingham, MA, USA) was used for the mass analysis of the fractions. The parameters of the system involving acceleration voltage (20 kV), grid voltage (18.8

kV), guide wire voltage (10 V) were optimised before analysis. Masses of samples in fractions were determined according to their mass-to-charge ratios, and then compared with calculated theoretical values.

3.2 RESULTS

3.2.1 Identification and purification of the synthesized QUB-2040

The synthetic version of the peptide QUB-2040 was successfully obtained through SPPS. Then the reverse-phase HPLC and MALDI-TOF mass spectrometry were both used to identify the synthesised peptide. Depending on the obtained results, the chemically synthesised peptide was pure to a relatively high extent.

As determined by MALDI-TOF mass spectrometry of the crude synthesised peptide, the main peak shown in the mass spectrum (Figure 3.1) had a calculated mass-to-charge ratio of 2043.39, which was similar to the desired value (2040). Meanwhile, the intensity of unwanted impure peaks was under 20%, indicating a certain high degree of purity. Thus, the synthesised peptide was suitable for the subsequent bioactivity assessments. Also, the synthetic peptide could be purified to a higher degree of purity by using reverse-phase HPLC (Figure 3.2).

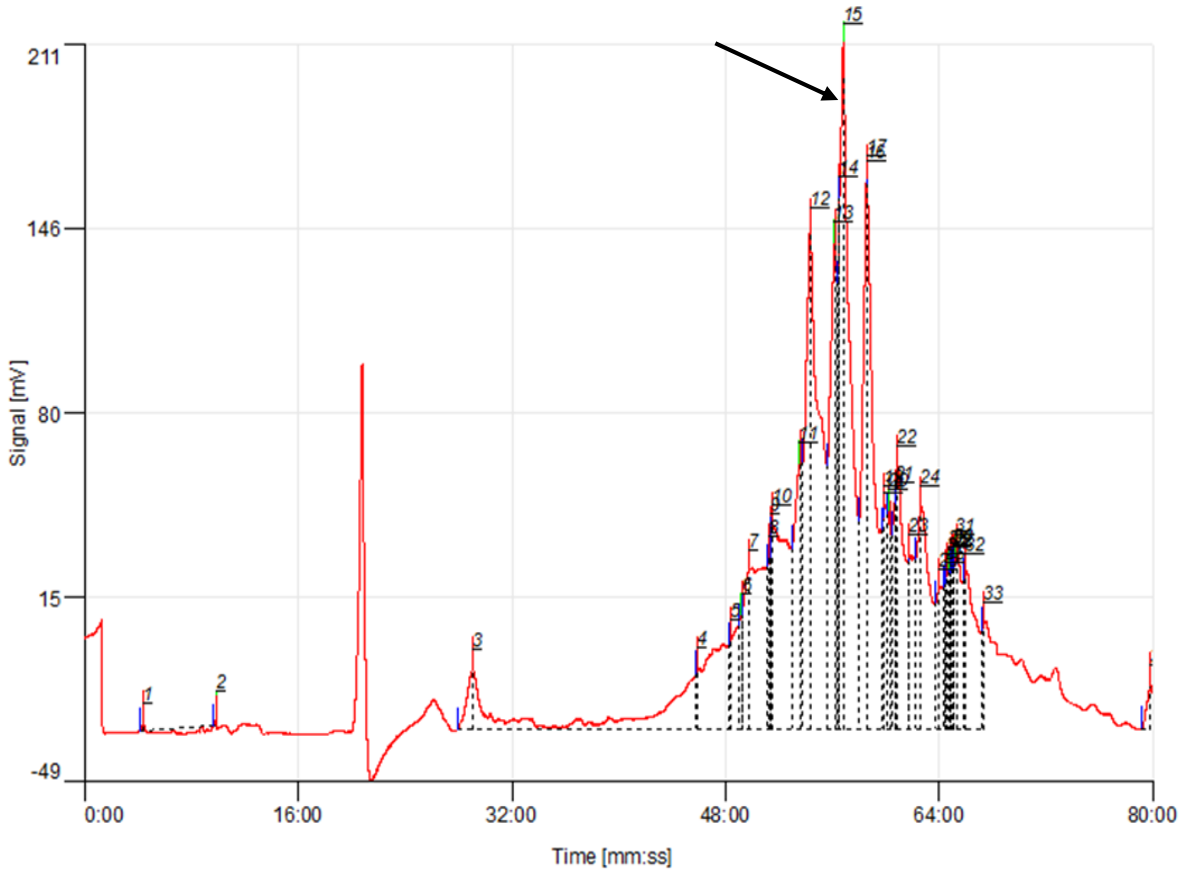

Figure 3.2 RP-HPLC chromatogram of the chemically synthesised peptide showing the elution position and retention time of authentic QUB-2040 (arrow). The Y-axis indicates the relative absorbance at 214 nm, whilst the X-axis represents the retention time in minutes/seconds.

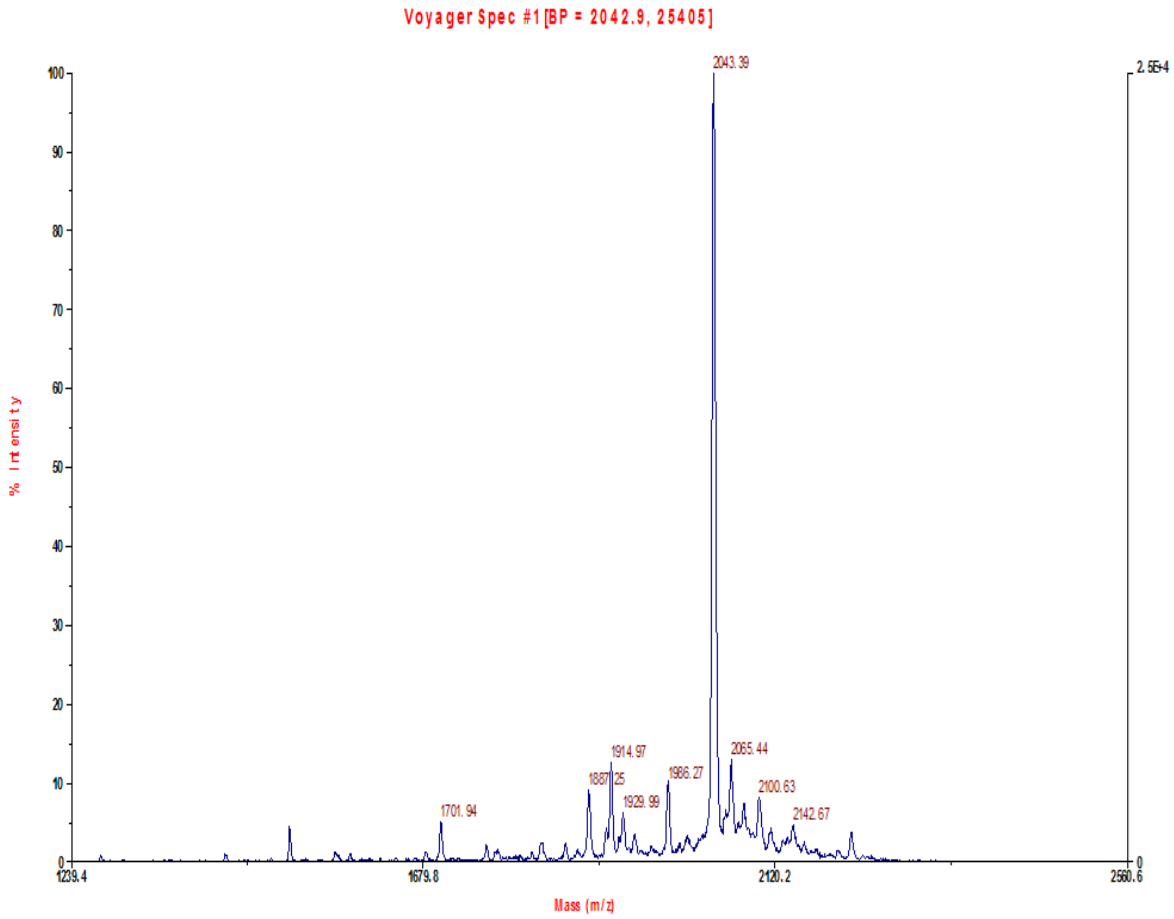

Figure 3.1 MALDI-TOF mass spectrum of the synthesised peptide (QUB-2040). The major singly-charged ion was resolved at an m/z of 2043.39.

3.2.2 Predicted secondary structure of QUB-2040

With the help of the online server I-TASSER, the secondary structure of QUB-2040 was predicted successfully. The results indicated that the peptide QUB-2040 may have a major region of coil with little α-helical region and little region of strand (Figure 3.3). The final models were also predicted by I-TASSER and the optimal one with a C-score (confidence score) of -1.75 is shown in Figure 3.4. The C-score ranging from -5 to 2 is a measurement to evaluate the confidence of each model. The higher value signifies a model with a higher confidence and *vice-versa.*

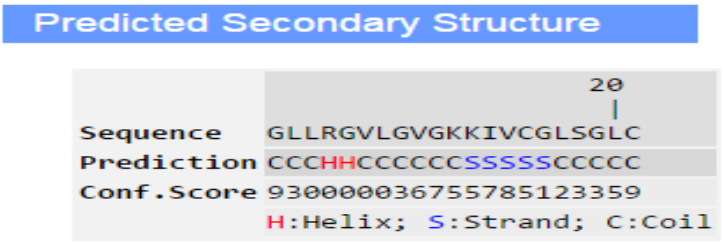

Figure 3.3 Predicted secondary structure using I-TASSER which is considered as a suitable online server for the protein structure prediction. The result is available at: *http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S317454/dkjpic/*.

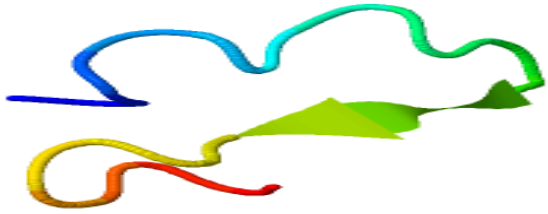

Figure 3.4 The optimal predicted secondary structure model of QUB-2040 using the online server I-TASSER with a C-score (confidence score) of -1.75. The estimated TM-score is 0.50±0.15, while the estimated RMSD is 4.3±2.9 Å. The result is available online at: *http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S317454/dkjpic/*.

3.2.3 Predicted physicochemical properties of QUB-2040

The physicochemical properties of QUB-2040 were predicted using the online tool ExPASy ProtParam and PepCalc.com including net charge, extinction coefficient, iso-electric point and theoretical pI (Table 3.3).

Table3.3 Predicted physicochemical properties of QUB-2040

Peptide	Net charge	Extinction coefficient	Iso-electric point	Theoretical pI
QUB-2040	+3	$120M^{-1}cm^{-1}$	pH 11.58	9.5

Chapter4 Bioactivity assessments of the peptide QUB-2040

4.1 Materials and methods

4.1.1 Antimicrobial assays

To test the antimicrobial properties of the peptide on different types of microorganism, three typical microbes were employed in this study: (i) the Gram-positive bacterium, *Staphylococcus aureus (S. aureus*, NCTC 10788*)*, (ii) the Gram-negative bacterium, *Escherichia coli* (*E. coli*, NCTC 10418) and (iii) the yeast, *Candida albicans* (*C. albicans*, NCTC 1467).

Seeding microbes and culturing One preserved bacterial bead was transferred from frozen stock into a 100 ml flask containing Mueller Hinton Broth (MHB) medium, and then the labelled flask was incubated in the orbital incubator (Stuart, UK) at a speed of 150 rpm at 37 °C overnight (16-20 h).

Subculture 500 µl of cultured bacteria/yeast was transferred into a McCartney bottle with 20 ml pre-warmed MHB. Then the sub-cultured medium was placed in the shaking incubator at 37 °C to continue growing until reaching the logarithmic phase of growth. OD was measured by a UV spectrophotometer at 550 nm wavelength. The optical concentration of microbes, corresponding culture time and OD values of the three microorganisms are shown in Table 4.1.

Table 4.1 The optical concentration of microbes, corresponding culture time and OD values of the three microorganisms used

Organism	Subculture incubation time	OD	Concentration (cfu/ml)
S. aureus	1.5 h	0.23	1×10^{8}
E. coli	1.0 h	0.41	1×10^{8}
C. albicans	45 min	0.15	5×10^{6}

*cfu/ml: colony forming units per millilitre

(3) Dilution After reaching pertinent OD values, the organisms were diluted with MHB to 5×10^5 cfu/ml as dilution stock. In detail, 100 µl *S. aureus* and *E. coli* were diluted by 19.9 ml MHB, while 2 ml *C. albicans* was mixed with 18 ml MHB.

Viable cell count

Once the desired density of microorganisms had been achieved and diluted, 100 µl of culture was added into 900 µl of sterile PBS solution and further diluted with PBS at a dilution of 1:10 in the sterile tubes. It was replicated five times until it reached a dilution of 10-6 -fold. 20 µl of each dilution (10-1 to 10-6) was plated onto the MHA plates. Three replications of each dilution were made. Then the plates were incubated at 37 °C for 16-20 h.

After incubation, appropriate spots were observed and the colonies of each area were counted and averaged. Then, the original CFU of the subculture was calculated according to the following formula:

$$N = (C \times 10D \times 50)/ 3$$

where N = cfu L-1; C = number of colonies per dilution (for 3 spots); D = number of the 1:10 dilution.

Minimum inhibitory concentration (MIC) assays

(1) Preparation of peptide solution. Lyophilised peptide was weighed and dissolved in dimethyl sulphoxide (DMSO) to make stock solution at a concentration of 512×10^2 µM. Then 10 µl stock solution was double-diluted in the ratio of 1:1 in DMSO to prepare a peptide solution with concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1×10^2 µM.

(2) Sample loading. A sterile 96-well plate was labelled and divided into sterile

control, vehicle control, growth control and experimental groups. All the samples and controls were arranged in the 96-well plate with 5 replications according to the following scheme shown in Figure 4.1. The final concentration of peptide in the single well is from 512 to 1 μM.

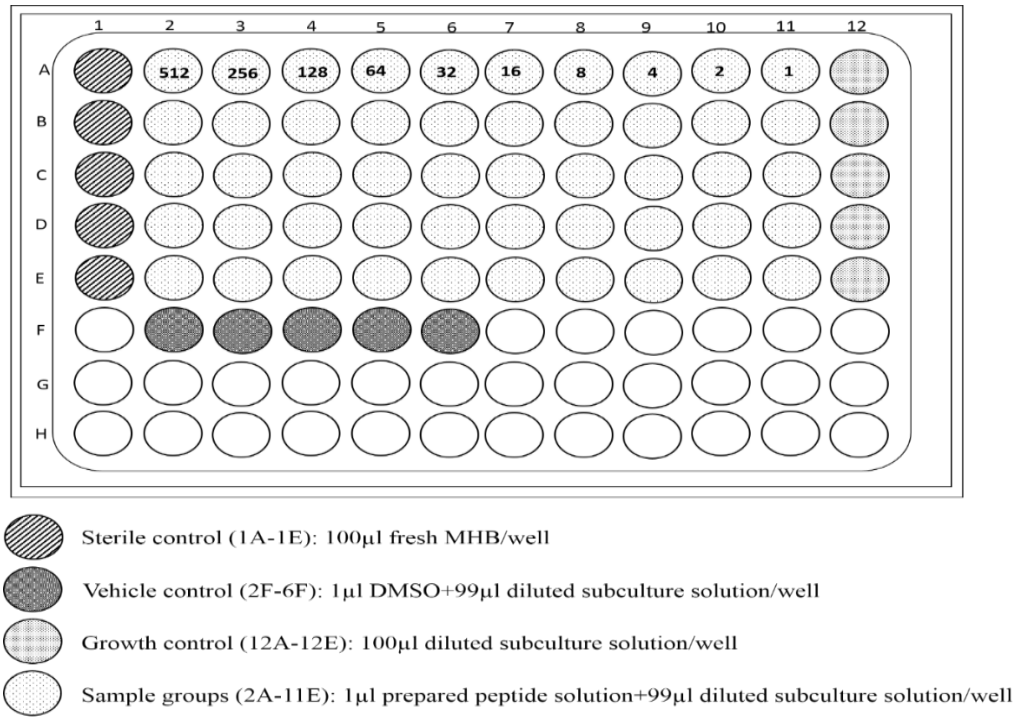

Figure 4.1 View of a 96-well plate used in the assay

Then the plate was transferred onto the shaker at 37 °C for 10 min to make the mixture well-distributed and after that, the plate was incubated at 37 °C overnight.

(3) MIC detection. A Synergy HT plate reader (BioTeK, USA) was used to detect the absorbance of each well at 550 nm and the data were analysed using Gen5^{TM} software (BioTeK, USA). The minimal concentration with no bacterial growth is the MIC. The MIC assays were carried out in triplicate for each tested microbial strain using the same methods.

Minimum bactericidal concentration (MBC) assays

The MBC assay should be performed when the peptide showed antimicrobial activity in MIC assays. MBC was defined as the lowest lethal concentration of peptide to the organism, which is generally close to or higher than the MIC value. To be specific, 20 µl of bacterial suspension from 5 replicates of MIC were dropped onto a pre-solidified MHA dish and dried before being incubated at 37°C for 16-20 h. For example, if the MIC was 8 µM, then 20 µl suspension in each of 5 replicates from 32, 16, 8 and 4 µM were dropped onto the MHA dish as shown in Figure 4.2. After incubation, the MHA dish was analysed. The lowest concentration without visible growth of microorganism was regarded as the MBC. Likewise, the MBC assay was repeated 3 times for each microbe as well.

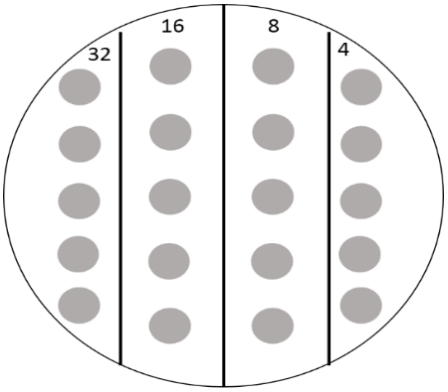

Figure 4.2 The view of a MHA dish in MBC assay

4.1.2 Haemolysis assays

Fresh whole horse blood (TCS Bioscience Ltd, UK) was mixed completely by gently shaking before 2 ml of this was centrifuged at 500 x g for 5min. After discarding the supernatant, the cell pellet was washed with 30ml autoclaved phosphate buffered saline (PBS) by gently mixing and then centrifugation (500 x g for 5min). The process of PBS washing, centrifugation and discarding of supernatant was repeated until the

supernatant became clear. Then sufficient PBS was added to 50ml to dilute the cell pellet.

Peptide solutions were prepared through dissolving synthetic peptide in PBS at twice the working concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2, 1μM) to compensate for on-plate dilution. 200μl of each peptide solution was added into corresponding 1.5ml tube and each concentration had 5 replicates. Both positive control (200μl PBS/1.5ml tube) and negative control (200μl 2% Triton X-100/1.5ml tube) were set with 5 replicates as references. 200μl of diluted blood solution was pipetted into each 1.5ml tube containing the same volume of peptide samples or control groups.

After being incubated at 37ºC for 2h, all tubes were centrifuged at 500 x g for 5min. Then 100μl supernatant of each sample was transferred into corresponding wells of a 96-well plate. The OD value of each well was measured with the help of a Synergy HT plate reader (BioTek, USA) at 550nm wavelength. The viability of cells was calculated according to the following formula---$(OD_{peptide} - OD_{Negative}) / (OD_{Positive} - OD_{Negative}) \times 100\%$.

4.1.3 Anticancer cell assays

Cancer cell preparation

Four types of cancer cell lines were used in this thesis and can be seen in Table 4.2. All cancer cell lines were obtained from the Centre for Cancer Research & Cell Biology (CCRCB), Queen's University Belfast.

Table 4.2 The four types of cancer cell lines and their corresponding basic media.

Cell line	Tumour type	Basic medium
PC-3	Human prostate carcinoma (GIV) cell line	RPMI-1640 (Invitrogen, USA)
NCI-H157	Non-small cell lung cancer	RPMI-1640
MDA-MB-435S	Melanoma	DMEM (Sigma, UK)
U251MG	Human neuronal glioblastoma	DMEM

(1) Resuscitation of frozen cell lines. The ampoule of cell lines was quickly defrosted from the freezer and pre-warmed in a water bath at 37 °C (2-3 min). All cell suspensions were transferred into a culture flask containing 15 ml pre-warmed growth medium [basic medium + FBS (Foetal Bovine Serum) + Penicillin and Streptomycin (PS)]. Then the cells were mixed with medium thoroughly but gently. After this, the flask was labelled with relevant information and placed into a 37 °C incubator under 5% CO_2.

(2) Medium changing. The cell lines were examined microscopically and the medium needed to be changed to promote their growth. The original medium was discarded by a sterilised pipette without touching the cells. 10 ml PBS were gently added to wash the cell monolayer and were then discarded. 15 ml fresh growth medium were transferred into the flask and incubated at 37 °C under 5% CO_2.

(3) Passage of cell lines. The cells were subcultured when the cell confluence was over 90%. The medium was removed from the flask and 10 ml PBS was used to wash the cell monolayer. 1 ml of 0.5% trypsin solution was added to the cell layer for digestion. Then the flask was incubated at 37 °C under 5% CO_2 until the cells were released from the flask wall. 10 ml of fresh complete growth medium was added to stop the digestion. The cell suspension was gently transferred into a 15 ml centrifuge

tube and centrifuged at 200 × g for 5 min (18-25 °C). After discarding the supernatant, an appropriate volume of fresh complete growth medium was added into the tube and mixed by vortexing. One fifth of the cell suspension was transferred to a prepared new flask containing 15 ml fresh complete medium. Finally, the flask was placed in the incubator (37 °C, 5% CO2) to subculture the cells.

MTT cell viability assay

(1) Viable cell counting. Cell counting was performed using an AS1000 Improved Neubauer haemocytometer (Hawksley, UK) just after the step of resuspending the centrifuged cell pellet described in the previous section. A 50 µl sample from the cell suspension was transferred to a test plate and mixed with an equal amount of trypan blue. 50 µl of this mixture was loaded onto the haemocytometer and the sample was viewed immediately under an inverted microscope. The number of cells was counted in the specific chamber with assistance of a counter (Figure 4.3).

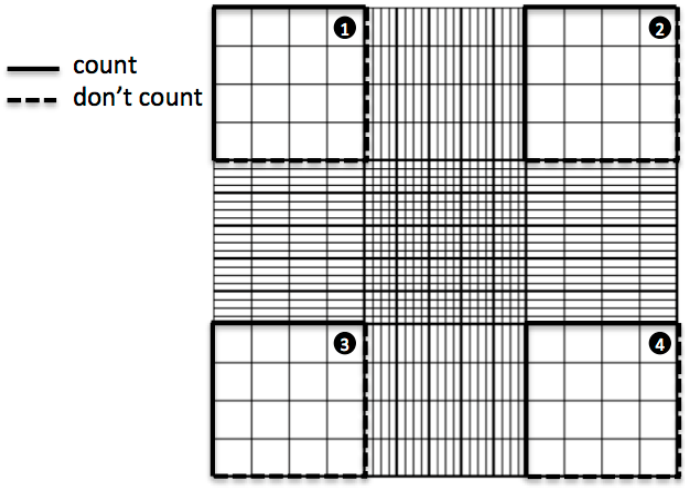

Figure 4.3 View of a haemocytometer. Only 3-4 squares were used for cell counting. The concentration could be calculated according to the following formula: C=n/number of squares×2×10^4 cells/ml. 'n' referred to the total number of counted cells and '2' is the dilution factor.

(2) Seeding cells. Each well of the plate used later should contain 5×10^3 cells/100 µl, thus the volume of cells and medium were calculated based on the concentration of cells. Then 100 µl of the diluted cell suspension were added to each well of 96-well plate. When the seeding was finished, the plate was labelled with relevant information and incubated at 37 °C under 5% CO_2 for 24 h.

(3) Starvation. Serum starvation was performed after the 24 h of seeding. The spent complete medium was removed and replaced by 100 µl serum-free medium for each well of the plate. Then the cells were starved for 6-12 h in the CO_2 incubator.

(4) Loading of peptide solutions /gradient screening. After the starvation, the peptide QUB-2040 was loaded into wells to test the antiproliferation activity on cancer cells. First, sufficient peptide was weighed and dissolved in a calculated volume of DMSO to achieve the concentration of 10^{-5} M. Then, the concentration of QUB-2040 was used to screen the ability to inhibit proliferation of cancer cell lines.

(5) MTT cell proliferation assay. Ten microliters of MTT (5 mg/ml) were added to each well (peptide groups and control groups) using a multi-channel pipette under low lighting. After that, the plate with MTT was incubated at 37 °C under 5% CO_2 for 4-6 h. Before the detection, a vacuum needle was used to remove all liquid from the well and 100 µl DMSO was added to each well line-by-line. Then the plate was kept in a 37 °C shaking incubator for 10 min to lyse cells and dissolve the formazan crystals completely. The changes of colour could be observed visually and directly. The absorbance of each well was detected by a Synergy HT plate reader (BioTek, USA) at 570 nm wavelength.

4.2 RESULTS

4.2.1 Antimicrobial activity of QUB-2040

The chemically synthetic QUB-2040 exhibited antimicrobial activity against all the three types of microorganisms tested in the experiments (Figure 4.4).

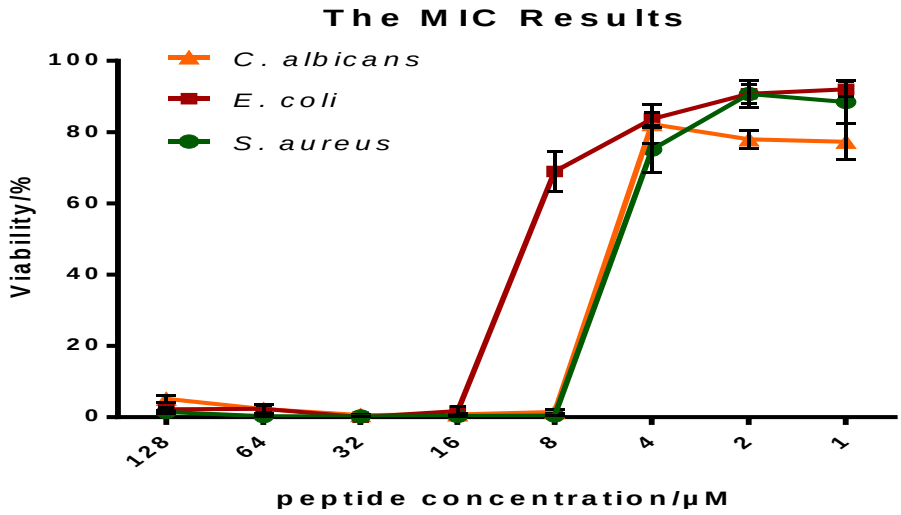

Figure 4.4 The antimicrobial activity of QUB-2040 against *S. aureus*, *E. coli* and *C. albicans*. The viabilities were compared with the growth control group, and each data point indicates the mean of the three replicates. Error bars indicate the mean ± SEM.

QUB-2040 demonstrated a broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and yeast at low concentrations. It revealed more potent inhibitory effects against *S. aureus* and *C. albicans*. The MICs of QUB-2040 against *S. aureus*, *E. coli* and *C. albicans* were 8, 16 and 8 μM, respectively (Table 4.3). In addition, the MBCs of QUB-2040 against the three microorganisms were 8, 32 and 8 μM (Table 4.3).

Table 4.3 MIC and MBC values of QUB-2040 against the tested microorganisms

Peptide	MIC/ μM			MBC/ μM		
	S. aureus	*E. coli*	*C. albicans*	*S. aureus*	*E. coli*	*C. albicans*
QUB-2040	8	16	8	8	32	8

4.2.2 Haemolytic activity of QUB-2040

The haemolytic activity of QUB-2040 evaluated using horse blood cells, is shown in Figure 4.5. Through the assay, QUB-2040 caused less than 30% lysis of the horse erythrocytes at the effective concentration of 32 μM at which QUB-2040 exhibited potent antimicrobial and antifungal activities.

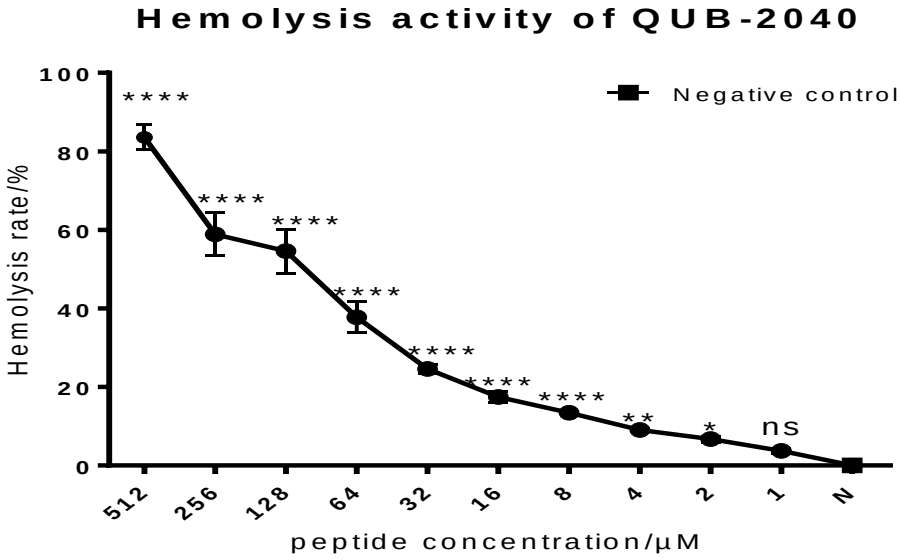

Figure 4.5 The haemolysis activity of QUB-2040 at various concentrations. The X-axis shows the concentrations of tested peptide and the Y-axis represents the percentage of haemolysis. Error bars indicate the mean ± SEM. (*student t-test of each individual concentration contrast with the negative group, and each data point indicates the mean of the five replicates, **** $p<0.0001$, *** $0.0001<p<0.001$, ** $0.001<p<0.01$, * $0.01<p<0.05$, ns $p\geq0.05$)

The HC_{50} which is the mean concentration of peptide causing 50% haemolysis of red blood cells, was acquired using GraphPad and was used to calculate therapeutic index (TI) with MIC values in section 4.4.1 (Table 4.4). Then the therapeutic index is calculated by the ratio of HC_{50} and MIC. Larger values indicate greater antimicrobial potency with lower haemolytic activity.

Table 4.4 HC_{50} of QUB-2040 and the value of TI against the three tested microorganisms

Peptide	HC_{50}/μM	Therapeutic Index (TI)=HC_{50}/MIC		
		S. aureus	*E. coli*	*C. albicans*
QUB-2040	123.8	15.475	7.7375	15.475

4.2.3 Anticancer cell activity of QUB-2040

The results of MTT screening on the peptide QUB-2040 (10^{-5}M) were listed below. According to the peptide screening results, QUB-2040 showed no inhibitory effect on the proliferation of human cancer cells, NCI-H157, PC-3, U251MG and MDA-MB-435S (Figure 4.6).

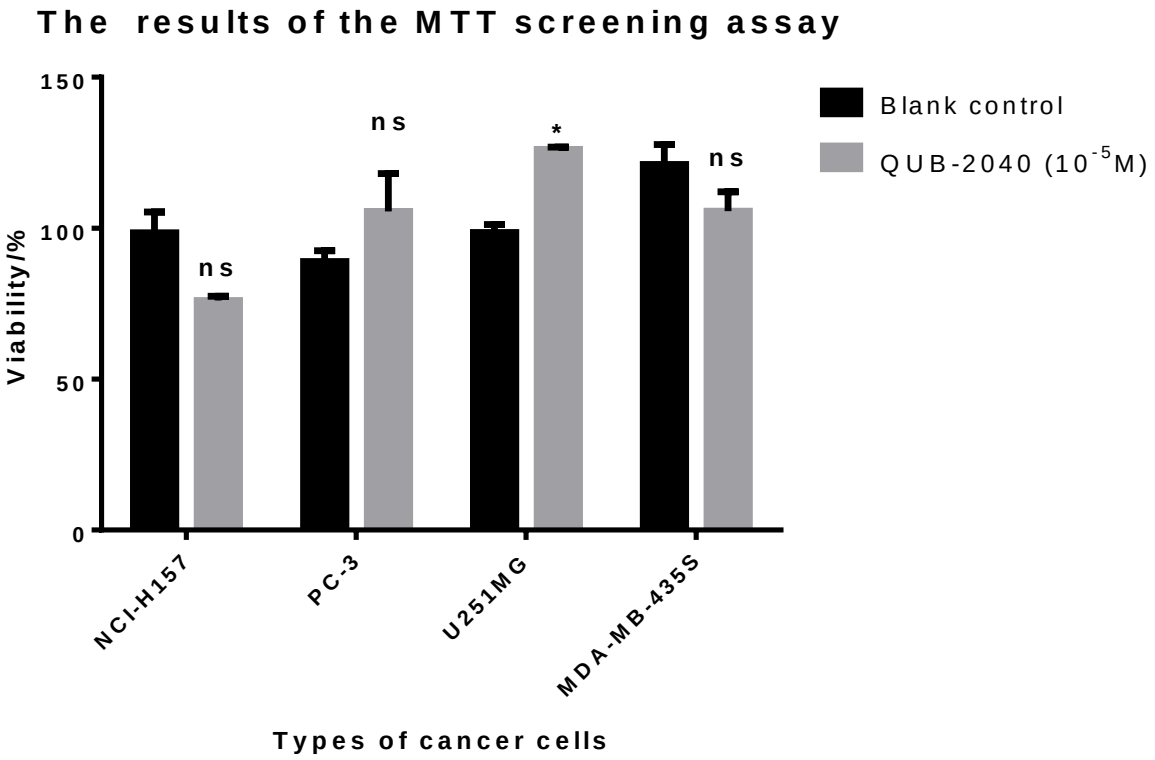

Figure 4.6 The results of the MTT screening assay. (* student t-test of each individual concentration contrast with the blank control, **** $p<0.0001$, *** $0.0001<p<0.001$, ** $0.001<p<0.01$, * $0.01<p<0.05$, ns $p\geq0.05$. Error bars indicate the mean ± SEM.)

Chapter5 Discussion

The occurrence of antibiotic resistance which has shortened or eliminated the efficacy of currently applied antibiotic agents against an increasing number of pathogenic microorganisms, constitutes a critical threat to human health and society. The urgent problem has necessitated an array of research on novel antimicrobial molecules to which the bacteria or fungi have not been able to be resistant (Conlon et al., 2008b). Nowadays, as top candidates of expanding the current limited therapeutic methods, AMPs have attracted increased attention from scientists and are widely recognised to play a crucial role in host immune systems against potential pathogens. Research has revealed that these host defence peptides possess outstanding physiological functions, such as a broad-spectrum of antimicrobial activity, anti-proliferative activity against cancer cells, smooth muscle pharmacological effects, even anti-HIV activity, and so on (Silva et al., 2011).

Undoubtedly, amphibians are endowed with an amazing defence system consisting of numerous antimicrobial chemical compounds. Granular glands in the skin of anuran amphibians are rich resources for screening novel antimicrobial peptides (AMPs), especially those of frogs from the families Ranidae, Pipidae, Hylidae, Pseudidae, and Hyperoliidae (Che et al., 2008). Numerous AMPs with various structures and different bioactivities have been isolated and identified from amphibian defensive skin secretions. The frog, *Odorrana versabilis*, studied in this thesis, belongs to the genus *Odorrana* which consists of more than 20 members. Considering that the other species in this genus, for example *O. grahami*, have shown a remarkable diversity of AMPs (Che et al., 2008), *O. versabilis* was anticipated to be another excellent resource for AMPs.

5.1 Structural aspects

In the present study, a bioactive AMP named QUB-2040 was isolated and identified from skin secretions of *Odorrana versabilis* using 'shotgun' molecular cloning. The sequenced primary structure of the mature peptide is GLLRGVLGVGKKIVCGLSGLC. Based on the matching results of the mature peptide sequence using the online tool BLAST, the peptide QUB-2040 is structurally related to an antimicrobial peptide family called nigrocin-2 which was first identified in the skin secretion of the Korean frog, *Rana nigromaculata* (Park et al., 2001), and shows almost 100% and 95% of sequence identity with nigrosin-RA4 and nigrosin-RA3 from *Odorrana andersonii*, respectively.

In general, the nucleotide sequences of cDNAs encoding open reading frames are similar to high degrees in many AMP precursor transcripts which especially encode structurally related analogues (Wang et al., 2010), and so are the nigrocin-2-related peptide transcripts. Likewise, the open reading frames are highly conserved in their domain organisation. Further into the sequence, the mature peptide sequences of nigrocin-2-related peptides share some limited similarities. The nigrocin-2-related peptides are usually rich in lysine residues with net positive charges, similar to other AMPs found in frogs (Park et al., 2001). The first reported nigrocin-2 was isolated from the dark-spotted frog, *Rana nigromaculata* which now is reclassified as *Pelophylax nigromaculatus* (Conlon et al., 2008b). An array of nigrocin-2-related peptides, named nigrocin-OG (1-23), were purified and characterised from the skin secretion-derived cDNA library of *Odorrana grahami*, a diskless-fingered odorous frog (Li et al., 2007). The various amino acid sequences are listed below in Table 5.1.

Table 5.1 Comparison of primary structures of QUB-2040 and several other nigrocin-2-related peptides.

Peptide	Source	Primary structure
QUB-2040	*Odorrana versabilis*	GLLRGVLGVGKKIVCGLSGLC
nigrosin-RA4	*Odorrana andersonii*	GLLRGVLGVGKKIVCGLSGLC
nigrosin-RA3	*Odorrana andersonii*	GLLSGVLGVGKKIVCGLSGLC
nigrocin-2	*Rana nigromaculata*	GLLSKVLGVGKKVLCGVSGLC
nigrocin-OG1	*Odorrana grahami*	GLLSGILGAGKHVVCGLSGLC
nigrocin-OG3	*Odorrana grahami*	GLLSGILGVGKHIVCGLSGLC
nigrocin-OG4	*Odorrana grahami*	GLLSGILGAGKHIICGLSGLC
nigrocin-OG8	*Odorrana grahami*	GLLSGILGAGKHIVCGLSRLC
nigrocin-OG14	*Odorrana grahami*	GLLKGILGAGKHIVCGLSGLC
nigrocin-OG15	*Odorrana grahami*	GLLRGILGAGKHIVCGLSGLC
nigrocin-OG20	*Odorrana grahami*	GLLSGILGAGKHIVCGLSGLC
nigrocin-OG23	*Odorrana grahami*	GLLSGVLGVGKKVVCGLSGLC

Obviously, peptide QUB-2040 has an equivalent size and exhibits structural similarities to the known nigrocin-2 AMPs. Several amino acid residues are conserved among the species of the same genus. The mature peptide sequence of QUB-2040 is identical with that of nigrocin-RA4 precursor derived from *Odorrana andersonii*. However, the biological functions of nigrocin-RA4 have not been reported yet. Therefore, the bioactivity assessment of QUB-2040 may provide certain reference signatures for activity research on nigrocin-RA4. Meanwhile, in comparison with nigrocin-RA3 from the skin secretion of *Odorrana andersonii*, the peptide QUB-2040 is highly similar with only one single substitution (Arg11→Ser11). Generally, at the C-terminus, the nigrocin-2-related peptides share a conserved disulphide-bridged

heptapeptide segment, which is a typical structural motif of some AMPs from Ranidae (Li et al., 2007, Park et al., 2001).

Although the peptides from ranid frogs which can be classified together in the nigrocin-2 family, the variations in amino acid sequences are considerable. Considering the limited similarities in structures, they may however share a common evolutionary origin (Conlon et al., 2008b). The various habitats of individual species with diverse types of pathogenic microorganisms and the rapid changes in microbial pathogens may serve to explain the diversification in peptide sequences. Each species of ranid frog possibly has adapted differently in response to the survival pressure. The lysine residues and the disulphide bridge in nigrocin-2 peptides appear to be retained thus conserving a vital element of their primary structure (Conlon et al., 2008b).

5.2 Bioactivity aspects

The chemically synthetic version of QUB-2040 was subject to a series of bioactivity assessments, including antimicrobial assays, haemolysis assays and anticancer cell assays. It was found that QUB-2040 exhibits inhibiting activities not only against Gram-positive and Gram-negative bacteria but also against fungi with similar antimicrobial potencies, but shows no cell lysis activities against the four tested types of cancer cells. QUB-2040 was proven to be lethal for all the three tested microbes and of these three microorganisms, *S. aureus* and *C. albicans* were more sensitive than *E. coli*. Besides, QUB-2040 displays a low haemolytic activity on horse red blood cells at its effective concentration.

In common with many other AMPs, the peptide QUB-2040 appears to be more potent against the model Gram-positive bacterium, *S. aureus*, than against the model Gram-negative bacterium, *E. coli*. The different cell conformations of Gram-positive and

Gram-negative bacteria can be used to explain such phenomenon to some degree. Gram-positive bacteria have a cell wall composed of several layers of peptidoglycan, making it thick and tough, while Gram-negative bacteria only have a single layer of peptidoglycan. However, the Gram-negative bacterial cell wall consists of an outer membrane which surrounds the peptidoglycan layer, making it difficult for some antimicrobial molecules to enter.

Nigrocin-2HSa, b, which belong to the nigrocin-2 family of AMPs, were isolated from Hose's frog, *Odorrana hosii*, by applying peptidomic analysis on the skin secretions (Conlon et al., 2008b). The measured minimum inhibitory concentration (MIC) values against reference strains of Gram-positive bacteria, *S. aureus* and Gram-negative bacteria, *E. coli* of the two nigrocins are listed in Table 5.2 and are compared with QUB-2040 which appears to possess a stronger potency of antimicrobial activities. Considering the amino acid sequence of the mature peptide, QUB-2040 possesses three basic amino acid residues (R4, K11, K12), while both nigrocin-2HSa and b have only two lysines, indicating that the net positive charge of QUB-2040 is the highest among the three peptides. Based on current well-developed mechanisms, AMPs interact with the negatively charged membrane of bacteria mainly through electrostatic forces. Peptides with higher cationicity bind to the phospholipid head groups of the bacterial cell membranes more easily and tightly, and then cause cell permeabilisation by forming transmembrane pores. The differences among the three peptides support the deduction that basic amino acid residues in peptides play a crucial role in the growth-inhibition of bacteria, even though the mechanisms by which AMPs induce bacterial cell death are not clear yet and may be diverse in different peptides against disparate kinds of pathogenic microorganisms.

Table 5.2 Minimum inhibitory concentrations (μM) of the peptide QUB-2040 isolated from *Odorrana versabilis*, Nigrocin-2HSa and b were isolated from *Odorrana hosii*. The conserved amino acid residues are shaded.

Peptide	Amino acid sequence	*S. aureus*	*E. coli*
QUB-2040	GLLRGVLGVGKKIVCGLSGLC	8	16
Nigrocin-2HSa	GLLGSLFGAGKKVACALSGLC	56	28
Nigrocin-2HSb	GLLGSIFGAGKKIACALSGLC	28	14

Two grahamin peptides, which are highly similar to nigrocins, were isolated and identified from the skin secretion of *Rana grahami,* and showed more potent antimicrobial activities against *S. aureus*, *E. coli* and *C. albicans* with MIC values all under 8 μg/ml than QUB-2040 (Xu et al., 2006). However, the two grahamin peptides only have one positively charged residue while nigrocins usually contain more than one positive amino acid. This indicates that there might be several types of mechanisms co-operating together to exhibit the antimicrobial activity. The research on the phylogenetic relationship between *Odorrana versabilis* and *Rana grahami* may be inspired by comparing the difference between grahamins and nigrocins.

Obviously, research about differences of antimicrobial activities among the paralogous peptides can bring an insight into the relationship between structures and bioactivities, for instance, the potential importance of cationic properties in antimicrobial potency. Depending on the online prediction of secondary structure, the peptide QUB-2040 possibly has a main region of random coil with little α-helix and a small region of strand. Therefore, it may be assigned into the extended class of peptides which usually lack classical secondary structures due to the high content of glycine and/or proline (Powers and Hancock, 2003), while QUB-2040 is rich in glycine.

Generally, the structure of an α-helix is considered as an important requirement of the antimicrobial activity. Thus, a question that why the peptide QUB-2040 with little α-helical structure manifests such strong potency in inhibiting the growth of bacteria arises. A few hypotheses are listed below.

As mentioned before, the interaction between negatively charged bacterial cell membranes and cationic AMPs is the common initial step in both membrane disruptive and non-disruptive mechanisms of peptide antimicrobial activity (da Cunha et al., 2016). The relatively high cationicity of QUB-2040 probably plays a decisive role in the cytolytic function against microbes. On the other hand, some peptides appear to adopt different secondary structures in various solution environments (Li et al., 2007). A number of peptides have the tendency to change into an amphipathic α-helical structure in a membrane mimetic environment, whilst they exist as random coils in aqueous solution, such as brevinin-1 (Giangaspero et al., 2001, Conlon et al., 2004). The peptide QUB-2040 might have an altered secondary structure which contributes to the bioactivities in the cell membrane environment as well. Circular Dichroism (CD) spectroscopy can be used to analyse the secondary structure of QUB-2040 in different solutions, especially in the membrane-mimetic solvent. With respect to the structural motif, the conserved disulphide-bridged heptapeptide segment in the peptide is hypothesised to be of importance in manifesting antimicrobial activities. Although the exact function of disulphide bonds remains unclear, the positive charge of the Rana box motif which alone could cause mast cell degranulation is likely to be fundamental in binding with the anionic bacterial membrane (Wang et al., 2010). In addition, the 'Rana box'-like structure may not be required absolutely for the antimicrobial activity, they could be needed to induce cell membrane translocation in several model systems (Powers and Hancock, 2003). Comparing the discrepancy of

antimicrobial potency between the native QUB-2040 and the reduced form of QUB-2040 could provide some evidence whether the disulphide bond is necessary for the high antimicrobial efficacy.

In all, the relationships between the α-helical conformation and the antimicrobial activity is not absolute, since the cytolytic activities of amphibian AMPs against various pathogenic microbes are influenced by a complicated interaction between hydrophobicity, amphipathicity, cationicity and α-helicity (Conlon et al., 2008b). To confirm these premature conjectures, further research is needed.

Therefore, the peptide QUB-2040 may provide a potential template for studying the structure-function relationships of the nigrocin-2 peptide family. Considering that there are not many effective antifungal agents available for medicinal options, the antifungal activity of QUB-2040 should also be valued (Wang et al., 2016a). Thus, it may be developed not only as a promising antibacterial agent but also as a novel antifungal candidate.

5.3 Further study

To develop such promising antimicrobial molecules, there is a large amount of work remaining to be carried out.

Given the incompletely studied mechanisms of action of AMPs, QUB-2040 may manifest its antimicrobial activities via various strategies depending on different kinds of microbes. Not only against the cell walls or cell membranes of bacteria but also the cytoplasm or other intracellular sites could be the target of the peptide (Che et al., 2008). AMPs usually manifest their physiological functions with different specificities, indicating various action modes which are worthy to be studied in order to exert their optimal function in clinic use. Thus, the analysis of the antimicrobial

mechanisms of QUB-2040 should be commenced. For this purpose, transmission electron microscopy can be applied in searching for clues to possible action modes of QUB-2040 against the tested microorganisms, according to the protocol described by Friedrich *et al* (Friedrich et al., 2000). The same method was applied to investigate the antimicrobial mechanisms of *Odorrana grahami* peptides and the visible phenomena have revealed several mechanisms, such as forming transmembrane pores or laminar mesosoma-like structures, inducing DNA condensation, and peeling off the cell walls (Li et al., 2007).

Although the feasibility of applying the AMPs in clinical practice has been put forward because of the diversity bioactivities presented by AMPs, the process of development is relatively slow and cautious. In order to optimise the function of current AMPs, some modifications of structures can be examined. According to the published research, the antimicrobial potency increases with the increasing cationicity of the peptide up to a certain limit (Conlon et al., 2004, Powers and Hancock, 2003). The cationicity of the peptide could be increased using selective substitutions by lysine with retained amphipathicity. The similar strategy has been applied successfully to design analogues of several therapeutically useful AMPs from amphibian skin secretions (Conlon et al., 2007).

In the present thesis, although QUB-2040 exhibits a relatively low haemolytic activity at the lowest concentration inhibiting the growth of microorganisms, the potential safety hazard of applying the peptide on human beings still exists. The calculated TI value of QUB-2040 against *S. aureus*, *E. coli* and *C. albicans* were 15.5, 7.7, and 15.5, respectively. The TI refers to the ratio of HC_{50} to MIC against bacteria, so larger values indicate greater antimicrobial potency with lower haemolytic activity. The TI values of QUB-2040 against these three microorganisms are not high enough because

of the haemolytic activity. However, from published research, the mutated peptide [K2, K16] of the *Xenopus tropicalis* peptide XT-7 was found to possess experimental TI values more than 120 against *S. aureus* or *E. coli*, while the parent peptide XT-7 only had TI values less than 20 (Kamech et al., 2012). Therefore, considering the low MIC of QUB-2040, the TI value could be expected to increase a lot by minimizing the toxicity on red blood cells with retained antimicrobial activity. Fortunately, the haemolytic activity of the peptide could be decreased through appropriate changes in conformation. Research on magainin analogues has drawn a conclusion that the increasing hydrophobicity increases haemolytic activity (Conlon et al., 2007). Obviously, the modification is mainly based on the structure-activity relationships. An ideal modification can make the peptide stronger in the battle against pathogens with decreasing cell lysis ability on red blood cells.

Therefore, appropriate modifications could be made to QUB-2040 to increase its antimicrobial potency while reducing undesirable cytolytic effects on erythrocytes in subsequent research. Besides, proper guidelines for the rational design of modified peptides should be proposed in the future so that the modification on peptides could be rigorous and logical.

In addition, it is worthy to continue to isolate and identify more AMPs from the Chinese bamboo leaf odorous frog to increase the diversity of AMPs from *Odorrana versabilis*. Considering that nigrocin-2 peptides are rich in the defensive skin secretion of Asian ranid frogs, especially in those of the genus *Odorrana* (Wang et al., 2010), *Odorrana versabilis* is expected to have more nigrocin homologues. These additional peptides could be potential templates for searching for clues to structure-activity relationships and drug candidates for future therapeutic applications. However, the number of amphibians is in decline and the numbers of extinct and threatened species

becomes larger and larger (Stuart et al., 2004). Although the frog *Odorrana versabilis* is a common species with regards to its relatively wide distribution, it is in serious decline because of many factors, such as the habitat loss and polluted environment. In the period of developing AMPs as new therapeutic medicines, the reduction of amphibians is not the only challenge we are faced with, but also the restrictions of AMPs, and difficulties in developing AMPs.

Further studies mentioned above should be performed to obtain a more in-depth knowledge about the peptide QUB-2040. It could be used as a potential template in studying the nigrocin peptide family, as well as the structure-activity relationships in order to design novel AMPs. Unfortunately, further research for this thesis has stopped because of time limitations.

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