MASTER OF PHILOSOPHY

The discovery and functional study of a bioactive peptide, QUB-3000, from the defensive skin secretion of the northern leopard frog, Rana pipiens

Xu, Wei

Award date:
2018

Awarding institution:
Queen's University Belfast

The discovery and functional study of a bioactive peptide, QUB-3000, from the defensive skin secretion of the northern leopard frog, *Rana pipiens*

Miss Wei Xu

School of Pharmacy

Faculty of Medicine, Health and Life Sciences

Queen's University Belfast

A thesis submitted to Queen's University Belfast for the degree of

Master of Philosophy (MPhil)

July 2018

Contents

Acknowledgements

It is my honour to obtain this opportunity to pursue my academic degree of MPhil at Queen's University Belfast. In the past year, I have learned a lot and I would like to express my gratitude to those who have helped me. I want to thank my supervisors, Dr Yuxin Wu, Dr. Tianbao Chen, Professor Chris Shaw, Dr. Lei Wang and Dr. Mei Zhou. Their profound professional knowledge, rigorous academic attitude and meticulous work style have inspired me deeply. Under the guidance of my supervisors, I learned how to conduct scientific thinking and apply it to my own research. During the experiments, I encountered many difficulties that were hard to deal with, but all of them have been solved under their guidance and help. Then, I would like to thank my colleagues. After arriving Belfast, we helped each other in our studies and daily life, and we made progress together. Finally, I want to thank my family and my friends for their understanding, support, tolerance and encouragement. It is because of them that I have the courage and determination to pursue this research.

Declaration

I hereby declare that the research reported in this thesis is my own work except where acknowledgement has been made. All of the work was carried out in the Molecular Therapeutics Research Group, School of Pharmacy, Faculty of Medicine, Health and Life Sciences, Queen's University Belfast.

I hereby declare that for five years following the date on which this thesis is deposited in the Library of Queen's University, Belfast, the thesis shall remain confidential with access or copying prohibited. Following expiry of this period, I permit the librarian of the University to allow this thesis to be copied in the whole or in part without reference to me on the understanding that such authority applies to the provision of single copies made for study purposes or for inclusion within the stock of another library. This restriction does not apply to the British Library Thesis Service. IT IS A CONDITION OF USE OF THIS THESIS THAT ANYONE WHO CONSULTS IN MUST RECOGNISE THAT THE COPYRIGHT RESTS WITH THE AUTHOR AND THAT NO QUOTATION FROM THE THESIS AND NO INFORMATION DERIVED FROM IT MAY BE PUBLISHED UNLESS THE SOURCE IS PROPERLY ACKNOWLEDGED.

Abstract

To solve the drug-resistant microorganism problem, antimicrobial peptides (AMPs) have been studied as alternatives for traditional antibiotics. AMPs can inhibit and kill gram-negative bacteria, gram-positive bacteria, fungi and even cancer cells. Compared with traditional antibiotics, it is much more difficult for pathogens to develop drug-resistance towards AMPs, which is because of the distinct mechanisms of action of AMPs.

In this study, an AMP precursor encoded by a cDNA was isolated and identified from the lyophilised skin secretion of the northern leopard frog, *Rana pipiens*, by "shotgun" cloning. According to its molecular mass, the mature peptide was named QUB-3000. Then, QUB-3000 was synthesised by solid phase peptide synthesis (SPPS), purified by RP-HPLC. The purified synthetic QUB-3000 was then subjected to several functional experiments analysis.

QUB-3000 exhibited strong antimicrobial ability against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. The minimum inhibitory concentration (MIC) values and minimum bactericidal concentration (MBC) values were 16 μM and 8 μM, respectively. While QUB-3000 possessed weak antimicrobial ability against the fungus *Candida albicans* with the MIC value at 256 μM and no MBC value. At the same time, QUB-3000 showed low haemolytic activity on horse blood cells. However, the 10^{-5} M QUB-3000 had very weak effects in inhibiting the growth of cancer cells (H-157, PC-3, U251MG), and it even could promote the growth of certain cancer cells (MCF-7). In the future, more

functional investigations can be performed to develop QUB-3000 into a clinical drug. For example, circular dichroism (CD) can be used to confirm the secondary structure of QUB-3000. Furthermore, more drug-resistant bacteria can be used for assessing the antimicrobial activity of QUB-3000.

Chapter 1: Introduction

1.1. Amphibians

Amphibians, include frogs, toads, newts, salamanders, and caecilians, are ectotherms and can live both on land and in water. The larvae of amphibians usually live in the water and breathe through the gills. However, after they grow up, their breathing method changes. Most amphibians breathe using lungs with the skin as a secondary breathing method. A small number of amphibians which do not have lungs only use the skin as their respiratory organ(FULL et al. 1988).

Amphibians can be divided into three categories: Anura, Caudata (or Urodela) and Gymnophiona (or Apoda). Anura, means "no tail", contains both frogs and toads. Anura usually can be divided into three suborders: Archaeobatrachia, Mesobatrachia, and Neobatrachia. Neobatrachia is the largest suborder, containing 96% of the frog species. Caudata is a group of amphibians with tails, such as salamanders. The larvae and adults of Caudata do not have significant differences in their appearances. The last type-Gymnophiona, mainly consists of the caecilian, unlike other amphibians, they do not have any limbs (Schmidt et al. 1996).

1.1.1. The defence mechanism of amphibians

In the long evolutionary process, amphibians developed their unique defence mechanisms. Some salamanders and frogs can produce special secretions that not only keep skin moist but also help them escape from predators. Some secretions are toxic, which are released by glands located on the neck or back of the body, where predators prefer to attack. For example, Siphonop is a kind of Caecilians, and its skin secretions were found to possess cardiac toxicity (Schwartz et al. 1999). These toxins

can help amphibians to kill their enemies. These poisonous species can also use bright colours, body languages and voices to warn and threaten their enemies. Additionally, some frogs can camouflage themselves and mimic their surroundings(Nunes and Costa 2011).

1.1.2. The skin of amphibians

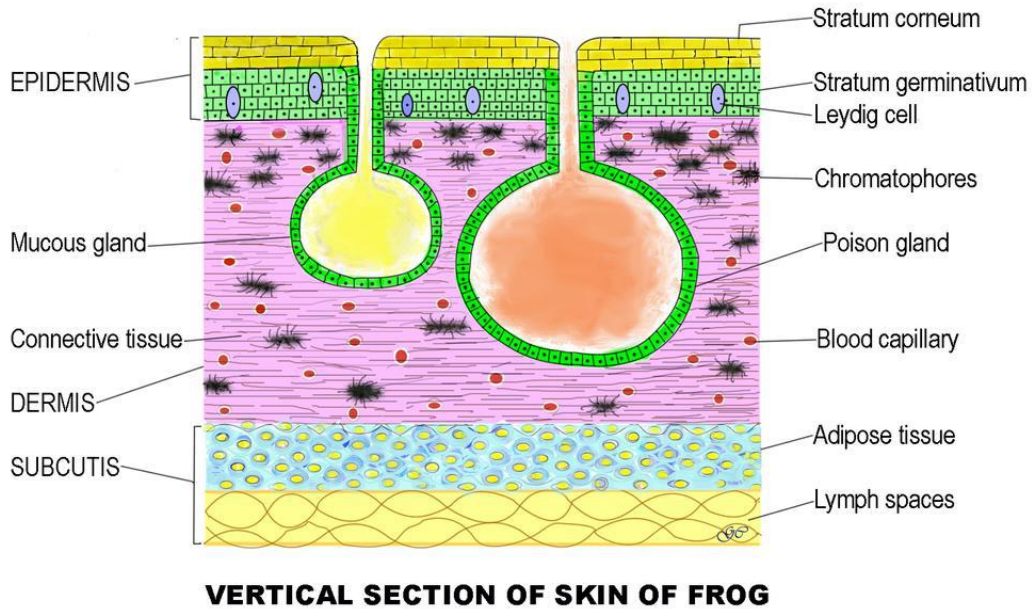


Figure 1.1 Vertical section of skin of frog ('Skin The Integument') From the skin surface to interior, the first part is the epidermis which consists of stratum corneum and stratum germinativum, and the dermis includes mucous gland, poison gland, blood capillaries, chromatophores and connective tissue. Below dermis is the subcutis, which contains adipose tissue and lymph spaces.

1.1.2.1 Amphibian skin structures and functions

The skin of amphibians is naked, with no scales (except for some caecilians) and hair. However, and it has glands with various functions. Taking frog skin as an example, from the skin surface to interior, the first part is the epidermis which consists of stratum corneum and stratum germinativum, and then the dermis includes mucous gland, poison gland, blood capillaries, chromatophores and connective tissue. Below dermis is the subcutis, which contains adipose tissue and lymph spaces.

The skin of amphibians is thin, like a semi-permeable membrane that allows water to pass through, and distributed with different functional glands. The skin of an amphibian is rich in colours, this is determined by hormonal control that could be used to conceal creature themselves or warn the enemy(Nilsson Sköld et al. 2013, Forsman and Hagman 2009).

1.1.2.2 Amphibian skin secretions

The amphibian skin has two basic glands - mucus glands and granular glands (Clarke 1997). The secretions of mucous gland can keep skin moist, help them to breath and regulate body temperature. Mucus can also make their bodies slippery, which reduce the resistance to swimming and increase the possibility of escaping from predators. The granular (poison) gland is located on the upper surface of the amphibian's skin, it produces the most effective tools for their self-protection. As mentioned above, the secretions produced by the mucus gland have fundamental functions, such as protecting the surface of the skin. There have not been well studied by scientists yet. However, the granular gland is very popular among researchers because it contains a large number of different kinds of chemicals in its secretions. The main components in the secretions include amines, peptides, proteins, alkaloids and steroids. They all have potential medicinal values(Clarke 1997).

1.2. Frogs

Figure 1.2 The northern leopard frog, *Rana pipiens*('Northern Leopard Frog Care Sheet')

1.2.1. *Rana pipiens*

The northern leopard frog, *Rana pipiens*, is a large frog and just like its name. Its whole body is covered with huge dark spots in its green or brown skin. It has two parallel raised folds on its outer back which extends from the eye to the back.The larvae of the northern leopard frog are the same as the normal tadpoles, but its lower surface has some light colour spots.

1.2.2. Regional distribution

The northern leopard frogs are widely distributed in ponds, swamps and streams, and in forests or cities. They usually like to live in water that is rich in aquatic plants, but in the summer, they come ashore and live on the grass. They are not afraid of cold, and they can live even in high-altitude areas. Their habitats include parts of the United States and parts of Canada. Research has shown that the peptides isolated from the skin secretions from northern leopard frogs which lived in Minnesota and Vermont are more effective in inhibiting the growth of *Batrachochytrium*

dendrobatidis, which is a fungus that causes the massive deaths or extinctions of amphibians, compared to the same peptides from Michigan frogs (Tennessen et al. 2009).

1.2.3. Medicinal research

As *Rana pipiens* holds great research value, especially for therapeutic potential, researchers have studied it extensively. For example, Onconase, which is isolated from its oocytes or early embryos, is an amphibian RNase and also the only animal RNase that effectively treats malignant mesothelioma in the phase iii of the clinical trial (Ardelt et al. 2009).

1.3. Peptides

The peptides can be divided into natural peptides and artificial peptides, it is a chain compound that consists of two or more amino acids linked through peptide bonds.Many aspects of peptides are worth to be studied. Due to their biological functions, peptides can be used in clinical research to treat multiple diseases. It also can be used as antigens to diagnose diseases, which is more sensitive and accurate than protein antigen, and is more economic and easier to produce (Kouzmitcheva et al. 2001). Since that proteins can be hydrolysed into peptides, peptide research is also useful for providing information to study the structures and functions of proteins.

1.3.1. Bioactive peptides

1.3.1.1. The classification of bioactive peptides

Bioactive peptides can be classified into exogenous peptides and endogenous peptides, according to source classification. Depending on the different functions of

the peptides, they can also be divided into the following types: Antimicrobial peptides, Antithrombotic peptides, Antihypertensive peptides, Immunomodulatory peptides, Antioxidative peptides, Tachykinin peptides, Vasoactive intestinal peptides, Pancreatic polypeptide related peptides, Opioid peptides and others(Drew et al. 2014, Korhonen 2009, Hamley 2017).

1.3.2. Peptides from*Rana pipiens*

After years of research, a large number of antimicrobial peptides, which belong to different families-Brevinin, Esculentin, Ranatuerin and Temporin, have been extracted from frog *Rana pipiens*(Goraya et al. 2000, Tennessen et al. 2009). In addition, in retina and other tissues of *Rana pipiens*, opioid peptides-β-Endorphin was isolated (Jackson et al. 1980), and researchers also identified insulinotropic peptides in *Rana pipiens* skin secretions (Marenah et al. 2005).

1.4. Antimicrobial peptides(AMPs)

AMPs are also called host-defence peptides. They are composed of 12 to 80 amino acids and have a positive net charge (Mikut et al. 2016). They have many advantages compared to those commonly used traditional antibiotics. Firstly, AMPs have a wide range of antimicrobial spectrum that can inhibit and kill Gram-negative bacteria, Gram-positive bacteria and fungi. Secondly, it is believed that the antimicrobial mechanisms of AMPs are related to the interaction of the phospholipid bilayer of the targets, which can induce the cell membrane to rupture that can make AMPs play an antimicrobial role (Epand and Vogel 1999). Resistance to AMPs is not easy for pathogens to develop, that is because AMPs have different mechanisms of action

compared to most antibiotics. Also, a previous report showed that AMPs could be used as bactericidal agents to avoid the contamination of blood products (Mohan et al. 2010). Currently, researchers believe that AMPs can overcome some of the disadvantages caused by traditional antibiotics, such as the problem of lung infections caused by drug resistance (Hancock and Sahl 2006). Experiments by Zhang et al. demonstrated that aerosol administration of AMPs could be used to treat lung infections in rats (Zhang et al. 2005), which suggests that the AMPs can be used to improve immunity as well. Mouse experiments by Bals et al. also showed that AMP enhances the ability of mice to prevent infection (Bals et al. 1999).Therefore, research on AMPs is helpful for the development of new drugs.

1.4.1. The structure of AMPs

The peptide has to go through four steps: assembly, folding, packing, and interaction, to produce the three-dimensional structure, which makes them occupy a hydrophilic side and a hydrophobic side (Hancock 1997). According to the structure of peptides analysed by nuclear magnetic resonance (NMR), AMPs can be divided into the following categories: peptides with α-helix, peptides with β-sheet, peptides with specific amino acids such as proline, arginine and tryptophan, peptides with the loop formed by a disulfide bond and peptides with extended structure(Batoni et al. 2011). Most AMPs mainly contain α-helix or β-sheet structures. Some specific AMPs may contain both structures.

1.4.1.1. α-helix

The α-helix is the most common type of peptide secondary structure. It is connected by hydrogen bonds. Each of the 20 amino acids has a different side chain and this could affect their ability to form an α-helix. The AMPs containing this structure are represented by cecropins from insects and magainins from frogs (Carnicelli et al. 2013).

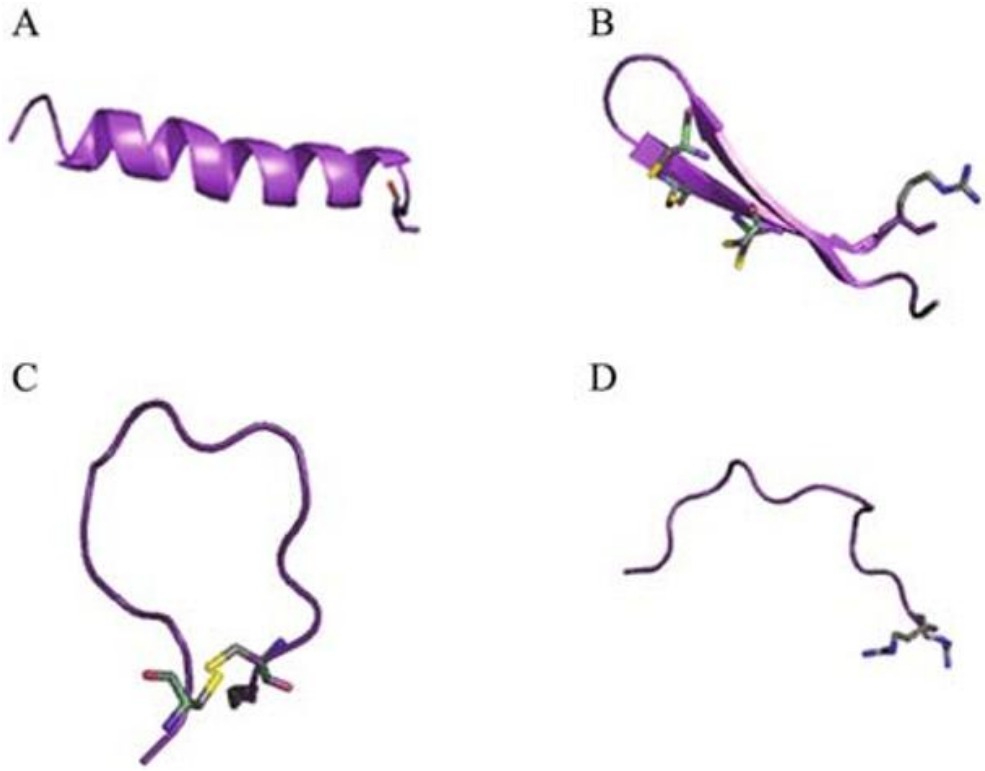


Figure 1.3 The normal models of AMPs structures. These models are from the NMR structural database. (A) The amphipathic α-helix structure (B) Two-stranded antiparallel β-sheet (C)The β-turn loop structure. (D) The random coil structure (Huang et al. 2010)

1.4.1.2. β-sheet

Unlike α-helix, the β-sheet does not occur within the peptide chain but forms hydrogen bonds between two adjacent peptide chains. In addition, β-sheet has two forms - parallel and anti-parallel arrangement. In theory, the parallel arrangement of β-sheet is more stable, because it produces the hydrogen bond in a flat structure.

Research indicated that proteins could be kept stable by hydrophobic and hydrogen bonds (Doig et al. 2001). It is obvious that β-sheet has more hydrogen bonds than α-helix, thus, β-sheet should be more stable than α-helix. The experiment of Henzler Wildman et al. also proved this hypothesis (Henzler Wildman et al. 2002). The β-sheet AMPs presented in aqueous solution in the form of dimer, and they are amphipathic due to the β-strand structure, which comes from the disulfide bond and the cyclization of main peptide strand (Yeaman and Yount 2003). The typical representative of peptides with β-sheet is the defensins, which can be found in mammals (Selsted et al. 1983).

1.4.2. The antimicrobial mechanism of AMPs

As mentioned above, AMPs mainly work by acting on cell membranes (Hancock and Rozek 2002). The cell membranes of prokaryotes and eukaryotes have different phospholipids that result in different electronegativity. The bacterial cell membranes have negative charges, because they mainly contain phosphatidylglycerol (PG) and cardiolipin (CL). In contrast, cell membranes of eukaryotes mainly carry neutral charges, because that the phospholipid composition mainly includes phosphatidylethanolamine (PE) and sphingomyelin (SM)(Yeaman and Yount 2003). Thus, the bacterial cell membranes can combine with positive-charged amino acid residues in the AMP. For example, the outer membrane of Gram-negative bacteria is rich in lipopolysaccharide, which carries negative charges, and the phosphoric acid on the surface of Gram-positive bacteria also contains negative charges (Brogden 2005). They both can be combined with cationic AMPs.

The AMPs can be divided into membrane disruptive and non-membrane disruptive categories, depending on whether the cell membrane is destroyed (Powers and Hancock 2003). Usually, non-membrane disruptive peptides have the antimicrobial mechanisms similar to those of general antibiotics. On the other hand, membrane disruptive peptides can permeate the cell membrane and cause cell death (Huang et al. 2010). Researchers have proposed a variety of models for the permeation by membrane disruptive peptides, such as barrel-stave model, carpet model and toroidal-pore model (Brogden 2005). However, Bechinger and Lohner believed that the same AMP can utilise different models to exert function under different conditions (the properties of cell membranes, environmental factors) (Bechinger and Lohner 2006).

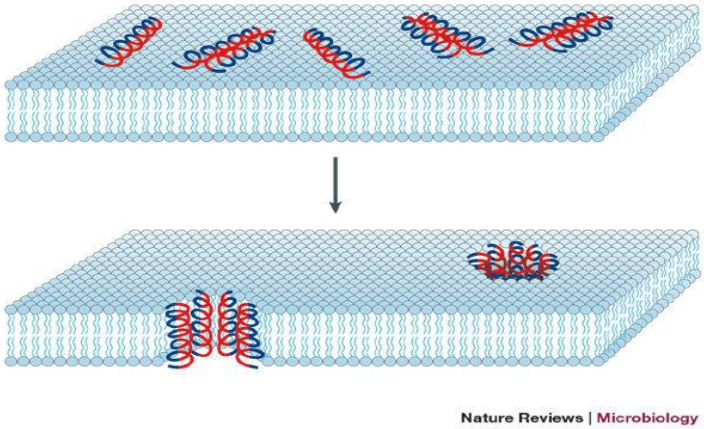


Figure 1.4 Barrel-stave model (Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue.) (Brogden 2005) In this model, the hydrophobic surface of the peptide is directed towards the cell membrane, and the hydrophilic surface is formed into a pore, which produced a transmembrane channel.

1.4.2.1. Barrel-stave model

The barrel-stave model was proposed initially in 1974 by Baumann and Mueller, and was the first model to explain the interaction between peptide and cell membrane (Baumann and Mueller 1974). It is mainly used to explain the zwitterionic membrane

related interactions (Huang et al. 2010). In this model, the hydrophobic surface of the peptide is directed towards the cell membrane, and the hydrophilic surface is formed into a pore (Yeaman and Yount 2003), which produced a transmembrane channel. At first, the peptide monomer binds to the surface of the cell membrane. Then the hydrophobic part of the peptide interacts with the hydrophobic part of the cell membrane to insert into the cell membrane. In the end, peptides accumulate through the interaction of the hydrophilic amino acid residues and form the barrel structure (Figure 1.4) (Huang et al. 2010). When the theory was firstly proposed, the influence of ion concentration gradient around the pore was ignored, then it was added and revised by Laver (Laver 1994).

1.4.2.2. Carpet model

As shown in Figure 1.5, the peptides bind to the membrane surface and were evenly distributed on the membrane surface. The phospholipids are replaced by peptides to break the stability of the cell membrane. When the concentration of the peptide increased to threshold level, the cell membrane will be destroyed (Huang et al. 2010). Unlike the barrel-stave model, the peptide does not insert into the cell membrane in carpet model. The interaction with cell membrane is only via the attraction of charges, so it mainly acts on the cell membranes with the negatively charged residues (Shai 1999).

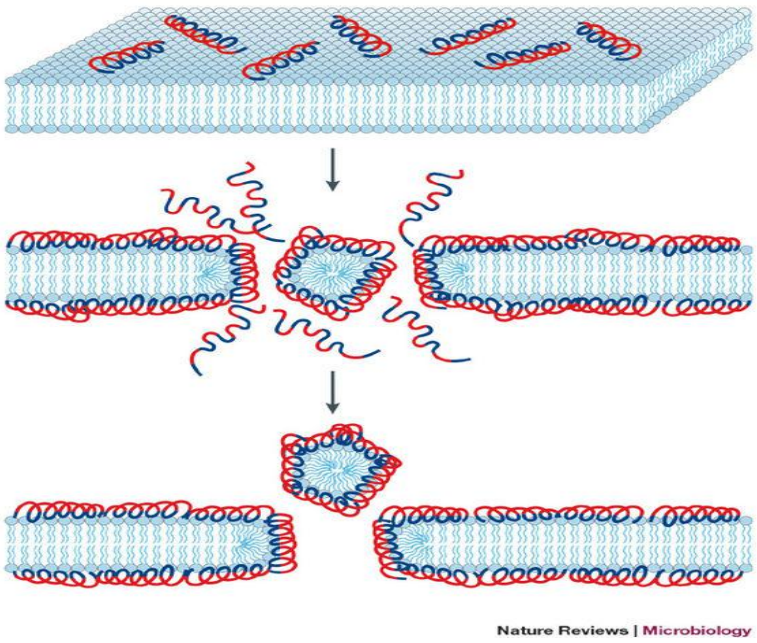


Figure 1.5 Carpet model (Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue.) (Brogden 2005) In this model, The phospholipids are replaced by peptides to break the stability of the cell membrane. When the concentration of the peptide increased to threshold level, the cell membrane will be destroyed.

1.4.2.3. Toroidal-pore model

This model is similar to the barrel-stave model. The difference is that the hydrophobic ends of the membrane surface - lipids are bent with the binding of peptides, which is possible to block the charge of cationic peptides (Yang et al. 2001). However, in the barrel-stave model, membrane lipids do not participate in the formation of pores (Huang et al. 2010). A variety of peptides, such as maganins and LL-37, can produce such pores (Henzler Wildman et al. 2003, Hallock et al. 2003).

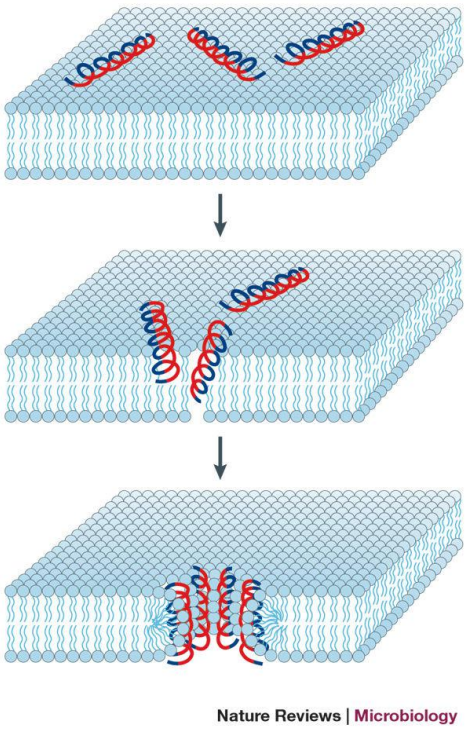


Figure 1.6 Toroidal-pore model (Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue.) (Brogden 2005) In this model, the hydrophobic ends of the membrane surface - lipids are bent with the binding of peptides, which is possible to block the charge of cationic peptides.

1.4.3. The haemolytic activity of AMPs

The haemolytic activity of AMPs is the same important as their antimicrobial and immunomodulatory ability, because haemolytic activity represents the toxicity of peptides, and it determines whether it can be applied clinically. Whether the peptide produces haemolysis mainly depends on its hydrophobicity and the interactions with the phosphatidylcholine on the surface of the cell membrane of mammal (Tachi et al. 2002). Researchers found that haemolytic activity could be reduced by destroying the hydrophobic surface of the peptides while not affecting other activities (Hawrani et al. 2008). In addition, altering the structure of the peptide can also change the haemolytic activity. Oren and Shai transformed the linear AMPs into cyclic peptides to reduce the haemolysis (Oren and Shai 2000). However, this approach may also

reduce the antimicrobial activity of peptides, which depends on the types of peptide and their structures (Unger et al. 2001).

1.4.4. Factors influencing the activity of AMPs

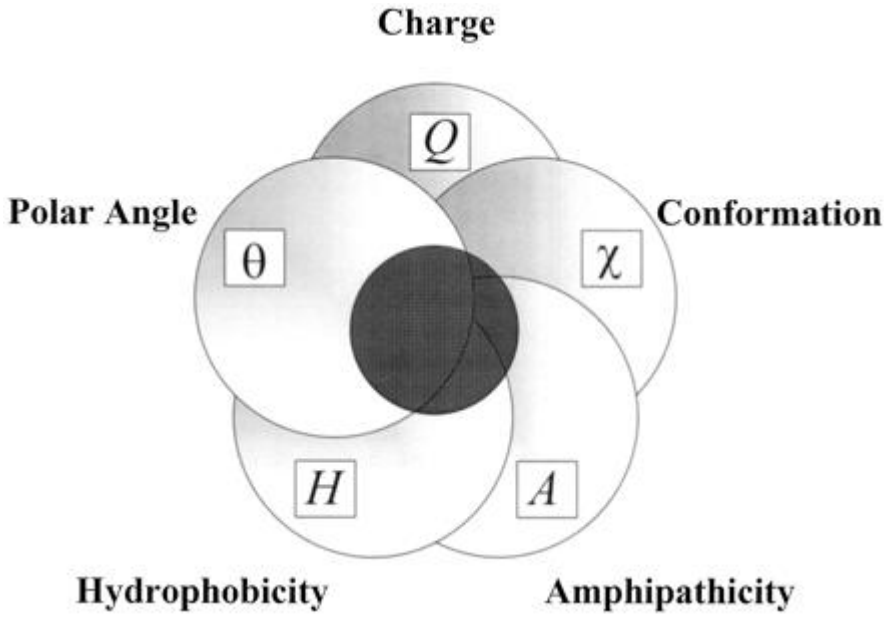


Figure 1.7 Factors influencing the activity of AMPs (Optimal antimicrobial peptide efficacy lies in the relevant coordination of these relationships (shaded area) as they relate to microbial target versus host cells in a particular context of infection.) (Yeaman and Yount 2003)

Many factors can affect AMPs activity, including charge, hydrophobicity, amphipathicity, conformation and polar angle (Figure 1.7) (Yeaman and Yount 2003). In addition, the number of amino acid residues is one of the factors that affect the antimicrobial potency. The antimicrobial activity was assessed experimentally by increasing the length of the lytic base unit (LBU) peptide and the results showed that the antimicrobial capacity increased with the increased length, and the peptide with 24 residues exhibited the best antimicrobial activity (Deslouches, Phadke et al. 2005). Researchers can improve the activity of AMPs by studying and modifying these factors.

1.4.4.1. Charge

Most of the AMPs have positive charges. Thus, the positively charged residues of AMPs play an important role in antimicrobial mechanisms (Giangaspero et al. 2001). Conlon also suggested that if somebody wants to increase the activity of antimicrobial peptides, more cationic residues should be included in the peptide sequence (Conlon et al. 2004a). In general, antibacterial ability increases with the increased net charge, but the study of Dathe pointed out that there exist a threshold, and if the net charge of AMPs increases to above the threshold, it will cause the increased haemolysis and decline in the antimicrobial action (Dathe et al. 2001).

1.4.4.2. Amphipathicity and hydrophobicity

Matsuzaki reported that the anti-Gram-negative bacteria activity is mainly determined by the net charge, whereas the activity against Gram-positive bacteria is mainly determined by the hydrophobicity and hydrophobic moment (Matsuzaki 2009). The hydrophobic moment is a parameter that is used to quantify amphipathicity (Eisenberg 1984). Most AMPs bind to the cell membrane and then form amphipathic structures. Increasing hydrophobic moment would result in a stronger effect on haemolytic activity compared to antimicrobial activity (Dathe and Wieprecht 1999). Hydrophobicity is indispensable for AMPs, because it determines the degree of peptides binding to cell membranes (Lee et al. 2016). Another study showed that hydrophobicity had little effect on the permeable activity of AMP on anionic membrane, but it increased the interactions between peptide and neutral phospholipids (Wieprecht et al. 1997a). In addition, higher hydrophobicity can

induce stronger haemolytic activity, decreased antibacterial activity antifungal ability, this may be related to the self-association of peptides (Chen et al. 2007).

1.4.4.3. Conformation

Among the secondary structure of AMPs, the α-helix is closely related to the antimicrobial mechanism of AMPs, nonetheless, if AMPs form α-helix before interacting with the cell membrane, it will reduce their antimicrobial ability (Tossi et al. 2000). Substitution of amino acids to prevent the α-helix structure formation can greatly reduce the antimicrobial activity (Lee et al. 2016). In addition, Shin et al. reported that the α-helix did not only affect the antimicrobial activity of peptides, it can also affect their anticancer and haemolytic abilities (Shin et al. 2001). However, the interaction of cell membrane with β-sheet AMPs depends largely on the hydrophobic moment and amphipathicity of peptides (Yeaman and Yount 2003).

1.4.4.4. Polar Angle

The polar angle represents the proportion of the polar surface and non-polar surface in helical AMPs, and the polar angle will decrease if the area of non-polar parts increases (Yeaman and Yount 2003). Wieprecht et al have found that increasing the polar angle would increase the antimicrobial and haemolytic capabilities of AMPs, while reducing the polar angle would increase the antimicrobial selectivity and membrane permeability (Wieprecht et al. 1997b). Uematsu and Matsuzaki also demonstrated that the polar angle could affects the AMPs, the smaller polar angle could lead AMPs to penetrate the cell membrane easier, and the pores formation on the surface of the cell membrane become faster (Uematsu and Matsuzaki 2000).

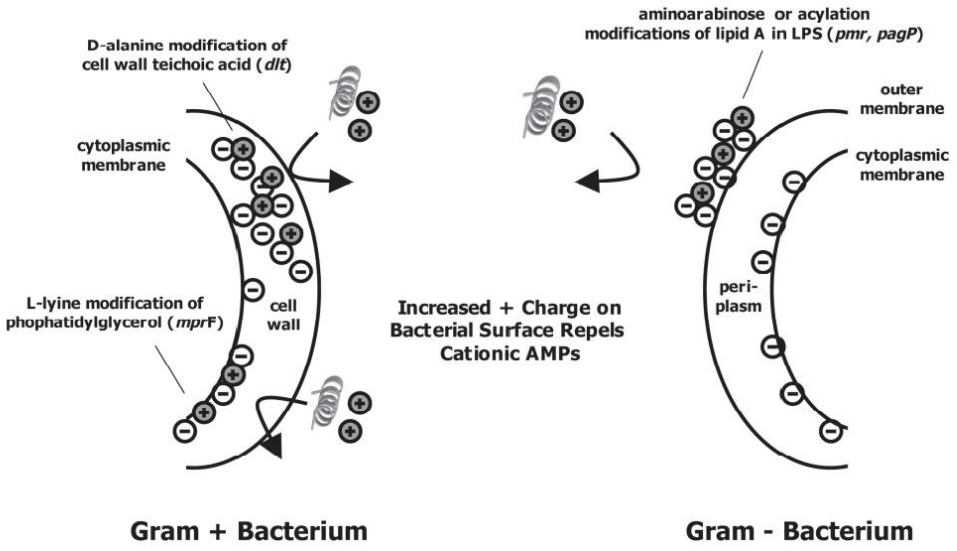


Figure 1.8

Figure 1.8 Different modifications on the surface of Gram-positive and negative bacteria (Nizet 2006) The D-alanine was incorporated into the phosphoacid on the surface of the cell membrane of Gram-positive bacteria by an ester bond, which is used to expose the positively charged amino group, thus rejecting cationic AMPs . In Gram-negative bacteria, the addition of 4-aminoarabinose to the phosphate groups in the lipid A of lipopolysaccharides (LPS) can reduce the total negative charge of cell membrane and enhance the resistance of AMPs.

1.4.5. The mechanisms of AMPs resistance

The overuse of antibiotics results in the emergence of superbugs as a consequence, proving that the microorganism can be resistant to drugs. Similarly, microorganism can also develop AMP resistance. Therefore, understanding the mechanism of AMP resistance is very helpful for the application of AMPs in clinic.

As mentioned before, one of the antimicrobial mechanisms of AMPs is the combination of cationic AMPs with anions on the surface of bacterial cell membranes. As a result, bacteria can increase their resistance to AMPs by specifically modifying the cell surface to reduce negative charges (Nizet 2006). For example, Peschel indicated that the D-alanine was incorporated into the phosphoacid on the surface of the cell membrane of Gram-positive bacteria by an ester bond,

which is used to expose the positively charged amino group, thus rejecting cationic AMPs (Peschel 2002). In Gram-negative bacteria, the addition of 4-aminoarabinose to the phosphate groups in the lipid A of lipopolysaccharides (LPS) can reduce the total negative charge of cell membrane and enhance the resistance of AMPs (Nizet 2006). Figure 1.8 shows the modification methods for the two Gram-positive and Gram-negative bacteria mentioned above. In addition, cell membrane energy regulation can also be an effective approach to resist AMPs. Gyurko et al. found that fungi lacking the mitochondrial respiration (due to genetic mutation) were better resistant to AMPs (Yeaman and Yount 2003).

1.4.6. The anticancer activity of AMPs

AMPs can target multiple types of cells due to the characteristics of their cell membranes. Thus, the cancer cells could also be the potential target of AMPs (Hancock and Diamond 2000). AMPs with anticancer activity can be classified into two types, cytotoxic and non-cytotoxic (Hoskin and Ramamoorthy 2008). The cell membrane of cancer cells is usually negatively charged because of the overexpression of the PS on the surface of the cell membrane (Utsugi et al. 1991). Therefore, AMPs can also bind to the cell membrane of cancer cells, which may be the main mechanism that how AMPs kill cancer cells. Additionally, Hoskin and Ramamoorthy also suggested that AMPs might also be internalised into the cytoplasm of cancer cells to induce apoptosis (Hoskin and Ramamoorthy 2008). For example, D-peptides A, B, C and D are selective for human cervix, glioma, lung, mouse myeloma, and African green monkey kidney cancer cells by disrupting cell

membrane (Iwasaki et al. 2009).

1.4.7. The mechanism of immunomodulation of AMPs

AMPs can bind to receptors of various immune cells on the surface of the membrane, and then trigger the intracellular actions. Some AMPs can also enter the cells through the membrane receptor and bind to the intracellular receptor, which stimulates the innate immune pathway and signal transduction (Hilchie et al. 2013). For example, an innate defense–regulator peptide (IDR-1) can act through mitogen-activated protein kinase and other signalling pathways, enhances the levels of monocyte chemokines while reducing pro-inflammatory cytokine responses. In addition, AMPs can induce the differentiation of macrophages to produce anti-inflammatory properties (Pena et al. 2013), and they have different effect on different kinds of macrophages (Brown et al. 2011).

1.4.8. Clinical application of AMPs

According to the unique antimicrobial mechanism of AMPs, AMPs can be used as a new type of drug for treatment of infections. Andres and Dimarcq listed all 8 AMPs that have underwent clinical trials before 2004, and this data shows that most of AMPs have entered phase ii and iii clinical trials such as Pexiganan (MSI-78),Iseganan (IB-367),Peptides MBI (MBI-226), Histatine variants and Neuprex (RBPI 21) (Andres and Dimarcq 2004), but failed to be used as a medicine (Gordon et al. 2005). The first antimicrobial peptide used in clinical trials was MSI-78, it is an analogue of the natural AMP Magainin, which is a class of AMPs with broad antimicrobial spectrum isolated from the frog *Xenopus laevis* (Zasloff 1987).The

MSI-78 completed two phase iii clinical trials in 1998, it showed that it has similar, but not stronger effects with ofloxacin in the treatment of diabetic foot ulcers (Lamb and Wiseman 1998).Therefore, the Food and Drug Administration (FDA) rejected the application of MSI-78 in 1999 (Moore 2003).Currently, it is known that one of the most potent natural AMPs is the protegrin from pigs and the derivative IB-367 of protegrin, which failed in phase iii clinical trial to treat oral mucositis (Fjell et al. 2012).Although AMPs have wide developmental prospects, no breakthrough has been achieved in clinical trials. Hancock and Sahl believed that the main reason may be the potential toxicity *in vivo*, the protease instability and the exorbitant cost of manufacturing AMP as drugs (Hancock and Sahl 2006).Therefore, scientists need to find new approaches to reduce the costs, or to modify AMPs to minimise toxicity. In summary, the study on AMPs must be carried on.

1.4.9. AMP families from genus *Rana*

A large number of AMPs have been isolated from the genus *Rana*, with more than 250 species, and according to the similarity of their structures and sequences, they could be divided into 13 families, named brevinin-1, brevinin-2, esculentin-1, esculentin-2, japonicin, melittin-related peptides, nigrocin, palustrin peptides, ranalexin, ranatuerin-1, ranatuerin-2, temporin peptides and tigerinin (Conlon et al. 2004a).

1.4.9.1. Ranatuerin-2

The ranatuerin-2 family was firstly isolated from *Rana catesbeiana* (Goraya et al. 1998) and also identified from such frogs in North America(Conlon et al. 2004a).

Unlike the previously mentioned peptides families of brevinin-1, esculentin-1, esculentin-2, ranatuerin-1, which have a representative "*Rana* box" structure formed by the seven residues at the end of their C-terminus, the "*Rana* box" structure of ranatuerin-2 family peptides has only 6 residues(Xu and Lai 2015).

Although the ranatuerin-2 peptides have certain genetic similarity (Figure 1.9), the primary structures of them are variable, with only five residues are conserved, which is essential for their broad antimicrobial spectrum (Conlon et al. 2004a). The MICs (minimum inhibitory concentration) and haemolytic activities of the ranatuerin-2 peptides isolated from genus *Rana* until 2011 are summarised in Table 1.1. Table 1.1 shows that ranatuerin-2 peptides are effective on microorganisms. They showed stronger effect against Gram-negative bacteria than fungi (Rollins-Smith et al. 2006). Conlon et al. confirmed that the more cationic of ranatuerin-2 peptide has, the better antimicrobial activity it will possess (Conlon et al. 2011).

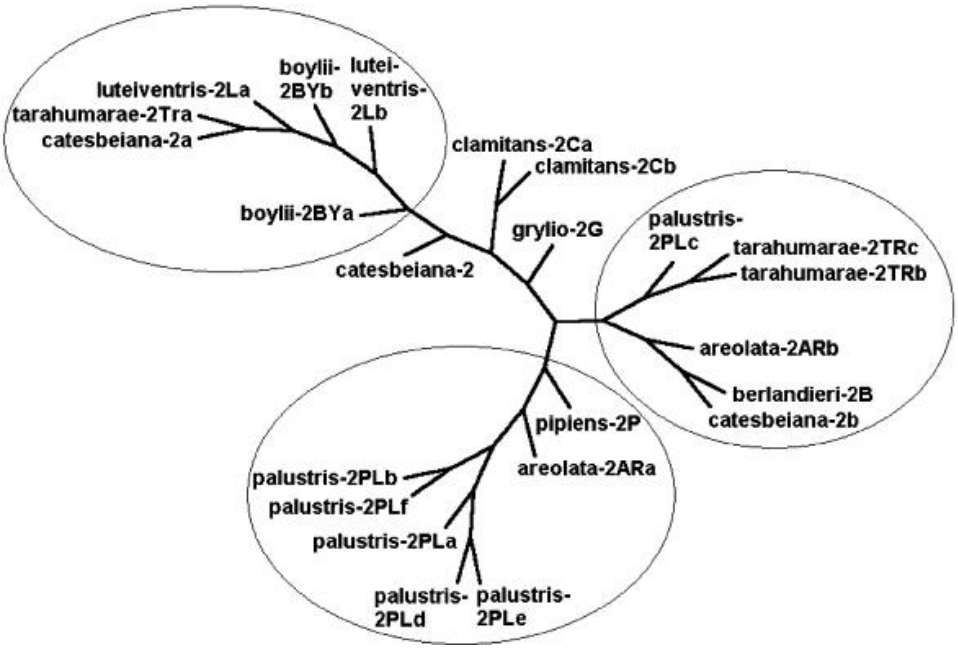


Figure 1.9 The genetic similarity of ranatuerin-2 family peptides in north American frogs (Conlon et al. 2004a)

Table 1.1 The MICs and haemolytic activities of the ranatuerin-2 family peptides isolated from *Rana* genus (In this table, HC_{50}, LD_{50} and LC_{50} represent the same hemolysis rate)

The name of peptide	MICs(μM)			Haemolytic activities (human erythrocytes) (μM)
	S. aureus	*E. coli*	*c. albicans*	
Ranatuerin-2ARa and Ranatuerin-2ARb (Ali et al. 2002)	70- not active up to 200	17-30	150- not active up to 200	HC_{50}: 100-150
Ranatuerin-2AUa(Conlon et al. 2005a)	20	5	>40	HC_{50}: 290
Ranatuerin-2B (Goraya et al. 2000)	2	2	35	No data
Ranatuerin-2BYa and Ranatuerin-2BYb (Conlon et al. 2003)	27- not active at 50	7-17	not active at 50	HC_{50}: 120-＞200
Ranatuerin-2CSa (Conlon et al. 2007)	8	4	No data	LD_{50}: 150
Ranatuerin-2Cb (Halverson et al. 2000)	40	2	46	No data
Ranatuerin-2G (Kim et al. 2000)	150	19	not active at a concentration of 150	The concentrations of peptides producing significant haemolysis 35
Ranatuerin-2La and Ranatuerin-2Lb (Goraya et al. 2000)	4-11	4	62-＞150	No data
Ranatuerin-2PLa plus -2PLe, Ranatuerin-2PLb, Ranatuerin-2PLc, Ranatuerin-2PLd and Ranatuerin-2PLf (Basir et al. 2000)	not active at a concentration <150	3-25	130, not active at a concentration <150 and ND	No data
Ranatuerin-2P (Goraya et al. 2000)	50	13	67	No data
Ranatuerin-2PRa (Conlon et al. 2004b)	13	＞100	100	HC_{50}: 150
Ranatuerin-2PRa, Ranatuerin-2PRb, Ranatuerin-2PRd and Ranatuerin-2PRe (Conlon et al. 2011)	6-100	6->50	50-ND	LC_{50}: 90-＞100
Ranatuerin-2SKa (Suzuki et al. 2007)	>50	50	>50	No data
Ranatuerin-2Toa and Ranatuerin-2Tob (Conlon et al. 2010)	>160	80	160-ND	No data

ND, not determined.

1.4.9.2. Other families of AMPs from genus *Rana*

Brevinin-1 can be isolated from a variety of frog species in North America and

Europe(Conlon et al. 2004a, Conlon et al. 2005b). The original source that for isolation of Brevinin-1 was *Rana brevipoda porsa,* and it was discovered by Japanese researchers (Morikawa et al. 1992). In addition to the antimicrobial action, this peptide family has strong haemolytic function (Bulet et al. 2004). Their secondary structures change with the environments. For example, in water solution, they normally exhibit random coil structures, while in the simulated cell membrane environment, they form α-helix structure (Kwon et al. 1998).

The Esculentin-1 family peptides consist of 46 amino acid residues. Their antimicrobial ability mainly depends on the 1-18 residues from the N-terminus and the positive charges. In addition, the selectivity of cells and the lengths of peptides are also important factors that influence the antimicrobial activity (Mangoni et al. 2003). The Esculentin-1 family peptides possess extremely strong antimicrobial ability (Simmaco et al. 1994). The esculentin-2 family peptides have 37 amino acid residues, and they does not possess highly conserved primary structure (Attoub et al. 2013). Esculentin-2 family peptides also have a broad-spectrum antimicrobial activity, but not as strong as esculentin-1 family peptides (Simmaco et al. 1994).

The ranalexin family peptides have 20 residues. They were originally found in the larva of *Rana* genus (Clark et al. 1994). It is one of the most effective peptides against Gram-positive bacteria, but has little effect on Gram-negative bacteria (Giacometti et al. 1998). Recent studies showed that ranalexin and lysostaphin have synergistic effect in the treatment of drug-resistant strains such as MRSA (Desbois et al. 2010).

Ranatuerin-1 family peptides existed only in three frog species *Rana catesbeiana, Rana clamitans* and *Rana grylio* (Sonnevend et al. 2004), they consist of 25 amino acid residues. The Ranatuerin-1 has three different secondary structures: the α-helix formed by the first to the eighth residues, the β-sheet, and the β-turn structure which is generated by the disulfide bond at the C-terminus (21-25 residues) (Conlon et al. 2004a). Furthermore, the glycine is essential for the β-sheet structure, and if glycine is replaced, the β-sheet structure could be destroyed (Conlon et al. 2004a, Sonnevend et al. 2004).

Temporin family is the smallest class of AMPs that have been found, they have only 10 to 13 residues (Simmaco et al. 1996). The Temporin peptide was initially found in European frogs, but subsequent studies showed that they are also existed in many frogs from North America and Asia (Conlon et al. 2004a). The structure of Temporin does not have "*Rana* box", and their C-terminus are always amidated (Mangoni 2006). One of the temporin family peptides has a unique antiparasitic ability and it does not have haemolytic activity, which makes it great potential to be developed as new drug in the future (Mangoni et al. 2005).

1.5. Aims and objectives of this thesis

To solve the drug-resistant microorganism problem, AMPs have been studied as alternatives for traditional antibiotics. AMPs from frog skin secretions are a promising source to study.

- The mRNAs will be extracted from skin secretions of frog *Rana pipiens*, and reverse-transcription will be used to construct cDNA library, by which will be

used to obtain the nucleotide precursor sequence of mature peptide using "shotgun" cloning.

- The peptide will be synthesised by Solid Phase Peptide Synthesis (SPPS).
- Use matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to identify the synthetic peptide, and the peptidewill be purified by reverse phase high performance liquid chromatography (rp-HPLC).
- Use the synthetic peptide to perform various functional experiments to assess its bioactivities.

Chapter 2: Identification of peptide precursor cDNA by “shotgun” cloning

2.1. Materials and methods

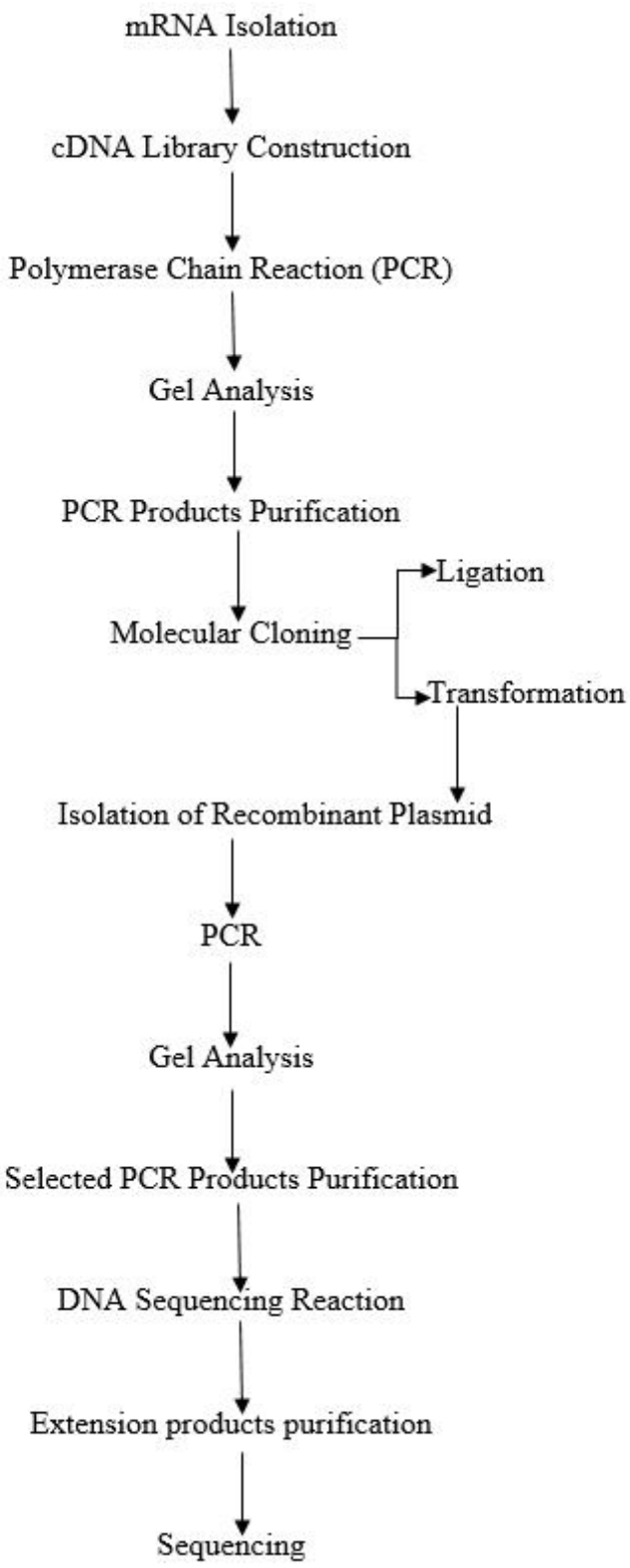


Figure 2.1 Molecular cloning strategy

2.1.1. Acquisition of skin secretion

The northern leopard frog, *Rana pipiens*, was bought from America. All frogs were adults. The frogs were placed in a simulated natural environment which contained 12 h of light -12 h of dark cycles in 18 to 25 ℃ temperature conditions for at least 4 weeks before extracting skin secretions. During this time, frogs were fed vitamin-rich crickets every few days.

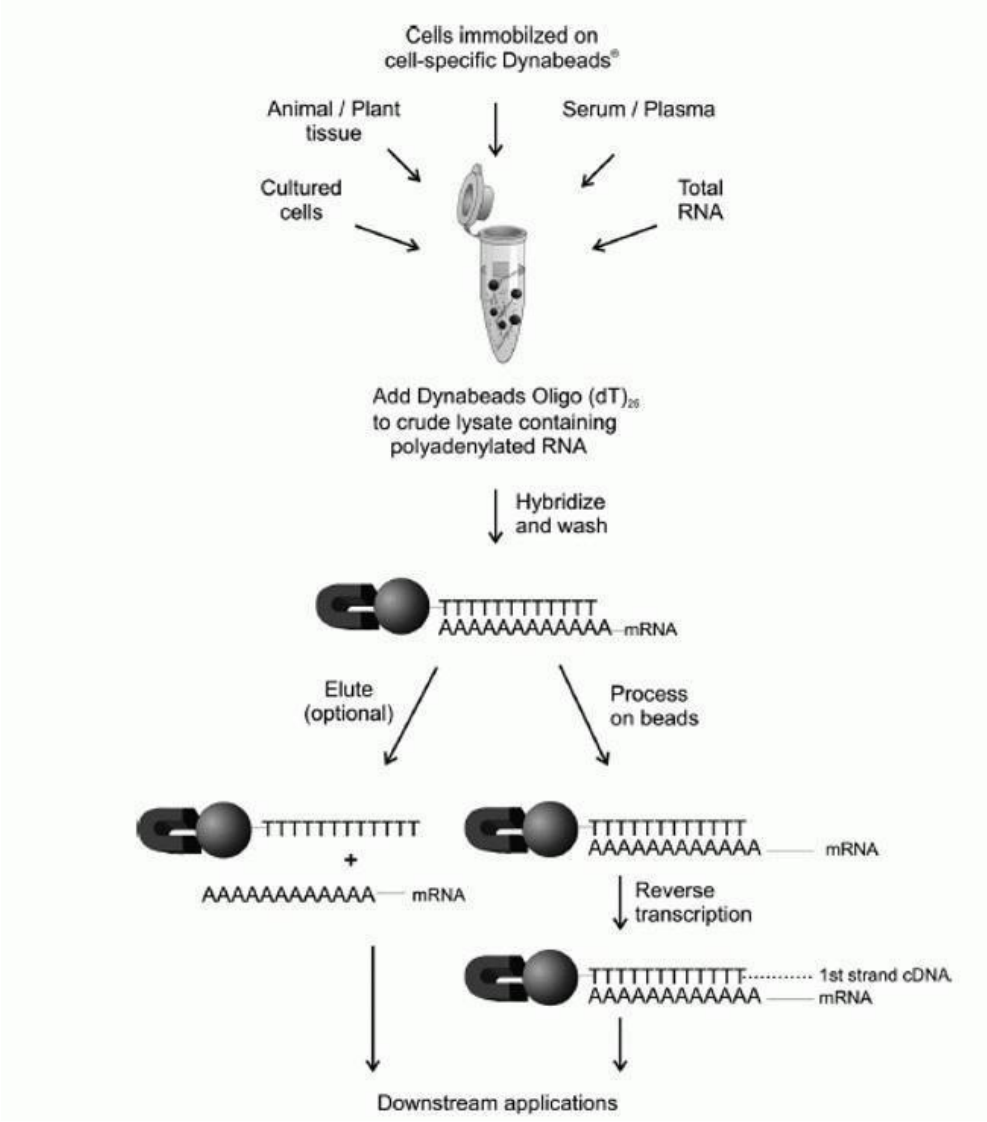
Skin secretions were obtained by massaging frog skin or stimulating the dorsal gland with gentle electrical stimulation (5 V, 100 Hz, 140 m pules width), and the secretions were washed into containers by using distilled deionized water, using liquid nitrogen for rapid freeze-drying and stored at -20 °C.

2.1.2. mRNA isolation

Five milligrams of lyophilised *Rana pipiens* skin secretion were dissolved in 1 ml Lysis/Binding Buffer supplied in a Dynabeads® mRNA DIRECT™ Kit (Invitrogen, Lithuania) (Figure 2.2) and then vortexed for 1 min, kept on ice for 1 min for a total of 10 times over 20 min. The sample containing tube was then centrifuged at 18,000 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany). During centrifugation, the Dynabeads Oligo $(dT)_{25}$ were first resuspended by gently rotating and 250 µl beads were transferred from the stock tube to an RNase-free 1.5 ml micro-centrifuge tube, which was then placed on a Dynal MPC-S magnet device. After the suspension was clear, the supernatant was discarded. Next, the tube was removed from the magnet, and the beads were washed by resuspending in an equivalent volume of fresh Lysis/Binding Buffer. As drying of the Dynabeads

Oligo$(dT)_{25}$ may lower their capacity, the buffer should be removed gently before the sample was transferred to the tube containing beads. The components were gently shaken for 1 min and kept on ice for 30 s for a total of 18 min afterwards. The sample-containing tube was placed on the magnet for 2 min, following which, the supernatant was removed completely by pipetting and the beads remained. The mRNA/beads complex was washed three times with 500 µl Washing Buffer A and twice with 500 µl Washing Buffer B at room temperature. The washing steps used the magnetic rack as well, discarding the buffer each time.

After washing, mRNA was eluted from Dynabeads with 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 eluate which contains mRNA. Finally, the elution was pipetted into a PCR (polymerase chain reaction) tube and the isolated mRNA was immediately placed on



ice.

Figure 2.2 Isolation of mRNA('Dynabeads™ mRNA DIRECT™ Purification Kit')

2.1.3. cDNA library construction

This was performed by using a SMART™ RACE cDNA Amplification Kit (BD Clontech, UK). Two tubes of 5`-RACE-Ready cDNA reaction samples were prepared by combining 3 µl mRNA sample, 1 µl 5`-CDS primer (10 mM) and 1 µl BD SMART II A oligo (10 mM) together, while three tubes of 3`-RACE-Ready cDNA reaction samples were mixed by 4 µl mRNA sample and 1 µl 3`CDS primer (10 mM). All the tubes of reaction mixes were incubated in a 70 °C heating block for 2 min and cooled on ice for 2 min. The master mix was prepared and consisted of 2 µl 5 ×

First-strand buffer, 1 µl DTT (20 mM), 1 µl dNTP Mix (10 mM), 1 µl PowerScript Reverse Transcriptase and 5 µl of mRNA sample. The solution was mixed by gently pipetting and the tubes were briefly centrifuged to make the liquid gather at the bottom of the tubes. The tubes were then put into the 96-well Thermal Cycler (ThermoFisher Scientific, USA) to be amplified by PCR for 90 min at 42 °C (the lid was set at 105 °C to prevent sample liquidation there). After that, 50 µl of PCR-Grade Water were added into each tube for dilution and they were mixed and briefly centrifuged. Finally, the tubes were placed into the Thermal Cycler again for 7 min at 72 °C (the lid was 105 °C), and the 3`- and 5`-RACE-Ready cDNA samples were obtained. The products could be stored at -20 °C for up to 12 months.

Table 2.1 The compositions of mRNA sample

Tube	mRNA (µl)	3'-RACE CDS Primer A (µl)	5'-RACE CDS Primer (µl)	BD SMART™ II A Oligonucleotide (µl)
1	4	1	-	-
2	4	1	-	-
3	4	1	-	-
4	3	-	1	1
5	3	-	1	1

Table 2.2 The sequences of primers for cDNA library construction

Primer	Sequence
3'-RACE CDS Primer A	5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N-3' (N = A, C, G, or T; V = A, G, or C)
5'-RACE CDS Primer	5'-$(T)_{25}$V N-3' (N = A, C, G, or T; V = A, G, or C)
BD SMART™ II A Oligonucleotide	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'

2.1.4. Rapid Amplification of cDNA Ends (RACE) PCR

The library was subjected to 3'-rapid amplification of cDNA ends (RACE) procedures using a BD SMARTTM RACE cDNA Amplification Kit (Clontech, UK) (Figure 2.3). A Master Mix, containing 12.4 µl PCR-Grade Water, 6 µl 10X BD Advantage 2 PCR Buffer, 0.8 µl dNTP Mix, 2 µl NUP, 2 µl designed 3'-primer and

0.8 µl 50 × BD Advantage™ 2 Polymerase Mix, was prepared and divided equally into two PCR tubes. Afterwards, 10 µl of 3'-RACE-Ready cDNA libraries was added into one tube as sample groups whilst 10 µl PCR-Grade Water was added into the other one (negative control groups). Both tubes were divided equally into another two tubes. The volume and final concentration of the components in the RACE PCR reactions are shown in Table 2.3 and Table 2.4.

3'-RACE was facilitated by a nested universal primer (NUP) (supplied by the kit) and a sense primer (5'-ATGTCACCTGAAGAAATCCCTC-3'). The 3'-RACE PCR programme was as follows: initial denaturation at 96 °C for 1 min; followed by additional 40 cycles: denaturation at 96 °C for 20 s; primer annealing at 55 °C for 10 s; extension at 60 °C for 4 min; and ended with a final extension at 72 °C for 10 min.

Table 2.3 The compositions of one sample

Reagent	Volume (µl)	Final concentration
PCR-Grade Water	3.1	-
10 × BD Advantage 2 PCR Buffer	1.5	1.5 ×
dNTP Mix (10 mM)	0.2	0.2 mM
NUP	0.5	1 µM
Sense Primer	0.5	1 µM
50 × BD Advantage 2 Polymerase Mix	0.2	1 ×
3'-RACE-Ready cDNA	5	-

Table 2.4 The compositions of one negative control

Reagent	Volume (µl)	Final concentration
PCR-Grade Water	3.1	-
10 × BD Advantage 2 PCR Buffer	1.5	1.5 ×
dNTP Mix (10 mM)	0.2	0.2 mM
NUP	0.5	1 µM
Sense Primer	0.5	1 µM
50 × BD Advantage 2 Polymerase Mix	0.2	1 ×
PCR-Grade Water	5	-

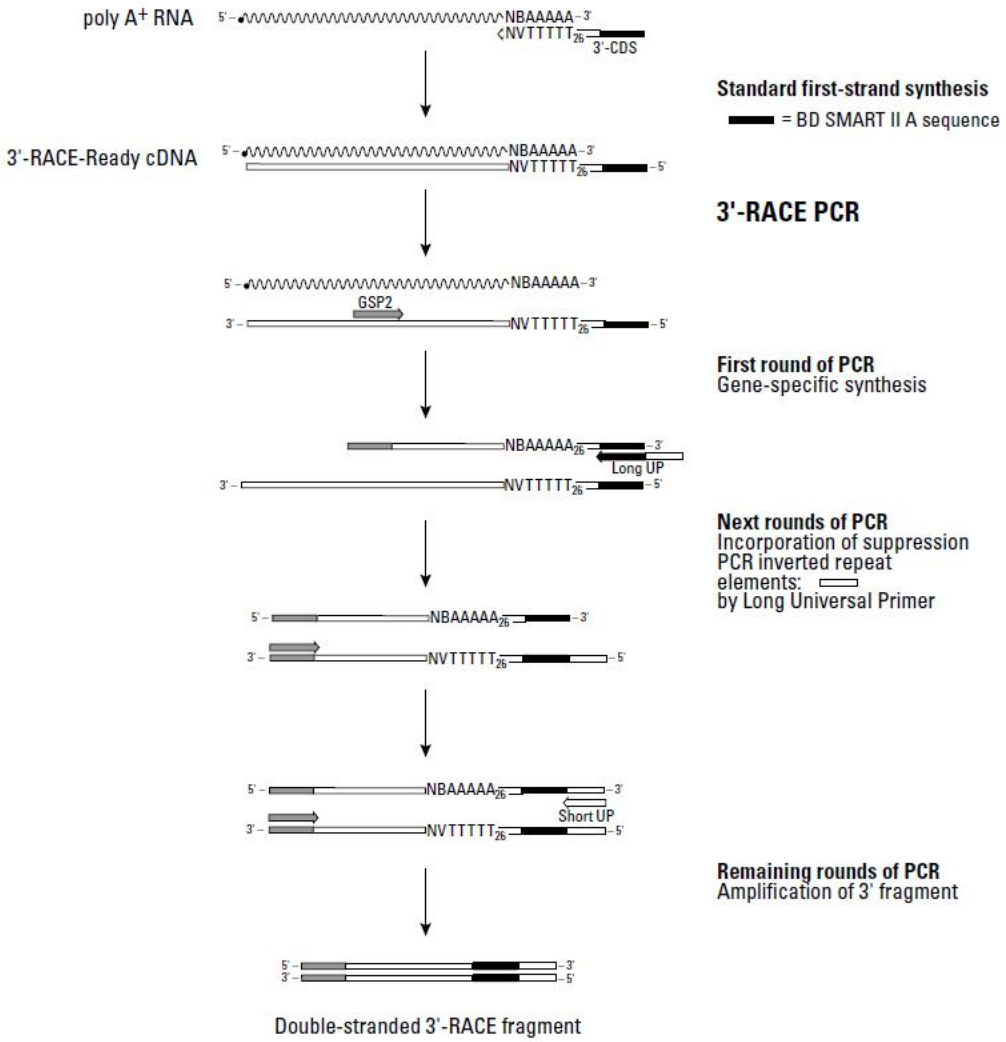


Figure 2.3 The procedure of 3'-RACE PCR

2.1.5. Agarose gel analysis of RACE products

DNA fragments of a larger size move at a slower rate than those of a smaller size in an electrical field due to the pores in the gel matrix, which play a sieving or sorting role. The sizes of the DNA or RNA sequences can be estimated by comparing with standard DNA bands on the solid gel or the distances that DNA fragments have travelled in the agarose gel. The percentage of agarose in the gel decides the positions of DNA fragments of different sizes after separation. The more agarose in the gel, the slower the DNA fragments move in the electrical field. 0.48 g of agarose

was dissolved in 35 ml of 1 × Tris-borate-EDTA (TBE) buffer and ethidium bromide (EB), was added into this mixed solution as a fluorescent marker for DNA band visualisation in a UV trans illuminator. The mixed solution was poured into a casting tray and an 8-toothed comb was inserted. Solidified agarose gel with pores was formed in the casting tray after 30 min. Then the solid gel was placed in the correct orientation and was soaked in 1 ×TBE buffer. Subsequently, 1.5 µl of DNA Ladder was loaded into one of the wells and 1.5 µl of PCR products were mixed with 0.5 µl of loading dye for other wells individually. A 90 V electrical current was applied and ran until the smallest sized dye band reached the border of the gel.

2.1.6. PCR product purification

The product generated from the PCR step contains some impurities, such as redundant dNTPs, enzymes, non-specific PCR products and inaccurate or unused primers. Purification of PCR product is essential for the following transcription step and one must avoid side reactions caused by impurities. A silica-based membrane was used to rapidly isolate target PCR product based on the size of DNA fragment.

The samples in sample 1 and sample 2 were mixed together in a 1.5 ml tube (total 19 µl). 95 µl CP Buffer was added (CP Buffer: Sample = 5:1(v/v)) All the liquid was removed to the purification column, centrifuged at 13,000 × g for 60 s and the flow-through was discarded. The samples were washed in the same way by 700 µl and 500 µl washing buffer respectively and after adding washing buffer, tubes were centrifuged at 13,000 × g for 1 min. The flow-through was discarded and the column was transferred to a new 1.5 ml tube. 30 µl PCR-Grade Water was added to the

column, and the column was kept for 2 min, centrifuged at 14,000 × g for 1 min, and the column was discarded finally. Those tubes were placed in the Eppendorf concentrator plus for 1 h to dry, then stored at -20 ℃.

2.1.7. Ligation

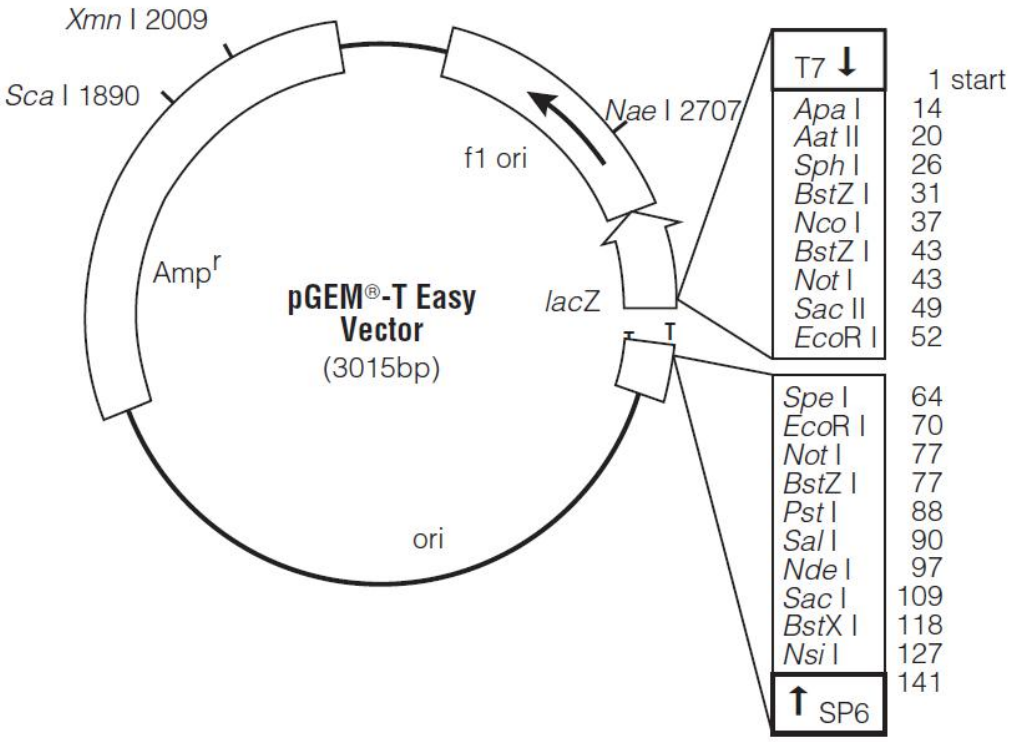


Figure 2.4 The pGEM®-T Easy Vector circle map ('pGEM®-T Easy Vector Systems')

This step employed a pGEM-T Easy vector system (Promega, USA) (Figure 2.4). PCR product was removed from storage and 8 µl PCR-Grade Water was added followed by vortexing and centrifugation then placed on ice. The reagents were added as shown in Table 2.5 and pipetted without bubbles. Tubes were kept for 1 h at room temperature and then stored in the fridge at 4 ℃ overnight.

Table 2.5 The reagent of ligation

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

2.1.8. Transformation

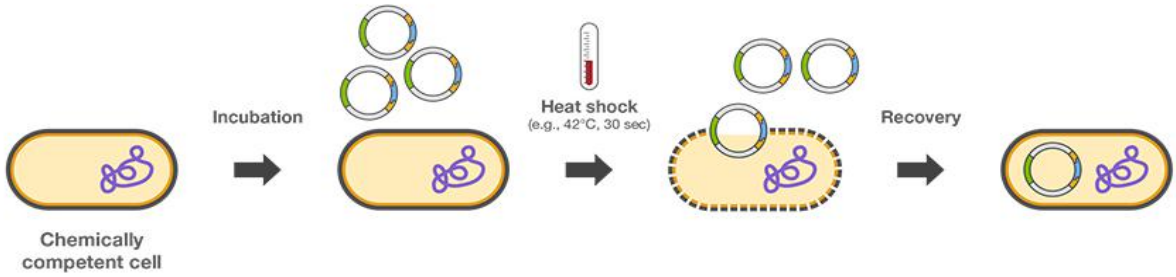


Figure 2.5 The mechanism of transformation ('Bacterial Transformation and Competent Cells–A Brief Introduction')

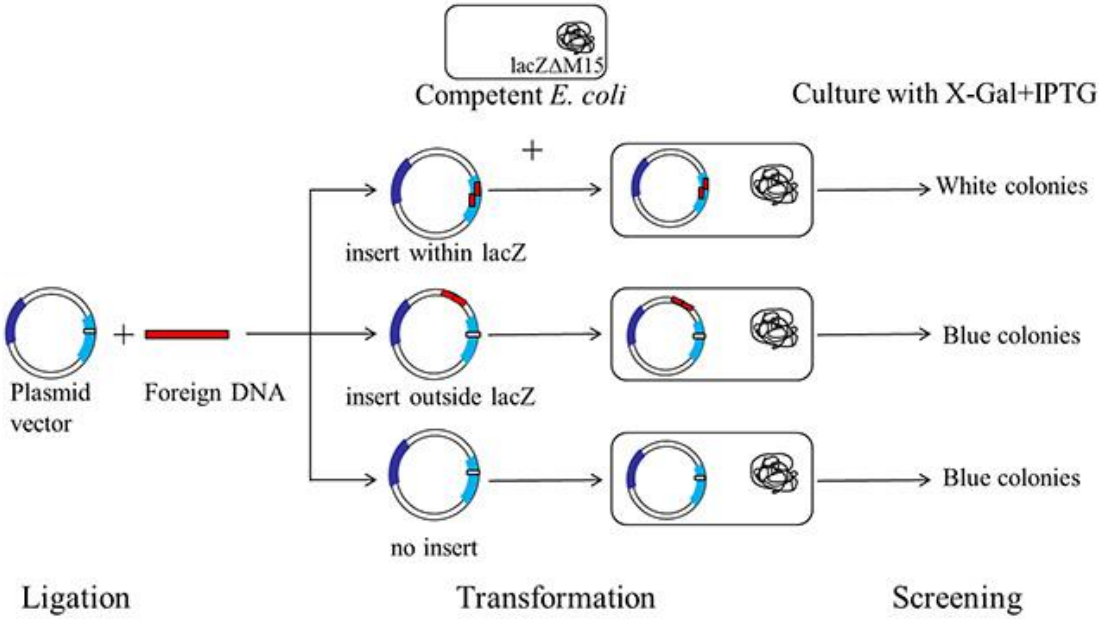


Figure 2.6 The blue-white screening ('Introduction to Blue-White Screening – Background and Protocols for Colony Selection')

Blue and white colony screening was used for transformation and a schematic representation of a typical blue and white screening procedure is shown in Figure 2.6. For experimental use, 3.2 g LB Agar was dissolved in 100 ml deionised water in a

glass bottle and sent for autoclaving. When the solution cooled to nearly 65 °C, 50 mg/ml ampicillin (Roche, USA), an antibiotic, was added to achieve a final concentration of 100 μg/ml and shaken well. About 12 ml LB Agar with ampicillin was poured into each Petri dish, and the Petri dishes were swirled in a circular motion to distribute agar on the bottom evenly. 4 °C storage was recommended for all Petri dishes after total solidification. 2.5 μl ligation products were added to a sterile 1.5 ml tube on ice and 50 μl JM109 cells were removed from -80 °C storage and thawed on ice. Then cells were carefully transferred into the tube with the ligation samples. The reaction tube was flicked gently to mix and placed on ice for 20 min, after which, the cells were heat-shocked for 47 s in a heating block at exactly 42 °C, and the tube was immediately returned to ice for at least 2 min (Figure 2.5). 950 μl room-temperature S.O.C medium was added to the tube and the tube was incubated at 37 °C for 1.5 h with a shaking rate of 150 rpm. To four LB plates, 100 μl ITPG (0.1 M, Promega, USA) plus 20 μl X-Gal (50 mg/ml, Promega, USA) were added and spread symmetrically with a disposable spreader, followed by activation in an incubator for 30 min at 37 °C. After these steps, 70 μl, 80 μl, 90 μl and 100 μl of transformation products were pipetted into each plate respectively and spread symmetrically and gently with a spreader. All the Petri dishes were placed upside down to incubate at 37 °C for 14-15 h.

Two LB plates with ampicillin/ITPG/X-Gal were prepared as described above after the back of their bottoms were lined and divided into eighteen plots. The 4 plates with transformation product were carried out of the incubator and the blue-white

screening was performed near the Bunsen burner. The white colonies (recombinants) were easily differentiated from the blue colonies (non-recombinants) and appropriate areas were chosen and transferred to the newly activated plate through drawing the continuous line resembling a 'Z' with a sterile inoculating loop. Two 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 two plates were placed upside down in a 37 °C incubator overnight.

2.1.9. Isolation of recombinant plasmid

Twenty microliters of PCR-GradeWater were added to the 0.5 ml tube. The tips were used to pick out and transfer the eight white colonies to the tubes. The cells were broken by vortexing for 15 s and then incubation in a 100 ℃ heating block for 4.5 min. The tube was then put on ice for an extra 5 min and vortexed for 20 s.

2.1.10. Cloning PCR

The isolation products were vortexed briefly and centrifuged at 18,000 × g for 5 min before use. 47.25 µl of Master Mix and 2.5 µl supernatant of cell lysate were added to each PCR tube. All the components of Master Mix are shown in Table 2.6. The cloning PCR programme was as follows: initial denaturation at 94 °C for 60 s; followed by additional 31 cycles: denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; extension at 72 °C for 180 s; and ended with a final extension phase at 72 °C for 180 s and storage in the fridge at 4 °C.

Table 2.6 The compositions of Master Mix per tube

Reagent	Volume (µl)	Final concentration
PCR-Grade Water	31	-
5 × Cloning Buffer	10	1 ×
dNTP Mix (10 mM)	1	0.2 mM
M13F (M13 Reverse Sequencing Primer) (20 µM) (Sense primer)	2.5	1 µM
M13R (M13 Reverse Sequencing Primer) (20 µM) (Antisense primer)	2.5	1 µM
Taq polymerase (5 unit/µl)	0.25	0.025 unit/µl

2.1.11. Gel analysis

The operation of this step was exactly the same as the section 2.1.5, except that two combs were put in the gel and the PCR sample was not mixed with the Loading Dye because the 5 × Cloning Buffer contained the dye.

2.1.12. Selected PCR products purification

Similarly, the procedure for this step was the same as the previous section 2.1.6, but without the need to concentrate the purified products.

2.1.13. DNA sequencing reaction

DNA sequencing was carried out by means of using a 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 Master Mix was mixed with 2.5 µl of each purified DNA product. The components of Master Mix are shown in Table 2.7. After this, all the tubes were subjected to a thermal cycler and the sequencing reaction PCR programme was as follows: initial denaturation at 96 °C for 60 s; followed by additional 26 cycles: denaturation at 96 °C for 20 s; annealing at 55 °C for 10 s; extension at 60 °C for 240 s; and ended with preservation at 4 °C.

Table 2.7 The compositions of Master Mix in each tube

Reagent	Volume (µl)	Final concentration
PCR-Grade Water	12.4	-
2.5 × Ready reaction mix	2.86	0.33 ×
5 × BigDye Sequencing Buffer	3.57	0.81 ×
M13F/M13R (3.2 µM)	1.14	0.16 µM

2.1.14. Extension products purification

Ten microliters of PCR-GradeWater were added to each fresh 1.5 ml tube first, and 72 µl of 95% ethanol (Sigma-Aldrich, USA) were added to the tubes containing sequencing reaction product, followed by being transferred together with extension products to the 1.5 ml tubes. These tubes were then vortexed briefly and placed at room temperature for 20 min, after which they were centrifuged at 18,000 × g for 20 min, and the supernatants were discarded immediately. Next, 260 µl of 70 % ethanol was added into the tubes and the tubes were vortexed for 30 s, then centrifuged for another 10 min. The supernatant was discarded quickly and the redundant liquid in the pellet was evaporated in a concentrator for at least 3 h to generate the dried DNA fragments.

2.1.15. Sequencing

First, all samples were dried in a concentrator for 45 min to remove ethanol thoroughly before sequencing.10 µl HiDi (highly deionised-formamide) was added to each tube containing dried DNA fragments. Then, the tubes were vortexed for 30 s, centrifuged briefly and placed in a heating block at 95 °C for 4.5 min, and then cooled on ice for3.5 min. The tubes were centrifuged briefly and 10 µl of each sample was transferred to a 96-well sequencing plate. Finally, DNA was sequenced by an ABI 3730 automated sequencer (Applied Biosystems, USA).

2.2. Results

The DNA fragments that amplified by 3'RACE PCR were examined by gel electrophoresis, the bands with size >500 bp (data not shown) were selected for molecular cloning and sequencing. The results of "shotgun" cloning were analysed by Basic Local Alignment Search Tool (BLAST). The biosynthetic precursor that encodes the peptide was successfully cloned from the cDNA library derived from the skin secretion of the northern leopard frog, *Rana pipiens*, by using sense Nested Universal Primer (NUP) and anti-sense specific degenerate primer. The synthetic precursor consists of 71 amino acids. The results showed that at the N-terminal of open reading frame, there is a 22-residue signal peptide domain, followed by an acidic spacer domain and a canonical convertase processing site -K-R-. The putative mature peptide is GLMDTVKNVAKNLAGHMLDKLKCKITGC, and followed by 3'-untranslated region (Figure 2.7). It was named as QUB-3000, according to its molecular mass. Clustal Omega alignment tool was used to compare the primary structure similarity of QUB-3000 peptide precursor with 5 ranatuerin-2 family peptide precursors, which were the top 5 sequences generated from by analysing the QUB-3000 peptide precursor using the BLAST tool. As shown in Figure 2.8, the peptide precursor of QUB-3000 was found to share highly-conserved domains with the peptide precursors of the ranatuerin-2 family. In the signal peptide region, there are 19 highly-conserved amino acids, and in the mature peptide region, 14 amino acids are highly-conserved. Through the analysis of the open reading frame and Clustal Omega alignment, the mature peptide was confirmed as:

GLMDTVKNVAKNLAGHMLDKLKCKITGC, and it belongs to the ranatuerin-2 family.

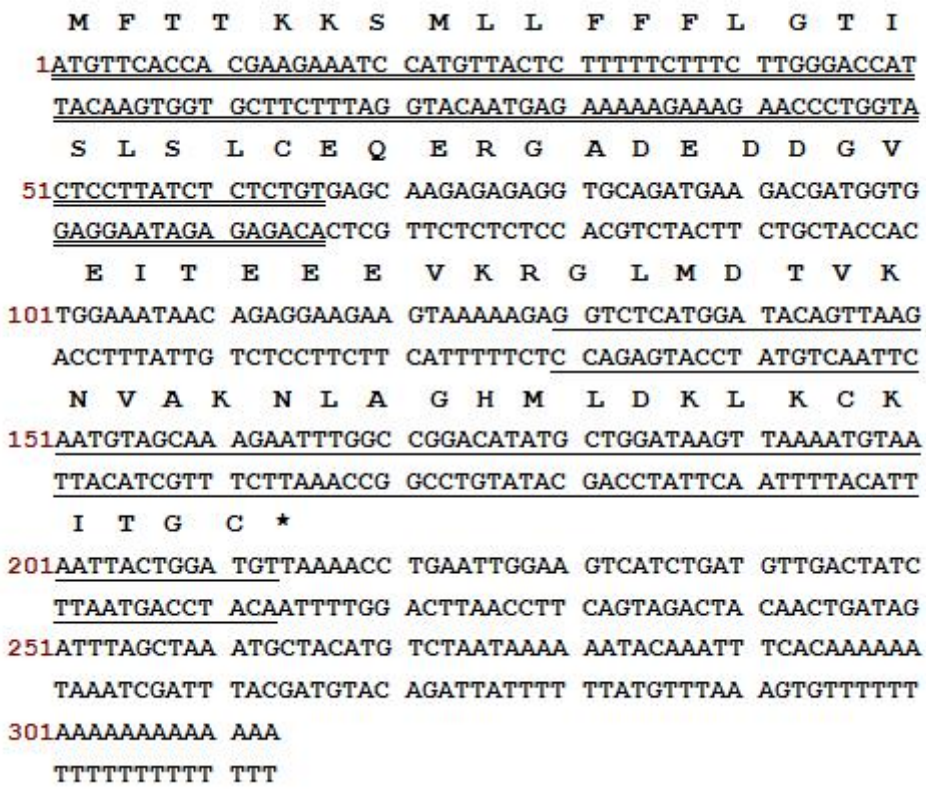


Figure 2.7 Nucleotide sequence and corresponding translated open reading frame amino acid sequence of the biosynthetic peptide precursor of QUB-3000 encoded by cDNA cloned from *Rana pipiens* skin secretion. The putative signal peptide is doubled-underlined, the putative mature peptide is single-underlined, and the stop colon is indicated by an asterisk.

```
Ranatuerin-2Va      MFTLKKSFLLLFFLGTITLSLCEQERGADEDDGVEMTEEEVKRGLLDTIKN----TAKNL
Ranatuerin-2DR      MFTLKKSLLLFFFLGTISLSLCEEERGADEDDGVELTEEEVKRGIMDTFKGIAKGVAKNL
Ranatuerin-2SRb     MFTLKKSLLLFFFLGTISLSLCEEERGADEDDDVEMTEEEVKRGIMDSVKG----VAKNL
Ranatuerin-2PLa     MFTTKKSMLLFFFLGTISLSLCEQERGADEDDGVEMTEEEVKRGIMDTVKN----VAKNL
Ranatuerin-2P       MFTMKKSLLLFFFLGTISLSLCEQERGADEDDGVEITEEEVKRGLMDTVKN----VAKNL
QUB-3000            MFTTKKSMLLFFFLGTISLSLCEQERGADEDDGVEITEEEVKRGLMDTVKN----VAKNL
                    *** *** ** ****** ***** ******** ** ********  *  *      ****

Ranatuerin-2Va      AVGLLDKIKCKMTGC
Ranatuerin-2DR      AGKLLDELKCKMTGC
Ranatuerin-2SRb     AAKLLEKLKCKITGC
Ranatuerin-2PLa     AGQLLDKLKCKITAC
Ranatuerin-2P       AGHMLDKLKCKITGC
QUB-3000            AGHMLDKLKCKITGC
                    *   *   *** * *
```

Figure 2.8 Alignments of the translated open reading frame amino acid sequence of the biosynthetic peptide precursor of QUB-3000 with that of the top 5 sequences of Ranatuerin-2 family precursors using the BLAST analysis in NCBI. The high conserved amino acids are shown with "*" underline.

Chapter 3: The synthesis and purification of QUB-3000

3.1. Materials and methods

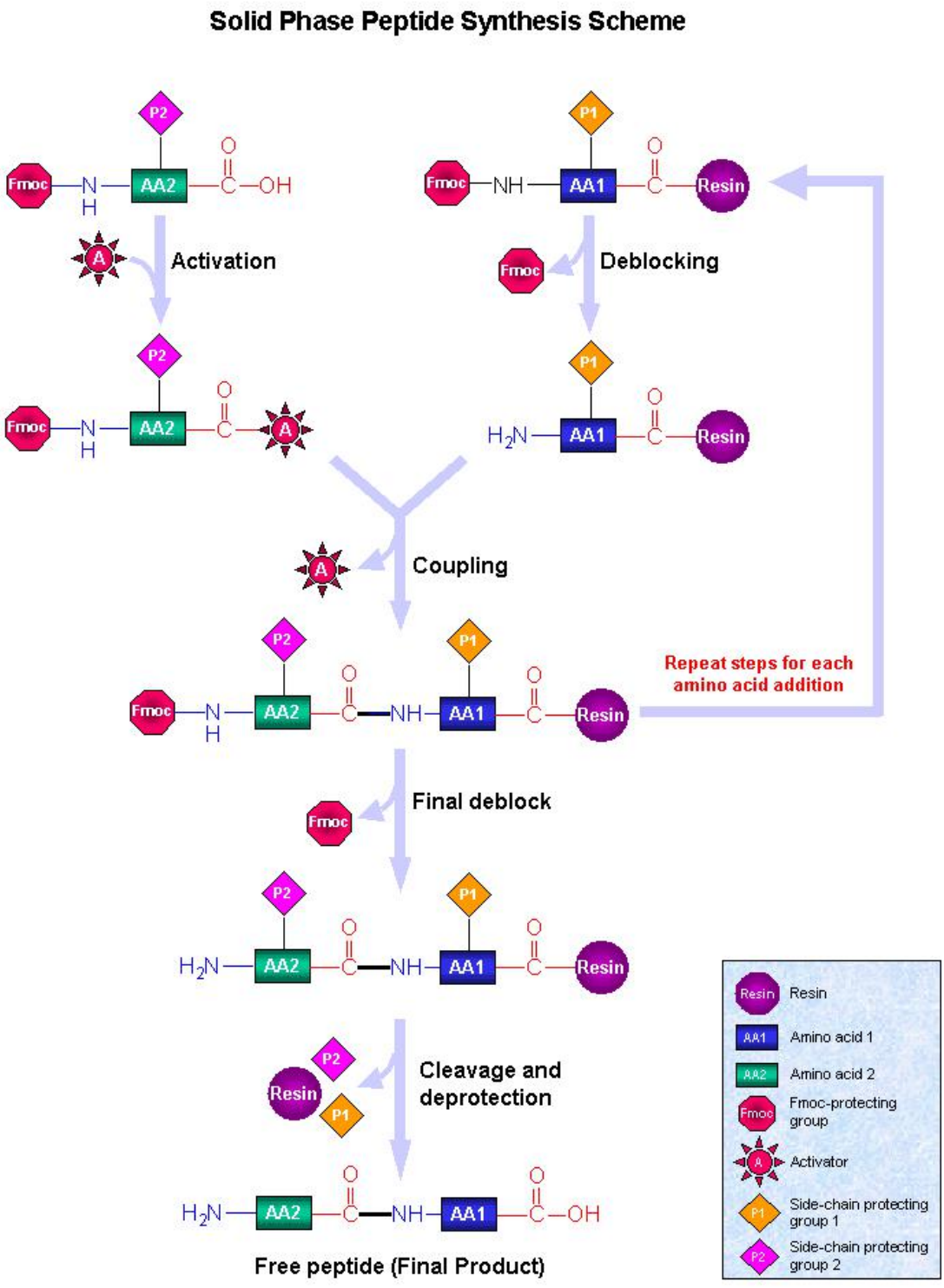


Figure 3.1 The Solid Phase Peptide Synthesis strategy ('Solid Phase Synthesis')

3.1.1. Solid Phase Peptide Synthesis (SPPS) of QUB-3000

SPPS is a standard method to synthesise peptides in the laboratory, producing peptides in high yield (typically around 70 %) in a user-friendly manner. Peptide synthesis is achieved through several stages.

Wang Resin is employed as a load target at the carboxyl (C-) terminal to synthesise the peptides from C-terminal to N-terminal ends. The Wang Resin was combined with the last amino acid at the C-terminal end of the target synthesised sequence, while the Rink Amide Resin was utilised to provide the amide at the C-terminal end.

According to the sequence, an amino acid was combined with the last one through coupling and when this was completed, then deprotection was performed by removing the 9-fluorenylmenthoxycarbonyl (Fmoc) protecting group at current amino terminal end and activation of the next amino acid. The whole process was performed under vacuum. After all amino acids were coupled in order, a completely synthesised peptide replicate was cleaved from the resin as were its side chain protecting groups. The brief procedure is described as follows (Figure 3.1)

3.1.1.1. The weighting of amino acids

The desired quantity of peptide was 0.15 mmol and each amino acid should be at least 4 times more than the desired quantity. Thus 0.6 mmol of each amino acid were weighed into amino acid vials to synthesise the sequence. Correspondingly, HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)was used at the same ratio for catalysing each coupling. The amino acids with different protecting groups were weighed based on the sequence, and loaded into pre-

cleaned vials containing HBTU. According to its loading capacity, Wang Resin was weighed into a 30 ml reaction vessel.

3.1.1.2. The synthesis of peptide

This procedure was performed using a Tribute peptide synthesiser (Protein Technologies, Inc. AZ, USA). As shown in Figure 3.2, there were five bottles containing reagents as listed in Table 3.1. The nitrogen source was inspected before starting the synthesis procedure. All the bottles were pressurised and it was confirmed that they contained enough reagents. After loading the reaction vessel and the vials onto the machine, the programme was set and the button RUN was pressed to start the synthesis.

Figure 3.2 The Tribute™ Peptide Synthesizer

Table 3.1 The contents and functions of bottles in machine

Bottle	Content	Function
1&2	DMF (N,N-Dimethylformamide)	Provide a reaction environment
3	Piperidine/DMF, 1/4, v/v	Make the deprotection of Fmoc
4	NMM (4-Methylmorpholine)/DMF, 11/89, v/v	Work together with HBTU to activate carboxyl
5	Dichloromethane	Help remove the DMF

3.1.1.3. Cleavage and deprotection of peptide

The purpose of this step was to cleave the side chain protection groups and, simultaneously, to isolate the resin and peptide. The products from the last step were weighed and transferred into a round bottomed flask. Then they were cleaved and deprotected by using cleavage cocktail (94% TFA (Trifluoroacetic acid), 2% EDT (Ethanedithiol), 2% TIS (Thioanisole), 2% distilled deionized water) by stirring at room temperature for 2 h 30 min. When this process was finished, the cleavage mixture was filtered through a Buchner funnel into a 50 ml round bottomed flask. The filtrate was concentrated by a rotary evaporator at a temperature of no more than 30 ℃ to near dryness. After that, 45 ml of Et_2O was added and the mixture was transferred to a 50 ml centrifuge tube and kept at -20 ℃ for precipitation and to remove TFA from the peptide.

3.1.1.4. The washing, oxidation and lyophilization of peptide

To wash the synthesised peptide, the tube was centrifuged at 2500 × g for 5 min. The supernatant was discarded carefully in order to avoid the loss of peptide pellet at the bottom. Then these steps were repeated ---another 45 ml Et_2O was added to wash the peptide and the tube was centrifuged under the same parameters again. Before the next addition, the supernatant was discarded completely. In all, Et_2O was employed

to wash the peptide a total of 3 times. After washing, the tube was covered using tinfoil with holes in it and was placed in a fume cabinet at room temperature overnight for Et_2O volatilisation.

The following step was used to oxidise the two cysteines in the synthesised peptide to form an intramolecular disulphide bond. When the peptide was dry, Buffer 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 with the presence of 40 µl H_2O_2 and operated at room temperature with stirring on a magnetic stirrer.

After the oxidation of peptide, the peptide solution was frozen in liquid nitrogen immediately and then lyophilised using an Alpha 1-2 freeze-dryer (SciQuip, UK) for about 62 h.

3.1.2. Reverse phase high performance liquid chromatography (RP-HPLC)

As one of the most popular techniques in chemical and biomolecular analysis, Reverse Phase High Performance Liquid Chromatography (RP-HPLC) has been applied in various areas, including manufacture, supervision, research, food safety, pharmaceutical, chemical and biological products etc. It works based on the different hydrophobicity/ polarity of each component in the sample, i.e. each component in the mobile phase interacts differently with the stationary phase, causing disparate elution rates, and thus they could be separated according to their specific elution rate from the column (Figure 3.3). The detector can then perform a qualitative or even quantitative analysis of those components.

Before separation, the column was washed with Buffer B (TFA/distilled deionized water/Acetonitrile, 0.05/19.95/80.00, v/v/v) for 30 min, and then it was equilibrated

with Buffer A (TFA/distilled deionized water, 0.05/99.95, v/v) for another 30 min. 8 mg lyophilised QUB-3000 was dissolved in 1ml of Buffer A and Buffer B, which was then centrifuged for 15 min at maximum speed, and transferred to another universal tube. A Cecil Adept CE4200 HPLC system (Amersham Biosciences) was employed to purify the synthetic peptide which was injected manually into the six-port micro sampling valve and eluted by a C5 300Å column (250 × 10.00 mm, Phenomenex, UK)with a linear gradient formed from 100% Buffer A: 0% Buffer B to 0% Buffer A: 100% Buffer B in 80 min at a flow rate of 4 ml/min. The column effluent was continuously monitored to detect peptide bonds at a wavelength of 214nm. The fractions were collected once the peaks were shown and the tubes were labelled with the corresponding time. After 80 min, Buffer B was pumped to wash the column for 30 min for the column protection.

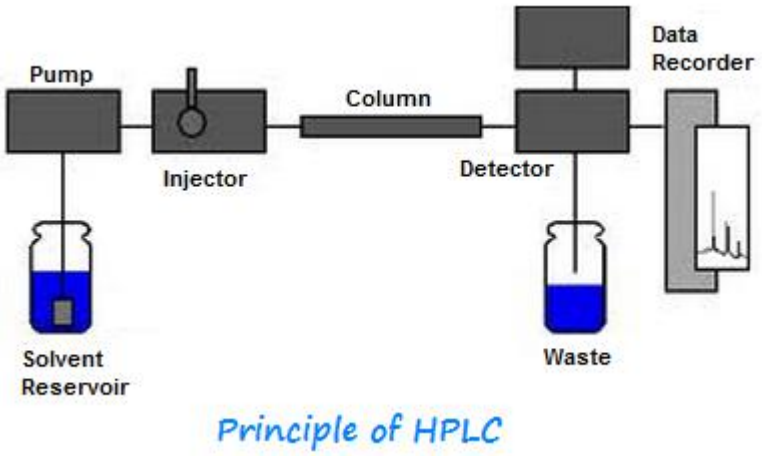


Figure 3.3 The working principle of RP-HPLC ('Principle of HPLC (Liquid Chromatography)')

3.1.3. Identification of peptide by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The molecular masses of peptides in reversed-phase HPLC fractions were obtained

by a MALDI-TOF mass spectrometer (Voyager DE, Perspective Biosystems, and Framingham, MA, USA). Figure 3.4 shows how the instrument works. A volume of 2 µl from each HPLC fraction was loaded into a well of the MALDI-TOF plate and left to dry. Then 1 µl of matrix solution [α-cyano-4-hydroxycinnamic acid (CHCA) in Acetonitrile/Water/TFA, 50.00/49.95/0.05, v/v/v] at a concentration of 10 mg/ml, was added to the spot of each sample and left to dry. After total drying, the plate was inserted into the instrument, and subsequently a pulsed nitrogen laser (337 nm) was used to irradiate the sample spot triggering the molecules to be ionised and protonated. Based on the time-of-flight of arrival at the detector, the ions were detected according to their mass-to-charge ratio (m/z). Molecules desorbed from the sample typically are singly protonated, giving an ion at $[M+H]^+$, where M is the molecular mass (Caprioli et al. 1997).

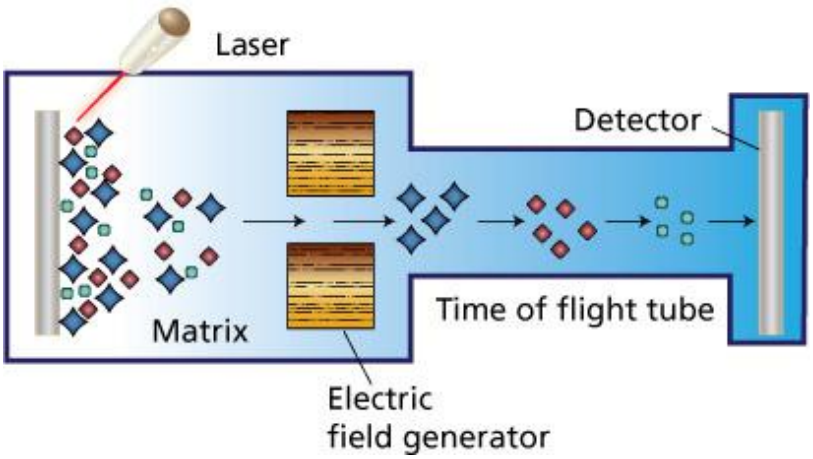


Figure 3.4 The working principle of MALDI-TOF MS('MALDI-Tof-MS ')

3.2. Results

3.2.1. Identification and purification of QUB-3000

Synthesis of QUB-3000 was successfully performed by SPPS. After synthesis, QUB-3000 was analysed and purified by using RP-HPLC. The chromatogram is shown in

Figure 3.7. Figure 3.8 showed that the molecular mass of the peak was 3001.07 Da, which was the ion at $[M+H]^+$ of QUB-3000. In addition, the online website Peptide property calculator (https://pepcalc.com) was used to predict the physicochemical properties of QUB-3000 (Table 3.2). It showed that QUB-3000 has 3 positive charge at pH 7, iso-electric point is pH 9.7, indicating good water solubility.

Table 3.2 The physicochemical properties of QUB -3000

	Extinction coefficient	Iso-electric point	Net charge at pH 7	Estimated solubility
QUB-3000	0 $M^{-1}cm^{-1}$	pH 9.7	+3	Good water solubility

3.2.2. Secondary structure prediction for QUB-3000

The online toolI-TASSER (Iterative Threading ASSEmbly Refinement) was used to predict the secondary structure of QUB-3000. The results showed that the secondary structure of QUB-3000 mainly consists of α-helix (Figure 3.5). Figure 3.6 showed the optimal predicted structural conformation of QUB-3000.

```
                                        20
                                        |
Sequence    GLMDTVKNVAKNLAGHMLDKLKCKITGC
Prediction  CHHHHHHHHHHHHHHHHHHHHHHCCCCCC
Conf. Score 935789889998899999988623201 69
            H:Helix; S:Strand; C:Coil
```

Figure 3.5 The predicted secondary structure of QUB-3000 using the online protein secondary structure prediction tool I-TASSER.

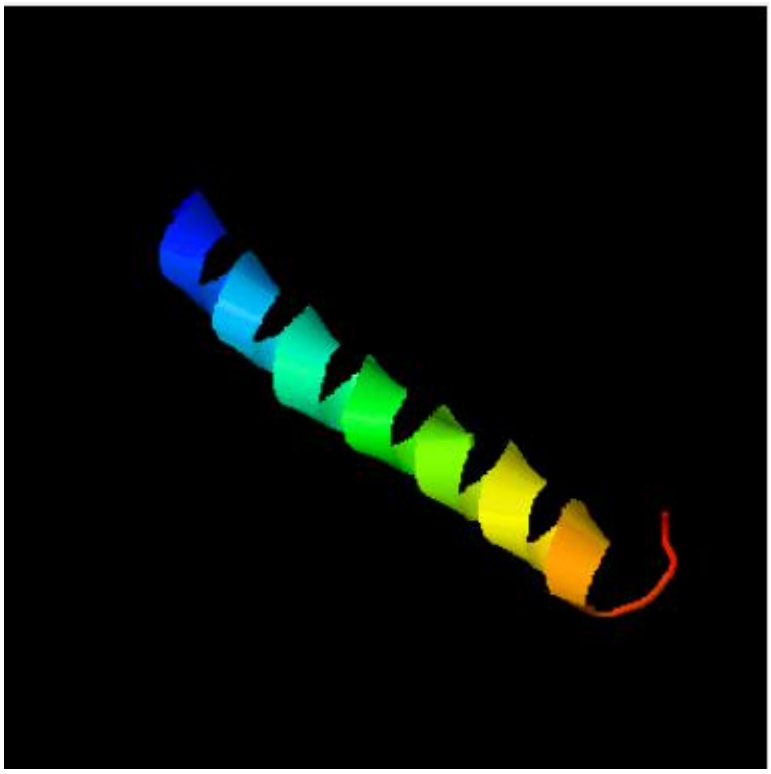


Figure 3.6 The optimal predicted structural conformation of QUB-3000 using the online tool. The confidence score (C-score) is 0.14. The estimated TM-score is 0.73±0.11.

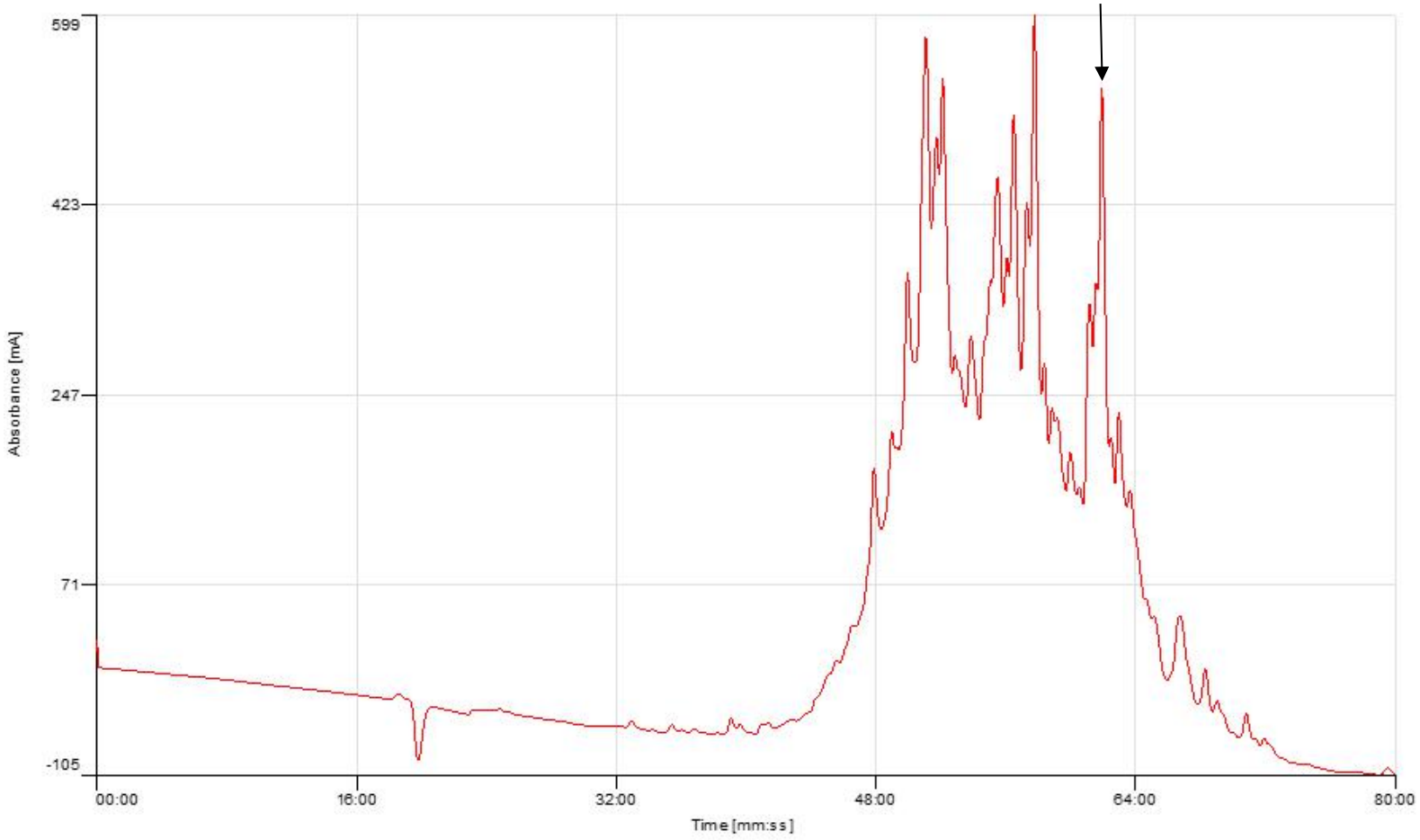


Figure 3.7 The RP-HPLC chromatogram of crude synthetic QUB-3000. Region of reverse-phase HPLC chromatogram of synthetic crude peptide QUB-3000 with arrows indicating the retention times of the purified potion of QUB-3000. The detection wavelength was 214 nm with a flow rate of 4 ml/min in 80 min.

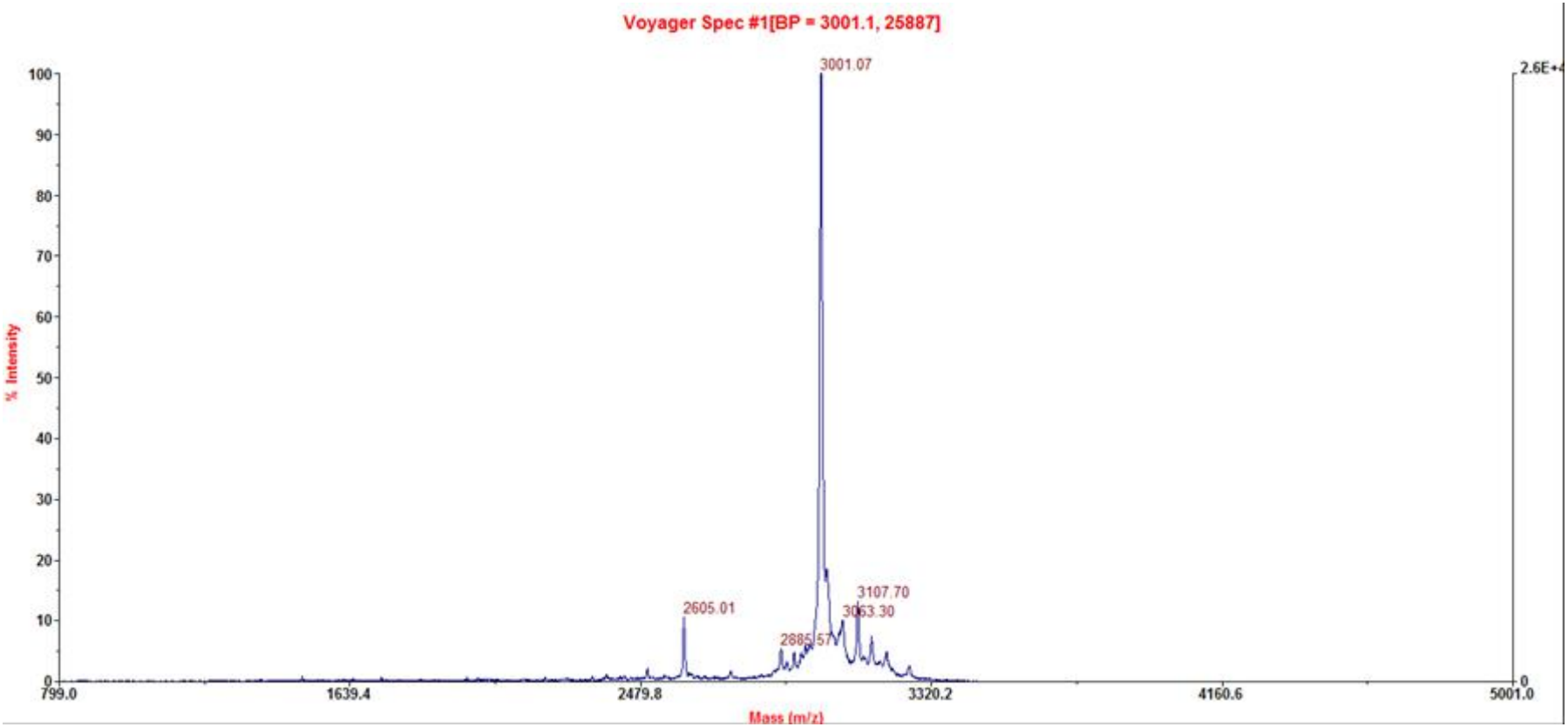


Figure 3.8 The MALDI-TOF MS of synthetic of QUB-3000 after purification. MALDI-TOF (Voyager DE, Perspective Biosystems, and Framingham, MA, USA) mass spectrum of synthetic peptide QUB-3000. Singly protonated molecule [M+H]+: 3001.07Da, where M is the initial neutral molecule, were observed.

Chapter 4: Assessment of biological functions of QUB-3000

4.1. Materials and methods

4.1.1. Antimicrobial assay

In this experiment, three microorganisms including the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*,NCTC 10788), the Gram-negative bacterium *Escherichia coli* (*E. coli*, NCTC 10418) and the fungus *Candida albicans* (*C. albicans*, NCPF 1467) were used to determine the antimicrobial activity of peptides. Mueller Hinton Agar (MHA, 38 g/l) and Mueller Hinton Broth (MHB, 21 g/l) were prepared to culture the microorganism. Phosphate-buffered saline (PBS) or Dimethyl sulfoxide (DMSO) was used to dissolve the peptide. All materials which used in this experiment were required for sterilization.

4.1.1.1. Germiculture

One bead of each microbial culture was transferred to a McCartney bottle of 100 ml of MHB and placed in the shaking incubator (150-200 rpm) at 37 ºCovernight (16-20 h). For each microbe, at least two McCartney bottles of 20 ml MHB were placed in the 37 ºC incubator to warm up overnight.

4.1.1.2. Bacterial plate preparation

The MHA was heated by microwave oven or at 50 °C in the boiling water bath (Grant, UK) and used to make the agar plates (11-12 ml MHA per plate). The plates were kept at 4 °C.

4.1.1.3. Peptide preparation

The peptide was weighed in a 1.5 ml tube and added to PBS or DMSO to make the concentration at 51200 µM, which was afterwards vortexed and briefly centrifuged. Then this was two-fold diluted to produce a series of concentrations of peptide solutions as follows: 25600, 12800, 6400, 3200, 1600, 800, 400, 200 and 100 (µM).

4.1.1.4. Subculture

Five hundred microliters of initial growth culture was transferred into a new pre-warmed 20 ml MHB McCartney bottle. Then the sub-cultured medium was placed in the shaking incubator at 37 °C to continue growing until reaching the logarithmic phase of growth. A UV spectrophotometer at 550 nm wavelength was employed to check the optical density and 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 OD values for antimicrobial text

Microorganism	Subculture incubation time	OD	Concentration (CFU/ml)
S. aureus	2.0h	0.23	10^8
E. coli	1.0h	0.41	10^8
C. albicans	1.0h	0.15	5×10^6

CFU=colony-forming units

Once Log Phase of growth had been reached, 100 µl subculture product of *S. aureus* and *E. coli* was transferred to each labelled Petri dish and 19.9 ml fresh MHB was measured for diluting to 5×10^5 CFU/ml. As for *C. albicans*, 2 ml subculture product was diluted with 18 ml fresh MHB and gently shaken to disperse.

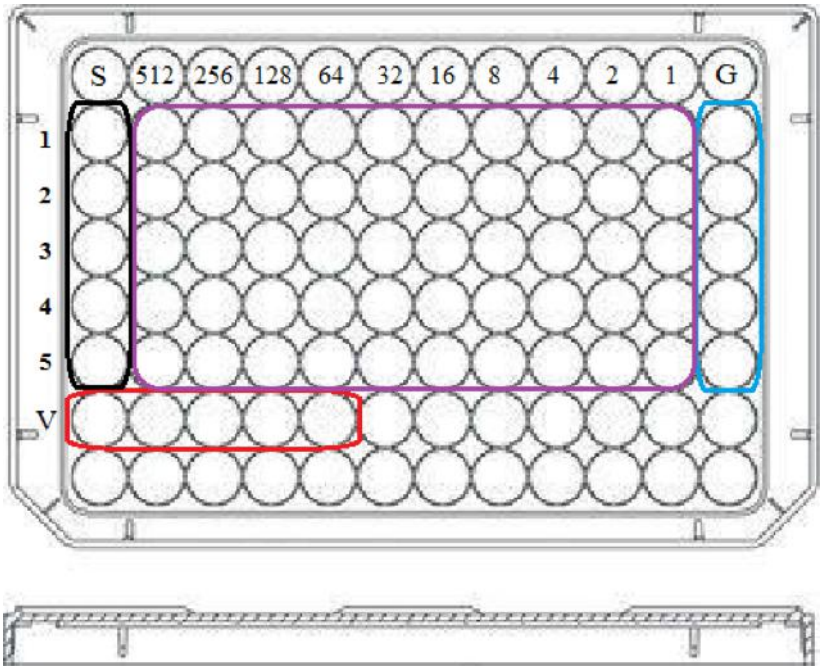


Figure 4.1 The 96-well plate ("S" stands for the blank control, "G" stands for the growth control and "V" stands for the vehicle control. The numbers "512-1" stand for 512-1 µM concentration of peptide)

Table 4.2 The compositions in the 96-well plate

Sample	Contents
512-1 μM concentration of peptide	1 μl 51200-100 μM concentration of peptide and 99 μl microorganism solution
S: Blank control	100 μl MHB
G: Growth control	100 μl microorganism solution
V: Vehicle control	1 μl DMSO and 99 μl microorganism solution

4.1.1.5. The minimum inhibitory concentration (MIC) assay

A 96-well plate was prepared and marked number (Figure 4.1). 5 replicates would be included in this step. 1 μl of different concentrations of peptide solution were added into each well respectively, and 99 μl of diluted bacterial subculture which had reached the standard optical density was subsequently added into each well to mix with the peptide solution. Thereafter, 100 μl of MHB was added into the blank group and the same volume of diluted bacterial subculture was added into the growth group as the growth control. The plate was incubated at 37 °C overnight (16-20 h), after mixing in the shaking incubator for 5-7 min.

4.1.1.6. Viable cell counts

Six 1.5 ml tubes were prepared and marked as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. 900 μl PBS was added in each tube. 100 μl of bacterial subculture from the Petri dish was pipetted. Then, 10-fold dilutions of this were prepared including 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. A Petri dish was prepared and the lines were drawn and marked as in (Figure 4.2). 20 μl of sample from each tube was added onto the Petri dish in small spots, 3 times. The dish was then transferred to the incubator overnight at 37 ℃. A suitable region of the viable cell count Petri dish was chosen to count the numbers of the bacterial colonies and then back calculate the concentration using the formula: Concentration (CFU/ml) = Spots number ÷ Drops number × D × 50 ($D=10^n$, n represents diluted times).

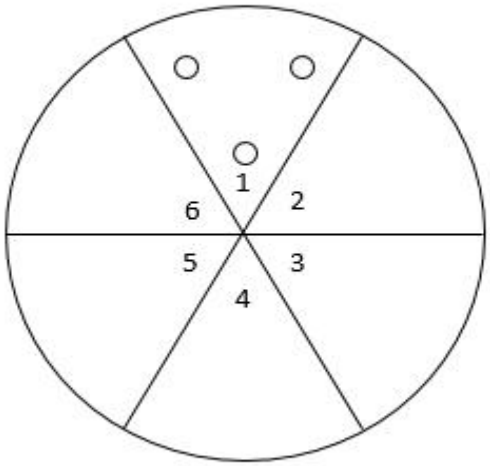


Figure 4.2 The viable count plate (The numbers "1-6" stand for the 10-fold dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.)

4.1.1.7. The detection of plate

The plate was observed directly, slots containing clear liquid represented no bacterial growth. On the other hand, the absorbance of each well was monitored by an EL× 808^{TM} Absorbance Microplate Reader (Bio Tec, USA) at 550 nm. Both results were considered in determining the MIC.

Viability% = (OD-peptide – OD-blank) / (OD-growth –OD-blank) ×100%

4.1.1.8. The minimum bactericidal concentration (MBC) assay

MBC refers to the lowest concentration of an antimicrobial agent that will successfully kill bacteria. After determining the MIC of the peptide against an organism, 20 µlmixture from the clear wells were dropped on the Petri dish with MHA, followed by drying. The Petri dish was placed in the 37 ºCincubator overnight and then observed, the concentration with no bacterial growth was the MBC.

4.1.2. Haemolytic assay

4.1.2.1. Peptide preparation

QUB-3000 was weighed and dissolved in PBS to a concentration of 1024 μM. The peptide solution was then double-diluted to form a series of concentrations: 512, 256, 128, 64, 32, 16, 8, 4 and 2 μM.

4.1.2.2. Horse blood preparation

Whole horse blood was mixed thoroughly by a rotating mixer and 2 ml of this blood was centrifuged at 930 × g for 5 min in an Eppendorf Centrifuge 5430 (Eppendorf, Germany). After centrifugation, the supernatant was removed slowly and PBS was added slowly to avoid breaking erythrocytes. Then cells were centrifuged at 930 × g for 5 min after they were mixed thoroughly. The PBS washing and centrifugation procedures were repeated until the supernatant became colourless and transparent. Finally, enough PBS was added to reach a volume of 50 ml.

4.1.2.3. Haemolysis assay

Positive control: 4% Triton X-100

Sufficient 1.5 ml tubes were prepared in advance and loaded with 100 μl of peptide solutions at different concentrations. Each concentration was replicated 5 times. Control groups were prepared as well. Finally, 100 μl of erythrocyte suspension was added to each tube drop by drop. The components in each group are shown in Table 4.3.

After loading erythrocytes, all tubes were incubated at 37 ℃ for 2 h. Then the tubes were centrifuged at 900 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany). After that, 100 μl of supernatant from each tube was transferred to the corresponding wells in a 96-well plate. Subsequently, the 96-well plate was examined at a wavelength of 450 nm.

Haemolysis% = (OD-peptide – OD-negative) / (OD-positive –OD-negative) ×100%

Table 4.3 The compositions of haemolysis assay

Sample	Contents
512-1 μl concentration of peptide	100 μl 1024-2 μl concentration of peptide and 100 μl blood cells
Positive control	100 μl 4% Triton X-100 and 100 μl blood cells
Negative control	100 μl PBS and 100 μl blood cells

4.1.3. Anticancer activity assay

4.1.3.1. Resuscitation of Frozen Cell Lines

All materials which used in this experiment were required sterilization. The cancer cells (Table 4.4) were stored at -80 °C, removed to 37 °C water bath immediately and gently shaken to unfreeze the cancer cells as quickly as possible. Ten to twelve milliliters preheated medium including 10% foetal bovine serum (FBS) (Sigma, UK) which provided nutrition for cells growing and 1% penicillin streptomycin solution (Sigma, UK) was transferred to a medium-sized culture flask (sigma-aldrich, USA), and all the thawed cells were also transferred into the flask. The flask was placed in an incubator at 37 ℃ under 5% CO_2. Medium was removed every two days, and 10 ml PBS was added to the flask to wash and dropped. Then the flask was added 12 ml full growth medium.

Table 4.4 Cancer cell lines and their basic culture media

Cell line	Tumour type	Medium
MCF-7	Breast cancer non-tumorigenic mammary gland	DMEM medium (high glucose), 10%PBS
PC-3	Human prostate carcinoma (GIV) cell line	RPMI-1640 medium, 10% PBS
U251MG	Human neuronal glioblastoma (Astrocytoma)	DMEM medium (high glucose), 10%PBS
H157	Non-small cell lung cancer	RPMI-1640 medium, 10% PBS

4.1.3.2. Subculture of Adherent Cell Lines

The spent medium was removed, and 10 ml PBS was added to the flask to wash and removed. One milliliter of trypsin/EDTA (Invitrogen, UK) solution was added to cover cells. Then the flask was stored in the incubator at 37 ℃ to remove the cells

from the flask wall. Ten milliliters of full growth medium was added to the flask and used to flush the wall 4-5 times. Subsequently, the liquid in the flask was completely transferred to the 15 ml centrifuge tube, centrifuged at 300 × g for 5 min at room temperature. The supernatant was abandoned after centrifugation, and cells were added 2-3 ml medium and uniformly mixed. Meanwhile 10 ml PBS was used to wash the flask and removed. One milliliter of prepared cells was transferred to the flask with 10-12 ml medium and incubated at 37 ℃.

4.1.3.3. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay

Cell Quantification:

The amount of medium used for mixed cells was changed to 4 ml. Fifty microliters cells and 50 µl Trypan Blue were mixed evenly in one well of the 96-well plate, then transferred to a haemocytometer. The microscope and counter were used to count the number of cells.

Cell density (cells/ml) = the number of counted cells/the number of counted chambers × dilution factor × 10^4.

Cell seeding:

When the concentration reached 5×10^4 cells/ml, the cells are added to the 96-well plate at the concentration of 100 µl/well and incubated for 24 h.

Cell starving:

FBS-free medium (starvation medium) was use to replace the full growth medium and cells were incubated for 6-12 h.

Dosing:

PBS or DMSO was used to dissolve the peptide to get concentrations of 10^{-2} M. The starvation medium was used to dilute the peptide to 10^{-5} M concentration. The 10^{-5} M

concentration of peptide was added to the 96-well plate with 3 replicates (100 µl/well) to take place with original starvation medium of each well. Theblank controls did not require any changes. In the vehicle control, 100µl of the mixture of 6 µl DMSO and 594µl starvation medium was used totake place with original starvation medium of each well. PBS did not need a solvent control because it has been shown to have no anticancer effect. The plate was kept in the incubator for 24 h at 37 °C.

MTT addition & detection

Each well of 96-well plate was added to 10µl MTT (5 mg/ml) and incubated for 3-4 h. Afterwards, 100µl DMSO was used to take place of all the solution of each well, and then the plate was placed in the orbital incubator (Stuart, UK) for 10 min. The Synergy HT plate reader (BioTek, USA) was used to detect the absorbance of each well of the plate at 570 nm wavelength.

Statistical analysis

Sample number (n) indicate the number of independent biological sample in each experiment. Sample number and experimental repeats are usually three times, otherwise are shown in the figure or methods. Data are shown as mean S.E.M and t-test was used to assess statistical significance. Analysis was preformed using GraphPad Prism.

4.2. Results

4.2.1. Antimicrobial activity of QUB-3000

The antimicrobial activity of QUB-3000 was investigated and the results were shown in Figures 4.3, 4.4 and 4.5, the MIC and MBC values are listed in Table 4.5. QUB-3000 exhibited strong antimicrobial ability against *S. aureus* and *E. coli* with the identified MIC and MBC values at 16 µM and 8 µM. While QUB-3000 possessed

weak antimicrobial ability against *C. albicans* with the MIC value at 256 μM and no MBC value. The value of viability% increased at high concentration might be due to that the peptide would precipitate at high concentration.

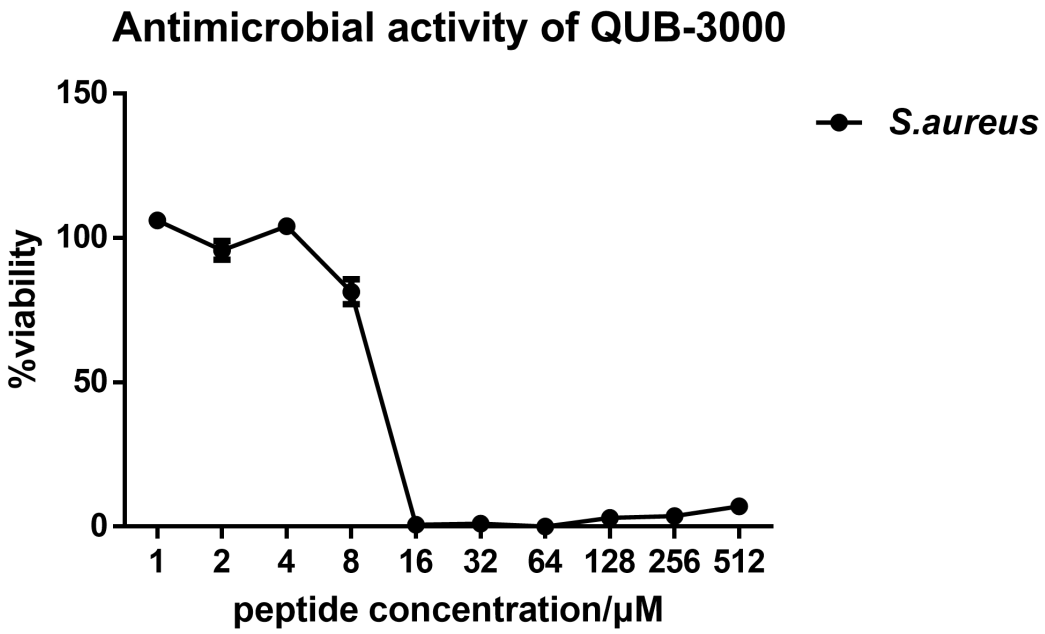


Figure 4.3 The antimicrobial activity of QUB-3000 against *S. aureus.* The error bars indicate the mean ± S.E.M of three replicates.

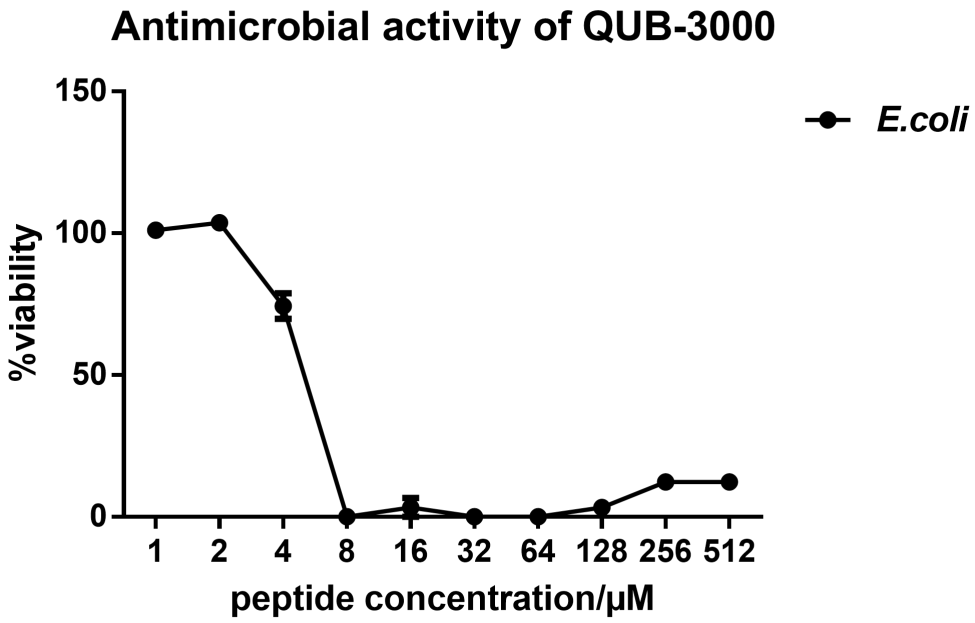


Figure 4.4 The antimicrobial activity of QUB-3000 against *E. coli.* The error bars indicate the mean ± S.E.M of three replicates.

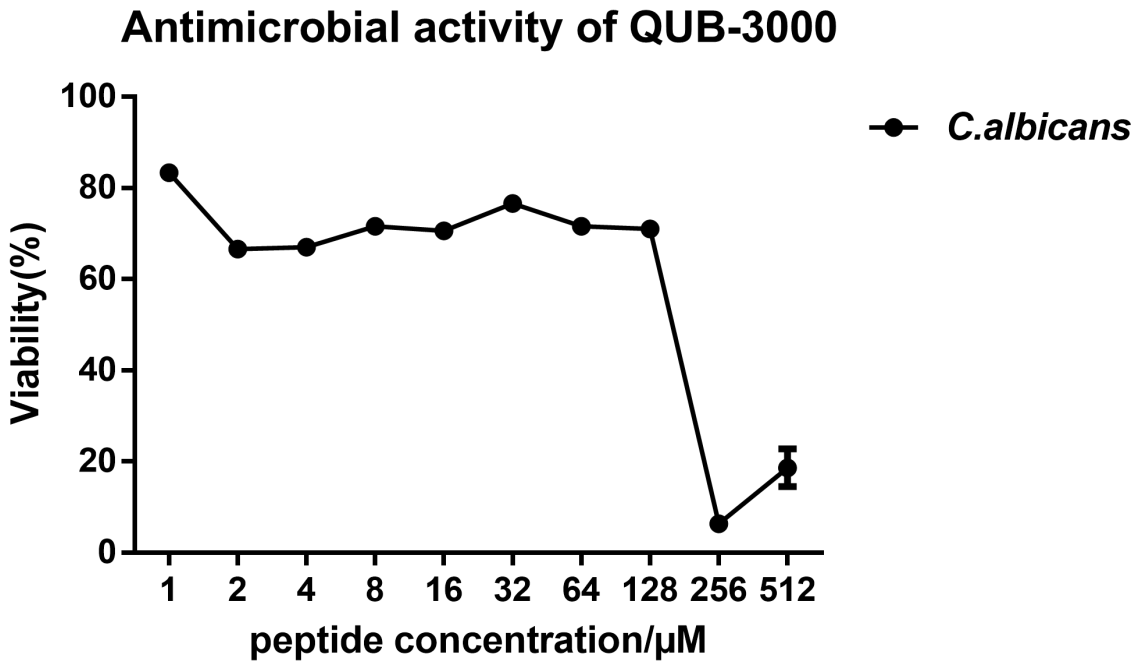


Figure 4.5 The antimicrobial activity of QUB-3000 against *C. albicans.* The error bars indicate the mean ± S.E.M of three replicates.

Table 4.5 The MIC and MBC values of QUB-3000 against *S. aureus*, *E. coli* and *C. albicans*

Microorganisms	MIC	MBC
S. aureus	16	16
E. coli	8	8
C. albicans	256	-

4.2.1. Haemolytic activity of QUB-3000

QUB-3000 showed less than 20% haemolytic rate at 128 μM concentration -1 μM concentration, and more than 50% haemolytic rate at 256 μM concentration on the horse blood cells (Figure 4.6), which suggested that QUB-3000 has low cytotoxicity compared with antibacterial activity. At 512 μM concentration, QUB-3000 was more effective than the positive control 4% Triton X-100.

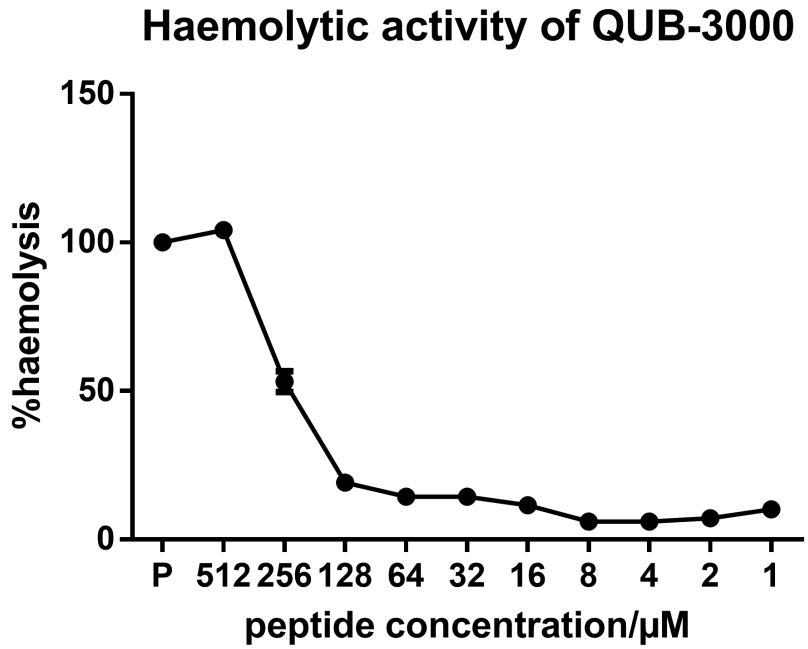


Figure 4.6 The haemolytic activity of QUB-3000 on the horse blood cells. "P" stands for the positive control: 4% Triton-100. The error bars indicate the mean ± S.E.M of five replicates.

4.2.1. Anticancer activity assessment of QUB-3000

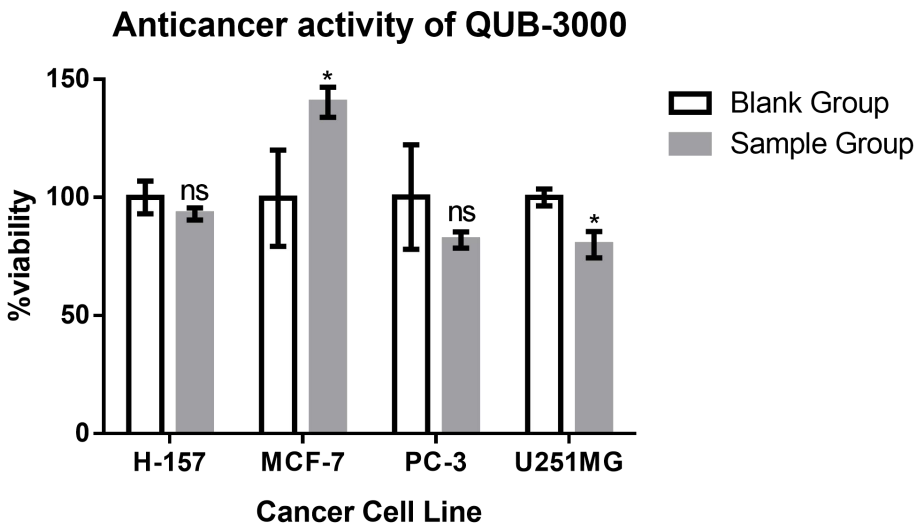


Figure 4.7 The screening results of MTT assay. The error bars indicate the mean ± S.E.M of three replicates, and * stands for t-test of each sample group against the corresponding blank group (* $p<0.05$,).

The 10^{-5} M concentration was used in MTT assay to determine whether the peptide QUB-3000 possess anticancer effects on those four cancer cells. The screening results were shown in Figure 4.7, it indicated that QUB-3000 had very weak effects in inhibiting the growth of cancer cells, and even could promote the growth of certain cancer cells (MCF-7).

Chapter 5: Discussion

5.1. Finding in this study

In this study, QUB-3000, GLMDTVKNVAKNLAGHMLDKLKCKITGC, was successfully isolated from frog skin secretions of the northern leopard frog, *Rana pipiens* by using "shotgun" cloning, synthesised by SPPS, purified by RP-HPLC, identifiedbyMALDI-TOF MS and its biological functions were examined. This discussion will focus on the results of biological functions. In functional experiments, QUB-3000 showed antimicrobial capability, which is consistent with ranatuerin-2 family peptides, but their potencies are not uniformed in bacteria and fungi. The main reason for the differences might be the mechanisms of action of AMPs against these three types of microorganisms (van der Weerden et al. 2013). In addition, the components on the cell membrane surface of Gram-positive bacteria and Gram-negative bacteria are different. Gram-positive bacteria have a thick cell wall and contains a large amount of peptidoglycan and a unique component of teichoic acids. However, the cell wall of Gram-negative bacteria is relatively thin and has a unique outer membrane with the main component lipopolysaccharide (LPS) (De Kimpe et al. 1995). Although LPS is negatively-charged in Gram-negative bacteria, which can be the target of AMPs, it can also be used as a barrier to prevent AMPs from inserting into the cell membrane. AMPs could combine with LPS to form oligomers which prevent AMPs from bonding to the cell membrane (Papo and Shai 2005). So, in theory, AMPs should have a stronger effect on Gram-positive bacteria. Nonetheless, most of the peptides of the ranatuerin-2 family possess different effects, and QUB-3000 also has a stronger effect on Gram-negative bacteria, but this trend was not

obvious, the mechanism of which is not yet clear. The mechanism of which is not yet clear. Matsuzaki et al. had shown that the increase of AMPs positive charge contributed to the greater permeability to the outer membrane of Gram-negative bacteria and stronger antibacterial activity(Matsuzaki et al. 1997). This could be a possible explanation. Furthermore, the ranatuerin-2 family peptides are poorly-conserved, but they have a unique feature compared to other family peptides: the "*Rana* box" at the C-terminal only has 6 residues (Xu and Lai 2015). Kim et al. found that the "*Rana* box" at the end of an AMP of gaegurin family -Gaegurin 4 (GGN4) had important effect on the effective perforation of the cell membrane (Kim et al. 2004). This proved that "*Rana* box" may also have a relevant influence on antibacterial ability, but the specific mechanism needs to be explored.

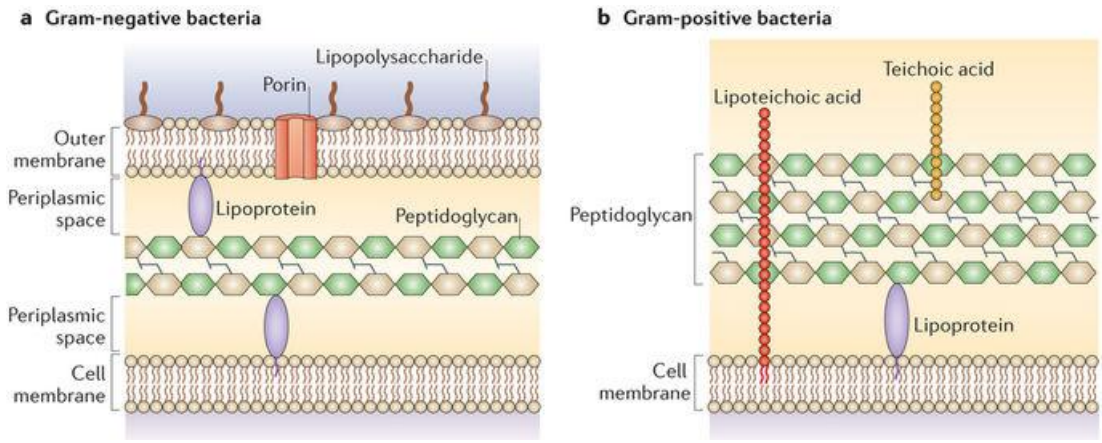


Figure 5.1 The cell wall structures of Gram-negative bacteria and Gram-positive bacteria (Brown et al. 2015)

Since that the QUB-3000 could be precipitated at high concentrations, it cannot be determined that whether the peptide has antifungal activity at 256 μM concentration. The cell surface of the fungi is different from those of Gram-positive and Gram-negative bacteria. The cell wall composition of fungi is glycosylated protein, β-glucan and chitin layer (van der Weerden et al. 2013). Unlike Gram-positive and

Gram-negative bacteria cell membranes, anionic lipid phosphatidylserine (PS) and zwitterionic lipidphosphatidylinositol (PI) are more common in fungal cell membranes (Theis and Stahl 2004), and sterols is the unique component of eukaryotic cell membrane (Bloch 1983). These different surface components and charges may be the factors that affect the QUB-3000 antifungal ability compared to the antibacterial ability. Among the AMP families from amphibians, only temporin and brevinin-1 family have strong antifungal activity (van der Weerden et al. 2013).

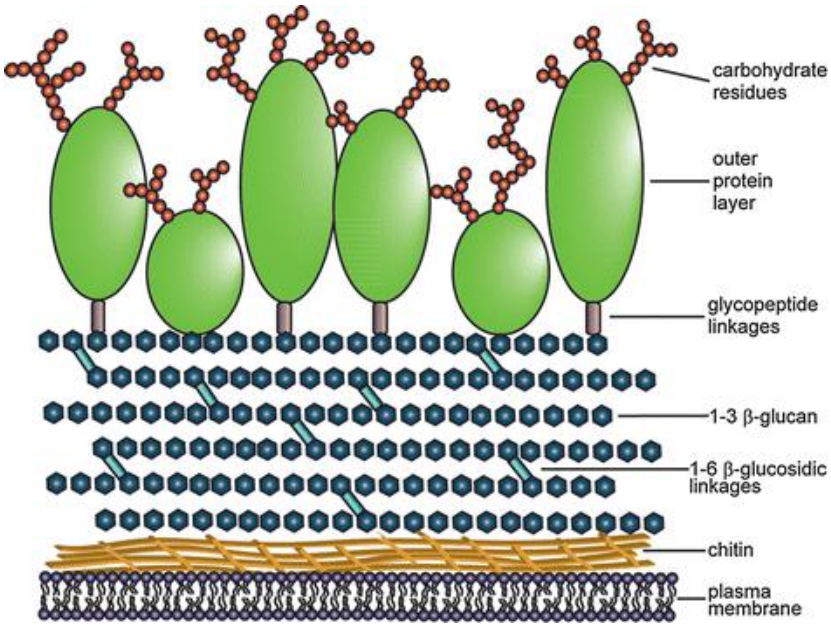


Figure 5.2 The cell wall structure of fungi (van der Weerden et al. 2013)

Besides, the antimicrobial activities of QUB-3000 were compared with Ranatuerin-2B (Goraya et al. 2000), which is from ranatuerin-2 family and share similar amino acid sequence with QUB-3000 (Table 5.1). According to Table 5.1, Ranatuerin-2B shows better antimicrobial effect than QUB-3000, and its hydrophobicity is a bit higher than that of QUB-3000.

Table 4.1 Comparison of the MIC values of QUB-3000 and Ranatuerin-2B as determined for specified microorganisms.

Peptide name	MIC/μM		
	S.aureus	*E.coli*	*C.albicans*
Ranatuerin-2B (GLLDTIKGVAKTVAASMLDKLKCKISGC)	2	2	35
QUB-3000 (GLMDTVKNVAKNLAGHMLDKLKCKITGC)	16	8	256

The online website Peptide property calculator (https://pepcalc.com) showed that QUB-3000 just has 3 positive charge at pH 7, while it has 19 hydrophobic amino acid residues. According to Dathe et al., the membrane of erythrocytes is mainly composed of zwitterionic phosphatidylcholine and phosphatidylethanolamine, which is not easily to combine cationic AMPs, so the intensity of haemolytic activity may not closely relate to the net charge of AMPs (Dathe et al. 1997). That is also may be why the QUB-3000's haemolytic activity is weak compared with antibacterial activity. However, hydrophobicity may be a significant factor to influence haemolytic activity, since the correlation between hydrophobicity and haemolytic activity has already been observed from some AMPs (Dathe et al. 1997). This means that hydrophobicity could be an important factor for future research.

In addition, QUB-3000 had very weak effects in inhibiting the growth of cancer cells like PC-3, H157 and U251MG. The selectivity may owes to the negative charge of cancer cell membrane, which differs from the membrane of normal mammalian cells (Gaspar et al. 2013). As a result, the positive charge of QUB-3000 can bind to the cancer cell membrane, and when the outer membrane peptides reach a certain concentration, the membrane may be split, which leads to the death of cell, just like

the carpet, barrel-stave and toroidal pore model (Gaspar et al. 2013). The weak differences in cell membranes between different types of cancer cells can also affect the anticancer activity of peptides (Hoskin and Ramamoorthy 2008). For example, some breast and prostate cancer cells have been found to contain more cholesterol-rich lipid rafts, which could protect cancer cells from AMPs (Li et al. 2006). Cuttitta et al. have proven that peptides can function as autocrine growth factors for human small-cell lung cancer (SCLC) (Cuttitta et al. 1985). That might be why QUB-3000 could even promote the growth of certain cancer cells (MCF-7).

5.2. Future work

The secondary structure of QUB-3000 has not been identified, and I-TASSER server was only used for prediction. Due to the close relationship between structure and function of AMPs (Powers and Hancock 2003), NMR and circular dichroism (CD) are recommended to obtain the secondary structure of QUB-3000, which can provide more information in studying the functions of QUB-3000.

The high antibacterial activity and relatively low haemolytic activity of QUB-3000 make it promising to be developed as a clinical drug. But more functional investigations are needed before clinical trials. Drug-resistant bacteria can be used for further assessment of the antimicrobial activity of QUB-3000, and haemolytic assay can also employ human blood cells instead of horse blood cells to observe the effect. Additional experiments, such as the animal experiments can be introduced to evaluate the ability of QUB-3000 to inhibit bacteria *in vivo*.

Primary structure modification of QUB-3000 can be helpful to enhance its

antibacterial ability and reduce toxicity.Several factors, such as charge, hydrophobicity, amphipathicity, conformation and polar angle that affecting the activity can all be changed for examination. Since QUB-3000 has good water solubility, replace hydrophilic amino acid with hydrophobic amino acids to increase the hydrophobicity of QUB-3000 for the purpose of enhancing the antibacterial activity is a good option. But the increase of haemolytic activity needs to be avoided. The chirality of amino acids is also important. Studies have shown that the incorporation of D-amino acids could help to enhance the stability of peptides, and natural AMPs also use this mechanism to avoid the negative effects of LPS of the outer membrane of Gram-negative bacteria to improve the antimicrobial activity (Papo and Shai 2005, Mignogna et al. 1993, Huang et al. 2010). The study of Huang et al. also showed that the amidation of the peptide at C-terminus can stabilise the structure of peptide and improve the antimicrobial ability (Huang et al. 2010). Therefore, the amidation of the peptide at C-terminus is also a good modification method to increase the activity.

In conclusion, QUB-3000, a bioactive peptide isolated and identified from the skin secretion of the northern leopard frog, *Rana pipiens*, by using “shotgun” cloning showed strong antibacterial activity and relatively low haemolytic activity. However, weak antifungal activity and anticancer activity were detected. More functional investigations can be taken to make QUB-3000 as a clinical drug. For example, circular dichroism (CD) can be used to confirm the secondary structure of QUB-3000. Then some modifications of QUB-3000 can be used to improve the

antibacterial ability and reduce toxicity. What's more, more drug-resistant bacteria can be used for further assessment of the antimicrobial activity of QUB-3000.

References

Ali, M. F., Lips, K. R., Knoop, F. C., Fritzsch, B., Miller, C. and Conlon, J. M. (2002) 'Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, Rana areolata', *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* 1601(1), 55-63.

Andres, E. and Dimarcq, J. (2004) 'Cationic antimicrobial peptides: update of clinical development', *Journal of internal medicine,* 255(4), 519-520.

Ardelt, W., Ardelt, B. and Darzynkiewicz, Z. (2009) 'Ribonucleases as potential modalities in anticancer therapy', *European journal of pharmacology,* 625(1), 181-189.

Attoub, S., Mechkarska, M., Sonnevend, A., Radosavljevic, G., Jovanovic, I., Lukic, M. L. and Conlon, J. M. (2013) 'Esculentin-2CHa: a host-defense peptide with differential cytotoxicity against bacteria, erythrocytes and tumor cells', *Peptides,* 39, 95-102.

'Bacterial Transformation and Competent Cells–A Brief Introduction', [online], available: https://www.thermofisher.com/uk/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/transformation/competent-cell-basics.html].

Bals, R., Weiner, D. J., Moscioni, A. D., Meegalla, R. L. and Wilson, J. M. (1999) 'Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide', *Infection and immunity,* 67(11), 6084-6089.

Basir, Y. J., Knoop, F. C., Dulka, J. and Conlon, J. M. (2000) 'Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, Rana palustris', *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1543(1), 95-105.

Batoni, G., Maisetta, G., Lisa Brancatisano, F., Esin, S. and Campa, M. (2011) 'Use of antimicrobial peptides against microbial biofilms: advantages and limits', *Current medicinal chemistry,* 18(2), 256-279.

Baumann, G. and Mueller, P. (1974) 'A molecular model of membrane excitability', *Journal of Cellular Biochemistry,* 2(5-6), 538-557.

Bechinger, B. and Lohner, K. (2006) 'Detergent-like actions of linear amphipathic cationic antimicrobial peptides', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1758(9), 1529-1539.

Bloch, K. E. (1983) 'Sterol, structure and membrane function', *Critical Reviews in Biochemistry,* 14(1), 47-92.

Brogden, K. A. (2005) 'Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?', *Nature reviews microbiology,* 3(3), 238-250.

Brown, K. L., Poon, G. F., Birkenhead, D., Pena, O. M., Falsafi, R., Dahlgren, C., Karlsson, A., Bylund, J., Hancock, R. E. and Johnson, P. (2011) 'Host defense peptide LL-37 selectively reduces proinflammatory macrophage responses', *The Journal of Immunology,* 186(9), 5497-5505.

Brown, L., Wolf, J. M., Prados-Rosales, R. and Casadevall, A. (2015) 'Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi', *Nature Reviews Microbiology,* 13(10), 620.

Bulet, P., Stöcklin, R. and Menin, L. (2004) 'Anti-microbial peptides: from invertebrates to vertebrates', *Immunological reviews,* 198(1), 169-184.

Caprioli, R. M., Farmer, T. B. and Gile, J. (1997) 'Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS', *Analytical chemistry,* 69(23), 4751-4760.

Carnicelli, V., Lizzi, A., Ponzi, A., Amicosante, G., Bozzi, A. and Di Giulio, A. (2013) 'Interaction between antimicrobial peptides (AMPs) and their primary target, the biomembranes'.

Chen, Y., Guarnieri, M. T., Vasil, A. I., Vasil, M. L., Mant, C. T. and Hodges, R. S. (2007) 'Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides', *Antimicrobial agents and chemotherapy,* 51(4), 1398-1406.

Clark, D. P., Durell, S., Maloy, W. L. and Zasloff, M. (1994) 'Ranalexin. A novel antimicrobial peptide from bullfrog (Rana catesbeiana) skin, structurally related to the bacterial antibiotic, polymyxin', *Journal of Biological Chemistry,* 269(14), 10849-10855.

Clarke, B. T. (1997) 'The natural history of amphibian skin secretions, their normal functioning and potential medical applications', *Biological Reviews,* 72(3), 365-379.

Conlon, J., Sonnevend, A., Patel, M., Davidson, C., Nielsen, P., Pal, T. and Rollins-Smith, L. (2003) 'Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog Rana boylii', *Chemical Biology & Drug Design,* 62(5), 207-213.

Conlon, J. M., Al-Dhaheri, A., Al-Mutawa, E., Al-Kharrge, R., Ahmed, E., Kolodziejek, J., Nowotny, N., Nielsen, P. F. and Davidson, C. (2007) 'Peptide defenses of the Cascades frog Rana cascadae: implications for the evolutionary history of frogs of the Amerana species group', *Peptides,* 28(6), 1268-1274.

Conlon, J. M., Coquet, L., Jouenne, T., Leprince, J., Vaudry, H. and Iwamuro, S. (2010) 'Evidence from the primary structures of dermal antimicrobial peptides that Rana tagoi okiensis and Rana tagoi tagoi (Ranidae) are not conspecific subspecies', *Toxicon,* 55(2), 430-435.

Conlon, J. M., Kolodziejek, J. and Nowotny, N. (2004a) 'Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents', *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* 1696(1), 1-14.

Conlon, J. M., Mechkarska, M., Ahmed, E., Coquet, L., Jouenne, T., Leprince, J., Vaudry, H., Hayes, M. P. and Padgett-Flohr, G. (2011) 'Host defense peptides in skin secretions of the Oregon spotted frog Rana pretiosa: implications for species resistance to chytridiomycosis', *Developmental & Comparative Immunology,* 35(6), 644-649.

Conlon, J. M., Sonnevend, A., Davidson, C., Demandt, A. and Jouenne, T. (2005a) 'Host-defense peptides isolated from the skin secretions of the Northern red-legged frog Rana aurora aurora', *Developmental & Comparative Immunology,* 29(1), 83-90.

Conlon, J. M., Sonnevend, A., Jouenne, T., Coquet, L., Cosquer, D., Vaudry, H. and Iwamuro, S. (2005b) 'A family of acyclic brevinin-1 peptides from the skin of the Ryukyu brown frog Rana okinavana', *Peptides,* 26(2), 185-190.

Conlon, J. M., Sonnevend, Á., Patel, M., Al-Dhaheri, K., Nielsen, P. F., Kolodziejek, J., Nowotny, N., Iwamuro, S. and Pál, T. (2004b) 'A family of brevinin-2 peptides with potent activity against Pseudomonas aeruginosa from the skin of the Hokkaido frog, Rana pirica', *Regulatory peptides,* 118(3), 135-141.

Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A. and Minna, J. D. (1985) 'Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer', *Nature,* 316(6031), 823.

Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M. and Bienert, M. (2001) 'Optimization of the antimicrobial activity of magainin peptides by modification of charge', *FEBS letters,* 501(2-3), 146-150.

Dathe, M. and Wieprecht, T. (1999) 'Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1462(1), 71-87.

Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W. L., MacDonald, D. L., Beyermann, M. and Bienert, M. (1997) 'Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides', *FEBS letters,* 403(2), 208-212.

De Kimpe, S. J., Kengatharan, M., Thiemermann, C. and Vane, J. R. (1995) 'The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure', *Proceedings of the National Academy of Sciences,* 92(22), 10359-10363.

Desbois, A. P., Gemmell, C. G. and Coote, P. J. (2010) 'In vivo efficacy of the antimicrobial peptide ranalexin in combination with the endopeptidase lysostaphin against wound and systemic meticillin-resistant Staphylococcus aureus (MRSA) infections', *International journal of antimicrobial agents,* 35(6), 559-565.

Doig, A. J., Andrew, C. D., Cochran, D. A., Hughes, E., Penel, S., Sun, J. K., Stapley, B. J., Clarke, D. T. and Jones, G. R. (2001) *Structure, stability and folding of the α-helix,* translated by Portland Press Limited, 95-110.

Drew, J., Woodward, D. T. and Ward, S. (2014) 'Stabilisation of polypeptides',

'Dynabeads™ mRNA DIRECT™ Purification Kit', [online], available: https://www.thermofisher.com/order/catalog/product/61011].

Eisenberg, D. (1984) 'Three-dimensional structure of membrane and surface proteins', *Annual review of biochemistry,* 53(1), 595-623.

Epand, R. M. and Vogel, H. J. (1999) 'Diversity of antimicrobial peptides and their mechanisms of action', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1462(1), 11-28.

Fjell, C. D., Hiss, J. A., Hancock, R. E. and Schneider, G. (2012) 'Designing antimicrobial peptides: form follows function', *Nature reviews Drug discovery,* 11(1), 37-51.

Forsman, A. and Hagman, M. (2009) 'Association of coloration mode with

population declines and endangerment in Australian frogs', *Conservation Biology,* 23(6), 1535-1543.

FULL, R. J., ANDERSON, B. D., FINNERTY, C. M. and FEDER, M. E. (1988) 'Exercising with and without lungs: I. The effects of metabolic cost, maximal oxygen transport and body size on terrestrial locomotion in salamander species', *Journal of experimental biology,* 138(1), 471-485.

Gaspar, D., Veiga, A. S. and Castanho, M. A. (2013) 'From antimicrobial to anticancer peptides. A review', *Frontiers in microbiology,* 4, 294.

Giacometti, A., Cirioni, O., Greganti, G., Quarta, M. and Scalise, G. (1998) 'In vitro activities of membrane-active peptides against gram-positive and gram-negative aerobic bacteria', *Antimicrobial agents and chemotherapy,* 42(12), 3320-3324.

Giangaspero, A., Sandri, L. and Tossi, A. (2001) 'Amphipathic α helical antimicrobial peptides', *The FEBS Journal,* 268(21), 5589-5600.

Goraya, J., Knoop, F. C. and Conlon, J. M. (1998) 'Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, Rana catesbeiana', *Biochemical and biophysical research communications,* 250(3), 589-592.

Goraya, J., Wang, Y., Li, Z., O'Flaherty, M., Knoop, F. C., Platz, J. E. and Conlon, J. M. (2000) 'Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs Rana luteiventris, Rana berlandieri and Rana pipiens', *The FEBS Journal,* 267(3), 894-900.

Gordon, Y. J., Romanowski, E. G. and McDermott, A. M. (2005) 'A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs', *Current eye research,* 30(7), 505-515.

Hallock, K. J., Lee, D.-K. and Ramamoorthy, A. (2003) 'MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain', *Biophysical journal,* 84(5), 3052-3060.

Halverson, T., Basir, Y. J., Knoop, F. C. and Conlon, J. M. (2000) 'Purification and characterization of antimicrobial peptides from the skin of the North American green frog Rana clamitans☆', *Peptides,* 21(4), 469-476.

Hamley, I. W. (2017) 'Small bioactive peptides for biomaterials design and therapeutics', *Chemical reviews*.

Hancock, R. E. (1997) 'Peptide antibiotics', *The Lancet,* 349(9049), 418-422.

Hancock, R. E. and Diamond, G. (2000) 'The role of cationic antimicrobial peptides in innate host defences', *Trends in microbiology,* 8(9), 402-410.

Hancock, R. E. and Rozek, A. (2002) 'Role of membranes in the activities of antimicrobial cationic peptides', *FEMS microbiology letters,* 206(2), 143-149.

Hancock, R. E. and Sahl, H.-G. (2006) 'Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies', *Nature biotechnology,* 24(12), 1551-1557.

Hawrani, A., Howe, R. A., Walsh, T. R. and Dempsey, C. E. (2008) 'Origin of low mammalian cell toxicity in a class of highly active antimicrobial amphipathic helical peptides', *Journal of Biological Chemistry,* 283(27), 18636-18645.

Henzler Wildman, K. A., Lee, D.-K. and Ramamoorthy, A. (2003) 'Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37', *Biochemistry,* 42(21), 6545-6558.

Henzler Wildman, K. A., Lee, D. K. and Ramamoorthy, A. (2002) 'Determination of α-helix and β-sheet stability in the solid state: A solid-state NMR investigation of poly (L-alanine)', *Biopolymers,* 64(5), 246-254.

Hilchie, A. L., Wuerth, K. and Hancock, R. E. (2013) 'Immune modulation by multifaceted cationic host defense (antimicrobial) peptides', *Nature chemical biology,* 9(12), 761-768.

Hoskin, D. W. and Ramamoorthy, A. (2008) 'Studies on anticancer activities of antimicrobial peptides', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1778(2), 357-375.

Huang, Y., Huang, J. and Chen, Y. (2010) 'Alpha-helical cationic antimicrobial peptides: relationships of structure and function', *Protein & cell,* 1(2), 143-152.

'Introduction to Blue-White Screening – Background and Protocols for Colony Selection', [online], available: https://www.sigmaaldrich.com/technical-documents/articles/biology/blue-white-screening.html].

Iwasaki, T., Ishibashi, J., Tanaka, H., Sato, M., Asaoka, A., Taylor, D. and Yamakawa, M. (2009) 'Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface', *Peptides,* 30(4), 660-668.

Jackson, I. M., Bolaffi, J. L. and Guillemin, R. (1980) 'Presence of immunoreactive β-endorphin and enkephalin-like material in the retina and other tissues of the frog, Rana pipiens', *General and comparative endocrinology,* 42(4), 505-508.

Kim, H., Kim, S., Lee, M., Lee, B. and Ryu, P. (2004) 'Role of C-terminal heptapeptide in pore-forming activity of antimicrobial agent, gaegurin 4', *Chemical Biology & Drug Design,* 64(4), 151-158.

Kim, J. B., Halverson, T., Basir, Y. J., Dulka, J., Knoop, F. C., Abel, P. W. and Conlon, J. M. (2000) 'Purification and characterization of antimicrobial and vasorelaxant peptides from skin extracts and skin secretions of the North American pig frog Rana grylio', *Regulatory peptides,* 90(1), 53-60.

Korhonen, H. (2009) 'Milk-derived bioactive peptides: From science to applications', *Journal of functional foods,* 1(2), 177-187.

Kouzmitcheva, G. A., Petrenko, V. A. and Smith, G. P. (2001) 'Identifying diagnostic peptides for lyme disease through epitope discovery', *Clinical and diagnostic laboratory immunology,* 8(1), 150-160.

Kwon, M.-Y., Hong, S.-Y. and Lee, K.-H. (1998) 'Structure-activity analysis of brevinin 1E amide, an antimicrobial peptide from Rana esculenta', *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1387(1), 239-248.

Lamb, H. M. and Wiseman, L. R. (1998) 'Pexiganan acetate', *Drugs,* 56(6), 1047-1052.

Laver, D. (1994) 'The barrel-stave model as applied to alamethicin and its analogs reevaluated', *Biophysical journal,* 66(2), 355-359.

Lee, T.-H., N Hall, K. and Aguilar, M.-I. (2016) 'Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure', *Current topics in medicinal chemistry,* 16(1), 25-39.

Li, Y. C., Park, M. J., Ye, S.-K., Kim, C.-W. and Kim, Y.-N. (2006) 'Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents', *The American journal of pathology,* 168(4), 1107-1118.

'MALDI-Tof-MS ', [online], available: http://dna-barcoding.blogspot.com/2013/08/maldi-tof-ms.html].

Mangoni, M. (2006) 'Temporins, anti-infective peptides with expanding properties', *Cellular and molecular life sciences,* 63(9), 1060-1069.

Mangoni, M. L., Fiocco, D., Mignogna, G., Barra, D. and Simmaco, M. (2003) 'Functional characterisation of the 1–18 fragment of esculentin-1b, an antimicrobial peptide from Rana esculenta', *Peptides,* 24(11), 1771-1777.

Mangoni, M. L., Saugar, J. M., Dellisanti, M., Barra, D., Simmaco, M. and Rivas, L. (2005) 'Temporins, small antimicrobial peptides with leishmanicidal activity', *Journal of Biological Chemistry,* 280(2), 984-990.

Marenah, L., Flatt, P., Orr, D., Shaw, C. and Abdel-Wahab, Y. (2005) 'Characterization of naturally occurring peptides in the skin secretion of Rana pipiens frog reveal pipinin-1 as the novel insulin-releasing agent', *Chemical Biology & Drug Design,* 66(4), 204-210.

Matsuzaki, K. (2009) 'Control of cell selectivity of antimicrobial peptides', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1788(8), 1687-1692.

Matsuzaki, K., Sugishita, K.-i., Harada, M., Fujii, N. and Miyajima, K. (1997) 'Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1327(1), 119-130.

Mignogna, G., Simmaco, M., Kreil, G. and Barra, D. (1993) 'Antibacterial and haemolytic peptides containing D-alloisoleucine from the skin of Bombina variegata', *The EMBO journal,* 12(12), 4829-4832.

Mikut, R., Ruden, S., Reischl, M., Breitling, F., Volkmer, R. and Hilpert, K. (2016) 'Improving short antimicrobial peptides despite elusive rules for activity', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1858(5), 1024-1033.

Mohan, K. V., Rao, S. S. and Atreya, C. D. (2010) 'Evaluation of antimicrobial peptides as novel bactericidal agents for room temperature–stored platelets', *Transfusion,* 50(1), 166-173.

Moore, A. (2003) 'The big and small of drug discovery', *EMBO reports,* 4(2), 114-117.

Morikawa, N., Hagiwara, K. i. and Nakajima, T. (1992) 'Brevinin-1 and-2, unique antimicrobial peptides from the skin of the frog, Rana brevipoda porsa', *Biochemical and biophysical research communications,* 189(1), 184-190.

Nilsson Sköld, H., Aspengren, S. and Wallin, M. (2013) 'Rapid color change in fish

and amphibians–function, regulation, and emerging applications', *Pigment cell & melanoma research,* 26(1), 29-38.

Nizet, V. (2006) 'Antimicrobial peptide resistance mechanisms of human bacterial pathogens', *Current issues in molecular biology,* 8(1), 11.

'Northern Leopard Frog Care Sheet', [online], available: http://www.reptilesmagazine.com/Care-Sheets/Northern-Leopard-Frog/].

Nunes, I. and Costa, F. R. (2011) 'Hide and seek": diurnal refuge and camouflage of two anurans from the Atlantic Forest of Northeastern Brazil', *Herpetology Notes,* 4, 431-433.

Oren, Z. and Shai, Y. (2000) 'Cyclization of a cytolytic amphipathic α-helical peptide and its diastereomer: effect on structure, interaction with model membranes, and biological function', *Biochemistry,* 39(20), 6103-6114.

Papo, N. and Shai, Y. (2005) 'A molecular mechanism for lipopolysaccharide protection of Gram-negative bacteria from antimicrobial peptides', *Journal of Biological Chemistry,* 280(11), 10378-10387.

Pena, O. M., Afacan, N., Pistolic, J., Chen, C., Madera, L., Falsafi, R., Fjell, C. D. and Hancock, R. E. (2013) 'Synthetic cationic peptide IDR-1018 modulates human macrophage differentiation', *PloS one,* 8(1), e52449.

Peschel, A. (2002) 'How do bacteria resist human antimicrobial peptides?', *Trends in microbiology,* 10(4), 179-186.

'pGEM®-T Easy Vector Systems', [online], available: https://www.promega.co.uk/products/pcr/pcr-cloning/pgem-t-easy-vector-systems/?catNum=A1360].

Powers, J.-P. S. and Hancock, R. E. (2003) 'The relationship between peptide structure and antibacterial activity', *Peptides,* 24(11), 1681-1691.

'Principle of HPLC (Liquid Chromatography)', [online], available: https://www.pharmaguideline.com/2013/07/principle-of-hplc-liquid-chromatography.html].

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vredenburg, V. T., Briggs, C. J., Nielsen, P. F. and Conlon, J. M. (2006) 'Antimicrobial peptide defenses of the mountain yellow-legged frog (Rana muscosa)', *Developmental & Comparative Immunology,* 30(9), 831-842.

Schmidt, A., Wake, D. B. and Wake, M. H. (1996) 'Motor nuclei of nerves innervating the tongue and hypoglossal musculature in a caecilian (Amphibia: Gymnophiona), as revealed by HRP transport', *Journal of Comparative Neurology,* 370(3), 342-349.

Schwartz, E. N. F., Schwartz, C. A., Sebben, A., Largura, S. W. and Mendes, E. G. (1999) 'Indirect cardiotoxic activity of the caecilian Siphonops paulensis (Gymnophiona, Amphibia) skin secretion', *Toxicon,* 37(1), 47-54.

Selsted, M., Brown, D., DeLange, R. and Lehrer, R. (1983) 'Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages', *Journal of Biological Chemistry,* 258(23), 14485-14489.

Shai, Y. (1999) 'Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides', *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1462(1), 55-70.

Shin, S., Yang, S. T., Park, E., Eom, S., Song, W., Kim, J., Lee, S. H., Lee, M., Lee, D. and Hahm, K. S. (2001) 'Antibacterial, antitumor and hemolytic activities of α-helical antibiotic peptide, P18 and its analogs', *Chemical Biology & Drug Design,* 58(6), 504-514.

Simmaco, M., Mignogna, G., Barra, D. and Bossa, F. (1994) 'Antimicrobial peptides from skin secretions of Rana esculenta. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides', *Journal of Biological Chemistry,* 269(16), 11956-11961.

Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L. and Barra, D. (1996) 'Temporins, antimicrobial peptides from the European red frog Rana temporaria', *The FEBS Journal,* 242(3), 788-792.

'Skin The Integument', [online], available: http://www.iaszoology.com/skin/].

'Solid Phase Synthesis', [online], available: https://www.sigmaaldrich.com/life-science/custom-oligos/custom-peptides/learning-center/solid-phase-synthesis.html].

Sonnevend, A., Knoop, F. C., Patel, M., Pál, T., Soto, A. M. and Conlon, J. M. (2004) 'Antimicrobial properties of the frog skin peptide, ranatuerin-1 and its [Lys-8]-substituted analog', *Peptides,* 25(1), 29-36.

Suzuki, H., Iwamuro, S., Ohnuma, A., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., Taylor, C. K., Abel, P. W. and Conlon, J. M. (2007) 'Expression of genes

encoding antimicrobial and bradykinin-related peptides in skin of the stream brown frog Rana sakuraii', *Peptides,* 28(3), 505-514.

Tachi, T., Epand, R. F., Epand, R. M. and Matsuzaki, K. (2002) 'Position-dependent hydrophobicity of the antimicrobial magainin peptide affects the mode of peptide− lipid interactions and selective toxicity', *Biochemistry,* 41(34), 10723-10731.

Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., Caprioli, R. M., Blouin, M. S. and Rollins-Smith, L. A. (2009) 'Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (Rana pipiens) populations suggest intraspecies differences in resistance to pathogens', *Developmental & Comparative Immunology,* 33(12), 1247-1257.

Theis, T. and Stahl, U. (2004) 'Antifungal proteins: targets, mechanisms and prospective applications', *Cellular and Molecular Life Sciences CMLS,* 61(4), 437-455.

Tossi, A., Sandri, L. and Giangaspero, A. (2000) 'Amphipathic, α-helical antimicrobial peptides', *Peptide Science,* 55(1), 4-30.

Uematsu, N. and Matsuzaki, K. (2000) 'Polar angle as a determinant of amphipathic α-helix-lipid interactions: a model peptide study', *Biophysical journal,* 79(4), 2075-2083.

Unger, T., Oren, Z. and Shai, Y. (2001) 'The effect of cyclization of magainin 2 and melittin analogues on structure, function, and model membrane interactions: implication to their mode of action', *Biochemistry,* 40(21), 6388-6397.

Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D. and Fidler, I. J. (1991) 'Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes', *Cancer research,* 51(11), 3062-3066.

van der Weerden, N. L., Bleackley, M. R. and Anderson, M. A. (2013) 'Properties and mechanisms of action of naturally occurring antifungal peptides', *Cellular and molecular life sciences,* 70(19), 3545-3570.

Wieprecht, T., Dathe, M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L. and Bienert, M. (1997a) 'Peptide hydrophobicity controls the activity and selectivity of magainin 2 amide in interaction with membranes', *Biochemistry,* 36(20), 6124-6132.

Wieprecht, T., Dathe, M., Epand, R. M., Beyermann, M., Krause, E., Maloy, W. L.,

MacDonald, D. L. and Bienert, M. (1997b) 'Influence of the angle subtended by the positively charged helix face on the membrane activity of amphipathic, antibacterial peptides', *Biochemistry,* 36(42), 12869-12880.

Xu, X. and Lai, R. (2015) 'The chemistry and biological activities of peptides from amphibian skin secretions', *Chemical reviews,* 115(4), 1760-1846.

Yang, L., Harroun, T. A., Weiss, T. M., Ding, L. and Huang, H. W. (2001) 'Barrel-stave model or toroidal model? A case study on melittin pores', *Biophysical journal,* 81(3), 1475-1485.

Yeaman, M. R. and Yount, N. Y. (2003) 'Mechanisms of antimicrobial peptide action and resistance', *Pharmacological reviews,* 55(1), 27-55.

Zasloff, M. (1987) 'Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor', *Proceedings of the National Academy of Sciences,* 84(15), 5449-5453.

Zhang, L., Parente, J., Harris, S. M., Woods, D. E., Hancock, R. E. and Falla, T. J. (2005) 'Antimicrobial peptide therapeutics for cystic fibrosis', *Antimicrobial agents and chemotherapy,* 49(7), 2921-2927.