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QUB-1985: a bioactive peptide from the frog skin secretion of *Odorrana livida*

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Contents

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Declaration

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

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Abstract

Considering the severe problem of drug resistance developed by multiple pathogenic microorganisms, scientists have been working on developing novel therapeutic agents to replace conventional antibiotics. Antimicrobial peptides (AMPs), possessing broad-spectrum antimicrobial activity, have raised great interests among medical field for its potential bioactivity against bacteria, fungi, viruses and cancer cells and its multi-target mechanisms.

In this study, a cDNA encoding an AMP precursor was isolated and identified from the skin secretion of the wild green mountain frog, *Odorrana livida*, with the assistance of shotgun cloning. The obtained mature peptide consists of 21 amino acid residues, GLLSGILGVGKKIVCGLSGLC, and this peptide was named QUB-1985 according to its molecular mass. Afterwards, adequate peptides were acquired through SPPS, and several assays were carried out on QUB-1985 to determine its biological activities.

In antimicrobial assay, QUB-1985 exhibited potent antimicrobial activity against three model microorganisms, which are *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli* (Gram-negative bacteria) and *Candida albicans* (fungi), and the MICs are 4, 8 and 16 μM respectively. Meanwhile, the cytotoxicity of QUB-1985 on horse red blood cells was rather low with a haemolysis of about 30% at the concentration up to 256 μM, which is more than 16-fold of the MICs against the three model microorganisms. In anticancer assay, however, no obvious anticancer activity was observed in QUB-1985 in this study.

Chapter 1 Introduction

1.1 Defensive system of amphibians

The word amphibian consists of two Greek words which mean two modes of live (Crump, 2009). Their life cycle begins with aquatic eggs, then they become larvae living in water and eventually metamorphose into adults living on land (Vonesh and De la Cruz, 2002). Except the Antarctic Circle, amphibian habitats cover a large range of areas around the world, from the freezing cold subarctic areas to muggy tropical areas (Duellman and Trueb, 1994). It seems difficult for amphibians to live in such complex living conditions, because they have no hard shields or sharp claws, even their skin is permeable. Not only the extreme environments, but also predators are serious threats to the survival of amphibians (Barthalmus and Zielinski, 1988). Moreover, it is known that the humid environment is a perfect condition for microorganism growth, which means amphibians will face the danger of getting infected by pathogens (Preusser et al., 1975). However, it does not mean that amphibians are not capable of defending themselves from these terrible threats. During centuries of evolution, amphibians have developed a complicated defensive system to protect themselves (Carey et al., 1999).

1.1.1 Amphibian skin

Skin, the first defence of the amphibian, does not only protect them visually, but also produce some bioactive chemical compounds to help amphibians keep health and survive in complex living conditions. When amphibians run into predators, their skin may expose different showy colours to warn predators that they are able to produce poisonous skin secretions (Darst and Cummings, 2006). Additionally, there are three kinds of glands in the dorsal area of amphibian skin: alveolar glands, mucus glands, and granular glands. Alveolar glands are limited to some specific species and the latter two are the main

functional parts in amphibian skin (Clarke, 1997). As their skin is permeable, it is important for amphibians to keep normal water regulation between their bodies and the outer environments. Mucous, produced by mucous glands, can help to keep amphibian skin moist and slippery, which is vital to those species living in dry conditions. For those living in humid areas, like rainforests or in the water, mucous can help reduce the harm of long stay in the water and mechanical injuries (Barthalmus and Zielinski, 1988).

Moreover, the skin glands are also able to protect the permeable skin from the attacks of virus, bacteria and other pathogenic microorganisms (Clarke, 1997). The granular glands, which are also called venom glands, may secrete poisonous venom to protect amphibians. Skin is the first line of amphibian host-defensive system, and skin secretions are the most important part. The skin secretions are produced and stored in the sacs inside the granular glands (Simmaco et al., 1994). These sacs are surrounded by muscle fibres, which are connected to nerves. When the amphibians detect the stimuli from either predation or microbial infection, the nerves may send signals to make the muscles contract so that the stored venom will be secrete to the dermal surface of the amphibian skin. As a result, the pathogens on their skin surface can be killed. The venom proves to possess the property of killing microbial pathogens or trap them (Daly, 1995).

1.1.2 Skin secretions of Amphibians

These secretions secreted by mucus and granular glands possess high level of antimicrobial bioactivities and can protect the permeable skin layer from the risk of the microorganism infection. From former studies, the main components of amphibian skin secretions include biological amines, peptides, proteins, steroids, and alkaloids (Lazarus and Attila, 1993).

The derivatives of catecholamines and indolealkylamines are the main components of biogenic amines extracted from amphibian skin secretions. Some of these chemical compounds can be hallucinogenic and may induce high blood pressure, such as bufotenin, bufoviridin and other bufotenins derived from indoleakylamines. These biogenic amines can cause vasoconstriction and some can even induce eclampsia (Clarke, 1997).

The most famous constituents of skin secretion steroids are bufotalin and bufotoxins, both of which comprise a steroid nucleus of three 6- and one 5- membered rings. They are capable of dealing with heart diseases via the increase of heart beat strength and the decrease of heart rate (Clarke, 1997).

It has been indicated that alkaloids found in amphibian skin secretion are mainly derived from the diet of amphibians, which are composed of insects and millipedes (Clarke, 1997). Alkaloids have a wide distribution in natural resources, mainly found from poison dart frogs and also from salamander, newts, mantelline ranid frogs and so on (Daly et al., 2005).

In fact, the bioactive compounds in amphibian skin secretions are mainly proteins and peptides which may exhibit protection effect against predators as hormone-like peptides and against pathogenic microorganisms as antimicrobial peptides in amphibian defensive system (Roelants et al., 2013). Despite antimicrobial activity, these peptides can also possess other bioactivities, such as smooth muscle contraction activity, protease inhibition activity, insulin-releasing activity and so on (Xu and Lai, 2015). The identified Bowman-Birk-like peptides such as pLR, pYR, HV-BBI and ranacyclin-B-like peptides, which are from the ORB family, share a highly conserved primary structure (Yan et al., 2012). However, the bioactivities among these BBI family peptides may vary. For

example, pLR and pYR have immunomodulatory functions, while ranacyclin-B-like peptides exhibit trypsin-inhibitory activity and antimicrobial activity (Xu and Lai, 2015). Another instance is Bombesin, which was first isolated from the European toad *Bombina bombina*, demonstrated to induce strong contractile activity on rat smooth muscles including bladder, uterus and ileum (Zhou et al., 2017).

1.2 Antimicrobial peptides

Thousands of AMPs have been isolated from a wide range of natural resources, not only from amphibians, but also from human, insects and even bacteria and plants (Li et al., 2012). Antimicrobial peptides play a vital role in the innate immune system to defend the hosts from the infection of different kinds of pathogenic microorganisms (Mojsoska et al., 2015). AMPs have been identified to be of a size of 10-50 amino acids and possess potent and rapid antimicrobial activity, and AMPs isolated from amphibian skin secretion are confirmed to be the first line defence of the hosts (Xu and Lai, 2015).

The broad-spectrum activity of AMPs against bacteria and pathogenic fungi have raised great interest among this field and extensive studies have been done to determine the antimicrobial mechanisms of AMPs (Lee et al., 2016). There are three models of action: ‘carpet’ model, ‘barrel-stave’ model and ‘toroidal-pore’ model (Bechinger and Gorr, 2017).

Despite antimicrobial activity, AMPs have been proven to possibly possess antiviral, anticancer, anti-parasitic activity and so on, and they are considered to be promising therapeutic agents. However, cytotoxicity problems and other security issues are obstacles (Li et al., 2012).

1.2.1 Antimicrobial peptide structure features

Large amounts of researches on structure-activity relationship of peptides have demonstrated that structural factors like secondary structure, charge, hydrophobicity and so on have significant effects on the activity of AMPs. One change in one factor may induce dramatic alterations in one or more of the other factors, which may also lead to increase or decrease in antimicrobial activity or cell toxicity (Mojsoska et al., 2015).

1.2.1.1 Secondary structures

The secondary structures of peptides are formed due to the formation of hydrogen or disulfide bonds between amino acids (Kabsch and Sander, 1983). According to the secondary structures of antimicrobial peptides, AMPs can be divided into 4 different groups, which are α-helix, β-sheet, extended and loop (Powers and Hancock, 2003). In addition, the most common two groups are α-helix and β-sheet groups (Jin et al., 2005).

Peptides adapting α-helix structure have been thoroughly researched by scientists all over the world for their wide distribution among the nature (Huang et al., 2010). The α-helix peptides are of 20-40 residues long but without cysteine residues (Brogden et al., 2003). These peptides may possibly be unstructured in aqueous environment, however, they will adopt a complete helix structure when the electrostatic interaction was induced with negatively-charged microorganism membranes (Bechinger et al., 1993). A motif of highly amphipathic helix with positively-charged and hydrophobic surface was observed in peptides of this structure group (Reddy et al., 2004), which brings peptides broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Zelezetsky and Tossi, 2006). However, these helical structure features also increase the haemolysis activity and cytotoxicity (Huang et al., 2010).

The most famous peptide family of α-helix structure must be magainins. Magainins were first extracted from the skin secretion of the African clawed frog *Xenopus laevis* in 1987 by Zasloff (Zasloff, 1987). Peptides from this family are normally 23-residue long and positively charged, forming α-helical structure (Matsuzaki, 1999). The strong antimicrobial activity is provided by the amphipathicity of helical structure (Xu and Lai, 2015). Magainin peptides possess potent antimicrobial activity against bacteria and fungi but rather weak haemolysis activity on mammalian cells. These peptides possess high selectivity and target mainly on bacterial intracellular contents, leading to cytoplasm membrane disruption (Matsuzaki, 1999).

However, the isolation and identification of the first α-helix peptide was done by Steiner, and the peptide discovered from the cecropia moth was named cecropins (Steiner et al., 1981). The N-terminals of cecropins are normally amphipathic and the C-terminals are hydrophobic, which are connected by a flexible hinge region and form a helix-bend-helix structure. This unique structure is the key feature of cecreopin antimicrobial acitivty (Efimova et al., 2014).

Antimicrobial peptides which contains several cystine residues may form disulphide bridges between each 2 cystine residues, which may possess firm structures (Venkatraman et al., 2002). This kind of peptides are characterized as β-sheet peptides and are of 16-40 amino acid residues in length (Brogden et al., 2003). Extensive studies on these AMPs demonstrate that β-sheet peptides exert strong antimicrobial bioactivity on most of the bacteria, while they may exhibit dramatically high level of activity against Gram-negative bacteria *Pseudomonas aeruginosa* (Doig et al., 1988).

One example of β-sheet peptides is defensins. Defensins are the most wide-spread antimicrobial peptides characterised in insects, which was first isolated from the flesh fly

Sacrophaga peregrine (Čeřovský et al., 2010). Defensins are cationic cysteine-rich polypeptides, which may form three to four disulphide bridges in their structures (Li et al., 2012). These peptides are 33 to 46 amino acids long, which may adopt β-sheet structures, and they can exhibit activity against Gram-positive bacteria but less effective on Gram-positive bacteria and fungi (Čeřovský et al., 2010).

The basic mechanism of this class of peptides is believed to be membrane disruption. However, some β-sheet peptides adopt distinct mechanisms (Jin et al., 2005). For example, the peptide Tachyplesin, which was found in horseshoe crabs, can work on the DNA minor grooves and disrupt the DNA synthesis of bacteria cells (Yonezawa et al., 1992).

Additionally, some antimicrobial peptides do not form the regular α-helical or β-sheet structure because they contain a large portion of proline, tryptophan, arginine or histidine, and these peptides are classified as extended peptides (Wang et al., 2016). This kind of peptides do not exhibit amphipathicity, but tryptophan residues may enhance the penetrating ability of peptides through the membrane lipid bilayers, while arginine may help peptides obtain positive charge to interact with the negatively charged pathogenic membranes (Chan et al., 2006). Unlike the two classes peptides mentioned above, extended peptides form their final structure through hydrogen bond or Van der Waals interactions with target membrane lipids instead of the bond between amino acid residues. Indolicidin has been determined as an extended peptide and possess a more bent motif in zwitterionic solution than in ionic solution (Powers and Hancock, 2003).

Moreover, antimicrobial peptides of the loop structure are actually produced by bacteria. These AMPs possess small loop structure formed by the bond between thio-ether, disulphide, amide bond or isopeptide (Powers and Hancock). Nisin is one of the loop

AMPs. It contains 34 amino acid residues and is synthesised by *Lactococcus lactis* (Hasper et al., 2004). It is widely applied in food industry to prevent food deterioration because nisin exhibits strong antimicrobial activity but low toxicity to mammalian cells (Wang et al., 2016).

1.2.1.2 Charge

It is a vital feature that antimicrobial peptides possess net positive charges. In the previous studies, many peptides have been characterized to possess a net positive charge or contain cationic regions, which has a close relationship with the antimicrobial activity to the microorganisms (Hancock and Diamond, 2000). Generally, the cell membranes of pathogenic microorganisms are negatively-charged, which may induce electrostatic interactions between peptides and the membrane (Zhang and Gallo, 2016). Moreover, the positive charge of antimicrobial peptides may help them to attract on the membranes of pathogenic microorganisms and reach the valid bactericidal concentration (Malanovic and Lohner, 2016). Then, the peptides may interrupt the membranes through dissolving the membranes or forming pores and channels on the membranes. During the range of +3 to +5, there is a positive correlation between the positive charge and the bioactivity against the target pathogens. As the positive charge rises in this range, it will become easier for the positive charged peptides to induce electrostatic interaction with the negatively-charged pathogenic cell membranes (Lee et al., 2016). With the raising of the positive charge, the peptides show more potent antimicrobial activity. However, the increase of positive charge to more than +6 may not result in an increase of antimicrobial activity against pathogenic microorganisms, while the haemolytic bioactivity may be increased (Dathe et al., 2001).

1.2.1.3 Amphipathicity

Amphipathicity is a significant feature of α-helical peptides which helps peptides obtain antimicrobial bioactivity (Rothemund et al., 1995). In this structure, hydrophilic and hydrophobic residues may distribute respectively at N-terminals or C-terminals, while in another situation hydrophilic residues distribute on both terminals and are separated by hydrophobic residues. In addition, peptides with the former motif usually exhibit activity only against Gram-negative bacteria, while peptides in the motif of the latter style show potent bioactivity against both Gram-positive and Gram-negative bacteria (Dominguez et al., 2016). The bioactivity of α-helical peptides may exhibit through the hydrophilic regions interacting with the hydrophilic lipid heads and the hydrophobic regions associating with the hydrophobic core of lipid bilayers (Wimley, 2010). Generally, the hydrophobic moment is used to describe the amphipathicity of antimicrobial peptides, which is calculated as the sum of amino acid vectorial hydrophobicities (Dathe et al., 1997). The antimicrobial activity of peptide can be enhanced by the increase of hydrophobic residues, because the higher hydrophobicity may contribute to the interaction between peptides and membranes. However, high hydrophobicity may cause an increase of haemolytic activity (Kuroda et al., 2009).

1.2.2 Mechanisms of AMPs

It has been proven in previous studies that AMPs exert broad-spectrum antimicrobial activities against Gram-positive bacteria, Gram-negative bacteria and fungi (Li et al., 2012). The main mechanism of peptides killing microorganisms is that peptides firstly attach to the cell membrane surface of microorganisms, then form pores that inducing

the leakage of cytoplasmic components (Yeaman and Yount, 2003). Furthermore, it has been demonstrated that the formation of pores on the membranes is not the only way that peptides perform bioactivity. The inhibition of cell membrane synthesis, nucleic-acid synthesis, protein synthesis and enzymatic activity is also proposed in studies (Brogden, 2005).

There are three steps before the peptides kill pathogenic microorganisms (Huang et al., 2010). Firstly, AMPs may be attracted to the microorganism cell surface due to the negative charge of the cell membranes, including the anionic phospholipids and phosphate groups on Gram-negative bacteria outer membranes and the teichoic acid on the surface of Gram-positive bacteria which possess obvious net negative charge. When peptides are approaching the surface of Gram-negative bacteria, in order to interact with the target membrane, they need to go through the capsular polysaccharides covering the outer membranes. When it comes to the Gram-positive bacteria, peptides need to get through capsular polysaccharides, teichoic acids and lipoteichoic acids first (Brogden, 2005). In the beginning, peptides only bind to the membrane surface parallel when it is at a low peptide/lipid ratio, whilst more and more peptides concentrating on the membrane surface, peptides may turn to orientate vertically to the membranes at a high peptide/lipid ratio and then insert into the lipid bilayers (Wimley, 2010). Secondly, the peptides binding to the membrane lipids will form pores or ion channels on the cell membranes, which can let more peptides get into the cell or leak the intracellular contents out. Finally, the cell membrane will be lysed by antimicrobial peptides or the cytoplasmic contents leak out, which was followed by cell death (Guilhelmelli et al., 2013).

The antimicrobial bioactivity is exhibited through two modes of actions, one of which is membrane disruption mode and the other one is non-membrane disruption mode. The membrane disruption mode is more discussed and can be divided into 3 models (Lee et

al., 2016). From extensive studies, the effect of dissolving microbial cell membrane is named as ‘carpet’ model. In the carpet model, the positively charged peptides will firstly be attracted by the net negatively charged cell membranes and associated with the membrane parallel on the membrane surface and cover it like a carpet. Then, the hydrophilic part of peptides may combine with the lipid head of the phospholipid bilayer. In this interaction, the stability of the phospholipid membrane will be decreased because the phospholipids are replaced by peptides. As a result, the peptides will disrupt the cell membranes in a detergent-like manner when the peptides reach a threshold concentration and allow more peptides to access the membranes. Finally, the pathogenic cells will die because of the disruption of cell membranes (Marquette and Bechinger, 2018). However, among the actions of the carpet model, there is no further interaction between peptides and the membrane hydrophobic core. The peptides only align with the lipid heads and alter the membrane fluidity to destabilize the cell membrane. Without insertion into the membrane core, there is no channel or pore formation (Brogden, 2005).

The pore or channel formation action mode can be divided into two slightly different manners (Yang et al., 2001). One of the three modes is ‘barrel-stave’ model. Different from the carpet model, when peptides come to the lipid bilayer, the peptides may firstly aggregate and then insert into the membrane bilayers. In this action, the hydrophobic regions of peptides may associate with the hydrophobic core of the membrane while the hydrophilic regions may face each other and make a pore or channel in the middle of these peptides. These peptides will form bundles on the membranes and look like several staves forming a barrel. Overall, pores and channels are formed on the surface of the membrane. Differently, while peptides bind to cell membrane parallel in the carpet model, peptides in barrel-stave model may insert into membrane vertically and combine with the hydrophobic core (Lee and Lee, 2015).

Another different action mode is ‘toroidal-pore’ model. Similarly, the peptides will insert into the lipid bilayer and interact with the hydrophobic membrane core. However, while the hydrophobic regions of the peptides bind to the hydrophobic core, the hydrophilic regions may combine with the hydrophilic lipid heads. Thus, the phospholipid head groups covered by peptides will tilt to the membrane core so that aqueous channels could be formed on the membrane (Shenkarev et al., 2011). Additionally, the pores and channels are not only made of antimicrobial peptides, but also the bending lipid head groups (Brogden, 2005).

The cell membrane of bacteria and fungi are different since ones are prokaryotic cells and the other ones are eukaryotic cells (Matsuzaki et al., 1995). Whilst, the membrane structure of Gram-positive and Gram-negative bacteria are slightly different from each other. The structure of peptides and the target microorganism membranes are highly relevant with the antimicrobial effect and the selectivity of AMPs. Furthermore, the microbial cell membranes contain large scales of phosphatidylglycerol (PG), cardiolipin and phosphatidylserine (PS), which are acidic phospholipids and lead to the negative charge of membranes (Trombetta et al., 2005). However, eukaryotes mammalian cell membranes are mainly composed of amphipathic phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamines (PE) and sphingomyelin (Carey et al.) (Ilić et al., 2013). Moreover, the mammalian cell membranes are rich in cholesterol, which may enhance the stability and integrity of membranes. It is the difference between pathogenic cell membranes and host cell membranes that contribute to the selectivity of antimicrobial peptides (Brender et al., 2012).

1.2.3 Resistance to AMPs

While there have been large quantities of studies and improvement of antibiotics, pathogenic microorganisms have also developed resistance mechanisms against the antimicrobial activity of AMPs. The most common resistance mechanisms maybe the pathogenic cell surface modification and the AMP blockage (Walsh, 2000).

Firstly, the negative charge on the pathogenic cell membrane surface can be reduced to decline the attraction to cationic AMPs. For instance, the net negative charge of LPS lipid A in Gram-negative bacteria can be reduced through the addition of ethanolamine, 4-amino-4-deoxy-T-arabinose and other amine-containing compounds (Yeaman and Yount, 2003). Then the capsule production is another surface modification mechanism to resist antimicrobial bioactivity. With the sense of the presence of AMPs, pathogens may produce the capsular polysaccharide to act as a shield to reduce the interaction between the peptides and membranes and avoid the binding or penetration into pathogenic cells (Wang-Lin et al., 2017). Moreover, pilus, exopolysaccharides and lipopolysaccharides are also able to associate with antimicrobial peptides to neutralize the antimicrobial activity of peptides (Banemann et al., 1998).

Biofilm is another way for microbes to resist antimicrobial peptides. Biofilms are communities of cells attached to each other or to biotic and abiotic surfaces, which have an extraordinary effect on the resistance of AMPs (Stewart and Costerton, 2001). Additionally, Gram-positive and Gram-negative bacteria may release enzymes, such as peptidases and proteases, which are capable of cause peptide degradation (Belas et al., 2004). AMPs are rich in alkaline amino acid residues, which makes it easy for proteases to degrade peptides (Plumb et al., 2000). Furthermore, when peptides have already

inserted into the cell membranes, pathogens can also extrude them through efflux pumps (Soto, 2013).

Although there are several different mechanisms of resistant the antimicrobial effect, one kind of pathogens may adopt more than one way at the same time. The drug resistance not only increase the possibility of microorganism infection, but also threat the global human health.

1.2.4 AMPs as novel antibiotics

Although. it is a great revolution of medicine field that antibiotics were discovered and put into volume production (Huang et al., 2010), bacterium drug resistance has become a raising problem since the last decades. In fact, antibiotic resistance of pathogens is a natural evolution, but the medical abuse of antibiotics has dramatically accelerated the progress of resistance evolution (Woodhead et al., 2004). Effective actions must be taken to prevent further disaster in human health. Numerous studies have been carried out on antimicrobial peptides as a novel therapeutic to fight against microbial drug resistance due to their broad-spectrum antimicrobial bioactivity against Gram-positive, Gram-negative bacteria and pathogenic fungi (Waghu et al., 2014). Moreover, AMPs carry out their bioactivity against pathogens through different action mechanisms from that of conventional antibiotics, which may decrease the chance for pathogens to develop resistance. Meanwhile, AMPs may not do harm to normal host cells or the health of hosts when they carry out the antimicrobial activity. Its immunomodulation function can also provide new methods in solving infection problems (Hancock, 1997).

Antimicrobial peptides not only possess a broad-spectrum antimicrobial bioactivity, but also exhibit the activity against parasites, virus and cancer cells (Papo and Shai, 2005).

Due to the drug resistance of parasites and the toxicity of existing drugs, it is urgent to find a new treatment against parasite diseases. Some AMPs are able to treat hosts which have been infected by parasites and can prevent diseases caused by parasites transmission (Li et al., 2012). For example, Temporins A and Temporins B have been proven possessing antiparasitic activity against *Leishmania infantum* and *Leishmania mexicana* respectively, and the parasite stages they attack on are promastigotes and amasitgotes (Torrent et al., 2012).

Herpes simplex virus 1 (HSV-1) is one of the most widespread pathogens in human body. HSV-1 infection can be immediately controlled among the bodies of normal patients and only short-term antiviral therapy is needed. However, it is difficult for immunocompromised patients to control HSV-1 infections and long-term therapies are urgently needed, which may induce drug resistance (Marcocci et al., 2018). Chemokines, which is a group of leukocyte chemotactic cytokines, have been proven to be capable of killing not only HSV-1, but also dengue virus serotype 2 and respiratory syncytial virus (Vanheule et al., 2016). In addition, another virus causing serious disease in human body, human immunodeficiency virus (HIV), can be killed by indolicidin (Robinson et al., 1998).

Antimicrobial peptides can inhibit the growth of various cancer cells without doing any harm to normal healthy human cells (Čeřovský et al., 2009). It has been reported that cervical cancer and bladder cancer can be remarkably inhibited by antimicrobial peptides (Nguyen et al., 1994). AMPs may not only induce cell death through lysing cell membrane, but also target on the mitochondrial in the presence of negatively-charged lipids (Jiang et al., 2015).

Furthermore, antimicrobial peptides also have the bioactivity of immunomodulation. For instance, AMPs can dissolve the microbial membranes and help release inflammatory stimulus signal, promote the growth of fibroblasts to accelerate the recovery of the wounds, and inhibit specific enzymes to decrease further damage to the wounds (Steinstraesser et al., 2011).

With such amazing properties, AMPs have raised great interests among the whole pharmaceutic field, and are thought to be a promising potent therapeutic to a variety of diseases (Reuther et al., 2018).

All these advantages above demonstrate the promising potential of AMPs as a novel therapeutic agent. In recent years, AMPs have raised great interests among the pharmaceutical field for its potential to bring great revolution in human medicine (Marr et al., 2006). Unfortunately, AMPs are not rich in natural resources, so it is difficult to extract or purify AMPs from plants or animals or to put them into volume production (Mahlapuu et al., 2016). Moreover, the potential haemolytic activity and other safety problems still remain to be solved before AMPs used as a therapeutic agent (Wong et al., 2000).

1.3 Aims and objectives of this thesis

The aim of this study is to discover novel antimicrobial peptides from the frog skin secretion of *Odorrana livida* and to evaluate the biological activities.

Firstly, a peptide precursor-encoding cDNA from the skin secretion of *Odorrana livida* was isolated, acquired and identified using shotgun cloning, reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS).

Secondly, solid phase peptide synthesis (SPPS) was used to obtain adequate synthetic peptides and RP-HPLC and MS were used to identify and purify the synthetic peptide.

Then, the synthetic peptide was assessed on its biological activities, such as antimicrobial activity, anticancer activity and haemolysis activity. It is possible to reveal the mechanisms of these actions and structure-activity relationships during this study.

Chapter 2 Materials and Methods

2.1 Molecular Cloning

2.1.1 mRNA isolation

2.1.1.1 Preparation of skin secretion

Firstly, five mg of lyophilised skin secretion from *Odorrana livida* were dissolved in 1 ml of lysis/binding buffer in a 1.5 ml tube. Afterwards, the tube was vortexed for 1 min and then kept on ice for 1 min, and this step was repeated for 20 min in total. Finally, the tube was centrifuged for 5 min at 18,000 × g in an Eppendorf Centrifuge 5425 (Eppendorf, Germany) to obtain a clear cell lysate. The supernatants were prepared and stored cold until the beads were ready.

2.1.1.2 Preparation of Dynabeads® Oligo (Rothemund et al.)$_{25}$ beads

At first, 250 µl of thoroughly suspended Dynabeads® Oligo (Rothemund et al.)$_{25}$ beads were transferred into a 1.5ml sterilised tube and put on a magnetic rack. When the liquid was clear, the storage buffer was discarded thoroughly. Subsequently, 250 µl of fresh Lysis/Binding Buffer was added into the 1.5 ml tube and shaken gently. Overall, the supernatants in the 1.5 ml tube were removed thoroughly when the lysate was prepared.

2.1.1.3 Hybridisation between the poly A tail of mRNA and bead-bound oligo-dT

The supernatants from the lysate tube were transferred into the tube containing the prepared beads. The mixture of lysate and beads were shaken gently for blend for 1 min and kept on ice for 30s. This step was repeated for 18 min in total. Finally, the tube was placed on the magnetic rack after the supernatants were discarded completely.

2.1.1.4 Washing

The beads/mRNA complex was washed slowly and gently using 500 µl of Washing Buffer A for three times. The beads were separated from the washing solution and the supernatants were discarded with the help of the magnetic rack. Similarly, the beads/mRNA complex was washed slowly with 500 µl of Washing Buffer B for 2 times.

2.1.1.5 Elution

At first, 18 µl of Tris-HCl (10 mM) was added drop by drop into the 1.5 ml tube containing the well-washed beads and the tube was flicked until all the solution ran through. Then, the 1.5 ml tube was heated in the heating block at 80°C for 2 min. All supernatant containing the mRNA was carefully transferred into a 0.2 ml PCR tube on the magnetic rack immediately to avoid recombination between mRNA and beads. Finally, the 0.2 ml PCR tube was placed on ice to cool for 2 min and then the solution was allocated into 5 chilled-prepared 0.2 ml PCR tubes which contained 4 µl of mRNA product for three PCR tubes and 3 µl of mRNA product for two PCR tubes respectively.

2.1.2 cDNA library construction

A BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK) was applied to construct the first strand cDNA and amplify primary cDNA. 5'-RACE Ready cDNA was synthesised using a 5'-RACE CDs Primer (5'–$(T)_{25}$VN–3') and the BD SMART II^{TM} A Oligonucleotide which contained a terminal stretch of G residues to pair dC-rich cDNA tail at the end. 3'-RACE Ready cDNA was synthesised using 3'-RACE CDs Primer (5'–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN–3') by a reverse transcription reaction.

2.1.2.1 Preparation of sample mixture

Preparation for 3'-RACE Ready cDNA synthesis

The following components were combined and mixed completely by pipetting in three 0.2 ml PCR tubes respectively. One extra volume of reagents was calculated and added to ensure sufficient volume for the reaction.

Table 2.1 The components of 3'-RACE cDNA reaction.

Reagent	Volume	Final concentration
mRNA sample	4 µl	40%
3'-RACE CDS primer	1 µl	1 µM
5X First-Strand Buffer	2 µl	1X
DTT	1 µl	2 mM
dNTP Mix	1 µl	1 mM
BD RTase	1 µl	10 Unit/µl

*The Master Mix includes dNTP Mix, DTT and 5X First-Strand Buffer for 5 reactions.

Preparation for 5'-RACE Ready cDNA synthesis

The following components were combined and mixed completely by pipetting in two 0.2 ml PCR tubes. One extra volume of reagents was calculated and added to ensure sufficient volume for the RT-PCR reaction.

Table 2.2 The components of 5'-RACE cDNA reaction.

Reagent	Volume	Final concentration
mRNA sample	3 µl	40%
5'-CDS primer	1 µl	1 µM
SMART II	1 µl	1 µM
5X First-Strand Buffer	2 µl	1X
DTT	1 µl	2 mM
dNTP Mix	1 µl	1 mM
BD RTase	1 µl	10 Unit/µl

*The Master Mix includes dNTP Mix, DTT and 5X First-Strand Buffer for 5 reactions.

2.1.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

All of five tubes containing sample mixture were firstly centrifuged briefly, then incubated in the heating block at 70°C for 2 min to combine the primer and templates, and cooled on ice for 2 min. Afterwards, 4 µl of prepared Master Mix was divided into each PCR tube and blended completely with a pipette. Additionally, 1 µl of Reverse Transcriptase was added into each 0.2 ml PCR tube and pipetted thoroughly. After all reagents were added, 5 tubes were micro-centrifuged to collect all contents at the bottom without bubbles and incubated in the thermal cycler (Applied Biosystems, UK) at 42°C for 1.5 h to complete the reverse transcription reaction.

2.1.2.3 Concentration dilution and fault correcting

Fifty µl of PCR water was added into each 0.2 ml PCR tube to lower the concentration. Subsequently, the five 0.2 ml tubes were pipetted, centrifuged briefly and then incubated in the thermal cycler at 72°C for 7 min to correct faults in the reaction and kill some enzymes such as Reverse Transcriptase. Ultimately, 3'-and 5'-RACE Ready cDNA templates were obtained and stored at -20°C in the freezer.

2.1.3 Rapid amplification of cDNA ends (RACE) PCR

A BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK) was employed in RACE-PCR**.** The following components were mixed completely by pipetting in a PCR tube: 12.4 µl PCR water, 6 µl 10 × advantage 2 PCR Buffer, 0.8 µl dNTP, 2 µl sense primer (5'-GAWYYAYYHGADHCBAAAGATGTTCA-3'), 2 µl NUP and 0.8 µl 50 × advantage 2 Polymerase Mix, and an extra volume has been calculated and added to ensure sufficient volume for the RACE-PCR reaction.

Firstly, 12 µl of Master Mix was transferred from the original PCR tube (Tube 1) into another one (Tube 2). 10 µl of 3' RACE-Ready cDNA templates were added into Tube 1 and 10 µl PCR water was added into Tube 2. Eleven µl of Tube 1 components were

transferred into another PCR tube (Tube 3) and 11 µl of Tube 2 reagents were added into a new PCR tube (Tube 4). All of the reagents were mixed thoroughly and centrifuged briefly. Three steps of PCR reaction with different conditions were set up and each cycle included 96°C denaturation for 20s, primer annealing at 67°C (Tube 1 and 2) and 69°C (Tube 3 and 4) for 30s and extension at 60°C for 4 min. All the procedures were repeated over 40 thermal cycles for double-stranded DNA amplification. The annealing temperature in one group included one 3' RACE-Ready cDNA template and one negative control at 67°C, whereas it was set at 69°C in another group. All of the four samples were stored at -20°C in the freezer after RACE-PCR reaction.

2.1.4 Agarose gel electrophoresis

At first, 0.45 g of agarose powder (Invitrogen, UK) was transferred into a 200 ml flask with 35 ml of freshly prepared 1 × Tris/Borate/EDTA (TBE) buffer (Invitrogen, UK) (Agarose gel: ≥ 1% w/v). The flask was heated in a microwave oven until all the agarose powder was dissolved completely, and then it was cooled to around 65°C. Next, 2.5 µl of 10 mg/ml Ethidium Bromide (EB) (Sigma-Aldrich, USA) was added into the flask and mixed. Then, the gel box was set with two well-placed blocks in the gel electrophoresis tank, before the melted agarose was poured in. And then the comb was vertically inserted into the melted gel. After the solidification of the agarose gel, the comb was vertically removed and the loading wells were obtained. Ultimately, recycling 1 × TBE Buffer was poured into the gel tank no more than the maximum lines.

Then, 2 µl of DNA ladder (BioLabs, UK) composed of several fragments of known molecular weight, was loaded into the first loading well of the agarose gel. Then, 1.5 µl of the four samples and 0.5 µl of 6 × Loading Dye (Promega, USA) were mixed evenly and loaded in the other wells in order. Next, the electrophoresis was turned on at 90 V and the samples ran through the gel from the negative electrode to the positive electrode

until the yellow colour indicator reached two-thirds of the gel. Finally, the electrophoresis was turned off and the gel was transferred for detection of bands. The 1 × TBE Buffer was collected for recycle and the samples were stored in the freezer at -20°C.

The gel was placed under the UV trans-illuminator BioDoc-It® Imaging System (NVP, Cambridge, UK) and recorded in the form of a picture as the result. The DNA bands of the samples were compared with the ladder to determine whether the DNA amplification was successful or not.

2.1.5 PCR products purification

An E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, USA) was employed for PCR product purification, and in this kit, the sample DNA was bound to silica-based filter membranes during washing steps and eluted for collection.

2.1.5.1 DNA binding with the filter membranes

Firstly, five times the sample volume of CP Buffer was added into Tube 1 and pipetted thoroughly. Then all the contents in Tube 1 was transferred into Tube 2 and mixed with a pipette. A filter cartridge was placed into a 2 ml wash tube and all the solution in Tube 2 was transferred onto the filter drop by drop. The whole cartridge was centrifuged at 13,000 × g for 1 min and the liquid in the wash tube was discarded.

2.1.5.2 DNA washing

At first, 700 µl of DNA Washing Buffer diluted in 100% ethanol was added into the cartridge and the cartridge was centrifuged at 13,000 × g for 1 min. Afterwards, the solution in the wash tube was discarded. Similarly, 500 µl of DNA Washing Buffer was added into the cartridge and the actions above was repeated. Then the cartridge with wash tube was centrifuged at 14,000 × g for 2 min.

2.1.5.3 DNA elution

The wash tube was discarded and replaced by a new clean 1.5 ml tube. Subsequently, 20 µl PCR water was added directly into the filter of the cartridge and the cartridge was incubated at room temperature for 2 min. After that, the cartridge with the 1.5 ml tube was centrifuged at 13,000 × g for 1 min. The cartridge was then discarded and the 1.5 ml tube with DNA purification products was retained. Finally, the DNA purification products were placed in a concentrator (Eppendorf, Hamburg, Germany) for 1 h to dry the DNA sample and drive the ethanol away thoroughly. After evaporation, the DNA sample was sealed with parafilm and stored at -20°C.

2.1.6 Ligation

A pGEM®-T and pGEM®-T Easy Vector kit (Promega, USA) was employed for ligation, transformation, blue and white colony screening and isolation of recombinant DNA reactions. The DNA with A at both ends of the strand could bind to and insert into the site of the pGEM®-T Easy Vector (50 ng/µl) with T through A-T based pairing.

2.1.6.1 Reagent preparation

At the beginning, 6 µl of PCR water were added into the 1.5 ml tube containing DNA purification product to dissolve DNA. Then tube was vortexed intermittently for 30 seconds, centrifuged briefly and placed on ice to cool for 30 s. This step was repeated 3 times. The 2 × Rapid Ligation Buffer was pipetted vigorously and 2.5 µl of it was transferred into a 0.2 ml tube. Also, pGEM®-T Easy Vector were micro-centrifuged briefly without pipetting to avoid damaging the fragile vectors and 0.5 µl of the vector was added into the 0.2 ml tube gently. Then 1.5 µl sample was transferred into the tube. The T4 DNA Ligase was also micro-centrifuged briefly without pipetting and 0.5 µl was added into the tube. There were no bubbles in the liquid and no liquid drops at the tube

wall. The tube was incubated at room temperature for 1 hour and then transferred into 4°C incubator overnight.

2.1.7 Transformation

The recombinant vectors were transformed into the competent cells and selected by ampicillin, IPTG and X-Gal using the pGEM®-T and pGEM®-T Easy Vector kit (Promega, USA).

2.1.7.1 Preparation of LB/Ampicillin/ ITPG/ X-Gal plates

LB agar (Invitrogen, UK) was weighed and dissolved in 200 ml double deionised water in a 400 ml glass bottle to obtain the LB agar solution (100 Unit/ml). The bottle was then autoclaved for sterilisation. Next, 550 µl of Ampicillin (Roche, USA) was added into the heated agar solution and mixed completely. Then 10 ml of melted agar solution was allocated and added into each Petri dish. After the solidification of agar, 110 µl of Isopropyl β-D-thiogalactoside (IPTG) (Promega, USA) and 20 µl of 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Promega, USA) were added on order and spread symmetrically and lightly over the surface completely. Ultimately, all the plates were incubated upside down at 37°C for 30 min for activation, and then the plates were ready for cell or bacterial culture.

2.1.7.2 Transformation

At first, 2.3 µl of ligation products were transferred into a 1.5 ml tube without pipetting. And then the JM109 cells (Promega, USA) were taken out from -80°C storage and put on ice for defrosting for 4 min until all were thawed and clarified. Then, 50 µl of JM109 cells were transferred quickly into the 1.5 ml tube containing ligation products. Both of the contents were mixed by gentle flicking and tapping at the bottom of the tube and then kept on ice for 20 min. After that, the 1.5 ml tube was heat-shocked at exactly 42°C for 47s and placed on ice immediately for 2 min. Finally, 950 µl of S.O.C medium

(Invitrogen, USA) was added to the 1.5 ml tube gently and the tube was then incubated at 37°C for 2.5 h at a shaking rate of 150 rpm in the shaking incubator.

2.1.7.3 Plating and culture for amplification

In this step, 80 µl, 90 µl and 100 µl of transformation suspensions were transferred and spread over the surface of LB/Ampicillin/ ITPG/ X-Gal plates respectively in two replicates and all plates were incubated upside down at 37°C overnight (16-24 h) for bacterial culture and DNA amplification.

2.1.8 Blue and white colony screening

Three new LB/Ampicillin/ ITPG/ X-Gal plates were divided into 17 squares by drawing lines at the bottom, then the pure white colonies containing recombinant plasmid DNA were picked up and transferred onto plates by streaking without touching the edge of the lines using an inoculating loop under a sterile environment. All the three plates were incubated upside down at 37°C overnight (16-24 h) for subculture.

2.1.9 Isolation of recombinant DNA by cloning PCR

The bacteria in the white colonies were harvested from the plates and transferred into each 1.5 ml tube containing 20 µl deionised water for dispersion. After that, 10 samples of bacteria were selected for recombinant DNA isolation. These 1.5 ml tubes containing samples were incubated in the 100°C heating block and then cooled on ice for 5 min immediately. Subsequently, each tube was vortexed for 30 s and centrifuged at the maximum speed of 8000 × g for 5 min. Finally, all the tubed were stored at -20°C.

2.1.10 Cloning PCR of plasmid DNA

The following components were added into a tube and mixed completely by pipetting.

Table 2.5 The components of each tube in cloning PCR reaction.

Reagent	Volume	Final concentration
5× Cloning Buffer	10 µl	1×
dNTP Mix	1 µl	0.2 mM
M13F	2.5 µl	1 µM
M13R	2.5 µl	1 µM
PCR-Grande water	31 µl	-
Taq polymerase	0.25 µl	0.025 Unit/µl
DNA template	2.5 µl	10-1000 ng

At first, 47.25 µl of Master Mix was added into 14 PCR tubes respectively and mixed completely. The samples were centrifuged at 18000 × g for 5 min and 2.5 µl of the suspensions were added into each tube. Finally, the tubes were applied into cloning PCR and the reaction programme was set as: denaturation at 94°C for 30s, annealing at 55°C for 30 s and extension at 72°C for 3 min, repeated 31 times over a total in 3 h 15 min. After this reaction, the sample tubes were stored at -20°C in the freezer.

2.1.11 Agarose gel electrophoresis analysis

The products of cloning PCR were mostly subjected to gel electrophoresis as described in former section 2.1.4 except for no need to add loading dye in this section.

2.1.12 Selected PCR products purification

The selected PCR products were then purified and washed out according to the procedure described for PCR products purification in section 2.1.5. Instead of 30 µl of PCR water, 20 µl of PCR water was utilised in the DNA elution.

2.1.13 DNA sequencing reaction

A BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used in the DNA sequencing reaction in which the sequence was detected by fluorescence during DNA extension and termination process.

2.1.13.1 Preparation of mixture for sequencing PCR reaction

Seven optimal DNA samples were chosen for the sequencing reaction. The following components were combined and mixed completely by pipetting with an extra volume added to ensure sufficient volume.

Table 2.6 Components in each sequencing reaction tube.

Reagent	Volume	Final concentration
PCR-Grande water	12.4 µl	-
Diluted M13F or M13R	1.14 µl	0.8 µM
2.5× Ready reaction mix	2.86 µl	13.68%
5× BigDye Sequencing Buffer	3.57 µl	1×
Purified cloned PCR products	2.5 µl	10-1000 ng

2.1.13.2 DNA sequencing reaction

Master Mix and 2.5 µl of sample were allocated into 0.2 ml PCR tubes. The sequencing PCR reaction was set and commenced using the following programme and each cycle in the thermal cycler included: 96°C denaturation for 20s, 55°C annealing for 10 s and 60°C extension for 4 min, repeated 26 times over a total in 2 h 5 min. After this reaction, the 7 samples tubes were stored at -20°C in the freezer.

2.1.14 Extension product purification by ethanol

2.1.14.1 Reagent preparation

Six ml of PCR-Grade water and 14 ml of ethanol were mixed for the 70% ethanol preparation and 1 ml of PCR-Grade water and 19 ml of ethanol were mixed completely to produce a 95% ethanol preparation.

2.1.14.2 Ethanol purification

At the beginning, 72 µl of 95% ethanol (Sigma-Aldrich, USA) were added into the PCR tube with sequencing reaction products and pipetted vigorously. Subsequently, all the solutions are transferred into a 1.5 ml tube with10 µl PCR-Grade water. Each of the 4 tubes was vortexed for 30 s and incubated at room temperature for 20 min and then centrifuged at the maximum 20,000 × g for 20 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany). Immediately after this, the supernatants were discarded completely. Similarly, 260 µl of 70% ethanol were added into each 1.5 ml tube with sequencing reaction products and mixed, vortexed for 30 s and then centrifuged at 20,000 × g for 10 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany). Then the supernatants were discarded immediately. Afterwards, all 1.5 ml tubes were heated in the 95°C heating block for 1 min and cooled on ice for another 1 min with the lid of each tube open. This thermal cycle was repeated 3 times. After thermal cycling, the contents of the 1.5 ml tubes were concentrated for 3 h to dry the DNA and to drive the ethanol off. Finally, the 4 samples were stored at -20°C in the freezer.

2.1.15 Sequencing

At first, 10.3 µl HiDi (highly deionised-formamide) was added to each sample which had been concentrated for 1 h before use. Then, all the tubes were vortexed for 30 s and then centrifuged briefly. Afterwards, the tubes were heated in the 95°C heating block for 4.5 min and cooled on ice for 3 min. Subsequently, 9 µl of well-prepared sample mixture

was loaded into a 96-well sequencing plate in odd or even rows. Ultimately, the DNA was sequenced using an ABI 3730 automated sequencer (Applied Biosystems, USA). The elongation of DNA strands in the solution was terminated by the modified ddNTPs randomly and detected by fluorescence.

2.2 Solid-phase Peptide Synthesis (SPPS)

Solid-phase peptide synthesis was carried out employing a Tribute Peptide Synthesiser (Protein Technologies, Inc., AZ, USA). The peptide was synthesised from C-terminal to N-terminal, which is opposite to the synthesis in cells. Resin was located at the C-terminal of the first amino acid to protect it. This resin was insoluble in the solvents used for synthesis, making it relatively simple and fast to wash away excess reagents and by-products. And each amino acid contained an Fmoc as the protecting group in the N-terminal, which was stable in acid but removable in base. Side chain functional groups were protected with groups which were stable in base but labile in acid. And these protecting groups were remove in the final deprotection.

There were 3 steps in the solid-phase peptide synthesis, deprotection of free amine groups, coupling of peptide bonds and cleavage from the resin. Firstly, the Fmoc amino acids were dissolved and 20% piperidine in DMF was used to remove the Fmoc groups from the amine groups. Then HBTU was used to activate the carbonyl group of the next amino-protected amino acid, so that the activated monomer could react with the free amidogen to connect to the peptide chain. Finally, the peptide was cleaved from the resin and the side-chain protecting groups were removed by cleavage cocktail.

2.2.1 Preparation

Three things constitute an amino acid vial: a vial, a cap and a septum. The vials were washed with detergent, rinsed with acetone and dried in a fume cupboard before use.

Each amino acid was used in a 4-fold molar excess to synthesise 0.3 mmol peptide, thus 1.2 mmol of each amino acid in the sequence was weighed. 1.2 mmol HBTU, as the activator, was also weighed and added into each amino acid containing vial to catalyse each coupling. The resin was weighed just before the synthesis and transferred into a reaction vessel, and the weight should follow this formula: the weight (g) = (peptide mmol)/ (loading capacity mmol/g).

2.2.2 Peptide synthesis

This procedure was performed using a Tribute peptide synthesiser (Protein Technologies, Inc. AZ, USA).

There are 5 bottles of reagents involved in the procedure of synthesis with dimethylformamide (DMF) in the bottle 1 and 2, 20% piperidine and 80% DMF in the bottle 3, 11% NMM and 89% DMF in the bottle 4 and Dichloromethane (DCM) in the bottle 5.

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.

2.2.3 Cleavage and deprotection

The synthesised peptide with its support resin was weighed and then removed into a 50 ml round-bottomed flask. Then the peptide/resin was incubated with the following cocktail recipe to cleave the side-chain protecting groups and resin, 94% Trifluoroacetic acid (TFA), 2% Thioanisole (TIS), 2% 1,2-Ethanedithiol (EDT) and 2% H_2O v/v. The cocktail volume was based on 1 g per 25 ml. The flask was then left on a magnetic stirrer

for 6 h to allow the reaction to go to completion. After this, the solution was placed on a filter device and washed three times with DCM. The peptide solution was collected at the bottom of the filter apparatus. A Rotovap apparatus was used to remove the solvent and 45 ml of diethyl ether (EtO_2) was added to precipitate the peptide from solution in a 50ml tube and the tube was then stored in the freezer at -20℃ overnight.

2.2.4 Oxidation

The sequence of QUB-1985 contains two cysteine residues, so the 50 ml tube was placed at room temperature with a porous cover for oxidation for 3 days. The mixture was shaken once every hour.

2.2.5 Precipitate washing and lyophilisation

Firstly, 45 ml EtO_2 was added into the tube and the tube was centrifuged at 2500 × g for 5 min, and the EtO_2 was discarded carefully. Then another 45 ml of EtO_2 were added and the tube was centrifuged again. This step was repeated 3 times, and then the tube was placed at room temperature overnight to dry the peptide. The peptide was dissolved in appropriate volume of HPLC Buffer B (80% acetonitrile, ACN, 19.95% H_2O and 0.05% TFA) and Buffer A (99.95% H_2O and 0.05% TFA). The final volume was about 20-25 ml and the peptide was completely dissolved. The tube was placed into an Alpha 1-2 freeze-drying system (Martinchrist, Germany) for lyophilisation. After 24-48 h, the peptide was dried, and the tube was sealed with the lid and stored at -20°C in the freezer.

2.3 MALDI-TOF identification

A MALDI-TOF mass spectrometer (Voyager DE, PerSeptive Biosystems, Framingham, MA, USA) was used for mass analyses. For MALDI ionisation, a matrix solution (α-cyano-4-hydroxycinnamic acid, CHCA) was prepared as a 10 mg/ml solution of CHCA

in acetonitrile/TFA/water (50/0.05/49.95, v/v/v). Subsequently, 1 µl of the samples of each fraction were spotted onto a MALDI plate and left to dry, then 1 µl of the matrix solution was added to the same spot and left to dry.

The target plate was then placed into the Linear MALDI-TOF mass spectrometer (Voyager DE, PerSeptive Biosystems, Framingham, MA, USA). The masses were recorded as mass/charge ratio (m/z) against abundance and the masses observed were compared with the theoretical mass values that had been calculated earlier.

The lyophilised peptide was subjected to MALDI-TOF MS analysis to examine the purity of product and accuracy of the solid-phase synthesis.

2.4 Reversed-phase high performance liquid chromatography (RP-HPLC)

Firstly, 8 mg of crude lyophilised peptide was weighed and transferred into a 1.5 ml tube and mixed with 500 µl Buffer B (TFA/water/ACN, 0.05/19.95/80) and 500 µl Buffer A (TFA/water, 0.05/99.95). Then, the 1.5 ml tube was vortexed and centrifuged at the maximum speed for 15 min. The clear supernatant was transferred into a new 1.5 ml universal tube. An analytical reverse phase HPLC Jupiter C5 column (250nm × 10 mm, Phenomenex, UK) was prewashed with Buffer B for 40 min and equilibrated in Buffer A for 30 min before use. Subsequently, 1 ml of clear supernatant was pumped onto the Jupiter C5 column on a Cecil Adept CE4200 HPLC system (Cecil, Cambridge, UK). The peptide was eluted from the column with a linear gradient from 100% Buffer A with no Buffer B to 100% Buffer B over 80 min at a flow rate of 1 ml/min. The fractions were collected in polypropylene tubes (Sarstedt, Germany) at every peak and utilised for identification.

2.5 Antimicrobial assay

Three model microorganisms, including Gram-positive bacteria (*Staphylococcus aureus*, NCTC 10788), Gram-negative bacteria (*Escherichia coli*, NCTC 10418) and the fungi (*Candida albicans*, NCPF 1467) were applied in this section to assay the antimicrobial potent of the QUB-1985.

2.5.1 Microorganism inoculation

Each microorganism stock culture was taken from frozen storage, and one bead from each stock was placed into separate marked flasks containing 100ml Mueller Hinton Broth (MHB). The cultures were incubated overnight (16-20 h) in the 37°C orbital incubator.

2.5.2 Subculture

After inoculation, 500 µl of microorganism culture was transferred into a pre-warmed 50 ml tube with 20 ml MHB medium. Then, the tube was placed into the orbital incubator (Stuart, UK) for incubation at 37°C for several hours until the sub-cultured microorganisms reached their respective logarithmic growth phases. A spectrometer (λ=550nm) was used to obtain the optical density (OD) to determine whether the log phase of growth was reached. In the following table, the appropriate OD values of the three kinds of microorganism cultures and their corresponding concentrations are given. 100 µl of subculture suspensions of *S. aureus* or *E. coli* were transferred into a Petri dish with 19.9 ml of pre-warmed MHB medium and shaken gently to mix. In terms of *C. albicans*, 2 ml of subculture suspension was mixed with 18 ml of pre-warmed MHB medium in the Petri dish.

Table 2.7 The appropriate OD values for the three microorganisms used in this assay.

Organism	Incubation	OD(λ=550nm)	Concentration(cfu/ml)
S. aureus	1.5 h	0.23	1×10^8
E. coli	1 h	0.41	1×10^8
C. albicans	45 min	0.15	1×10^6

2.5.3 Peptide preparation

Two and a half mg of lyophilised peptide (QUB-1985) were dissolved in an appropriate volume of the reagent dimethyl sulphoxide (DMSO) to the concentration of 512×10^2 µM and double-diluted in the ratio of 1:1 in DMSO to produce a series of concentrations - 256, 128, 64, 32, 16, 8, 4, 2, 1($\times 10^2$ µM), which were diluted 100-fold when added to the required plate, for achieving the final concentration from 512 to 1 µM.

2.5.4 MIC measurements

In one well of a 96-well plate, 1 µl of peptide solution was added and repeated 5 times at each concentration, followed by 99 µl adjusted microorganism suspension. While 100 µl of adjusted microorganism suspension were added in 5 replicates as growth controls, 100 µl of pre-warmed MHB medium were added in 5 replicates as negative blank controls. Additionally, 1 µl DMSO with 99 µl adjusted microorganism suspensions in 5 replicates were added as vehicle controls. And then each 96-well plate was incubated in the orbital incubator (Stuart, UK) for 5 min and transferred into the incubator (Genlab Limited, UK) to culture at 37°C overnight (16-20 h). Afterwards, the plate was measured by the Synergy HT plate reader (BioTek, USA) at 550 nm wavelength to obtain the absorbance of each well. Finally, the MIC value was determined as the lowest concentration wells in which no growth of organism was detected.

2.5.5 Viable cell counts

One hundred µl adjusted microorganism suspension was mixed with 900 µl PBS in a 1.5 ml sterile tube using a pipette. Then, 10-fold dilutions were prepared from this tube into 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} 10^{-6} into another 5 new tubes. Next, 20 µl of each concentration culture were spotted onto the dried Mueller Hinton Agar (MHA) plate in 3 replicates. After the spots of microorganism culture were dried, the plate was put into the incubator at 37°C for overnight (16-20 h) incubation. Then the numbers of the microorganisms in each drop were counted. Finally, the exact concentrations of microorganisms were calculated using the following formula:

$$C = N/3 \times 50 \times 10^{n},$$

where N represented the total quantity of the bacteria at each concentration and n was the ratio of dilution.

2.5.6 Minimum bactericidal concentration (MBC) measurement

Five µl was taken from each well of the 5 at clear concentrations and transferred onto a pre-sterilised MHA plate and incubated overnight to determine the MBC. After 24 h, these plates were examined for microorganism growth and the wells that showed no colonies growth were considered to be the values for the MBC.

2.6 Haemolysis assay

2.6.1 Preparation of horse red blood cell suspension

Firstly, 2 ml fresh horse blood was transferred into a 50 ml centrifuge tube. Then 30 ml autoclaved phosphate buffered saline (PBS) was transferred into the tube and the tube was gently shaken to mix the liquid on a rotating mixer. Next, the tube was centrifuged at 1000 × g for 5 min and the supernatant was discarded thoroughly. This process was

repeated until the supernatant was completely clear. Overall, 50 ml of autoclaved PBS was added into the tube to make a 4% (v/v) erythrocyte suspension.

2.6.2 Peptide preparation

Peptide was dissolved in sterilised PBS to make peptide solutions at concentrations from 512 µM to 2 µM. Next, 200 µl of each concentration of peptide solution were mixed with 200 µl of prepared red blood cell suspension to achieve the final concentration of peptide from 256 to 1 µM. Then 4 µl Triton X-100 were mixed with 196 µl of PBS and these were added into 200 µl RBC suspension as the positive control of 1% TritonX-100. Two hundred µl RBC suspension was mixed with 200 µl PBS as the negative control. All these solutions were added into 1.5 ml tubes and each concentration and control had 5 replicates. All tubes were then incubated at 37ºC for 2 h.

2.6.3 Measurements and calculation

After incubation, all samples were centrifuged at 1000 × g for 5 min and then 200 µl of supernatant of each tube were transferred into the walls of a 96-well plate. The plate was scanned at λ=550 nm with an ELx880^{TM} Absorbance Microplate Reader (BioTek, USA).

2.7 Anticancer assay

2.7.1 Resuscitation of frozen cell lines

Firstly, a water bath was preheated to 37ºC and kept at this temperature. Then the human cancer cells were taken out from -80 ºC storage and put into the water bath immediately. A 15 ml centrifuge tube was prepared and 10 ml pre-warmed complete medium was transferred into it. After thawed, all cells were transferred in to the 15 ml centrifuge tube and centrifuged at 200 × g for 5 min. The supernatant containing DMSO was discarded while the cells at bottom were not disturbed. After that, 1 ml of medium was added into

the tube and mixed with the cells by gentle pipetting, and then all the solution was transferred into 75 cm^2 culture flasks. Finally, the flask was incubated at 37°C with 5% CO_2 atmosphere after the cells were examined by an inverted microscope.

2.7.2 Subculture of Adherent Cell Lines

First of all, the degree of confluence was evaluated using an inverted microscope before the subculture of cells. The spent medium in the flask was discarded and 10 ml sterile PBS was added into the flask to wash he cell monolayer, and then the PBS was removed. Next, 1 ml of trypsin/EDTA (Invitrogen, UK) solution was added into the flask covering the cell monolayer and then the flask was stored in the incubator for 2–10 min at 37 ºC. After incubation and the cells were ensured detached and floating by an inverted microscope, 10 ml complete medium was added onto the flask wall which might have any cells remained attached. Then all the solution was transferred into a sterile 15 ml centrifuge tube and centrifuged at 350 × g for 5 min. After the centrifuge, the supernatant was discarded, while 10 ml complete medium was added in and mixed with cells by vortexing. Finally, a specific volume was transferred and diluted to 10 ml into a new flask, and put into incubation at 37°C under 5% CO_2.

The medium was changed when the pH indicator in the medium changed colour or every 2 days.

2.7.3 MTT cell proliferation assay

The MTT cell viability assay was performed when 90% of the culture flask surface was covered by secondary passaged cells.

2.7.3.1 Cell quantification

The steps of cell washing, digestion, transfer, centrifugation and medium remove were the same as previous steps in cell passage. An appropriate volume of pre-warmed medium was added into a 15 ml tube. After that, 50 µl of cell suspension and an equivalent volume of 0.4% (w/v) trypan blue (Invitrogen, UK) were mixed in the tube. Then the mixture was loaded onto the AS1000 Improved Neubauer haemocytometer (Hawksley, UK). A microscope was used to observe and count the living cells and 3 squares were adopted for cell quantification. All living cells existing in the culture could be quantified according to the formula:

$$\text{concentration of cell lines (cells/ml)} = N/3 \times D \times 10^3,$$

while N represented the number of living cells and D represented dilution factor (D=2, Trypan Blue: cells= 1:1, v/v). And a desired concentration of 5×10^3 cells/well/100 µl was achieved by calculating the volume of cell suspension and fresh complete medium.

2.7.3.2 Plate seeding

Cell suspension and medium were mixed in an appropriate volume and diluted to a final concentration of 5×10^4 cells/ml. Next, 100 µl of the mixed cell culture were added into each well of the 96-well plated and then the plated was incubated in a 37°C, 5% CO_2 incubator for 24 h.

2.7.3.3 Cell starvation

The complete medium in the 96-well plate was discarded and 100 µl of FBS-free medium was refilled in each well. Then the cells were starved for 6-12 hours.

2.7.3.4 Dosing

Firstly, 5 mg of QUB-1985 was dissolved in sufficient PBS to acquire a stock solution with the concentration of 10^{-2} M. Then FBS-free medium was applied to dilute the stock solution into concentrations of 10^{-4} M and 10^{-5} M.

All the suspensions in the cultured plate were discarded thoroughly and 100 µl peptide dilutions at both concentrations were added in five replicates in the plate. Five replicates of FBS-free medium were added as blank control, while an equal volume of 0.1% of PBS solution was added as vehicle control. The status of cancer cells was observed under the microscope before the plated was put into a 37°C, 5% CO_2 incubator for 24 h incubation.

2.7.3.5 MTT assay

In a dark environment, 10 µl MTT solution (5mg/ml) was added into each well and the plate was put into the same incubator again for another 4-6 h. After the incubation, all the suspensions were discarded and 100 µl DMSO was added in the wells to dissolve the purple formazan crystals. Overall, the Synergy HT plate reader (Biotek, USA) was used to measure the absorbance of each well at 570 nm wavelength.

Chapter 3 Results

3.1 Molecular Cloning of the cDNA encoded precursor of QUB-1985

The “shotgun” cloning strategy was used in this project to discover peptide-encoded cDNAs, where a degenerate primer was used to seize highly-conserved sequences in similar species. A novel peptide encoded cDNA was cloned from the skin secretion library of *Odorrana livida*, encoding a single copy of an antimicrobial peptide. The open reading frame of the obtained peptide QUB-1985 consists of 67 amino acids, and the first 22 amino acids are the putative signal peptide. Following the signal peptide, there is an acidic amino acid rich peptide domain of 22 amino acids. The mature peptides consist of 21 amino acids, starting from the N-terminal Gly residue, which is cleaved at a typical propeptide convertase processing site, -KR- (Figure 3.1).

The results of A NCBI-BLAST search and a sequence alignment performed by CLUSTAL OMEGA show that the full-length open-reading frame amino acid sequence of QUB-1985 precursor exhibits a high degree of structural similarity with Nigrosin peptides (Figure 3.2A). The similarity between QUB-1985 and Nigrosin-OG17 is 85%, while the similarity between QUB-1985 and Nigrosin-OG20 is 87%. Their signal peptides exhibit a high degree of similarity with only 3 amino acid differences. Additionally, only 2 amino acids are different between each other in the mature peptide domain. The Ala at position 9 in Nigrosin-OG17 is replaced by a Val in QUB-1985, while the Asn at position 12 is replaced by a Lys. In Nigrosin-OG20, the position 9 Ala is replaced by Val as well in QUB-1985 and the His at position 12 is replaced by Lys.

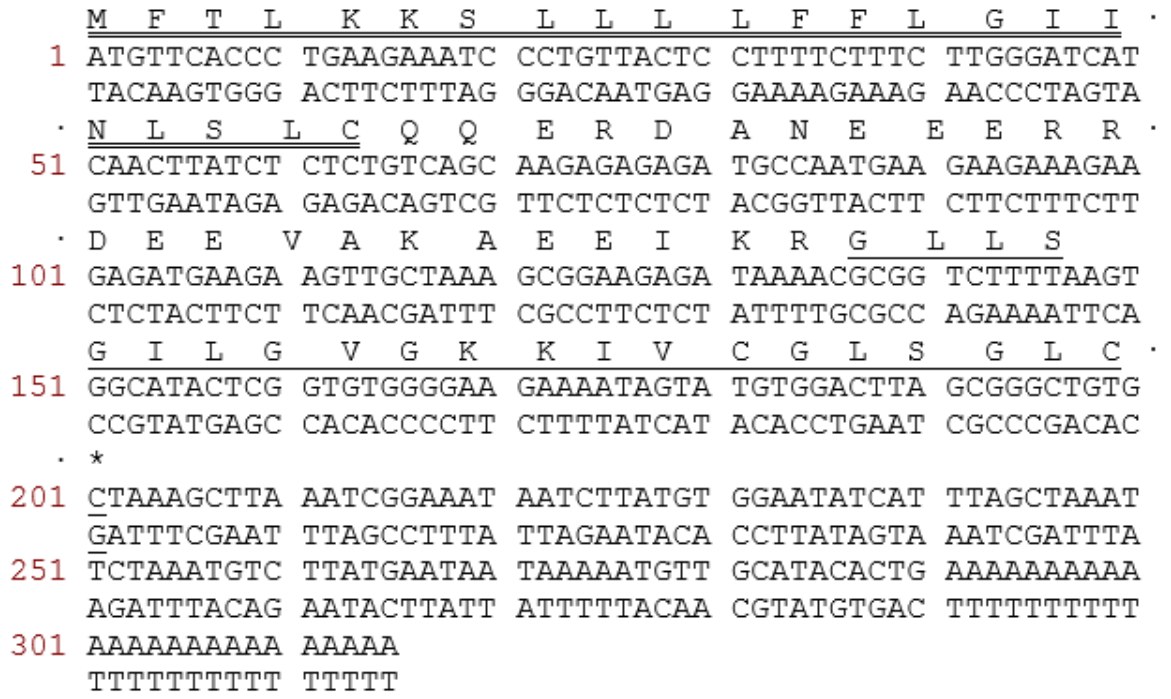


Figure 3.1 Nucleotide sequence and corresponding open-reading frame of the QUB-1985 peptide precursor encoding cDNA cloned from an *Odorrana livida* skin secretion-derived cDNA library. The double-underlined sequence at the beginning is the putative signal peptide and the mature peptide sequence is single-underlined, with an asterisk indicating the stop codon.

```
QUB-1985       1  MFTLKKSLLLLFFLGIINLSLCQQERDANEEERRDEEVAKAEEIKRGLLSGILGVGKKIV 60
Nigrosin-OG17  1  MFTLKKSMLLLFFLGTISLSLCEQERNADEEERRDGEVAKMEEIKRGLLSGILGAGKNIV 60
Nigrosin-OG20  1  MFTLKKSLLLPFFLGTINLSLCQDETNA-EEERRDEEVAKMEEIKRGLLSGILGAGKHIV 59
                  ******* ** **** * ****  *  * ****** **** ************ ** **

QUB-1985       61 CGLSGLC 67
Nigrosin-OG17  61 CGLSGLC 67
Nigrosin-OG20  60 CGLSGLC 66
                  *******
```

Figure 3.2 The alignment of the peptide precursor-encoding cDNAs of QUB-1985 and two prepropeptides discovered from the *Odorrana* species. The similarity between QUB-1985 and Nigrosin-OG17 is 85%, and the similarity between QUB-1985 and Nigrosin-OG20 is 87%. The identical amino acids are indicated by asterisks.

3.2 Peptide synthesis and purification of QUB-1985

After determination of the mature peptide sequence of QUB-1985 through the combination of shotgun cloning and BLAST analysis, the solid phase synthesis of this peptide was carried out. The MALDI-TOF analysis was subsequently performed to examine the products in the crude peptide replicates and the result turns out that the synthesis was successful due to the observation of the consistent mass of 1986.8 Da with the computed molecular weight of QUB-1985, though there were a variety of impurities produced along with the expected peptide sequence (Figure 3.3). The following RP-HPLC analysis of the crude peptide shows that there are three main impurities synthesised, which is consistent with the MALDI-TOF spectrum (Figure 3.4A). After that all the peaks were collected and subjected to MALDI-TOF, the peak eluted around 68 min was proved to be the purified QUB-1985 as the represented singly-charged ion was observed in the spectrum (Figure 3.4B). The retention position of QUB-1985 is pointed by an arrow.

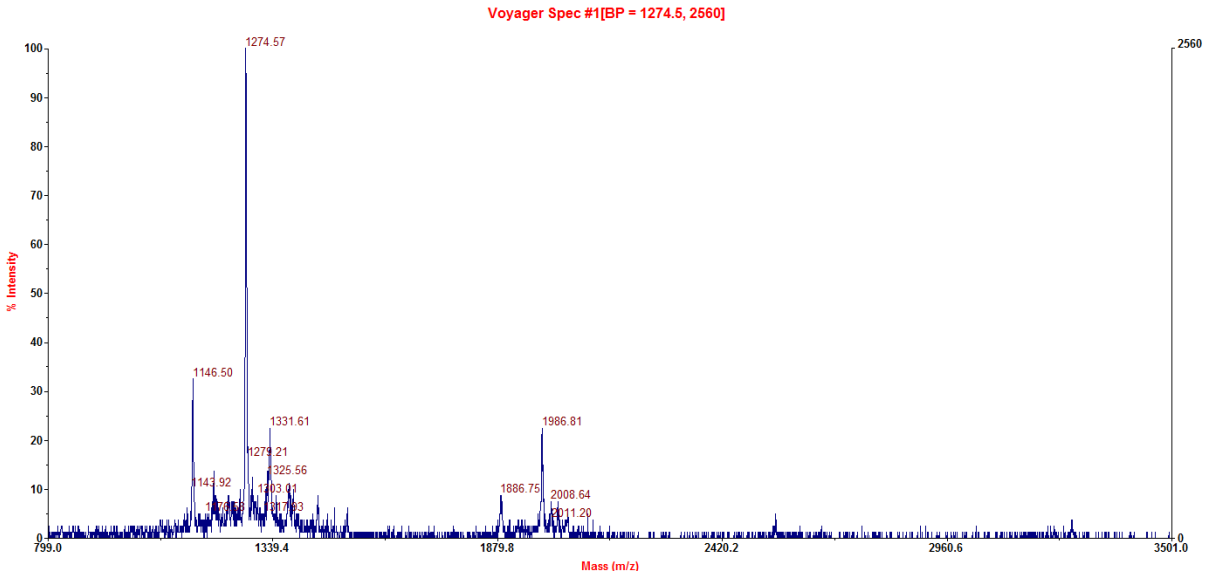
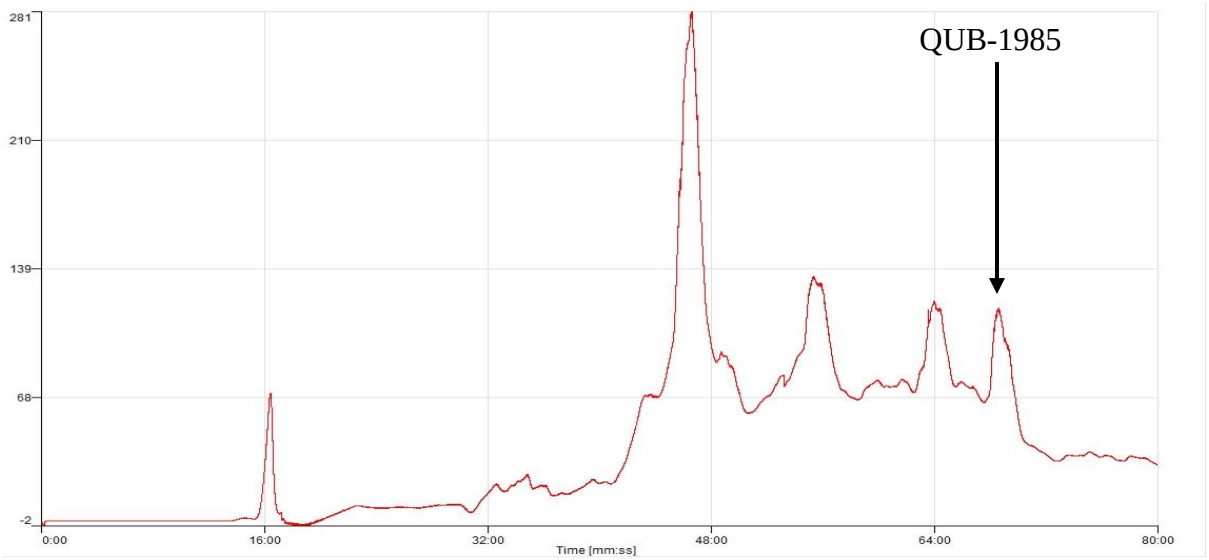


Figure 3.3 The MALDI-TOF MS analysis result of the crude peptide QUB-1985. The observed ion mass of synthetic QUB-1985 is 1986 Da.

A.



B.

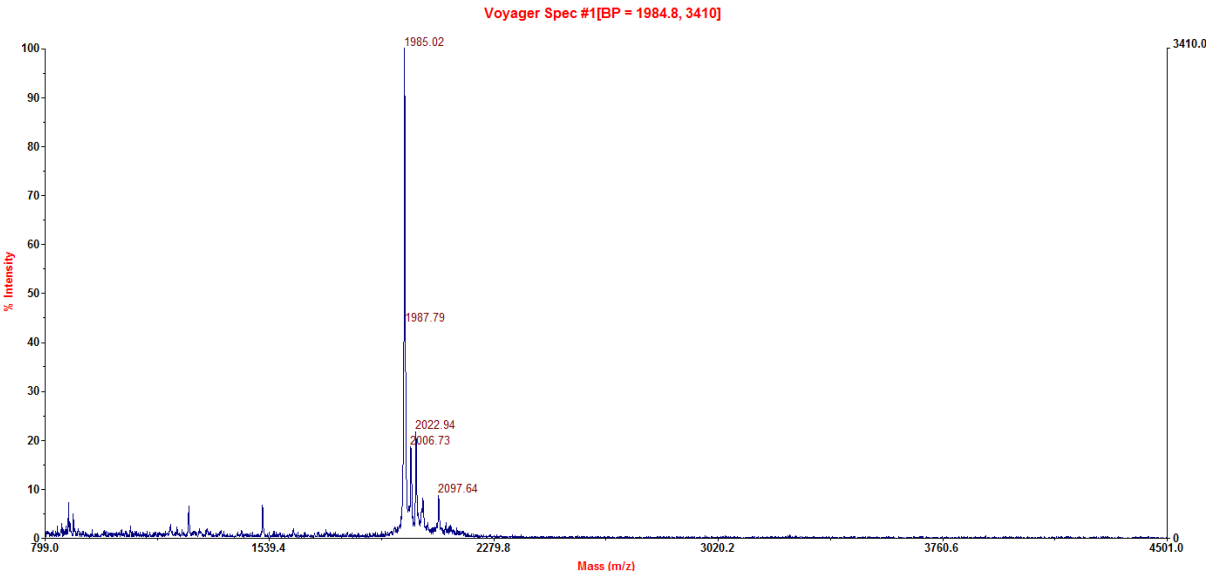
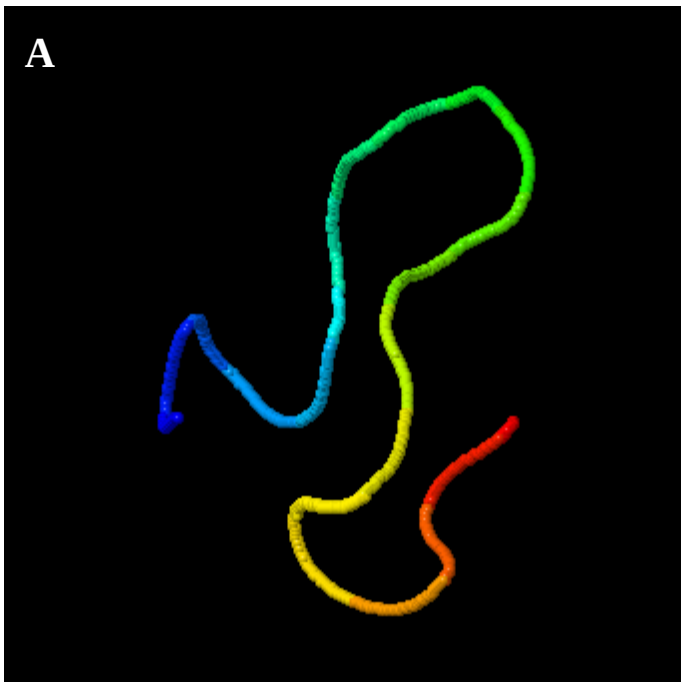


Figure 3.4 The RP-HPLC purification process for QUB-1985. (A) The 80 min chromatogram of crude peptide QUB-1985 with a gradient from 0 % ACN to 80% ACN. The retention time of QUB-1985 is 68 min. (B) The MALDI-TOF MS analysis of the HPLC fraction indicated in (A) by an arrow, which contains QUB-1985 with a high degree of purity. The observed mass is 1985.02 Da.

3.3 The Prediction of Secondary Structure of QUB-1985

The secondary structure of the QUB-1985 was predicted by I-TASSER. The optimal 3D structure prediction shows that the peptide possesses a structure of mainly random coil conformation, but a strand turn structure in the middle region (green region) (Figure 3.5A). Meanwhile, the secondary structure prediction also indicates that QUB-1985 possesses only random coil and strand structure without any helix (Figure 3.5B). However, the confidence scores of coil structure at N-terminal is quite low, which may indicate that there can possibly be a helical structure as the literature indicated (Conlon et al., 2006).The hydrophobicity is 0.741 and the hydrophobic moment is 0.474. The net charge of the peptide is +2, which is provided by the 2 lysine residues.



B

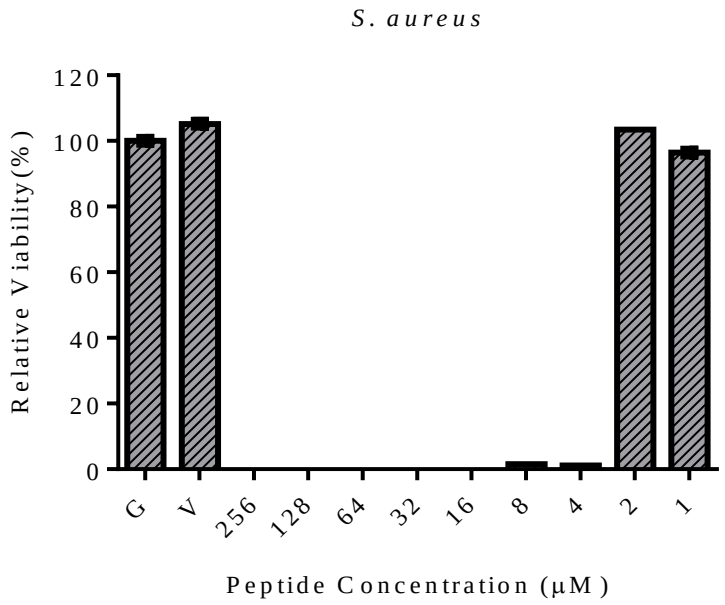
```
                    20
                    |
Sequence    GLLSGILGVGKKIVCGLSGLC
Prediction  CCCCCCCCCCSSSSSCCCCC
Conf.Score  94200003685478512335 9
            H:Helix; S:Strand; C:Coil
```

Figure 3.5 The computational structure modelling of QUB-1985. (A) The predicted 3D structure of the peptide QUB-1985 (C score: -1.74; TM-score = 0.50±0.15; RMSD = 4.3±2.9Å) (B) The secondary structure of QUB-1985. The grey "C" represents the random coil structure, while the blue "S" represents strand structure. The confidence score demonstrates the confidence level of the predicted structures.

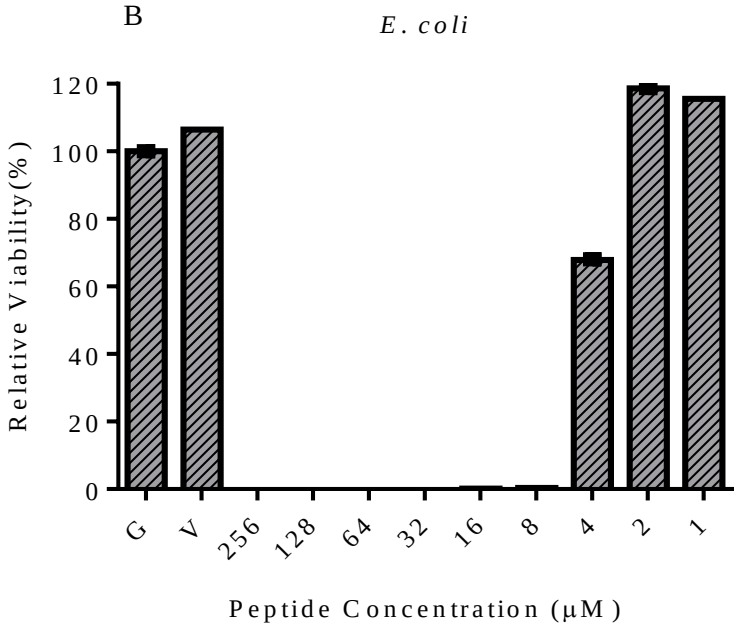
3.4 Antimicrobial Activity of QUB-1985

In order to examine the bioactivity of the peptide QUB-1985, Gram-positive bacteria, *S. aureus*; Gram-negative bacteria, *E. coli* and the pathogenic yeast, *C. albicans* were selected for the evaluation of the antimicrobial property of QUB-1985 (Figure 3.7). The MIC of QUB-1985 against *S. aureus* is 4 µM, while the MBC is 8 µM. The MIC against Gram-negative bacteria *E. coli* is two times higher than which of *S. aureus*, at 8 µM and its MBC is 16 µM. However, the peptide shows the lowest antimicrobial potency against the yeast *C. albicans* at 16 µM, and the MBC is 128 µM (Table 3.1).

A


S. aureus
Relative Viability(%)
120
100
80
60
40
20
0
G
V
256
128
64
32
16
8
4
2
1
Peptide Concentration (μM)

B


E. coli
Relative Viability(%)
120
100
80
60
40
20
0
G
V
256
128
64
32
16
8
4
2
1
Peptide Concentration (μM)

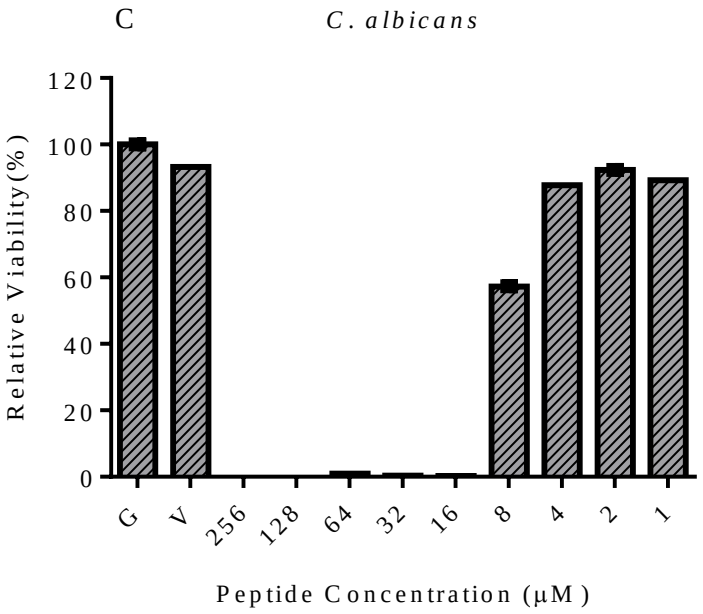


Figure 3.7. The antimicrobial activity of a series concentrations of QUB-1985 against *S. aureus* (A), *E. coli* (B) and *C. albicans* (C). G represents growth control group, which consists of only microorganism culture. V represents vehicle control group, which contains 1% DMSO with microorganism culture. The relative viability was calculated against the growth control group. The error bars represent the SD of five replicates.

Table 3.1. MICs and MBCs of peptide QUB-1985 against three microorganisms.

Peptide	MIC(μM)			MBC(μM)		
	S. aureus	*E. coli*	*C. albicans*	*S. aureus*	*E. coli*	*C. albicans*
QUB-1985	4	8	16	8	16	128

3.5 Haemolysis of QUB-1985 on horse red blood cells

Through the haemolysis assay, the side effect of the peptide QUB-1985 was evaluated using horse red blood cells. The result in Figure 3.9 demonstrates that QUB-1985 exhibits about 30% haemolysis activity at the highest peptide concentration at 256 μM in this study. The peptide QUB-1985 exhibits mild haemolysis activity at each MIC against the three pathogenic microorganisms in antimicrobial assays.

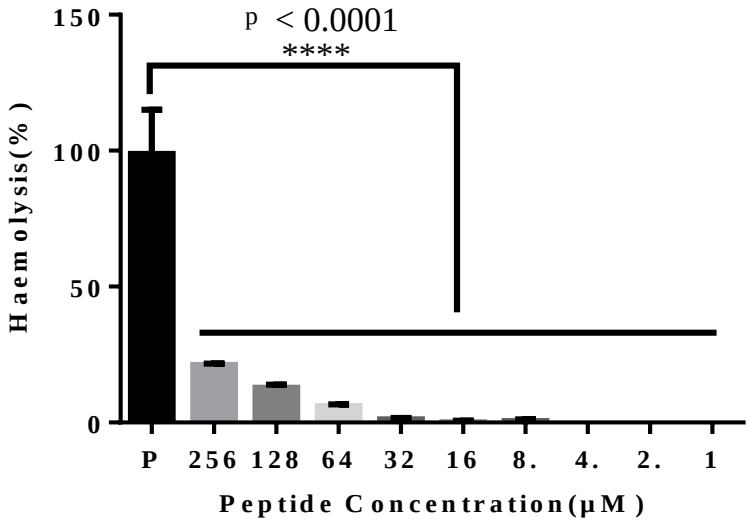


Figure 3.8. The haemolysis of QUB-1985 against horse red blood cells. The composition of the positive control is 1% Triton x-100 with red blood cell suspension. The error bars represent the SD of five replicates.

3.6 Cytotoxicity of QUB-1985 on selected human cancer cell lines

The anticancer activity of QUB-1985 against four human cancer cells, H-157, MCF-7, PC-3 and U251MG, was screened at a peptide concentration of 10 μM. After the treatment of QUB-1985, the growth of these four cancer cell lines was not affected significantly, indicating QUB-1985 is not able to inhibit the proliferation of those human cancer cells (Figure 3.9).

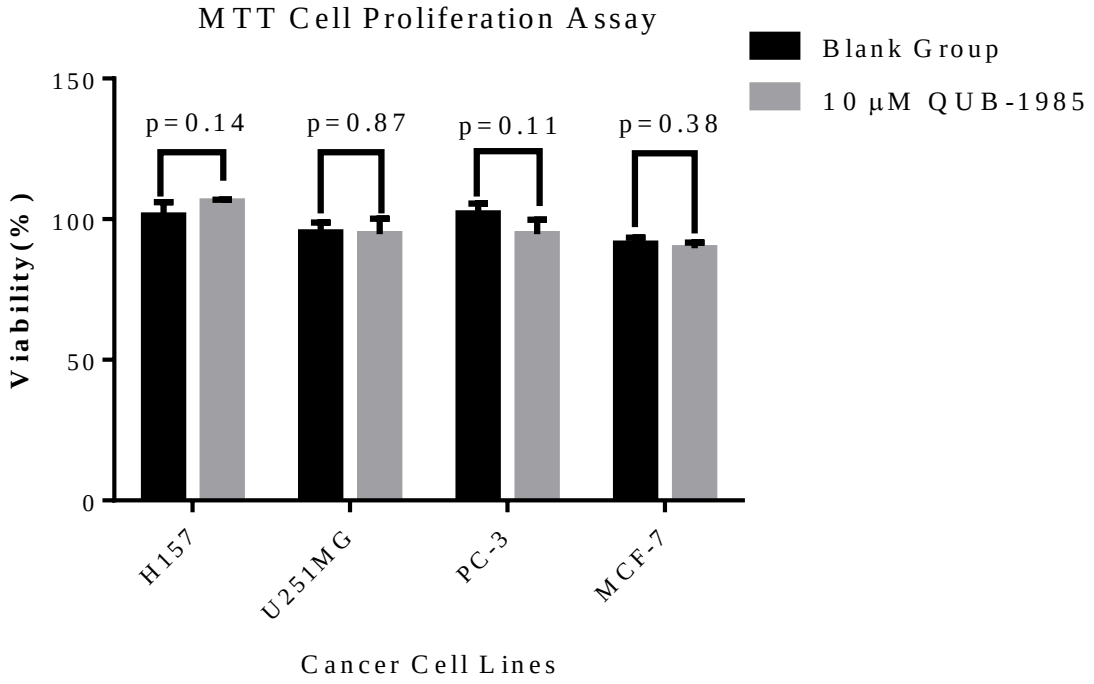


Figure 3.9. The cell viability of selected cancer cell lines in the treatment of QUB-1985 at 10 μM for 24 h. The error bars in the figure were calculated as the SD of five replicates. The 100% cell viability used the blank group, which only consists of medium and cancer cells.

Chapter 4 Discussion

Drug resistance has become a severe worldwide problem in medical field, due to the abuse of antibiotics. The simplex mechanism of conventional antibiotics, targeting at the intracellular parts of pathogens, is no longer effective enough (Blair et al., 2015). Scientists have been working on a solution to this serious clinical problem.

Amphibians, such as frogs, live in complex environments. The complicated living conditions are full of risks from not only predators but also pathogenic infections (Xu and Lai, 2015). In order to defend themselves, amphibians have developed a complete defensive system. The skin secretions produced by their glands play a vital role defending the hosts, and large quantities of antimicrobial peptides can be found in them (Pukala et al., 2006). Antimicrobial peptides from amphibian skin secretions have raised great interests for their rapid and broad spectrum antimicrobial activity. The mechanisms of antimicrobial peptides killing pathogens are different from the intracellular-targeting mechanism of conventional antibiotics. Whilst, they target mainly on the lysis of the membrane, which makes it difficult for microorganisms to develop drug resistance (Bechinger and Gorr, 2017).

The sequence of the peptide QUB-1985 precursor was achieved through cloning from the skin secretion of *Odorrana livida* using a degenerate primer designed from highly-conserved sequences of characterised peptide cDNAs from *Odorrana* species. The sequence of QUB-1985 showed high degree of similarity to the peptides from the Nigrosin peptide family, and possesses the same amount of 21 amino acids. Most Nigrosin peptides share conserved motifs. In details, they all possesses several Gly residues at position 1, 5, 8 and 10, and a hydrophilic Ser residue at position 4. Whilst, the rest of amino acid residues at N-terminus are Leu or Ile residues, which makes the N-terminal domain hydrophobic. Following the hydrophobic domain, there is a di-hydrophilic peptide domain, usually consisting of Lys, Asn and His residues. Moreover,

the unique C-terminal "*rana* box" structure (-CGLSGLC) is highly-conserved in Nigrosin peptides, which is consistent with QUB-1985 (Figure 4.1). As we known, most AMPs isolated from *Rana* species contain the "*rana* box" structure, though the amino acids constitutions are varied between different peptide families (Nissen-Meyer and Nes, 1997). We usually named those peptides as brevinin superfamily, which made confusions sometimes for distinguishing the peptides belonging to this superfamily. Therefore, we actually could define the peptide family based on the conservation of the typical "*rana* box" domain.

QUB-1985	GLLSGILGVGKKIVCGLSGLC
Nigrosin-OG17	GLLSGILGAGKNIVCGLSGLC
Nigrosin-OG20	GLLSGILGAGKHIVCGLSGLC
Nigrosin-MG2	GLLSGILGAGKNIVCGLSGLC
Nigrosin-OG3	GLLSGILGVGKHIVCGLSGLC
Nigrosin-RA3	GLLSGVLGVGKKIVCGLSGLC
Nigrosin-OG12	GPLSGILGAGKHIVCGLSGLC

Figure 4.1 The alignment of the mature peptide, QUB-1985, and the Nigrosin peptides.

According to the result of antimicrobial assay, the peptide QUB-1985 shows the stronger activity against Gram-positive bacteria *S. aureus*, while its activity against Gram-negative bacteria *E. coli* is two-fold weaker. This result can be possibly due to the membrane structure differences between Gram-positive and Gram-negative bacteria. Despite the capsules, S-layer and sheaths, it is known that Gram-negative bacteria possess the structure of outer membrane, which mainly consists of Lipopolysaccharide (LPS), while Gram-positive bacteria only contain peptidoglycan on the outer layer (Beveridge, 1999). More than 90% of the outer leaflet cell surface of Gram-negative bacteria is covered by LPS, which provides physical protection to the Gram-negative

bacteria from the antimicrobial agents. The negatively-charged LPS can attract and bind to the positively-charged antimicrobial peptides, and the neutralization would occur when peptides bind to Gram-negative bacteria cell wall (Rosenfeld and Shai, 2006). Moreover, the LPS is composed of outer hydrophobic lipid heads and inner hydrophilic core, so that the amphipathic QUB-1985 may combine the hydrophobic regions to the lipid head groups and hydrophilic regions to the LPS hydrophilic core (Lee et al., 2016). Therefore, the peptides are fixed on the outer LPS and failed of the translocation of inner layer of cell membrane. By contrast, the peptidoglycan layer of Gram-positive bacteria is hydrophilic and can be easily crossed by AMPs. Therefore, QUB-1985 could get easy access to the plasma membrane of Gram-positive bacteria and induce the membrane lysate.

Meanwhile, QUB-1985 shows the weakest activity against *C. albicans*, which is 4-fold weaker than that against *S. aureus*. The MBC against this fungus is 128 μM, which is much higher than which against bacteria. In fact, many antifungal agents possess potent fungistatic activity but weak fungicidal activity against pathogenic fungi (Maurya et al., 2011). The reason could be explained by the difference of the fungus cell wall structure. The inner layer of fungus cell wall contains structural polysaccharides, chitin and β-1,3 glucan, which add to the cell wall strength and shape of fungus cells. Meanwhile, the cell wall outer layer contains mannans, which may not contribute to shape keeping or cell walls strength but helps lower the permeability and porosity of the cell wall (Bowman and Free, 2006). As a result, it may be difficult for QUB-1985 to insert into the cell wall and cause cell lysate, and fungus cells can obtain high degree of drug resistance. So it is easy for QUB-1985 to inhibit the growth of *C. albicans*, but it needs high concentration to kill the cells.

Compared with the structure-similar peptide Nigrosin-OG20, the peptide QUB-1985 shows stronger antimicrobial activity. The antimicrobial activity of Nigrosin-OG20 against Gram-positive bacteria *S. aureus* is more than 6-fold weaker than which of QUB-1985, while its activity against Gram-negative bacteria *E. coli* is more than 7-fold weaker. The difference in antimicrobial activity between these two peptides may result from their structural differences. The net charge of Nigrosin-OG20 is +1, while QUB-1985 possesses a net charge of +2. Because the position 12 amino acid in Nigrosin-OG20 is His, while the position 12 in QUB-1985 is Lys, which provides more positive charge. The main mechanism of antimicrobial activity is that the cationic peptides induce electrostatic interaction with the anionic pathogenic cell membranes. It can be easier for peptides possessing higher net charge to be attracted to cell membranes and interact with them (Bechinger and Gorr, 2017). Therefore, QUB-1985, which contains higher net charge than Nigrosin-OG20, may induce electrostatic interaction with the negatively-charged pathogenic membrane easier than Nigrosin-OG20.

However, another Nigrosin peptide, Nigrosin-2VB, which possesses a same net charge with QUB-1985, shows much weaker antimicrobial activity against bacteria than QUB-1985. The bactericidal activity against *S. aureus* and *E. coli* are both more than 3-fold weaker than which of QUB-1985. According to the online server HeliQuest, the structural difference between these two peptides is that the hydrophobic face of Nigrosin-2VB is shorter than that of QUB-1985 with 4 amino acids less. Therefore, hydrophobicity is another key factor which has remarkable impact on peptide antimicrobial activity. In order to form pores and channels on bacterium cell membranes, peptides may use their hydrophobic regions to bind to the hydrophobic lipid head groups on the cell membrane and their hydrophilic parts are combined with the hydrophilic cores (Lee et al., 2016). The long hydrophobic face of QUB-1985 may contribute to a tighter coupling of the lipid

head groups than which of Nigrosin-2VB, and it is easier for QUB-1985 to cause the collapse of bacterium cells.

According to the result of antimicrobial and haemolysis assays, QUB-1985 showed really mild haemolysis activity at the MICs against Gram-positive and Gram-negative bacteria. The secondary structure prediction made by I-TASSER indicates that QUB-1985 does not possess the α-helical structure. The high amphipathicity provided by the α-helical structure may help peptides exhibit potent antimicrobial activity, but it may also induce strong haemolysis activity. The membrane of erythrocytes is composed mainly of zwitterion phosphatidylcholine and phosphatidylethanolamine with few acidic lipids in the outer leaflet. And the helical structure often brings high amphipathicity to peptides, which may cause severe cytotoxicity to human cells. Maybe the absence of α-helical structure helps QUB-1985 decrease the haemolysis activity (Conlon et al., 2006). With potent antimicrobial activity but low haemolysis activity at the same time, the peptide QUB-1985 can be considered as a promising therapeutic agent. Additionally, intravenous administration can be considered as its rapid and potent administration route.

It is predicted that there is a ‘rana box’ structure at C-terminal of QUB-1985 according to its sequence. And this prediction has been confirmed because the molecular mass of synthesised QUB-1985 is 2 Da less than the calculated one. Rana box is a cyclic structure at C-terminal constructed by disulphide bond (Simmaco et al., 1994). It has been assumed that the existence of rana box is related with peptide antimicrobial activity. With the reduction of the rana box, the antimicrobial activity of the modified peptide may be weakened (Clark et al., 1994). However, this hypothesis was dismissed because it has been proven that sometimes antimicrobial activity may not be affected with the deletion of rana box motif (Vignal et al., 1998). It still needs more research to figure out the role of rana box structure playing in the influence of antimicrobial activity.

However, there is a long way to go before the peptide become a novel antibiotic agent. Low haemolysis activity does not mean that the peptide is completely safe to human body. The allergy problem should be considered and solved before peptides develop into therapeutic agents. As large amounts of people are allergic to protein, peptide-related antibiotics may not suitable for them. And some other people may be potentially allergic to peptide-related antibiotics, which may add to the risk of peptide application (Guaní-Guerra et al., 2010). Despite the allergy problem, antimicrobial mechanisms, structure-activity relationship and some other problems still remain to be solved, which may need massive financial support. Additionally, according to biosynthesis, the manufacture cost of peptide therapeutics can be dramatically high (Marr et al., 2006). Therefore, financial conundrum may impede the extensive application of these antibiotics.

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