DOCTORAL (Ph.D.) DISSERTATION

Synthesis and Antimicrobial Activity Investigation of Peptides Derived from NCR169 and NCR147

Dian Herlinda Octorina Howan

Supervisor:

Prof. Gábor K. Tóth

DOCTORAL SCHOOL OF CHEMISTRY

University of Szeged

Department of Medical Chemistry

2023

Table of Contents

Table of Contents 1
List of Abbreviations 3
1. Introduction 6
2. Literature Review 10
2.1. Antimicrobial Peptides 10
2.2. Antimicrobial Peptides Classification 16
2.3. Antimicrobial Peptides Mechanisms 16
2.3.1.1. Direct Killing: Membrane Disrupting Mechanisms 17
2.3.1.2. Direct Killing: Non-Membrane Disrupting Mechanisms 18
2.3.2. Immune Modulation Mechanisms 19
2.4. Existing Antimicrobial Peptide Drugs 19
2.5. Nodule-Cysteine-Rich Peptides 27
2.6. Solid-Phase Peptide Synthesis 29
2.6.1. The Solid Support 30
2.6.2. The Linker 31
2.6.3. Protecting Groups 32
2.6.4. Coupling 34
3. Objectives 36
4. Experimental Methods 37
4.1. Synthesis of Peptides 37
4.1.1. Materials 37
4.1.2. Synthesis Procedures 37
4.1.3. Analysis and Purification 38
4.2. Antimicrobial Activity Assays 39
4.2.1. Bacterial Strains 39
4.2.2. Antibacterial Activity Assay 40
4.2.3. Hemolysis Assay 40
4.2.4. Fungi Strains and Growth Conditions 40

4.2.5. Antifungal Activity Assay 41
4.2.6. Biofilm Formation Assay 42
4.2.7. Morphological Analysis 42
4.2.8. Combined Treatment of *C. auris* with Fluconazole and NCR169C$_{17-38}$ 43
4.2.9. Assessment of the Viability of Human Keratinocytes 43
5. Synthesis & Antimicrobial Activity of NCR169 & Its Derivatives 44
5.1. Synthesis of NCR169 and Its Derivatives 44
5.2. Antibacterial Activity of NCR169 and Its Derivatives 45
5.3. Synthesis of NCR169C$_{17-38}$ Derivatives 47
5.4. Antibacterial Activity of NCR169C$_{17-38}$ and Its Derivatives 49
5.5. Antifungal Activity of NCR169C$_{17-38}$ and Its Derivatives 50
5.6. Synthesis of NCR169C$_{17-38}$C$_{12-17}$/S Derivatives 50
5.7. Antimicrobial Activity of NCR169C$_{17-38}$C$_{12,17}$/S Derivatives 52
5.8. Hemoloysis Activity of NCR169C$_{17-38}$C$_{12,17}$/S Derivatives 55
5.9. Discussion 56
6. Synthesis & Antimicrobial Activity of NCR147 & Its Derivatives 61
6.1. Synthesis of NCR147 and Its Derivatives 61
6.2. Antimicrobial Activity of NCR147 and Its Derivatives 63
6.3. Synthesis of NCR147$_{25-36}$ Derivatives 64
6.4. Antibacterial Activity of NCR147$_{25-36}$ Derivatives 65
6.5. Discussion 66
7. Summary 69
List of References 74
Acknowledgement 91
List of Publications 92
Appendices 94

List of Abbreviations

AMPs	: antimicrobial peptides
AMR	: antimicrobial resistance
APD	: antimicrobial peptide database
ATCC	: American type culture collection
BOC	: tert-butyloxycarbonyl
BSA	: birdseed agar
CBS	: central bureau voor schimmelcultures
CCK	: cyclic cystine knot
chCATH	: chicken cathelicidin B1
COMU	: (1-cyano-2-ethoxy-2-oxoethyl-idenaminooxy) dimethylaminomor-pholino carbenium hexafluorophosphate
CRPs	: cysteine-rich peptides
CSαβ	: cysteine-stabilized αβ
DCC	: N-N'-dicyclohexylcarbodiimide
DCM	: dichloromethane
DIC	: N,N'-diisopropylcarbodiimide
DMF	: dimethylformamide
DMSO	: dimethylsulfoxide
DNA	: deoxyribonucleic acid
DTT	: dithiothreitol
ESI-MS	: electrospray ionization mass spectroscopy
FDA	: food and drug administration
FIC	: fractional inhibitory concentration
FMOC	: 9-fluorenylmethyloxycarbonyl
GLASS	: global antimicrobial resistance and use surveillance system
GDP	: gross domestic product

GRAVY	: grand average hydropathy
HATU	: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*] pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
hBD	: human beta-defensin
HIV	: human immunodeficiency virus
HNP	: human neutrophil peptides
HOAt	: 1-hydroxy-7-azabenzotriazole
HOBt	: 1-hydroxybenzotriazole
HPLC	: high-performance liquid chromatography
HSQC	: heteronuclear single quantum coherence
IDR	: immunomodulatory host defense peptides
IRLC	: inverted repeat-lacking clade
LB	: luria broth
LC-MS	: liquid-chromatography mass spectroscopy
LfcinB	: lactoferricin bovine
LTPs	: lipid transfer proteins
MBC	: minimum bactericidal concentration
MDR	: multi-drug resistance
MIC	: minimal inhibitory concentration
MRSA	: methicillin resistance *Staphyloccus aureus*
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NCR	: nodule-cysteine-rich peptides
NCTC	: national collection of type cultures
NMP	: N-methylpirrolidone
NMR	: nuclear magnetic resonance
OD_{560}	: optical density measured at 560 nm wavelength
OD_{600}	: optical density measured at 600 nm wavelength
OH-CATH	: OH-cathelicidin

OXYMA	: ethyl 2-cyano-d-(hydroxyamino) acetate
PBB	: potassium-phosphate buffer
PbF	: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
PET-G	: polyethylene terephthalate glycol-modified
pI	: isoelectric point
PrAMP	: proline-rich antimicrobial peptide
PyBOP	: benzotriazole-1-yloxytri(pyrrolidino) phosphonium hexafluorophosphate
RAM	: rink amide linker
RNA	: ribonucleic acid
RP-HPLC	: reverse-phase high performance liquid chromatography
SC	: squibb institute for medical research
SEM	: scanning electron microscopy
SPPS	: solid-phase peptide synthesis
SZMC	: szeged microbiological collection
TBS	: tris-buffered saline
TBTU	: N-[(1H-benzotriazol-1-yl) (dimethyl-amino) methylene]-N-methylmethan-aminium tetrafluorovorate N-oxide
TFA	: trifluoro acetic acid
THPdb	: therapeutic protein database
TIS	: triisopropylsilane
TNF	: tumor necrosis factor
VAPGHs	: virion-associated peptidoglycan hydrolases
WHO	: world health organization
XTT	: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
YNB	: yeast nitrogen base
YPD	: yeast extract peptone dextrose

Chapter 1

Introduction

Sir Alexander Fleming's discovery of Penicillin in 1928 marked the initial breakthrough in the development of antibiotics for the prevention and treatment of bacterial infections. Subsequently, antibiotics have found widespread application in various medical treatments. The most significant percentage is the intestines, the heaviest organ in the body. Five antibiotic classes are widely used to generate successive generations of antibiotics: penicillins, cephalosporins, quinolones, macrolides, and tetracyclines. Only quinolone is synthetic among this group, whereas the other scaffolds are natural product derivatives[1]. Antibiotics have also been used in agriculture, animal farming, and veterinary medicine to produce healthier and more productive farm animals and warrant the well-being and health of humans and animals[2]. In addition, antibiotics can be found in cleaning products as additives[3].

The extensive use of antibiotics subsequently raised a new threat called antimicrobial resistance (AMR), a condition in which microorganisms, such as bacteria, fungi, and viruses, are developing the ability to fight against the drugs initially designed to extinguish them. Another crucial factor that enhanced the progression of AMR was antibiotics over-prescription, in which antibiotics in some countries can easily be purchased without a doctor's recipe[4]. In contrast, in the countries that assign antibiotics as prescription-only drugs, most of the antibiotics' prescriptions are misguided[5,6].

According to the U.S. Centers for disease control and Prevention report in 2013, at least 2,049,442 illnesses and 23,000 deaths were related to AMR[7,8]. In 2017, AMR was causing the death of nearly 700,000 per year, which may increase to 10 million deaths by 2050, close to the overall mortality caused by all significant diseases (Figure 1.1 A)[7]. Moreover, it is predicted that the number of deaths prompted by AMR would decrease in the continents with better economic climates and stricter antibiotic regulations (Figure 1. B)[9].

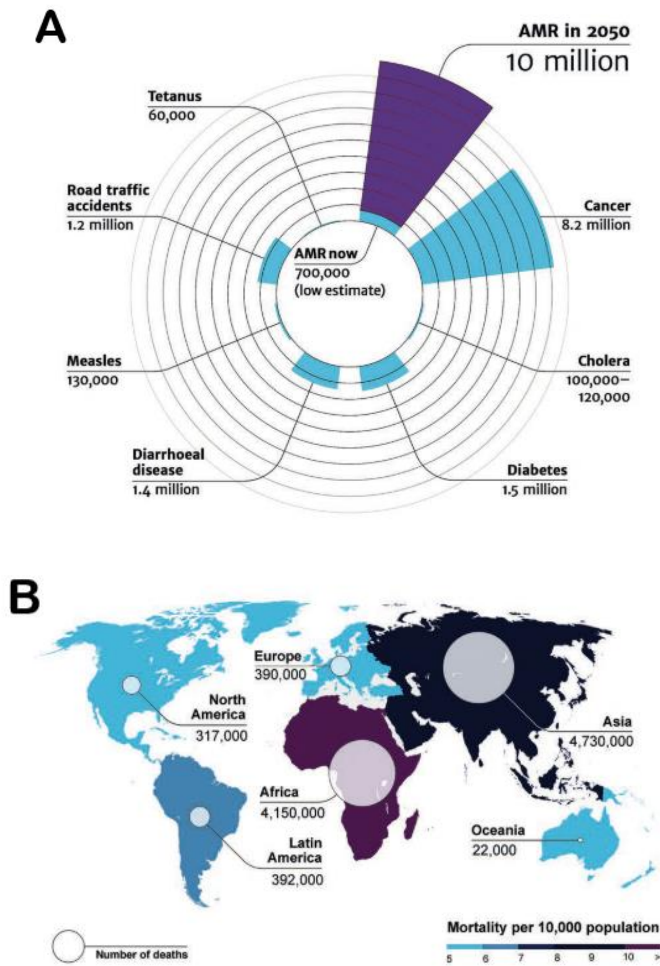


Figure 1.1 Prediction of Antimicrobial Resistance Impact in 2050. A). AMR mortality rate contrasted with other leading causes of death nowadays. B) Projection of mortality rate attributed to AMR throughout the world. It is predicted to decline immortality number in prospering continents like North America and Europe.[9]

Infection with AMR leads to increases in terms of healthcare costs, second-line drugs, and treatment failures[10]. Above nine billion euros per year is estimated in Europe, which is correlated with AMR[8,11]. At the same time, in the United States, the number is a 20 billion dollars surplus in healthcare costs directly caused by AMR[12]. It is predicted that around 100 trillion US dollars of gross domestic product (GDP) will be lost between the period 2014 to 2050 for battling AMR[9].

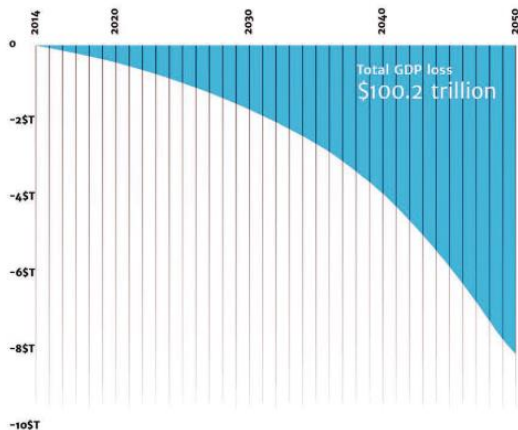


Figure 1.2 Projection of AMR's Impact on the Global Economy from 2014-2050[9].

The World Health Organization (WHO) declared AMR as one of the top 10 international public health threats and, together with authorities all around the globe, initiated a program called GLASS (global antimicrobial resistance and use surveillance system) in 2015 as a response to this phenomenon[13]. WHO lists global priority pathogens, whereas some bacterial strains, including *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli*, are on the top of the list as priority bacteria. This bacteria group is called the ESKAPE group since they can "escape" conventional antibiotics and medication, causing multi-drug resistance (MDR)[14,15]. The mechanism of drug resistance, specifically in the ESKAPE pathogens group, could be specified into several broad categories, namely drug inactivation/alteration, drug binding sites/targets modification, changes in cell permeability which then decreased the accumulation of intracellular drug, and formation of biofilm[16–18].

The development of new antibiotics is crucial to combat bacterial pathogens. A promising type of antimicrobial agent is antimicrobial peptides (AMPs). AMPs retain some plus points because they are less likely to create resistance towards bacteria due to their favorable action at the membrane level compared to conventional antibiotics[19]. AMPs, such as colistin, can also be fused with other antimicrobial agents to gain a better synergistic impacts[20]. Other advantages of AMPs are a broad spectrum of targets, excellent chemical and biological diversity, and lesser toxicity compared to small molecules[21]. However, AMPs possess several drawbacks, such as

inadequate metabolic stability/short half-life, membrane permeability issues, low activity in physiological salts, bewildering biological roles, and high costs of productions[21,22].

Some approaches have been conducted to tackle the issues related to AMPs, such as activity, production cost, and resistance. One of these strategies is generating shorter AMPs since it is believed to be more economical and enhances the activity of the peptides. For instance, bovine lactoferricin (LfcinB) is more active in its "shorter form" than full version of it[23]. Few shorter analogs of human β-defensins-3 showcased better activity against *E. coli* contrasted with the longer analogs[24]. Hence, this dissertation focuses on synthesizing plant-derived AMP and its shorter fragment to enhance the activity against pathogens microorganisms. In addition, the investigation would also incorporate modification of peptide sequences with unnatural amino acids, such as D-amino acids, as well as modified amino acids.

Chapter 2

Literature Review

2.1 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are natural antibiotics synthesized in ribosomes of nearly all organisms, from bacteria to animals and plants. AMPs can be defined as peptides that can terminate microbes, but not the enzymes that eradicate microbes through hydrolytic activities[25]. AMPs have been known as evolutionary primordial arsenals that delivered a vital function in the successful evolution of complex multicellular organisms[26]. AMPs also acted as host defense peptides, leading them as the front runners' drug candidates for infectious disease treatment and novel immunomodulatory therapies[27]. A unique feature of AMPs that distinguishes them from other antimicrobial agents is their capability to attack multiple low-affinity targets, such as bacterial membranes, which is considered to abbreviate the development of antimicrobial resistance[27]. AMPs are also responsive to mutagenesis and peptide engineering, properties that have developed in the production of numerous compounds with improved bioactivity and reduced cytotoxicity[28,29].

More than 3000 AMPs from diverse sources have been reported and characterized; however, only a small amount of these peptides can pass the clinical trial to be approved as an acceptable drug for humans[30]. From the U.S. FDA Therapeutic Protein Database (THPdb), 852 peptide drugs have been reported[31], and only six small AMPs have been successfully approved by the FDA[30]. Those peptides are gramicidin, daptomycin, vancomycin, oritavancin, and telavancin (these peptides will be discussed thoroughly in the current AMPs drugs in the market section). The antimicrobial peptide database (APD) has 3156 AMPs, whereas most are natural peptides with different structures and sequence motifs[32]. The mean length of peptides in the APD is 33 amino acids, and above 90% of the peptides have less than 50 amino acids in their sequences[30].

2.2 Antimicrobial Peptides Classification

AMPs can be found ubiquitously in living organisms. Based on their sources, AMPs can be classified into five major groups[33–35]. Those groups are:

i. Bacteriophage or viral AMPs. This group consists of viruses that demonstrate antibacterial activity, such as endolysins, depolymerase, virion-associated peptidoglycan hydrolases (VAPGHs), and holins[36–38]. There are two bacteriophage/phage AMPs: phage-tail complexes and phage-encoded lytic factors, ranging from 25 to 40 kDa[39]. The first type of viral AMPs has a cylindrical structure similar to a phage tail with high-molecular weight[33], whereas the second type is smaller (25-40 kDa) and known as peptidoglycan-hydrolyzing enzyme[38]. In addition, this enzyme can hydrolyze the wall of microbial cells, enabling the discharge of progeny[40].

ii. Bacterial AMPs. This broad group can be further divided into AMPs from Gram-positive bacteria and AMPs from Gram-negative bacteria. The AMPs from Gram-positive can be synthesized in ribosomes (ribosomal AMPs) or via an enzymatic process (non-ribosomal AMPs)[35,41]. There are four classes of Gram-positive bacteria AMPs: class I (lantibiotics); class II (non-lantibiotics); class III (large-sized bacteriocins); IV (uniquely structured bacteriocins)[42,43], whereas the AMPs from Gram-negative bacteria can be classified into colicins, colicin-like bacteriocins, phage-tail-like bacteriocins, and microcins[43].

iii. Fungal AMPs, divided into fungal defensins and peptaibols[33,35,44], are part of the cysteine-rich AMPs family. These peptides are characterized by short, cationic disulfide bridges[45]. The fungal defensins are grouped into defensins-like peptides because of their structural resemblances and high sequence[46]. Furthermore, the "peptaibol" term is created from the combination of three names "peptide," "α-amino isobutyrate," and "amino alcohol"[47]. The peptaibols are short peptides (5-21 amino acids) with a high percentage of non-proteinogenic amino acids and stereotypically comprise an acylated *N*-terminal residue and an amino alcohol (i.e., leucenol or phenylalaninol) attached to the *C*-terminal[48]. These peptides are mostly produced by the soil fungi *Trichoderma*[49].

iv. Plant-derived AMPs are the vanguard of plant defense versus infections caused by pathogenic microorganisms. The plant defense system primarily forms from cysteine-rich AMPs. Therefore, this group could develop several disulfide bridges, creating a dense configuration and maintaining stability against thermal, proteolytic, and chemical degradation[50]. The classification of plant AMPs is as follows:

   a. Thionins, cationic peptides with 45-48 amino acids and 3-4 disulfide bridges. This group has antibacterial and antifungal activities[50,51].

b. Hevein-like peptides, a basic peptides group containing 29-45 amino acids, with 3-5 disulfide bridges in charge of stabilizing the antiparallel β-sheets and short α-helix[52] and plentiful glycine, cysteine, and aromatic residues. This group has antifungal activity due to the presence of a chitin-binding domain[53].
c. Defensins, cationic peptides comprise 45-54 amino acids and 4-5 disulfide bridges. The secondary structure of defensins consists of an antiparallel β-sheet which is surrounded by an α-helix and is constrained by intramolecular disulfide bonds[54], so-called cysteine-stabilized αβ (CSαβ) motifs[55]. Defensins are unaffected by proteolysis and unchangeable in varied temperatures and pH. Moreover, these peptides inhibit microbial growth and trypsin and lessen abiotic stress[56]. Defensins are known for their antibacterial and antifungal activities[57,58].
d. Knottin-type peptides, known as cysteine-know peptides, are amphipathic peptides containing around 30-39 amino acids, conserved cysteine residues, and disulfide bridges. This group has two conformations: linear and cyclic[50,59], stable at high temperatures, inhibitor of several enzymes (i.e., trypsin, carboxypeptidase, α-amylase, cysteine protease), and withstand proteolytic action[60,61]. Knottins exhibit antiviral and antibacterial activities[50,62]. However, this group is not selective regarding contact with membranes, which generate toxic properties in human cells[33].
e. Stable-like peptides or known as α-hairpins due to their helix-loop-helix secondary structure. Lysine and arginine are abundant in this group, with a typical cysteine motif of XnC1X3C2XnC3X3C4Xn, whereas -X is an amino acid residue of cysteine. The three-dimensional structure of these peptides is conserved even though their amino acid sequence is varied. These peptides can act as antifungal, antibacterial, and ribosome-inactivating agents, plus trypsin inhibitors[50,63]. Their biological activities correlate with the loop region that connects the two α-helices[64].
f. Lipid transfer proteins (LTPs) are cationic peptides rich in cysteine residue and consist of 70-90 amino acids in their sequence. In their secondary structure, 4 to 5 helices are present and steadied by hydrogen bonds. There are five types of LTPs based on the conserved location, the distance between the cysteine residues, and the characteristics of the amino acid sequence. Those types are LTP1, LTP2, LTPc, LTPd, and LTPg[59]. These

peptides are known as lipids distributors among membranes[33] and act as antibacterial and antifungal agents[50,65,66].

g. Snakins are small-sized cationic proteins with the unique characteristic of 12 conserved residues. This group has antimicrobial, antinematode, and antifungal properties[67], although its mechanism of action is still debatable. Some publications claimed snakins could stimulate immune responses by disrupting the active site via interaction with the negatively charged constituent[67,68]. At the same time, another paper stated that snakins take action on the biosynthesis of phytohormone and transduction processes[69].

a. Cyclotides are macrocyclic peptides characterized by their structural motif of cyclic cystine knot (CCK)[70] and can be categorized into two sub-type, mobius, and bracelets[71]. Cyclotides' mode of action is controlled by the cystine knot motif, which advocates contact with the surface of hydrophobic residues. They possess anticancer, antibacterial, and anti-HIV properties[33].

i. Animal-derived AMPs are the most abundant AMPs group from natural resources. By far, more than 2500 AMPs have been identified from this kingdom[72]. Animal-based AMPs have four sub-divisions such as following:

a. Invertebrates. AMPs are synthesized in invertebrate organisms as part of their humoral defense due to their shortage of adaptive immune response. The major AMPs in invertebrates are defensins, cationic peptides, with 6 or 8 cysteine residues and display an αβ/ cysteine-stabilized motif[73]. Based on the abundance of cysteine, invertebrate defensins are categorized into two groups, in which the largest group comprises six cysteine residues (mainly from arthropods, insects, and mollusks). The smaller group is mostly found in nematodes and mollusks and has eight cysteine residues[74]. The invertebrate AMPs mode of action is similar to the vertebrate defensins, involving the target bacteria's cytoplasmic or inner mitochondrial membrane permeabilization. However, these two defensins share no sequence homology or structural resemblance[35,75]. Several members of invertebrate defensins have various biological activities, such as 'big defensin,' originally isolated from the Horseshoe crab (*Trachypleus tridentatus*), which displayed antibacterial and antifungal activity[35].

b. Fish and Amphibian AMPs. The sequence in vertebrates AMPs is from 15 to 200 amino acids. As part of vertebrates' AMPs, fish AMPs are vital in the instant defense response

to microorganisms[35,76]. Cathelicidins, β-defensins, piscidins, hepicidins, and histone-derived peptides are examples of peptides produced by fish[33,77] Cathelicidins are cationic peptides initiated by elastase and other proteases[78], and able to combat bacteria, fungi, parasites and enveloped viruses[79–82]. Fish defensins are part of the β-defensin-like proteins family with six conserved cysteine motifs[83,84], which are potential antibacterial and fish-specific viruses agents[77,85,86]. Piscidins are amphipathic linear AMPs containing histidine residues and an α-helix similar to magainins and cecropins[87,88]. The piscidin leurocidin, a member of the piscidin group, is known as an active antibacterial peptide[89]. Amphibians are the richest source of AMPs according to APD, in which 1117 registered peptides were from frogs and toads[35]. Cancrin, magainin, buforin, and phylloxin are some of the amphibians AMPs reported for their chemical properties and biological activities[90].

c. Reptile- and Avian-Derived Peptides are part of the cathelicidin and defensin families. An example of reptile AMPs is OH-CATH peptide, isolated from king cobra, containing 34 amino acids and active against several pathogen bacteria[91]. In addition, fowlicidins and chCATH B1 are avian AMPs isolated from chicken that demonstrated activity against some Gram-positive and Gram-negative bacteria[81,92,93].

d. Mammalian-Derived AMPs mainly belong to the defensins and cathelicidin groups, although not all mammalian AMPs are in both groups, such as platelet antimicrobial proteins, dermcidin, and hepcidins[94]. The only cathelicidin found in humans is LL-37, an amphipathic peptide that shaped an α-helical structure during membrane interaction and interestingly disheveled it in an aqueous solution[95]. There are three sub-classes of vertebrate defensins: α, β, θ, which are synthesized as 'prepropeptides,' and the mature peptides revealing numerous typical features such as short polypeptide sequences (between 18 to 45 residues), a cationic net charge (+1 - +10) and no glycosyl or acyl side-chain modification[35]. Several vertebrate defensins peptides showcased antimicrobial activity, such as human α-defensins HNP1, lingual AMP, and tracheal AMP[96–98].

There are four structural classes of AMPs (Figure 2.1), namely (a) extended peptide chain forming amphipathic α-helical structures; (b) peptide chain arranged in β-sheet structure with internal two or more disulfide bonds; (c) extended linear peptide chain structure; (d) hybrid peptide chain structure in a loop structure that is formed via a single internal disulfide bond[33,99]. Numerous

AMPs in these groups would lose their structure in free solution and regain their original conformation through the partitioning into the membranes of the host cell[99].

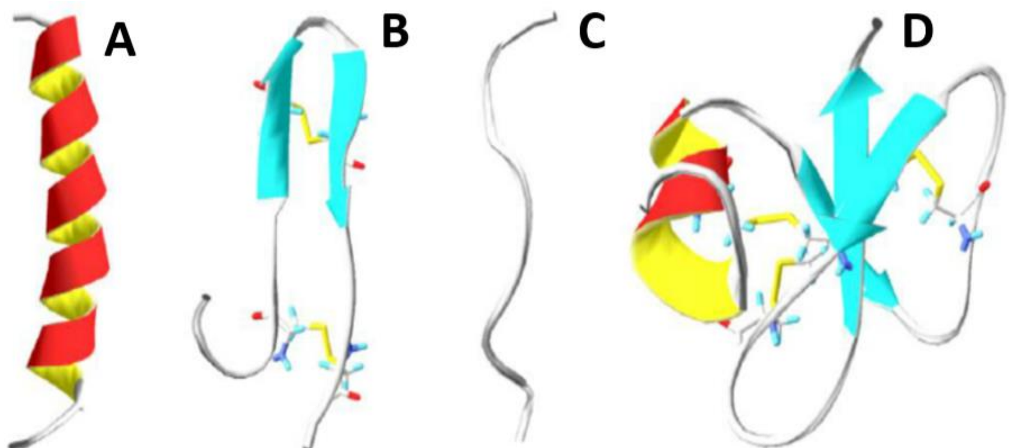


Figure 2.1. Structure-based AMPs. (A) AMPs with α-helix structure; (B) AMPs with β-sheet structure; (C) AMPs with linear extension structure; (D) AMPs with α-helix and β-sheet mix structure[99].

Another way to classify AMPs is by their sequence abundance of certain amino acids. The amount and characteristics of amino acids would define the pharmacological appliances of AMPs. For example, the glycine-rich peptides group can be considered a versatile therapeutic agent for its antimicrobial, antimycotic, and anticancer activities, and it also has excellent selectivity[100–102]. In addition, cysteine-rich peptides initiate the pores forming in membranes[103] and can enhance the antimicrobial activity of AMP by steadying the β-hairpin structures or sheet[104]. Normally, amino acids with an excessive helical propensity, like alanine, arginine, lysine, and leucine, could generate novel antimicrobial peptides because α-helical structure stimulates interaction with membranes and regulates the cellular disruption[105–107]. Other properties that influence AMP activity are hydrophobicity and amphipathicity. AMPs with high hydrophobicity possess antimicrobial and hemolytic activities. The antimicrobial activity of high hydrophobicity AMP improves by the blockage of peptide passage through the microbial cell wall because of the self-association of peptides[108].

Nevertheless, AMPs with low hydrophobicity could also have antimicrobial activity due to the peptide self-association that inhibits peptide passage through the cell wall[109]. Furthermore, amphipathic AMPs have bactericidal and cytotoxic activities related to their capacity to construct

an α-helix[110]. These peptides can interact with intracellular targets, ravaging the membrane structure and shaping transient pores[109].

Finally, AMPs can be classified based on their biological activities. In this category, there are 4 broad groups of AMPs[34]. Firstly, antibacterial AMPs, such as AMPs P5 and P9, could inhibit methicillin resistance *Staphylococcus aureus* (MRSA). Secondly is antifungal AMPs, like AurH1, which can treat the infection of *C. albicans*. Thirdly, anticancer AMPs. For example, indolicidin and puroindoline A. Lastly, antiviral AMPs, for instance, AMP EPI-1, trigger the virus particles' inactivation and display good inhibition activity towards the food-and-mouth disease virus.

2.3 Antimicrobial Peptides Mechanisms

Numerous AMPs from varied sources have been identified successfully, and several studies have already been conducted to investigate their mechanisms of action. Despite all these efforts, the wide gap in details of how AMPs interrupt the membrane needs to be unraveled. Understanding the behavior of peptides toward the targeted cells would make the design of effective peptide-based drugs more convenient. AMPs' modes of action are classified into two broad classes: direct killing and immune modulation (Figure 2.2). The first class can be further divided into membrane-disrupting and non-membrane targeting groups[35]. Nevertheless, several AMPs might perform in both paths.

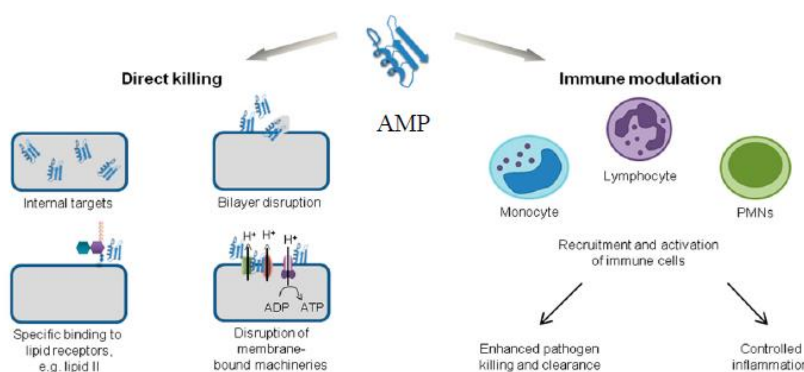


Figure 2.2. Assorted AMPs mode of actions[111]. ADP: adenosine diphosphate; ATP: adenosine triphosphate; PMNs: polymorphonuclear neutrophils.

2.3.1.1 Direct Killing: Membrane Disrupting Mechanisms

Initially, AMPs are considered to play a role merely by disordering the bacterial layer integrity through several modes of action. Those modes of action are:

1. Barrel-stave model. This model has a "barrel-like" ring construction inside the membrane channels by the AMPs, and each transmembrane in the barrel is suggested as a "stave"[112]. In this mode, the AMPs are initially parallel to the membrane. The parallel position caused the membrane surface to become stiff and reedy, which enabled the peptides to enter the lipid bilayer perpendicularly. The insertion of peptides into the membrane ultimately will create pore formation (Figure 2.3 A)[41,113]. Some AMPs demonstrating the barrel-stave model are alamethicin, pardaxin, and protegrins[111,114].
2. Toroidal pore model. The AMPs in this toroidal model are placed vertically intersect a lipid layer with no peptide-peptide interactions in the lipid membrane (Figure 2.3 B)[115]. The pores formed through this model are transitory and less firm compared to the previous model[116]. Magainin, aurein 2.2, lacticin Q, and melittin are a few of the AMPs that displayed toroidal pore model[111,117,118].
3. Carpet model. Unlike the previous models, the AMPs in the carpet model do not require to be inserted into the hydrophobic core of the membrane nor assemble their hydrophilic surfaces against each other. The permeation of the membrane transpires only when a high local concentration of membrane-bound peptide is present. This condition can be achieved if the peptide monomers are enclosed in the entire membrane surface or, instead, after there is a connection between membrane-bound peptides, constructing a confined 'carpet'(Figure 2.3 C)[119]. Furthermore, it is continued with a detergent-like action stimulated by the peptides that trigger the pores conformation[120]. Examples of AMPs that act following the carpet model are indolicidin, aurein 1.2, and LL-37[111,121].
4. Molecular electroporation model. This model was proposed for cationic AMPs that bonded to cell membranes and generated electrical potential differences throughout the membrane. When the value of potential difference expands to 0.2 V, it initiates the formation of pores in the membrane(Figure 2.3 D)[122]. NK-lysin is a peptide that displays molecular electroporation mechanism[123].

5. Sinking raft model. This model advised that the amphipathic AMPs binding creates a mass imbalance and subsequently boasts local curvature of the membrane. Upon the peptides' self-association, they sink into the cell membrane, forming transient pores(Figure 2.3 E); hence the sinking raft model[122]. An example of AMPs with a sinking raft mode of action is δ-lysin[124].
6. Channel forming or aggregate model. According to this model, the binding of AMPs to the anionic cytoplasmic membrane drives the lipids and peptides to develop a lipid-peptide micelle-like complex. Moreover, lipid-peptide aggregation creates ion leakage inside the membrane, consequently leading to cell termination(Figure 2.3 F)[125]. Polyphemusin and indolicidin are AMPs that operate with channel-forming model[126].

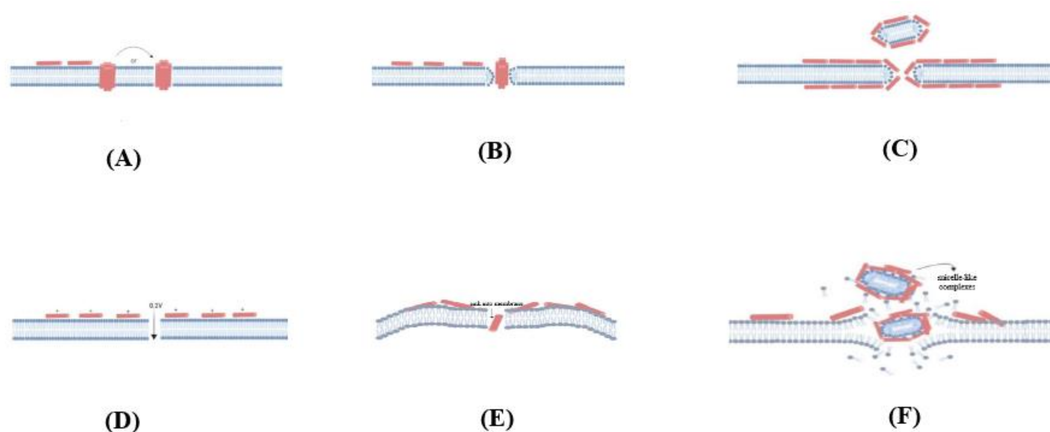


Figure 2.3. Membrane Disruption Mechanisms. (A) Barel-Stavel Model; (B) Toroidal Pore Model; (C) Carpet Model; (D) Molecular Electroporation Model; (E) Sinking Raft Model; (F) Aggregate Model[112]

2.3.1.2. Direct Killing: Non-Membrane Disrupting Mechanisms

Several AMPs have been reported to constrain protein folding (i.e., droscosin and pyrrhocoricin)[127] or enzyme activity (i.e., PrAMP pyrrhocoricin, microcin J25, and NP-6)[127–130] or to target some intracellular components such as mitochondria, protein synthesis, DNA/RNA, or essential enzymes. Buforin II is an example of AMP that acts on nucleic acids[131]. It is suggested that buforin II entered the lipid vesicles without disrupting membrane permeability. Instead, it binds directly to DNA and RNA. A few papers have shown that besides this motif, AMPs can inhibit DNA replication or transcription indirectly[132,133]. PR-39, an AMP rich with proline and arginine, hinders protein synthesis and initiates protein degradations needed for DNA synthesis,

which ultimately interrupts DNA synthesis[134]. Other AMPs that stunt protein synthesis are oncocin-type peptide, an apidaecin-derived peptide APi137, and proline-rich AMP Bac5[72,135,136].

2.3.2. Immune Modulation Mechanisms

In conjunction with the direct killing mechanism, several AMPs can augment microbial extermination and regulate inflammation by recruiting and activating immune cells[137–139]. For example, IDR-1002, a host defense peptide derived from bovine bactenecin, has the potency to diminish the production of nitric oxide, TNF-α, and IL-8[140–142]. Moreover, β-defensin human endogenous peptide (hBD-2) and its derivative A-hBD2 are AMPs that have been described to enhance the keratinocytes proliferation and movement of keratinocytes *in vitro*, as well as recuperate lesion recovery *in vivo*[143].

Alongside their direct killing mechanism, several antimicrobial peptides (AMPs) can enhance microbial eradication and control inflammation by attracting and activating immune cells[137–139]. For example, IDR-1002, a host defense peptide derived from bovine bactenecin, has the potency to diminish the production of nitric oxide, TNF-α, and IL-8[140–142]. Moreover, β-defensin human endogenous peptide (hBD-2) and its derivative A-hBD2 are AMPs that have been described to enhance the keratinocytes proliferation and movement of keratinocytes *in vitro*, as well as recuperate lesion recovery *in vivo*[143].

2.4 Existing Antimicrobial Peptides Drugs

As mentioned above, 6 FDA-approved AMP drugs are listed in the THPdb: gramicidin, daptomycin, vancomycin, dalbavancin, oritavancin, and telavancin. A few commercial AMP drugs not listed in THPdb are teicoplanin, colistin, and polymyxin B[144].

- ✓ The FDA approved Gramicidin D (Figure 2.4) in 1955 as a constituent in Neosporin ®, a drug for bacterial conjunctivitis treatment[145]. This peptide was first isolated from *Bacillus brevis* and characterized in 1941 as a heterogenous mixture of three pentadeca peptides, which were gramicidins A (~80%), B(~6%), and C(~14%)[30,146]. The mechanism of action of this linear AMP is through forming a dimeric channel straddling the bilayer and accelerating the uncontrolled transmission of sodium cations (Na^+) amongst the intra- and extracellular environment, which in turn causes cell termination[146].

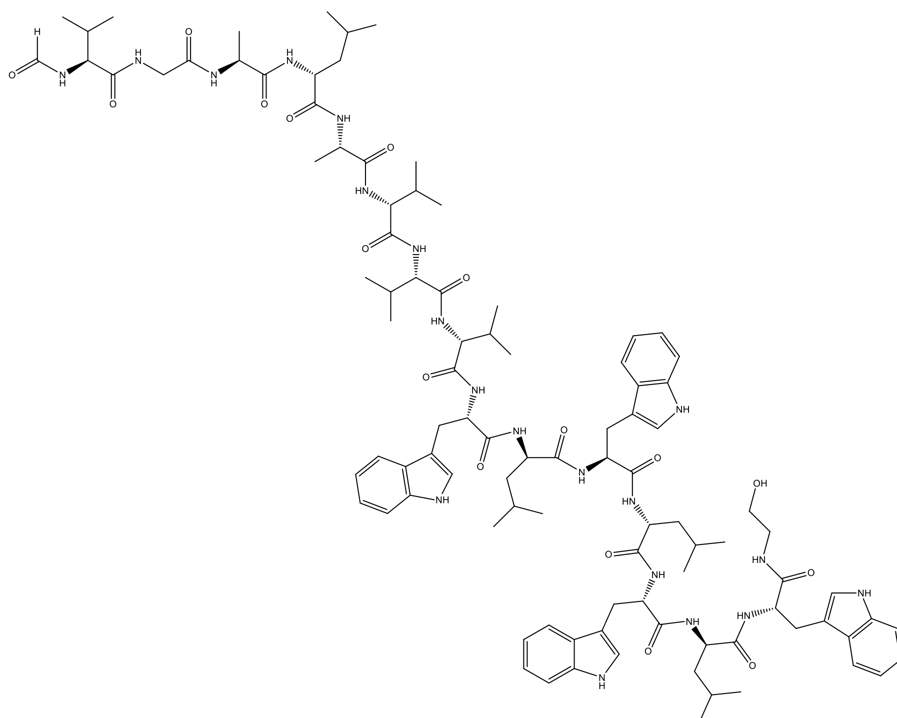


Figure 2.4. Chemical structure of Gramicidin

- ✓ Daptomycin (Figure 2.5) is a cyclic lipopeptide antibiotic with 13 amino acids in a sequence that connects to the bacterial cell membrane, aggregates, and disturbs the membrane. Daptomycin was synthesized by *Streptomyces roseoporus*, a soil bacterium with a half-life ($T_{1/2}$) of 8-9 hours. The FDA accepted this AMP and its derivative, Cubicin, in 2003 for clinical use specifically for infectious disease treatment or prevention[147].

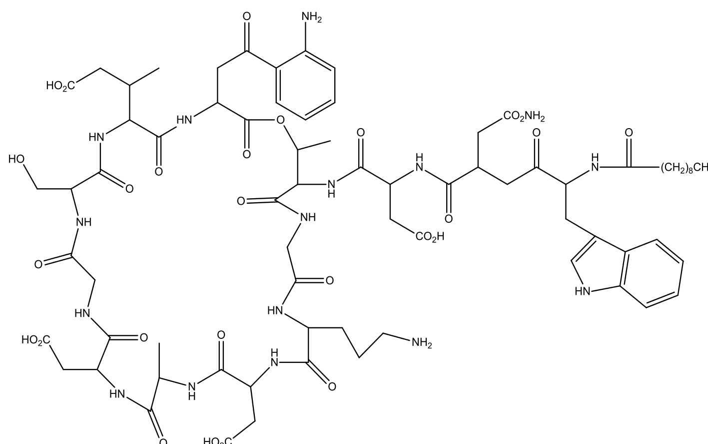


Figure 2.5. Chemical Structure of Daptomycin

- ✓ Vancomycin (Figure 2.6) is one of the oldest antibiotics still on the market for clinical purposes nowadays. It was isolated from *Streptomyces orientalis*, a fungus from Borneo jungles, in 1957 and accepted by FDA in 1958, although the original version of vancomycin

was contaminated by impurities[148]. Over the years, the contaminations have been successfully removed, leading to fewer vestibular side effects. However, this antibiotic is still lethal if dispensed at higher doses or administered for extended periods of time[149]. Vancomycin remains the leading agent for methicillin-resistant coagulase-negative and coagulase-positive staphylococcal infections such as bacteremia, pneumonia, and osteomyelitis. Moreover, this AMP drug is also administered for semi-synthetic penicillins or cephalosporins allergic patients[150].

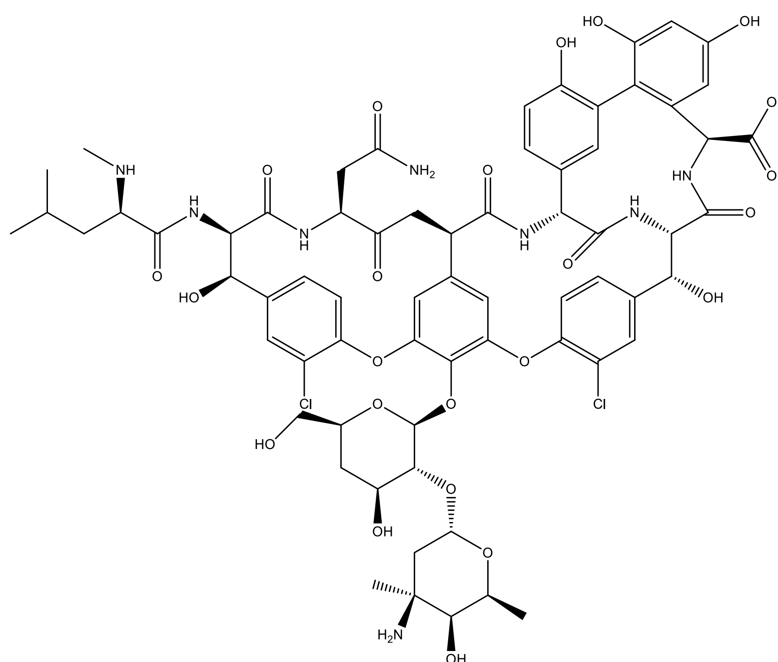


Figure 2.6. Chemical Structure of Vancomycin

- ✓ Dalbavancin (Figure 2.7), also known by trade names Dalvancan and Xydalba, is a semisynthetic glycopeptide vancomycin-derivative that has antimicrobial activity against Gram-positive bacteria[151,152] and long elimination half-life ($T_{1/2}$ = 14 days)[30]. This antibiotic blocks the formation of bacteria cell walls as well as the interrupts cell membrane and influences membrane permeability[152–154]. Dalbavancin was approved by the FDA in 2014 as an intravenous drug for severe bacterial skin infection treatment[151].

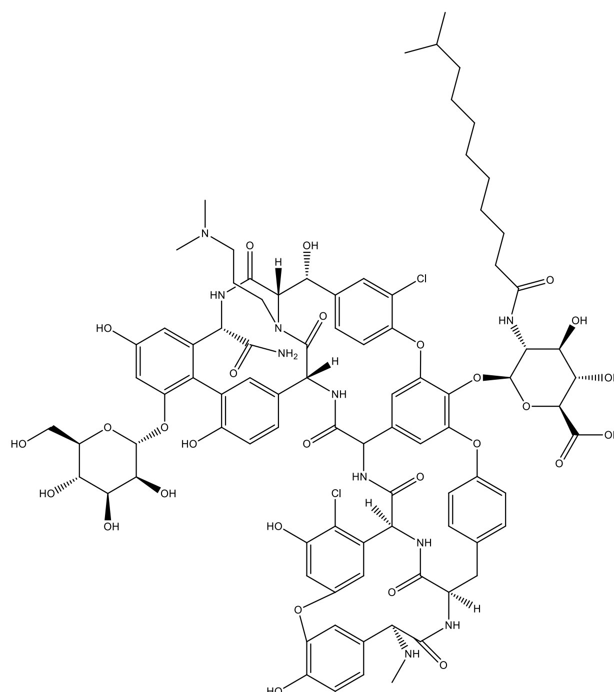


Figure 2.7. Chemical Structure of Dalbavancin

- ✓ Oritavancin is a small vancomycin-derived lipoglycopeptide (Figure 2.8), along with dalbavancin and telavancin. Orbactiv is the trade name of oritavancin, and it was accepted by the FDA in 2014 as an injection drug against *Staphylococcus aureus* infection[30]. As part of the second generation of glycopeptides, oritavancin is a semisynthetic derivative with outstanding pharmacokinetic and target engagement profiles to combat infection caused by vancomycin-resistant bacteria[155].

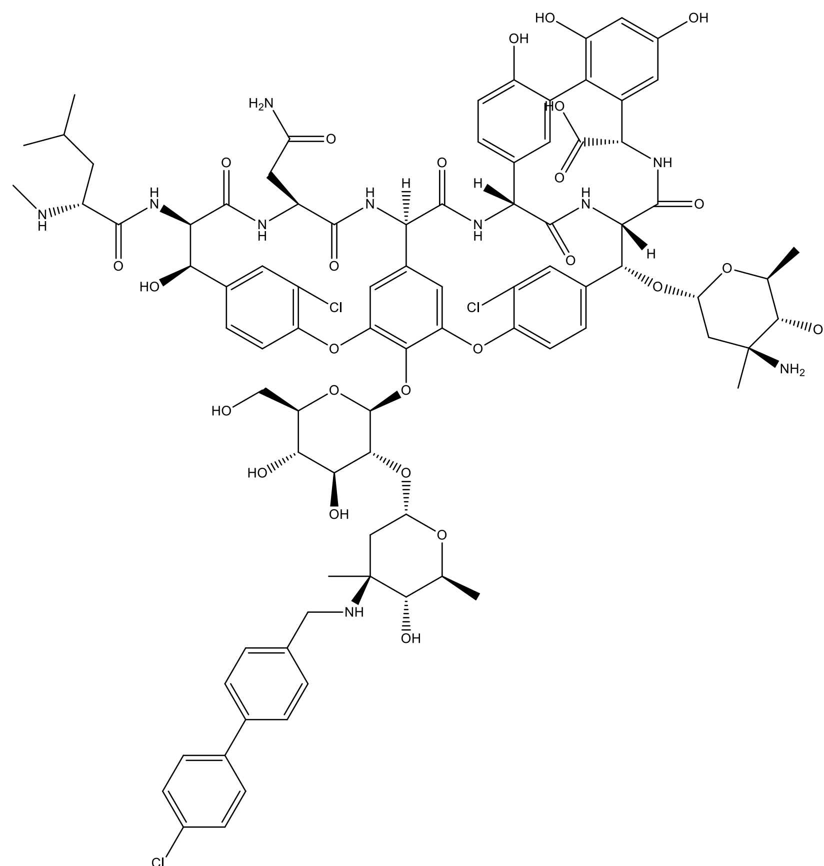


Figure 2.8. Chemical Structure of Oritavancin

- ✓ Telavancin is another lipoglycopeptide derived from vancomycin (Figure 2.9). Telavancin acts in a dual mode of action. Firstly, connect to the peptidoglycan precursor lipid-linked N-acetyl-glucosamine-N-muramylpentapeptide of the D-Ala-D-Ala terminus. This interaction inhibits the polymerization of peptidoglycan, followed by cross-linking (transpeptidation) phases. Telavancin is a powerful peptidoglycan synthesis inhibitor at the transglycosylase stage. The activity of telavancin is ten times better than its precursor, vancomycin[156]. Secondly, telavancin can initiate prompt concentration-dependent dissipation of cell membrane potential through bonding with lipid intermediate II molecules, creating membrane pores. Potassium ions and cytoplasmic adenosine triphosphate leakage then follow depolarization of the membrane. This second mechanism is specifically for bacterial membranes and seems to promote the speedy bacterial activity of telavancin, in contrast with vancomycin[154]. The FDA approved telavancin in September 2009 as an intravenous drug for patients with complicated skin and skin structure infections. This antibiotic is also suggested for susceptible or highly suspected Gram-positive bacterial infection patients[157]. Telavancin

demonstrates a serum half-life of 7 to 9 hours. Therefore it can be administered in once-a-day dosage[158,159].

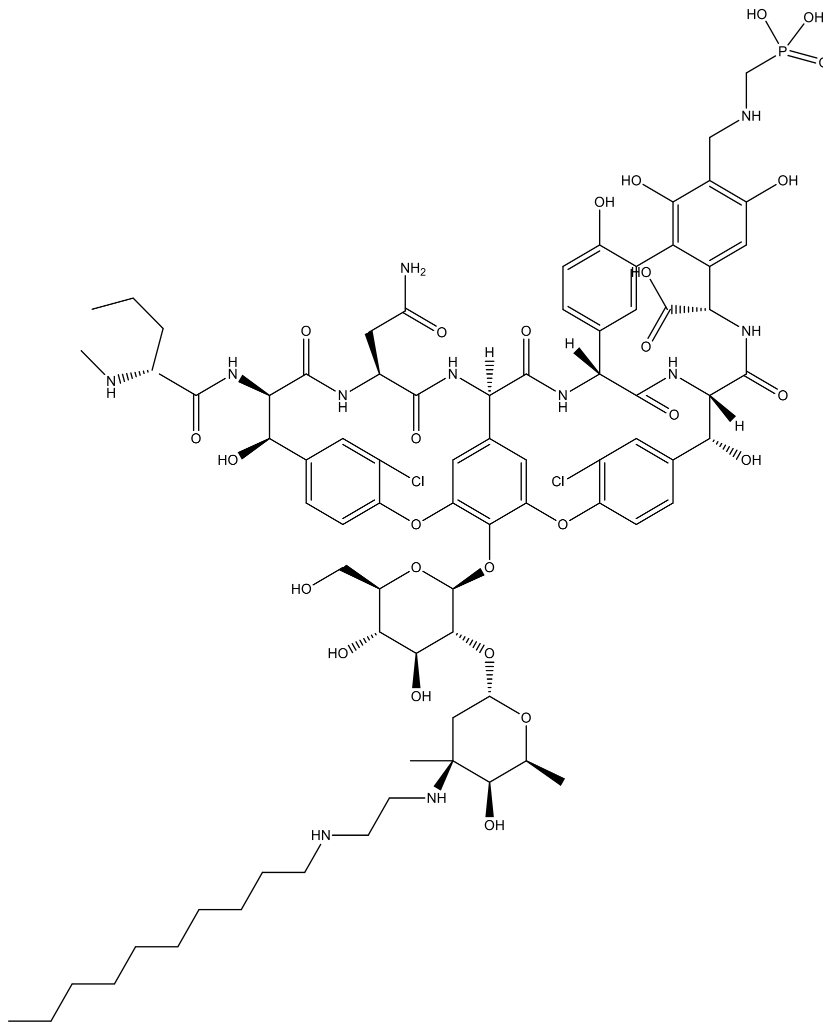


Figure 2.9. Chemical Structure of Telavancin

✓ Teicoplanin (Figure 2.10), a glycopeptide synthesized by the bacterium *Actinoplanes teichomyceticus,* has six major elements and a similar mechanism of action with vancomycin through inhibition of cell wall synthesis. However, it has different pharmacokinetic characteristics[160]. For instance, teicoplanin has a longer serum half-life (88-182 hours) and can be administered intravenously or intramuscularly. This antibiotic target some Gram-positive bacteria, specifically against methicillin-resistant *Staphylococcus aureus*.

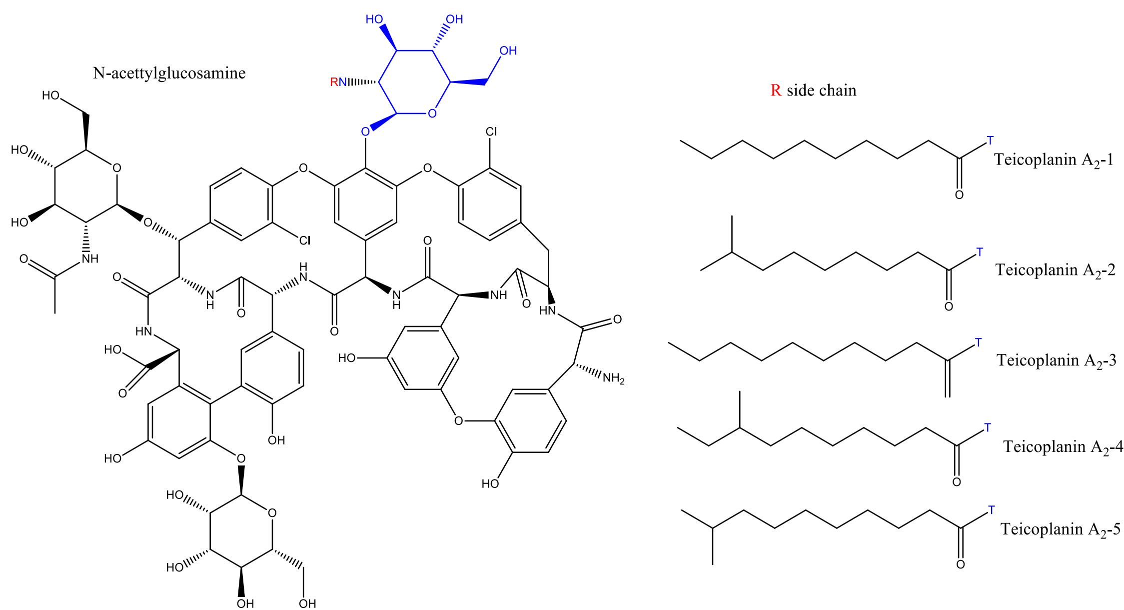


Figure 2.10. Chemical Structure of Teicoplanin

- ✓ Colistin or polymyxin E is a cyclic lipopeptide antibiotic containing ten residues and a fatty acid, 6-methyl octanoic acid (Figure 2.11). This peptide is generated by *P. polymyxa* var. *colistinus* and is active against some Gram-negative bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*[30]. It was accepted by the FDA in 1959 as an antimicrobial drug for treating several forms of diseases caused by Gram-negative bacteria, such as diarrhea and infections in the urinary system[161]. This antibiotic is considered the ultimate resource to fight against infections of carbapenem-resistant *Enterobacteriaceae*[9], even though the use of colistin has been limited due to its antagonistic toxic effects and resistance matter[161].To solve the resistance issue, an alternative approach of endolysin-antibiotics combination for bacterial treatment has been recommended[40]. For instance, the combination of endolysin from *Acinetobacter baumanii* (Lys-ABP-01) and colistin in the therapy of multi-drug resistant strains of *Acinetobacter baumanii* improved the antimicrobial activity compared to either of the treatments alone[162].

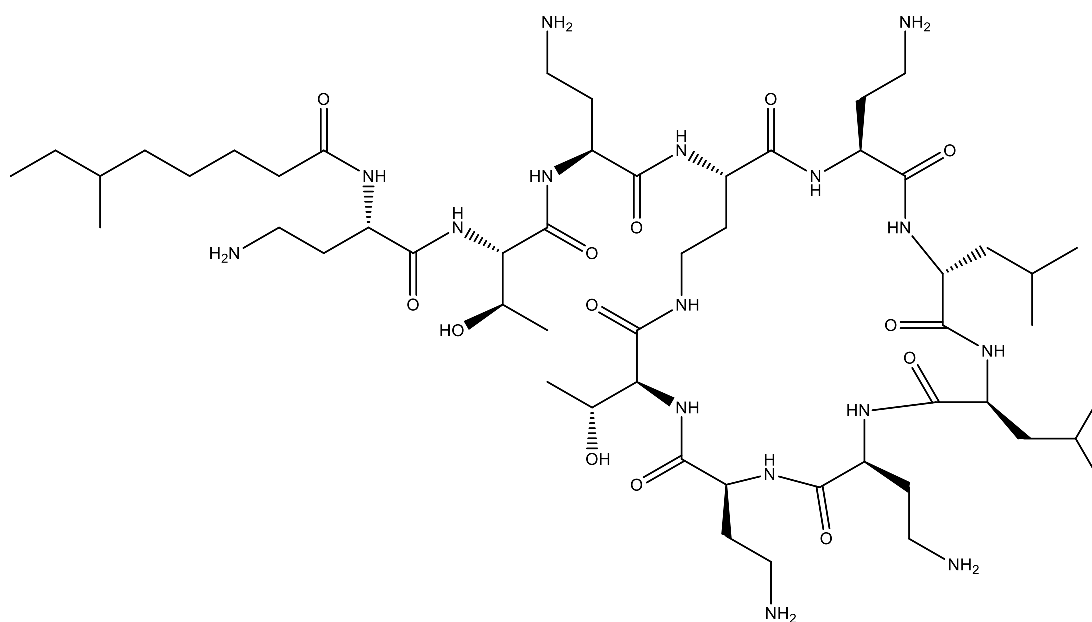


Figure 2.11. Chemical Structure of Colistin

- ✓ Polymyxin B is a cyclic lipopeptide like colistin containing diamino butyric acid and D-phenylalanine liable for its planar ring structure (Figure 2.12)[163]. This antibiotic was synthesized by *Paenibacillus polymyxa* and had six different isoforms that varied in the primary fatty acid and in one of the amino acids that shape the planar ring. Polymyxin B is lethal to a wide array of Gram-negative bacteria, including the *Enterobacteriaceae* family, by acting as a surfactant that penetrates the bacterial cell membrane[35].

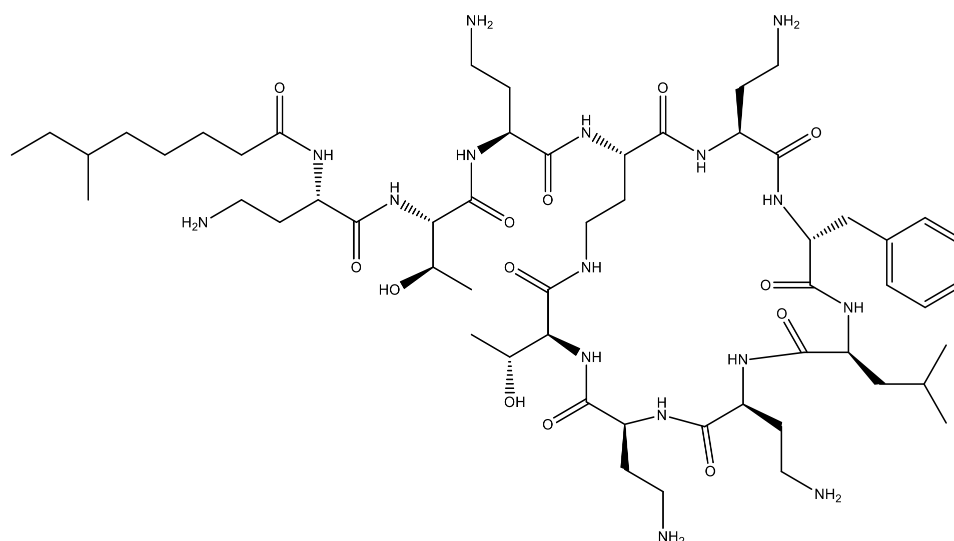


Figure 2.12. Chemical Structure of Polymyxin B

2.5 Nodule-Cysteine-Rich Peptides

Many cysteine-rich peptides (CRPs) are plant-based [164–166], and most have antimicrobial activity[50,167,168]. One interesting member of the CRPs group is the NCR peptides, which are generated through the nitrogen-fixing symbiosis between legumes and rhizobia in nodule cells and then exhilarate rhizobia into terminal differentiation[169,170]. A unique characteristic of the NCR peptides is that they contain four or six cysteine residues, which the cysteines are vital for the symbiotic functions of the legume plants[171]. One well-known model legume that establishes symbioses with *Sinorhizobium meliloti* as a symbiotic partner is *Medicago truncatula*, an inverted repeat-lacking clade (IRLC) legume. From this model, 639 NCR peptides have been encoded from over 700 NCR genes[172,173]. These are secretory peptides expressed specifically in the nodules and played a significant role in symbiosomes to generate suitable bacteroid differentiation[174,175].

NCR peptides contain conserved cysteine residues, categorizing them into defensin-like families. Several publications have validated that some members of the NCRs family (NCR247 and NCR335) possessed antimicrobial activity, like defensins[176,177]. Moreover, extensive research utilizing 19 NCR peptides discovered that NCR peptides with high isoelectric points property (pI >9), such as NCR044, NCR147, and NCR247, possess in vitro antimicrobial activity against *Candida albicans*[178]. Hence, the NCR net charge is supposed to be a crucial component of antimicrobial activity.

NCR044 is a well-known NCR member that has been reported for its three-dimensional structure. This peptide has 36 amino acids in its sequence, a net charge of +9, pI of 10.32, and 38% of hydrophobic residues[178,179]. A report from Velivelli et al. revealed the secondary structure of NCR044, which displayed an antiparallel β-sheet between A23 to R25, and G28 to C30 at the C-terminal region and a short α-helix (S11 to E14). At the same time, half of the N-terminal was disordered and dynamic (Figure 2.13)[179]. In addition, the disulfide bridges formed at C1 – C4 and C2 – C3 corresponded to conformational stability. The synthetic NCR044 displayed activity against some plant fungal pathogens, such as *Candida albicans*, *Botrytis cinerea*, *Fusarium graminearum*, *F. virguliforme*, and *F. oxysporum*[178,179].

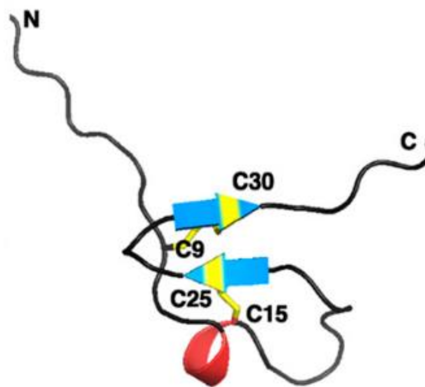


Figure 2.13. The secondary structure of NCR044 with four cysteine side chains is colored in yellow, β-strand highlighted in blue, and α-helix is tinted in red[179].

NCR 169 peptide is a member of the NCR family comprised of 38 amino acids, including four cysteine residues, and is crucial for bacteroid differentiation[171]. NCR 169 is usually located in the peribacteroid space between the symbiosome membrane and bacteroid, hence the function of NCR 169 in bacteroid differentiation[171,180,181]. Nonetheless, it is also believed that NCR 169 might have other functions besides bacteroid differentiation, such as in nitrogen fixation and bacterial survival in the symbiotic cell since it has been discovered in isolated bacteroid extracts, and the mutant form of NCR 169 provokes early senescence of the nodule[182–184]. A recent publication by the Isozumi group in 2021 revealed the structure of NCR 169 in two oxidized forms, NCR169-ox 1 and -ox2, utilizing ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) NMR technique (Figure 2.14)[185]. The NCR169-ox1 has one anti-parallel β-sheet at the C-terminal. This secondary structure was similar to the NCR169-ox2 even though their disulfide bridge motifs were diverse, in which NCR169-ox1 comprises two disulfide bridges at C1-C2 and C3-C4, and NCR169-ox2 has C1-C3 and C2-C4 disulfide patterns. Furthermore, the investigated peptides in this study have also been tested for their antimicrobial activity against *Escherichia coli* K12 and *Sinorhizobium meliloti*, in which NCR 169-ox 1 has slightly better activity against both bacterial strains compared to NCR 169-ox 2.

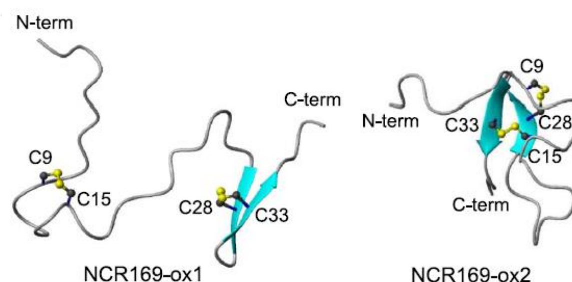


Figure 2.14. Secondary Structure of NCR169-ox 1 (left) and -ox2 (right)[185]

2.6 Solid-Phase Peptide Synthesis

The isolation and introduction of insulin as a therapeutic agent in 1923 were pioneering peptides as potential drug sources. However, the interest in peptide synthesis was depleted in the second half of the 20th century because "small molecules" were favored as biologically active agents, considering their advantages, such as the simplicity of the synthesis process, which enabled to obtain more products in less budget compared to the production of peptide-based drugs. Nevertheless, peptide synthesis has gained more attention over the last two decades, propelled by some genetic engineering and molecular biology inventions that enabled a simpler polypeptide synthesis[186].

Solid-phase peptide synthesis (SPPS) is a well-known procedure that can be carried out on solid support to formulate an expansive scope of synthetic compounds. Bruce Merrifield developed this method in 1963, simplifying the peptide synthesis process. Merrifield was awarded the Nobel Prize in 1984 for this innovation. SPPS became a popular choice in many peptides synthesis research due to several advantages: all reactions in SPPS are conducted in one single container; an excessive amount of amino acid and reagents can be used to drive the reactions until completed and consequently can be removed by washing and lacking intermediates purification in each step; high yields can be achieved; shorter and faster-reacting cycles compared to other methods; and application of repetitive steps which allowed to automatize the whole process[187,188].

The SPPS was conducted linearly from the C-terminus to the N-terminus (the C → N strategy). The carboxyl group of the amino acid to be integrated reacts with the N^{α}-amino group of the peptidyl chain anchored to the solid support. The success of SPPS is determined by the proper

selection of the solid support, the linker, the coupling reagents, and the protecting groups. Furthermore, there are four main phases in SPPS (Figure 2.15)[189]. Those phases are:

1) The first amino acid (appropriately protected) is coupled to the solid support. This process typically utilizes a linker to assist in releasing the peptide at the end of the synthesis.
2) Scrupulous deprotection of the $N^{\alpha-}$amino group of the first amino acid coupled to the solid support.
3) Cycles of coupling and deprotection steps of the related protected amino acids until the coveted peptide sequence is attained.
4) Deprotection and discharge of the peptide from the solid support to yield the desired product.

2.6.1. The Solid Support

A solid support is frequently used to designate the matrix in which peptide synthesis is staged. Specific physical and chemical requirements for solid support to be beneficial in the SPPS process are as follows: have a good swelling capacity and display low cross-linking for a good approachability, and the distribution of reagents must be inert in all environments used in the whole synthesis; must display great mechanical constancy, must be insoluble in all solvents utilized in the synthesis, and must comprise functional groups so that a linker might be attached to it[110].

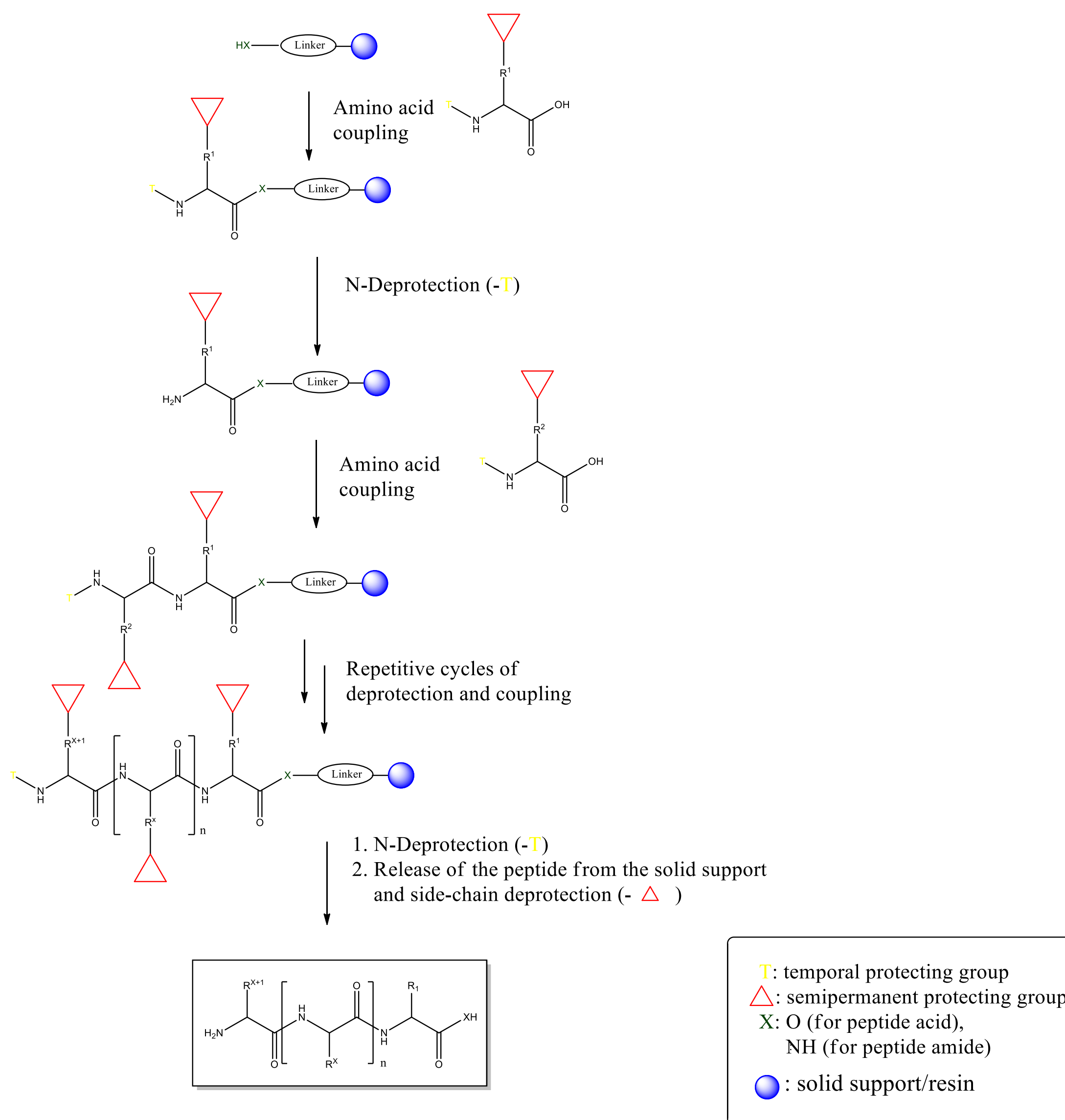


Figure 2.15. A general protocol for SPPS

At present, two foremost groups of supports are utilized for SPPS, namely polystyrene (PS), a slightly cross-linked with divinylbenzene, and polyethylene glycol. Based on the strategy used in solid-phase peptide synthesis, TentaGel® resin, a member of the polyethylene glycol group, was selected in this research.

2.6.2. The Linker

The linker is a bifunctional molecule with two sides, one side is bonded to the first amino acid via a bond labile to cleavage conditions, and another is connected to the solid support. The linker denotes the reversible bond between the peptide and the resin. Linker will affect peptide-resin

arrangement, resin loading, and C-terminal functionality of the synthesized peptide[187,188]. Various linkers are commercially available; nonetheless, the most used are Wang and Rink linkers (Figure 2.16), whereas Wang linker is a peptide acid linker, and the Rink linker is an amide linker. Their linker commonly acknowledges the resins, hence the name Wang Resin and the Rink Amide Resin. As for this project, since PEG resins with polystyrene were used, only a Rink amide linker (RAM) was utilized[188].

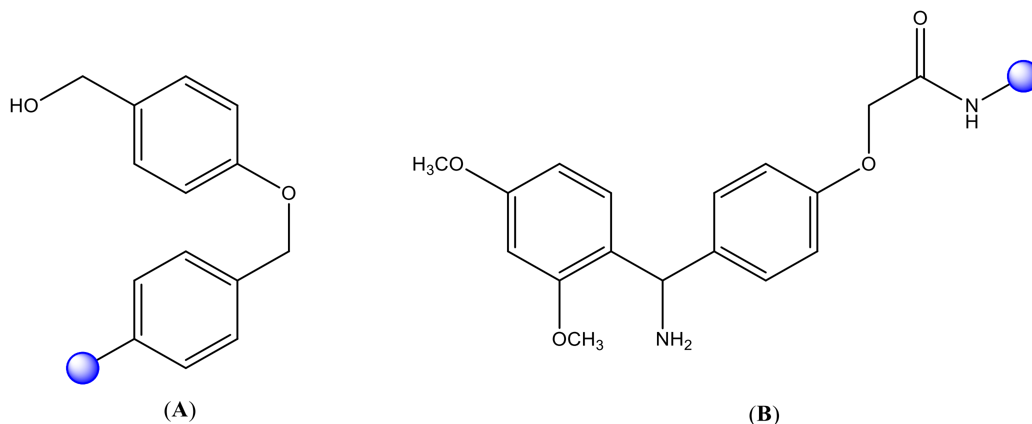


Figure 2.16. Structures of Common Linkers Utilized in SPPS. (**A**) Wang Linker and (**B**) Rink Amide Linker

2.6.3. Protecting Groups

The protecting groups play a vital role in SPPS to avoid unwanted side reactions during the amino acid coupling process, in which the protecting groups are sheltering the functional groups that are not participating in the reaction. The main requirements for a good protecting group are: the introduction of protecting group to the functional group should be easily executed; the functional group should be stable in varied reaction conditions; it should be safely removed at the end of the synthesis or when modification in the functional group is necessary[187].

Two types of protecting groups involved in SPPS are the temporary protecting group which protects the N^{α}-amino group, and the permanent protecting group, which shields the side-chain functionality of trifunctional amino acids. The temporary group is removed before adding an amino acid, while the permanent group is detached during cleaving. The removal conditions for these two types of protecting groups must be different. Thus the discouragement process could be selectively completed.

The main approaches applied in SPPS are Boc/benzyl and Fmoc/tBu. The Boc/benzyl approach involves the tert-butyloxycarbonyl (Boc) group (Figure 2.17 A) as the protecting group for the α-amino group, and benzyl groups act as the side chains protecting groups. The Boc has acid-labile characteristics, and it can be removed with trifluoroacetic acid (TFA) in dichloromethane (DCM). Furthermore, the side chain protecting groups are detached with hydrogen fluoride (HF) treatment [187].

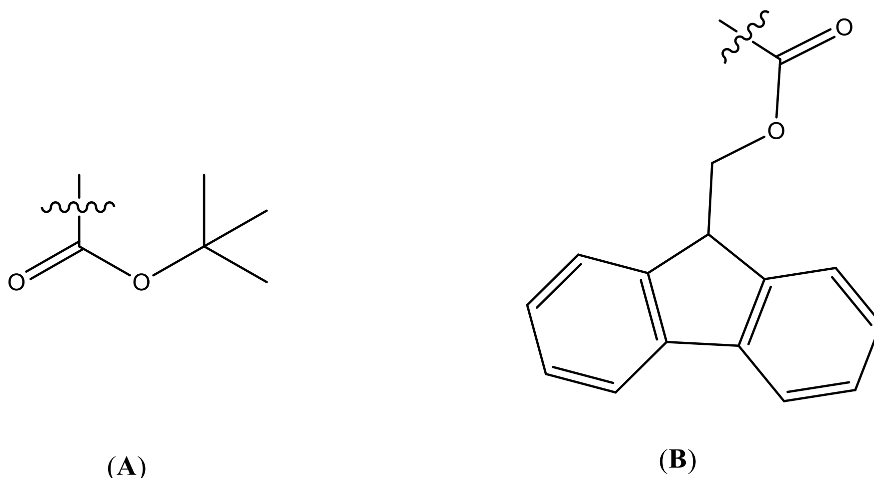


Figure 2.17. Chemical Structure of Common Temporary Protecting Group used in SPPS. (A). Boc Group and (B) Fmoc Group.

The Fmoc/tBu approach includes the use of the 9-fluorenylmethyloxycarbonyl (Fmoc) group (Figure 2.17 B) as the protecting group for the α-amino group and tBu (*tert*-Butyl) or trityl as the side chain protecting groups. The Fmoc group is a base labile; therefore, it can be removed in the presence of secondary amines (Figure 2.18), such as piperidine and piperazine in a relative polar solvent such as *N*-*N*-dimethylformamide (DMF) or *N*-methyl pirrolidone (NMP)[190]. In contrast, the side chain protecting groups are acid-labile. Consequently, acid solvents like TFA can be used for their removal[187].

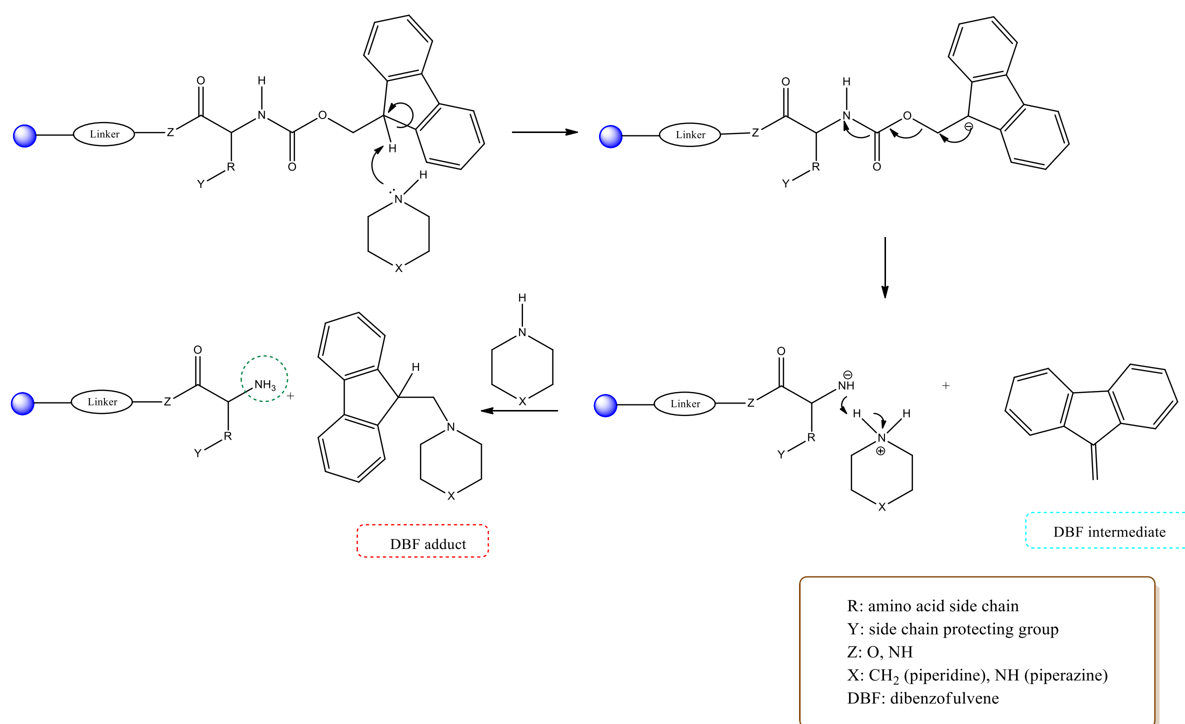


Figure 2.18. Mechanism of Fmoc Group Removal by Secondary Amines for the Formation of a Free-NH_2 in SPPS[190]

Both Boc and Fmoc approaches are equivalently acceptable. However, the Fmoc/tBu strategy is the most advantageous because this strategy facilitates the removal of the protecting group selectively under different chemical environments and cleavage procedures, which subsequently certifies milder total reactions[187]. The work reported in this dissertation utilizes Fmoc/tBu strategy.

The type of amino acid determines the selection of side chain protecting groups; not all amino acids require protection. For instance, the trityl group is used for amino acids like asparagine, cysteine, glutamine, and histidine. In contrast, the tertiary butyl is used for aspartic acid, glutamic acid, serine, threonine, and tyrosine. The Boc group protects lysine and tryptophan amino acids, while the Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl) protects arginine[187]. The amino acids used in this project were properly protected in the side chain.

2.6.4. Coupling

The amide bond formation between two amino acids does not occur spontaneously. The activation of carboxylic acids by coupling reagents is needed for this bond formation. The amide bond formation can precede the loss of chiral α-carbon integrity, allowing racemization. The activated species, such as benzotriazole ester, is mostly in ester form, affording an efficient leaving

group that supports the amide bond formation. At present, many coupling reagents are available, though the most used coupling reagents can be divided into three groups: aminium salts, for instance, HOBt (1-hydroxy-benzotriazole) and HOAt (1-hydroxy-7-azabenzotriazole),; carbodiimides, such as DCC (N-N'-dicyclohexylcarbodiimide) and DIC (N,N'-diisopropylcarbodiimide) and phosphonium salt, like PyBOP (benzotriazole-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate) (Figure 2.19)[187,191].

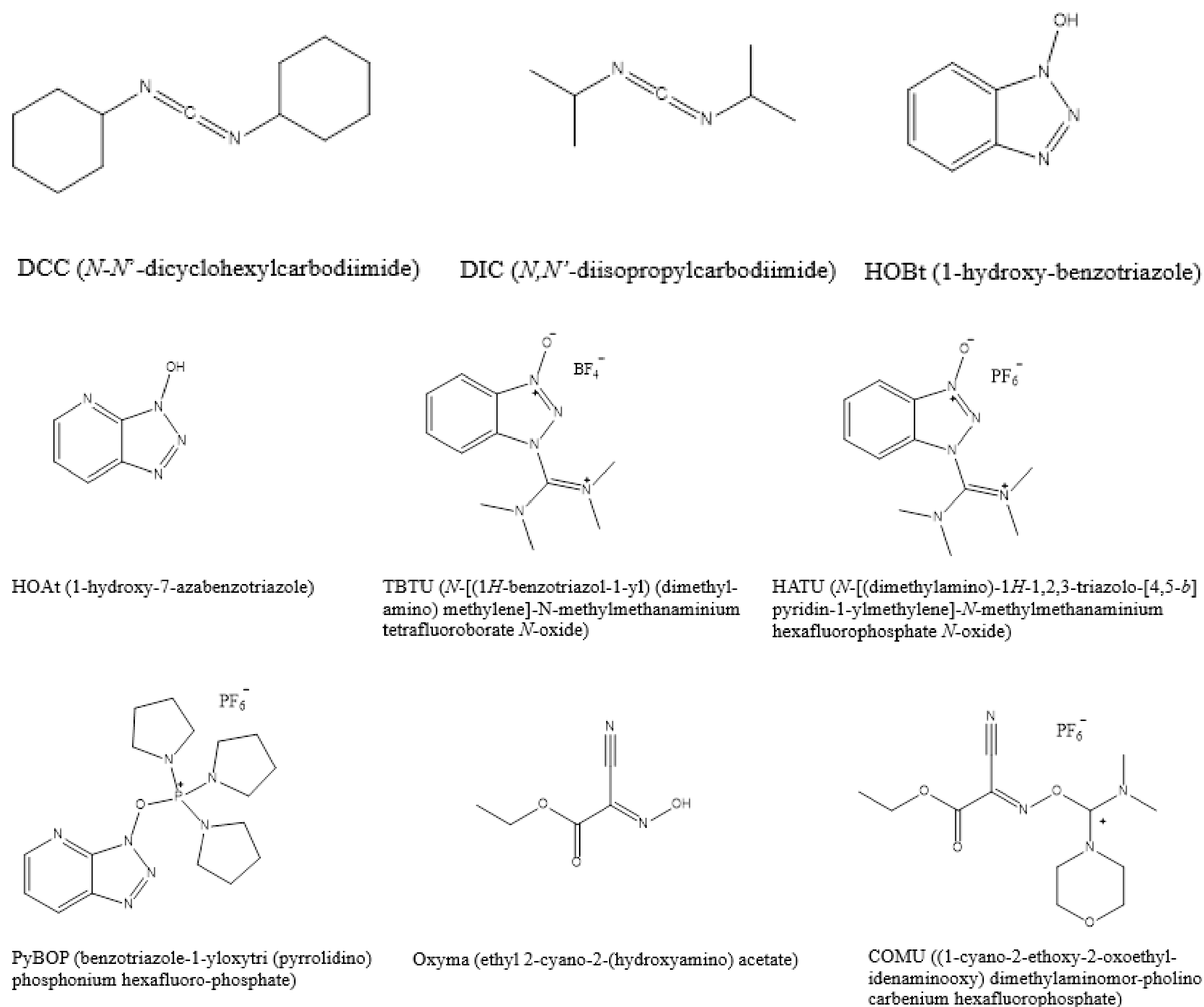


Figure 2.19 Examples of Coupling Reagents for SPPS

Chapter 3

Objectives

The main objective of this project was to synthesize AMPs derived from NCR169 and NCR147 with enhanced antimicrobial activity. Determining the active part from each parent peptide's sequence is important for developing the antimicrobial profile of each peptide. Another key factor addressed in this study is the role of certain amino acids in the antimicrobial profiles of peptides.

In the first part of our research, a full sequence of NCR169 was synthesized along with its derivatives, including the smaller fragments, followed by a biology assay for antimicrobial activity. We aimed to determine the active core fragment of NCR169 against microorganisms such as bacteria and fungi. Second, we utilized our findings from the first step and synthesized the active sequence of NCR169 with some modifications to the initial sequence to investigate the role of certain amino acids in the stability of the peptide and how they could affect the activity. The best sequence would then be the parent compound in the next phase. Third, following the result of our previous stage, we synthesized analogs with modification of amino acid at a specific position with a modified amino acid that contains D- and L- forms and an unnatural side chain. This approach investigates the effect of different isoforms and side chain types on antimicrobial activity.

Lastly, we synthesized the full sequence of NCR147 and its derivatives, followed by a biology assay for antimicrobial activity. We aimed to determine the active core fragment of NCR147 and to enhance the antimicrobial activity of the peptide by conducting modifications to the lead sequence.

Chapter 4

Experimental Methods

4.1. Synthesis of Peptides

4.1.1. Materials

The Amino acids derivatives utilized in this project were bought from Bachem AG (Bubendorf, Switzerland), Chem-Impex (Wood Dale, IL, USA), and Irish Biotech GMBH (Marktredwitz, Germany). Solvents and reagents for microwave-assisted peptide synthesis were obtained from the following suppliers: diisopropylcarbodiimide (DIC) and Oxyma from Fluorochem Ltd. (Hadfield, Derbyshire, UK), dimethylformamide (DMF) from Merck KGaA (Darmstadt, Germany), N-methylpyrrolidone (NMP) from Iris Biotech GMBH (Marktredwitz, Germany). Solvents for manual SPPS were purchased from the following companies: dichloromethane (DCM), dimethylformamide (DMF), methanol, and piperazine from Alfa Aesar (Thermo Fisher Scientific GmbH, Kandel, Germany), and trifluoroacetic acid (TFA) and dithiothreitol (DTT) from Fluorochem Ltd. (Hadfield Derbyshire, UK). TentaGel S RAM resin was acquired from Rapp Polymere GmbH (Tübingen, Germany), and anhydrous 1-hydroxybenzotriazole (HOBt) from Abcr GmbH (Karlsruhe, Germany). HPLC grade TFA and acetonitrile (can) were procured from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of the highest grade available.

4.1.2. Synthesis Procedures

The synthesis process followed the standard protocol of SPPS (see Figure 2.21) using the automated peptide synthesizer CEM Liberty Blue at the Department of Medical Chemistry, University of Szeged, Hungary. The Fmoc-protected amino acids involved in the synthesis were dissolved in DMF to form a 0.2 M solution. The prepared amino acid solutions were placed in the reaction vessels and loaded into the CEM Liberty Blue SPPS instrument. We applied TentaGel S RAM (loading rate: 0.23mmol/g) as resin for this work, as well as diisopropylcarbodiimide (DIC) as an activator (0.5 M) and Oxyma (1 M) as a base activator in 5-fold excess of volume. A mixture of 10% piperazine and 0.1 mol of 1-hydroxy benzotriazole in DMF/ethanol (90:10 % *v/v*) was used

for removing the Fmoc group during the synthesis process. The removal process was conducted in two cycles. The ultimate peptide was cleaved out of the resin with a cleaving cocktail consisting of 95:5 (*v/v*) trifluoroacetic acid (TFA)/water, added with dithiothreitol (DTT) 3% (*w/v*) and triisopropylsilane (TIS) 3% (*w/v*). The cleaving cocktail and peptide-resin complex mixture were shaken for 3 hours at room temperature. The resin was separated through filtration followed by evaporation with Heidolph Rotary Evaporator, and the crude peptides were precipitated in cold diethyl ether. Then, the precipitate was filtered, and dissolved with water, followed by lyophilization in S-Biotech Christ Beta 2-8 LD to gain a white solid peptide.

4.1.3. Analysis and Purification

Following the synthesis process, analytical HPLC (high-performance liquid chromatography) and electrospray ionization mass spectroscopy (ESI-MS) were utilized to analyze the peptides. RP-HPLC is a technique that enables the separation, identification, and quantification of the various constituents in a sample. This separation technique is based on the distinct affinities of each sample component to a mobile phase and a stationary phase. For this work, we used RP-HPLC (reverse-phase high-performance liquid chromatography), a separation technique based on hydrophobicity. Several advantages of RP-HPLC for peptides analysis include the finest resolution that can be gained on a broad scope of chromatographic circumstances; the easiness of experiment in which the selectivity of the chromatography system can be adjusted by changing the mobile phase properties; high recoveries in general, therefore it could yield more product; and the outstanding reproducibility of recuring separations that conducted in a lengthy period[192]. In RP-HPLC, the mobile phase is polar, whereas water-based solutions are mainly used, and the stationary phase is hydrophobic, such as C8- or C18-based sorbent. Consequently, constituents with smaller affinity/more polar to the stationary phase will be primarily eluted, followed by a higher affinity/non-polar constituent. The analytical HPLC in this project was accomplished with Agilent 1200 HPLC system on a 250 x 4.60 mm Luna 5µ C8 silica column, 220 nm wavelength, and a solvent system of 0.1% (*v/v*) TFA in water (buffer A) and 80% (*v/v*) acetonitrile and 0.1% TFA (*v/v*) in water (buffer B) at a flow rate of 1.0 ml/min. The studied peptide was measured around 0.5-1 mg and dissolved in water or acetonitrile until the concentration of 0.25-1 mg/ml. It was then injected into the HPLC instrument to obtain a chromatogram.

Electrospray ionization mass spectrometry (ESI-MS) is a common technique used for peptide characterization. For this method, an electric field is directed to the analyte solution streaming through a capillary. Then, the solution is secreted toward the counter electrode at the tip of a fine capillary. Hence, the evaporation of the solvent caused the size of the droplets to be reduced, which then generated slighter droplets by Coulomb explosions and ultimately produced charged ions. The charged ions are then tested into the high-vacuum part of the mass spectrometer to analyze and detect mass[193]. The ESI-MS method enables multiple-charged peptide analysis. Therefore it is beneficial to utilize this method for peptide molecular weight analysis[194–196]. In this project, the ESI-MS used a Waters SQ detector coupled with an Agilent 1200 HPLC system, and the capillary voltage was 3.51 volts, in which the analytes from the analytical HPLC are transported straight to MS for analysis.

In the purification step, preparative HPLC was utilized to separate pure peptide fractions. The methodology for preparative HPLC is the same as analytical HPLC. Nonetheless, preparative HPLC aims to isolate the interested compound and collect the pure fractions. The preparative HPLC was performed with Shimadzu LC-20AD Liquid Chromatography on a 250 x 10 mm Jupiter 10µ C12 Proteo 90Å with the same solvents as the analytical HPLC and flow rate of 3.0 ml/min. In this purification step, we applied a gradient elution system until it reached the isocratic zone, based on the solvents system obtained from analytical HPLC. The fractions were collected based on their retention time. Then they were analyzed and identified with LC-MS. The pure fractions were combined, lyophilized, and examined for their biological activity.

4.2. Antimicrobial Activity Assay

4.2.1. Bacterial Strains

In this project, we utilized bacterial strains attained from the American Type Culture Collection (ATCC – United States) and NCTC (National Collection of Type Cultures - England). Gram-negative strains were *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 8739, ATCC 35218, and ATCC 25922), *Salmonella enterica* (ATCC 13076), *Klebsiella pneumoniae* (NCTC 13440), *Acinetobacter baumannii* (ATCC 17978) and the Gram-positive strains were *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (HNCMO112011 and ATCC 25923). In the last part of this work, we utilized an additional 8 Gram-negative bacteria for antibacterial assay. Those bacteria are

Pseudomonas syringae pv. tomato, *Pseudomonas syringae* pv. tabaci, *Pseudomonas gladioli*, *Xanthomonas campestris*, *Xanthomonas malvaceae*, *Erwinia chrysanthemi*, *Erwinia carotovora*, and *Agrobacterium tumefaciens*.

4.2.2. Antibacterial Activity Assay

To investigate the antibacterial activity of our synthesized peptides, we conducted a minimum bactericidal concentration (MBC) assay using potassium-phosphate buffer (PPB) at pH 7.4. This assay was performed at the Biological Research Center, Szeged, Hungary. The bacterial cultures were incubated overnight in LB and then diluted and raised to OD_{600} = 0.20-0.60 at 37°C with shaking. The grown bacteria were reaped and washed in PPB, then amended to OD_{600} = 0.1 (equivalent to 10^7 bacteria/mL). The studied peptides were diluted in sterile water and utilized between 25-0.125 µM in a two-fold dilution series. They were then incubated for 3 hours with ~ 10^7 log-phase bacteria in PBB at pH 7.4. Furthermore, 5µ from each sample were collected and arranged on LB agar. The growth of bacteria was supervised after overnight incubation at 37°C. The lowest concentration of the peptides that eradicated viable bacteria was regarded as the MBC.

4.2.3. Hemolysis Assay

Human blood was purchased from the Regional Blood Centre in Szeged. The use of human blood for the hemolysis assay has been authorized by the Regional Hungarian Ethics Committee and approved by the Ethics Review Sector of DG RTD (European Commission) in connection with EK's ERC AdG SymBiotics. The protocol was done as described in Lima et al. [197]. Baseline OD_{560} values were determined with cells in TBS buffer, while 0.5% Triton X-100 (Serva) was added to the cells at the same time as NCRs represented 100% of hemolysis. The hemolytic activity was calculated as % of red blood cell disruption relative to the positive control sample lysed with detergent Triton X-100.

4.2.4 Fungi Strains and Growth Conditions

In this work, we utilized the fungi strain from ATCC, *Centraal bureau voor Schimmelcultures* (CBS), Squibb Institute for Medical Research (SC), New Brunswick, NJ, US, and Szeged Microbiological Collection (SZMC). The *Candida* strains are *Candida albicans* (ATCC 10231, SC 5314, and SZMC 1458), *Candida auris* (0381), *Candida glabrata* (CBS 138), *Candida parapsilosis* (CBS 604), and *Candida tropicalis* (CBS 94).

All the *Candida* strains utilized for the assay were grown overnight on YPD medium (1% peptone, 1% dextrose, 0.5% yeast extract) at 30°C in a water bath shaker before each experiment. The cells were harvested by centrifugation (5 min, 3000x g) followed by two times washing with sterile distilled water and suspended in Difco Yeast Nitrogen base *w/o* Amino Acids medium (Becton, Dickinson and Company, Sparks, MD, USA) five times excess added with1% dextrose (YNB). Cells were calculated with the Burker chamber and diluted to the proper concentration. YNB medium was altered for eight-fold diluted AIM-V+AlbuMAX (BSA) medium (1/8AIM) from Gibco, Thermo Fisher Scientific, Dublin, Ireland, with the aim for induction of morphological change of *C. albicans* and *C. tropicalis*.

4.2.5. Antifungal Activity Assay

We conducted an antifungal assay to investigate the antifungal activity of our synthesized peptides. This assay was performed at the Biological Research Center, Szeged, Hungary. The growth inhibition of the studied peptides was examined in 96-well microtiter plates on *Candida* strains. The minimal inhibitory concentration (MIC) was determined with the micro-dilution method by adding 5 µL serially two-fold-diluted peptide solution to a YNB medium containing 95 µL cell suspension (4×10^4 cell/mL). The optical density of the cultures was assessed at 620 nm in SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany) after 48 h of incubation at 30°C. Minimal inhibitory concentration was defined as growth inhibition ≥90% compared to 100% growth of the untreated control. The experiments were performed thrice through biological repetition, always in triplicates.

The same primary experimental setting was applied to establish the peptides' fungicide effect. After 24 h of incubation, five µL samples were acquired from the cultures and combined with 95 µL sterile distilled water, followed by 10- and 100-fold dilution. Five µL from each dilution were stationed on a solid YPD medium, and after 48 h incubation at 30°C, the strains' growth was measured.

4.2.6. Biofilm Formation Assay of *C. albicans* and *C. tropicalis* Strains

A total of 95 µL of cell suspensions at 4 x 10^4 cells/mL in 1/8AIM medium were added into the wells of microplates and supplemented with 5 µL of two-fold dilution series of the peptides or 5 µL of the medium as control. Plates were incubated at 37°C at a 5% CO_2 level for 72 h, and the formed biofilms were washed twice with phosphate-buffered saline (PBS) to remove the slightly attached cells. The viability of the biofilm-embedded cells was measured with the XTT reduction assay. XTT was solved in PBS at 0.5 mg/mL concentration and supplemented with 1 µM menadion. After adding 100 µL XTT solution to each well, the plates were incubated for 2 h at 37°C in the dark. Subsequently, 80 µL of each supernatant was transferred to new 96-well plates, and the absorbance was measured at 490 nm using a SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany). The experiments were carried out in 5 biological replicates in duplicates.

4.2.7. Morphological Analysis

Bright-field microscopy. The *C. albicans* ATCC 10231 and *C. tropicalis* cells morphology was investigated after 48 h incubation with the NCR $169C_{17\text{-}38}$ and its derivatives by Zeiss Axio Observer inverted microscope (Carl Zeiss AG, Jena, Germany). The concentration of $NCR169C_{17\text{-}38}$ was 1.56 or 3.12 µM, while $NCR169C_{17\text{-}38ox}$ was 0.78 or 3.12 µM, and the initial cell concentration was 4 x 10^4 cell/mL. The control for this test is cells cultivated without peptides.

Scanning electron microscopy. Into the 12-well microtiter plate wells were stationed the polyethylene terephthalate, glycol-modified (PET-G) coverslips (Sarstedt, Nümbrecht, Germany). The suspension of 600 µL of *C. albicans* ATCC 10231 and *C. tropicalis* at 4 x 10^4 cells/mL were filled into the wells and processed with the tested peptides for 72 h at 37°C in 5% CO_2 level. The cultivation medium was separated, and the samples were cleansed with PBS. Cells were then fixed at room temperature for 2 h with 2.5% glutaraldehyde in PBS. After completing the fixation, the cells of *C. tropicalis* were strained using poly-L-lysine-coated polycarbonate filters. Then both samples of *C. albicans* and *C. tropicalis* samples were desiccated in aqueous solutions of escalating ethanol concentrations, critical point arid, enclosed with 15 nm gold by a Quorum Q150T ES sputter (Quorum, Laughton, UK) and examined in a JEOL JSm-7100F/LV scanning electron microscope (Jeol Ltd, Tokyo, Japan).

4.2.8. Combined Treatment of *C. auris* with Fluconazole and $NCR169C_{17-38}$

The products of the varied mixtures of fluconazole, $NCR169_{17-38}$, and $NCR169_{17-38ox}$ were generated via standard checkerboard titration technique. The fluconazole was assessed with a concentration of $NCR169_{17-38}$ span from 6.25 to 75 µM and $NCR169_{17-38ox}$ from 0.78 to 12.50 µM. The initial cell concentration in every well was 4 x 10^4 cells/mL. The optical density of the cultures was identified using a SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany) at 620 nm after 72 h of incubation at 30°C. The inhibition concentrations were characterized for each compound separately and in combinations. The experiments were conducted at least three times. The effect of combinations was counted using the fractional inhibitory concentration index (FIC). $FIC = FIC_A + FIC_B$. FIC_A = (MIC_A in combination)/(MIC_A alone); FIC_B = (MIC_B in combination)/(MIC_B alone).

4.2.9. Assessment of the Viability of Human Keratinocytes

The human keratinocytes (HaCaT) viability was recognized once the peptide treatments were completed and measured towards the untreated control. A 96-well micro-plate comprising 10,000 cells per well was embedded with HaCaT cells and then incubated at 37°C with 5% CO_2 in 95% humidity. The cells were then treated with the NCR169C derivatives in expanding concentrations the following day. Furthermore, HaCaT cells were rinsed with PBS after 48 h, followed by 69 min incubation at 37°C with tetrazolium salt (MTT) reagents (Sigma-Aldrich, St. Louis, MO, USA) at 0.5 mg/mL concentration diluted in the culture medium. Finally, the formazan crystals were dissolved in DMSO (Sigma-Aldrich, St Louis, MO, USA). The absorption was assessed at 570 nm using a SPECTROstar Nano plate reader (BMG Lab Tech, Offenburg, Germany). The experiments were conducted at least three times using four independent biological duplicates.

Chapter 5

Synthesis & Antimicrobial Activity of NCR169 and Its Derivatives

5.1.Synthesis of NCR169 and Its Derivatives.

NCR169, a key cationic peptide (net charge at pH 7 = 1.91) in bacteroid differentiation, contains 38 amino acids with four cysteine residues in its sequence[171]. Nitrogen fixation will not be generated if NCR169 is absent, and an early nodule senescence will be formed[182–185,198]. As mentioned in Chapter 2, the structure of NCR169 in two oxidized forms had a short antiparallel β-sheet in the C-terminal, while the elongated N-terminal retained different properties[185]. These results conform to our structural predictions created by the AlphaFold program (Figure 5.1).

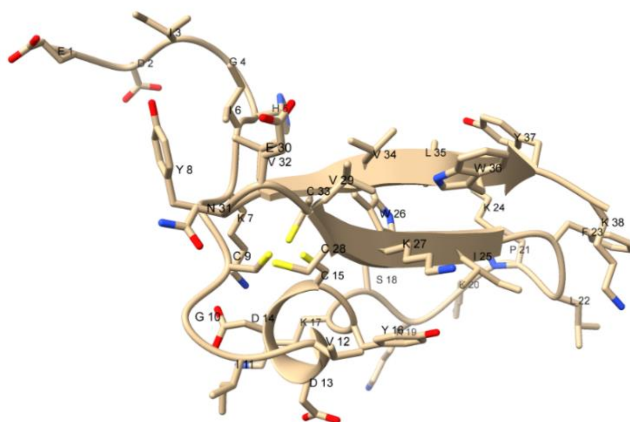


Figure 5.1 Structure Prediction of NCR169 (AlpaFold)

In the first part of the work, we synthesized the NCR169 full sequence with its shorter fragments to determine the active region of the peptide (Table 5.1). We also combined a short fragment called StrepII, consisting of eight amino acids (WSHPQFEK), into the NCR169, as this fragment enhanced the activity of NCR247[199]. The HPLC's chromatograms and the MS spectra from each synthesized peptide are available in Appendix 1. NCR169 peptide with its derivatives was then subjected to the antibacterial activity test.

Table 5.1 NCR169 and its derivatives. (A). Amino acid sequence and molecular mass values of the short peptide fragments. (B). Isoelectric point (pI), hydrophobicity (%), grand average hydropathy (GRAVY), and Boman index (kcal/mol) of the synthesized peptides.

A.

Peptides	Amino Acid Sequence	Molecular Mass Calculated	Molecular Mass Experimental[1]
NCR169	EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYK	4565.51	1522.9
NCR169-StrepII	EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYKWSHPQFEK	5605.66	1870.7
NCR169ox	EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYK	4565.51	1521.1
NCR169C$_{9,15,28,33}$/S	EDIGHIKY<u>S</u>GIVDD<u>S</u>YKSKKPLFKIWK<u>S</u>VENV<u>S</u>VLWYK	4521.24	1125.8
NCR169C$_{17-38}$	KSKKPLFKIWKCVENVCVLWYK	2740.44	914.1
NCR169C-StrepII	KSKKPLFKIWKCVENVCVLWYKWSHPQFEK	3780.60	1260.5
NCR169C$_{17–38}$ ox	KSKKPLFKIWKCVENVCVLWYK	2739.4	913.5
NCR169$_{21-38}$	PLFKIWKCVENVCVLWYK	2268.84	756.8
NCR169$_{27-38}$	KCVENVCVLWYK	1483.83	907.5
NCR169$_{17-28}$	KSKKPLFKIWK	1402.80	468.2

[1]$(M + 3H)^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide consistent with the calculated mass

B.

Peptides	pI[1]	Hydrophobicity[1]	GRAVY[1]	Boman Index[2] (kcal/mol)
NCR169	8.6	53.18	-0.14	0.87
NCR169-StrepII	8.1	55.09	-0.43	1.17
NCR169ox	8.6	53.18	-0.14	0.87
NCR169C$_{9,15,28,33}$/S	9.7	52.28	-0.48	1.37
NCR169C$_{17-38}$	10.2	48.27	-0.15	0.6
NCR169C-StrepII	10.1	51.19	-0.60	1.12
NCR169$_{21-38}$	9.1	51.93	0,51	-0.38
NCR169$_{27-38}$	8.1	33.79	0.37	0.23
NCR16917-28	11.3	28.55	-1.06	1.45

[1]values from https://www.thermofisher.com/hu/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html *[2]values from* https://aps.unmc.edu/prediction/predict

5.2 Antibacterial Activity of NCR169 and Its Derivatives

The lowest concentration that can eliminate the growth of bacteria was defined as the minimal bactericidal concentration (MBC). The antibacterial activities of NCR169 and its derivatives NCR169 are shown in Table 5.2. As for control, we tested two commercial antibiotics, Carbenicillin and Levofloxacin, against the same bacterial strains (Table 5.2). NCR169 and its derivatives are generally more effective than existing antibiotics. A concentration of 3.1 mM of

NCR169 eradicated *A. baumannii* and showed similar effectiveness against *E. coli* and *S. enterica*. However, its potency against *S. aureus* was lower (MBC: 6.3 µM), and higher concentrations of 12.5 mM were required to eliminate *K. pneumoniae*, *P. aeruginosa*, and *L. monocytogenes*. In contrast, it did not exhibit any activity against *E. faecalis*, as reported in Table 5.2. The MBC values presented here are the mean of four biological repeats. Still, there was significant variance in reproducibility, which may be attributed to the peptide's reduced solubility in PPB at pH 7.4.

Table 5.2. The minimum bactericidal concentrations (MBC; measured in µM) of the investigated peptides against various pathogens following a 3-hour treatment in phosphate buffer (PPB). Gram-negative *E. c., Escherichia coli* (ATCC 8739)*; S. e., Salmonella enterica* (ATCC 13076)*; K. p., Klebsiella pneumoniae* (NCTC 13440)*; A. b., Acinetobacter baumannii* (ATCC 17978)*; P. a., Pseudomonas aeruginosa* (ATCC 27853). Gram-Positive: *E. f., Enterococcus faecalis* (ATCC 29212)*; L. m., Listeria monocytogenes* (ATCC 19111)*; S. a., Staphylococcus aureus* (HNCMO112011).

Peptides	**Gram-Negative**					**Gram-Positive**		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
NCR169	3.1*	3.1*	12.5*	3.1	12.5*	-	12.5*	6.3*
NCR169-StrepII	3.1	3.1	12.5	12.5	12.5	25	-	6.3
NCR169ox	6.3	12.5	25	1.6	6.3	-	12.5	25
NCR169$C_{9,15,28,33}$/S	6.3	-	-	3.1	25	-	-	-
NCR169C_{17-38}	1.6	3.1	3.1	3.1	3.1	6.3	3.1	3.1
NCR169C-StrepII	1.6	3.1	3.1	3.1	3.1	3.1	3.1	3.1
NCR169$_{21-38}$	25	-	-	12.5	-	-	-	-
NCR169$_{27-38}$	25	-	-	-	-	-	-	-
NCR169$_{17-28}$	-	-	-	NT	-	-	-	-
Carbenicillin	1280	640	>10240	5120	10240	5120	80	640
Levofloxacin	5.0	1.3	320	20	1.3	160	320	2.5

**The best MBC value was measured, but there were more than two dilution step differences in replicate experiments.*

Using PPB at pH 5.8 resolved the solubility problem. It provoked the reproducible killing of *S. aureus, A. baumannii, E. coli,* and *S. enterica* at 3.1 mM and the termination of *P. aeruginosa* and *L. monocytogenes* at 6.3 mM (Table 5.3). The oxidized form of NCR169 at pH 7.4 did not improve the activity (except for *A. baumannii*); at pH 5.8, it was more active, particularly on *K. pneumoniae.* Replacement of the four cysteines with serines decreased the activity except for *A. baumannii* at pH 7.4. In contrast, the activity was better at pH 5.8 due probably to the better solubility of the peptide. The equal or two-fold differences in the MBCs of NCR169 and its serine-

substituted derivative indicated that cysteines are not essential for the peptide's antimicrobial activity. However, NCR169 was more effective on *L. monocytogenes*.

Table 5.3. The MBCs (µM) of the investigated peptides against various pathogens following a 3-hour treatment in PPB with modified pH. 5.8.

Peptides	Gram-Negative					Gram-Positive		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
NCR169	3.1	3.1	12.5	3.1	6.3	-	6.3	3.1
NCR169ox	1.6	3.1	3.1	3.1	6.3	-	3.1	3.1
NCR169$C_{9,15,28,33}$/S	1.6	6.3	12.5	3.1	6.3	-	25	6.3

It can be seen from this result that NCR169C_{17-38} is the active region of NCR169 compared to the full sequence of NCR169 and other shorter fragments. Therefore, we decided to explore more on this sequence more.

5.3 Synthesis of NCR169C_{17-38} Derivatives.

The next part of this project was to synthesize NCR169C_{17-38} with C-terminal amidation derivatives with various replacements of certain amino acids listed in Table 5.4 A [200]. The HPLC chromatograms and MS spectra of these peptides can be found in Appendix 1. The modifications in the NCR169C_{17-38} sequence slightly influenced the physicochemical properties of the peptides (Table 5.4 B). Replacing the two tryptophans with two alanines led to a slight decline in hydrophobicity (less than 10%) and altering the two cysteines to serine or alanine led to a 0.5 growth in the calculated pI value. The Gravy and Boman index, in all cases, have modest rates. Moreover, the 3D structures predictions of NCR169C_{17-38} and all its derivatives acquired with the AlphaFold program displayed a similar alpha-helical structure instead of the presence of β-sheet structure in the C-terminal part of the unmodified peptide (Figure 5.2).

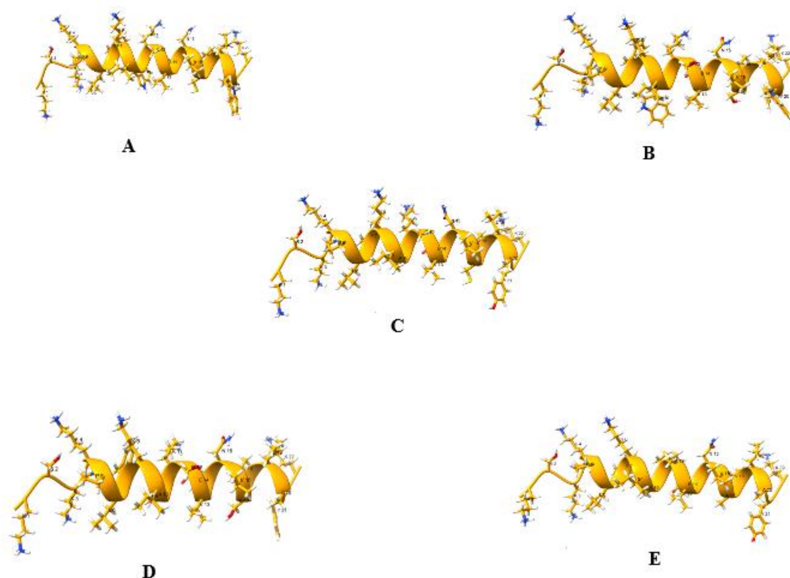


Figure 5.2 Structure Prediction of NCR169$C_{17,38}$ and its Derivatives (AlphaFold). **A**. NCR169C_{17-38}; **B**.NCR169C_{17-38} $C_{12,17}$/S; **C**. NCR169C_{17-38} $W_{10,20}$/A; **D**. NCR169C_{17-38} $W_{10,20}$/A, $C_{12,17}$/S; **E**. NCR169C_{17-38} $W_{10,20}C_{12,17}$/A

Table 5.4. NCR169C_{17-38} substitution derivatives[200]. **A**. Amino acid sequence and molecular mass values of the NCR169C_{17-38} derivatives. Substituted amino acids are underlined. **B**. Isoelectric point (pI), hydrophobicity (%), grand average hydropathy (GRAVY), and Boman index (kcal/mol) of the NCR169C_{17-38} and its derivatives.

A.

Peptides	**Amino Acid Sequence**	**Molecular Mass Calculated**	**Molecular Mass Experimental**[1]
NCR169$C_{17–38}C_{12,17}$/S	KSKKPLFKIWK**S**VENV**S**VLWYK	2707.3	903.6
NCR169$C_{17–38}W_{10,20}$/A	KSKKPLFKI**A**KCVENVCVL**A**YK	2509.1	837.3
NCR169$C_{17–38}W_{10,20}$/A, $C_{12,17}$/S	KSKKPLFKI**A**K**S**VENV**S**VL**A**YK	2477	826.8
NCR169$C_{17–38}W_{10,20}C_{12,17}$/A	KSKKPLFKI**A**K**A**VENV**A**VL**A**YK	2445	815.6

[1]*$(M + 3H)^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide, consistent with the calculated mass*

B.

Peptides	**pI**[1]	**Hydrophobicity**[1]	**GRAVY**[1]	**Boman Index**[2] **(kcal/mol)**
NCR169$C_{17–38}C_{12,17}$/S	10.6	47.64	-0.45	1.02
NCR169$C_{17–38}W_{10,20}$/A	10.1	38.74	0.09	0.64
NCR169$C_{17–38}W_{10,20}$/A, $C_{12,17}$/S	10.6	38.04	-0.21	1.07
NCR169$C_{17–38}W_{10,20}C_{12,17}$/A	10.6	41.04	0.03	0.59

[1]*values from* https://www.thermofisher.com/hu/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html [2]*values from* https://aps.unmc.edu/prediction/predict

5.4 Antibacterial Activity of NCR169C$_{17-38}$ and its Derivatives

All synthesized peptides retained antimicrobial activities against ESKAPE bacteria: *Enterococcus faecalis; Staphylococcus aureus; Klebsiella pneumoniae; Acinetobacter baumannii; Pseudomonas aeruginosa; Escherichia coli,* as well as *Listeria monocytogenes* and *Salmonella enterica* (Table 5.5)[200]. NCR169C$_{17-38}$ could terminate most tested bacteria at 3.1 µM, except *E. faecalis* and *E. coli,* requiring 6.3 µM and 1.6 µM, respectively. Substitution of the two cysteines with serine residues (NCR169C$_{17–38}$C$_{12,17}$/S) did not weaken. It even increased the activity in the case of *E. faecalis, S. aureus, A. baumannii,* and *S. enterica*, indicating that cysteines and the formation of a disulfide bridge are not compulsory for antimicrobial activity (Table 5.5). Tryptophan is suggested to play a crucial role in the activity and interaction of AMPs with bacterial membranes. Switching of the two tryptophan residues with alanine in NCR169C$_{17–38}$ (NCR169C$_{17–38}$W$_{10,20}$/A) eradicated or intensely lessened the activity against *E. faecalis, S. aureus, S. enterica, L. monocytogenes,* and *K. pneumoniae* whereas it continued effective against *A. baumannii, P. aeruginosa,* and *E. coli.* A mixture of these two kinds of replacements (NCR169C$_{17–38}$W$_{10,20}$/A, C$_{12,17}$/S) or substitution of both tryptophan and cysteine residues with alanine (NCR169C$_{17–38}$W$_{10,20}$C$_{12,17}$/A), reserved or condensed further the activities: both peptides were equally effective against *P. aeruginosa* and *L. monocytogenes* at 3.1 µM, and *S. aureus, E. coli,* and *S. enterica* at 6.3 µM, and *A. baumannii* (12.5 µM), nonetheless NCR169C$_{17–38}$W$_{10,20}$C$_{12,17}$/A became sedentary against *K. pneumoniae* (25 µM).

Table 5.5. The MBCs (µM) of peptides against Gram-negative: *E. c., Escherichia coli* (ATCC 8739)*; S. e., Salmonella enterica* (ATCC 13076)*; K. p., Klebsiella pneumoniae* (NCTC 13440)*; A. b., Acinetobacter baumannii* (ATCC 17978)*; P. a., Pseudomonas aeruginosa* (ATCC 27853)*, and* Gram-Positive: *E. f., Enterococcus faecalis* (ATCC 29212)*; L. m., Listeria monocytogenes* (ATCC 19111)*; S. a., Staphylococcus aureus* (HNCMO112011)[200].

Peptides	Gram-Negative					Gram-Positive		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
NCR169C$_{17–38}$	1.6	3.1	3.1	3.1	3.1	6.3	3.1	3.1
NCR169C$_{17–38}$C$_{12,17}$/S	1.6	1.6	3.1	1.6	3.1	3.1	3.1	1.6
NCR169C$_{17–38}$W$_{10,20}$/A	3.1	-	12.5	3.1	3.1	-	25	-
NCR169C$_{17–38}$W$_{10,20}$/A, C$_{12,17}$/S	6.3	6.3	25	12.5	3.1	25	3.1	6.3
NCR169C$_{17–38}$W$_{10,20}$C$_{12,17}$/A	6.3	6.3	25	12.5	3.1	-	6.3	6.3

-: inactive up to 25 µM

To investigate the effect of tested peptides on different strains of bacterial species, we measured the MBC of the two most active peptides, NCR169$C_{17–38}$ and NCR169$C_{17–38}C_{12,17}$/S against two additional strains of *E. coli* (ATCC 25922 and ATCC 35218) plus *S. aureus* (ATCC 25923), which are frequently used for antibiotic susceptibility assays. All these strains were comparably sensitive to these peptides (Table 5.6).

Table 5.6. MBCs (μM) of peptides against additional strains of *E. coli* (ATCC 25922 and ATCC 35218) and *S. aureus* (ATCC 25923)[200].

Peptides	***E.c.* ATTC** 25922	***E.c.* ATTC** 35218	***S.a.* ATTC** 25923
NCR169$C_{17–38}$	1.6	1.6	3.1
NCR169$C_{17–38}C_{12,17}$/S	1.6	3.1	3.1

5.5 Antifungal Activity of NCR169C17-38 and Its Derivatives

NCR169$C_{17\text{-}38}$ with its oxidized form (NCR169$C_{17\text{-}38}$ox) and NCR169$C_{17\text{-}38}W_{10,20}$/A were tested for their antifungal activity against some human pathogen *Candida* species and strains in the Department of Microbiology, University of Szeged. The peptides' minimal inhibitory concentration was determined by incubating the strains in the two-fold serial dilutions of the peptides, resulted a MIC value between 3.12 and 25 μM[201].

5.6. Synthesis of NCR169$C_{17\text{-}38}C_{12\text{-}17}$/S Derivatives

Substituting two cysteines with the isosteric serine residues (NCR169$C_{17–38}C_{12,17}$/S) has proved to boost the antimicrobial effect of the NCR169$C_{17\text{-}38.}$ Therefore, we decided to explore this peptide by synthesizing the NCR169$C_{17–38}C_{12,17}$/S peptide analogs to examine their antimicrobial properties by integrating some modified tryptophan into the sequence at residue numbers 10 and 20 (Table 5.7)[200]. The next chemically modified tryptophans (Figure 5.3) were applied to evaluate whether they could cause a significant impact on the antimicrobial activities of the peptide: 5-methyl tryptophan ($W^{5\text{-Me}}$), 5-fluoro tryptophan ($W^{5\text{-F}}$), 6-fluoro ($W^{6\text{-F}}$) tryptophan, 7-aza tryptophan ($W^{7\text{-Aza}}$), and 5-methoxy tryptophan ($W^{5\text{-MeO}}$). 5-fluoro tryptophan was utilized in the L- and D- enantiomeric forms for the synthesis, hence these two peptides are named NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-F-L}}$ and NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-F-D}}$. All other analogs of NCR169$C_{17\text{-}38}$C12,17/S were synthesized utilizing modified tryptophans in racemic forms, then separated into two peptides, excluding NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-MeO}}$. The separated peptides

were identified with codes I and II indicating either D- or L- conformations of the substituted tryptophan, which could be further verified if an exciting antimicrobial influence validates it[200]. The HPLC chromatograms and MS spectra of these peptides are presented in Appendix 1.

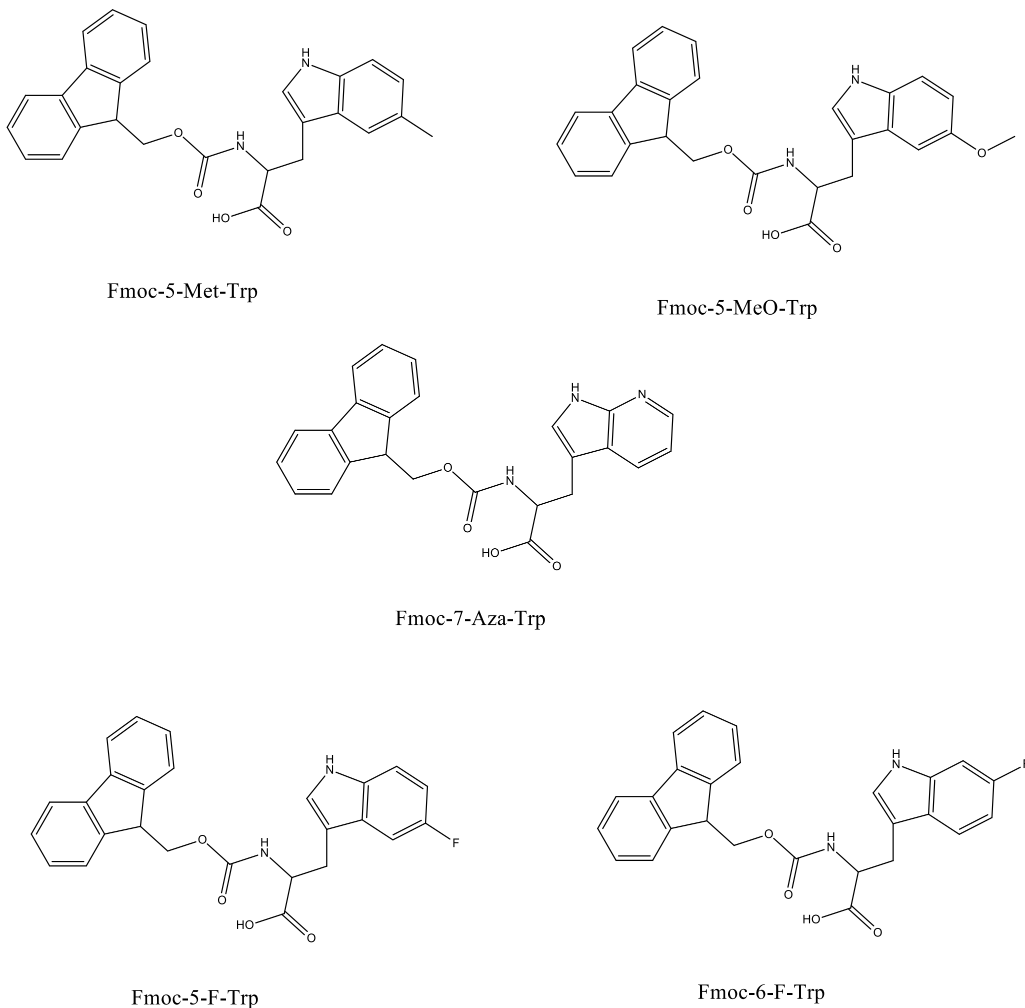


Figure 5.3. Structures of Modified Tryptophans

Table 5.7. List of $NCR169C_{17\text{-}38}C_{12,17}/S$ Derivatives[200]

Peptide Analogs	**Amino Acid Sequence**	**Molecular Mass Calculated**	**Molecular Mass Experimental** [1]
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}Me}$ I*	$KSKKPLFKIW^{5\text{-}Me}KSVENVSVLWYK$	2721.26	907.6
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}Me}$ II*	$KSKKPLFKIW^{5\text{-}Me}KSVENVSVLWYK$	2721.26	907.9
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{5\text{-}Me}$ I	$KSKKPLFKIWKSVENVSVLW^{5\text{-}Me}YK$	2721.26	908.6
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{5\text{-}Me}$ II	$KSKKPLFKIWKSVENVSVLW^{5\text{-}Me}YK$	2721.26	907.8
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}F\text{-}L}$	$KSKKPLFKIW^{5\text{-}F\text{-}L}KSVENVSVLWYK$	2725.47	908.9
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}F\text{-}D}$	$KSKKPLFKIW^{5\text{-}F\text{-}D}KSVENVSVLWYK$	2725.47	909.7
$NCR169C_{17\text{-}38}C_{12,17}/S\text{-}20W^{5\text{-}F\text{-}L}$	$KSKKPLFKIWKSVENVSVLW^{5\text{-}F\text{-}L}YK$	2725.47	909.0
$NCR169C_{17\text{-}38}C_{12,17}/S\text{-}10W^{6\text{-}F}$I	$KSKKPLFKIW^{6\text{-}F}KSVENVSVLWYK$	2725.47	909.6
$NCR169C_{17\text{-}38}C_{12,17}/S\text{-}10W^{6\text{-}F}$II	$KSKKPLFKIW^{6\text{-}F}KSVENVSVLWYK$	2725.47	909.4
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{7\text{-}Aza}$ I	$KSKKPLFKIW^{7\text{-}Aza}KSVENVSVLWYK$	2706.46	903.3
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{7\text{-}Aza}$ II	$KSKKPLFKIW^{7\text{-}Aza}KSVENVSVLWYK$	2706.46	903.6
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{7\text{-}Aza}$ I	$KSKKPLFKIWKSVENVSVLW^{7\text{-}Aza}YK$	2706.46	903.2
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{7\text{-}Aza}$ II	$KSKKPLFKIWKSVENVSVLW^{7\text{-}Aza}YK$	2706.46	903.1
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}MeO}$ I	$KSKKPLFKIW^{5\text{-}MeO}KSVENVSVLWYK$	2737.49	913.1
$NCR169C_{17–38}C_{12,17}/S\text{-}10W^{5\text{-}MeO}$ II	$KSKKPLFKIW^{5\text{-}MeO}KSVENVSVLWYK$	2737.49	913.1
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{5\text{-}MeO}$	$KSKKPLFKIWKSVENVSVLW^{5\text{-}MeO}YK$	2737.49	913.1

[1]$(M + 3H)^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide, consistent with the calculated mass.

**I and II correspond to the D- or L- configurations of the tryptophan-substituted peptides, as the modified tryptophans were initially used in racemic forms during synthesis, with subsequent separation of the resulting peptides.*

5.7. Antimicrobial Activity of $NCR169C_{17\text{-}38}C_{12,17}/S$ Derivatives

The synthesized $NCR169C_{17\text{-}38}C_{12,17}/S$ derivatives were subjected to an antibacterial assay to define their antibacterial activity against the same bacterial strains in the previous test (Table 5.8 A).

Table 5.8. MBCs (µM) of the altered peptides against various pathogens following a 3-hour treatment in PPB[200]. **A.** *E. f., Enterococcus faecalis* (ATCC 29212)*; S. a., Staphylococcus aureus* (HNCMO112011)*; K. p., Klebsiella pneumoniae* (NCTC 13440)*; A. b., Acinetobacter baumannii* (ATCC 17978)*; P. a., Pseudomonas aeruginosa* (ATCC 27853)*; E. c., Escherichia coli* (ATCC 8739)*; L. m., Listeria monocytogenes* (ATCC 19111); *S. e., Salmonella enterica* (ATCC 13076). The two most potent peptides are in bold. **B**. MBCs (µM) of peptides against supplementary strains of *E. coli* (ATCC 25922 and ATCC 35218) and *S. aureus* (ATCC 25923).

A.

Peptides	**Gram-Negative**					**Gram-Positive**		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$ I	3.1	3.1	6.3	6.3	3.1	3.1	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$ II	3.1	3.1	6.3	3.1	3.1	3.1	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-Me}}$ I	6.3	6.3	12.5	6.3	6.3	12.5	6.3	6.3
NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-Me}}$ II	1.6	3.1	3.1	3.1	3.1	25	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-F-L}}$	1.6	1.6	3.1	1.6	1.6	1.6	1.6	1.6
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-F-D}}$	1.6	3.1	3.1	1.6	3.1	1.6	6.3	3.1
NCR169$C_{17\text{-}38}C_{12,17}$/S-20$W^{5\text{-F-L}}$	3.1	3.1	3.1	3.1	6.3	3.1	3.1	1.6
NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-F}}$ I	**0.8**	**1.6**	**1.6**	**1.6**	**0.8**	**1.6**	**1.6**	**0.8**
NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-F}}$ II	**0.8**	6.3	**1.6**	**0.8**	**1.6**	**0.8**	**1.6**	**0.8**
NCR169$C_{17–38}C_{12,17}$/S-10$W^{7\text{-Aza}}$ I	3.1	3.1	6.3	3.1	3.1	6.3	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-10$W^{7\text{-Aza}}$ II	3.1	3.1	6.3	6.3	3.1	3.1	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-Aza}}$ I	3.1	6.3	6.3	6.3	6.3	25	25	6.3
NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-Aza}}$ II	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I	3.1	1.6	1.6	1.6	1.6	1.6	1.6	1.6
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ II	3.1	3.1	3.1	3.1	25	3.1	3.1	3.1
NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-MeO}}$	3.1	3.1	3.1	3.1	0.8	3.1	3.1	3.1

B.

Peptides	***E.c.* ATTC** 25922	***E.c.* ATTC** 35218	***S.a.* ATTC** 25923
NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-F}}$I	0.8	0.8	1.6
NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-F}}$II_	0.8	0.8	1.6

NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$ I and II possess similar antibacterial activities against most of the tested pathogen bacteria apart from *A. baumannii*, whereas NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$ II has somewhat better activity (3.1 µM) compares to NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$ I (6.3 µM). The activity of these two peptides, compared to NCR169$C_{17–38}C_{12,17}$/S, displayed that the lead compound had advanced antibacterial activity on *S. aureus*, *K. pneumoniae*, *E. coli*, and *S. enterica*. The same modification in tryptophan at residue 20 (NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-Me}}$) had

a more severe effect by significantly decreasing its capacity to exterminate most tested bacteria, especially for form I, which had 12.5 µM MBC against *E. faecalis* and *K. pneumonia* and 6.3 µM against the rest of bacteria, while form II demonstrated modest activity only towards *E. faecalis* (25 µM).

Replacement of tryptophan at position 10 with 5-fluoro-L-tryptophan enhanced the antibacterial activity versus several bacteria. NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}F\text{-}L}$ could kill all tested pathogens with 1.6 µM, with an exemption for *K. pneumoniae* (MBC: 3.1 µM). The same alteration with another racemic form of 5-fluoro-D-tryptophan (NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}F\text{-}D}$) exhibited a general antibacterial activity like the parent peptide, the same as when the tryptophan at position 20 was changed to 5-fluoro-tryptophan. The finest antibacterial effects were discovered for those peptide analogs, whereas 6-fluoro-tryptophan was deployed at position 10. The NCR169C17-38C12,17/S-10W6-F I and II peptides displayed a remarkable ability to eliminate various pathogens, including *E. faecalis, S. aureus, A. baumannii, P. aeruginosa*, and *E. coli*, even at a concentration as low as 0.8 µM. This feature was unique among the tested peptide analogs. Additionally, these two peptides were effective against other strains, such as *E. coli* ATCC 25922 and ATCC 35218, as well as *S. aureus* ATCC 25923 (Table 5.8 B).

The substitution of 7-Aza tryptophan at the 10th residue of the NCR169$C_{17–38}C_{12,17}$/S peptide yet again developed two peptides, NCR169$C_{17–38}C_{12,17}$/S-10$W^{7\text{-}Aza}$ I and II, which held a similar antibacterial activity to one another (mostly at 3. 1 µM), and little lower than the parental peptide. The smallest MBC values against all tested pathogens were detected when 7-Aza-tryptophan was at the 20th position in the lead sequence. In this context, NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-}Aza}$ I peptide displayed an MBC value of 6.3 µM against most bacteria excluding *E. faecalis* and *L. monocytogenes,* which was only 25 µM. Nonetheless, NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-}Aza}$ II analog revealed an equal MBC value of 12.5 µM for all tested bacteria.

NCR169$C_{17\text{-}38}C_{12,17}$/S analogs were also synthesized by switching to 5-methoxy tryptophan in either position 10 (NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}MeO}$ I and II) or position 20 (NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-}MeO}$ containing both racemic forms) and assessed for their antibacterial activity. Intriguingly, NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}MeO}$ II and NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-}MeO}$ displayed identical overall activity indicated by the MBC values of 3.1 µM, while NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}MeO}$ I had a tad greater ability to terminate these pathogens at 1.6 µM. Nevertheless, they

concealed very specific activity against *P. aeruginosa*: NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ II could only eradicate this bacterium with a concentration of 25 µM, though NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I was successful in killing this pathogen at a concentration of 1.6 µM. Moreover, NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-MeO}}$ even eliminates *P. aeruginosa* at a 0.8 µM.

5.8. Hemolysis Activity of NCR169C17-38 and Its Derivatives

A recent publication demonstrated that when NCR169 is in its oxidized state, it has the ability to attach to bacterial phospholipids that carry a negative charge[185]. This finding suggests that NCR169 and its derivatives could have antimicrobial activity by causing harm to the bacterial membrane. However, it is important to note that the interaction between these peptides and bacterial membranes does not necessarily harm human cells.

For that reason, we examined the potential hemolytic activity of NCR169$C_{17–38}$, NCR169$C_{17-38}C_{12,17}$/S, and the two most active derivatives, NCR169$C_{17-38}C_{12,17}$/S-10$W^{6\text{-F}}$I and NCR169$C_{17-38}C_{12,17}$/S-10$W^{6\text{-F}}$II, against human red blood cells. None of the peptides triggered hemolysis in the MBCs spectrum and not even close to 100 µM concentration, excluding NCR169$C_{17-38}C_{12,17}$/S-10$W^{6\text{-F}}$II which initiated minor hemolysis at greater concentrations (Figure 5.4).

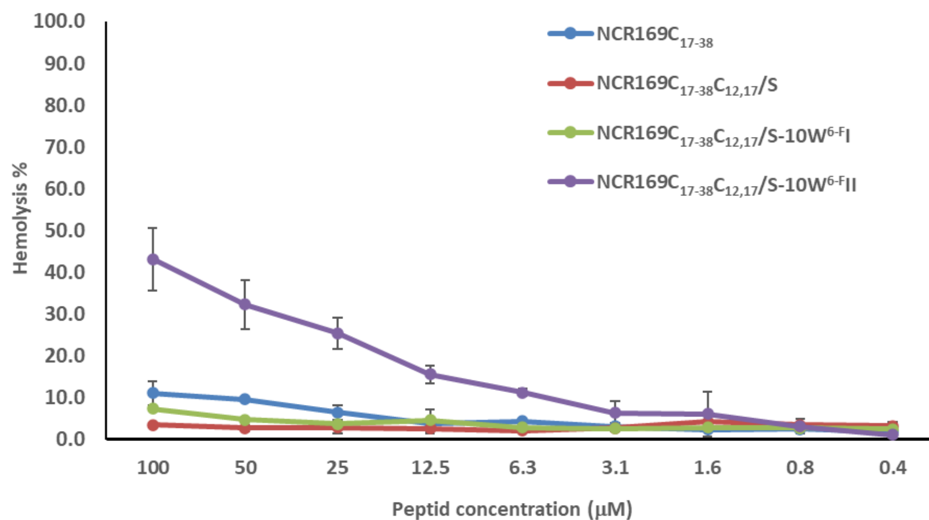


Figure 5.4. Assessment of Hemolytic Activity of the NCR169C_{17-38} and its Most Potent Derivatives[200]

5.9. Discussion

The expanding number of global AMR incidents, the inadequate resources, and the side effects of antimicrobial drugs constrain the exploration of new effective antimicrobial medicines. Antimicrobial peptides are potential antimicrobial agents considering that this group possessed a few valuable properties, such as a wide range of activity, small toxicity, adequate immunogenicity, excellent penetration ability, small as they have several beneficial characteristics, i.e., broad-spectrum activity, low toxicity, moderate immunogenicity, good penetration capability, the small tendency of resistance development, and specific mechanism of actions[151,201–203].

Many AMPs have been isolated from natural resources, such as bacteria, fungi, plants, and animals. These natural peptides showcased similarities in a compact size, the positive charge in general, and amphipathic character[204]. NCR peptides are mainly isolated from plants as part of the cysteine-rich peptides family. Several investigated NCR peptides, like defensin, have shown remarkable antimicrobial activity, considering their sequences' presence of conserved cysteine residues. One interesting NCR peptide that became the highlight of our project is NCR169, a peptide consisting of 38 amino acids with four cysteine residues and involved in bacteroid differentiation. The first objective of this work is to determine the part of NCR169 that is essential for its antimicrobial potency. Our evaluation indicated that the C-terminal region of NCR169 is the most active part of the sequence compared to other short fragments. Our result aligns with the publication from Isozumi group[185], which suggested that lysin residues close to the C-terminal have greater solvent availability and flexibility. These features are likely important factors in the action of the Lys-rich region.

The second objective of this study was to investigate the antimicrobial activity of the NCR169 active region, which is the C-terminal. Firstly, we tested the C-terminal part of the NCR169 sequence for its anticandidal activity. The result ratified our hypothesis that NCR169C$_{17–38}$ was a potential antifungal agent and was active against all tested *Candida* species and strains except for *C. parapsilosis.* The next part of this study was to investigate the antibacterial activity of the C-terminal region of NCR169 since this short fragment displayed better activity than other short fragments of NCR169. Our results display that NCR169C$_{17-38}$ could terminate all eight pathogenic bacterial species tested, indicating that the C-terminal region of NCR169 is crucial and adequate for bactericidal activity. The substitution of cysteine or tryptophan residues with different amino

acids or the utilization of various chemically modified tryptophan developed a few changes in the antimicrobial profile of the studied peptide.

The substitution of cysteine residues with serines in NCR169C_{17-38} conserved the activity of the peptide. Moreover, this change even promoted the activity, signifying that the presence of cysteine and creating a disulfide bridge is not vital for the NCR169C_{17-38} antimicrobial activity. The production of disulfide bridges in peptide sequence was also replaceable for several AMPs, and dropping the disulfide bonds from the peptide sequence proved to strengthen the antimicrobial activity[205–208]. Adding serine into the mixture with some antibacterial medications improved the antimicrobial activity [209–211].

Additionally, we examined the function of tryptophans in the given peptide, considering that they may be needed for interacting AMPs with bacterial membranes [120,212–214]. In accord, the exchange of tryptophan residues with alanine in NCR169C_{17-38} weakened the antimicrobial activity in all three derivatives (NCR169$C_{17-38}W_{10,20}$/A, NCR169$C_{17-38}W_{10,20}$/A, $C_{12,17}$/S, NCR169$C_{17-38}W_{10,20}C_{12,17}$/A). The MBCs of NCR169$C_{17-38}$ and NCR169$C_{17-38}C_{12,17}$/S were between 1.6 and 3.5 µM against most tested pathogen bacteria. NCR169C_{17-38} and its four derivatives in Table 5.4 impart identical physicochemical properties and an alpha-helical 3D structure unveiled by AlphaFold prediction (Figure 5.2).

The NCR169C_{17-38} peptide exhibits a great ability to form an alpha-helix structure aside from the four N-terminal amino acids. Surprisingly, the two cysteines are not in the vicinity, as constructing the disulfide bridge would abolish the high helicity (Figure 5.2 **A**). The non-essentialness of the disulfide bond is also advocated by the exchange of cysteines with serines, which developed a similar alpha-helical structure (Figure 5.2 **B**) and biological activity. Swapping the tryptophans and cysteines to alanine (NCR169$C_{17-38}W_{10,20}C_{12,17}$/A) did not change the steric structure (Figure 5.2 **C-E**). Nonetheless, this structure diverged from the full sequence of NCR169 peptide, which shows mostly disordered structure along with two short anti-parallel beta-pleated sheets at the C-terminal region predicted by AlphaFold (Figure 5.1), which then matches perfectly with the published NMR structures of the original conformation[185]. Hence, the alpha-helical structure might be a key factor for the antimicrobial activity of the studied peptides. Yet, the distinct alterations in their activities cannot be connected to variations in the 3D structure; the quality of individual amino acids is somewhat more significant, as presented in the previous

examples. While the alpha-helical 3D structure was a distinctive feature of NCR169C17-38 and its four derivatives, it cannot be generalized to all varied NCR peptides, for instance, in the case of NCR335, whereas both the N- and C-terminal part demonstrates antimicrobial activity, one part has β-pleated sheet structure, while the other has alpha-helical configuration[201].

We composed new $NCR169C_{17\text{-}38}C_{12,17}/S$ analogs based on these results by integrating five modified tryptophans available in the market in racemic forms or, in the case of 5-fluoro tryptophan, in L- and D-forms. Introducing modified amino acids into the $NCR169C_{17\text{-}38}C_{12\text{-}17}/S$ sequence would generate new chemical and biological properties, fulfilling our third research objective.

Substitution of a normal tryptophan with 5-methyl-tryptophan at position 10 caused an increment of MBC (6.3 µM) towards two Gram-negative bacteria (*K. pneumoniae* and *A. baumannii*), as for the rest of the tested bacteria, it was active at 3.1 µM. Altering the position from 10 to 20 significantly degraded the active concentration for $NCR169C_{17\text{–}38}C_{12,17}/S\text{-}20W^{5\text{-}Me}$ I to a scope of 6.3 µM to 12.5 µM. Intriguingly, the antimicrobial profile of $NCR169C_{17\text{–}38}C_{12,17}/S\text{-}20W^{5\text{-}Me}$ II was equivalent to the lead peptide except for *E. faecalis* (25 µM). These data confirmed that changing one hydrogen substituent at the position of the indole ring with one methyl group vaguely decreased the antibacterial activity. It is possible because of the inability of methyl to form a hydrogen bond. Therefore the peptides containing 5-methyl tryptophan were not active[215,216].

Due to its distinct characteristics, such as little polarizability, and high electronegativity, fluorine is an attractive option for developing peptide-based drugs. Presently, above 20% of commercial drugs are fluorine-containing drugs[217–219]. Correspondingly, $NCR169C_{17\text{–}38}C_{12,17}/S$ analogs comprised of modified fluoro tryptophan were the most effective antibacterial agents among all analogs. The eradicating ability of these peptides validated that the analogs containing 6-fluoro tryptophan condensed MBCs the most. The MBC values of both $NCR169C_{17\text{-}38}C_{12,17}/S\text{-}10W^{6\text{-}F}$ I and II span from 0.8 µM to 1.6 µM, with only one exemption (6.3 µM for *S. enterica*). Likewise, peptides with 5-fluoro tryptophan, $NCR169C_{17\text{-}38}C_{12,17}/S\text{-}10W^{5\text{-}F\text{-}L,}$ and $NCR169C_{17\text{-}38}C_{12,17}/S\text{-}10W^{5\text{-}F\text{-}D}$, also have MBCs of 1.6 – 6.3 µM, with the highest MBC for *P. aeruginosa*, Gram-negative bacterium and L. monocytogenes, a Gram-positive one. The antibacterial activity improvement of these $NCR169C_{17\text{–}38}C_{12,17}/S$ analogs comprising modified fluoro tryptophan could

be caused by the chemical and thermal stability of the peptides, as well as grander resistance of the peptides towards proteolysis [217–219].

The integration of one methoxy group to tryptophan did not influence extensively in the antibacterial abilities, except that it provoked remarkable changes in the peptides MBC values against *P. aeruginosa*. NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I showed an overall antimicrobial activity at 1.6 µM, while NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I and NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-MeO}}$ were at a concentration of 3.1 µM. Nonetheless, NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I had the biggest MBC value of 25 µM against *P. aeruginosa,* whereas NCR169$C_{17–38}C_{12,17}$/S-20$W^{5\text{-MeO}}$ owned an MBC of 0.8 µM, which is the smallest MBC between these analogs for this Gram-negative bacterium. The oxygen in the methoxy group may affect the antibacterial activity through a hydrogen bond formation with the targeted bacterial membrane. The influence of 5-methoxy tryptophan on peptide activity is published, for instance, on an argyrin analog that consisted of modified 5-methoxy tryptophan and was active against *P. aeruginosa* and *Proteus mirabilis* [216].

Surprisingly, the introduction of 7-Aza tryptophan in the sequence did not improve the biological activity of the NCR169$C_{17–38}C_{12,17}$/S analogs. This outcome is unusual since it was assumed that 7-Aza tryptophan, competed with unmodified tryptophan, has a better ability to generate hydrogen bonds [220]. Nevertheless, the existence of 7-Aza tryptophan in peptides or proteins may weaken the activity by the intrusion of the 7-Aza with the side chain of other amino acids, and the replacement of one carbon at position 7 in the indole ring with nitrogen lowers the hydrophobicity of the amino acid, hence demotes the activity of the peptide [221,222].

Amidst the short and chemically modified peptide derivatives of NCR169, some can terminate all or some of the tested bacterial species with a very small MBC (0.8 - 3.1 µM). In contrast, the MBCs of carbenicillin were 2-4 orders of magnitude greater, while the MBCs of levofloxacin were either alike or 200-fold higher for the same bacterial species[199]. Furthermore, none of the four active tested peptides displayed hemolytic activity at the MBC values, and just one showed signs of hemolytic activity at bigger concentrations, which is encouraging following possible future applications. The mechanism of action of NCR169 and its derivatives must be clarified. A comprehensive examination of the mechanism of active NCR169C derivatives from this series would be necessary to comprehend these analogs' function in peptide-microbe interactions. The great benefit of these peptides is that they are not cytotoxic to human cells, unlike many other

AMPs. This work provides highly powerful peptide antimicrobials and boosts activity by replacing tryptophan with mass-produced modified tryptophan at a particular position.

Chapter 6

Synthesis & Antimicrobial Activity of NCR147 & Its Derivatives

6.1 Synthesis of NCR147 and its derivatives

NCR147 is a noncationic peptide (pI = 7) consisting of 36 amino acids and four cysteine residues. The matured NCR147 displayed antifungal activity against a couple of *Candida albicans* strains[178]. However, no publication has shown the part of this peptide responsible for its activity. In our preliminary investigation of the antimicrobial activity of some legume plant peptides, we discovered that the NCR147 with -COOH at the C- terminus displayed good activity against *E. coli* (MBC: 25 µM), *P. aeruginosa* (MBC: 12.5 µM), *L. monocytogenes* (MBC: 12.5 µM), and *A. baumannii* (MBC: 3.125)[197]. We expanded our findings by synthesizing the NCR147 series with an amide functional group (-$CONH_2$) at the C-terminus. We focused on synthesizing the whole sequence of NCR147 along with its shorter fragments to identify its active region. The synthesized NCR147 peptide and its derivatives are listed in Table 6.1 below. The HPLC chromatograms and MS spectra of these peptides can be found in Appendix 2.

Table 6.1. NCR147 and its derivatives. (**A**). Amino acid sequence and molecular mass values of the short peptide fragments. (**B**). Isoelectric point (pI), hydrophobicity (%), grand average hydropathy (GRAVY), and Boman index (kcal/mol) of the synthesized peptides.

A

Peptides	Amino Acid Sequence	Molecular Mass Calculated	Molecular Mass Experimental[1]
NCR147	AYIECEVDDDCPKPMKNSHPDTYYKCVKHRCQWAWK	4389.05	1464.7
$NCR147_{13-36}$	KPMKNSHPDTYYKCVKHRCQWAWK	3035.60	1012.4
$NCR147C_{20-36}$	PDTYYKCVKHRCQWAWK	2212.60	738.5
$NCR147_{25-32}$	KCVKHRCQ	1001.25	501.1
$NCR147_{25-36}$	KCVKHRCQWAWK	1572.93	525.1
$NCR147_{28-36}$	KHRCQWAWK	1242.48	414.6
$NCR147_{25-36}W_{11}/A$	KCVKHRCQWAAK	1457.80	486.6
$NCR147_{25-36}W_{9-11}/A$	KCVKHRCQAAAK	1342.66	448.3
$NCR147_{25-36}W_{9}/A$	KCVKHRCQAAWK	1457.80	486.3

[1]$(M + 3H)^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide, consistent with the calculated mass.

B

Peptides	pI[1]	Hydrophobicity[1]	GRAVY[1]	Boman Index[2] (kcal/mol)
NCR147	7.0	32.35	-1.17	2.50
$NCR147_{13-36}$	10.1	28.58	-1.55	2.65
$NCR147C_{20-36}$	9.5	29.05	-1.30	2.36
$NCR147_{25-32}$	9.9	3.96	-1.23	3.70
$NCR147_{25-36}$	10.3	21.47	-1.14	2.39
$NCR147_{28-36}$	10.6	19.87	-1.83	3.16
$NCR147_{25-36}W_{11}/A$	10.3	11.12	-0.92	2.43
$NCR147_{25-36}W_{9,11}/A$	10.3	3.21	-0.69	2.47
$NCR147_{25-36}W_{9}/A$	10.3	12.17	-0.92	2.43

[1]values from https://www.thermofisher.com/hu/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html *[2]values from* https://aps.unmc.edu/prediction/predict

At present, confirmation about the structure of NCR147 is not available. Therefore, we made structural predictions of this peptide and its derivatives using the AlphaFold program (Figure 6.1). Based on these predictions, there is a short antiparallel β-sheet between T22 to V25 and R30 to W35 at the C-terminal of NCR147 and different properties at the elongated N-terminal, including a β strand between D8 to D10. The β sheet structure is still present in $NCR147_{13-36}$ and $NCR147C_{20-36}$.

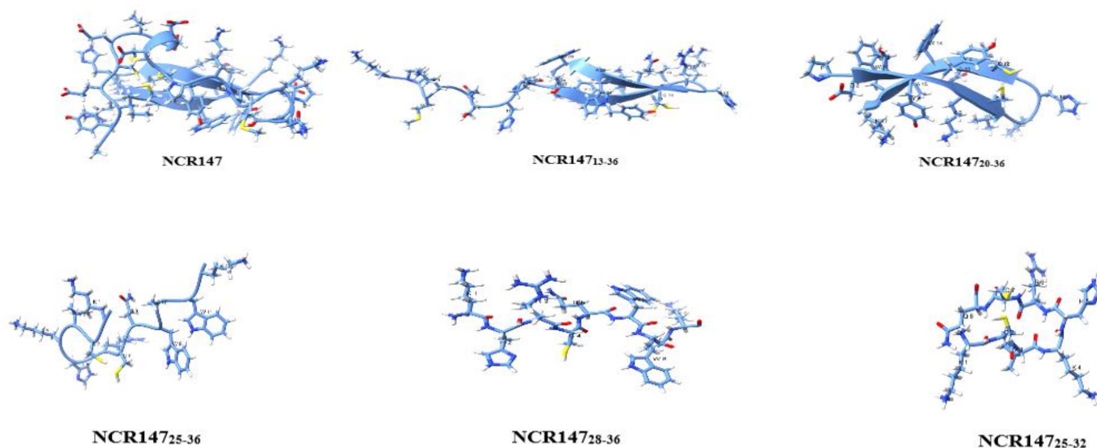


Figure 6.1. Structure Prediction of NCR147 and Its Derivatives

6.2 Antimicrobial Activity of NCR147 and Its Derivatives

The NCR147 peptide and its analogs were assessed for antimicrobial activity against the same bacterial strain used for the NCR169 biological evaluation. The result of this assay is presented in Table 6.2.

Table 6.2. Minimal bactericidal concentrations (MBC; in µM) of the studied peptides on different pathogens after 3 h of treatment in PPB (20 mM). Gram-negative: *E. c., Escherichia coli* (ATCC 8739)*; S. e., Salmonella enterica* (ATCC 13076)*; K. p., Klebsiella pneumoniae* (NCTC 13440)*; A. b., Acinetobacter baumannii* (ATCC 17978)*; P. a., Pseudomonas aeruginosa* (ATCC 27853). Gram-Positive: *E. f., Enterococcus faecalis* (ATCC 29212)*; L. m., Listeria monocytogenes* (ATCC 19111)*; S. a., Staphylococcus aureus* (HNCMO112011).

Peptides	Gram-Negative					Gram-Positive		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
NCR147	50	50	50<	50	50	50<	25	50<
NCR147$_{13-36}$	12.5	50	100<	12.5	6.25	50	12.5	50
NCR147C$_{20-36}$	50	100<	100<	12.5	25	12.5	6.25	100<
NCR147$_{25-32}$	50<	50<	50<	50<	50<	50<	50<	50<
NCR147$_{25-36}$	25	50	50<	25	25	50<	50<	50
NCR147$_{28-36}$	6.25	25	100<	6.25	12.5	100<	100<	25
NCR147$_{25-36}$W$_{11}$/A	50<	50<	50<	50<	50<	50<	50<	50<
NCR147$_{25-36}$W$_{9,11}$/A	50<	50<	50<	50<	50<	50<	50<	50<
NCR147$_{25-36}$W$_{9}$/A	50<	50<	50<	50<	50<	50<	50<	50<

[1](M + 3H)$^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide, consistent with the calculated mass.

The minimum bactericidal concentration (MBC) was used to assess the antibacterial activity of the NCR147 peptide and its derivatives. We utilized the same bacterial strains as the previous work on the NCR169 analogs. The antibacterial activities of NCR147 and its derivatives are presented in Table 6.2. The whole sequence of NCR147 displayed a low antimicrobial concentration of 50 µM against the tested bacterial strains. Among the short fragments, $NCR147_{25-36}$ demonstrated slightly better activity than its lead compound, with an MBC concentration exceeding 50 µM. $NCR147_{13-36}$ showed better activity against *P. aeruginosa*, with an MBC of 6.25 µM, the lowest tested peptides, and 12.5 µM MBC against *E. coli, A. baumannii,* and *L. monocytogenes*. However, this peptide was weak against *K. pneumoniae* with MBC around 100 µM and 50 µM against *S. enterica*, *E. faecalis*, and *S. aureus*. The C-terminal region of NCR147 ($NCR147C_{20-36}$) has better activity against Gram-positive bacteria of *L. monocytogenes* (MBC: 6.25 µM), and *E. faecalis* (MBC: 12.5 µM), but not so good against most of the Gram-negative bacteria. Shorter fragments of $NCR147C_{20-36}$ exhibited similar declining MBC values, except for the $NCR147_{28-36}$. This peptide eliminated *E. coli* and *A. baumannii* with 6.25 µM, better than the lead peptide. Substitution of tryptophans in the sequence of $NCR147_{25-36}$ with alanines weakened the activity.

6.3. Synthesis of $NCR147_{25-36}$ Derivatives

Following the result of the antibacterial activity, we synthesized analogs of $NCR147_{25-36}$ incorporating a modified tryptophan. We used a fluoro-modified tryptophan in these analogs because, in our previous work on the NCR169 series, we discovered that this type of modified tryptophan could enhance the antibacterial activity. The synthesized $NCR147_{25-36}$ analogs can be seen in Table 6.3. The HPLC chromatograms and the MS spectra are presented in Appendix 2. In these analogs, we substituted the tryptophan at the 9 and 11 positions separately and together with 5-fluoro-L-tryptophan.

Table 6.3. List of $NCR147_{25-36}$ Analogs

Peptides	Amino Acid Sequence	Molecular Mass Calculated	Molecular Mass Experimental[1]
$NCR147_{25-36}W_9/W^{5-F-L}$	$KCVKHRCQW^{5-F-L}AWK$	1591.15	531.3
$NCR147_{25-36}W_{11}/W^{5-F-L}$	$KCVKHRCQWAW^{5-F-L}K$	1591.15	531.2
$NCR147_{25-36}W_{9,11}/W^{5-F-L}$	$KCVKHRCQW^{5-F-L}AW^{5-F-L}K$	1609.37	537.1

[1]*$(M + 3H)^{3+}$ refers to the experimentally determined molecular mass of the triple charged peptide, consistent with the calculated mass.*

6.4 Antibacterial Activity of $NCR147_{25-36}$ Derivatives

The synthesized $NCR147_{25-36}$ derivatives were subjected to an antibacterial evaluation to characterize their antibacterial assay against the same bacterial strains in the previous test (Table 6.4). Generally, the $NCR147_{25-36}$ derivatives showcased better antibacterial profiles than the initial peptide. The analog with double substituting modified fluoro tryptophan ($NCR147_{25-36}W_{9,11}/W^{5-F-L}$) is the best among the derivatives. This peptide is the most active one from our NCR147 series, with an MBC value of 3.125 µM against most of the tested bacteria, except for *K. pneumoniae* and *E. faecalis* with MBC around 50 µM, and against *P. aeruginosa* with MBC of 6.25 µM. Substituting one tryptophan at residue number 11 ($NCR147_{25-36}W_{11}/W^{5-F-L}$) demonstrated better antibacterial activities than substituting tryptophan at the 9th position. The best MBC value of $NCR147_{25-36}$, W_{11}/W^{5-F-L} was against *E. coli*, *S. enterica*, *A. baumannii*, *P. aeruginosa*, and *S. aureus*, with a concentration of 6.25 µM.

Table 6.4. MBCs (µM) of the $NCR147_{25-36}$ derivatives on different pathogens after 3 h of treatment in PPB (20 mM). Gram-negative: *E. c., Escherichia coli* (ATCC 8739)*; S. e., Salmonella enterica* (ATCC 13076)*; K. p., Klebsiella pneumoniae* (NCTC 13440)*; A. b., Acinetobacter baumannii* (ATCC 17978)*; P. a., Pseudomonas aeruginosa* (ATCC 27853). Gram-Positive: *E. f., Enterococcus faecalis* (ATCC 29212)*; L. m., Listeria monocytogenes* (ATCC 19111)*; S. a., Staphylococcus aureus* (HNCMO112011).

Peptides	Gram-Negative					Gram-Positive		
	E. c.	***S. e.***	***K. p.***	***A. b.***	***P. a.***	***E. f.***	***L. m.***	***S. a.***
$NCR147_{25-36}W_9/W^{5-F-L}$	12.5	12.5	50<	25	12.5	50<	50	12.5
$NCR147_{25-36}W_{11}/W^{5-F-L}$	6.25	6.25	50<	6.25	6.25	50<	12.5	6.25
$NCR147_{25-36}W_{9,11}/W^{5-F-L}$	3.125	3.125	50<	3.125	6.25	50<	3.125	3.125

We then tested these analogs along with the NCR147 and its shorter fragments against 8 Gram-negative pathogen bacteria (Table 6.5). Those bacteria are *Pseudomonas syringae* pv. tomato, *Pseudomonas syringae* pv. tabaci, *Pseudomonas gladioli*, *Xanthomonas campestris*, *Xanthomonas malvaceae*, *Erwinia chrysanthemi*, *Erwinia carotovora*, and *Agrobacterium tumefaciens*. These pathogen bacteria cause some plant diseases, such as periwinkle leaf spots and stem lesions, tobacco wildfire disease, potato blackleg and soft rot, and crown gall disease[223–226].

Table 6.5. MBCs (µM) of the NCR147 and its derivatives against different pathogens after 3 hr of treatment in PPB (20 µM). *P. s. tom., Pseudomonas syringae* pv. tomato*; X.c., Xanthomonas campestris; E. ch., Erwinia chrysanthemi; A. t., Agrobacterium tumefaciens; E. ca., Erwinia carotovora; P. g., Pseudomonas gladioli; X. m., Xanthomonas malvaceae; P. s. tab., Pseudomonas syringae* pv. tabaci.

Peptides	*P. s. tom.*	*X. c.*	*E. ch.*	*A. t.*	*E. ca.*	*P. g.*	*X. m.*	*P. s. tab.*
NCR147	50<	50<	50<	50<	50<	50<	25	12.5
NCR147$_{13-36}$	12.5	50<	50<	50<	12.5	50<	12.5	6.25
NCR147$_{25-36}$	12.5	50<	50<	50<	12.5	50<	25	3.125
NCR147$_{28-36}$	6.25	50<	50<	50<	-	50<	6.25	6.25
NCR147$_{25-32}$	50<	50<	50<	50<	50<	50<	50<	50<
NCR147$_{25-36}$W$_{9}$/W$^{5\text{-F-L}}$	12.5	50<	50<	50<	12.5	50<	12.5	3.125
NCR147$_{25-36}$w$_{11}$/W$^{5\text{-F-L}}$	3.125	50<	50<	50<	6.25	50<	6.25	1.6
NCR147$_{25-36}$W$_{9,11}$/W$^{5\text{-F-L}}$	3.125	50<	50<	50<	12.5	50<	-	1.6

NCR147$_{25-36}$W$_{11}$/W$^{5\text{-F-L}}$ exhibited the best antibacterial activity against most tested pathogens. This peptide could terminate *P. syringae* pv. tabaci with a concentration of 1.6 µM, and *P. syringae* pv. tomato with a concentration of 3.125 µM. These values are similar to the concentration of NCR147$_{25-36}$W$_{9,11}$/W$^{5\text{-F-L}}$. However, the later peptide has a slightly weaker concentration against *E. carotovora* (MBC: 12.5 µM). The NCR147$_{25-36}$W$_{9}$/ W$^{5\text{-F-L}}$ was less potent than NCR147$_{25-36}$W$_{11}$/W$^{5\text{-F-L}}$, with an MBC value of 3.125 µM against *P. syringae* pv. tabaci, and MBC of 12.5 µM against *P. syringae* pv. tomato and *E. carotovora*. The NCR147 peptide demonstrated low antibacterial activity against most tested pathogens, except for *P. syringae* pv. tabaci (MBC: 12.5 µM). The NCR147$_{25-36}$ showed better activity against *P.syringae* pv. tabaci, with MBC of 3.125 µM. This peptide could terminate *P. syringae* pv. tomato and *E. carotovora* at a concentration of 12.5 µM, slightly better than its native peptide that has MBC around 50 µM.

6.5 Discussion

Our *in vitro* studies of NCR147 peptide and its derivatives showed that the native peptide has poor antibacterial activity against several pathogens' bacteria, including the ESKAPE group. This

peptide has a low isoelectric point (pI = 7) compared to its derivatives, which have pI between 9.5 and 10.6. Most AMPs possess pI around 9.2 to 9.6, and it is believed that a high positive net charge is necessary for this peptide group[227]. Another key parameter for AMPs' mechanism of action is their structure. Many AMPs are likely to form a random coil structure in solution, whereas the non-AMPs have the tendency of α-helix or β-sheet formation. From our structural predictions of NCR147 and its derivatives, we learned that there is an antiparallel β-sheet at the C-terminal region of NCR147. This structure is still present in the short fragmentation of NCR147 ($NCR147_{13-36}$ and $NCR147_{20-36}$). However, if we compared it with the structure of $NCR147_{25-36}$, only a random coil structure occurred, and this peptide is the most promising antibacterial agent among the NCR147 short fragments. Hence, the coil structure might be an essential element for the antibacterial activity of the studied peptides.

The GRAVY index of NCR147 and its derivatives are between -1.83 to -0.98, and the hydrophobic percentage of these peptides is below 50 %. These results indicate that the NCR147 and its derivatives tend to be hydrophilic instead of hydrophobic. Some studies suggest that the peptide's hydrophobicity may increase the antimicrobial activity[227,228], although this trait could also enhance the hemolytic activity[229]. Therefore, it is vital to notice the optimum level of hydrophobicity since exceeding this limit may cause a reduction of antimicrobial activity and escalation of toxicity[230].

Replacement of tryptophans with alanines in the $NCR147_{25-36}$ sequence weakened the activity of the peptide. The antibacterial activities of the sequence containing tryptophans against more than half of the tested bacteria were slightly better than the antibacterial activities of the peptides with substituted alanines. We concluded that the presence of tryptophan in the $NCR147_{25-36}$ sequence is crucial for its antibacterial profile. Tryptophan has been reported as an important amino acid for AMPs activity due to its capability to interact with bacterial membranes[120,212–214], and it is also highly favored for interfacial regions in the lipid bilayers[231].

Following these results, we synthesized derivatives of $NCR147_{25-36}$ incorporating 5-fluoro-L-tryptophan at the 9th and 11th positions. The results of the in vitro studies against the same pathogens bacteria showed that the presence of fluoro-modified tryptophan could improve the antibacterial activities of the peptides. Substitution of two tryptophans at the 9th and 11th positions with 5-fluro-L-tryptophan boasted the activity against *E. coli*, *S. enterica*, *A. baumannii*, *L.*

monocytogenes, and *S. aureus* with the MBC value = 3.125 µM. This result makes NCR147$_{25-36}$W$_{9,11}$/ W$^{5\text{-F-L}}$ the most promising antibacterial peptide among the NCR147 series. NCR147$_{25-36}$W$_{11}$/ W$^{5\text{-F-L}}$ has slightly weaker antibacterial activity compared to the NCR147$_{25-36}$W$_{9,11}$/ W$^{5\text{-F-L}}$, with MBC of 6.25 µM against *E. coli, S. enterica, A. baumannii*, *P. aeruginosa*, and *S. aureus*. The shift substitution of the modified fluoro tryptophan, from the 11th to 9th position, lowered the antibacterial concentration against these pathogens. We conclude that substituting tryptophans at specific positions with fluoro-modified tryptophan could enhance the antibacterial activity of the NCR147$_{25-36}$ peptide against the tested bacteria.

We then evaluated the antibacterial profiles of the NCR147$_{25-36}$ containing 5-fluoro-tryptophan derivatives, along with the NCR147 peptide and its short fragments against 8 Gram-negative bacteria that instigate several plant diseases. The results of this biological assay demonstrated that the native NCR147 peptide has low antibacterial activity towards the tested pathogens bacteria, with MBC around 50 µM, except for *P. syringae* pv. tabaci with concentration = 12.5 µM. Interestingly, two derivatives of NCR147$_{25-36}$, namely NCR147$_{25-36}$W$_{11}$/ W$^{5\text{-F-L}}$ and NCR147$_{25-36}$W$_{9,11}$/ W$^{5\text{-F-L}}$ exhibit the most potential activity against this bacterium (MBC: 1.6 µM). These two peptides also have similar activity against *P. syringae* pv. tomato, with MBC = 3.125 µM. NCR147$_{25-36}$W$_{11}$/ W$^{5\text{-F-L}}$ and NCR147$_{28-36}$ could terminate *X. malvaceae* with the same concentration of 6.25 µM. From this point of view, we might conclude that NCR14725-36 peptides containing 5-fluoro-L-tryptophans are promising candidates for antimicrobial agents. Modifications of these compounds to enhance the antimicrobial activity, structure elucidations, and study of the mechanism of actions from these peptides are crucial and need further investigation.

Chapter 7

Summary

The present work comprises the synthesis and antimicrobial assay of two NCR peptides, NCR169 and NCR147. Identifying the active core region of each peptide is essential to develop stronger AMPs as new antimicrobial agents. The principal conclusions constructed based on the findings of this study can be summarized as follows.

1) **We have synthesized the full sequence of NCR169, its elongated form, and an oxidized form. We also have generated 6 shorter fragments of NCR169 intending to identify the active core fragment of this peptide responsible for the antimicrobial activity. We discovered that the C-terminal region of NCR169 (NCR169C$_{17\text{-}38}$) is the most active sequence.**

   In the first part of this doctoral project, we predicted the structure of NCR169 and its shorter fragments using the AlphaFold program. The result showed the native peptide contained different properties in the elongated N-terminal, and the C-terminal preserved a short antiparallel β-sheet. This result was aligned with the publication from Isozumi group[185]. However, the structural prediction of NCR169C$_{17\text{-}38}$ displayed the absence of the β-sheet and was replaced by an alpha-helical structure. We then synthesized the full sequence of NCR169 and some of its derivatives, followed by an antibacterial assay against some pathogen bacteria, including the ESKAPE group. The NCR169C$_{17\text{-}38}$ demonstrated the highest antibacterial activity among a series of NCR169 derivatives, including 6 shorter fragments, an oxidized form, and an elongated form where a StrepII short fragment, consisting of 8 amino acids (WSHPQFEK), was attached to the C-terminal. This peptide effectively terminated most tested bacteria, exhibiting a minimum bactericidal concentration (MBC) of 3.1 μM. Notably, NCR169C$_{17\text{-}38}$ outperformed the full-length NCR169 sequence and other derivatives regarding potency. Additionally, the antibacterial effectiveness of NCR169C$_{17\text{-}38}$ surpassed that of the control antibiotics carbenicillin and levofloxacin.

2) **Several derivatives of NCR169$C_{17\text{-}38}$ have been synthesized, and we revealed that replacing the cysteines with serines and preserving the tryptophans in the peptide (NCR169$C_{17\text{-}38}C_{12,17}$/S) enhanced the antibacterial activities. Furthermore, the oxidized form of NCR169$C_{17\text{-}38}$ and the linear form of NCR169$C_{17\text{-}38}$ and NCR169$C_{17\text{-}38}W_{10,20}$/A also displayed good antifungal activities against several Candida strains.**

Using the AlphaFold program for structural prediction, we demonstrated that making specific modifications to the NCR169$C_{17\text{-}38}$ sequence, namely replacing cysteine and tryptophan residues with serine and alanine, had minimal impact on the native peptide's secondary structure. We then synthesized 5 modified NCR169$C_{17\text{-}38}$ and assessed their antibacterial profile.

In our in vitro antibacterial studies on the NCR169$C_{17\text{-}38}$ derivatives, we found that retaining the tryptophans in their original positions while replacing two cysteines with serines (NCR169$C_{17\text{-}38}C_{12,17}$/S) led to an enhanced MBC value for the peptide. The comparison between the structural predictions of these peptides and their antibacterial activity revealed that the peptide's backbone sequence did not significantly influence its biological activity. Instead, it appeared that certain amino acid side chains might play a crucial role in determining the peptide's activity. We demonstrated that the linear and oxidized forms of NCR169$C_{17\text{-}38}$ exhibited antifungal properties.

3) **We prepared a total of 16 derivatives of NCR$C_{17\text{-}38}C_{12,17}$/S, incorporating different commercially available tryptophan analogs. Our findings indicated that those containing modified fluoro tryptophans exhibited the most promising antibacterial activity among these derivatives.**

Using five commercial tryptophan derivatives, we developed new NCR169$C_{17\text{-}38}C^{12,17}$/S derivatives by modifying the tryptophans at the 10th and 20th positions. These modifications were carried out in both racemic forms, except for 5-fluoro tryptophan, where we used D- and L- enantiomeric forms. As a result of our efforts, we successfully synthesized and purified a total of 16 novel NCR169$C_{17\text{-}38}C_{12,17}$/S derivatives.

Our in vitro investigations found that substituting the tryptophan at the 10th position with 6-fluoro tryptophan significantly improved the antibacterial activity against all the tested bacteria. For NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-}F}$I, the minimum bactericidal concentration (MBC) ranged from 0.8 to 1.6 μM. Similarly, NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-}F}$II demonstrated

comparable antibacterial profiles, except for *Salmonella enterica*, which required a slightly higher MBC of 6.3 µM to terminate the bacterium. Additionally, peptides containing 5-fluoro tryptophan exhibited notable MBC activity, ranging from 1.6 to 6.3 µM against the pathogenic bacteria.

The results from our hemolysis assay provided evidence that $NCR169C_{17-38}$, as well as $NCR169C_{17-38}C_{12,17}/S$ and its two most potent derivatives ($NCR169C_{17-38}C_{12,17}/S\text{-}10W^{6\text{-}F}I$ and $NCR169C_{17-38}C_{12,17}/S\text{-}10W^{6\text{-}F}II$), did not induce any hemolysis in human red blood cells. This result suggests these peptides are unlikely to threaten human cells during their interaction with bacterial membranes.

4) **The full sequence of NCR147, its 5 shorter fragments, and 3 derivatives of $NCR147_{25-36}$ have been synthesized and evaluated for their antibacterial activity. Most of these peptides displayed relatively low antibacterial activity. The $NCR147_{25-36}$ has a slightly better MBC value than the native peptide.**

Our structural prediction of NCR147 and its shorter fragments displayed that NCR147 possesses a short antiparallel β-sheet structure at the C-terminal and various properties, including a β strand at the elongated N-terminal. This β-sheet structure was also observed in $NCR147_{13-36}$ and $NCR147_{20-36}$.

During our initial research, we made a significant discovery that the NCR147 derivative with a -COOH group at the C-terminal exhibited activity against *Escherichia coli* (MBC: 25 µM), *Pseudomonas aeruginosa* (MBC: 12.5 µM), *Listeria monocytogenes* (MBC: 12.5 µM), and *Acinetobacter baumannii* (MBC: 3.125 µM). Subsequently, we synthesized the amidated NCR147 peptide, 5 shorter fragments and 3 $NCR147_{25-36}$ derivatives, in which the tryptophan was replaced with alanine.

The antibacterial assay of the amidated NCR147 series unveiled that NCR147 has low antibacterial activity, with MBC around 50 µM. The $NCR147_{25-36}$ has slightly better antibacterial activity (MBC between 25 to 50 µM), whereas the $NCR147C_{20-36}$ could terminate *Listeria monocytogenes* with MBC of 6.25 µM, *Acinetobacter baumannii* and *Enterococcus faecalis* with MBC of 12.5 µM. However, the later peptide was weak against *Salmonella enterica*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (MBC around 100 µM).

5) **A few derivatives of $NCR147_{25-36}$ containing 5-fluoro-tryptophan have been synthesized. The antibacterial activity of $NCR147_{25-36}W_{9,11}/W^{5-F-L}$ is the most potent derivative against most tested bacteria.**

We synthesized some variations of $NCR147_{25-36}$ by introducing 5-fluoro-L-tryptophan at positions 9 and 11 in the sequence. The purpose of incorporating this modified tryptophan was to study the impact of fluorinated tryptophan on the antibacterial activity of $NCR147_{25-36}$. We tested the synthesized peptides against the ESKAPE group plus *Salmonella enterica* and *Listeria monocytogenes* in the antibacterial assay. Among the NCR147 derivatives, $NCR147_{25-36}W_{9,11}/W^{5-F-L}$ showed the most promising antibacterial activity. This peptide eradicated half of the tested pathogens, with a minimum bactericidal concentration (MBC) of 3.125 µM. While peptides with a single substitution of fluorinated tryptophan at the 9th or 11th residue exhibited good antibacterial activity, it was not as potent as the double substitution. However, it's worth noting that these $NCR147_{25-36}$ analogs displayed relatively weak activity against *Klebsiella pneumoniae* and *Enterococcus faecalis*, with MBC values around 50 µM.

Further investigations were carried out on the antibacterial profile of NCR147 peptide and its shorter fragments against eight Gram-negative bacteria known to cause some plant diseases. The results showed consistent trends, where the full sequence of NCR147 displayed low MBC values, while shorter fragments like $NCR147_{13-36}$ and NCR_{25-36} demonstrated activity against half of the tested bacteria. Interestingly, $NCR147_{25-36}W_{11}/W^{5-F-L}$ exhibited improved activity against *Pseudomonas syringae* pv. tabaci (MBC: 1.6 µM), *Pseudomonas syringae* pv. tomato (MBC: 3.125 µM), *Erwinia carotovora* (MBC: 6.25 µM), and *Xanthomonas malvaceae* (MBC: 6.25 µM). The $NCR147_{25-36}W_{9,11}/W^{5-F-L}$ also displayed comparable activity, though slightly less potent against *Erwinia carotovora* (MBC: 12.5 µM).

Following these results, we are directing our research on exploring the fluorinated tryptophans potency on the antimicrobial profile of our active peptides. Specifically, we focus on introducing multiple fluorine-substituted tryptophans, such as di- and tetra-fluoro-tryptophans, into our active peptides' sequences. However, using commercial multiple fluorinated tryptophan products is not economical; therefore, we decided to synthesize these noncanonical amino acids in our laboratory.

We utilized various indoles, such as 5- and 6-fluoro indole, 4,6- and 5,6-difluoro indole, and 4,5,6,7-tetrafluoro indole, as the precursors to create tryptophan derivatives. Our first attempt for this work was to apply a facile method to synthesis tryptophans[232]. In this protocol, we reacted the indole with L-serine in acetic acid and acetic anhydride mixture. The product of this reaction was then reacted with an acylase enzyme to produce tryptophan. The final product of this reaction was not satisfying since it was not a pure chiral compound, and removing the acetyl group from the product was difficult. We then attempted to apply a hydrogen borrowing approach for indole's alkylation. In this method, we alkylated indoles with alcohol, using [Cu]/ligand as catalyst[233]. This reaction was successful with indoles at 160°C but was not working at lower temperatures. Moreover, the final product was degraded when we used a protected serine.

We subsequently tried to perform alkylation on indoles through the Friedel-Craft reaction utilizing dipeptides containing dehydroalanine and various Lewis acids[234]. The reaction's condition was gentle (0°C), and we successfully synthesized Fmoc-Ile-dehydroAla-methylester dipeptide, and then the Fmoc-Ile-Trp-methylester with the addition of indole. However, the chiral purity can be lost here as well. We are currently investigating this reaction with Fmoc-Ser-ethylester. Our next attempt was to apply the hydrogen borrowing method with nickel catalyst[235]. This reaction was conducted at 110°C for 12 hours. It was successful with alcohol, and we are currently applying Fmoc-Ile-Ser-methylester in this reaction.

Following the enzymatic pathways, we utilized the reaction of indole and serine with the aid of the apotryptophanase enzyme as the catalyst. We reacted a mixture of L-Ser and pyridoxal-5-phosphate in KH_2PO_4/K_2HPO_4 buffer (pH 8) with the solution of fluoro-indole analogs in methanol plus the enzyme catalyst. This reaction was conducted at 40°C for several days up to 2 weeks. By far, we have successfully synthesized 5-6-difluoro tryptophan through this enzymatic method. However, we could only generate the tryptophan derivative in L- form instead of D- form.

List of References

(1) Fischbach, M. A.; Walsh, C. T. Antibiotics for Emerging Pathogens. *Science (80-.).* **2009**, *325* (5944), 1089–1093. https://doi.org/10.1126/science.1176667.

(2) Economou, V.; Gousia, P. Agriculture and Food Animals as a Source of Antimicrobial-Resistant Bacteria. *Infect. Drug Resist.* **2015**, *8*, 49–61. https://doi.org/10.2147/IDR.S55778.

(3) Davies, J.; Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **2010**, *74* (3), 417–433. https://doi.org/10.1128/mmbr.00016-10.

(4) Auta, A.; Hadi, M. A.; Oga, E.; Adewuyi, E. O.; Abdu-Aguye, S. N.; Adeloye, D.; Strickland-Hodge, B.; Morgan, D. J. Global Access to Antibiotics without Prescription in Community Pharmacies: A Systematic Review and Meta-Analysis. *J. Infect.* **2019**, *78* (1), 8–18. https://doi.org/10.1016/j.jinf.2018.07.001.

(5) Ierano, C.; Thursky, K.; Marshall, C.; Koning, S.; James, R.; Johnson, S.; Imam, N.; Worth, L. J.; Peel, T. Appropriateness of Surgical Antimicrobial Prophylaxis Practices in Australia. *JAMA Netw. Open* **2019**, *2* (11), 1–15. https://doi.org/10.1001/jamanetworkopen.2019.15003.

(6) Milani, R. V.; Wilt, J. K.; Entwisle, J.; Hand, J.; Cazabon, P.; Bohan, J. G. Reducing Inappropriate Outpatient Antibiotic Prescribing: Normative Comparison Using Unblinded Provider Reports. *BMJ Open Qual.* **2019**, *8* (1), 1–6. https://doi.org/10.1136/bmjoq-2018-000351.

(7) Shankar, Pr. *Book Review: Tackling Drug-Resistant Infections Globally*; 2016; Vol. 7. https://doi.org/10.4103/2045-080x.186181.

(8) Prestinaci, F.; Pezzotti, P.; Pantosti, A. Antimicrobial Resistance: A Global Multifaceted Phenomenon. *Pathogens and Global Health*. Maney Publishing October 1, 2015, pp 309–318. https://doi.org/10.1179/2047773215Y.0000000030.

(9) Ma, Y. X.; Wang, C. Y.; Li, Y. Y.; Li, J.; Wan, Q. Q.; Chen, J. H.; Tay, F. R.; Niu, L. N. Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections. *Adv. Sci.* **2020**, *7* (1). https://doi.org/10.1002/advs.201901872.

(10) Dadgostar, P. Antimicrobial Resistance: Implications and Costs. *Infect. Drug Resist.* **2019**, *12*, 3903–3910. https://doi.org/10.2147/IDR.S234610.

(11) Llor, C.; Bjerrum, L. Antimicrobial Resistance: Risk Associated with Antibiotic Overuse and Initiatives to Reduce the Problem. *Ther. Adv. Drug Saf.* **2014**, *5* (6), 229–241. https://doi.org/10.1177/2042098614554919.

(12) Prevention, C. for D. C. and. *Antibiotic Resistance Threats in the United States, 2013*; 2013.

(13) Organisation, W. H. *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2021*; 2021.

(14) Bhatia, P.; Sharma, A.; George, A. J.; Anvitha, D.; Kumar, P.; Dwivedi, V. P.; Chandra, N. S. Antibacterial Activity of Medicinal Plants against ESKAPE: An Update. *Heliyon* **2021**, *7* (2), e06310. https://doi.org/10.1016/j.heliyon.2021.e06310.

(15) Pandey, R.; Mishra, S. K.; Shrestha, A. Characterisation of Eskape Pathogens with Special Reference to Multidrug Resistance and Biofilm Production in a Nepalese Hospital. *Infect. Drug Resist.* **2021**, *14* (February), 2201–2212. https://doi.org/10.2147/IDR.S306688.

(16) Wilson, D. N. Ribosome-Targeting Antibiotics and Mechanisms of Bacterial Resistance. *Nat. Rev. Microbiol.* **2014**, *12* (1), 35–48. https://doi.org/10.1038/nrmicro3155.

(17) Li, X. Z.; Nikaido, H. Efflux-Mediated Drug Resistance in Bacteria. *Drugs* **2004**, *64* (2), 159–204. https://doi.org/10.2165/00003495-200464020-00004.
(18) Wright, G. D. Bacterial Resistance to Antibiotics: Enzymatic Degradation and Modification. *Adv. Drug Deliv. Rev.* **2005**, *57* (10), 1451–1470. https://doi.org/10.1016/j.addr.2005.04.002.
(19) Sierra, J. M.; Fusté, E.; Rabanal, F.; Vinuesa, T.; Viñas, M. An Overview of Antimicrobial Peptides and the Latest Advances in Their Development. *Expert Opin. Biol. Ther.* **2017**, *17* (6), 663–676. https://doi.org/10.1080/14712598.2017.1315402.
(20) Ni, W.; Shao, X.; Di, X.; Cui, J.; Wang, R.; Liu, Y. In Vitro Synergy of Polymyxins with Other Antibiotics for Acinetobacter Baumannii: A Systematic Review and Meta-Analysis. *Int. J. Antimicrob. Agents* **2015**, *45* (1), 8–18. https://doi.org/10.1016/j.ijantimicag.2014.10.002.
(21) Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. The Future of Peptide-Based Drugs. *Chem. Biol. Drug Des.* **2013**, *81* (1), 136–147. https://doi.org/10.1111/cbdd.12055.
(22) Kościuczuk, E. M.; Lisowski, P.; Jarczak, J.; Strzałkowska, N.; Jóźwik, A.; Horbańczuk, J.; Krzyżewski, J.; Zwierzchowski, L.; Bagnicka, E. Cathelicidins: Family of Antimicrobial Peptides. A Review. *Mol. Biol. Rep.* **2012**, *39* (12), 10957–10970. https://doi.org/10.1007/s11033-012-1997-x.
(23) Nguyen, L. T.; Chau, J. K.; Perry, N. A.; de Boer, L.; Zaat, S. A. J.; Vogel, H. J. Serum Stabilities of Short Tryptophan- and Arginine-Rich Antimicrobial Peptide Analogs. *PLoS One* **2010**, *5* (9), 1–8. https://doi.org/10.1371/journal.pone.0012684.
(24) Dhople, V.; Krukemeyer, A.; Ramamoorthy, A. The Human Beta-Defensin-3, an Antibacterial Peptide with Multiple Biological Functions. *Biochim. Biophys. Acta - Biomembr.* **2006**, *1758* (9), 1499–1512. https://doi.org/10.1016/j.bbamem.2006.07.007.
(25) Maróti Gergely, G.; Kereszt, A.; Kondorosi, É.; Mergaert, P. Natural Roles of Antimicrobial Peptides in Microbes, Plants and Animals. *Res. Microbiol.* **2011**, *162* (4), 363–374. https://doi.org/10.1016/j.resmic.2011.02.005.
(26) Zasloff, M. *Antimicrobial Peptides of Multicellular Organisms*; 2002; Vol. 415.
(27) Mahlapuu, M.; Björn, C.; Ekblom, J. Antimicrobial Peptides as Therapeutic Agents: Opportunities and Challenges. *Crit. Rev. Biotechnol.* **2020**, *40* (7), 978–992. https://doi.org/10.1080/07388551.2020.1796576.
(28) Henriques, S. T.; Lawrence, N.; Chaousis, S.; Ravipati, A. S.; Cheneval, O.; Benfield, A. H.; Elliott, A. G.; Kavanagh, A. M.; Cooper, M. A.; Chan, L. Y.; Huang, Y. H.; Craik, D. J. Redesigned Spider Peptide with Improved Antimicrobial and Anticancer Properties. *ACS Chem. Biol.* **2017**, *12* (9), 2324–2334. https://doi.org/10.1021/acschembio.7b00459.
(29) Wang, C. K.; Craik, D. J. Designing Macrocyclic Disulfide-Rich Peptides for Biotechnological Applications Perspective. *Nat. Chem. Biol.* **2018**, *14* (5), 417–427. https://doi.org/10.1038/s41589-018-0039-y.
(30) Chen, C. H.; Lu, T. K. Development and Challenges of Antimicrobial Peptides for Therapeutic Applications. *Antibiotics* **2020**, *9* (1). https://doi.org/10.3390/antibiotics9010024.
(31) Usmani, S. S.; Bedi, G.; Samuel, J. S.; Singh, S.; Kalra, S.; Kumar, P.; Ahuja, A. A.; Sharma, M.; Gautam, A.; Raghava, G. P. S. THPdb: Database of FDA-Approved Peptide and Protein Therapeutics. *PLoS One* **2017**, *12* (7), 1–12. https://doi.org/10.1371/journal.pone.0181748.
(32) Wang, G.; Li, X.; Wang, Z. APD3: The Antimicrobial Peptide Database as a Tool for

Research and Education. *Nucleic Acids Res.* **2016**, *44* (D1), D1087–D1093. https://doi.org/10.1093/nar/gkv1278.
(33) Dini, I.; De Biasi, M.-G.; Mancusi, A. An Overview of the Potentialities of Antimicrobial Peptides Derived from Natural Sources. *Antibiotics* **2022**, *11* (11), 1483. https://doi.org/10.3390/antibiotics11111483.
(34) Huan, Y.; Kong, Q.; Mou, H.; Yi, H. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front. Microbiol.* **2020**, *11* (October), 1–21. https://doi.org/10.3389/fmicb.2020.582779.
(35) Bin Hafeez, A.; Jiang, X.; Bergen, P. J.; Zhu, Y. Antimicrobial Peptides: An Update on Classifications and Databases. *Int. J. Mol. Sci.* **2021**, *22* (21). https://doi.org/10.3390/ijms222111691.
(36) Danis-Wlodarczyk, K. M.; Wozniak, D. J.; Abedon, S. T. Treating Bacterial Infections with Bacteriophage-Based Enzybiotics: In Vitro, in Vivo and Clinical Application. *Antibiotics* **2021**, *10* (12), 1–36. https://doi.org/10.3390/antibiotics10121497.
(37) Rodríguez-Rubio, L.; Martínez, B.; Donovan, D. M.; Rodríguez, A.; García, P. Bacteriophage Virion-Associated Peptidoglycan Hydrolases: Potential New Enzybiotics. *Crit. Rev. Microbiol.* **2013**, *39* (4), 427–434. https://doi.org/10.3109/1040841X.2012.723675.
(38) Yan, J.; Mao, J.; Xie, J. Bacteriophage Polysaccharide Depolymerases and Biomedical Applications. *BioDrugs* **2014**, *28* (3), 265–274. https://doi.org/10.1007/s40259-013-0081-y.
(39) Abril, A. G.; Carrera, M.; Notario, V.; Sánchez-Pérez, Á.; Villa, T. G. The Use of Bacteriophages in Biotechnology and Recent Insights into Proteomics. *Antibiotics* **2022**, *11* (5). https://doi.org/10.3390/antibiotics11050653.
(40) Abdelrahman, F.; Easwaran, M.; Daramola, O. I.; Ragab, S.; Lynch, S.; Oduselu, T. J.; Khan, F. M.; Ayobami, A.; Adnan, F.; Torrents, E.; Sanmukh, S.; El-shibiny, A. Phage-Encoded Endolysins. *Antibiotics* **2021**, *10* (124), 1–29. https://doi.org/https://doi.org/10.3390/antibiotics10020124.
(41) Tajbakhsh, M.; Karimi, A.; Fallah, F.; Akhavan, M. M. Overview of Ribosomal and Non-Ribosomal Antimicrobial Peptides Produced by Gram Positive Bacteria. *Cell. Mol. Biol.* **2017**, *6* (10). https://doi.org/10.14715/cmb/2017.63.10.4.
(42) Meade, E.; Slattery, M. A.; Garvey, M. Bacteriocins, Potent Antimicrobial Peptides and the Fight against Multi Drug Resistant Species: Resistance Is Futile? *Antibiotics* **2020**, *9* (1). https://doi.org/10.3390/antibiotics9010032.
(43) Simons, A.; Alhanout, K.; Duval, R. E. Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. *Microorganisms* **2020**, *8* (5). https://doi.org/10.3390/microorganisms8050639.
(44) Wu, J.; Gao, B.; Zhu, S. The Fungal Defensin Family Enlarged. *Pharmaceuticals* **2014**, *7* (8), 866–880. https://doi.org/10.3390/ph7080866.
(45) Qi, S.; Gao, B.; Zhu, S. A Fungal Defensin Inhibiting Bacterial Cell-Wall Biosynthesis with Non-Hemolysis and Serum Stability. *J. Fungi* **2022**, *8* (2). https://doi.org/10.3390/jof8020174.
(46) Zhu, S. Discovery of Six Families of Fungal Defensin-like Peptides Provides Insights into Origin and Evolution of the CSαβ Defensins. *Mol. Immunol.* **2008**, *45* (3), 828–838. https://doi.org/10.1016/j.molimm.2007.06.354.

(47) Evans, B. S.; Robinson, S. J.; Kelleher, N. L. Surveys of Non-Ribosomal Peptide and Polyketide Assembly Lines in Fungi and Prospects for Their Analysis in Vitro and in Vivo. *Fungal Genet. Biol.* **2011**, *48* (1), 49–61. https://doi.org/10.1016/j.fgb.2010.06.012.
(48) Leitgeb, B.; Szekeres, A.; Manczinger, L.; Vágvölgyi, C.; Kredics, L. The History of Alamethicin: A Review of the Most Extensively Studied Peptaibol. *Chem. Biodivers.* **2007**, *4* (6), 1027–1051. https://doi.org/10.1002/cbdv.200790095.
(49) Tyśkiewicz, R.; Nowak, A.; Ozimek, E.; Jaroszuk-ściseł, J. Trichoderma: The Current Status of Its Application in Agriculture for the Biocontrol of Fungal Phytopathogens and Stimulation of Plant Growth. *Int. J. Mol. Sci.* **2022**, *23* (4). https://doi.org/10.3390/ijms23042329.
(50) Tam, J. P.; Wang, S.; Wong, K. H.; Tan, W. L. Antimicrobial Peptides from Plants. *Pharmaceuticals* **2015**, *8* (4), 711–757. https://doi.org/10.3390/ph8040711.
(51) Stec, B. Plant Thionins - The Structural Perspective. *Cell. Mol. Life Sci.* **2006**, *63* (12), 1370–1385. https://doi.org/10.1007/s00018-005-5574-5.
(52) Odintsova, T.; Shcherbakova, L.; Slezina, M.; Pasechnik, T.; Kartabaeva, B.; Istomina, E.; Dzhavakhiya, V. Hevein-like Antimicrobial Peptides Wamps: Structure-Function Relationship in Antifungal Activity and Sensitization of Plant Pathogenic Fungi to Tebuconazole by WAMP-2-Derived Peptides. *Int. J. Mol. Sci.* **2020**, *21* (21), 1–26. https://doi.org/10.3390/ijms21217912.
(53) Slavokhotova, A. A.; Shelenkov, A. A.; Andreev, Y. A.; Odintsova, T. I. Hevein-like Antimicrobial Peptides of Plants. *Biochem.* **2017**, *82* (13), 1659–1674. https://doi.org/10.1134/S0006297917130065.
(54) Azmi, S.; Hussain, M. K. Analysis of Structures, Functions, and Transgenicity of Phytopeptides Defensin and Thionin: A Review. *Beni-Suef Univ. J. Basic Appl. Sci.* **2021**, *10* (1). https://doi.org/10.1186/s43088-020-00093-5.
(55) Gao, B.; Zhu, S. A Fungal Defensin Targets the Sars−cov−2 Spike Receptor−binding Domain. *J. Fungi* **2021**, *7* (7). https://doi.org/10.3390/jof7070553.
(56) Lima, A. M.; Azevedo, M. I. G.; Sousa, L. M.; Oliveira, N. S.; Andrade, C. R.; Freitas, C. D. T.; Souza, P. F. N. Plant Antimicrobial Peptides: An Overview about Classification, Toxicity and Clinical Applications. *Int. J. Biol. Macromol.* **2022**, *214* (April), 10–21. https://doi.org/10.1016/j.ijbiomac.2022.06.043.
(57) Fujimura, M.; Minami, Y.; Watanabe, K.; Tadera, K. Purification, Characterization, and Sequencing of a Novel Type of Antimicrobial Peptides, Fa-Amp1 and Fa-Amp2, from Seeds of Buckwheat (Fagopyrum Esculentum Moench.). *Biosci. Biotechnol. Biochem.* **2003**, *67* (8), 1636–1642. https://doi.org/10.1271/bbb.67.1636.
(58) Pelegrini, P. B.; Franco, O. L. Plant γ-Thionins: Novel Insights on the Mechanism of Action of a Multi-Functional Class of Defense Proteins. *Int. J. Biochem. Cell Biol.* **2005**, *37* (11), 2239–2253. https://doi.org/10.1016/j.biocel.2005.06.011.
(59) Santos-Silva, C. A. dos; Zupin, L.; Oliveira-Lima, M.; Vilela, L. M. B.; Bezerra-Neto, J. P.; Ferreira-Neto, J. R.; Ferreira, J. D. C.; Oliveira-Silva, R. L. de; Pires, C. de J.; Aburjaile, F. F.; Oliveira, M. F. de; Kido, E. A.; Crovella, S.; Benko-Iseppon, A. M. Plant Antimicrobial Peptides: State of the Art, In Silico Prediction and Perspectives in the Omics Era. *Bioinform. Biol. Insights* **2020**, *14*. https://doi.org/10.1177/1177932220952739.
(60) Hellinger, R.; Gruber, C. W. Peptide-Based Protease Inhibitors from Plants. *Drug Discov. Today* **2019**, *24* (9), 1877–1889. https://doi.org/10.1016/j.drudis.2019.05.026.

(61) Molesini, B.; Treggiari, D.; Dalbeni, A.; Minuz, P.; Pandolfini, T. Plant Cystine-Knot Peptides: Pharmacological Perspectives. *Br. J. Clin. Pharmacol.* **2017**, *83* (1), 63–70. https://doi.org/10.1111/bcp.12932.
(62) Pallaghy, P. K.; Norton, R. S.; Nielsen, K. J.; Craik, D. J. A Common Structural Motif Incorporating a Cystine Knot and a Triple-stranded Β-sheet in Toxic and Inhibitory Polypeptides. *Protein Sci.* **1994**, *3* (10), 1833–1839. https://doi.org/10.1002/pro.5560031022.
(63) Postic, G.; Gracy, J.; Périn, C.; Chiche, L.; Gelly, J. C. KNOTTIN: The Database of Inhibitor Cystine Knot Scaffold after 10 Years, toward a Systematic Structure Modeling. *Nucleic Acids Res.* **2018**, *46* (D1), D454–D458. https://doi.org/10.1093/nar/gkx1084.
(64) Haney, E. F.; Petersen, A. P.; Lau, C. K.; Jing, W.; Storey, D. G.; Vogel, H. J. Mechanism of Action of Puroindoline Derived Tryptophan-Rich Antimicrobial Peptides. *Biochim. Biophys. Acta - Biomembr.* **2013**, *1828* (8), 1802–1813. https://doi.org/10.1016/j.bbamem.2013.03.023.
(65) Carvalho, A. de O.; Gomes, V. M. Role of Plant Lipid Transfer Proteins in Plant Cell Physiology-A Concise Review. *Peptides* **2007**, *28* (5), 1144–1153. https://doi.org/10.1016/j.peptides.2007.03.004.
(66) Sels, J.; Mathys, J.; De Coninck, B. M. A.; Cammue, B. P. A.; De Bolle, M. F. C. Plant Pathogenesis-Related (PR) Proteins: A Focus on PR Peptides. *Plant Physiol. Biochem.* **2008**, *46* (11), 941–950. https://doi.org/10.1016/j.plaphy.2008.06.011.
(67) Su, T.; Han, M.; Cao, D.; Xu, M. Molecular and Biological Properties of Snakins: The Foremost Cysteine-Rich Plant Host Defense Peptides. *J. Fungi* **2020**, *6* (4), 1–17. https://doi.org/10.3390/jof6040220.
(68) Yeung, H.; Squire, C. J.; Yosaatmadja, Y.; Panjikar, S.; López, G.; Molina, A.; Baker, E. N.; Harris, P. W. R.; Brimble, M. A. Radiation Damage and Racemic Protein Crystallography Reveal the Unique Structure of the GASA/Snakin Protein Superfamily. *Angew. Chemie - Int. Ed.* **2016**, *55* (28), 7930–7933. https://doi.org/10.1002/anie.201602719.
(69) Rodríguez-Decuadro, S.; Barraco-Vega, M.; Dans, P. D.; Pandolfi, V.; Benko-Iseppon, A. M.; Cecchetto, G. Antimicrobial and Structural Insights of a New Snakin-like Peptide Isolated from Peltophorum Dubium (Fabaceae). *Amino Acids* **2018**, *50* (9), 1245–1259. https://doi.org/10.1007/s00726-018-2598-3.
(70) Zhang, S.; Wang, X. One New Kind of Phytohormonal Signaling Integrator: Up-and-Coming GASA Family Genes. *Plant Signal. Behav.* **2017**, *12* (2), 1–6. https://doi.org/10.1080/15592324.2016.1226453.
(71) De Veer, S. J.; Kan, M. W.; Craik, D. J. Cyclotides: From Structure to Function. *Chem. Rev.* **2019**, *119* (24), 12375–12421. https://doi.org/10.1021/acs.chemrev.9b00402.
(72) Zhang, Q. Y.; Yan, Z. Bin; Meng, Y. M.; Hong, X. Y.; Shao, G.; Ma, J. J.; Cheng, X. R.; Liu, J.; Kang, J.; Fu, C. Y. Antimicrobial Peptides: Mechanism of Action, Activity and Clinical Potential. *Mil. Med. Res.* **2021**, *8* (1), 1–25. https://doi.org/10.1186/s40779-021-00343-2.
(73) Froy, O. Convergent Evolution of Invertebrate Defensins and Nematode Antibacterial Factors. *Trends Microbiol.* **2005**, *13* (7), 314–319. https://doi.org/10.1016/j.tim.2005.05.001.
(74) Tassanakajon, A.; Somboonwiwat, K.; Amparyup, P. Sequence Diversity and Evolution of Antimicrobial Peptides in Invertebrates. *Dev. Comp. Immunol.* **2015**, *48* (2), 324–341.

https://doi.org/10.1016/j.dci.2014.05.020.
(75) Yang, D.; Zhang, Q.; Wang, Q.; Chen, L.; Liu, Y.; Cong, M.; Wu, H.; Li, F.; Ji, C.; Zhao, J. A Defensin-like Antimicrobial Peptide from the Manila Clam Ruditapes Philippinarum: Investigation of the Antibacterial Activities and Mode of Action. *Fish Shellfish Immunol.* **2018**, *80* (December 2017), 274–280. https://doi.org/10.1016/j.fsi.2018.06.019.
(76) Hancock, R. E. W.; Diamond, G. The Role of Cationic Antimicrobial Peptides in Innate Host Defences. *Trends Microbiol.* **2000**, *8* (9), 402–410. https://doi.org/10.1016/S0966-842X(00)01823-0.
(77) Masso-Silva, J. A.; Diamond, G. Antimicrobial Peptides from Fish. *Pharmaceuticals* **2014**, *7* (3), 265–310. https://doi.org/10.3390/ph7030265.
(78) Nam, B. H.; Moon, J. Y.; Kim, Y. O.; Kong, H. J.; Kim, W. J.; Lee, S. J.; Kim, K. K. Multiple β-Defensin Isoforms Identified in Early Developmental Stages of the Teleost Paralichthys Olivaceus. *Fish Shellfish Immunol.* **2010**, *28* (2), 267–274. https://doi.org/10.1016/j.fsi.2009.11.004.
(79) van Harten, R. M.; van Woudenbergh, E.; van Dijk, A.; Haagsman, H. P. Cathelicidins: Immunomodulatory Antimicrobials. *Vaccines* **2018**, *6* (3). https://doi.org/10.3390/vaccines6030063.
(80) Wessely-Szponder, J.; Majer-Dziedzic, B.; Smolira, A. Analysis of Antimicrobial Peptides from Porcine Neutrophils. *J. Microbiol. Methods* **2010**, *83* (1), 8–12. https://doi.org/10.1016/j.mimet.2010.07.010.
(81) Goitsuka, R.; Chen, C. L. H.; Benyon, L.; Asano, Y.; Kitamura, D.; Cooper, M. D. Chicken Cathelicidin-B1, an Antimicrobial Guardian at the Mucosal M Cell Gateway. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (38), 15063–15068. https://doi.org/10.1073/pnas.0707037104.
(82) Tossi, A.; Scocchi, M.; Zanetti, M.; Storici, P.; Gennaro, R. PMAP-37, a Novel Antibacterial Peptide from Pig Myeloid Cells: CDNA Cloning, Chemical Synthesis and Activity. *Eur. J. Biochem.* **1995**, *228* (3), 941–946. https://doi.org/10.1111/j.1432-1033.1995.tb20344.x.
(83) Wang, G.; Li, J.; Zou, P.; Xie, H.; Huang, B.; Nie, P.; Chang, M. Expression Pattern, Promoter Activity and Bactericidal Property of β-Defensin from the Mandarin Fish Siniperca Chuatsi. *Fish Shellfish Immunol.* **2012**, *33* (3), 522–531. https://doi.org/10.1016/j.fsi.2012.06.003.
(84) Cuesta, A.; Meseguer, J.; Esteban, M. Á. Molecular and Functional Characterization of the Gilthead Seabream β-Defensin Demonstrate Its Chemotactic and Antimicrobial Activity. *Mol. Immunol.* **2011**, *48* (12–13), 1432–1438. https://doi.org/10.1016/j.molimm.2011.03.022.
(85) Jin, J. Y.; Zhou, L.; Wang, Y.; Li, Z.; Zhao, J. G.; Zhang, Q. Y.; Gui, J. F. Antibacterial and Antiviral Roles of a Fish β-Defensin Expressed Both in Pituitary and Testis. *PLoS One* **2010**, *5* (12). https://doi.org/10.1371/journal.pone.0012883.
(86) Guo, M.; Wei, J.; Huang, X.; Huang, Y.; Qin, Q. Antiviral Effects of β-Defensin Derived from Orange-Spotted Grouper (Epinephelus Coioides). *Fish Shellfish Immunol.* **2012**, *32* (5), 828–838. https://doi.org/10.1016/j.fsi.2012.02.005.
(87) Chaturvedi, P.; Bhat, R. A. H.; Pande, A. Antimicrobial Peptides of Fish: Innocuous Alternatives to Antibiotics. *Rev. Aquac.* **2020**, *12* (1), 85–106. https://doi.org/10.1111/raq.12306.
(88) Fernandes, J. M. O.; Ruangsri, J.; Kiron, V. Atlantic Cod Piscidin and Its Diversification

through Positive Selection. *PLoS One* **2010**, *5* (3). https://doi.org/10.1371/journal.pone.0009501.
(89) Raju, S. V.; Sarkar, P.; Kumar, P.; Arockiaraj, J. Piscidin, Fish Antimicrobial Peptide: Structure, Classification, Properties, Mechanism, Gene Regulation and Therapeutical Importance. *Int. J. Pept. Res. Ther.* **2021**, *27* (1), 91–107. https://doi.org/10.1007/s10989-020-10068-w.
(90) Xu, X.; Lai, R. The Chemistry and Biological Activities of Peptides from Amphibian Skin Secretions. *Chem. Rev.* **2015**, *115* (4), 1760–1846. https://doi.org/10.1021/cr4006704.
(91) Zhao, H.; Gan, T. X.; Liu, X. D.; Jin, Y.; Lee, W. H.; Shen, J. H.; Zhang, Y. Identification and Characterization of Novel Reptile Cathelicidins from Elapid Snakes. *Peptides* **2008**, *29* (10), 1685–1691. https://doi.org/10.1016/j.peptides.2008.06.008.
(92) Qu, P.; Gao, W.; Chen, H.; Li, D.; Yang, N.; Zhu, J.; Feng, X.; Liu, C.; Li, Z. The Central Hinge Link Truncation of the Antimicrobial Peptide Fowlicidin-3 Enhances Its Cell Selectivity without Antibacterial Activity Loss. *Antimicrob. Agents Chemother.* **2016**, *60* (5), 2798–2806. https://doi.org/10.1128/AAC.02351-15.
(93) Xiao, Y.; Herrera, A. I.; Bommineni, Y. R.; Soulages, J. L.; Prakash, O.; Zhang, G. The Central Kink Region of Fowlicidin-2, an α-Helical Host Defense Peptide, Is Critically Involved in Bacterial Killing and Endotoxin Neutralization. *J. Innate Immun.* **2009**, *1* (3), 268–280. https://doi.org/10.1159/000174822.
(94) Ageitos, J. M.; Sánchez-Pérez, A.; Calo-Mata, P.; Villa, T. G. Antimicrobial Peptides (AMPs): Ancient Compounds That Represent Novel Weapons in the Fight against Bacteria. *Biochem. Pharmacol.* **2017**, *133*, 117–138. https://doi.org/10.1016/j.bcp.2016.09.018.
(95) Duplantier, A. J.; van Hoek, M. L. The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds. *Front. Immunol.* **2013**, *4* (JUL), 1–14. https://doi.org/10.3389/fimmu.2013.00143.
(96) Rieg, S.; Meier, B.; Fähnrich, E.; Huth, A.; Wagner, D.; Kern, W. V.; Kalbacher, H. Differential Activity of Innate Defense Antimicrobial Peptides against Nocardia Species. *BMC Microbiol.* **2010**, *10*, 1–3. https://doi.org/10.1186/1471-2180-10-61.
(97) De Smet, K.; Contreras, R. Human Antimicrobial Peptides: Defensins, Cathelicidins and Histatins. *Biotechnol. Lett.* **2005**, *27* (18), 1337–1347. https://doi.org/10.1007/s10529-005-0936-5.
(98) Diamond, G.; Zasloff, M.; Eck, H.; Brasseur, M.; Lee Maloy, W.; Bevins, C. L. Tracheal Antimicrobial Peptide, a Cysteine-Rich Peptide from Mammalian Tracheal Mucosa: Peptide Isolation and Cloning of a CDNA. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88* (9), 3952–3956. https://doi.org/10.1073/pnas.88.9.3952.
(99) Ibrahim, O. O. Classification of Antimicrobial Peptides Bacteriocins, and the Nature of Some Bacteriocins with Potential Applications in Food Safety and Bio-Pharmaceuticals. *EC Microbiol.* **2019**, *15* (7), 591–608.
(100) Tripathi, A. K.; Kumari, T.; Harioudh, M. K.; Yadav, P. K.; Kathuria, M.; Shukla, P. K.; Mitra, K.; Ghosh, J. K. Identification of GXXXXG Motif in Chrysophsin-1 and Its Implication in the Design of Analogs with Cell-Selective Antimicrobial and Anti-Endotoxin Activities. *Sci. Rep.* **2017**, *7* (1), 1–16. https://doi.org/10.1038/s41598-017-03576-1.
(101) Wang, J.; Chou, S.; Xu, L.; Zhu, X.; Dong, N.; Shan, A.; Chen, Z. High Specific Selectivity and Membrane-Active Mechanism of the Synthetic Centrosymmetric α-Helical

Peptides with Gly-Gly Pairs. *Sci. Rep.* **2015**, *5* (October), 1–19. https://doi.org/10.1038/srep15963.
(102) De Souza Cândido, E.; E Silva Cardoso, M. H.; Sousa, D. A.; Viana, J. C.; De Oliveira-Júnior, N. G.; Miranda, V.; Franco, O. L. The Use of Versatile Plant Antimicrobial Peptides in Agribusiness and Human Health. *Peptides* **2014**, *55*, 65–78. https://doi.org/10.1016/j.peptides.2014.02.003.
(103) Li, W.; Tailhades, J.; O'Brien-Simpson, N. M.; Separovic, F.; Otvos, L.; Hossain, M. A.; Wade, J. D. Proline-Rich Antimicrobial Peptides: Potential Therapeutics against Antibiotic-Resistant Bacteria. *Amino Acids* **2014**, *46* (10), 2287–2294. https://doi.org/10.1007/s00726-014-1820-1.
(104) Sonderegger, C.; Fizil, Á.; Burtscher, L.; Hajdu, D.; Muñoz, A.; Gáspári, Z.; Read, N. D.; Batta, G.; Marx, F. D19S Mutation of the Cationic, Cysteine-Rich Protein PAF: Novel Insights into Its Structural Dynamics, Thermal Unfolding and Antifungal Function. *PLoS One* **2017**, *12* (1), 1–21. https://doi.org/10.1371/journal.pone.0169920.
(105) Mojsoska, B.; Jenssen, H. Peptides and Peptidomimetics for Antimicrobial Drug Design. *Pharmaceuticals* **2015**, *8* (3), 366–415. https://doi.org/10.3390/ph8030366.
(106) Epand, R. M.; Vogel, H. J. Diversity of Antimicrobial Peptides and Their Mechanisms of Action. *Biochim. Biophys. Acta - Biomembr.* **1999**, *1462* (1–2), 11–28. https://doi.org/10.1016/S0005-2736(99)00198-4.
(107) Padmanabhan, S.; York, E. J.; Stewart, J. M.; Baldwin, R. L. Helix Propensities of Basic Amino Acids Increase with the Length of the Side-Chain. *J. Mol. Biol.* **1996**, *257* (3), 726–734. https://doi.org/10.1006/jmbi.1996.0197.
(108) Hollmann, A.; Martínez, M.; Noguera, M. E.; Augusto, M. T.; Disalvo, A.; Santos, N. C.; Semorile, L.; Maffía, P. C. Role of Amphipathicity and Hydrophobicity in the Balance between Hemolysis and Peptide-Membrane Interactions of Three Related Antimicrobial Peptides. *Colloids Surfaces B Biointerfaces* **2016**, *141*, 528–536. https://doi.org/10.1016/j.colsurfb.2016.02.003.
(109) Pace, C. N.; Scholtz, J. M. A Helix Propensity Scale Based on Experimental Studies of Peptides and Proteins. *Biophys. J.* **1998**, *75* (1), 422–427. https://doi.org/10.1016/s0006-3495(98)77529-0.
(110) Schmidtchen, A.; Pasupuleti, M.; Malmsten, M. Effect of Hydrophobic Modifications in Antimicrobial Peptides. *Adv. Colloid Interface Sci.* **2014**, *205*, 265–274. https://doi.org/10.1016/j.cis.2013.06.009.
(111) Kumar, P.; Kizhakkedathu, J. N.; Straus, S. K. Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility in Vivo. *Biomolecules*. MDPI AG March 1, 2018. https://doi.org/10.3390/biom8010004.
(112) Nayab, S.; Aslam, M. A.; Rahman, S. ur; Sindhu, Z. ud D.; Sajid, S.; Zafar, N.; Razaq, M.; Kanwar, R.; Amanullah. A Review of Antimicrobial Peptides: Its Function, Mode of Action and Therapeutic Potential. *Int. J. Pept. Res. Ther.* **2022**, *28* (1), 1–15. https://doi.org/10.1007/s10989-021-10325-6.
(113) Jenssen, H.; Hamill, P.; Hancock, R. E. W. Peptide Antimicrobial Agents. *Clin. Microbiol. Rev.* **2006**, *19* (3), 491–511. https://doi.org/10.1128/CMR.00056-05.
(114) Shai, Y. Mode of Action of Membrane Active Antimicrobial Peptides. *Biopolymers* **2002**, *66*, 236–248.
(115) Sengupta, D.; Leontiadou, H.; Mark, A. E.; Marrink, S. J. Toroidal Pores Formed by Antimicrobial Peptides Show Significant Disorder. *Biochim. Biophys. Acta - Biomembr.*

2008, *1778* (10), 2308–2317. https://doi.org/10.1016/j.bbamem.2008.06.007.
(116) Rapaport, D.; Shai, Y. Interaction of Fluorescently Labeled Pardaxin and Its Analogues with Lipid Bilayers. *J. Biol. Chem.* **1991**, *266* (35), 23769–23775. https://doi.org/10.1016/s0021-9258(18)54349-0.
(117) Cheng, J. T. J.; Hale, J. D.; Elliot, M.; Hancock, R. E. W.; Straus, S. K. Effect of Membrane Composition on Antimicrobial Peptides Aurein 2.2 and 2.3 from Australian Southern Bell Frogs. *Biophys. J.* **2009**, *96* (2), 552–565. https://doi.org/10.1016/j.bpj.2008.10.012.
(118) Wimley, W. C. Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. *ACS Chem. Biol.* **2010**, *5* (10), 905–917. https://doi.org/10.1021/cb1001558.
(119) Shai, Y. Mechanism of the Binding, Insertion and Destabilization of Phospholipid Bilayer Membranes by α-Helical Antimicrobial and Cell Non-Selective Membrane-Lytic Peptides. *Biochim. Biophys. Acta - Biomembr.* **1999**, *1462* (1–2), 55–70. https://doi.org/10.1016/S0005-2736(99)00200-X.
(120) Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and Arginine-Rich Antimicrobial Peptides: Structures and Mechanisms of Action. *Biochim. Biophys. Acta - Biomembr.* **2006**, *1758* (9), 1184–1202. https://doi.org/10.1016/j.bbamem.2006.04.006.
(121) Oren, Z.; Shai, Y. Mode of Action of Linear Amphipathic α-Helical Antimicrobial Peptides. *Biopolymers* **1998**, *47* (6), 451–463. https://doi.org/10.1002/(SICI)1097-0282(1998)47:6<451::AID-BIP4>3.0.CO;2-F.
(122) Liu, S.; Fan, L.; Sun, J.; Lao, X.; Zheng, H. Computational Resources and Tools for Antimicrobial Peptides. *J. Pept. Sci.* **2017**, *23* (1), 4–12. https://doi.org/10.1002/psc.2947.
(123) Moravej, H.; Moravej, Z.; Yazdanparast, M.; Heiat, M.; Mirhosseini, A.; Moosazadeh Moghaddam, M.; Mirnejad, R. Antimicrobial Peptides: Features, Action, and Their Resistance Mechanisms in Bacteria. *Microb. Drug Resist.* **2018**, *24* (6), 747–767. https://doi.org/10.1089/mdr.2017.0392.
(124) Pokorny, A.; Birkbeck, T. H.; Almeida, P. F. F. Mechanism and Kinetics of δ-Lysin Interaction with Phospholipid Vesicles. *Biochemistry* **2002**, *41* (36), 11044–11056. https://doi.org/10.1021/bi020244r.
(125) Hale, J. D. F.; Hancock, R. E. W. Alternative Mechanisms of Action of Cationic Antimicrobial Peptides on Bacteria. *Expert Rev. Anti. Infect. Ther.* **2007**, *5* (6), 951–959. https://doi.org/10.1586/14787210.5.6.951.
(126) Malanovic, N.; Leber, R.; Schmuck, M.; Kriechbaum, M.; Cordfunke, R. A.; Drijfhout, J. W.; De Breij, A.; Nibbering, P. H.; Kolb, D.; Lohner, K. Phospholipid-Driven Differences Determine the Action of the Synthetic Antimicrobial Peptide OP-145 on Gram-Positive Bacterial and Mammalian Membrane Model Systems. *Biochim. Biophys. Acta - Biomembr.* **2015**, *1848* (10), 2437–2447. https://doi.org/10.1016/j.bbamem.2015.07.010.
(127) Kragol, G.; Lovas, S.; Varadi, G.; Condie, B. A.; Hoffmann, R.; Otvos, L. The Antibacterial Peptide Pyrrhocoricin Inhibits the ATPase Actions of DnaK and Prevents Chaperone-Assisted Protein Folding. *Biochemistry* **2001**, *40* (10), 3016–3026. https://doi.org/10.1021/bi002656a.
(128) Otvos, L.; Insug, O.; Rogers, M. E.; Consolvo, P. J.; Condie, B. A.; Lovas, S.; Bulet, P.; Blaszczyk-Thurin, M. Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochemistry* **2000**, *39* (46), 14150–14159. https://doi.org/10.1021/bi0012843.
(129) Braffman, N. R.; Piscotta, F. J.; Hauver, J.; Campbell, E. A.; James Link, A.; Darst, S. A.

Structural Mechanism of Transcription Inhibition by Lasso Peptides Microcin J25 and Capistruin. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (4), 1273–1278. https://doi.org/10.1073/pnas.1817352116.
(130) Hou, X.; Feng, C.; Li, S.; Luo, Q.; Shen, G.; Wu, H.; Li, M.; Liu, X.; Chen, A.; Ye, M.; Zhang, Z. Mechanism of Antimicrobial Peptide NP-6 from Sichuan Pepper Seeds against E. Coli and Effects of Different Environmental Factors on Its Activity. *Appl. Microbiol. Biotechnol.* **2019**, *103* (16), 6593–6604. https://doi.org/10.1007/s00253-019-09981-y.
(131) Cardoso, M. H.; Meneguetti, B. T.; Costa, B. O.; Buccini, D. F.; Oshiro, K. G. N.; Preza, S. L. E.; Carvalho, C. M. E.; Migliolo, L.; Franco, O. L. Non-Lytic Antibacterial Peptides That Translocate through Bacterial Membranes to Act on Intracellular Targets. *Int. J. Mol. Sci.* **2019**, *20* (19). https://doi.org/10.3390/ijms20194877.
(132) Chaudhari, A. A.; Ashmore, D.; Nath, S. deb; Kate, K.; Dennis, V.; Singh, S. R.; Owen, D. R.; Palazzo, C.; Arnold, R. D.; Miller, M. E.; Pillai, S. R. A Novel Covalent Approach to Bio-Conjugate Silver Coated Single Walled Carbon Nanotubes with Antimicrobial Peptide. *J. Nanobiotechnology* **2016**, *14* (1), 1–15. https://doi.org/10.1186/s12951-016-0211-z.
(133) Galdiero, E.; Siciliano, A.; Maselli, V.; Gesuele, R.; Guida, M.; Fulgione, D.; Galdiero, S.; Lombardi, L.; Falanga, A. An Integrated Study on Antimicrobial Activity and Ecotoxicity of Quantum Dots and Quantum Dots Coated with the Antimicrobial Peptide Indolicidin. *Int. J. Nanomedicine* **2016**, *11*, 4199–4211. https://doi.org/10.2147/IJN.S107752.
(134) Boman, H. G.; Agerberth, B.; Boman, A. Mechanisms of Action on Escherichia Coli of Cecropin P1 and PR-39, Two Antibacterial Peptides from Pig Intestine. *Infect. Immun.* **1993**, *61* (7), 2978–2984. https://doi.org/10.1128/iai.61.7.2978-2984.1993.
(135) Florin, T.; Maracci, C.; Graf, M.; Karki, P.; Klepacki, D.; Berninghausen, O.; Beckmann, R.; Vázquez-Laslop, N.; Wilson, D. N.; Rodnina, M. V.; Mankin, A. S. An Antimicrobial Peptide That Inhibits Translation by Trapping Release Factors on the Ribosome. *Nat. Struct. Mol. Biol.* **2017**, *24* (9), 752–757. https://doi.org/10.1038/nsmb.3439.
(136) Mardirossian, M.; Barriere, Q.; Timchenko, T.; Muller, C.; Pacor, S.; Mergaert, P.; Scocchi, M.; Wilson, D. N. Fragments of the Nonlytic Proline-Rich Antimicrobial Peptide Bac5 Kill Escherichia Coli Cells by Inhibiting Protein Pynthesis. *Antimicrob. Agents Chemother.* **2018**, *62* (8), 1–15.
(137) Hilchie, A. L.; Wuerth, K.; Hancock, R. E. W. Immune Modulation by Multifaceted Cationic Host Defense (Antimicrobial) Peptides. *Nat. Chem. Biol.* **2013**, *9* (12), 761–768. https://doi.org/10.1038/nchembio.1393.
(138) Afacan, N. J.; Yeung, A. T. Y.; Pena, O. M.; Hancock, R. E. W. Therapeutic Potential of Host Defense Peptides in Antibiotic-Resistant Infections. **2012**, 807–819.
(139) Mader, J. S.; Hoskin, D. W. Cationic Antimicrobial Peptides as Novel Cytotoxic Agents for Cancer Treatment. *Expert Opin. Investig. Drugs* **2006**, *15* (8), 933–946. https://doi.org/10.1517/13543784.15.8.933.
(140) Levast, B.; Hogan, D.; Van Kessel, J.; Strom, S.; Walker, S.; Zhu, J.; Meurens, F. J.; Gerdts, V. Synthetic Cationic Peptide IDR-1002 and Human Cathelicidin LL37 Modulate the Cell Innate Response but Differentially Impact PRRSV Replication in Vitro. *Front. Vet. Sci.* **2019**, *6* (JUN), 1–12. https://doi.org/10.3389/fvets.2019.00233.
(141) Wuerth, K. C.; Falsafi, R.; Hancock, R. E. W. Synthetic Host Defense Peptide IDR-1002 Reduces Inflammation in Pseudomonas Aeruginosa Lung Infection. *PLoS One* **2017**, *12*

(11), 1–18. https://doi.org/10.1371/journal.pone.0187565.
(142) Wu, B. C.; Lee, A. H.-Y.; Hancock, R. E. W. Mechanisms of the Innate Defense Regulator Peptide-1002 Anti-Inflammatory Activity in a Sterile Inflammation Mouse Model. *J. Immunol.* **2017**, *199* (10), 3592–3603. https://doi.org/10.4049/jimmunol.1700985.
(143) Mi, B.; Liu, J.; Liu, Y.; Hu, L.; Liu, Y.; Panayi, A. C.; Zhou, W.; Liu, G. The Designer Antimicrobial Peptide A-HBD-2 Facilitates Skin Wound Healing by Stimulating Keratinocyte Migration and Proliferation. *Cell. Physiol. Biochem.* **2018**, *51* (2), 647–663. https://doi.org/10.1159/000495320.
(144) Browne, K.; Chakraborty, S.; Chen, R.; Willcox, M. D. P.; Black, D. S.; Walsh, W. R.; Kumar, N. A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *Int. J. Mol. Sci.* **2020**, *21* (19), 1–23. https://doi.org/10.3390/ijms21197047.
(145) Hallett, J. W.; Wolkowicz, M. I.; Leopold, I. H. Ophthalmic Use of Neosporin. *Am. J. Ophthalmol.* **1956**, *41* (5), 850–853. https://doi.org/10.1016/0002-9394(56)91781-0.
(146) Meikle, T. G.; Conn, C. E.; Separovic, F.; Drummond, C. J. Exploring the Structural Relationship between Encapsulated Antimicrobial Peptides and the Bilayer Membrane Mimetic Lipidic Cubic Phase: Studies with Gramicidin A′. *RSC Adv.* **2016**, *6* (73), 68685–68694. https://doi.org/10.1039/c6ra13658c.
(147) Carpenter, C. F.; Chambers, H. F. Daptomycin: Another Novel Agent for Treating Infections Due to Drug-Resistant Gram-Positive Pathogens. *Clin. Infect. Dis.* **2004**, *38* (7), 994–1000. https://doi.org/10.1086/383472.
(148) Moellering, R. C. Vancomycin : A 50-Year Reassessment. **2006**, *42* (Suppl 1), 3–4.
(149) Rubinstein, E.; Keynan, Y. Vancomycin Revisited - 60 Years Later. *Front. Public Heal.* **2014**, *2* (OCT), 1–7. https://doi.org/10.3389/fpubh.2014.00217.
(150) Liu, C.; Bayer, A.; Cosgrove, S. E.; Daum, R. S.; Fridkin, S. K.; Gorwitz, R. J.; Kaplan, S. L.; Karchmer, A. W.; Levine, D. P.; Murray, B. E.; Rybak, M. J.; Talan, D. A.; Chambers, H. F. Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin-Resistant Staphylococcus Aureus Infections in Adults and Children. *Clin. Infect. Dis.* **2011**, *52* (3). https://doi.org/10.1093/cid/ciq146.
(151) Lei, J.; Sun, L. C.; Huang, S.; Zhu, C.; Li, P.; He, J.; Mackey, V.; Coy, D. H.; He, Q. Y. The Antimicrobial Peptides and Their Potential Clinical Applications. *Am. J. Transl. Res.* **2019**, *11* (7), 3919–3931.
(152) Chen, A. Y.; Zervos, M. J.; Vazquez, J. A. Dalbavancin: A Novel Antimicrobial. *Int. J. Clin. Pract.* **2007**, *61* (5), 853–863. https://doi.org/10.1111/j.1742-1241.2007.01318.x.
(153) Zhanel, G. G.; Calic, D.; Schweizer, F.; Zelenitsky, S.; Adam, H.; Lagacé-Wiens, P. R. S.; Rubinstein, E.; Gin, A. S.; Hoban, D. J.; Karlowsky, J. A. New Lipoglycopeptides: A Comparative Review of Dalbavancin, Oritavancin and Telavancin. *Drugs* **2010**, *70* (7), 859–886. https://doi.org/10.2165/11534440-000000000-00000.
(154) Saravolatz, L. D.; Stein, G. E.; Johnson, L. B. Telavancin: A Novel Lipoglycopeptide. *Clin. Infect. Dis.* **2009**, *49* (12), 1908–1914. https://doi.org/10.1086/648438.
(155) Agarwal, R.; Bartsch, S. M.; Kelly, B. J.; Prewitt, M.; Liu, Y.; Chen, Y.; Umscheid, C. A. Newer Glycopeptide Antibiotics for Treatment of Complicated Skin and Soft Tissue Infections: Systematic Review, Network Meta-Analysis and Cost Analysis. *Clin. Microbiol. Infect.* **2018**, *24* (4), 361–368. https://doi.org/10.1016/j.cmi.2017.08.028.
(156) Higgins, D. L.; Chang, R.; Debabov, D. V.; Leung, J.; Wu, T.; Krause, K. M.; Sandvik, E.; Hubbard, J. M.; Kaniga, K.; Schmidt, D. E.; Gao, Q.; Cass, R. T.; Karr, D. E.; Benton,

B. M.; Humphrey, P. P. Telavancin, a Multifunctional Lipoglycopeptide, Disrupts Both Cell Wall Synthesis and Cell Membrane Integrity in Methicillin-Resistant Staphylococcus Aureus. *Antimicrob. Agents Chemother.* **2005**, *49* (3), 1127–1134. https://doi.org/10.1128/AAC.49.3.1127-1134.2005.
(157) Plotkin, P.; Patel, K.; Uminski, A.; Marzella, N. Telavancin (Vibativ), a New Option for the Treatment of Gram-Positive Infections. *P T* **2011**, *36* (3), 127–138.
(158) Laohavaleeson, S.; Kuti, J. L.; Nicolau, D. P. Telavancin: A Novel Lipoglycopeptide for Serious Gram-Positive Infections. *Expert Opin. Investig. Drugs* **2007**, *16* (3), 347–357. https://doi.org/10.1517/13543784.16.3.347.
(159) Van Bambeke, F. Glycopeptides and Glycodepsipeptides in Clinical Development: A Comparative Review of Their Antibacterial Spectrum, Pharmacokinetics and Clinical Efficacy. *Current Opinion in Investigational Drugs*. 2006, pp 740–749.
(160) Wilson, A. P. R. Clinical Pharmacokinetics of Teicoplanin. *Clin. Pharmacokinet.* **2000**, *39* (3), 167–183. https://doi.org/10.2165/00003088-200039030-00001.
(161) El-Sayed Ahmed, M. A. E. G.; Zhong, L. L.; Shen, C.; Yang, Y.; Doi, Y.; Tian, G. B. Colistin and Its Role in the Era of Antibiotic Resistance: An Extended Review (2000–2019). *Emerg. Microbes Infect.* **2020**, *9* (1), 868–885. https://doi.org/10.1080/22221751.2020.1754133.
(162) Thummeepak, R.; Kitti, T.; Kunthalert, D.; Sitthisak, S. Enhanced Antibacterial Activity of Acinetobacter Baumannii Bacteriophage Ï¿½ABP-01 Endolysin (LysABP-01) in Combination with Colistin. *Front. Microbiol.* **2016**, *7* (SEP), 1–8. https://doi.org/10.3389/fmicb.2016.01402.
(163) Hilpert, K.; McLeod, B.; Yu, J.; Elliott, M. R.; Rautenbach, M.; Ruden, S.; Bürck, J.; Muhle-Goll, C.; Ulrich, A. S.; Keller, S.; Hancock, R. E. W. Short Cationic Antimicrobial Peptides Interact with ATP. *Antimicrob. Agents Chemother.* **2010**, *54* (10), 4480–4483. https://doi.org/10.1128/AAC.01664-09.
(164) Marshall, E.; Costa, L. M.; Gutierrez-Marcos, J. Cysteine-Rich Peptides (CRPs) Mediate Diverse Aspects of Cell-Cell Communication in Plant Reproduction and Development. *J. Exp. Bot.* **2011**, *62* (5), 1677–1686. https://doi.org/10.1093/jxb/err002.
(165) Liu, X.; Zhang, H.; Jiao, H.; Li, L.; Qiao, X.; Fabrice, M. R.; Wu, J.; Zhang, S. Expansion and Evolutionary Patterns of Cysteine-Rich Peptides in Plants. *BMC Genomics* **2017**, *18* (1), 1–14. https://doi.org/10.1186/s12864-017-3948-3.
(166) Shelenkov, A.; Slavokhotova, A.; Odintsova, T. Predicting Antimicrobial and Other Cysteine-Rich Peptides in 1267 Plant Transcriptomes. *Antibiotics* **2020**, *9* (2), 1–8. https://doi.org/10.3390/antibiotics9020060.
(167) Kuddus, M. R.; Rumi, F.; Tsutsumi, M.; Takahashi, R.; Yamano, M.; Kamiya, M.; Kikukawa, T.; Demura, M.; Aizawa, T. Expression, Purification and Characterization of the Recombinant Cysteine-Rich Antimicrobial Peptide Snakin-1 in Pichia Pastoris. *Protein Expr. Purif.* **2016**, *122*, 15–22. https://doi.org/10.1016/j.pep.2016.02.002.
(168) Srivastava, S.; Dashora, K.; Ameta, K. L.; Singh, N. P.; El-Enshasy, H. A.; Pagano, M. C.; Hesham, A. E. L.; Sharma, G. D.; Sharma, M.; Bhargava, A. Cysteine-Rich Antimicrobial Peptides from Plants: The Future of Antimicrobial Therapy. *Phyther. Res.* **2021**, *35* (1), 256–277. https://doi.org/10.1002/ptr.6823.
(169) Pan, H.; Wang, D. Nodule Cysteine-Rich Peptides Maintain a Working Balance during Nitrogen-Fixing Symbiosis. *Nat. Plants* **2017**, *3* (May). https://doi.org/10.1038/nplants.2017.48.

(170) Zorin, E. A.; Kliukova, M. S.; Afonin, A. M.; Gribchenko, E. S.; Gordon, M. L.; Sulima, A. S.; Zhernakov, A. I.; Kulaeva, O. A.; Romanyuk, D. A.; Kusakin, P. G.; Tsyganova, A. V.; Tsyganov, V. E.; Tikhonovich, I. A.; Zhukov, V. A. A Variable Gene Family Encoding Nodule-Specific Cysteine-Rich Peptides in Pea (Pisum Sativum L.). *Front. Plant Sci.* **2022**, *13* (September), 1–22. https://doi.org/10.3389/fpls.2022.884726.
(171) Horvath, B.; Domonkos, A.; Kereszt, A.; Szucs, A.; Abraham, E.; Ayaydin, F.; Boka, K.; Chen, Y.; Chen, R.; Murray, J. D.; Udvardi, M. K.; Kondorosi, E.; Kalo, P. Loss of the Nodule-Specific Cysteine Rich Peptide, NCR169, Abolishes Symbiotic Nitrogen Fixation in the Medicago Truncatula Dnf7 Mutant. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (49), 15232–15237. https://doi.org/10.1073/pnas.1500777112.
(172) Montiel, J.; Downie, J. A.; Farkas, A.; Bihari, P.; Herczeg, R.; Bálint, B.; Mergaert, P.; Kereszt, A.; Kondorosi, É. Morphotype of Bacteroids in Different Legumes Correlates with the Number and Type of Symbiotic NCR Peptides. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (19), 5041–5046. https://doi.org/10.1073/pnas.1704217114.
(173) Roux, B.; Rodde, N.; Jardinaud, M. F.; Timmers, T.; Sauviac, L.; Cottret, L.; Carrère, S.; Sallet, E.; Courcelle, E.; Moreau, S.; Debellé, F.; Capela, D.; De Carvalho-Niebel, F.; Gouzy, J.; Bruand, C.; Gamas, P. An Integrated Analysis of Plant and Bacterial Gene Expression in Symbiotic Root Nodules Using Laser-Capture Microdissection Coupled to RNA Sequencing. *Plant J.* **2014**, *77* (6), 817–837. https://doi.org/10.1111/tpj.12442.
(174) Marx, H.; Minogue, C. E.; Jayaraman, D.; Richards, A. L.; Kwiecien, N. W.; Siahpirani, A. F.; Rajasekar, S.; Maeda, J.; Garcia, K.; Del Valle-Echevarria, A. R.; Volkening, J. D.; Westphall, M. S.; Roy, S.; Sussman, M. R.; Ané, J. M.; Coon, J. J. A Proteomic Atlas of the Legume Medicago Truncatula and Its Nitrogen-Fixing Endosymbiont Sinorhizobium Meliloti. *Nat. Biotechnol.* **2016**, *34* (11), 1198–1205. https://doi.org/10.1038/nbt.3681.
(175) Penterman, J.; Abo, R. P.; De Nisco, N. J.; Arnold, M. F. F.; Longhi, R.; Zanda, M.; Walker, G. C. Host Plant Peptides Elicit a Transcriptional Response to Control the Sinorhizobium Meliloti Cell Cycle during Symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (9), 3561–3566. https://doi.org/10.1073/pnas.1400450111.
(176) Farkas, A.; Maróti, G.; Kereszt, A.; Kondorosi, É. Comparative Analysis of the Bacterial Membrane Disruption Effect of Two Natural Plant Antimicrobial Peptides. *Front. Microbiol.* **2017**, *8* (JAN), 1–12. https://doi.org/10.3389/fmicb.2017.00051.
(177) Farkas, A.; Pap, B.; Kondorosi, É.; Maróti, G. Antimicrobial Activity of NCR Plant Peptides Strongly Depends on the Test Assays. *Front. Microbiol.* **2018**, *9* (October), 1–10. https://doi.org/10.3389/fmicb.2018.02600.
(178) Ördögh, L.; Vörös, A.; Nagy, I.; Kondorosi, É.; Kereszt, A. Symbiotic Plant Peptides Eliminate Candida Albicans Both in Vitro and in an Epithelial Infection Model and Inhibit the Proliferation of Immortalized Human Cells. *Biomed Res. Int.* **2014**, *2014*. https://doi.org/10.1155/2014/320796.
(179) Velivelli, S. L. S.; Czymmek, K. J.; Li, H.; Shaw, J. B.; Buchko, G. W.; Shah, D. M. Antifungal Symbiotic Peptide NCR044 Exhibits Unique Structure and Multifaceted Mechanisms of Action That Confer Plant Protection. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (27), 16043–16054. https://doi.org/10.1073/pnas.2003526117.
(180) Hawkins, J. P.; Oresnik, I. J. The Rhizobium-Legume Symbiosis: Co-Opting Successful Stress Management. *Front. Plant Sci.* **2022**, *12* (January). https://doi.org/10.3389/fpls.2021.796045.
(181) Roy, P.; Achom, M.; Wilkinson, H.; Lagunas, B.; Gifford, M. L. Symbiotic Outcome

Modified by the Diversification from 7 to over 700 Nodule-specific Cysteine-rich Peptides. *Genes (Basel).* **2020**, *11* (4), 1–16. https://doi.org/10.3390/genes11040348.
(182) Van de Velde, W.; Zehirov, G.; Szatmari, A.; Debreczeny, M.; Ishihara, H.; Kevei, Z.; Farkas, A.; Mikulass, K.; Nagy, A.; Tiricz, H.; Satiat-Heunemaitre, B.; Alunni, B.; Bourge, M.; Kucho, K.; Abe, M.; Kereszt, A.; Maroti, G.; Uchiumi, T.; Kondorosi, É.; Mergaert, P. Plant Peptides Govern Terminal Differentiation of Bacteria in Symbiosis. *Science (80-.).* **2010**, *327* (February), 1122–1126. https://doi.org/10.2307/j.ctv9zcj2n.53.
(183) Farkas, A.; Maróti, G.; Dürgo, H.; Györgypál, Z.; Lima, R. M.; Medzihradszky, K. F.; Kereszt, A.; Mergaert, P.; Kondorosi, É. Medicago Truncatula Symbiotic Peptide NCR247 Contributes to Bacteroid Differentiation through Multiple Mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (14), 5183–5188. https://doi.org/10.1073/pnas.1404169111.
(184) Lindström, K.; Mousavi, S. A. Effectiveness of Nitrogen Fixation in Rhizobia. *Microb. Biotechnol.* **2020**, *13* (5), 1314–1335. https://doi.org/10.1111/1751-7915.13517.
(185) Isozumi, N.; Masubuchi, Y.; Imamura, T.; Mori, M.; Koga, H.; Ohki, S. Structure and Antimicrobial Activity of NCR169, a Nodule-Specific Cysteine-Rich Peptide of Medicago Truncatula. *Sci. Rep.* **2021**, *11* (1). https://doi.org/10.1038/s41598-021-89485-w.
(186) Amblard, M.; Fehrentz, J. A.; Martinez, J.; Subra, G. Methods and Protocols of Modern Solid Phase Peptide Synthesis. *Mol. Biotechnol.* **2006**, *33* (3), 239–254. https://doi.org/10.1385/MB:33:3:239.
(187) Mäde, V.; Els-Heindl, S.; Beck-Sickinger, A. G. Automated Solid-Phase Peptide Synthesis to Obtain Therapeutic Peptides. *Beilstein J. Org. Chem.* **2014**, *10* (Scheme 1), 1197–1212. https://doi.org/10.3762/bjoc.10.118.
(188) Hansen, P. R.; Oddo, A. Fmoc Solid-Phase Peptide Synthesis. *Methods Mol. Biol.* **2015**, *1348*. https://doi.org/10.1007/978-1-4939-2999-3.
(189) Costa, I. G. SYNTHESIS OF ANTIMICROBIAL PEPTIDES DERIVED FROM BP100 AND BPC194, Universitat de Girona, 2011.
(190) Luna, O. F.; Gomez, J.; Cárdenas, C.; Albericio, F.; Marshall, S. H.; Guzmán, F. Deprotection Reagents in Fmoc Solid Phase Peptide Synthesis: Moving Away from Piperidine? *Molecules* **2016**, *21* (11), 1–12. https://doi.org/10.3390/molecules21111542.
(191) Münzker, L.; Oddo, A.; Hansen, P. R. Chemical Synthesis of Antimicrobial Peptides. *Methods Mol. Biol.* **2017**, *1548*, 35–49. https://doi.org/10.1007/978-1-4939-6737-7_3.
(192) Aguilar, M.-I. *HPLC of Peptides and Proteins: Methods and Protocols (Methods in Molecular Biology)*; 2004.
(193) Nadler, W. M.; Waidelich, D.; Kerner, A.; Hanke, S.; Berg, R.; Trumpp, A.; Rösli, C. MALDI versus ESI: The Impact of the Ion Source on Peptide Identification. *J. Proteome Res.* **2017**, *16* (3), 1207–1215. https://doi.org/10.1021/acs.jproteome.6b00805.
(194) Letzel, T. How to Couple and Handle Liquid Chromatography with Mass Spectrometry. In *Protein and peptide Analysis by LC-MS: Experimental Strategies*; Royal Society of Chemistry, 2011; pp 11–25.
(195) Zhang, G.; Annan, R. S.; Carr, S. A.; Neubert, T. A. Overview of Peptide and Protein Analysis by Mass Spectrometry. *Curr. Protoc. Protein Sci.* **2010**, No. SUPPL.62, 1–30. https://doi.org/10.1002/0471140864.ps1601s62.
(196) Trauger, S. A.; Webb, W.; Siuzdak, G. Peptide and Protein Analysis with Mass Spectrometry. *Spectroscopy* **2002**, *16* (1), 15–28. https://doi.org/10.1155/2002/320152.
(197) Lima, R. M.; Rathod, B. B.; Tiricz, H.; Howan, D. H. O.; Al Bouni, M. A.; Jenei, S.;

Tímár, E.; Endre, G.; Tóth, G. K.; Kondorosi, É. Legume Plant Peptides as Sources of Novel Antimicrobial Molecules Against Human Pathogens. *Front. Mol. Biosci.* **2022**, *9* (June), 1–12. https://doi.org/10.3389/fmolb.2022.870460.
(198) Mergaert, P. Role of Antimicrobial Peptides in Controlling Symbiotic Bacterial Populations. *Natural Product Reports*. Royal Society of Chemistry April 1, 2018, pp 336–356. https://doi.org/10.1039/c7np00056a.
(199) Jenei, S.; Tiricz, H.; Szolomájer, J.; Tímár, E.; Klement, É.; Al Bouni, M. A.; Lima, R. M.; Kata, D.; Harmati, M.; Buzás, K.; Földesi, I.; Tóth, G. K.; Endre, G.; Kondorosi, É. Potent Chimeric Antimicrobial Derivatives of the Medicago Truncatula NCR247 Symbiotic Peptide. *Front. Microbiol.* **2020**, *11* (February), 1–10. https://doi.org/10.3389/fmicb.2020.00270.
(200) Howan, D. H. O.; Jenei, S.; Szolomajer, J.; Endre, G.; Kondorosi, É.; Tóth, G. K. Enhanced Antibacterial Activity of Substituted Derivatives of NCR169C Peptide. *Int. J. Mol. Sci.* **2023**, *24* (3). https://doi.org/10.3390/ijms24032694.
(201) Szerencsés, B.; Gácser, A.; Endre, G.; Domonkos, I.; Tiricz, H.; Vágvölgyi, C.; Szolomajer, J.; Howan, D. H. O.; Tóth, G. K.; Pfeiffer, I.; Kondorosi, É. Symbiotic NCR Peptide Fragments Affect the Viability, Morphology and Biofilm Formation of Candida Species. *Int. J. Mol. Sci.* **2021**, *22* (7). https://doi.org/10.3390/ijms22073666.
(202) Fernández de Ullivarri, M.; Arbulu, S.; Garcia-Gutierrez, E.; Cotter, P. D. Antifungal Peptides as Therapeutic Agents. *Front. Cell. Infect. Microbiol.* **2020**, *10* (March). https://doi.org/10.3389/fcimb.2020.00105.
(203) De Cesare, G. B.; Cristy, S. A.; Garsin, D. A.; Lorenz, M. C. Antimicrobial Peptides: A New Frontier in Antifungal Therapy. *MBio* **2020**, *11* (6), 1–21. https://doi.org/10.1128/mBio.02123-20.
(204) Bondaryk, M.; Staniszewska, M.; Zielińska, P.; Urbańczyk-Lipkowska, Z. *Natural Antimicrobial Peptides as Inspiration for Design of a New Generation Antifungal Compounds*; 2017; Vol. 3. https://doi.org/10.3390/jof3030046.
(205) Ma, B.; Niu, C.; Zhou, Y.; Xue, X.; Meng, J.; Luo, X.; Hou, Z. The Disulfide Bond of the Peptide Thanatin Is Dispensible for Its Antimicrobial Activity in Vivo and in Vitro. *Antimicrob. Agents Chemother.* **2016**, *60* (7), 4283–4289. https://doi.org/10.1128/AAC.00041-16.
(206) Wu, Z.; Hoover, D. M.; Yang, D.; Boulègue, C.; Santamaria, F.; Oppenheim, J. J.; Lubkowski, J.; Lu, W. Engineering Disulfide Bridges to Dissect Antimicrobial and Chemotactic Activities of Human β-Defensin 3. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (15), 8880–8885. https://doi.org/10.1073/pnas.1533186100.
(207) Schroeder, B. O.; Wu, Z.; Nuding, S.; Groscurth, S.; Marcinowski, M.; Beisner, J.; Buchner, J.; Schaller, M.; Stange, E. F.; Wehkamp, J. Reduction of Disulphide Bonds Unmasks Potent Antimicrobial Activity of Human β 2-Defensin 1. *Nature* **2011**, *469* (7330), 419–423. https://doi.org/10.1038/nature09674.
(208) Sharma, H.; Nagaraj, R. Human β-Defensin 4 with Non-Native Disulfide Bridges Exhibit Antimicrobial Activity. *PLoS One* **2015**, *10* (3), 14–18. https://doi.org/10.1371/journal.pone.0119525.
(209) Wang, Q.; Lv, Y.; Pang, J.; Li, X.; Lu, X.; Wang, X.; Hu, X.; Nie, T.; Yang, X.; Xiong, Y. Q.; Jiang, J.; Li, C.; You, X. In Vitro and in Vivo Activity of D-Serine in Combination with β-Lactam Antibiotics against Methicillin-Resistant Staphylococcus Aureus. *Acta Pharm. Sin. B* **2019**, *9* (3), 496–504. https://doi.org/10.1016/j.apsb.2019.01.017.

(210) Makino, Y.; Oe, C.; Iwama, K.; Suzuki, S.; Nishiyama, A.; Hasegawa, K.; Okuda, H.; Hirata, K.; Ueno, M.; Kawaji, K.; Sasano, M.; Usui, E.; Hosaka, T.; Yabuki, Y.; Shirouzu, M.; Katsumi, M.; Murayama, K.; Hayashi, H.; Kodama, E. N. Serine Hydroxymethyltransferase as a Potential Target of Antibacterial Agents Acting Synergistically with One-Carbon Metabolism-Related Inhibitors. *Commun. Biol.* **2022**, *5* (1). https://doi.org/10.1038/s42003-022-03555-x.
(211) Wu, T.; Wang, X.; Dong, Y.; Xing, C.; Chen, X.; Li, L.; Dong, C.; Li, Y. Effects of l -Serine on Macrolide Resistance in Streptococcus Suis . *Microbiol. Spectr.* **2022**, *10* (4). https://doi.org/10.1128/spectrum.00689-22.
(212) Feng, X.; Jin, S.; Wang, M.; Pang, Q.; Liu, C.; Liu, R.; Wang, Y.; Yang, H.; Liu, F.; Liu, Y. The Critical Role of Tryptophan in the Antimicrobial Activity and Cell Toxicity of the Duck Antimicrobial Peptide DCATH. *Front. Microbiol.* **2020**, *11* (May), 1–14. https://doi.org/10.3389/fmicb.2020.01146.
(213) Bi, X.; Wang, C.; Ma, L.; Sun, Y.; Shang, D. Investigation of the Role of Tryptophan Residues in Cationic Antimicrobial Peptides to Determine the Mechanism of Antimicrobial Action. *J. Appl. Microbiol.* **2013**, *115* (3), 663–672. https://doi.org/10.1111/jam.12262.
(214) Mishra, A. K.; Choi, J.; Moon, E.; Baek, K. H. Tryptophan-Rich and Proline-Rich Antimicrobial Peptides. *Molecules* **2018**, *23* (4), 1–23. https://doi.org/10.3390/molecules23040815.
(215) Zhou, L.; Shao, J.; Li, Q.; Van Heel, A. J.; De Vries, M. P.; Broos, J.; Kuipers, O. P. Incorporation of Tryptophan Analogues into the Lantibiotic Nisin. *Amino Acids* **2016**, *48* (5), 1309–1318. https://doi.org/10.1007/s00726-016-2186-3.
(216) Chen, C. H.; Genapathy, S.; Fischer, P. M.; Chan, W. C. A Facile Approach to Tryptophan Derivatives for the Total Synthesis of Argyrin Analogues. *Org. Biomol. Chem.* **2014**, *12* (48), 9764–9768. https://doi.org/10.1039/c4ob02107j.
(217) Marsh, E. N. G.; Buer, B. C.; Ramamoorthy, A. Fluorine - A New Element in the Design of Membrane-Active Peptides. *Mol. Biosyst.* **2009**, *5* (10), 1143–1147. https://doi.org/10.1039/b909864j.
(218) Meng, H.; Kumar, K. Antimicrobial Activity and Protease Stability of Peptides Containing Fluorinated Amino Acids. *J. Am. Chem. Soc.* **2007**, *129* (50), 15615–15622. https://doi.org/10.1021/ja075373f.
(219) Mardirossian, M.; Rubini, M.; Adamo, M. F. A.; Scocchi, M.; Saviano, M.; Tossi, A.; Gennaro, R.; Caporale, A. Natural and Synthetic Halogenated Amino Acids—Structural and Bioactive Features in Antimicrobial Peptides and Peptidomimetics. *Molecules* **2021**, *26* (23). https://doi.org/10.3390/molecules26237401.
(220) Scherer, E. M.; Scherer, E. M. Inquiry : The University of Arkansas Undergraduate Research Properties of Modified Tryptophans in a Membrane- Spanning Channel. **2002**, *3*.
(221) Füller, J. J.; Röpke, R.; Krausze, J.; Rennhack, K. E.; Daniel, N. P.; Blankenfeldt, W.; Schulz, S.; Jahn, D.; Moser, J. Biosynthesis of Violacein, Structure and Function of L-Tryptophan Oxidase VioA from Chromobacterium Violaceum. *J. Biol. Chem.* **2016**, *291* (38), 20068–20084. https://doi.org/10.1074/jbc.M116.741561.
(222) Katragadda, M.; Lambris, J. D. Expression of Compstatin in Escherichia Coli: Incorporation of Unnatural Amino Acids Enhances Its Activity. *Protein Expr. Purif.* **2006**, *47* (1), 289–295. https://doi.org/10.1016/j.pep.2005.11.016.
(223) Basavand, E.; Firouzianbandpey, S.; Rahimian, H. Periwinkle Leaf Spots and Stem

Lesions Caused by Xanthomonas Campestris. *Eur. J. Plant Pathol.* **2022**, *164* (2), 167–176. https://doi.org/10.1007/s10658-022-02534-6.

(224) Ichinose, Y.; Watanabe, Y.; Tumewu, S. A.; Matsui, H.; Yamamoto, M.; Noutoshi, Y.; Toyoda, K. Requirement of Chemotaxis and Aerotaxis in Host Tobacco Infection by Pseudomonas Syringae Pv. Tabaci 6605. *Physiol. Mol. Plant Pathol.* **2023**, *124* (February), 101970. https://doi.org/10.1016/j.pmpp.2023.101970.

(225) Dilbar, S.; Sher, H.; Binjawhar, D. N.; Ali, A.; Ali, I. A Novel Based Synthesis of Silver/Silver Chloride Nanoparticles from Stachys Emodi Efficiently Controls Erwinia Carotovora, the Causal Agent of Blackleg and Soft Rot of Potato. *Molecules* **2023**, *28* (6), 2500. https://doi.org/10.3390/molecules28062500.

(226) Fürst, U.; Zeng, Y.; Albert, M.; Witte, A. K.; Fliegmann, J.; Felix, G. Perception of Agrobacterium Tumefaciens Flagellin by FLS2XL Confers Resistance to Crown Gall Disease. *Nat. Plants* **2020**, *6* (1), 22–27. https://doi.org/10.1038/s41477-019-0578-6.

(227) Torrent, M.; Andreu, D.; Nogués, V. M.; Boix, E. Connecting Peptide Physicochemical and Antimicrobial Properties by a Rational Prediction Model. *PLoS One* **2011**, *6* (2), 1–8. https://doi.org/10.1371/journal.pone.0016968.

(228) Jindal, M. H.; Le, C. F.; Mohd Yusof, M. Y.; Sekaran, S. D. Net Charge, Hydrophobicity and Specific Amino Acids Contribute to the Activity of Antimicrobial Peptides. *J. Heal. Transl. Med.* **2014**, *17* (1), 1–7. https://doi.org/10.22452/jummec.vol17no1.1.

(229) Yeaman, M. R.; Yount, N. Y. Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacol. Rev.* **2003**, *55* (1), 27–55. https://doi.org/10.1124/pr.55.1.2.

(230) Edwards, I. A.; Elliott, A. G.; Kavanagh, A. M.; Zuegg, J.; Blaskovich, M. A. T.; Cooper, M. A. Contribution of Amphipathicity and Hydrophobicity to the Antimicrobial Activity and Cytotoxicity of β-Hairpin Peptides. *ACS Infect. Dis.* **2016**, *2* (6), 442–450. https://doi.org/10.1021/acsinfecdis.6b00045.

(231) Sun, H.; Greathouse, D. V.; Andersen, O. S.; Koeppe, R. E. The Preference of Tryptophan for Membrane Interfaces: Insights from N-Methylation of Tryptophans in Gramicidin Channels. *J. Biol. Chem.* **2008**, *283* (32), 22233–22243. https://doi.org/10.1074/jbc.M802074200.

(232) Blaser, G.; Sanderson, J. M.; Batsanov, A. S.; Howard, J. A. K. The Facile Synthesis of a Series of Tryptophan Derivatives. *Tetrahedron Lett.* **2008**, *49* (17), 2795–2798. https://doi.org/10.1016/j.tetlet.2008.02.120.

(233) Nguyen, N. K.; Nam, D. H.; Phuc, B. Van; Nguyen, V. H.; Trịnh, Q. T.; Hung, T. Q.; Dang, T. T. Efficient Copper-Catalyzed Synthesis of C3-Alkylated Indoles from Indoles and Alcohols. *Mol. Catal.* **2021**, *505* (February). https://doi.org/10.1016/j.mcat.2021.111462.

(234) Gentilucci, L.; Cerisoli, L.; De Marco, R.; Tolomelli, A. A Simple Route towards Peptide Analogues Containing Substituted (S)- or (R)-Tryptophans. *Tetrahedron Lett.* **2010**, *51* (19), 2576–2579. https://doi.org/10.1016/j.tetlet.2010.03.017.

(235) Bains, A. K.; Biswas, A.; Adhikari, D. Nickel-Catalysed Chemoselective C-3 Alkylation of Indoles with Alcohols through a Borrowing Hydrogen Method. *Chem. Commun.* **2020**, *56* (98), 15442–15445. https://doi.org/10.1039/d0cc07169b.

Acknowledgment

First and foremost, I would like to convey my sincere gratitude to my supervisor, Prof. Gábor K. Tóth, for his wholehearted support and understanding throughout my research. His priceless guidance and counsel from the initiation of my doctoral study have led me to this point. Being his Ph.D. student has been an immense privilege.

Secondly, I would like to extend my appreciation to the peptide research group in the Department of Medical Chemistry, University of Szeged, especially to Dr. János Szolomajer and Dr. Zsolt Bozsó as my great mentors in the laboratory. Their acceptance and valuable knowledge really helped me to conduct my research, as well as their assistance in completing my doctorate project. I also gratefully appreciate the laboratory technicians (Ildikó Masa, Máté Orsós, and Nelli Albert), for their valuable practical experience and friendship since I started my laboratory work.

I would like to deeply thank Prof. Éva Kondorosi, Dr. Gabriella Endre, Sándor Jenei, and Dr. Hilda Tiricz, from the Biology Research Center, Szeged, for collaboration, and for measuring the antimicrobial activities that are essential to my Ph.D. work.

I would like to greatly thank Dr. Ágota Tóth the head of the Doctoral School of Chemistry, Prof. Tamás Martinek the head of the Medical Chemistry Department, and Dr. Györgyi Váradi for their professional advice that enabled me to accomplish my doctorate study.

I wish to extend my acknowledgment to the Hungarian Government through the Tempus Foundation which has granted me the scholarship to pursue Ph.D. study at the University of Szeged.

Finally, I am truly thankful for my family. No words can describe how much I owe my parents, my sister, my relatives, and my friends, for their encouragement, support, prayers, care, and love.

List of Publications

Publications Related to Dissertation:

1. **Dian H.O. Howan**, Sándor Jenei, János Szolomajer, Gabriella Endre, Éva Kondorosi, Gábor K. Tóth,
   Enhanced Antibacterial Activity of Substituted Derivatives of NCR169C Peptide
   International Journal of Molecular Sciences, 24 (2023) 1-13
   IF_{2022} = 5.57
2. Bettina Szerencsés, Attila Gácser, Gabriella Endre, Ildikó Domonkos, Hilda Tiricz, Csaba Vágvölgyi, János Szolomajer, **Dian H.O. Howan**, Gábor K. Tóth, Ilona Pfeiffer, Éva Kondorosi
   Symbiotic NCR Peptide Fragments Affect the Viability, Morphology and Biofilm Formation of Candida Species
   International Journal of Molecular Sciences, 22 (2021), 1-20
   IF_{2021} = 6.03
3. Rui M. Lima, Balaji B. Rathod, Hilda Tiricz, **Dian H.O. Howan**, Mohamad A. Al Bouni, Sándor Jenei, Edit Timár, Gabriella Endre, Gábor K. Tóth, Éva Kondorosi
   Legume Plant Peptides as Sources of Novel Antimicrobial Molecules Against Human Pathogens
   Frontiers in Molecular Biosciences, 9 (2022), 870460
   IF_{2022} = 5.0

   ΣIF = 16.6

Publication Unrelated to Dissertation

1. Attila Bajtai, István Ilisz, **Dian H.O. Howan**, Gábor K. Tóth, Gerhard K.E. Scriba, Wolfgang Lindner, Antal Péter
   Enantioselective Resolution of Biologically Active Dipeptide Analogs by High-Performance Liquid Chromatography Applying *Cinchona* Alkaloid-Based Ion-Exchange Chiral Stationary Phases
   Journal of Chromatography A, 1611 (2020), 460574
   IF_{2020} = 4.76

   Total IF = 21.36

Conference Participation Related to Dissertation

1. **Dian H.O. Howan**, Sándor Jenei, Mohamad Anas Al Bouni, Éva Kondorosi, Gábor K. Tóth
   Synthesis and Investigations of the Antimicrobial NCR169 Analogs
   Peptidkémiai Munkabizottság Tudományos Ülése, 11-13 October 2021, Hungary (oral presentation)

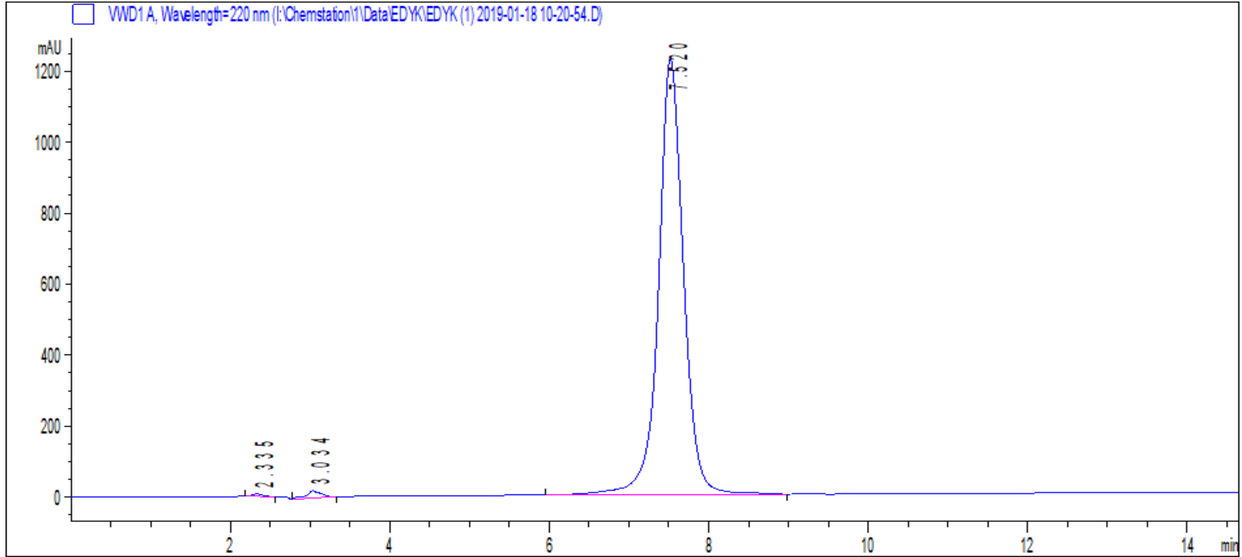
2. János Szolomajer, Bettina Szerencsés, **Dian H.O. Howan**, Ilona Pfeiffer, Éva Kondorosi, Gábor K. Tóth
   Növényi Eredetű Peptidek Antifungális Hatása
   Peptidkémiai Munkabizottság Tudományos Ülése, 11-13 October 2021, Hungary (oral presentation)

Appendix 1

HPLC Chromatograms and MS Spectra of NCR169 and Its Derivatives

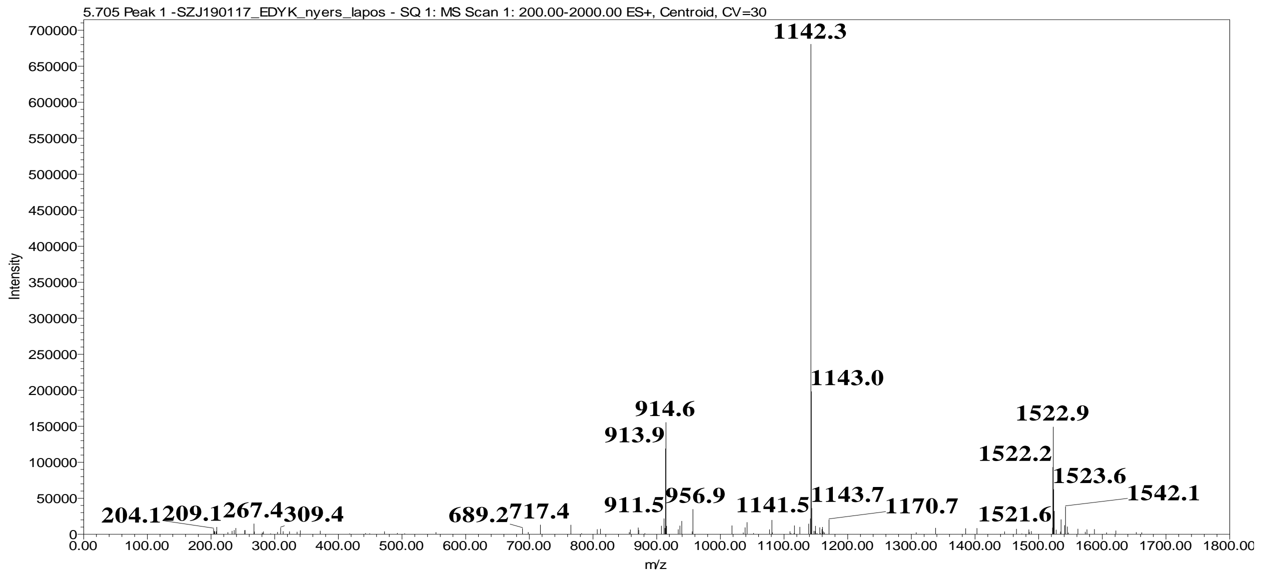
NCR169

HPLC



Purity : 98.8%

MS



NCR169-StrepII

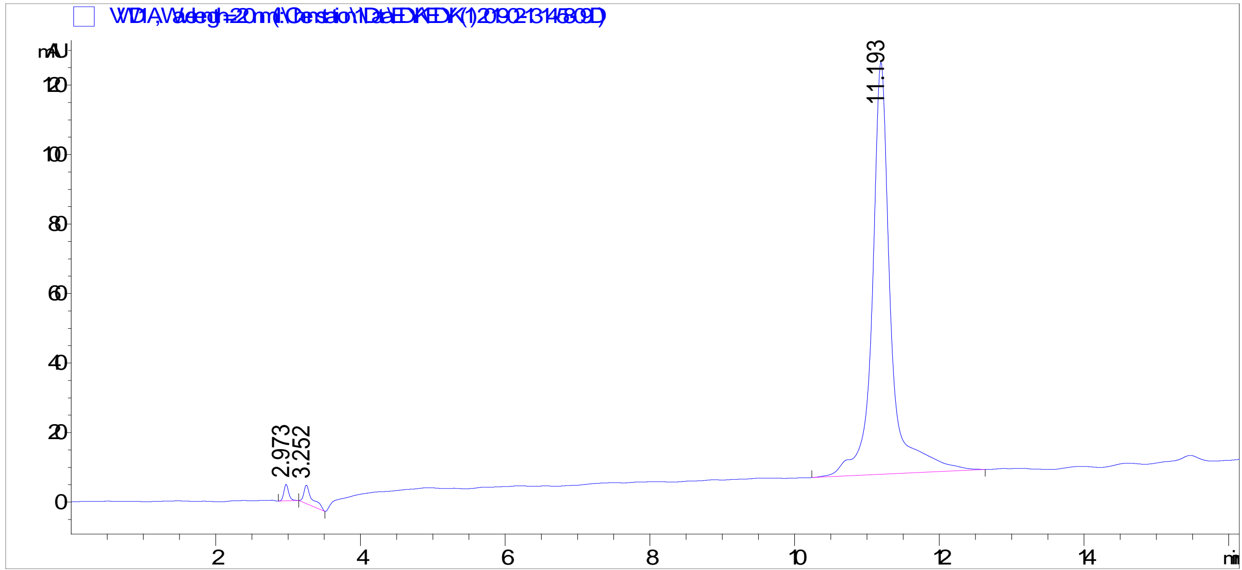
HPLC

Purity: 99.9%

MS

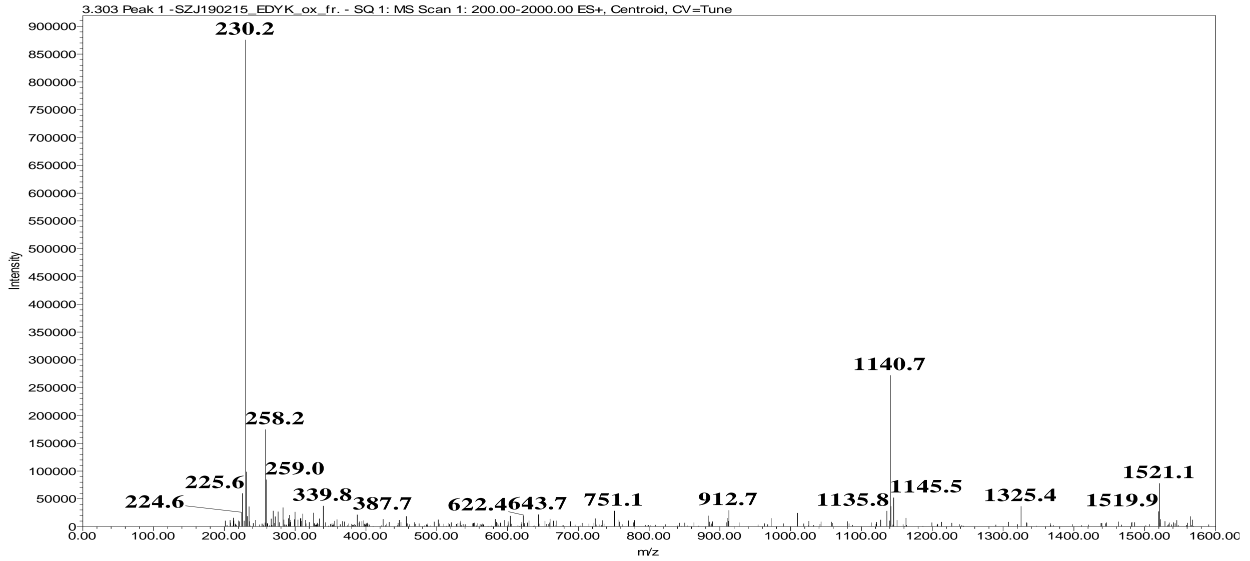
NCR169ox

HPLC



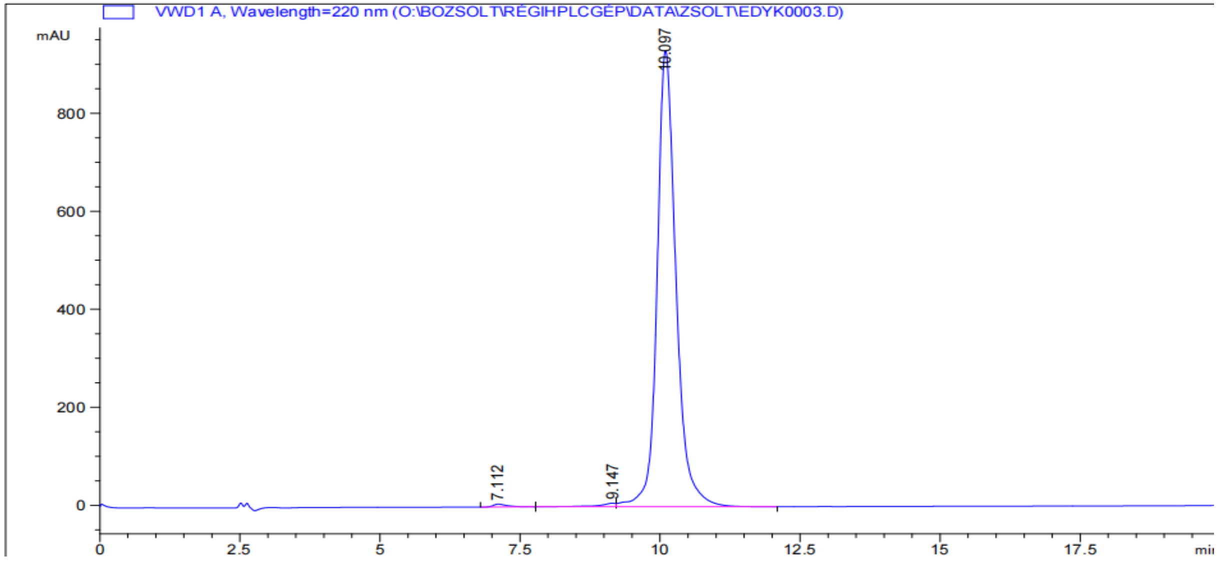
Purity: 97.02%

MS



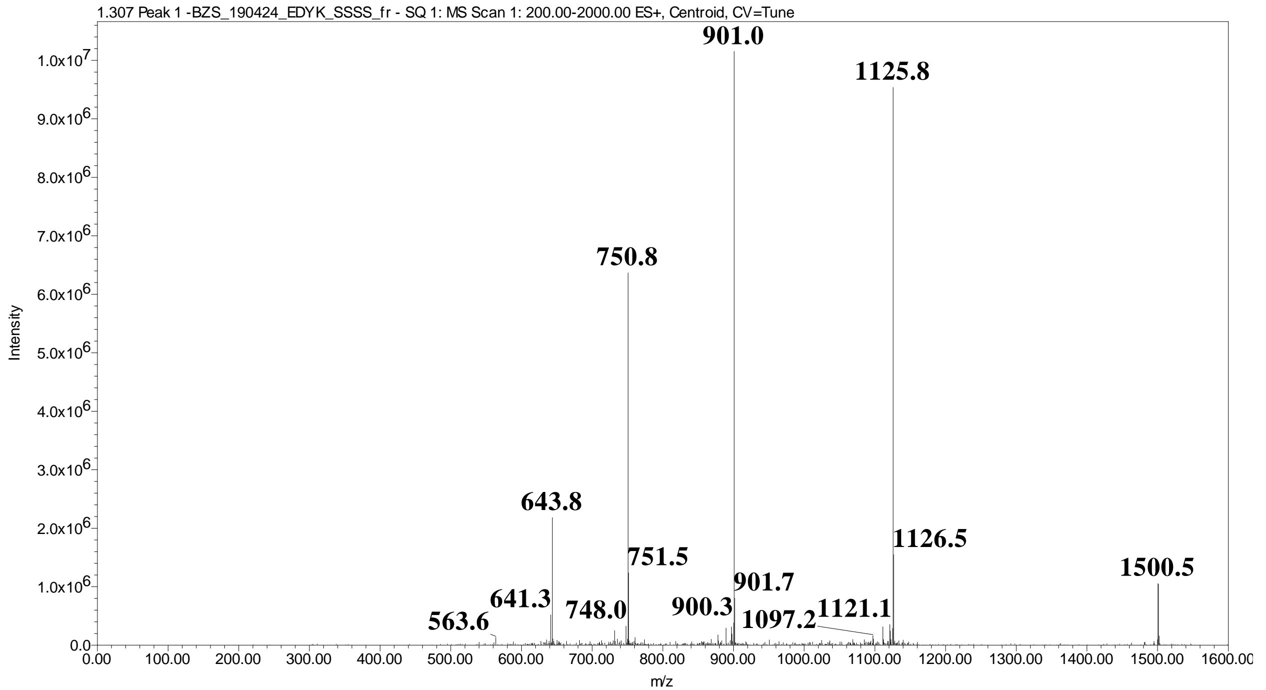
NCR169C$_{9,15,28,33}$/S

HPLC



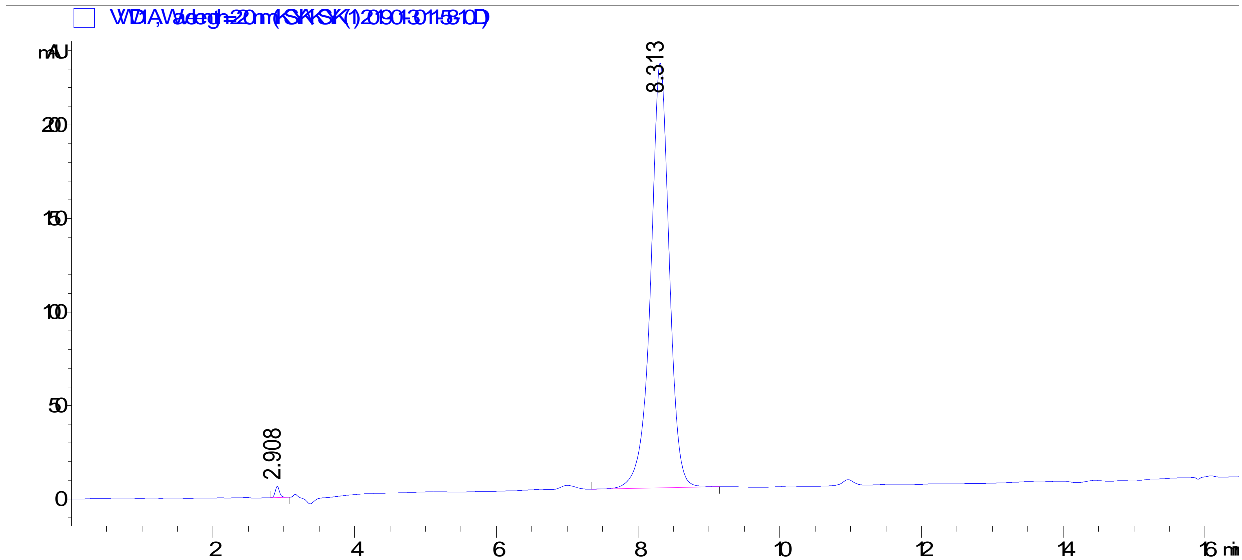
Purity : 99%

MS



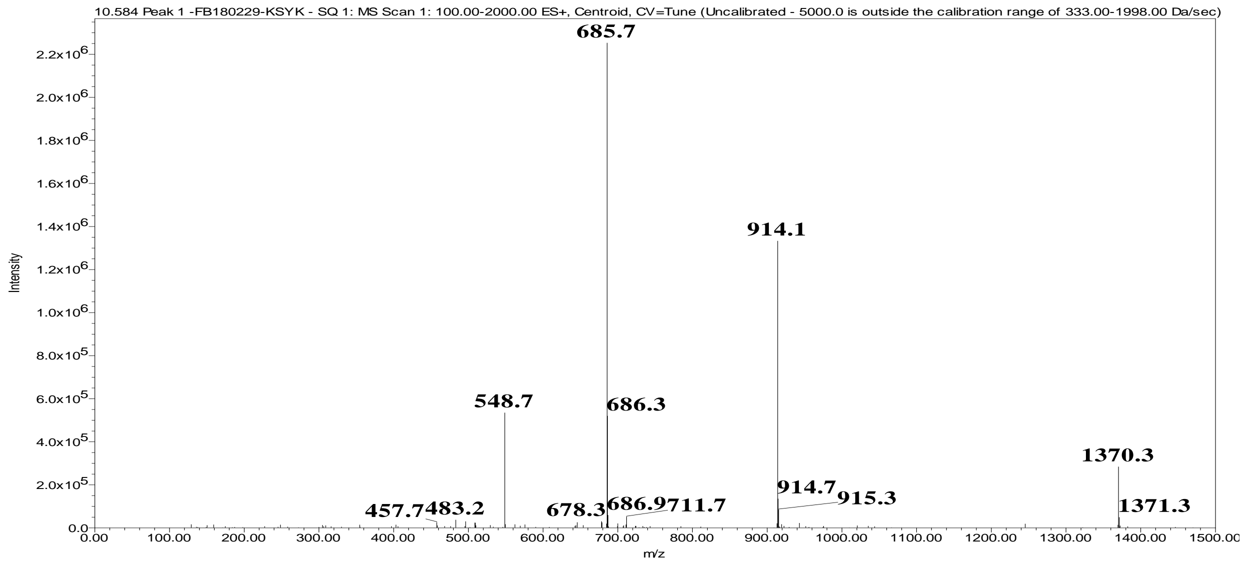
NCR169C$_{17-38}$

HPLC



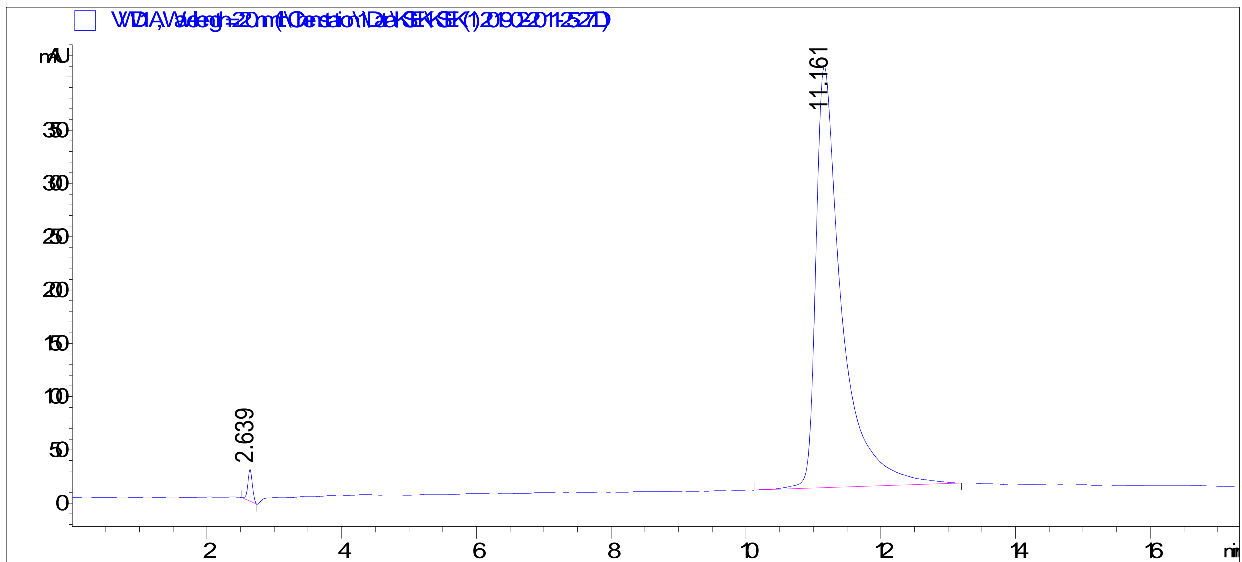
Purity: 99.4%

MS



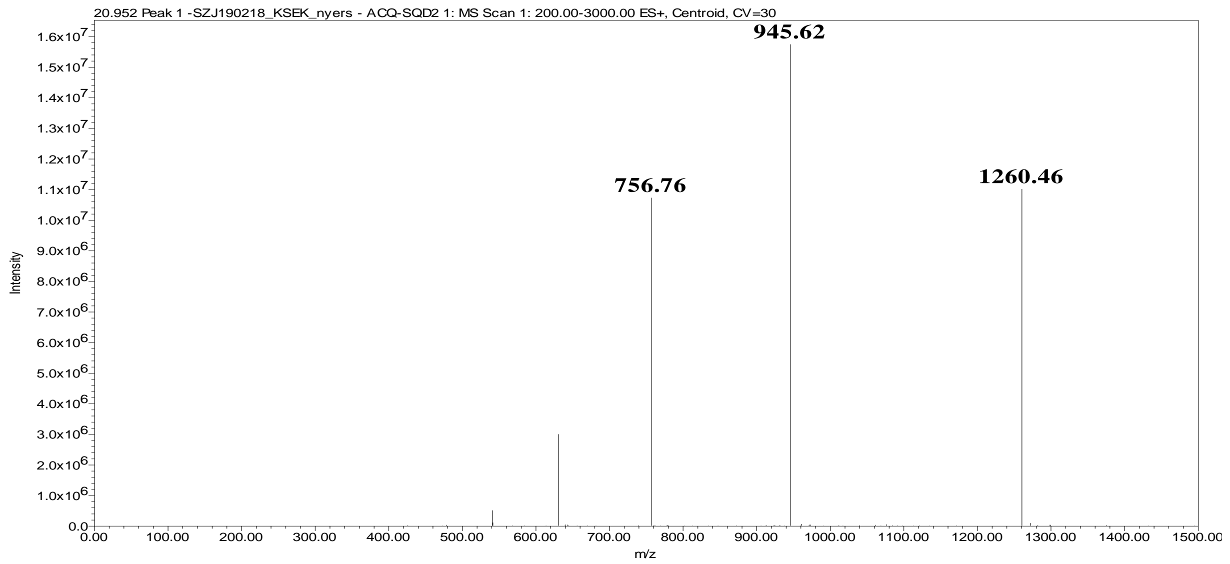
NCR169C-StrepII

HPLC



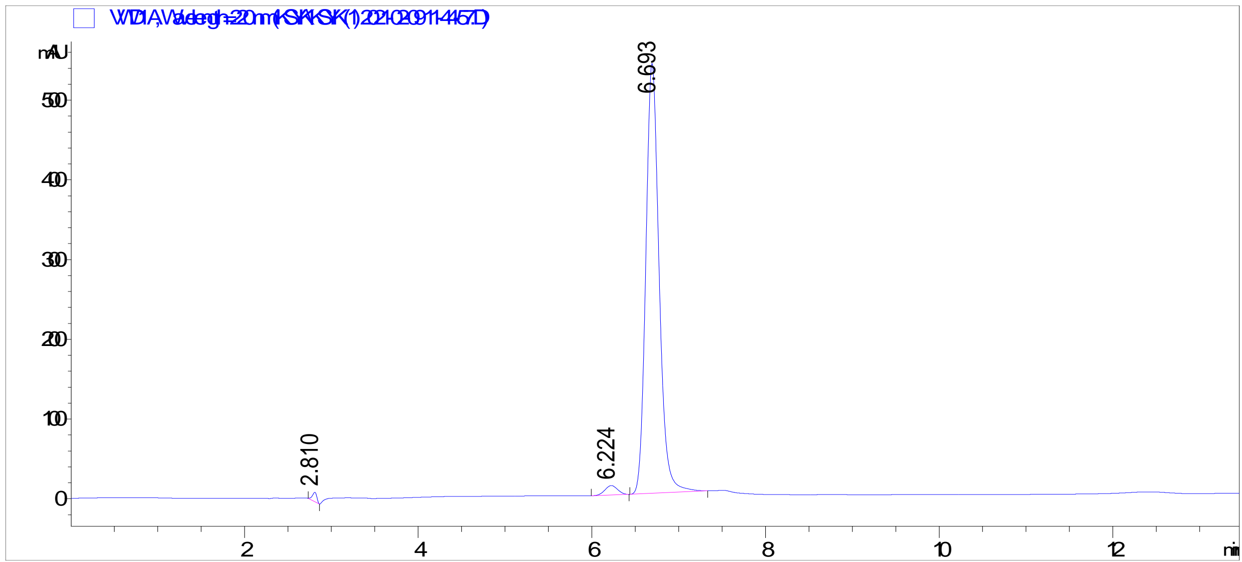
Purity : 98.7%

MS



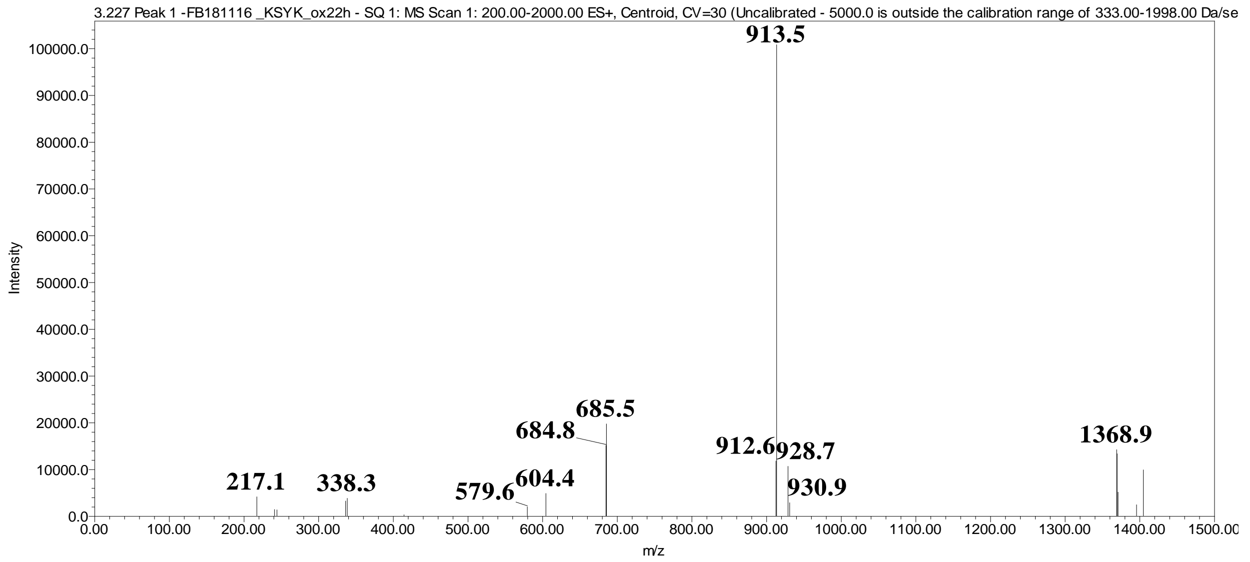
NCR169C$_{17–38}$ ox

HPLC



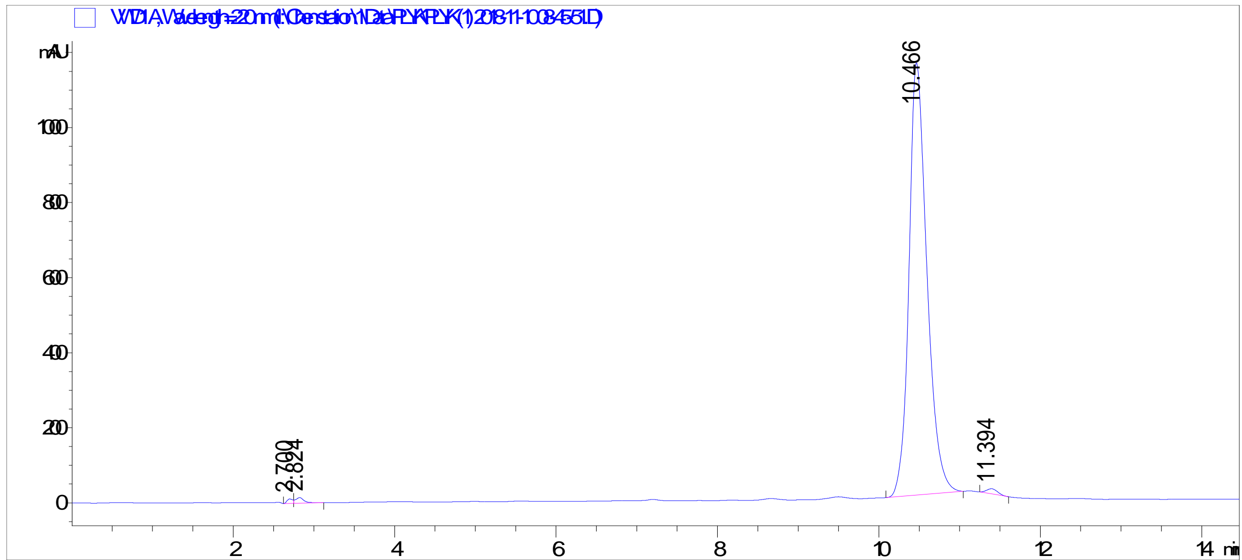
Purity : 97.24%

MS



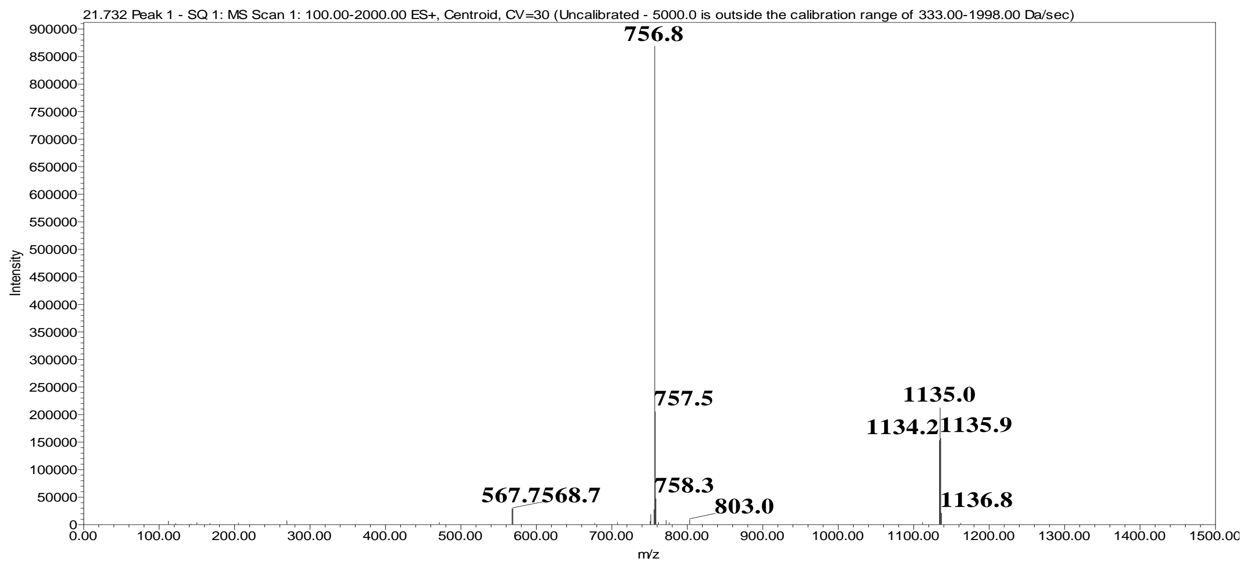
$NCR169_{21-38}$

HPLC



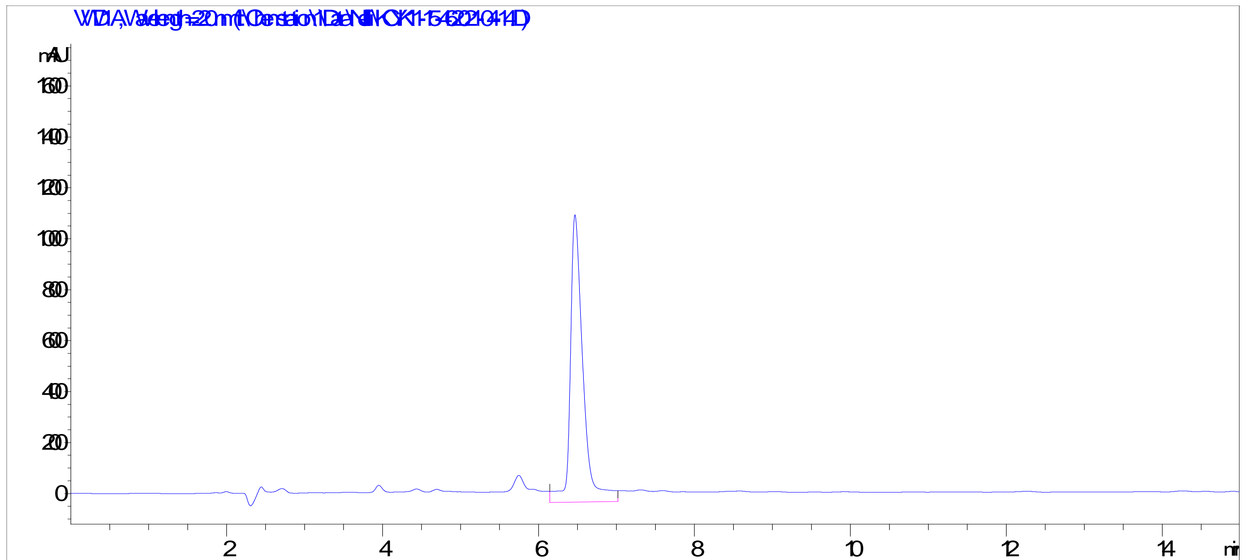
Purity : 99.3%

MS



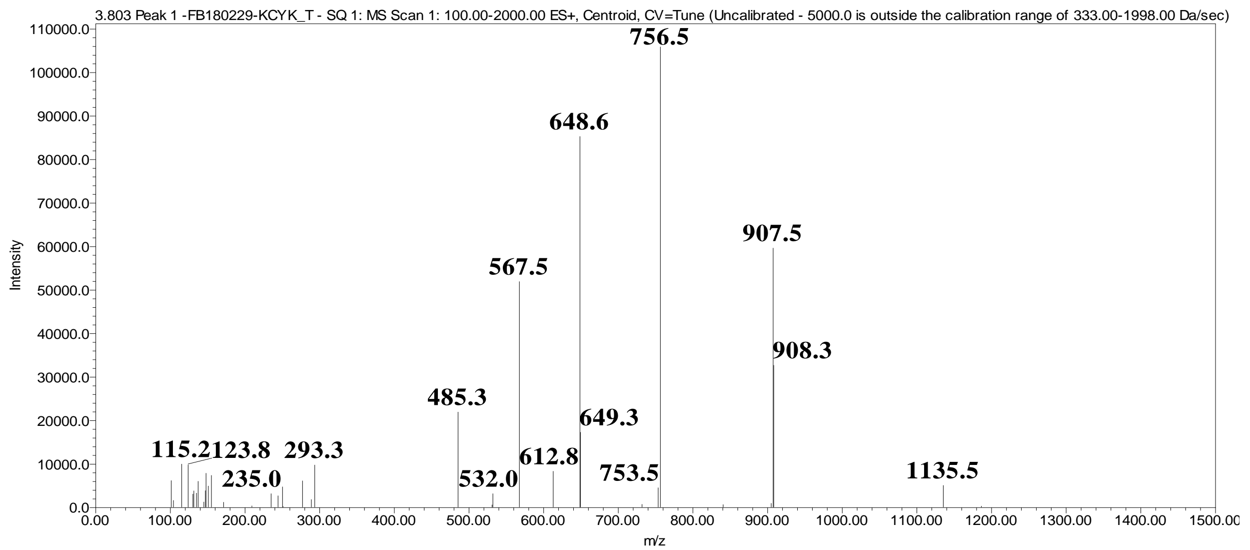
$NCR169_{27\text{-}38}$

HPLC



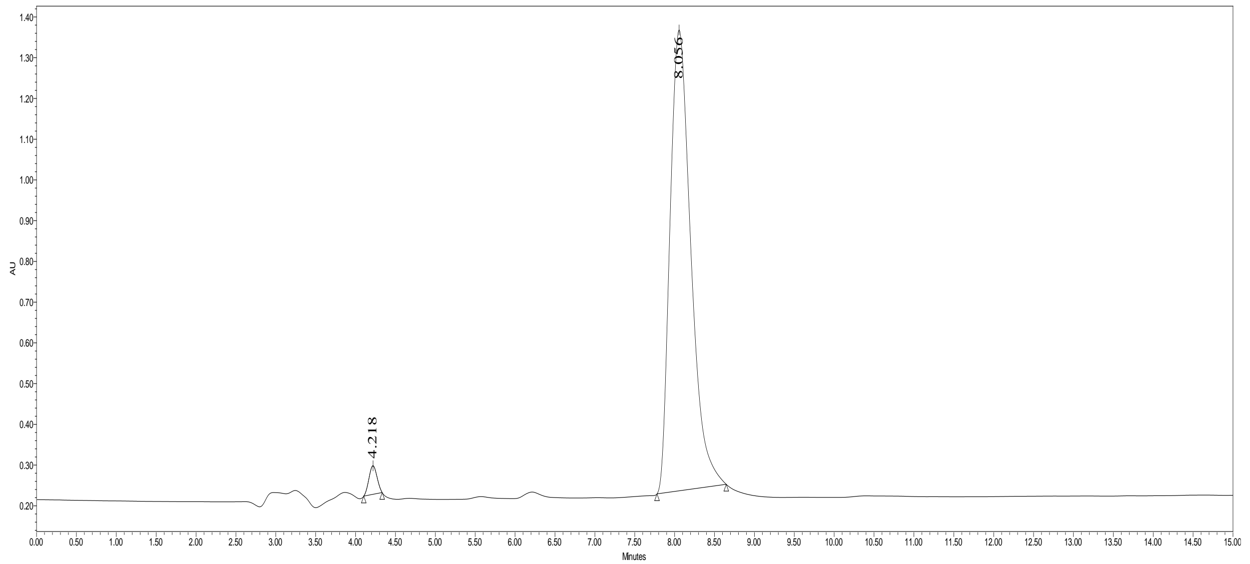
Purity : 96.4%

MS



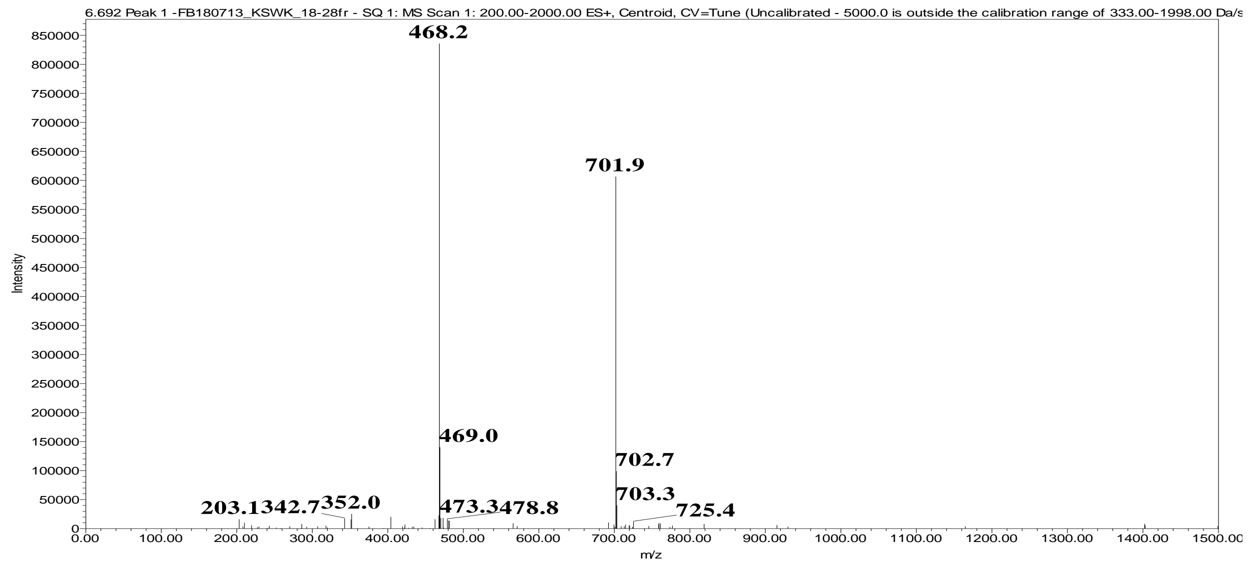
NCR169$_{17\text{-}28}$

HPLC



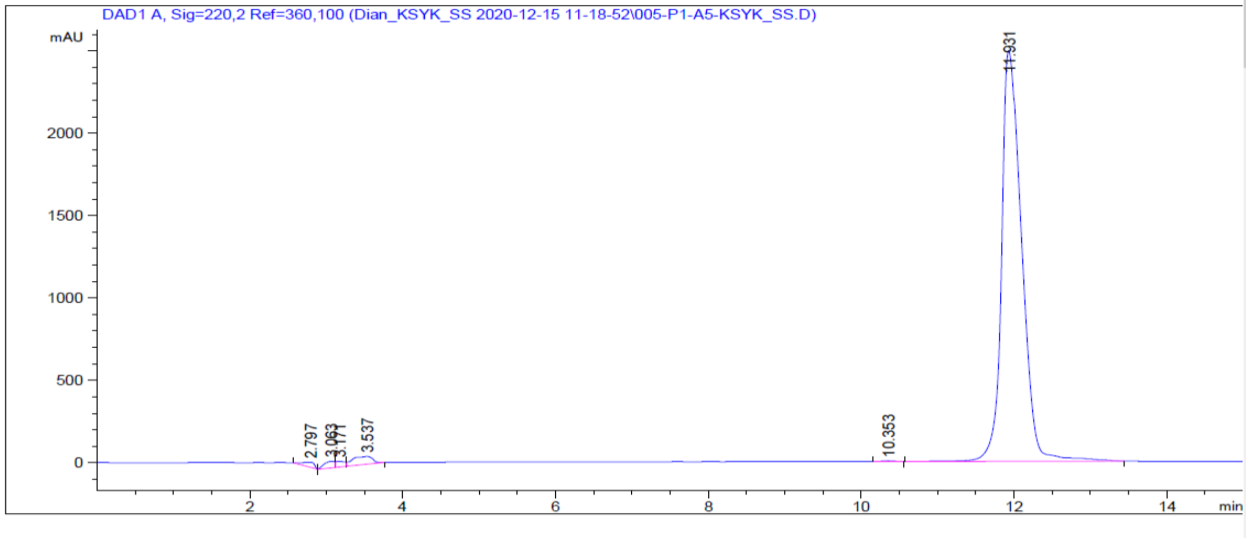
Purity: 97.7%

MS



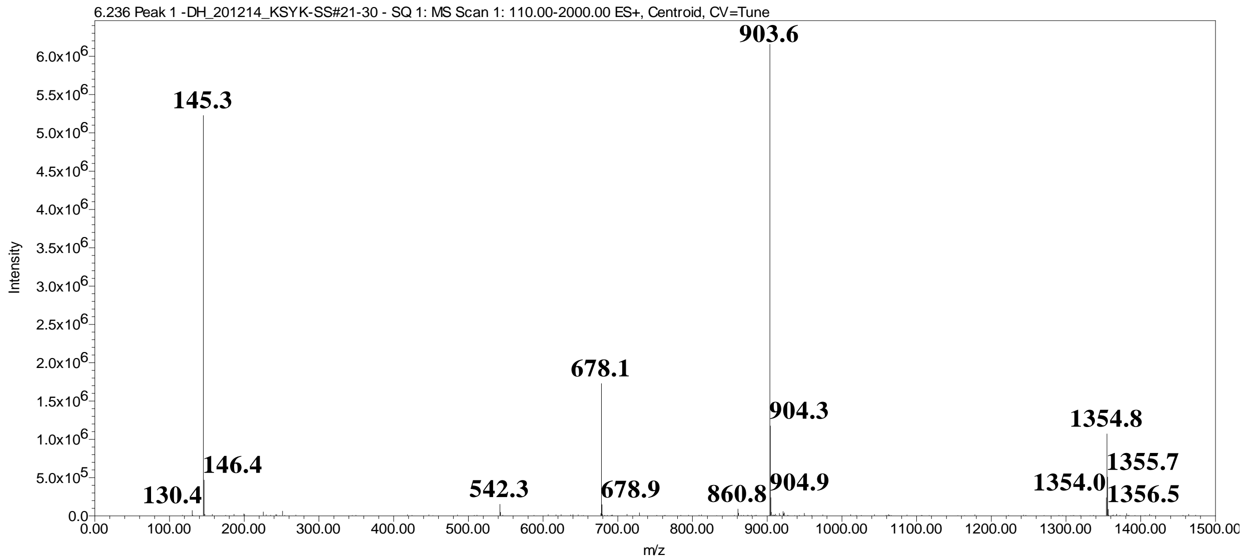
NCR169$C_{17–38}C_{12,17}$/S

HPLC



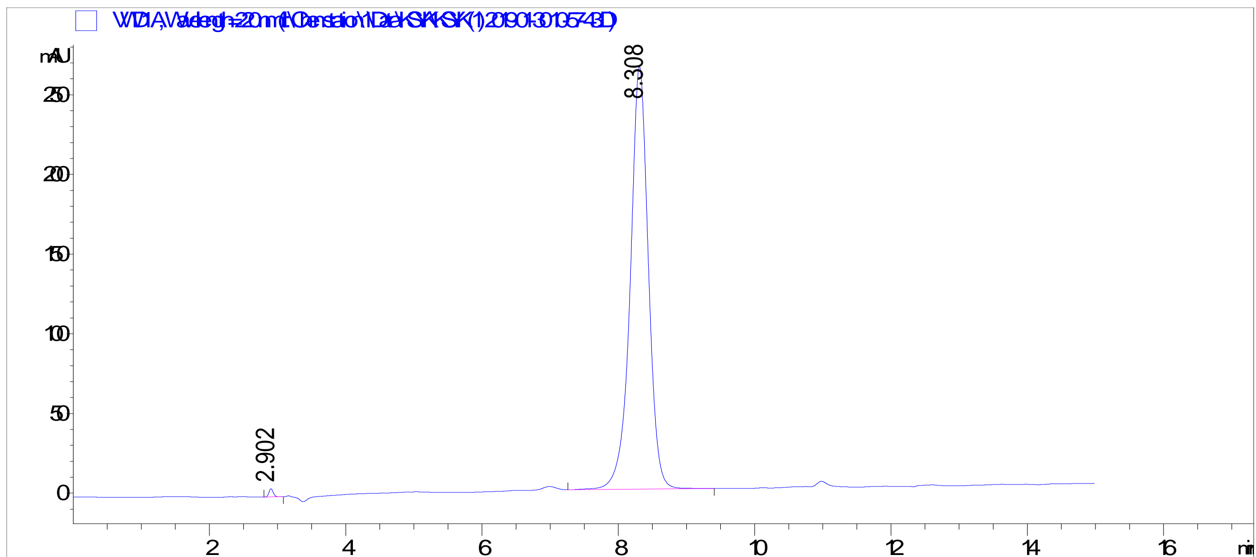
Purity : 95.9%

MS



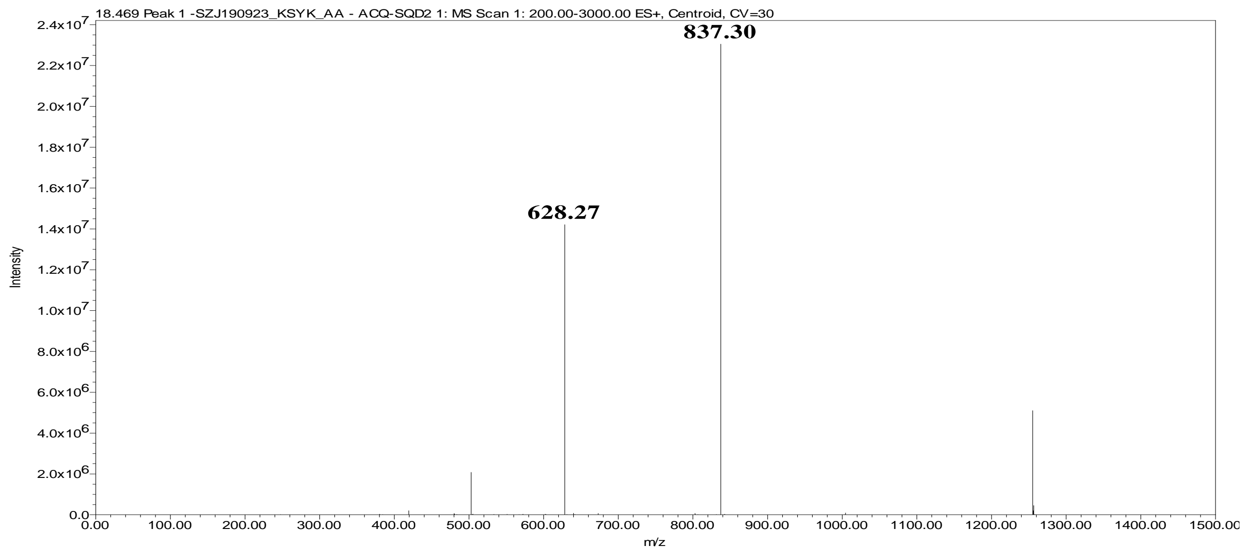
NCR169$C_{17-38}W_{10,20}$/A

HPLC



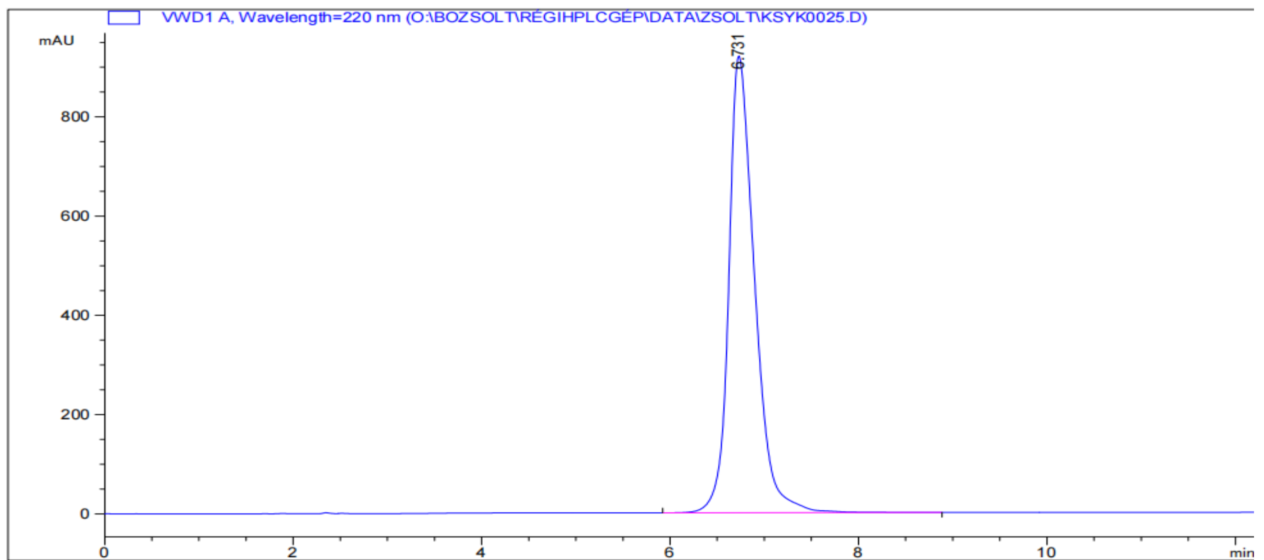
Purity : 99.5%

MS



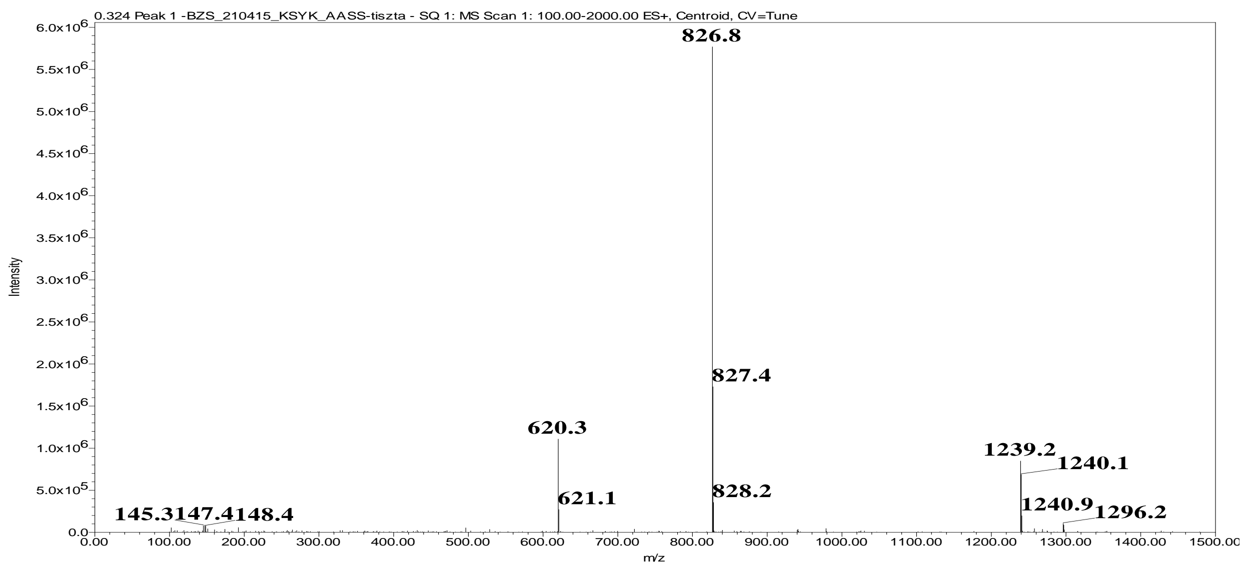
NCR169C$_{17–38}$W$_{10,20}$/A, C$_{12,17}$/S

HPLC



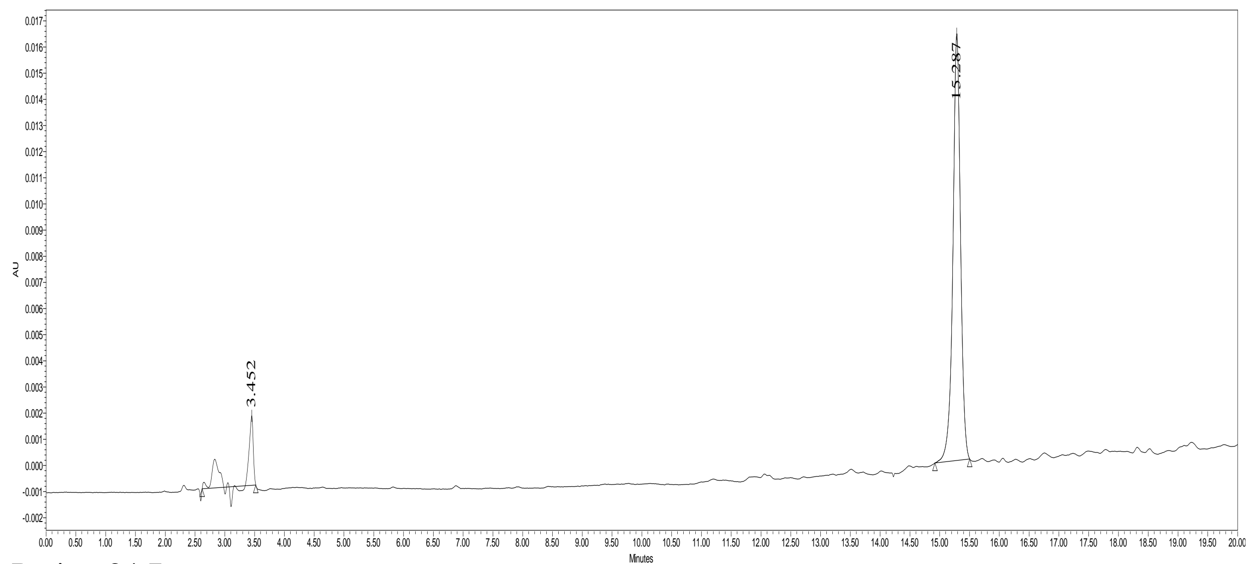
Purity: 100%

MS



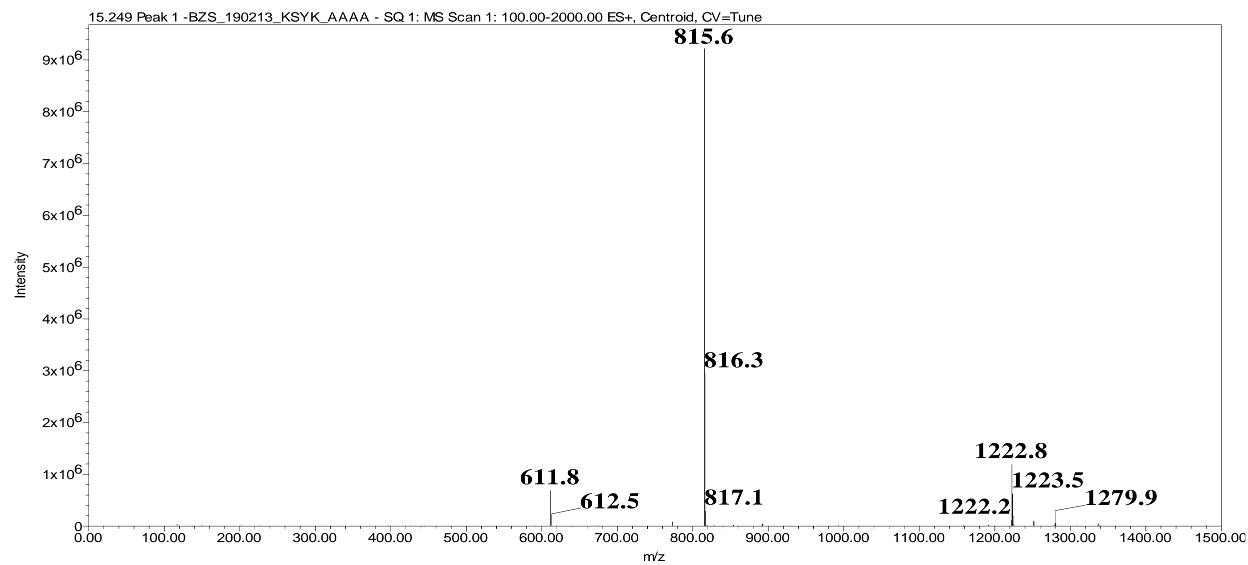
$NCR169C_{17–38}W_{10,20}C_{12,17}/A$

HPLC



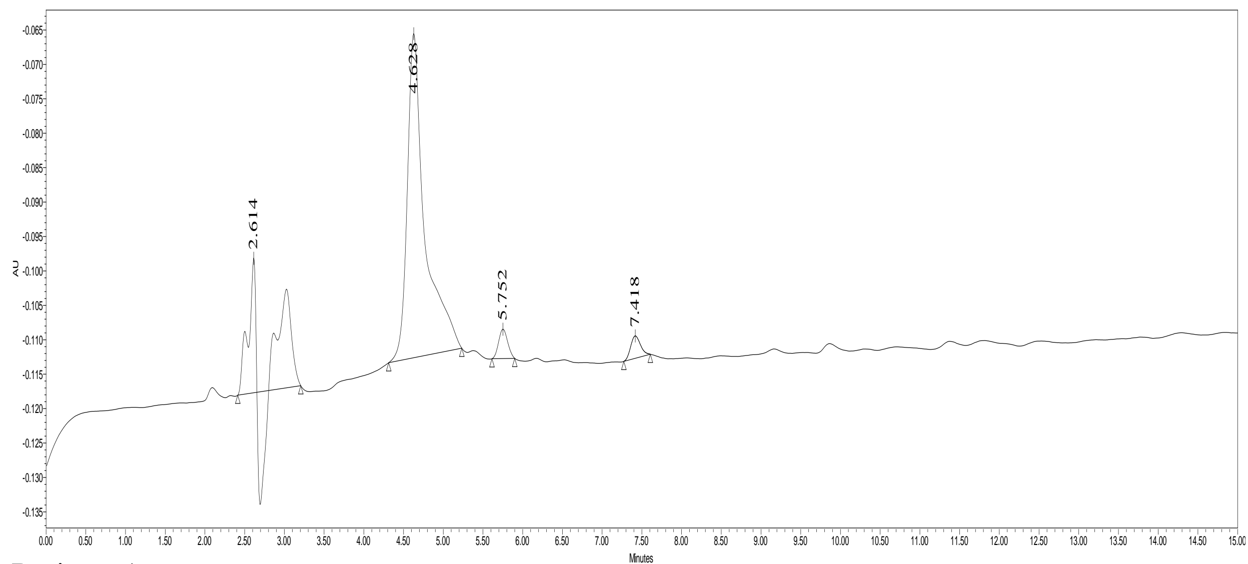
Purity: 84.7%

MS



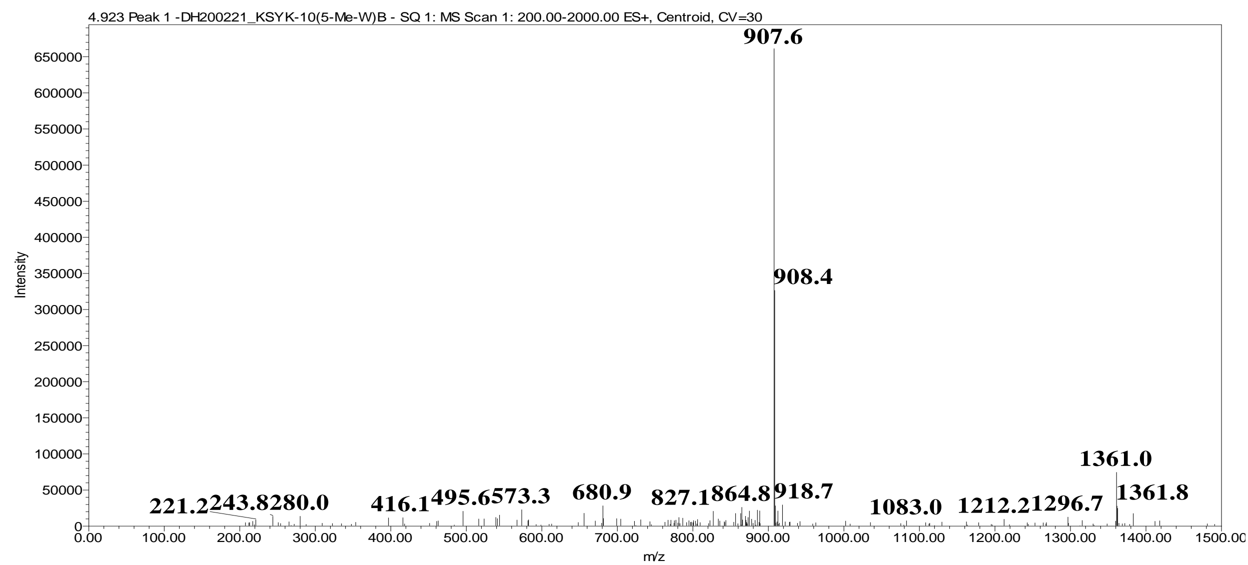
NCR169$C_{17\text{–}38}C_{12,17}$/S-10$W^{5\text{-}Me}$ I

HPLC



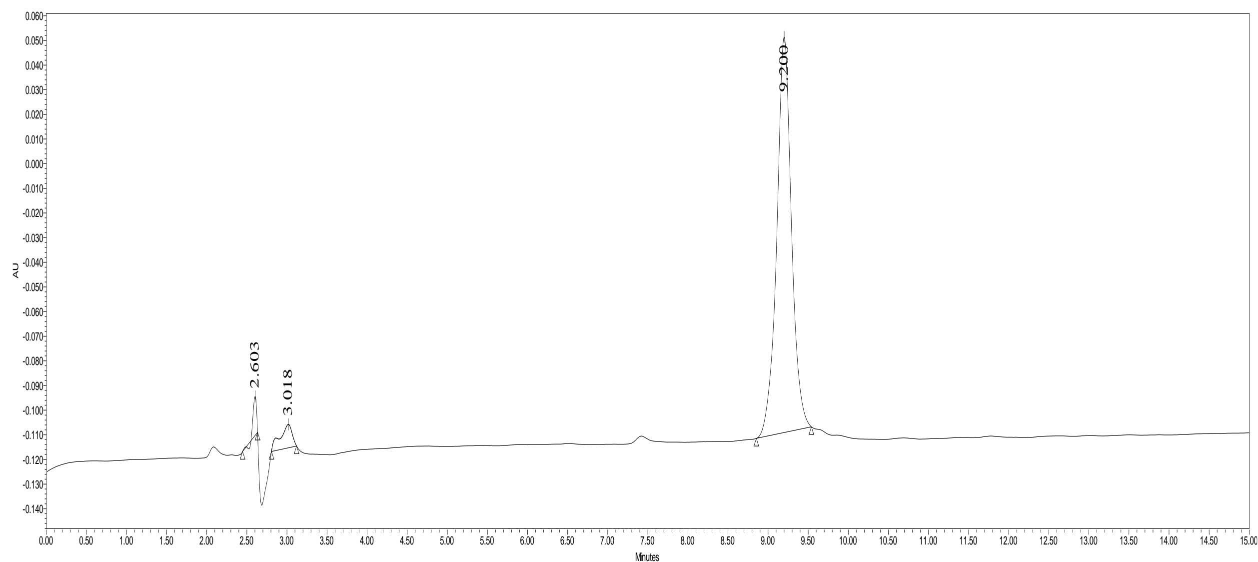
Purity: 61.6 %

MS



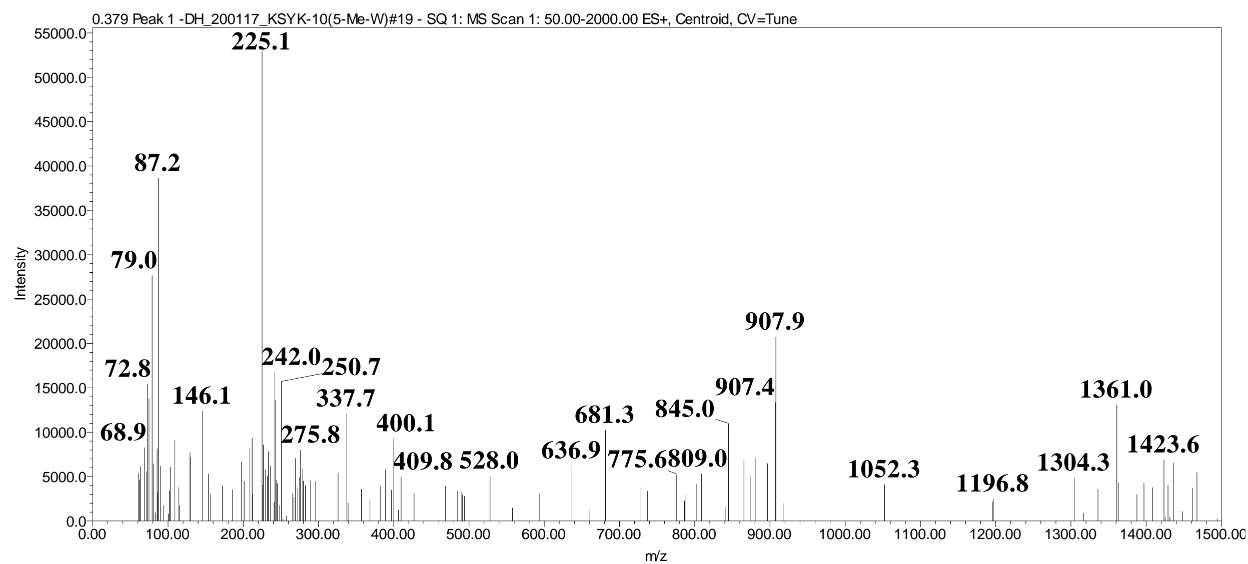
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-}Me}$ II

HPLC



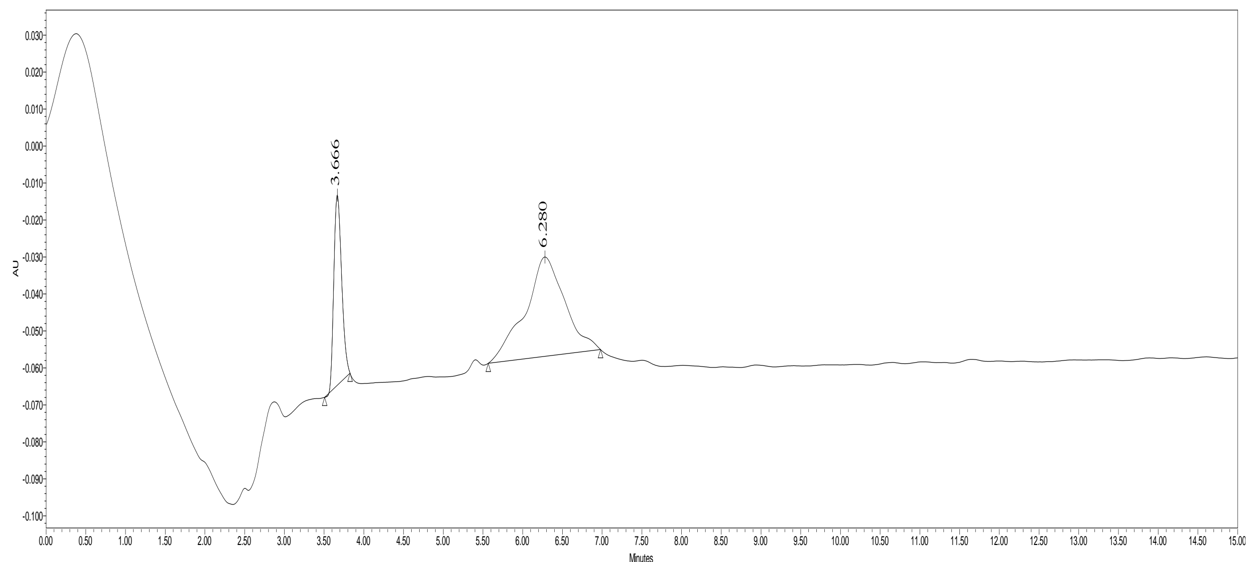
Purity: 93.0%

MS



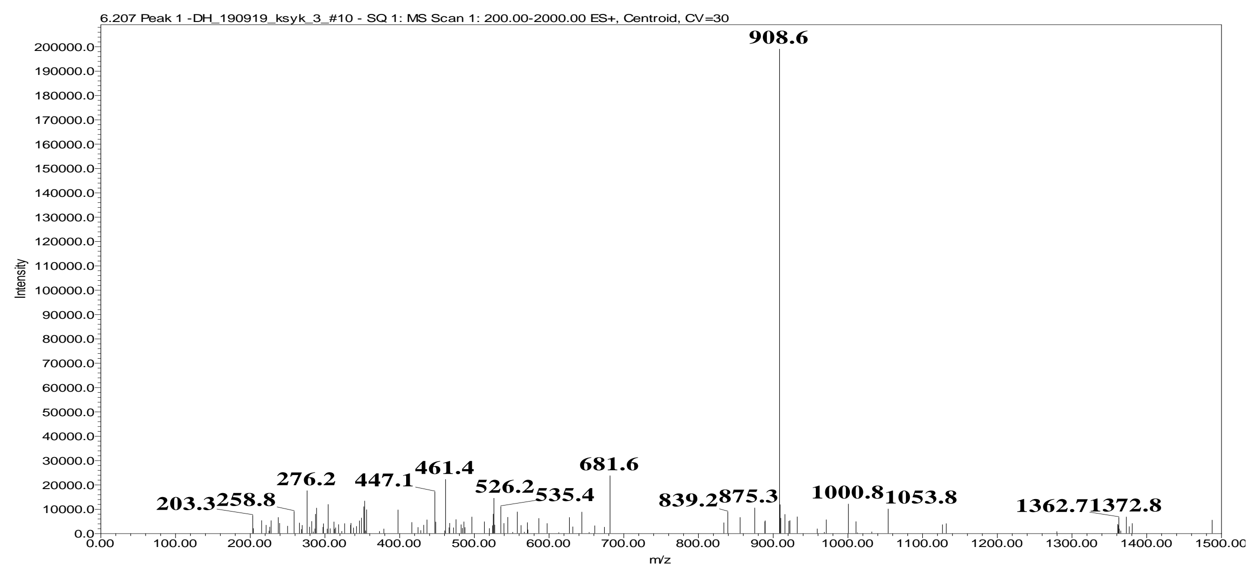
NCR169$C_{17\text{–}38}C_{12,17}$/S-20$W^{5\text{-Me}}$ I

HPLC



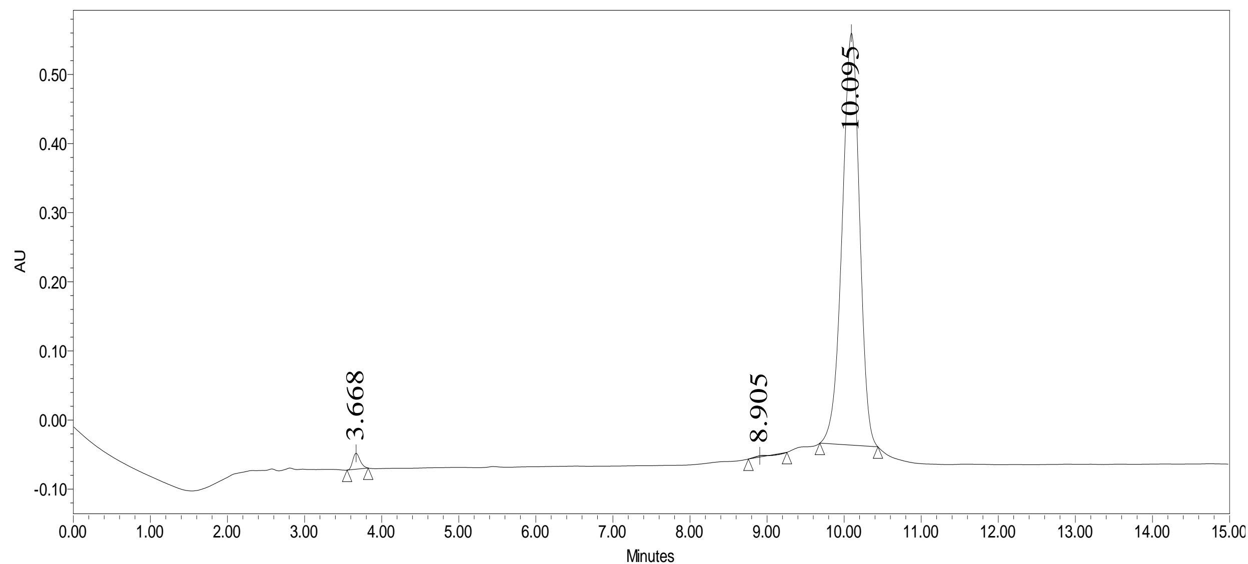
Purity: 67.2%

MS



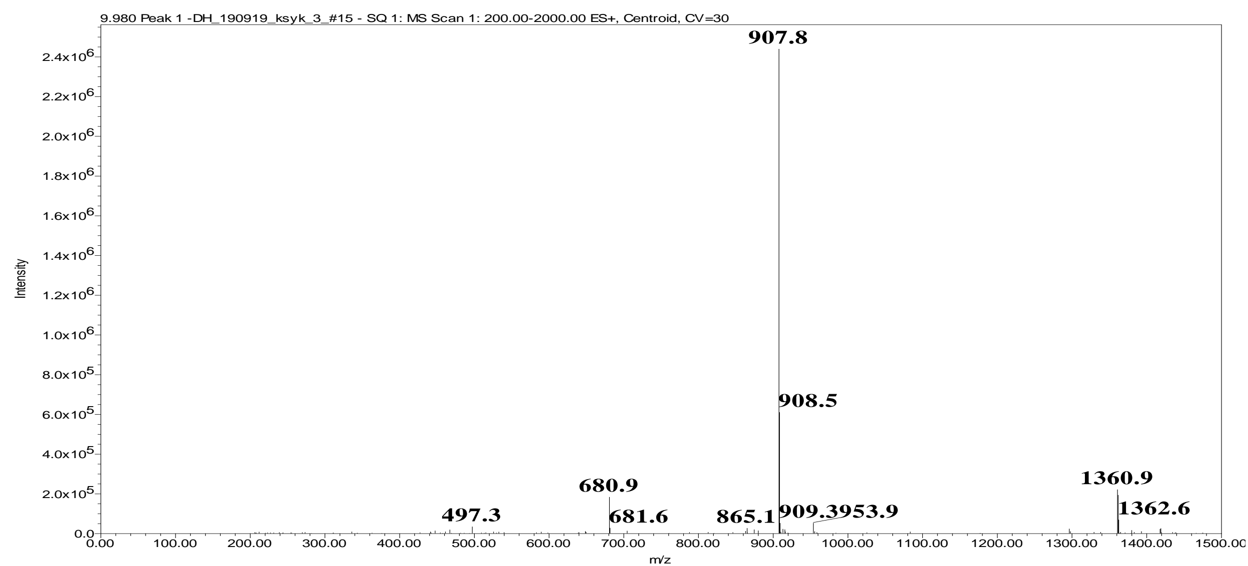
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{5\text{-}Me}$ II

HPLC



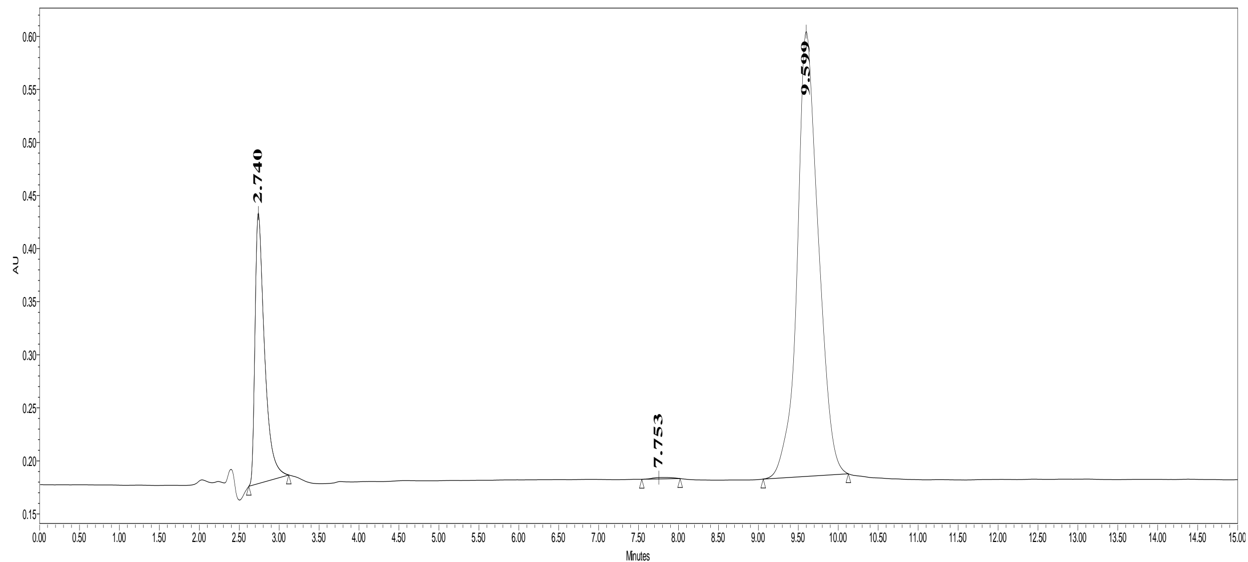
Purity: 98.5%

MS



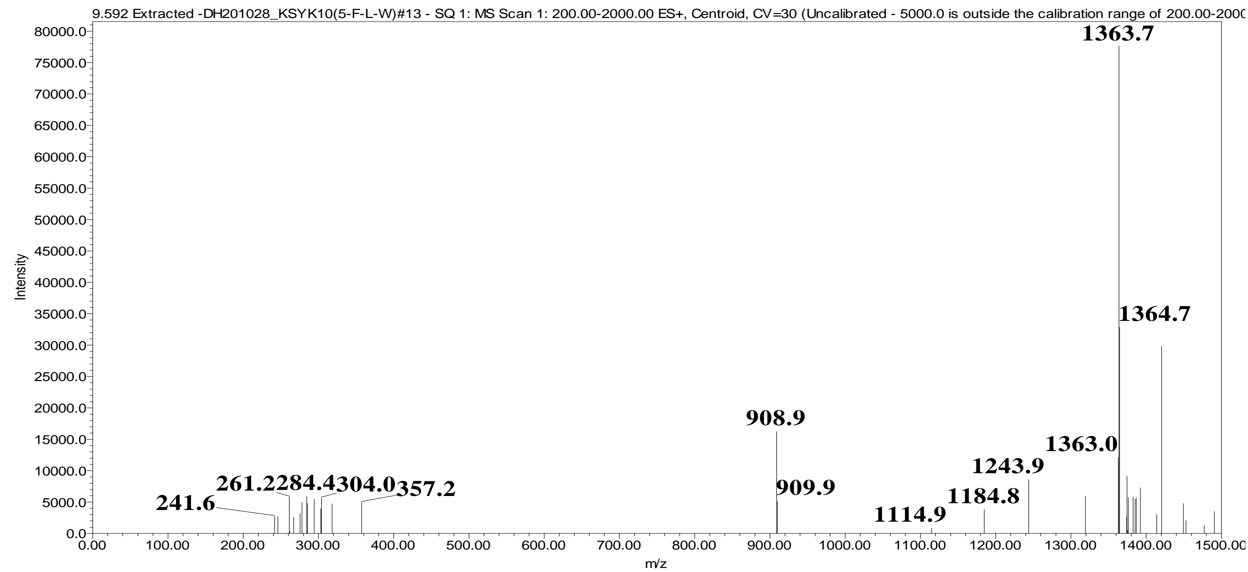
NCR169$C_{17-38}C_{12,17}$/S-10$W^{5\text{-}F\text{-}L}$

HPLC



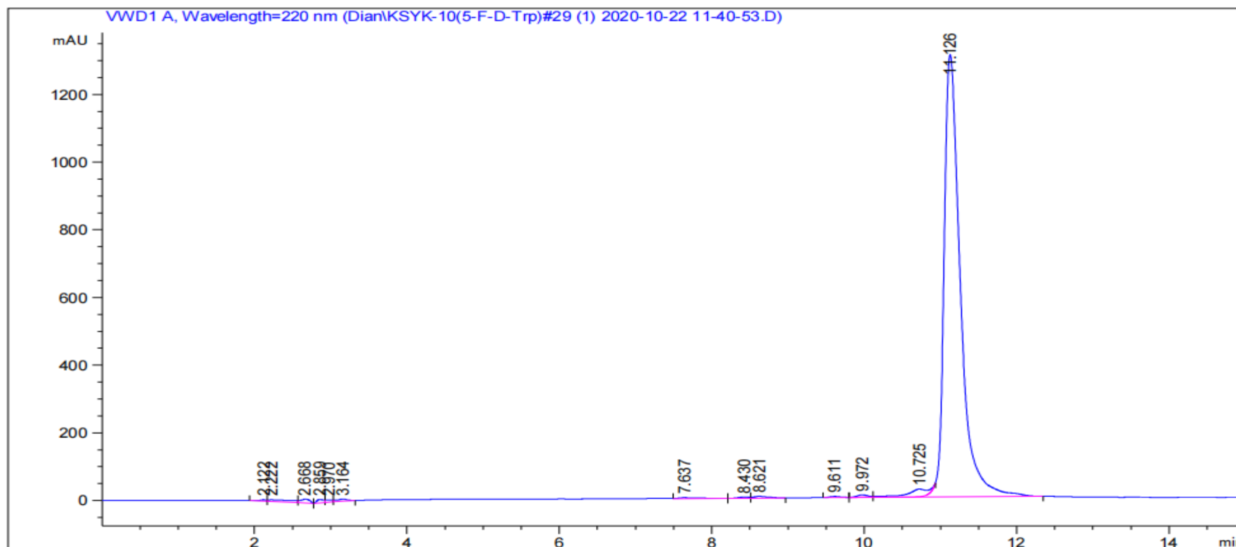
Purity : 93.4%

MS



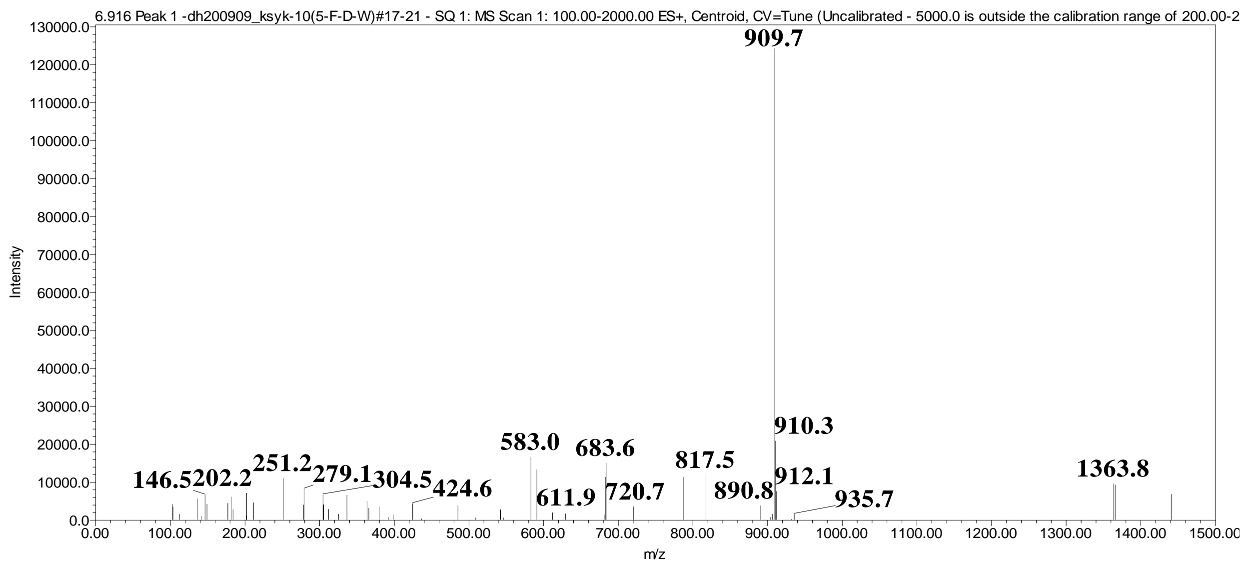
NCR169$C_{17-38}C_{12,17}$/S-10$W^{5\text{-F-D}}$

HPLC



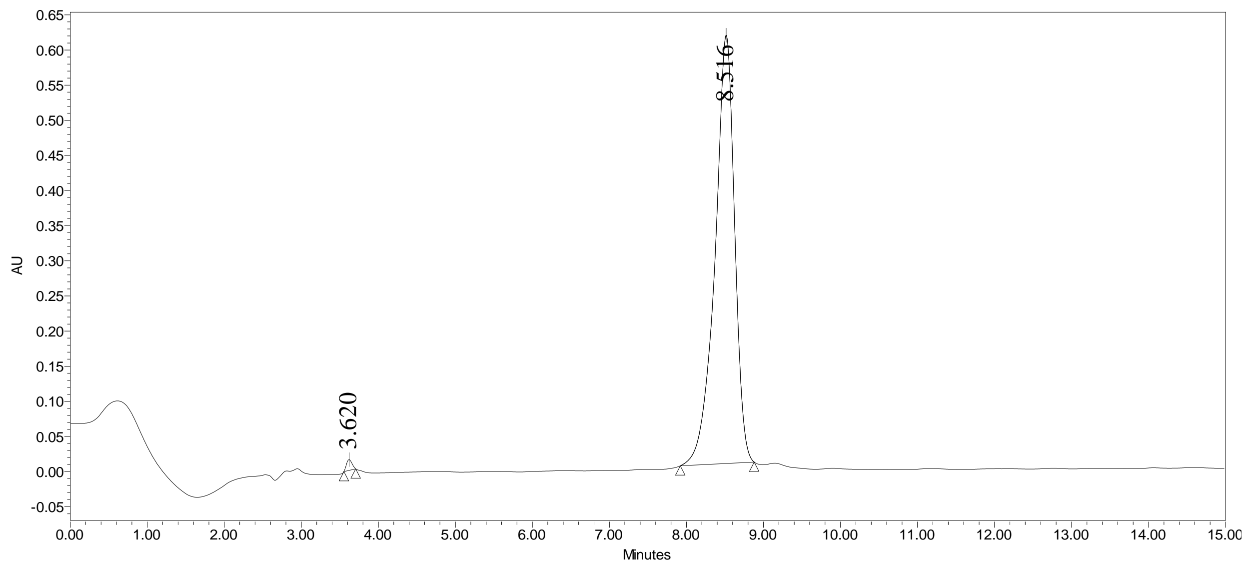
Purity: 94.6%

MS



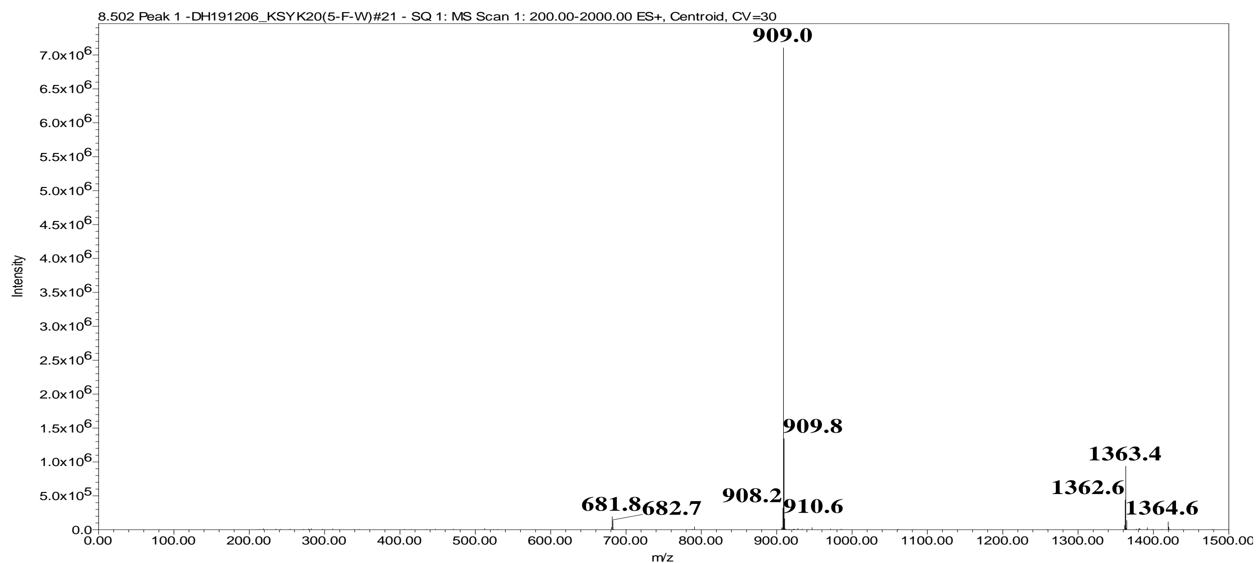
NCR169$C_{17\text{-}38}C_{12,17}$/S-20$W^{5\text{-}F\text{-}L}$

HPLC



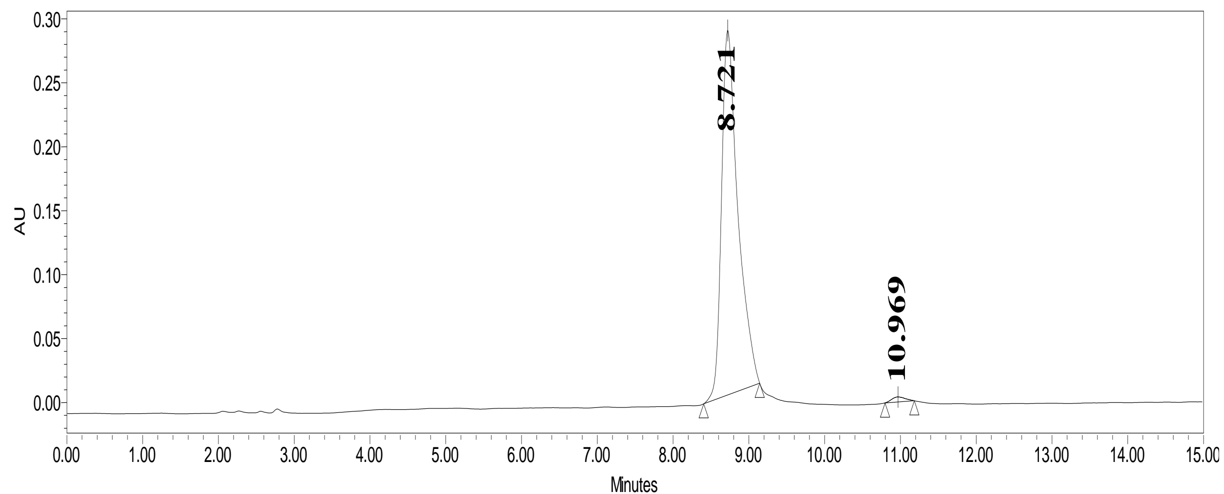
Purity: 99.3%

MS



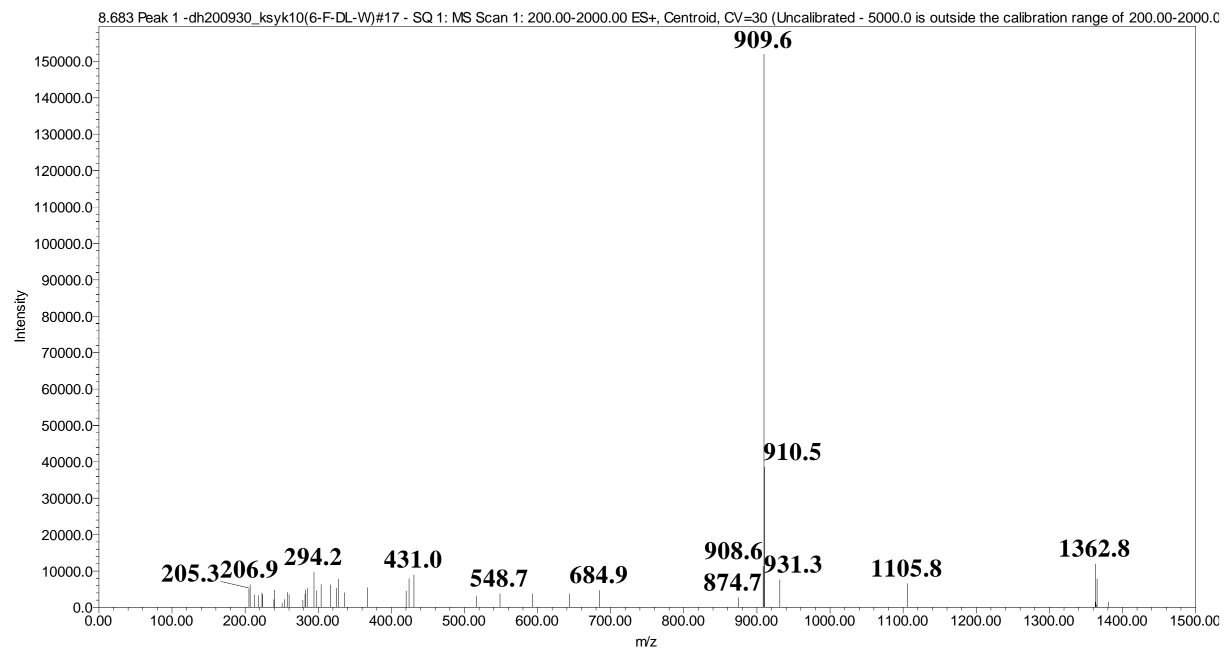
NCR169$C_{17\text{-}38}C_{12,17}$/S-10$W^{6\text{-}F}$ I

HPLC



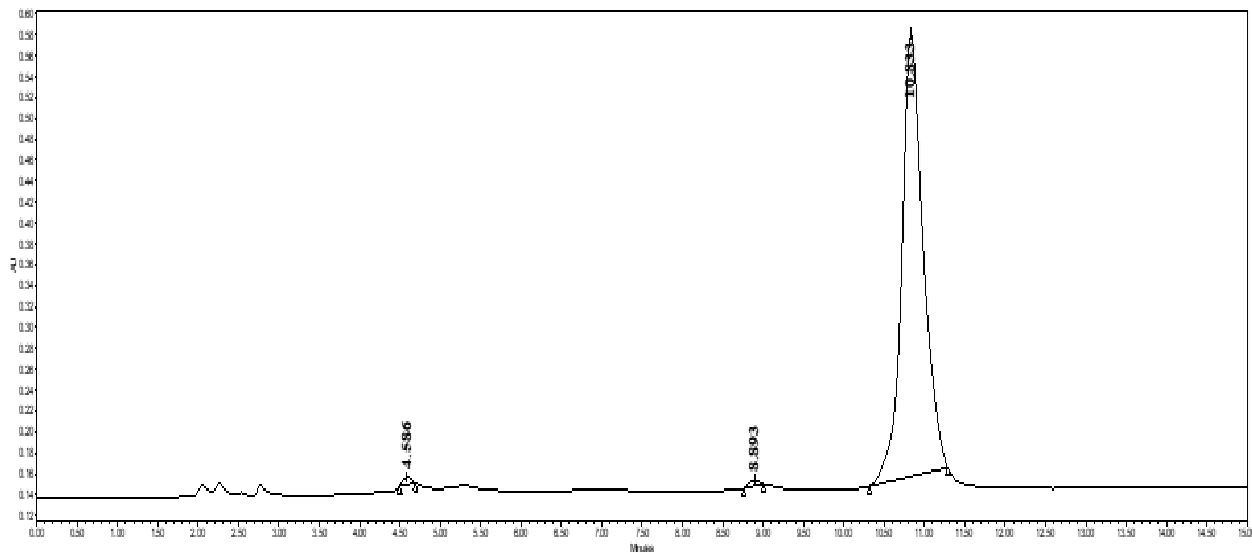
Purity : 98.89%

MS



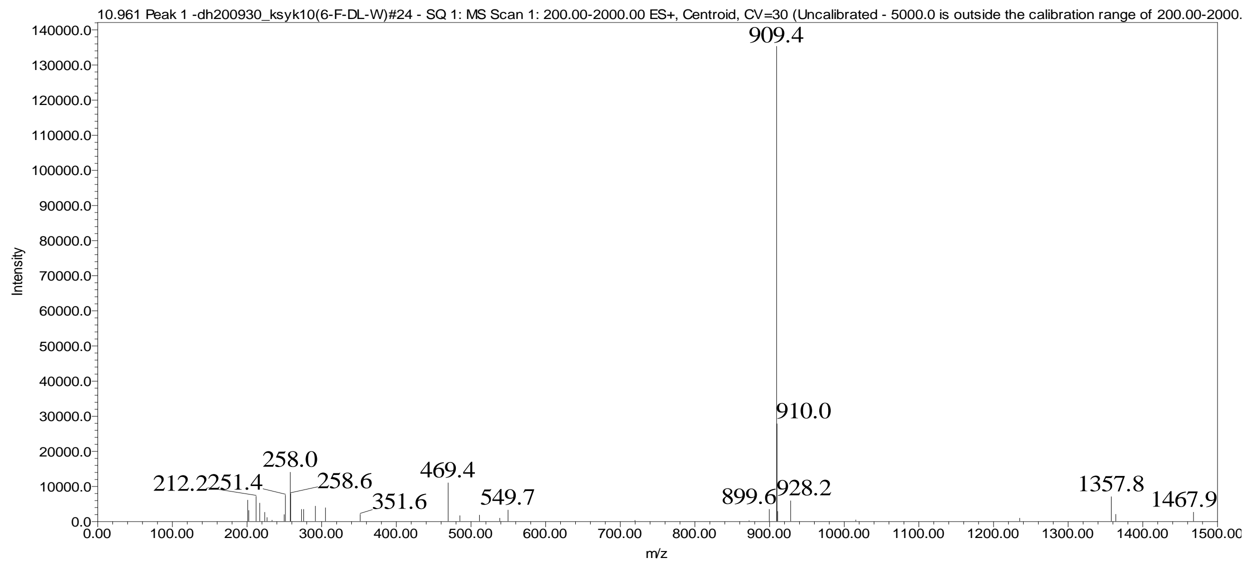
NCR169$C_{17-38}C_{12,17}$/S-10W^{6-F} II

HPLC



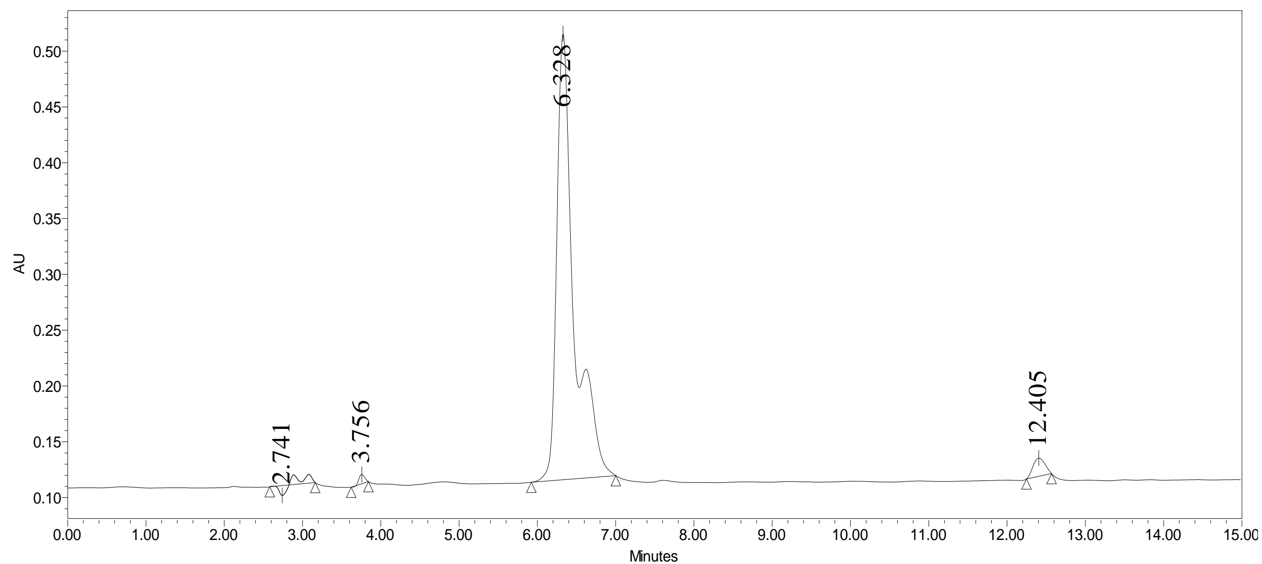
Purity : 98.77%

MS



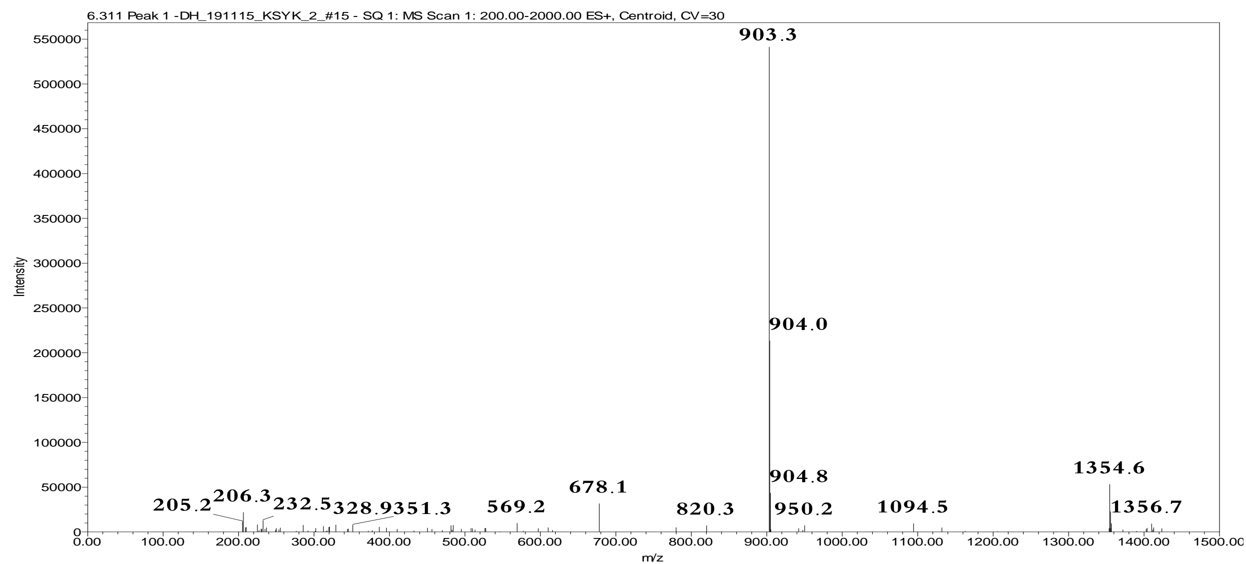
NCR169$C_{17–38}C_{12,17}$/S-10$W^{7\text{-Aza}}$ I

HPLC



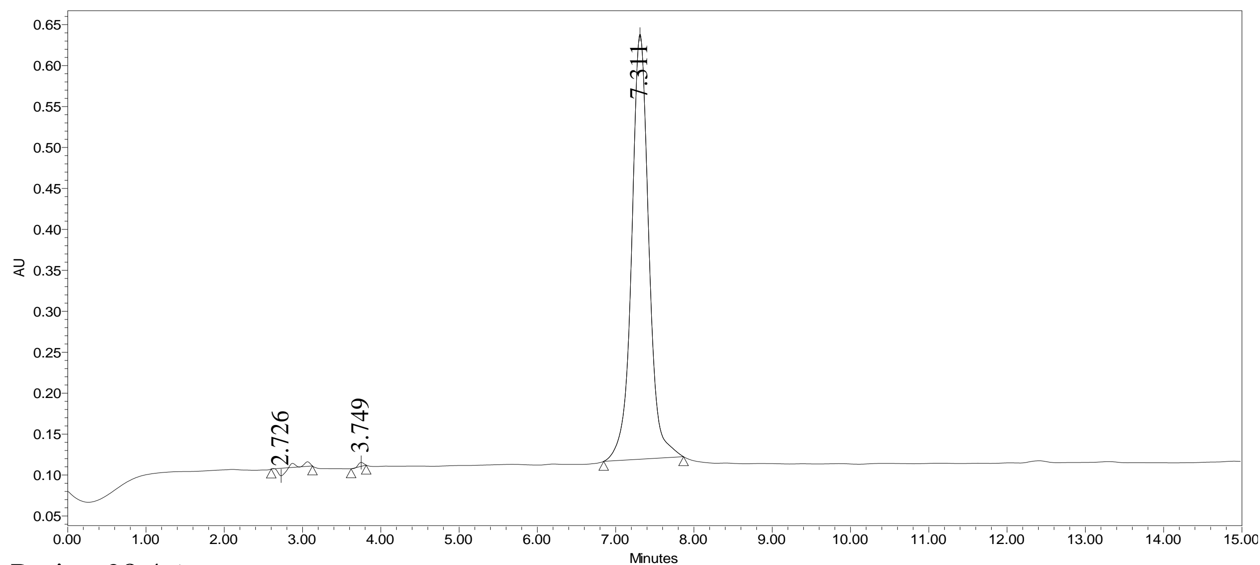
Purity: 94.4%

MS



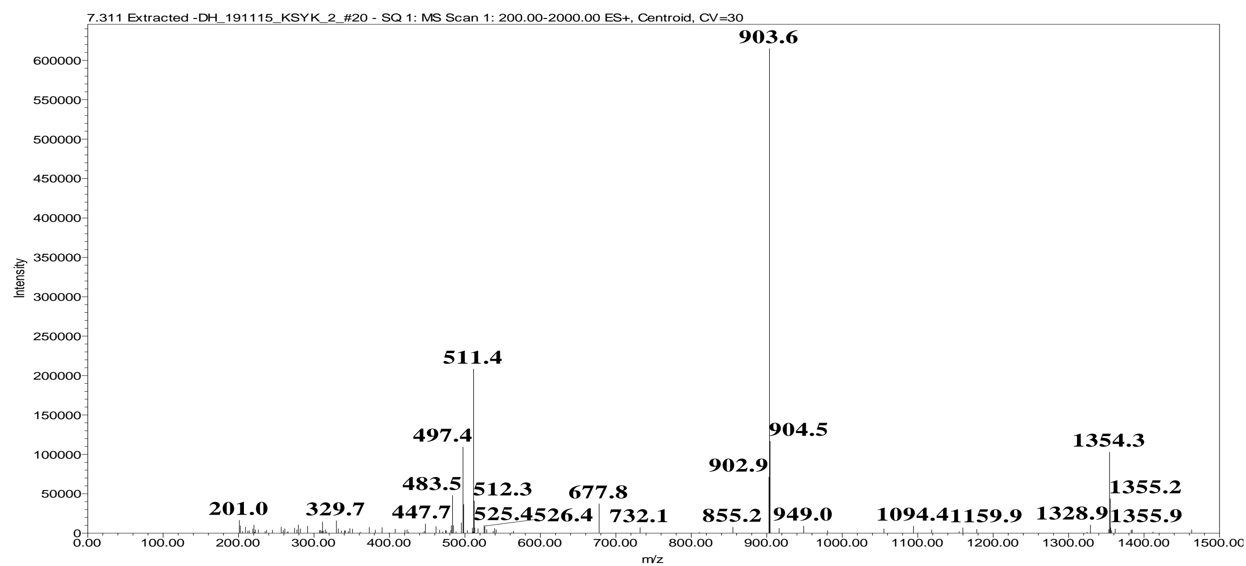
NCR169$C_{17–38}C_{12,17}$/S-10$W^{7\text{-Aza}}$ II

HPLC



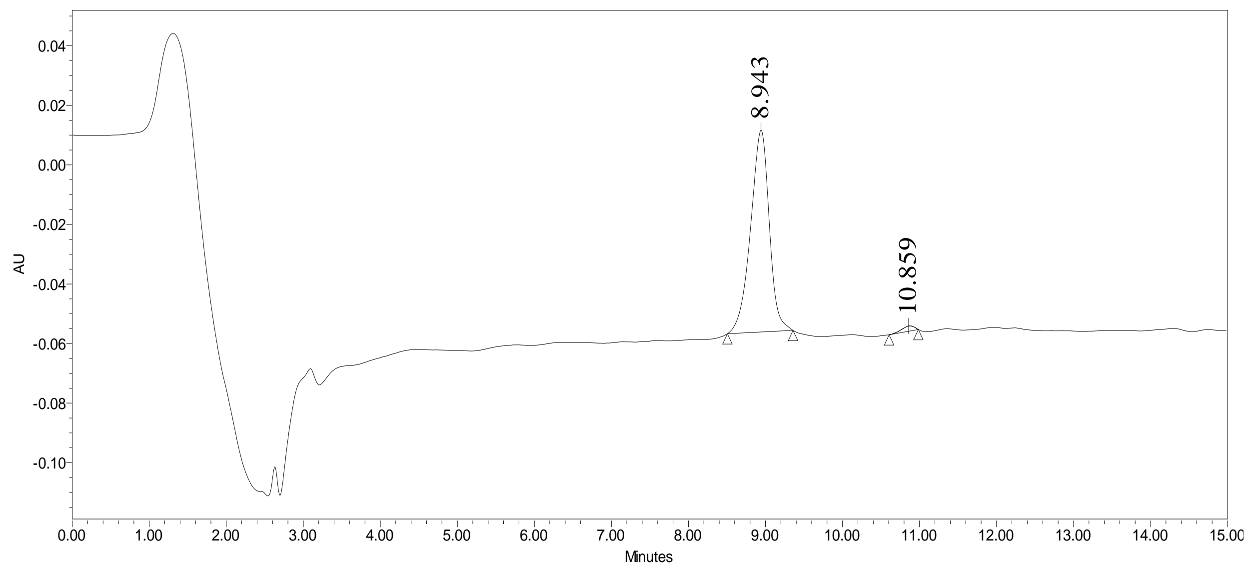
Purity: 98.4%

MS



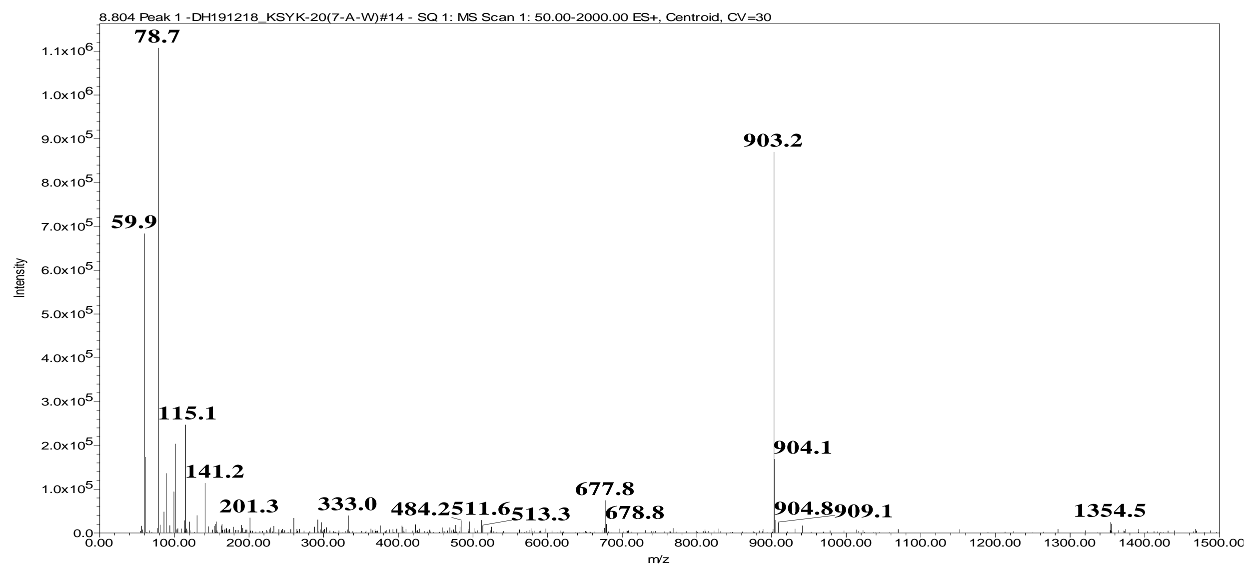
NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-}Aza}$ I

HPLC



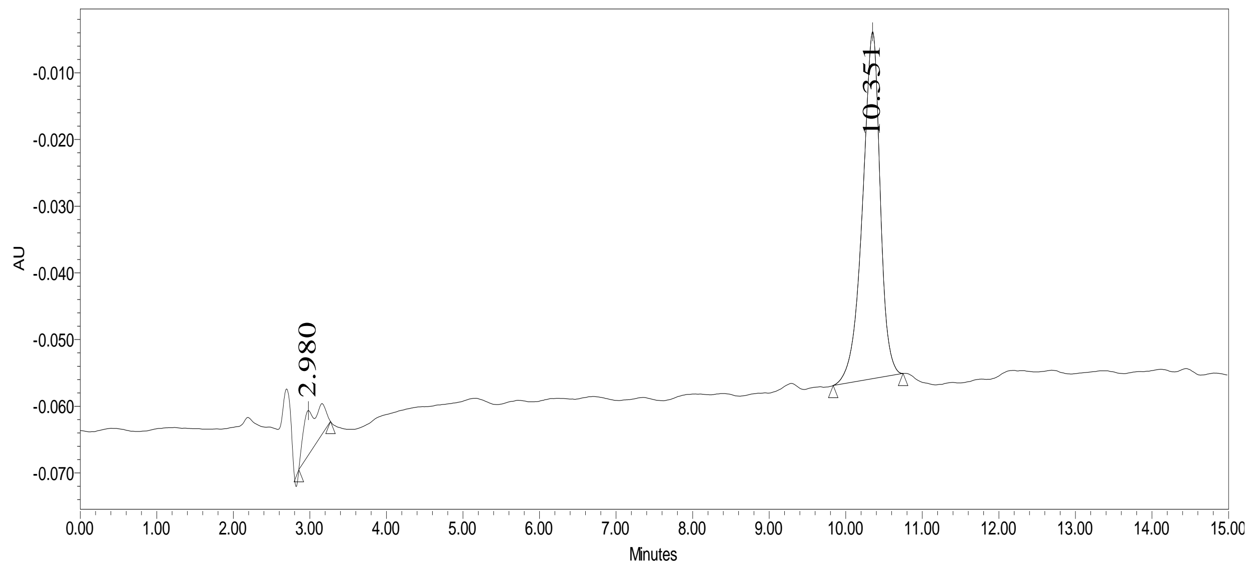
Purity: 98.3%

MS



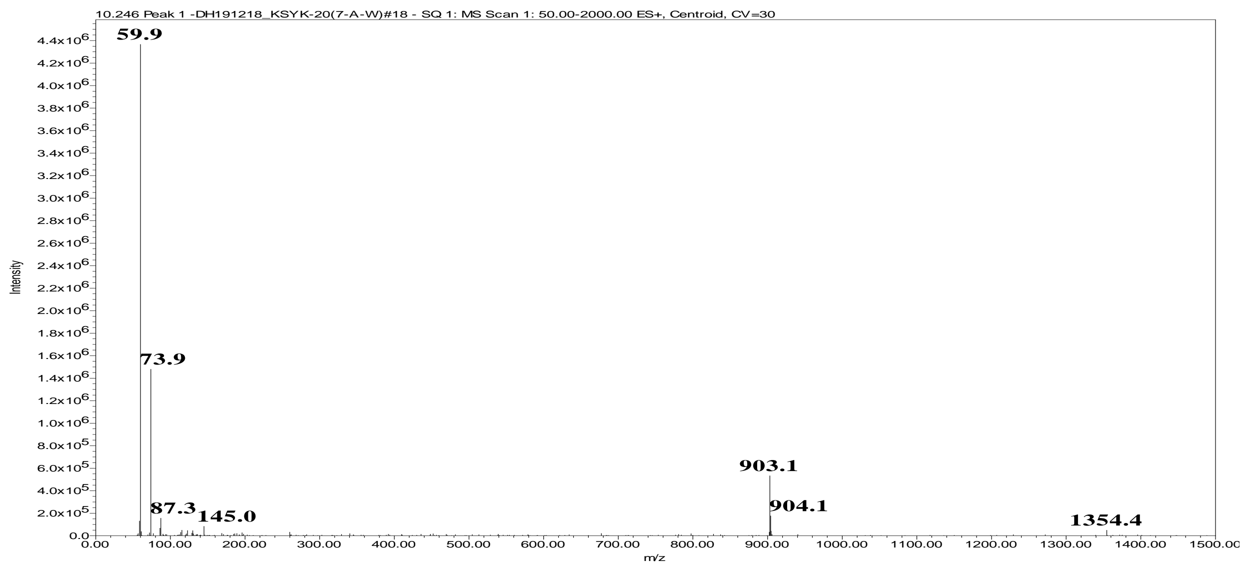
NCR169$C_{17–38}C_{12,17}$/S-20$W^{7\text{-}Aza}$ II

HPLC



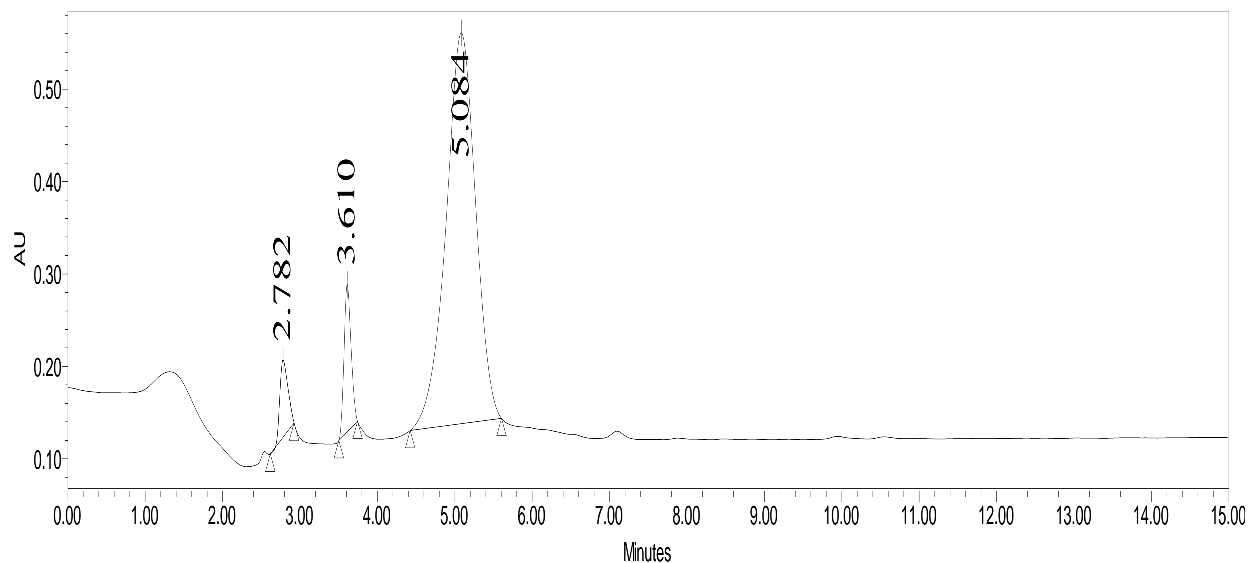
Purity: 89.24%

MS



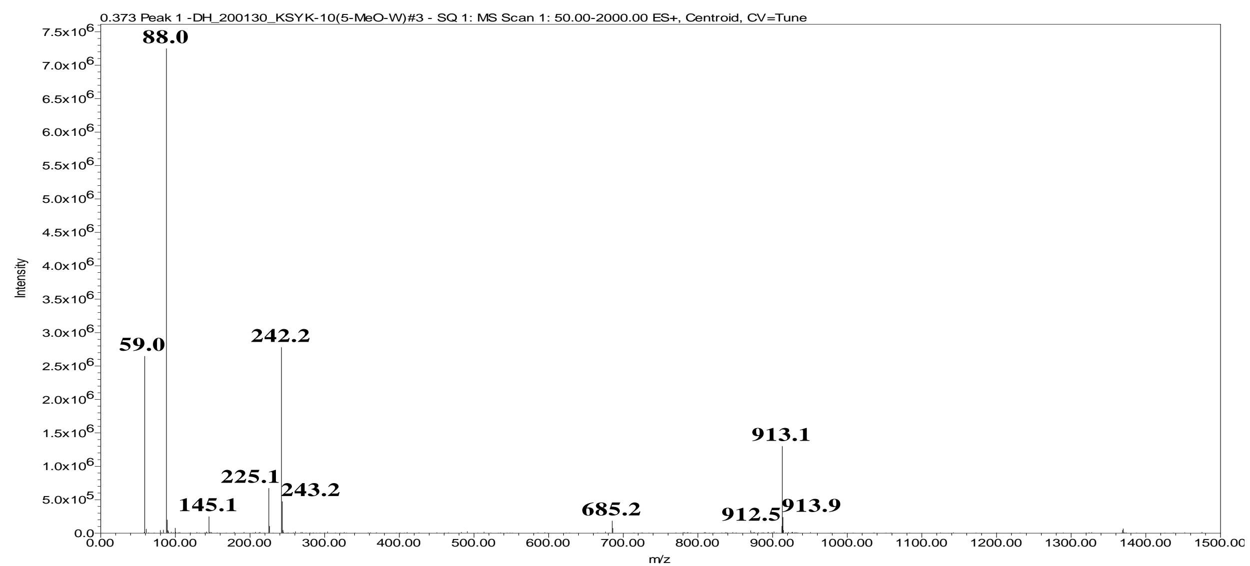
NCR169$C_{17–38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ I

HPLC



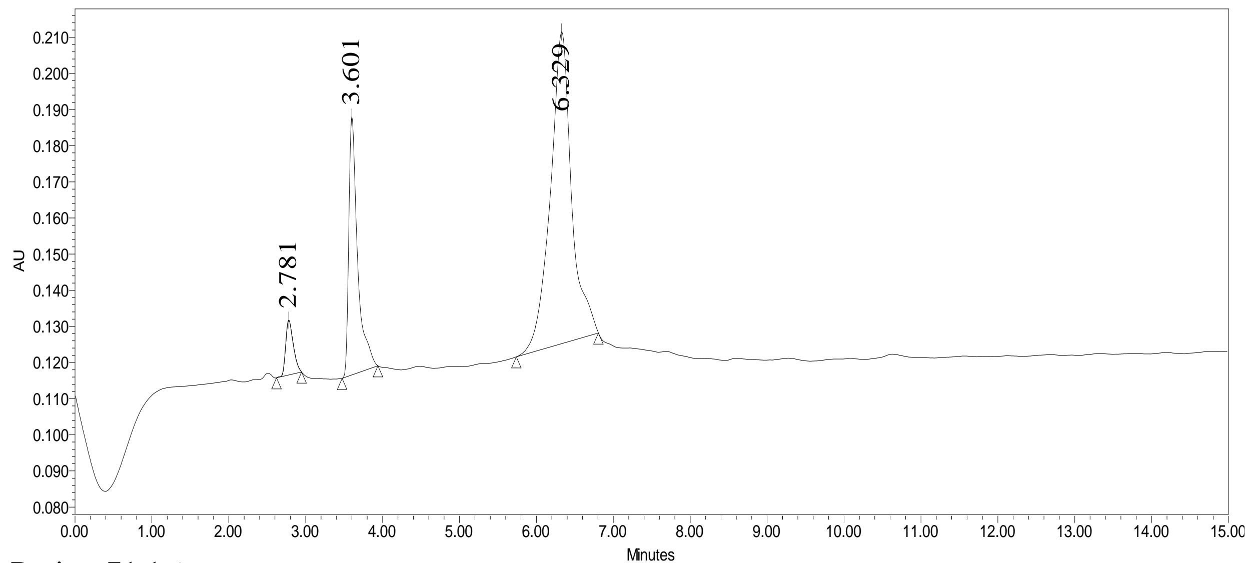
Purity: 87.5%

MS



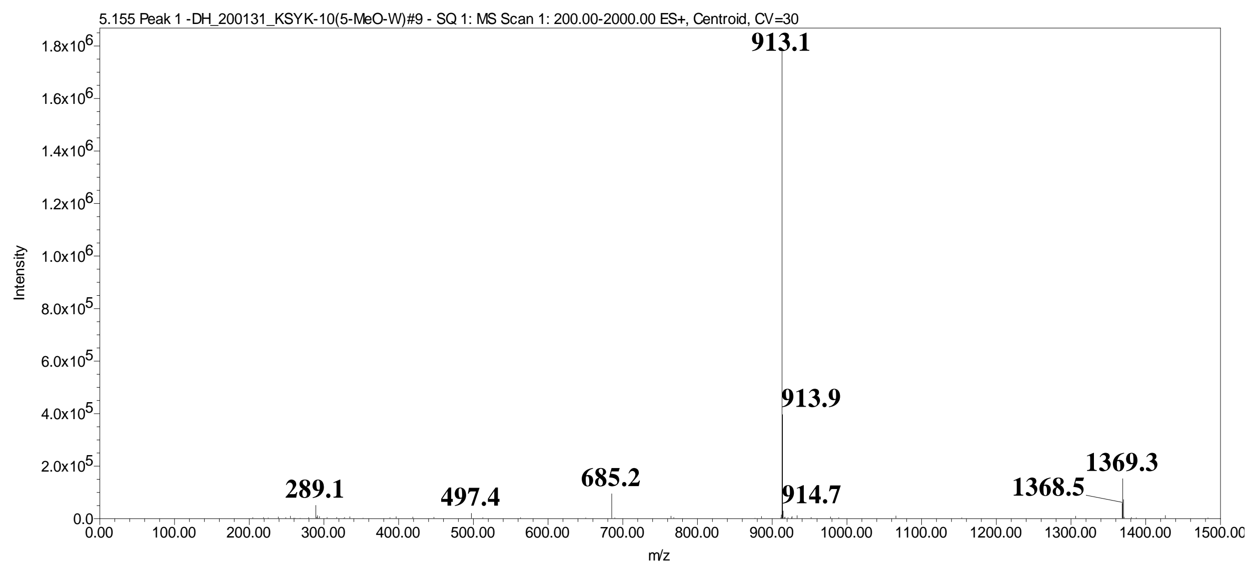
NCR169$C_{17-38}C_{12,17}$/S-10$W^{5\text{-MeO}}$ II

HPLC



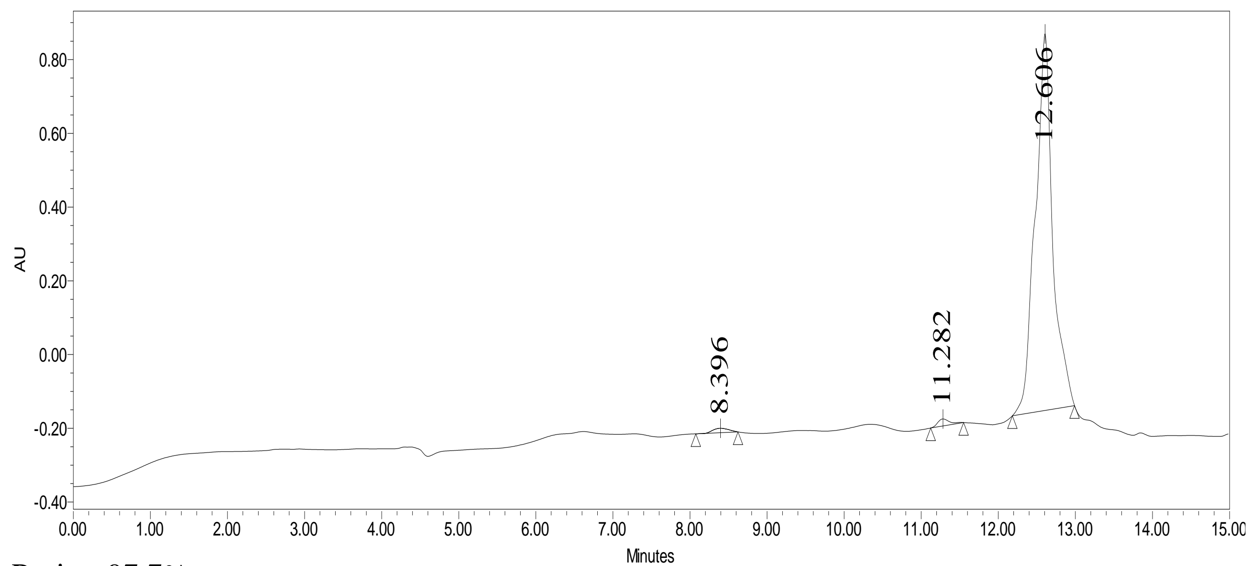
Purity: 71.1%

MS



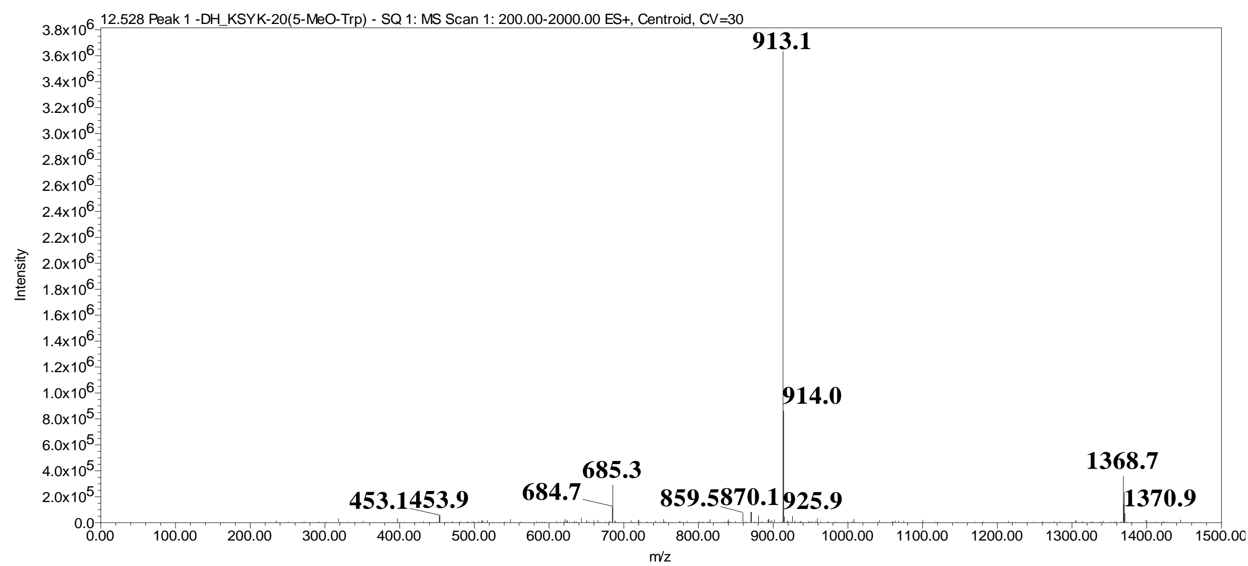
$NCR169C_{17–38}C_{12,17}/S\text{-}20W^{5\text{-}MeO}$

HPLC



Purity: 97.7%

MS

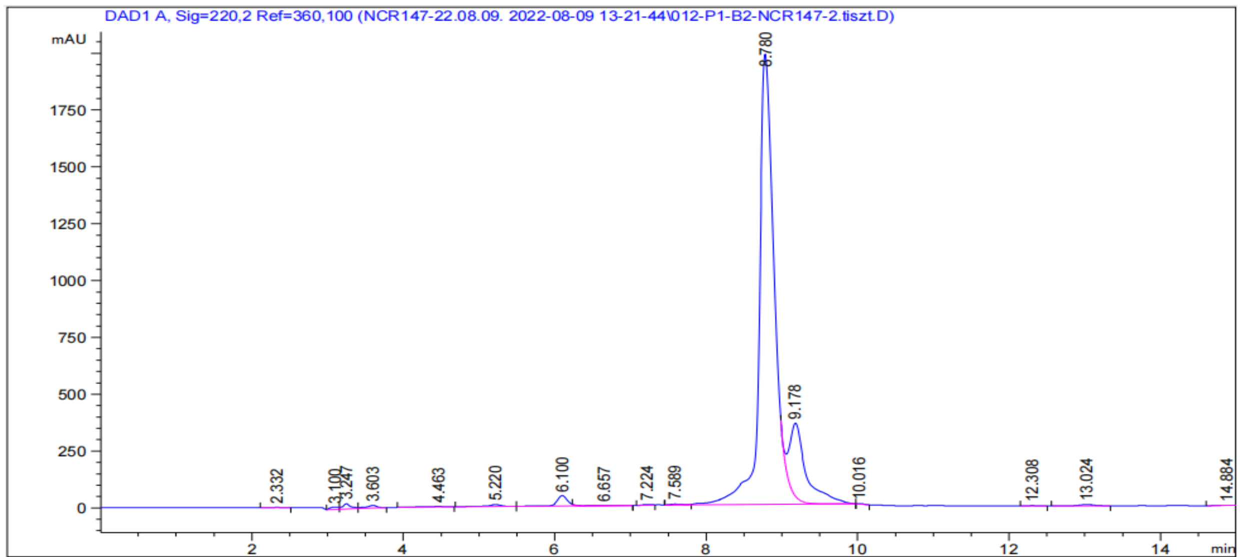


Appendix 2

HPLC Chromatograms and MS Spectra of NCR147 and Its Derivatives

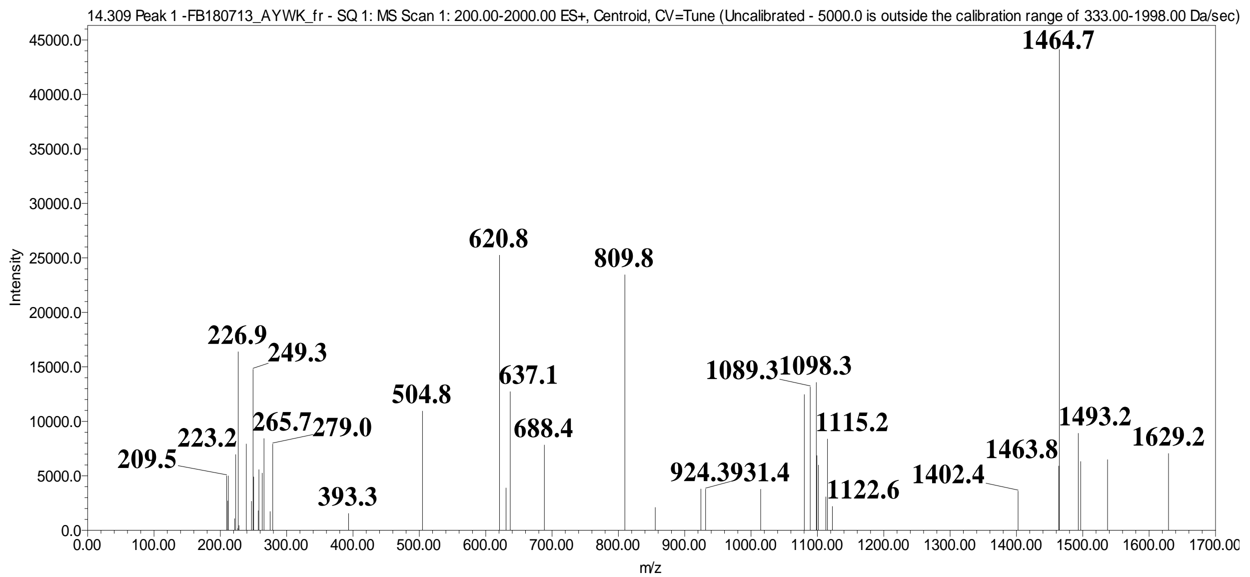
NCR147

HPLC



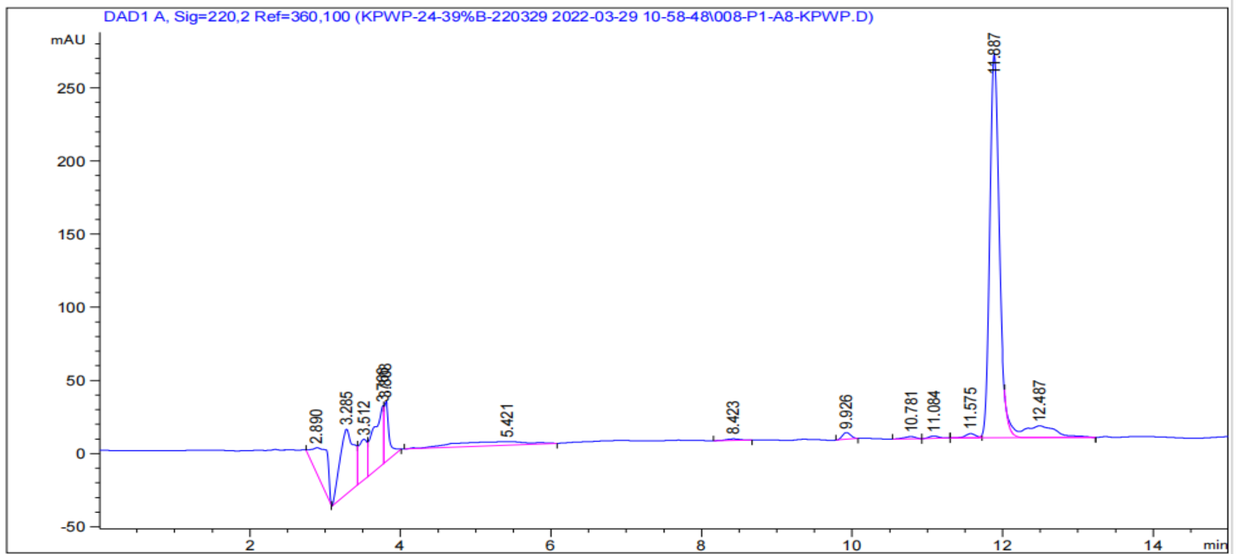
Purity: 91.4%

MS



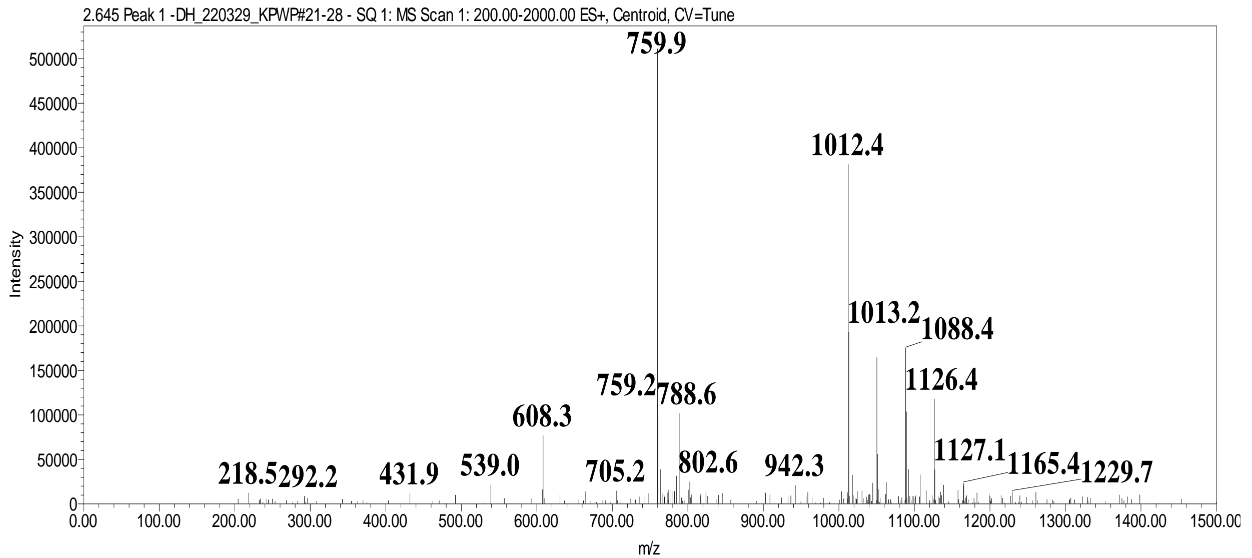
NCR147$_{13\text{-}36}$

HPLC



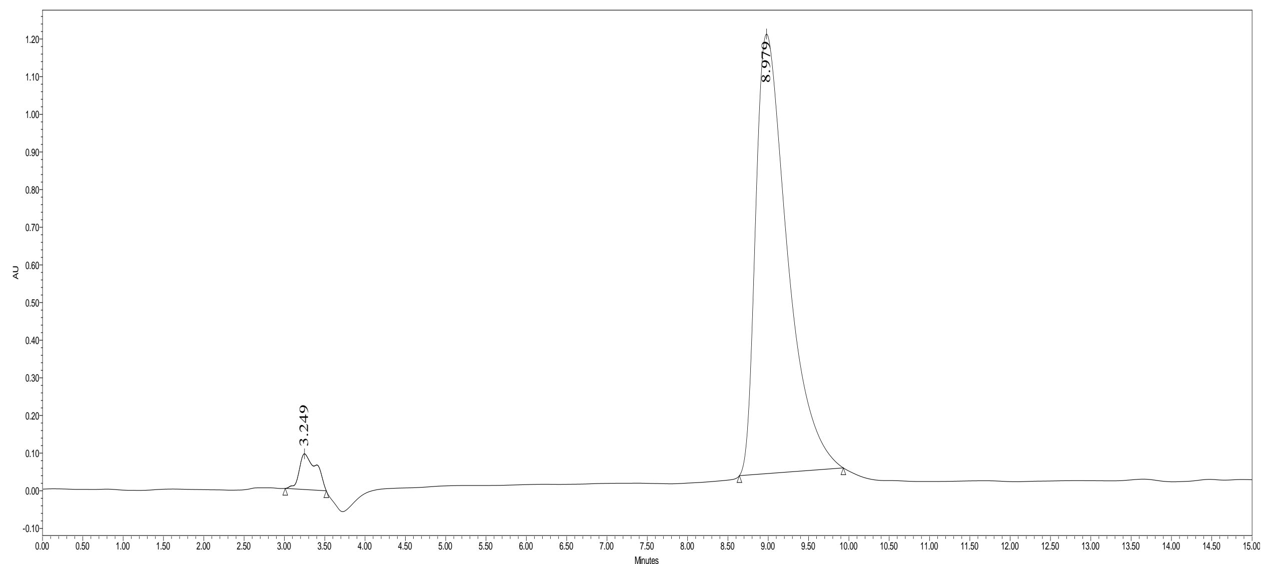
Purity: 91.5%

MS



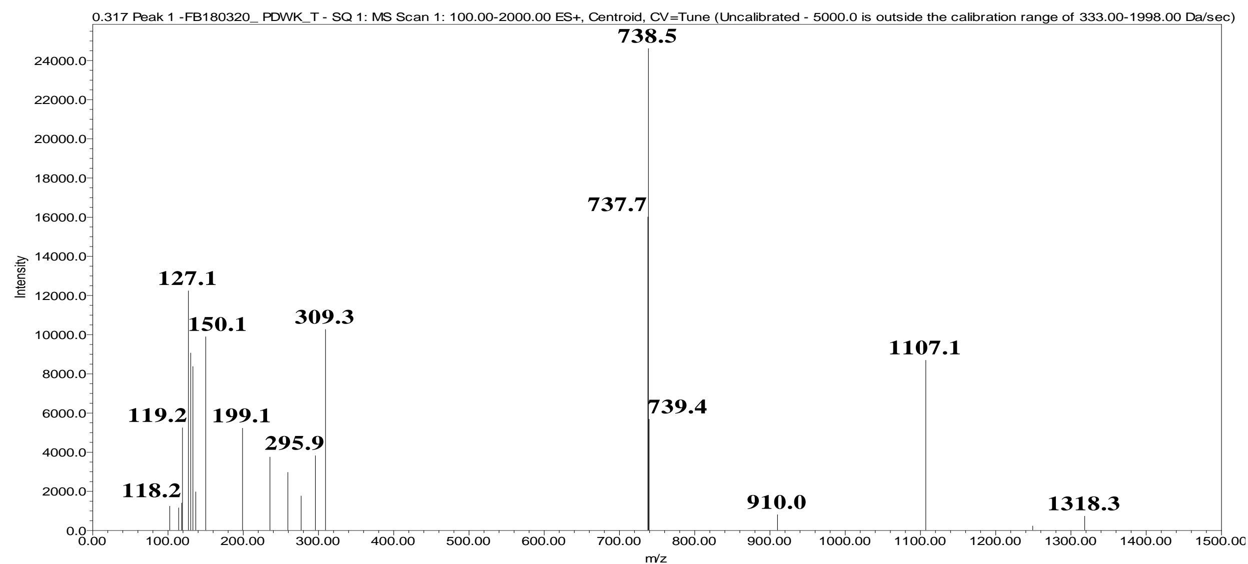
NCR147C$_{20-36}$

HPLC



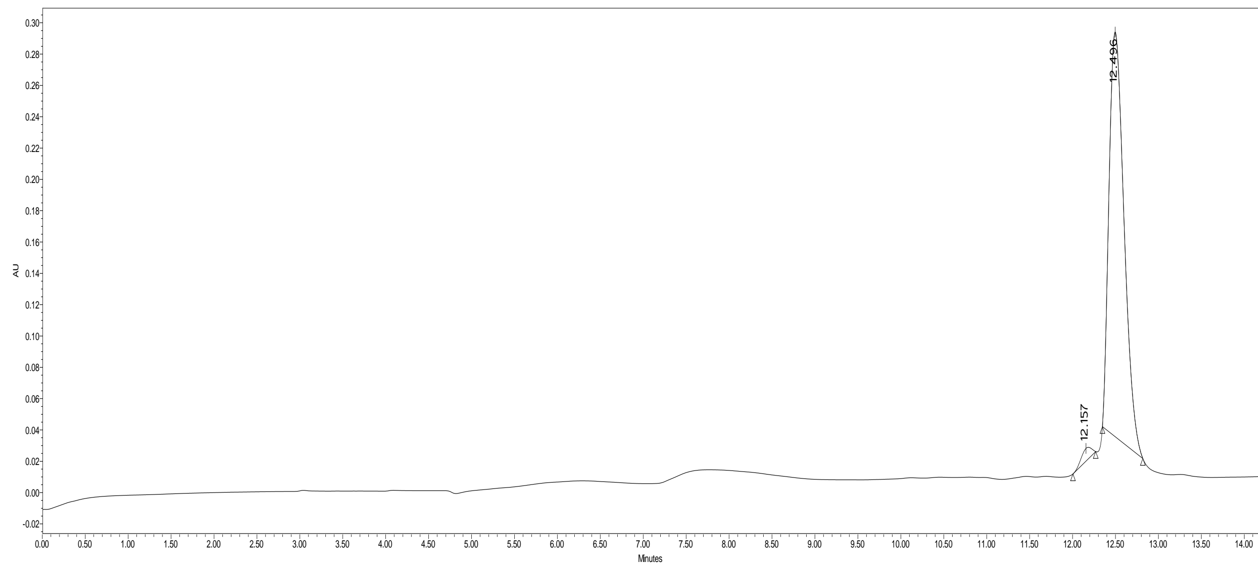
Purity: 95.8%

MS



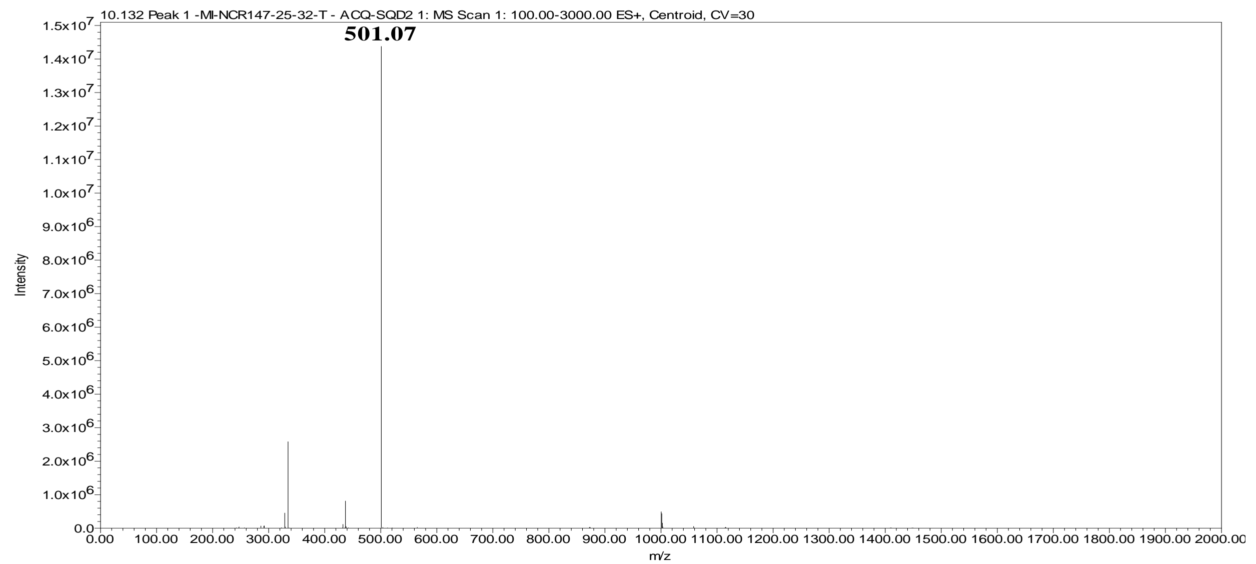
NCR147$_{25\text{-}32}$

HPLC



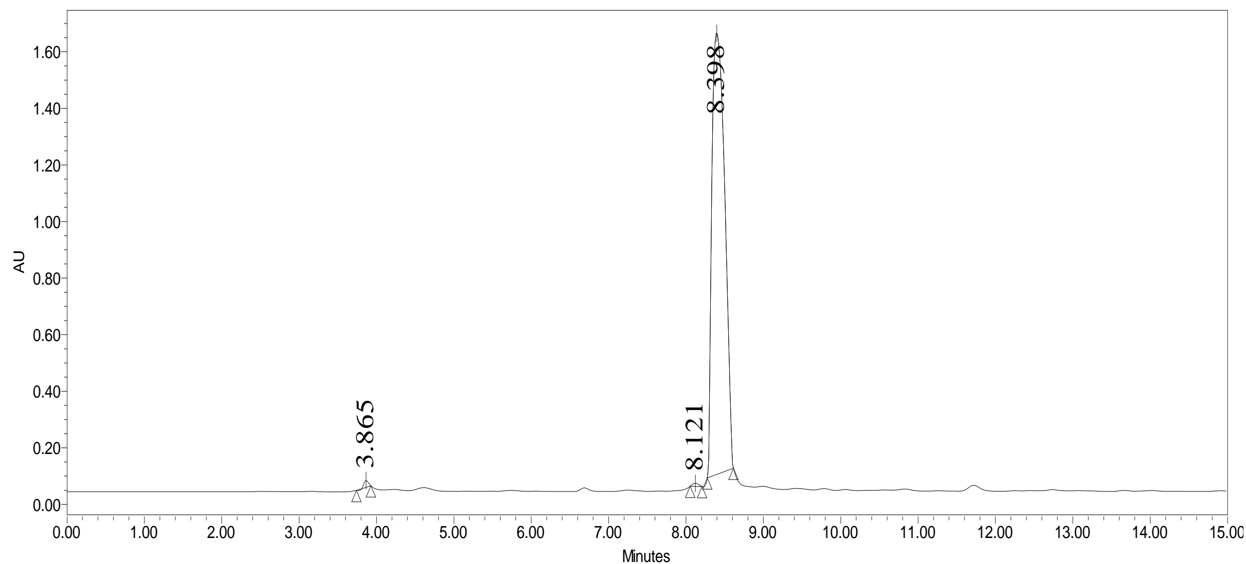
Purity: 99.35%

MS



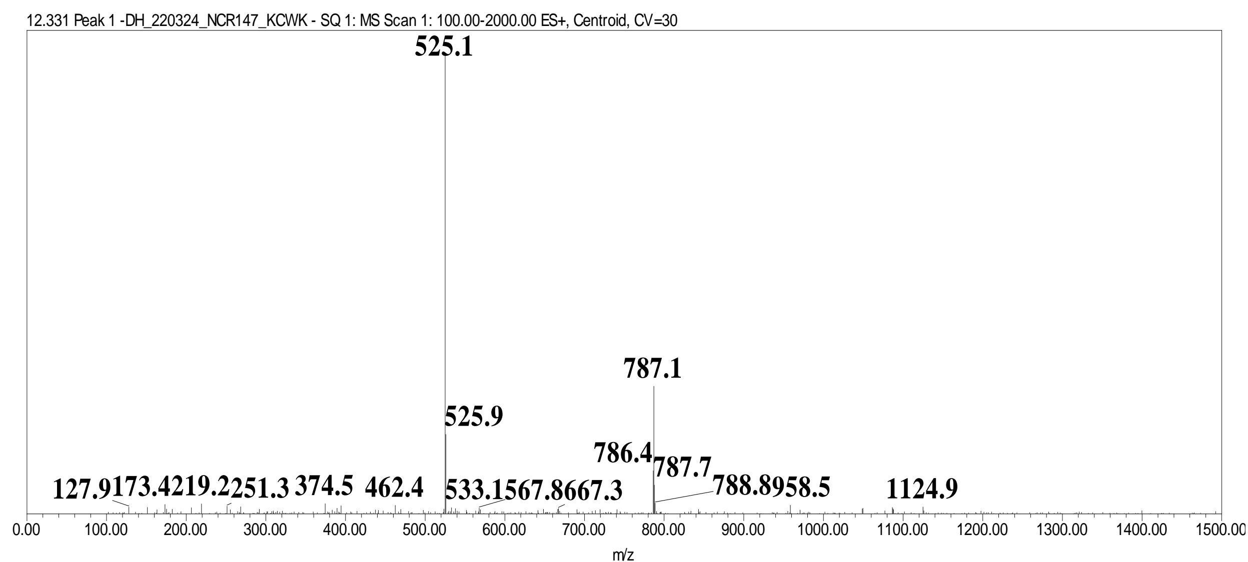
NCR147$_{25\text{-}36}$

HPLC



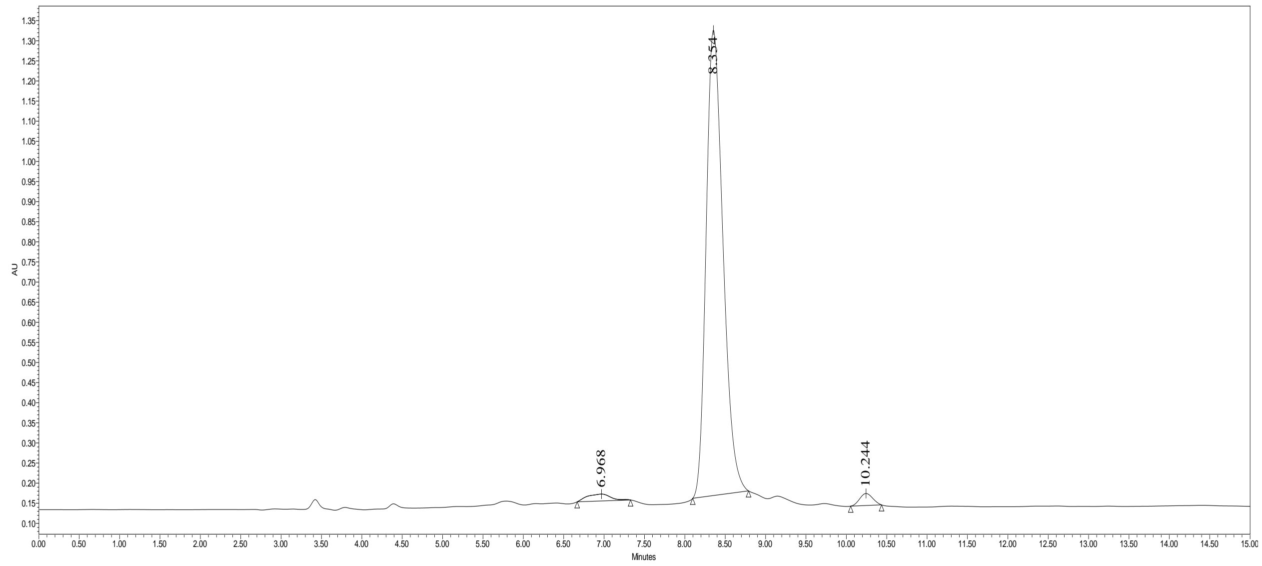
Purity: 99.1%

MS



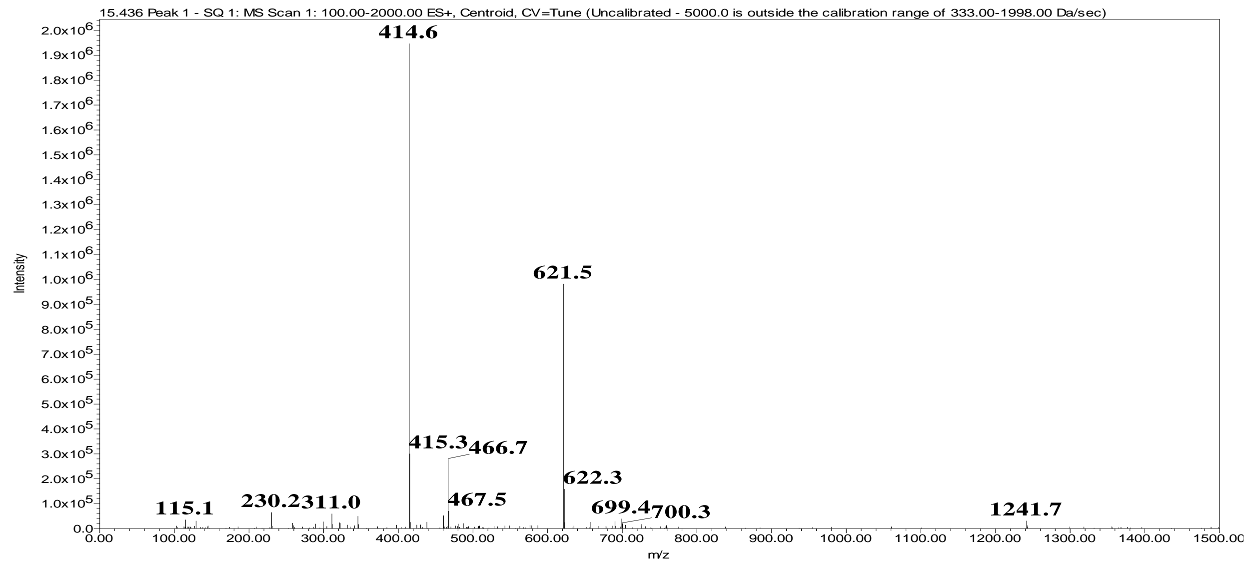
NCR147$_{28\text{-}36}$

HPLC



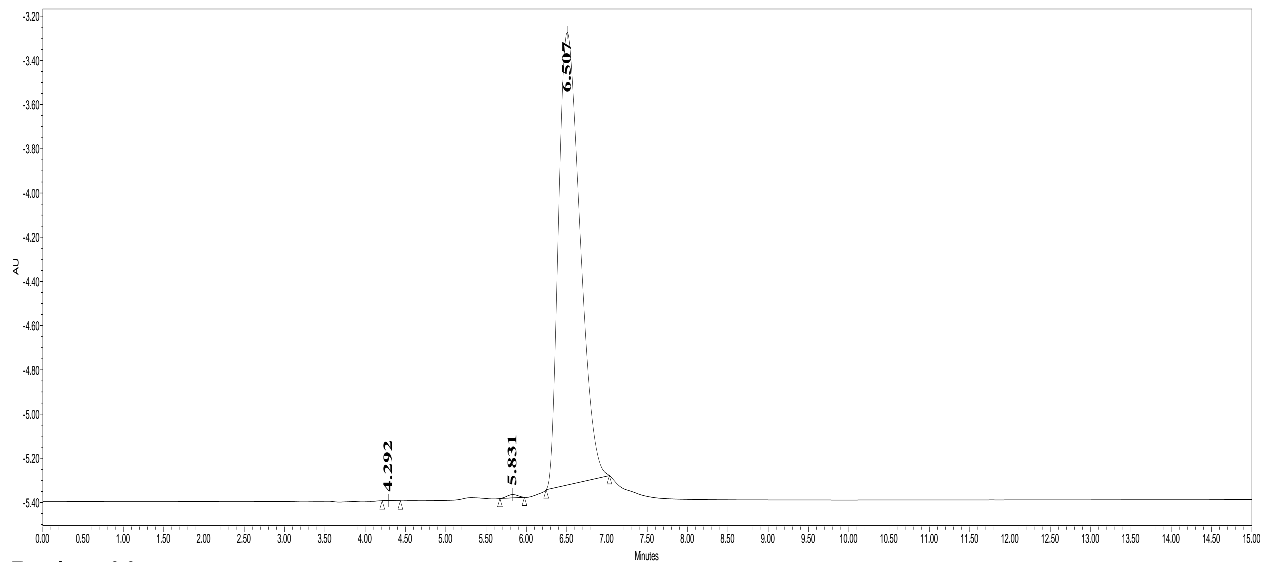
Purity: 96.1%

MS



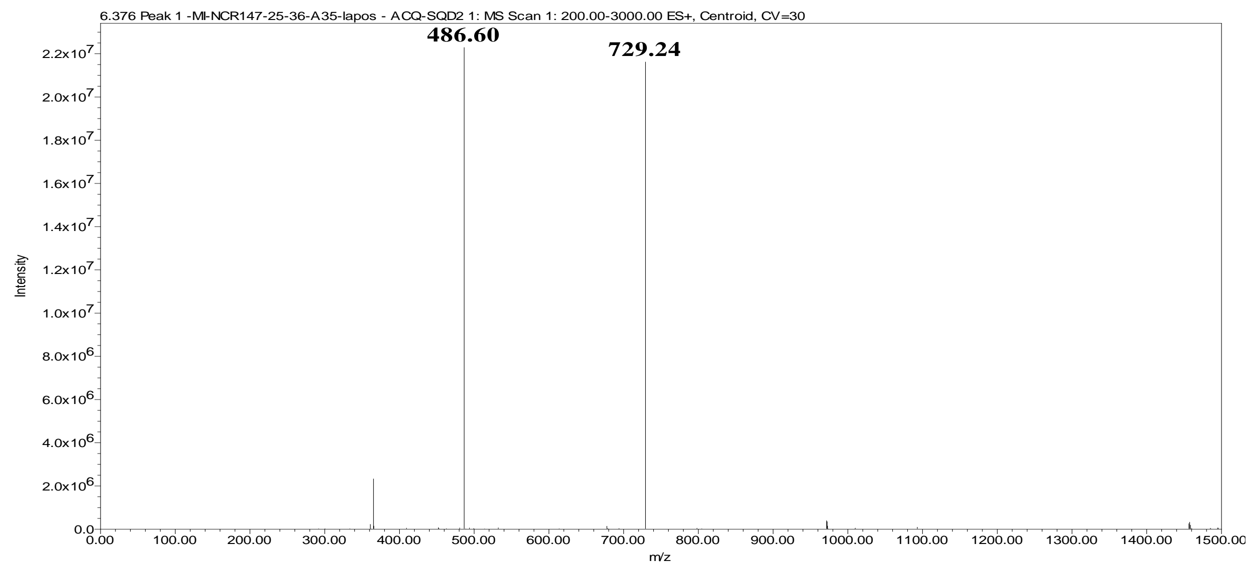
NCR147 $_{25\text{-}36}$W$_{11}$/A

HPLC



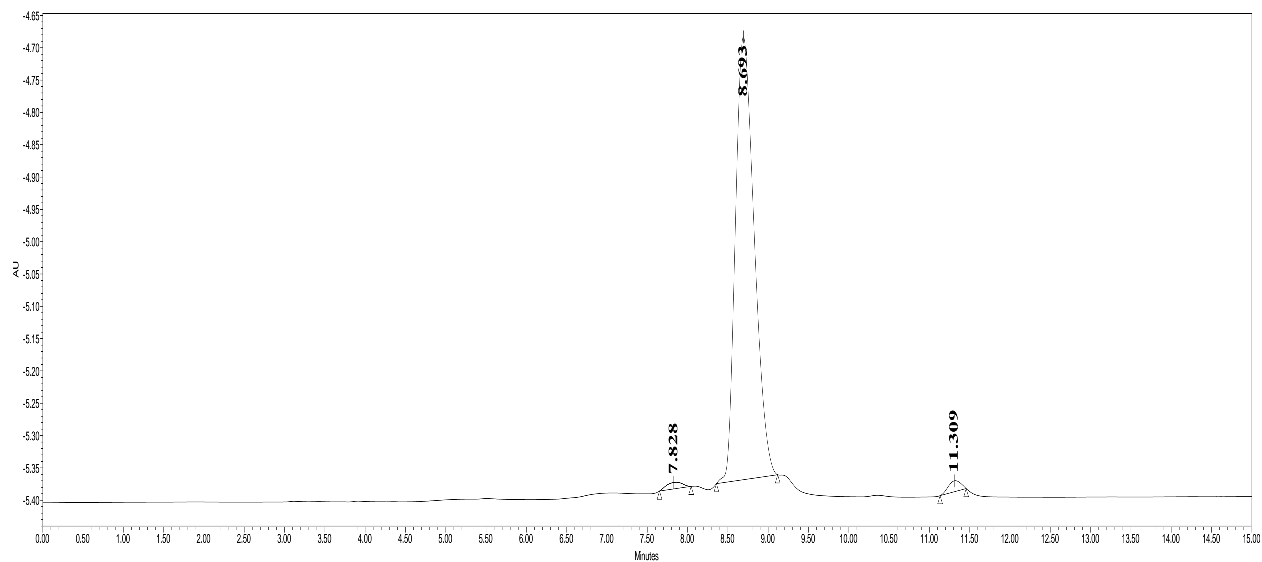
Purity: 99.6%

MS



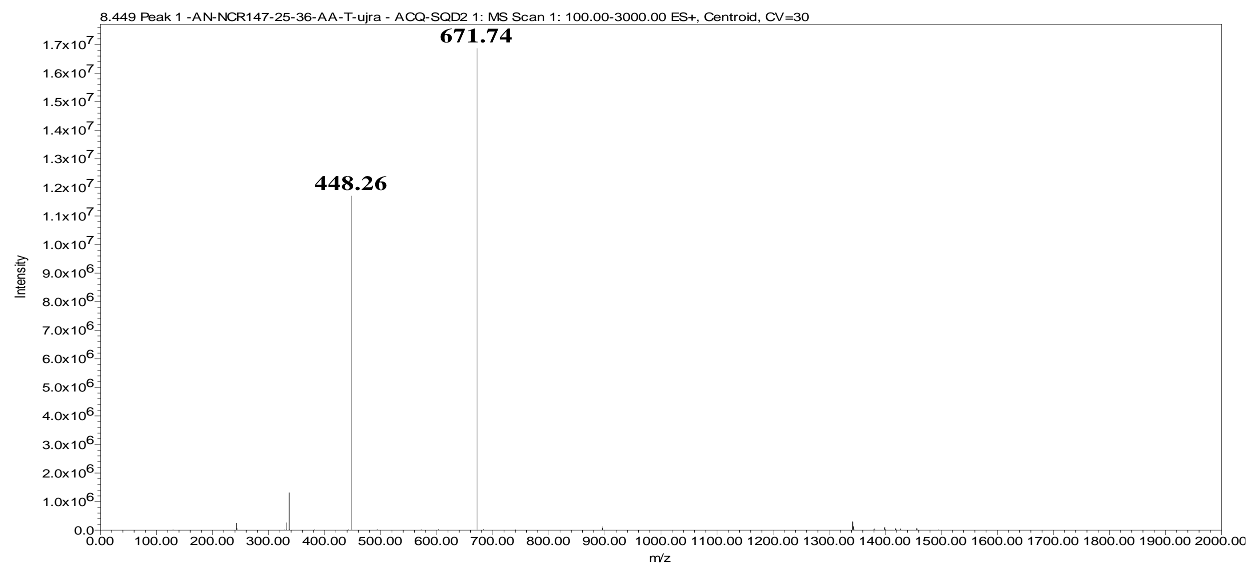
NCR147$_{25\text{-}36}$W$_{9,11}$/A

HPLC



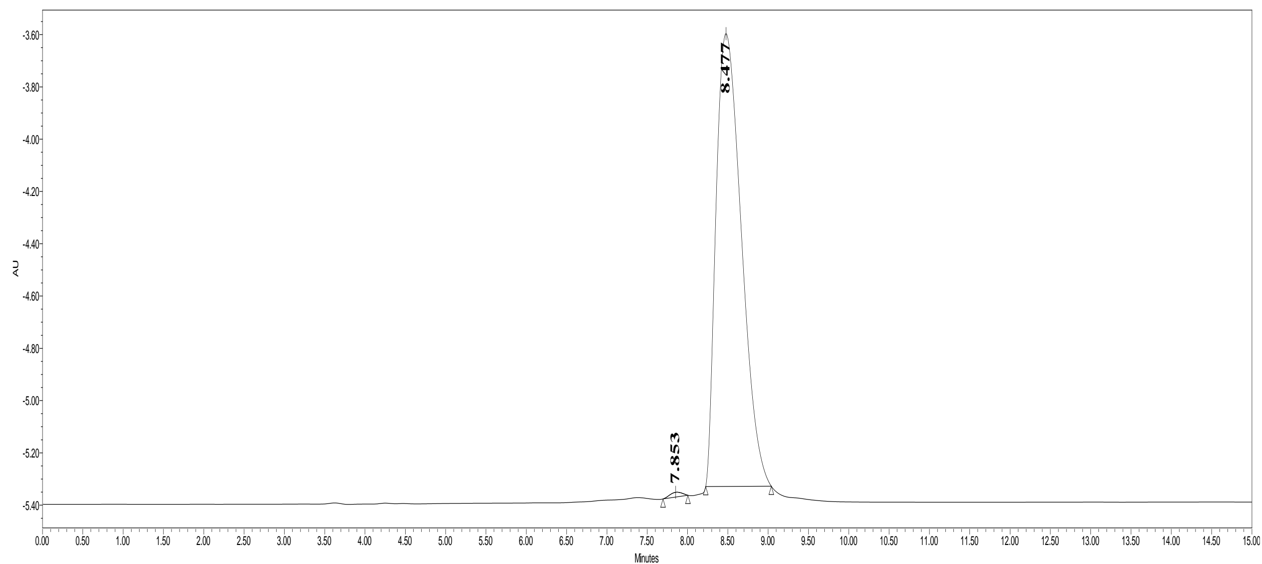
Purity: 97.21%

MS



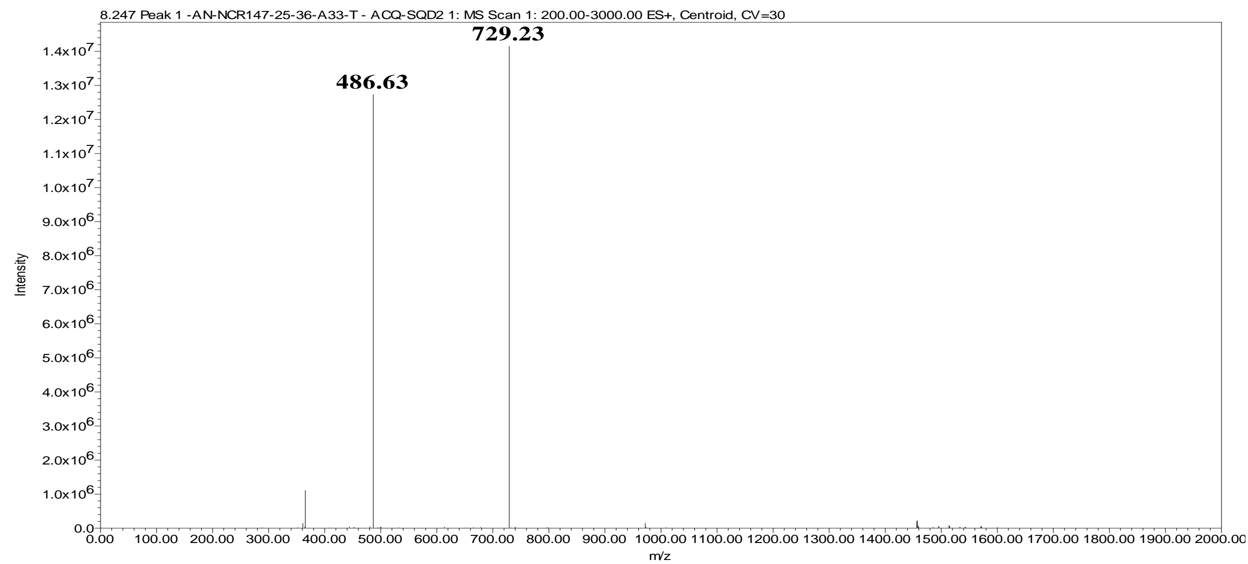
NCR147 $_{25\text{-}36}$W$_9$/A

HPLC



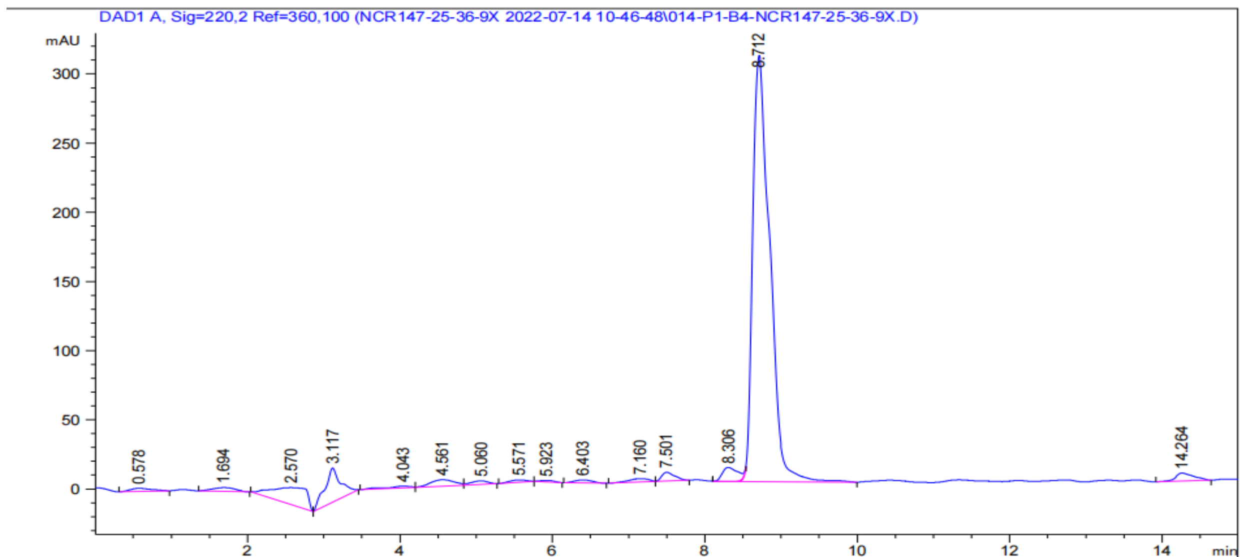
Purity: 99.5%

MS



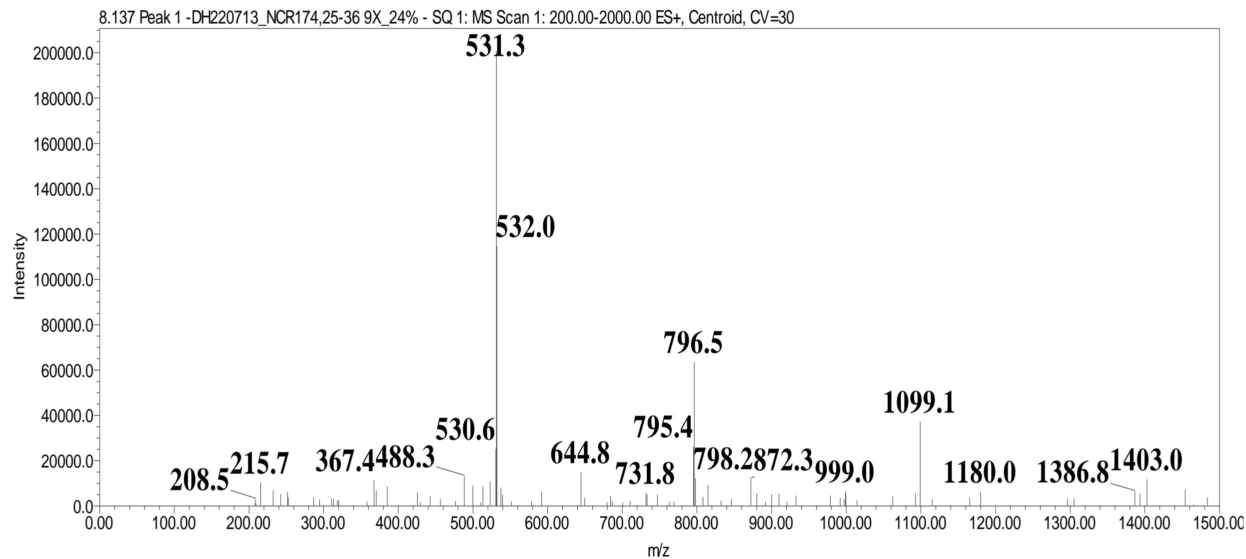
NCR147$_{25\text{-}36}$W$_9$/W$^{5\text{-F-L}}$

HPLC



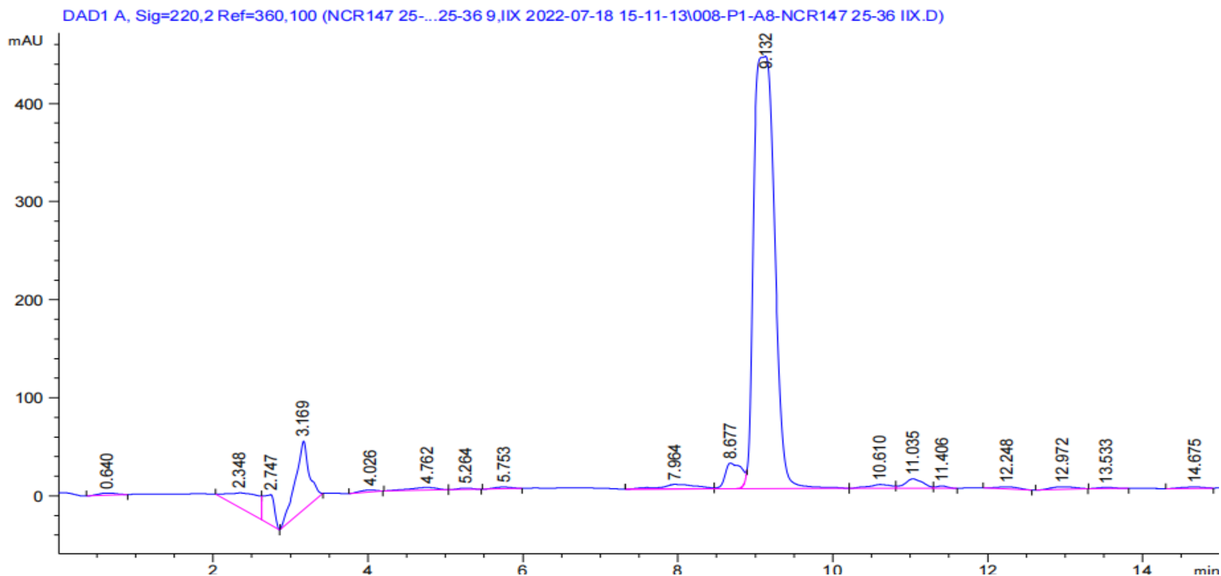
Purity: 76.9%

MS



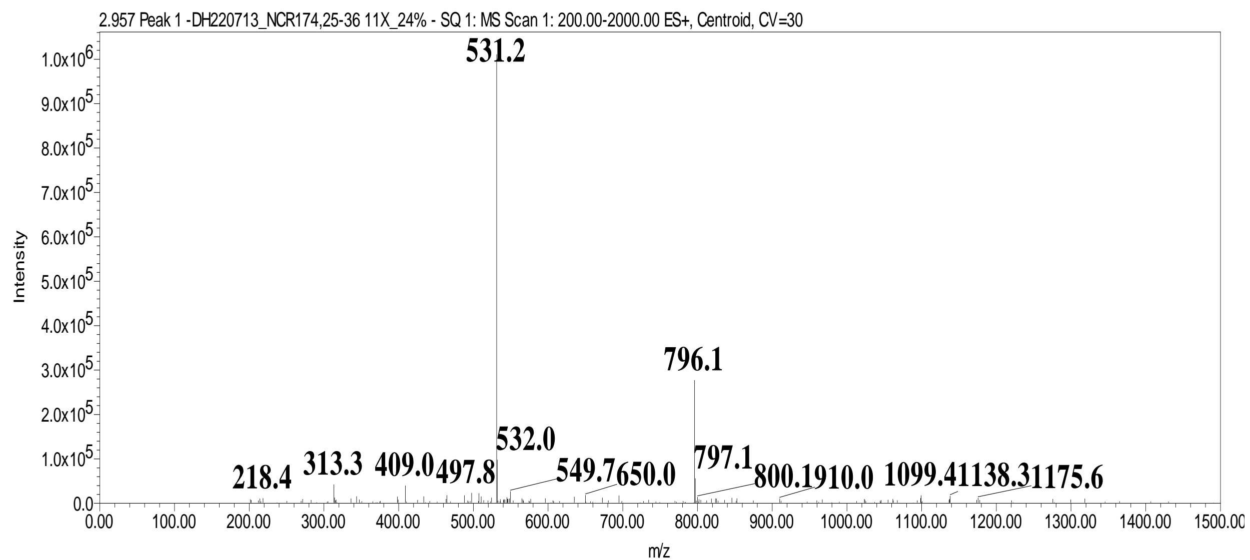
$NCR147_{25\text{-}36}W_{11}/W^{5\text{-}F\text{-}L}$

HPLC



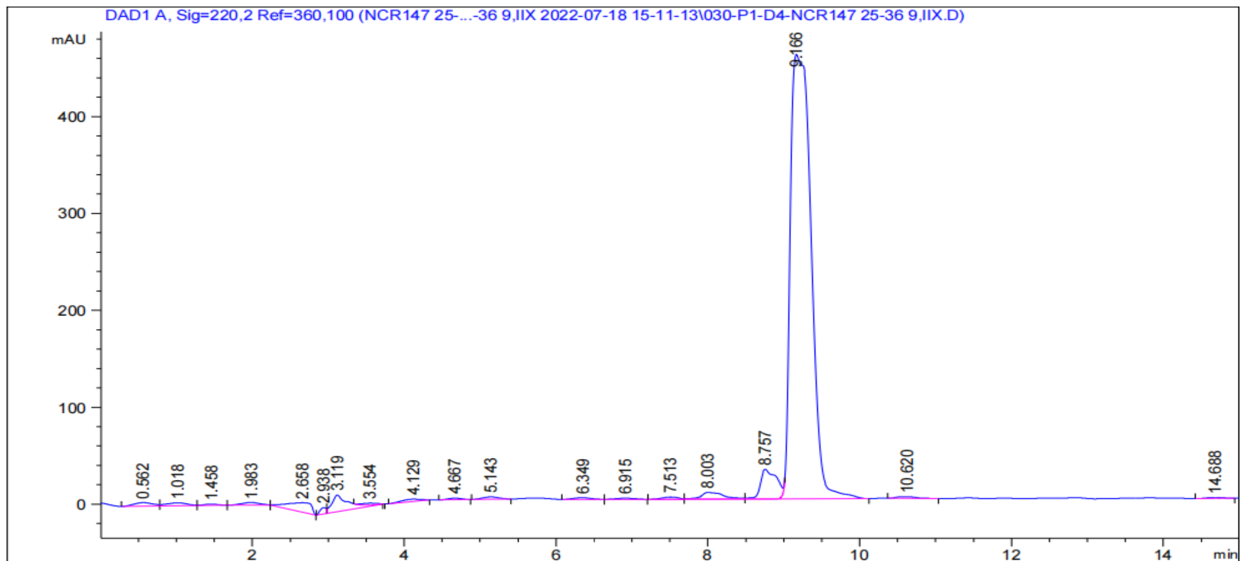
Purity: 90.8 %

MS



$NCR147_{25\text{-}36}W_{9,11}/W^{5\text{-}F\text{-}L}$

HPLC



Purity: 93.6%

MS